Emulsifier content and side effects of oil-based adjuvant vaccine in swine

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Accepted 10 October 2005

Abstract

Side effects caused by the excessive emulsifier in oil-based adjuvant vaccine were examined practically in swine using one oil-in-water type adjuvant vaccine against swine pleuropneumonia. The vaccine was prepared from cell-free-antigen of *Actinobacillus pleuropneumoniae*, liquid paraffin, and several polyoxyethylene sorbitan and sorbitan oleates. Based on findings about safety in mice and emulsion stability, 2 vaccines containing either 11.25% or 6.25% emulsifier content were injected intramuscularly twice in swine, as the highest and lowest limits, respectively, within the practical range. All pigs showed temporary fever and malaise with anorexia for several days after each injection. The fever of the higher emulsifier content group took significantly longer to recover than the lower. Malaise also showed a similar tendency. On the other hand, antibody response was sufficiently induced with no significant difference between the 2 groups. Lowering the emulsifier content is a very simple but effective solution for mitigation of side effects without the reduction of adjuvanticity. For safe and high-quality oil-based adjuvant vaccines, not only antigen and base-oil, but emulsifier content must be optimized.

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Keywords: Oil-based adjuvant; Vaccine component; Emulsifier; Side effect; *Actinobacillus pleuropneumoniae*; Swine

1. Introduction

Oil-based adjuvant contributes to the enhancement of veterinary vaccine potency and has brought great benefits to the prevention of many infectious diseases. At the same time, it has often caused several side effects, such as inflammation, granulomatous lesions, and sterile abscesses at the injection site (Straw et al., 1985), fever, and anorexia (Tizard, 2000). These are usually caused by 3 major components of the vaccine: antigen, base-oil, and emulsifier (Stewart-Tull, 1995; Aucouturier et al., 2001). The side effects caused by the former 2 components have been investigated intensely, and an outline of both theoretical and practical methods for preparation of safer oil-based adjuvant vaccines was established based on intense investigations (Stewart-Tull, 1995; Chang et al., 1998; Whitehouse et al., 1974). On the other hand, few reports about the relation between vaccine emulsifier and side effects in clinically treated animals have been published, while many such reports have been published about experimental animals (Barile and Hardegree, 1970; Berlin and Wyman, 1970; Bollinger, 1970; Berlin, 1968; Hardegree and Kirschstein, 1968).

Many excellent adjuvant formulations for oil-based vaccine have been commercially available and have been applied to practical situations (Montanide® ISA series, Seppic; TiterMax® series, CytRx Corp.); however, the details of the type and content of emulsifier were rarely
disclosed in public. The emulsifier often tends to be added excessively (Otsuka et al., 1987) because it can stabilize the emulsification manufacturing process and make emulsion of the vaccine easier (Aucouturier et al., 2001). It has important effects not only on stability but also on biological activities, and plays a role in the side effects of the vaccine emulsion (Stewart-Tull, 1995). Therefore, investigators and manufacturers should provide the information about the type and content of emulsifier to the public to enable the production of more efficacious and safer vaccines.

We were interested in this problem and investigated the relation between emulsifier content and toxicity in mice experimentally, using one oil-in-water (O/W) type oil-based adjuvant vaccine against swine pleuropneumonia caused by Actinobacillus pleuropneumoniae (Oishi et al., 1997). Furthermore, we made a practical investigation of the influence of excess emulsifier on clinical signs in swine with the aim of decreasing the side effects caused by the vaccine components.

2. Materials and methods

2.1. Vaccine

Vaccine was prepared as an O/W-type emulsifying mixture of cell-free-antigen (CFA) derived from the supernatant of A. pleuropneumoniae serotypes 1, 2, and 5 (strains Y-1, G-4, and E-3, respectively), according to the reports by Oishi et al. (1995a, b, 1997). The O/W-type adjuvants were prepared from high-refined light liquid paraffin (Nikko Chemicals Co. Ltd., Tokyo, Japan) and an O/W emulsifier. The emulsifier consists of 4 highly refined polyoxyethylenesorbitan and sorbitan oleates: polysorbate 85, polysorbate 80, PEG-6 sorbitan oleate, and sorbitan oleate (Nikko Chemicals Co. Ltd., Tokyo, Japan). Fifty milliliters of the light liquid paraffin was individually mixed with various amounts of emulsifier: 50, 45, 40, 35, 30, 25, 20, or 10 g, or with no emulsifier (0 g). PBS was added to each of the emulsifier/oil mixtures to give a final volume of 100 ml of the 9 adjuvant preparations. Each of the preparations was emulsified using a homogenizer, T.K. ROBOMICS® (Tokushu Kika Kogyo Co., Ltd., Osaka, Japan) with CFA at a ratio of 25:75, respectively, to make O/W-type vaccine emulsion. The final 9 emulsifier contents in each vaccine were 12.50%, 11.25%, 10.00%, 8.75%, 7.50%, 6.25%, 5.00%, 2.50%, and 0.00% (weight per volume%).

2.2. Stability of vaccine emulsions

Stability was observed at 4°C for 2 years, which corresponds to the general storage temperature and period (Aucouturier et al., 2001) of the vaccine. Stability of each vaccine was defined as the emulsion remaining homogeneous, and instability as the emulsion separating into oil and water phases.

2.3. Animal experiments

All animal experiments were carried out according to the guidelines provided by the Animal Ethics Committee, Kyoto Biken Laboratories, Inc.

2.3.1. Abnormal toxicity of vaccines in mice

The test followed an abnormal toxicity test in official standard examinations for veterinary vaccines introduced by the Ministry of Agriculture, Forestry and Fisheries in Japan (2002). Groups of 10 5-week-old male ddY mice (Nippon SLC, Shizuoka, Japan) were injected intraperitoneally with 0.3 ml/dose of each vaccine emulsion. The mice were weighed every day for 7 days after injection. The fraction of mice who weight recovered to over their weights at the time of injection was defined as the rate of weight recovery, an indicator originally defined in this study.

On the basis of preliminary studies of both emulsion stability and this toxicity test, the set of 2 vaccines prepared using the 2 highest and lowest emulsifier contents within the practical range was used for the following clinical test.

2.3.2. Swine clinical test

Ten 30-day-old specific-pathogen-free (SPF) pigs that had been weaned at 24 days old and had no serological evidence of exposure to A. pleuropneumoniae were obtained from a commercial farm in Japan. They were divided into 2 groups: 5 pigs in one group were immunized with a vaccine containing the highest emulsifier content (High-E group), and the other pigs were immunized with vaccine containing the lowest emulsifier content (Low-E) within the practical range previously defined on the basis of emulsion stability and toxicity test. (See Section 3 for these content values.) The pigs in every group were housed in isolation rooms and fed commercial feed equally. They were immunized twice intramuscularly with 0.5 ml/dose of each vaccine at 4-week intervals. The clinical signs after immunization were observed during this experiment. Body temperature was statistically compared between the High- and Low-E groups. The injection sites were also observed for injection scar at slaughter. Sera were collected every week during observation for the following immunological assay.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The specific antibody response in serum against CFA of each of the 3 serotypes of A. pleuropneumoniae were measured as total-IgG ELISA titers. ELISA was performed on polycarbonate round-bottomed 96-well plates (Greiner Bio-One, Frickenhausen, Germany), according to the previously reported method (Oishi et al., 1995b). One hundred microliters of 200 µg/ml of CFA in carbonate buffer (pH 9.4) was added to each well of the ELISA plates, and the plates were incubated at 4°C overnight. The plates were washed 3 times with 350 µl of PBS containing 0.05% Tween-20 (Tween PBS). They were...
incubated at 37 °C for 1 h with 100 µl of serial dilutions of test sera in Tween PBS containing 5% bovine serum albumin. After 3 washes, 100 µl of horseradish peroxidase-conjugated anti-swine total-IgG (Cappel, CA, USA) diluted with Tween PBS was added. The plates were incubated at 37 °C for 1 h. After 5 washes, 100 µl of substrate solution consisting of 0.003% H2O2 and 10 mg/ml of o-phenylenediamine in phosphate citrate buffer (pH 5.6) was added to each well, and the plates were incubated at 30 °C for 30 min. Finally, 50 µl of 1 M-H2SO4 was added to each well to stop the enzymatic reaction. The absorbance of each well was read using an Auto Reader III (Sanko Junyaku Co. Ltd., Tokyo, Japan) at a wavelength of 492 nm. ELISA titers were determined as the reciprocal of the highest serum dilution giving an Abs492 ≥ 0.5.

2.5. Statistical analysis

The statistical significance of differences was determined from the means and standard deviations by Student’s two-tailed t-test for comparison of body temperature and antibody titer of swine test, and by paired two-tailed t-test for the weight recovery of mice and for the swine test. P values of <0.05 were considered significant. They were calculated using the Microsoft Excel 98 software package installed in Apple Macintosh Performa 6420 (G3) and PowerBook 2400c (G3) personal computers.

3. Results

3.1. Stability of vaccine emulsions

Seven vaccines containing 12.50–5.00% of emulsifier were all homogeneous just after preparation. On the other hand, those with 2.50% and 0.00% emulsifier separated into oil and water phases immediately after homogenization. After 10 days, vaccine containing 5.00% emulsifier separated. The other vaccines remained as homogeneous emulsions for over 2 years. Only stable vaccines with emulsifier contents ranging from 6.25% to 12.50% were tested in the following experiment for toxicity, as the initial candidates for clinical comparison in swine (Table 1).

3.2. Weight recovery change of mice after vaccine injection

The weight of all 6 groups significantly decreased the day after injection of each vaccine. Two days after injection, the weight began to gradually recover from the decreased level. However, the degrees of recovery were different among the groups (Table 2).

The group with 6.25% emulsifier content, which was the lowest emulsifier content of all 6 groups tested in this experiment, recovered a weight that was not significantly different from the starting weight on the 3rd day after injection. Six mice recovered their individual starting weights by the 4th day, 9 mice by the 5th day, and all 10 mice by the 6th day after injection. This group recovered its starting weight the earliest of all the groups. The groups with 7.50%, 8.75%, and 10.00% emulsifier content significantly recovered their starting weights on the 3rd day after injection. The number of mice recovering their starting weights in this group showed a tendency to be inversely proportional to the emulsifier content. The group with 11.25% emulsifier content significantly recovered weight on the 4th day after injection. In the group with 12.50% emulsifier content, which was the highest emulsifier content of all 6 groups, the average weight gradually recovered; however, 1 mouse died on the 3rd day after injection. All mice excluding the dead mouse in the 12.50% emulsifier group grew normally after recovering their weight.

Based on the above results and the regulation of abnormal toxicity test (Ministry of Agriculture, Forestry and Fisheries, 2002), the vaccine containing 12.50% emulsifier in this experiment was judged not to be acceptable as a safe practical vaccine, since one of the mice died due to the vaccine injection. Therefore, the set of 2 vaccines containing 11.25% as the highest and 6.25% as the lowest limits within the practical range of emulsifier content in this formulation (corresponding to High- and Low-E groups, respectively, defined previously in Section 2) were injected into the swine

<table>
<thead>
<tr>
<th>Emulsifier (% in vaccine)</th>
<th>Stability of each vaccine emulsion at 4 °C after preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h 1 day 10 days 1 month 6 month 1 year 2 years</td>
</tr>
<tr>
<td>0.00</td>
<td>sep. b sep. sep. n.t. n.t. n.t. n.t.</td>
</tr>
<tr>
<td>2.50</td>
<td>sep. sep. sep. n.t. n.t. n.t. n.t.</td>
</tr>
<tr>
<td>5.00</td>
<td>– – – – – – –</td>
</tr>
<tr>
<td>6.25</td>
<td>– – – – – – –</td>
</tr>
<tr>
<td>7.50</td>
<td>– – – – – – –</td>
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<tr>
<td>8.75</td>
<td>– – – – – – –</td>
</tr>
<tr>
<td>10.00</td>
<td>– – – – – – –</td>
</tr>
<tr>
<td>11.25</td>
<td>– – – – – – –</td>
</tr>
<tr>
<td>12.50</td>
<td>– – – – – – –</td>
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</tbody>
</table>

a Detailed composition of emulsifier in this study was described in Section 2.
b Abbreviations are as follows: sep., separated into oil and water phases; –, stable (homogeneous) and n.t., not tested.
groups, for clinical evaluation of the effect of emulsifier content.

### 3.3. Swine clinical test

All pigs grew normally during this experiment. The body weight increased without any significant differences between the 2 groups. Neither local swelling just after each injection, nor vaccine scars at slaughter were observed at the injection sites in the 2 groups (data not shown).

