The adjuvant effect of Corynebacterium ovis

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Summary: Data in this study showed that Corynebacterium ovis has a clear immunopotentiating effect when used as an adjuvant with several antigens including egg albumin, Salmonella typhimurium and foot and mouth disease virus, inoculated into guinea pigs. The optimal dose was found to be 400 mg of C. ovis mixed with antigen. However, a dose of 300 mg of C. ovis when mixed with incomplete Freund’s adjuvant was enough to stimulate a sustained potent immune response which was superior to that obtained with the complete Freund’s adjuvant.

Immunopotentiation is a topic in immunology that is attracting increasing interest. In this field, oil adjuvants have proved their superiority and Freund et al. (10) have shown that the addition of some bacteria, such as mycobacteria, to such oil adjuvants improves their immunopotentiating effect.

Current studies carried out in our laboratory on crystal violet treated C. ovis have clearly shown its capacity as a non-specific immune stimulant, capable of raising the resistance of sheep to artificial infection with potential pathogens, in a manner comparable with that produced by BCG (5, 6).

It was decided to test the formalin-killed C. ovis for its immunopotentiating ability when used as an adjuvant with inert antigens and killed microbial vaccines.

MATERIALS AND METHODS

Animals.

Guinea pigs: 630 healthy albino adult guinea pigs, weighing approximately 400-500 grams, were used in this study. The animals were put in equal

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separate groups of 30 for each vaccine formula. At 10-days intervals, 5 animals from each group were sacrificed and their sera were pooled for determination of antibody levels by the suitable serological tests.

**Antigens.**

The following antigens were used:

(a) *Egg albumin,* from Sigma Chemical Company, USA, was used as a soluble antigen.

(b) *Foot and mouth disease (FMD) virus vaccine* type O, a local Egyptian strain that was inactivated by acetyleneimine according to Brown and Crick (7). Each guinea pig was inoculated with 2 ml of $10^7$ TCID$_{50}$ per ml subcutaneously.

(c) *Salmonella typhimurium,* a local isolate that was killed with 0.5% formalin. The adjuvant was added to a bacterial suspension of $10^9$ cells per ml, and 2 ml of the mixture were injected subcutaneously into guinea pigs according to Hunter and Peek (12) and Bairey (1).

**Adjuvants.**

(a) *Corynebacterium ovis,* a local strain that was isolated from a native sheep, identified morphologically, biochemically and biologically (9) and stored freeze-dried at the Animal Health Research Institute, Dokki, Giza, Egypt, as a reference strain.

This strain was injected subcutaneously into guinea pigs to produce abscess formation at the site of inoculation for activation of the microorganism. Pus from the abscess was streaked on blood agar that revealed pure *C. ovis.* For quantitative cell production, *C. ovis* was plated onto heart infusion agar in Roux bottles, incubated for 48 hours and swabbed into saline. The cells were killed by 0.5% formalin, washed thrice, weighed and stored in saline at 4°C until use.

Crystal violet treated *C. ovis* is an attenuated strain, which was used in other studies on the same principles as BCG, with the purpose to have a good substitute for BCG to avoid tuberculin sensitivity.

(b) *Incomplete and complete Freund's adjuvants* were obtained from Difco Laboratories, Detroit, Michigan, USA.

**Methods.**

(a) The immune response against FMD and egg albumin was measured by the complement fixation test (CFT) after Traub and Pyl (19). End points were calculated according to Kärber (14).

(b) The tube agglutination test, according to Kauffmann (15), was used to determine quantitatively the *Salmonella*-specific antibodies in sera of vaccinated animals.
RESULTS

The studies presented here include four experiments:

1. The first experiment was designed to test *C. ovis* for its immunopotentiating activity when used with a standard antigen like egg albumin, and to determine the optimal dose leading to the maximal immune response. The results of this experiment are shown in Table I.

2. The second experiment aimed, by adding an oil base to *C. ovis*, to improve its immunopotentiating effect. Freund's incomplete adjuvant was chosen for that purpose, and was mixed with different concentrations of killed *C. ovis*. The resulting immune responses were compared with that elicited when using Freund's complete adjuvant as an immunopotentiator. The results of this experiment are shown in Table II.

