Comparison of Tissue Reactions Produced by *Haemophilus pleuropneumoniae* Vaccines Made with Six Different Adjuvants in Swine

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ABSTRACT

Tissue damage caused by six different adjuvants incorporated in a *Haemophilus pleuropneumoniae* vaccine was compared in swine. The adjuvants compared were four mineral oil compounds, one peanut oil compound and aluminum hydroxide. Inoculations were given in the neck, quadriceps and semitendinosus muscles. The mineral oil adjuvants were highly irritant and caused extensive areas of granulomatous inflammation that were present at eight weeks after injection. The aluminum hydroxide produced smaller lesions that also persisted for eight weeks. Only the peanut oil adjuvant did not produce significant lesions at the site of injection. At two and four weeks, but not at eight weeks postinoculation, lesions in the quadriceps and semitendinosus muscles were approximately twice as extensive as those in the muscles of the neck.

Key words: Adjuvants, injection reactions, muscle condemnation.

RÉSUMÉ

Cette expérience visait à comparer, chez le porc, les dommages tissulaires attribuables à six adjuvants incorporés à un vaccin contre *Haemophilus pleuropneumoniae*. Ces adjuvants comprenaient quatre composés à base d'huile minérale, un composé à base d'huile d'arachides et l'hydroxyde d'aluminium. L'injection du vaccin se fit dans les muscles du cou, le quadriceps crural ou le semi-tendineux. Les adjuvants à base d'huile minérale se révélèrent très irritants et causèrent d'importants foyers d'inflammation granulomateuse qui existaient toujours, au bout de huit semaines après la vaccination. L'hydroxyde d'aluminium produisit des lésions plus discrètes, qui persistèrent néanmoins huit semaines. Seul l'adjuvant à base d'huile d'arachides ne produisit pas de lésions significatives au site d'injection. Au bout de deux et quatre semaines, mais non au bout de huit, après la vaccination, les lésions des quadriceps cruraux et des semitendineux s'avérèrent environ deux fois plus importantes que celles des muscles du cou.

Mots clés: adjuvants, réactions à une injection, condamnation des muscles.

INTRODUCTION

Since 1980 (1,2) *Haemophilus pleuropneumoniae* has been found with increasing frequency as a cause of severe pneumonia and has contributed markedly to pneumonia being a major source of decreased productivity in finishing pigs. This pneumonia is documented to result in high mortality (greater than 50%), decreased rate of gain, increased marketing of undersized or cull pigs and loss due to slaughter condemnation (3,4). This situation coupled with poor feed conversion of chronically affected pigs and high medication costs results in major financial losses of concern to producers.

Control of *H. pleuropneumoniae* has been implemented using vaccines. This approach is based on the finding of an inverse relationship between herd mortality and antibody titers (5,6,7). However, vaccines that stimulated a protective antibody response also caused much tissue reaction (abscesses and granulomas) (8). This resulted in an additional economic loss due to the trimming of condemned meat from the carcass.

The issue of the best adjuvant to use for a *Haemophilus* vaccine has not been clearly addressed. Oil and aluminum hydroxide, the most common adjuvants, both have drawbacks: the former producing a major tissue irritation and the latter producing a poor antibody response (9). No systematic evaluation has been undertaken to date to identify the best *Haemophilus* vaccine based on tissue reaction and antibody titers. This pilot study was the beginning of such an investigation focusing on the tissue reaction of six adjuvants employed to produce a *Haemophilus pleuropneumoniae* vaccine.

MATERIALS AND METHODS

Crossbred swine weighing approximately 25 kilograms were injected with 2 mL of vaccine produced in this laboratory with commercially available adjuvants or ones supplied by a USDA research laboratory. Injection sites varied, so that site variation could also be analyzed in association with specific adjuvants and tissue reaction. Injection sites were the neck, quadriceps and semitendinosus muscles.

For the aqueous phase of the vaccines, *Haemophilus pleuropneumoniae* serotype 1 was grown overnight on chocolate agar supplemented with nicotinamide adenine dinucleotide

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Guidelines as given in "Guide to the Care and Use of Experimental Animals, Volume I" were followed for animals in this study.

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(NAD). Bacterial colonies were harvested by washing plates with phosphate buffered saline. The concentration of harvested bacteria in PBS was adjusted to 10^9 per mL. Bacteria were killed by adding sufficient formalin to produce a 0.2% concentration. Sterility and absence of toxin were determined by plating the killed bacterial suspension on chocolate agar supplemented with NAD and by mouse inoculation.

The six adjuvants used in this study were 1) Freund's incomplete adjuvant (Difco Labs, Detroit, Michigan), 2) aluminum hydroxide gel (Amphogel, Wyeth Labs, Philadelphia, Pennsylvania), 3) Lipovant (Accurate Chemical & Scientific Corp., Westbury, New York), 4) 89% Drakeol 6 VR, 10% Arlacel A and 1% Tween 80, 5) 90% Drakeol 6 VR and 10% Arlacel 80, and 6) 90% Marcol 52, 5.4% Span 85 and 4% Tween 85 (Dr. H.D. Stone, Southeast Poultry Research Lab, U.S.D.A., Athens, Georgia). Adjuvants 4, 5 and 6 were prepared at the Southeast Poultry Research Lab, Athens, Georgia, as previously described (10,11). Freund's incomplete adjuvant was combined with the aqueous phase in a 1:1 ratio using an emulsion churn. Four parts aluminum hydroxide gel was combined with one part aqueous phase. Lipovant was combined with antigen at the maximum concentration recommended by the manufacturer, 1 g Lipovant with 4 mL of aqueous portion. The other three adjuvants were combined with aqueous portion using drop-wise addition of one part aqueous portion to four parts oil during two minutes while the oil was constantly stirred at low speed in a blender (Waring blender, Dynamics Corporation of America, New Hartford, Connecticut) (10,11). After the addition was complete, this mixture was emulsified by homogenization at high speed for 30 seconds. Throughout vaccine preparation, close attention was given to maintaining sterility of all components. All vaccine preparations were stored at 4°C until use.