However, the following clinical signs were temporarily observed for several days after each injection and were analyzed with regard to the significance of differences between the 2 groups.

#### 3.3.1. Fever

The body temperature of both groups significantly increased 1 h after the 1st injection (Table 3). It reached the 41–42 °C range at the peak 6 or 12 h after the injection. The Low-E group took 2 days to completely recover to the normal body temperature before injection. On the other hand, the High-E group took 4 days to recover, which was significantly lower than the 2 days taken by the Low-E group. One pig in the High-E group (No. 97) did not recover by the 4th day after the 1st injection; it took 5 days to recover. No significant difference was observed between the peak heights of the fever in the 2 groups.

Similar symptoms were observed at the 2nd injection (Table 3). The body temperature significantly increased at 1 h, and reached a peak 6 or 12 h after the 2nd injection. The High-E group took 3 days longer than the Low-E group to recover completely to the normal temperature before injection. The body temperature of the High-E group was significantly higher than that of the Low-E group during the recovery from 18 h to 4 days after the 2nd injection (Table 3).

No other fever than the temporary fever described above was observed during this experiment (data not shown).

#### 3.3.2. Malaise with anorexia

All pigs showed depression with proneness 1 h after the 1st injection and stayed in this condition for 24 h. They did not show a good appetite during this period. Three pigs of the Low-E group recovered from the malaise after 36 h, and all 5 pigs recovered by the 2nd day after the 1st injection. In contrast, none of the pigs of the High-E group recovered from malaise after 36 h. Three pigs recovered by the 2nd day, 4 pigs by the 3rd day, and all 5 pigs by the 4th day after the 1st injection (Table 4).

Malaise with anorexia after the 2nd injection was similar to that after the 1st injection. The Low-E group recovered earlier than the High-E group (Table 4).

#### 3.3.3. Antibody titer

The ELISA titer against serotypes 1, 2, and 5 markedly increased after the 2nd injection, and remained high until slaughter. No significant difference between the High- and Low-E groups was observed in the ELISA titers of any serotype during this experiment (Fig. 1).

### 4. Discussion

The emulsifier content of 11.25% tested as the highest limit in the vaccine in this study is a popular content used for commercial O/W type vaccine and many other drug preparations (Otsuka et al., 1987; Oshima and Hirata, 1997). This emulsifier content allows the vaccine emulsion...
Table 3
The change of body temperature after injection of each of 2 vaccines in swine

<table>
<thead>
<tr>
<th>Injection No.</th>
<th>Group</th>
<th>Pig No.</th>
<th>Body temperature score after each injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before injection</td>
</tr>
<tr>
<td>1st injection</td>
<td>Low-E</td>
<td>91</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93</td>
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<td>94</td>
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<tr>
<td></td>
<td></td>
<td>95</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>High-E</td>
<td>96</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97</td>
<td>–</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>100</td>
<td>–</td>
</tr>
</tbody>
</table>

P value: <0.001 <0.01

P value was statistically calculated between High- and Low-E groups, according to the actual value of each body temperature before scoring.

Body temperature (°C) was scored as follows: –, $t \leq 40.0$; +, $40.0 < t \leq 40.5$; ++, $40.5 < t \leq 41.0$; ++++, $41.0 < t \leq 41.5$ and ++++; $41.5 < t$.

Table 4
Malaise with diminution of appetite after injection of each of 2 vaccines in swine

<table>
<thead>
<tr>
<th>Injection No.</th>
<th>Group</th>
<th>Pig No.</th>
<th>Malaise with anorexia after each injection a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before injection</td>
</tr>
<tr>
<td>1st Injection</td>
<td>Low-E</td>
<td>91</td>
<td>–</td>
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<td></td>
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<td>92</td>
<td>–</td>
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<td>95</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>High-E</td>
<td>96</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td>97</td>
<td>–</td>
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<td></td>
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<td></td>
<td></td>
<td>100</td>
<td>–</td>
</tr>
</tbody>
</table>

P value: <0.05 <0.05 <0.05 <0.001 <0.01 <0.001

P value was statistically calculated between High- and Low-E groups, according to the actual value of each body temperature before scoring.