3. The third and fourth experiments were designed to illustrate the adjuvant effect of *C. ovis* when used with FMD and *Salmonella* killed vaccines. The results of using *C. ovis* alone or *C. ovis* with incomplete Freund’s adjuvant are presented in comparison with complete Freund’s adjuvant in Tables III and IV.

DISCUSSION

By immunopotentiation is meant an increase in the intensity of the immune response, prolongation of its duration or even the development of a response to an otherwise non-immunogenic substance. There is a continuous search for such immunopotentiators or adjuvants to enhance the immune response of killed vaccines in the human and veterinary medical fields.

*C. ovis* has been extensively studied in our laboratory, and its properties as a non-specific immune stimulant have been proved. In this paper, we introduced it as an adjuvant giving some light on its immunopotentiating capacity (2, 3, 4).

From Table I, it is clear that the addition of formalin-killed *C. ovis* to egg albumin increased the resulting immune response of inoculated guinea pigs, both in its intensity and duration. The best response was obtained when using 400 mg of *C. ovis*, while higher and lower doses were less satisfactory. In other words, the resulting immune response is roughly proportional to the amount of *C. ovis* incorporated in the vaccine till a certain optimal concentration is reached, in this case 400 mg, beyond which some sort of a blockage effect holds.

In the second experiment, we thought of adding an oil base to *C. ovis* in an attempt to increase its immunopotentiating effect. For this purpose, incomplete Freund’s adjuvant was chosen. Results given in Table II show that the incorporation of only 300 mg of *C. ovis* with incomplete Freund’s
### TABLE I

*The immunogenic potentiating effect of various doses of C. ovis added to egg albumin*

<table>
<thead>
<tr>
<th>Types of vaccines</th>
<th>Reciprocal of endpoint dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 DPV</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>0</td>
</tr>
<tr>
<td>Egg albumin + C 200</td>
<td>0</td>
</tr>
<tr>
<td>Egg albumin + C 300</td>
<td>47.8</td>
</tr>
<tr>
<td>Egg albumin + C 400</td>
<td>12.0</td>
</tr>
<tr>
<td>Egg albumin + C 500</td>
<td>0</td>
</tr>
<tr>
<td>Egg albumin + complete Freund's</td>
<td>0</td>
</tr>
</tbody>
</table>

C stands for *C. ovis* and is followed by the dose used in mg.

DPV : Days post vaccination.

### TABLE II

*The immunogenic potentiating effect of various doses of C. ovis added to incomplete Freund’s adjuvant and egg albumin*

<table>
<thead>
<tr>
<th>Types of vaccines</th>
<th>Reciprocal of endpoint dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 DPV</td>
</tr>
<tr>
<td>Egg alb. + incompl. Freund’s</td>
<td>12.0</td>
</tr>
<tr>
<td>Egg alb. + incompl. Freund’s + C 200</td>
<td>63.1</td>
</tr>
<tr>
<td>Egg alb. + incompl. Freund’s + C 300</td>
<td>0</td>
</tr>
<tr>
<td>Egg alb. + incompl. Freund’s + C 400</td>
<td>3.0</td>
</tr>
<tr>
<td>Egg alb. + incompl. Freund’s + C 500</td>
<td>0</td>
</tr>
</tbody>
</table>

C stands for *C. ovis* and is followed by the dose used in mg.

DPV : Days post vaccination.
TABLE III
The adjuvant effect of C. ovis and Freund's adjuvants on the antibody response to foot and mouth disease virus (FMDV)

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>CF antibody titres log_{10}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10 DPV</td>
</tr>
<tr>
<td>FMDV</td>
<td>0.90</td>
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<tr>
<td>FMDV + C 300</td>
<td>1.20</td>
</tr>
<tr>
<td>FMDV + compl. Freund's</td>
<td>0</td>
</tr>
<tr>
<td>FMDV + incompl. Freund's + C 300</td>
<td>0.30</td>
</tr>
<tr>
<td>FMDV + S. typhimurium + C 300</td>
<td>0</td>
</tr>
</tbody>
</table>

C stands for C. ovis and is followed by the dose used in mg.
DPV : Days post vaccination.

TABLE IV
The adjuvant effect of C. ovis and Freund's adjuvants on the antibody response to Salmonella typhimurium

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Agglutination titre log_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 DPV</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>1.6</td>
</tr>
<tr>
<td>S. typhimurium + C 300</td>
<td>2.5</td>
</tr>
<tr>
<td>S. typhimurium + compl. Freund's</td>
<td>2.5</td>
</tr>
<tr>
<td>S. typhimurium + incompl. Freund's + C 300</td>
<td>2.5</td>
</tr>
<tr>
<td>S. typhimurium + FMDV + C 300</td>
<td>2.2</td>
</tr>
</tbody>
</table>

C stands for C. ovis and is followed by the dose used in mg.
DPV : Days post vaccination,
adjuvant was quite enough to produce the maximal immune response. It is also clear that the C. ovis-incomplete Freund's adjuvant mixture elicited a better response than did the incomplete Freund's adjuvant alone or even the complete adjuvant. In other words, formalin-killed C. ovis seems to be superior to killed mycobacteria in its immunopotentiating effect, on some antigens at least.

In the third and fourth experiments, we studied the use of C. ovis as an adjuvant with killed vaccines for two potential pathogens of veterinary interest, namely FMD virus and Salmonella typhimurium. The results obtained, as shown in Tables III and IV respectively, demonstrate clearly the stimulatory effect of C. ovis. It is clear that the best response can be obtained by using 300 mg of C. ovis alone as an adjuvant or when mixed with incomplete Freund's adjuvant. The response was superior to that obtained by Freund's complete adjuvant. As shown in the same tables, mixing Salmonella and FMD antigens together with the optimal dose of C. ovis resulted in a potentiation in the immune response to Salmonella at the expense of FMD. This may be explained according to the antigenic concurrence phenomenon (18).

From the above data, it is clear that formalin-killed C. ovis has a good immunopotentiating effect on a standard soluble antigen as well as bacterial and viral particulate antigens, which justifies its proposal as an adjuvant to killed vaccines. The organism can be used either alone or mixed with incomplete Freund's adjuvant, the optimal dose being 400 and 300 mg, respectively. It seems here that the waxy material in C. ovis structure (13) contributes efficiently to the stimulation of antibody production in a similar way and rather superior to the waxes of mycobacteria (Malcolm et al., 1979) associated with Freund's complete adjuvant. Besides, the use of C. ovis did not entangle risks of antigenic denaturation as seen by the use of Freund's complete adjuvant (20).

Moreover, the use of Freund's complete adjuvant may sensitize the host to react non-specifically when the animal is tuberculin-tested as an essential step in the measures for tuberculosis control. Stimulation of cross-reactivity to tuberculin by C. ovis has not been established. Studies are in progress to investigate the sensitivity of C. ovis-infected sheep and bovines to tuberculin.

Another advantage is the specific immunization provoked also by C. ovis adjuvant against Corynebacterium ovis infection in sheep, cattle and buffaloes, taking into account that immunity to C. ovis is mainly cellular (11, 8), and that there is no reliable serological test until now for its diagnosis (17).

Finally, we think that a better immune response might be obtained if the principal adjuvant component of C. ovis could be isolated, identified and used in a pure form.

* * *

Résumé : Selon les résultats de cette étude, Corynebacterium ovis, utilisé comme adjuvant avec divers antigènes, dont l’albumine d’œuf, Salmonella typhimurium et le virus aphteux, exerce un net effet stimulant de l’immunité après inoculation aux cobayes. Les auteurs ont constaté que la dose optimale de C. ovis, en mélange avec un antigène, était de 400 mg. Toutefois, une dose de 300 mg de C. ovis additionnée d’adjuvant incomplet de Freund s’est montrée suffisante pour induire une réponse immunitaire puissante et soutenue, supérieure à celle obtenue en utilisant l’adjuvant complet de Freund.

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REFERENCES