Three pigs were euthanized at two weeks postinoculation (PI), three pigs at four weeks PI and four pigs at eight weeks PI. Injection sites and adjacent lymph nodes were examined and the extent of tissue damage measured. Injection sites were opened aseptically, swabbed onto blood agar and cultures incubated at 37°C for 24 h. Sections of affected muscle and adjacent lymph nodes were fixed in formalin. Tissue sections were embedded in parrafin, cut at 6 μm and stained with hematoxylin and eosin.

The statistical analysis employed in this study in order to compare the extent of lesions was a Kruskall-Wallis one-way ANOVA. This nonparametric test on ranks was used since the results were not anticipated to have a normal distribution. This was followed by multiple pairwise comparisons.

RESULTS

Table I indicates the amount of tissue damage in grams. The wide range noted proved to be statistically significant ($X^2 = 39$, $p < 0.001$). Multiple pairwise comparisons showed that five of the fifteen possible pairs were significantly different: Freund-Lipovant, arlacel-Lipovant, arlacel+tween-Lipovant, span-Lipovant, span-aluminum hydroxide. Lipovant produced a minor gross reaction in only one animal, whereas $AL(OH)_3$ produced some reaction in all subjects, although this difference was not statistically significant. The oil adjuvants all produced severe gross tissue reactions.

Affected muscle was paler and firmer than surrounding tissue. At two weeks PI, the lesions were globoid in shape and at four and eight weeks PI the lesions became more linear and tended to course between muscle groups. Interspersed within the larger area of firm, pale muscle were small, irregularly sized areas of abscessation that were often encapsulated.

Bacteriological examinations of injection sites were negative. Occasionally *Escherichia coli* and *Staphylococcus epidermis* were isolated in small numbers and were considered as contaminants.

Histologically two kinds of reactions were distinguishable in affected tissue. The four mineral oil adjuvants elicited similar reactions. These adjuvants caused extensive lesions characterized by replacement of skeletal muscle with tracts of fibrous connective tissue, large foci of pyogranulomatous inflammation, and caseation necrosis. Accumulations of lymphocytes and mineralization of tissue were also frequent findings. At four and eight weeks PI there was an increase in fibrosis and the inflammatory response became purely granulomatous. Lipid droplets were readily detected in all lesions caused by mineral oil, and were not diminished in numbers at eight weeks PI. Aluminum hydroxide was associated with accumulation of large numbers of macrophages with abundant foamy cytoplasm. The pyogranulomatous response, caseation necrosis, and fibrosis were less marked than those seen with mineral oil. Lymphocytes were seen with slightly greater frequency. The one lesion caused by Lipovant was pyogranulomatous.

At two and four weeks PI, but not at eight weeks PI the extent of muscle reaction differed between injection sites (Table II). At two and four weeks PI the amount of tissue involved when the injection was given in the quadri-

TABLE I. Extent of Lesions[a] Following Vaccination with Six Adjuvants

Pig I.D.	Weeks After Inject	Adjuvant: Freund's incomplete	Drakeol 6VR and Arlacel 80	Drakeol 6VR Arlacel A and Tween 80	Marcol 52, Span 85 and Tween 85	Lipovant	Aluminum hydroxide
2-1	2	193	38	5	224	0	11
2-2	2	16	240	100	90	1	18
2-3	2	56	56	53	126	0	14
4-1	4	2	108	50	648	0	6
4-2	4	53	50	144	180	0	4
4-3	4	44	36	108	450	0	16
8-1	8	60	24	80	45	0	20
8-2	8	16	28	48	240	0	40
8-3	8	36	30	6	27	0	20
8-4	8	16	56	18	36	0	6
Total		492	666	612	2,066	1	155

[a]Values represent the weight (g) of injured tissue

TABLE II. Extent of Adjuvant-induced Tissue Injury in Various Sites

Site	Mean Weight (g) of Affected Tissue at 2,4 and 8 Weeks Postinjection		
	2	4	8
Neck	40	45	29
Quadriceps	98	129	26
Semitendinosus	68	143	46

ceps or the semitendinosus was more than twice the amount that was seen when the injection was given in the muscles of the neck. At eight weeks PI there was not a significant difference between the extent of damage in the neck and leg muscles.

DISCUSSION

All four mineral oil adjuvants used in this study caused extensive, persistent granulomatous reactions in swine. The peanut oil adjuvant, Lipovant, was least irritant, producing a small area of tissue reaction in just one of the inoculated animals. Aluminum hydroxide was intermediate in its irritancy; however, lesions induced by $Al(OH)_3$ persisted throughout the eight weeks of the trial. Predicted economic loss from adjuvant-induced muscle condemnation in these pigs would be approximately \$3.00/pig for oil adjuvants, \$1.80/pig for $Al(OH)_3$ and 10¢/pig for Lipovant.

The neck was the site of choice for injections for two reasons. First, injections in this area initially caused reactions one-half the size of those in the rear limbs. Second, muscle removed from the neck is worth less than that removed from the hind leg.

Lipovant was the least irritant of the adjuvants tested; however, its ability to enhance antibody production in swine is unknown. In species such as mice, hamsters, monkeys and sheep, Lipovant is a potent adjuvant (12). If Lipovant exhibits similar activity in swine, it could be expected to provide significant advantages over currently available adjuvants such as $Al(OH)_3$.

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