Malaise with diminution of appetite (normal and +, malaise with anorexia).

* Score was represented as –, normal and +, malaise with anorexia.
to be stable and homogeneous more conveniently and reproducibly, regardless of the quality of antigen and base-oil, or the capacity of the instrument for emulsification. However, previous studies indicated the undesirable feature that the highest emulsifier content delayed recovery from several side effects that were typically caused by CFA of *A. pleuropneumoniae* as a gram-negative bacterial antigen (Oishi et al., 1997, 1995b; Straw et al., 1985; Tizard, 2000). The delay of side effects has a close relation with the emulsifier content of O/W type vaccine both directly and indirectly.

Excessive emulsifier contributes not only to reliable emulsification between the base-oil and antigen solution, but to the solubilization of aggregated toxins like haemolysins (Oishi et al., 1995a) and endotoxins derived from gram-negative bacterial antigen (Aucouturier et al., 2001; Tizard, 2000). The delay of side effects has a close relation with the emulsifier content of O/W type vaccine both directly and indirectly.

![Fig. 1. Comparison of swine specific antibody titers in serum against CFA of each of the 3 serotypes of *A. pleuropneumoniae* between High- and Low-E groups. The ELISA titers were determined as the reciprocal of the highest sample dilution giving an Abs₄₉₂ ≥ 0.5. They were plotted as the geometric mean values. Symbols are: High-E (■) and Low-E (○) groups.](image)

The excessive emulsifier temporarily induced the above-mentioned signs just after vaccine injection. The fever and malaise with anorexia may cause marked stress to animals even in good health on stable farms under SPF or equally well controlled conditions. Furthermore, the side effects may cause stress and induce latent infections more easily in animals which already have lowered resistance due to environmental factors and daily stresses, or in non-resistant piglets. The weaker animals may consequently show a decrease of daily gain in weight, or in the worst case, may occasionally die. Also, pregnant females may more frequently be provoked to abortion (Tizard, 2000) due to the stress caused by the excessive emulsifier content in such vaccines.

No remarkable relation was found between the emulsifier content and antibody response in this study. The adjuvanticity of O/W type vaccine is due to the condition of the oil droplets in the emulsion (Stewart-Tull, 1995; Cox and Coulter, 1997). The vaccine shows strong adjuvanticity when forming stable oil droplets, namely, a stable emulsion. The two vaccines used for comparison of the antibody response in the swine clinical test in this study were selected on the basis of stability first (Table 1). The results and the general theory of antibody response suggest that the stable vaccines will have equally sufficient adjuvanticity within the range of emulsifier content that allows formation of a stable emulsion.

Investigators and manufacturers have striven to produce more efficacious and safer vaccines. Especially, 2 components of the oil-based vaccine, antigen and base-oil, have been intensely investigated and their performance has been considerably improved. Improving these two components is effective for decreasing several side effects derived from these components, but does not make it possible to avoid all side effects. Reducing the emulsifier content to a minimum is also a very simple but essential solution for further decreasing side effects. Emulsifier should not be added so excessively beyond the necessary level as in the modern popular vaccines on the market, only for reasons of the convenience of the emulsification manufacturing process, emulsion stability, and easy injectability of the vaccine. In conclusion, all of these three vaccine components must be improved harmoniously with respect to the stability, efficacy, and safety of the emulsion, for the development of high-quality oil-based adjuvant vaccines.
Acknowledgements

We thank Aya Hasegawa, Akiko Haga, and Ikuko Yamamoto, three of the able assistants at Kyoto Biken Laboratories, Inc. in Kyoto, Japan, for assistance in carrying out animal experiments and collecting the experimental data on antibody titer. We are grateful to Kazuo Okamoto of Summit Medi-Chem. Ltd. in Tokyo, Japan and Kuniyoshi Mori of Nikko Chemicals Co. Ltd. in Tokyo, Japan, for providing a great many samples as well as useful information about adjuvant materials. We are greatly obliged to Dr. Shigeji Katayama of Kyoto Biken Laboratories for offering the opportunity to pursue this study. And we also thank Dr. Toshiaki Ohgitani, Dr. Shin-ichi Fukuyama, Dr. Morimasa Yamanaka, and Dr. Yukio Shimizu of Kyoto Biken Laboratories for valuable discussion and encouragement during this study.

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