Use of Acetone-Dried Vaccines for Preparing Capsular Antisera Against the *Klebsiella* