Enzyme-Linked Immunosorbent Assay for Evaluation of Immunity in Mice Vaccinated with Blackleg Vaccine

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Clostridium chauvoei is the causative agent of blackleg in cattle and causes wound infections in cattle, sheep, pigs and occasionally horses. Blackleg vaccine prepared from formalinized whole culture is used widely in enzootic areas for prevention of the disease.

To assay blackleg vaccine, such serological tests as agglutination [5], complement fixation [3] and indirect hemagglutination [9] have been reported. These tests, however, are not specific enough to evaluate the immunogenic properties of culture filtrate contributing much to the efficacy of the vaccine [4, 8], since these methods employ C. chauvoei cells or cellular components only as antigen.

This paper deals with application of enzyme-linked immunosorbent assay (ELISA) using an antigen obtained from the culture supernatant for evaluation of the immunogenicity of the vaccine in mice.

For ELISA, three antigens were prepared. C. chauvoei strain C6H was grown in VL medium containing 0.2% glucose [1]. After incubation for 3 days, the culture was inactivated with 1% formalin. Bacterial cells were removed by centrifugation and the supernatant was divided into two parts. One part was concentrated 200 times by ultrafiltration through PM-10 membrane (Amicon Co., USA); this concentrate was designated as CC-1 antigen. The second part was precipitated at 0.3 (CC-2) and 0.3-0.7 (CC-3) saturation of ammonium sulfate. Each precipitate was dialyzed against phosphate buffered saline (PBS).

Two kinds of C. chauvoei antisera were prepared in guinea pigs (PG) by injection of washed bacterial cells or a cell-free supernatant of a blackleg vaccine.

Three antigens, CC-1, CC-2 and CC-3, were examined for their reactivities to the antisera by indirect-ELISA [10]. Serial dilutions of each antigen in 0.05 M carbonate buffer (pH 9.5) were coated on polystyrene micro-ELISA plates (Dynatech, West Germany), which were kept standing overnight at 4°C. After washing with 0.15 M NaCl containing 0.02% Tween 20, adequate dilutions (100 μl) of the GP antisera were added to the wells and the plates were incubated for 15 min at room temperature. Then, the wells were sensitized with Anti-GP IgG rabbit serum and peroxidase-conjugated antirabbit IgG prepared by the method of Nakane [7]. As diluent for sera, PBS containing 1% bovine serum albumin and 0.02% Tween 20 was used. After washing, 100 μl of substrate (o-phenylenediamine dihydrochloride 12 mg, 0.1 M citric acid 15 ml, 0.2 M Na2HPO4 15 ml and H2O2 3 μl) was added and the plates were incubated for 5 min in dark. At the end of incubation, 2 N H2SO4 (100 μl) was added to stop the reaction. Optical density (OD) of each well was read at 492 nm. Each sample was tested in three wells and the arithmetical mean OD value was recorded as the titer.

The reactivities of the three antigens to the antisera are shown in Fig. 1. All antigens examined showed high reactivities against anti-C. chauvoei culture supernatant serum, irrespective of antigen concentration. There was, however, a marked difference in their reactivities against anti-cell serum from those against the anticulture supernatant. Although slightly lower OD values were observed with CC-2 antigen against anti-cell serum, the differences were much smaller than those of CC-1 and CC-3 antigens. From these results, CC-2 antigen was considered to be an appropriate antigen and was selected for titration of the antibodies in mice produced by blackleg vaccine.

Active protection tests were carried out in guinea pigs and mice. Washed bacterial cells and supernatant fluid were prepared from a blackleg vaccine by centrifugation as the immunogens for

guinea pigs. Thirteen guinea pigs (Hartley, male, 250 g) were inoculated subcutaneously with the cell suspension (1 ml) twice at a 7-day interval. Groups of 5 and 10 guinea pigs were given two or three successive subcutaneous doses of the supernatant fluid (1 ml) at 7-day intervals. Two weeks after the final injection, all animals were challenged intramuscularly with about 100 spores of C. chauvoei strain Okinawa, which is used for vaccine production in Japan, with 0.2 ml of 2.5% CaCl₂. Blood samples were collected before the first injection and the challenge.

In an experiment in mice, ICR mice (female, 5 weeks old) were divided into six groups of 10 each. Group I were given four successive intraperitoneal doses of the vaccine (0.25 ml) at 3-day intervals, and group II received three doses of the vaccine. In addition, groups III and IV were inoculated four times with heat-treated (100°C, 10 min) vaccine and with formalinized medium, respectively. The other two groups, V and VI, were inoculated respectively with physiological saline and CC-2 antigen (100 μg/ml) in the same way as were group I. Ten days after the final injection, each mouse was challenged intramuscularly with about 100 spores of the strain Okinawa with 0.2 ml of 3% CaCl₂. Blood samples were collected from the eye before the first and the third injections and the challenge, except for group II. Mice of group II were bled before the first and the second injections and the challenge.

Antibody responses of these guinea pigs and mice were examined by ELISA using CC-2 antigen at a concentration of 30 μg/ml. These sera were diluted 100-fold for titration. For mouse sera, peroxidase-conjugated anti-mouse immunoglobulin (IgA+IgG+IgM, Cappel, USA) was used at a 1:2,000 dilution.

As shown in Table 1, six of 13 guinea pigs immunized with the cells survived the challenge. Most survivors showed higher OD values than the dead. All guinea pigs having received two injections of supernatant died after the challenge. The survival ratio and the mean OD value, however, increased significantly by an additional injection. Although there was no significant difference in the mean OD value between the survivors and the dead since the OD values of survivors varied in a wide range, none of the dead showed a high OD value.

As in Fig. 2, the OD value of mice in this assay was highly correlated with the protective poten
cy. The OD values of the mice of groups I, II and VI increased gradually during the course of immunization, and these mice resisted C. chauvoei infection. On the contrary, all sera collected from the mice of groups III, IV and V continuously showed low OD values until the challenge, and no protection developed in these mice. These results indicate that the protective antibodies against C. chauvoei infection induced by the vaccine can be specifically detected by this assay.

To evaluate the efficacy of the vaccine, antibodies against not only the cellular components but also the extracellular components should be detected, since the protective antigens of C. chauvoei are carried by both the cells and the culture supernatant fluid [4, 8]. The cellular antigens are released easily into culture fluid by autolysis after cultivation for 48 hr [6], and such soluble protective antigens as hemolysin are precipitated from culture filtrate with ammonium sulfate at 0.4 saturation [4]. In the present study, CC-2 antigen, obtained by precipitation from culture supernatant at 0.3 saturation of ammonium sulfate, showed a high reactivity against the
ELISA FOR *C. CHAUVOEI* INFECTION

Table 1. Survival ratios and ELISA titers in the active protection test in guinea pigs

<table>
<thead>
<tr>
<th>Immunogens</th>
<th>Injection time</th>
<th>Survival ratio(^3)</th>
<th>OD value in ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Survivors</td>
<td>Dead</td>
</tr>
<tr>
<td>Cells from</td>
<td>2</td>
<td>6/13</td>
<td>0.36±.46(^b)</td>
</tr>
<tr>
<td>vaccine</td>
<td>2</td>
<td>0/5</td>
<td>0.27±.15</td>
</tr>
<tr>
<td>Supernatant</td>
<td>3</td>
<td>6/10</td>
<td>0.67±.23(^c)</td>
</tr>
<tr>
<td>fluid of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vaccine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Survival ratio represents: Number of animals having survived 5 days after challenge/Number of animals challenged. b) and c) There was no significant difference between survivors and the dead at P>0.05. d) There was significant difference at P<0.01.

![Fig. 2. Antibody responses and survival ratios in the active protection test in mice.](image)

Fig. 2. Antibody responses and survival ratios in the active protection test in mice. Mice of each group were immunized with blackleg vaccine (groups I & II), heat-treated vaccine (group III), medium (group IV), physiological saline (group V) and CC-2 antigen (group VI). The survival ratio was obtained by the following equation; (Number of mice having survived for 7 days after challenge/Number of mice challenged) \times 100 (%)..

Antisera to both the cells and culture supernatant. Moreover, this antigen showed an effective protective antigenicity like that of the vaccine, suggesting that it contains both cellular and extracellular protective antigens of *C. chauvoei*. Although the CC-2 antigen used was not characterized, the present study demonstrated that ELISA using culture fluid antigen can be used to titrate the protective immunity induced by blackleg vaccine in mice. There was a difference in the OD value between survivors immunized with the cells and those with the supernatant. Both qualitative and quantitative analyses of the protective antigens included in CC-2 antigen seem necessary.

REFERENCES

要約

酵素抗体法による気腫疽ワクチン接種マウスの抗体検出（短報）：浜岡隆文・森康行・寺門誠致（農林水産省家畜衛生試験場）--気腫疽ワクチン免疫マウスの血中抗体の検出法として酵素抗体法（ELISA）を検討した。抗原は気腫疽菌培養上清の30％飽和硫酸塩析出物が用いていた。感染防御試験におけるマウスの感染防御能の獲得とELISA抗体価の上昇とは良く相関していた。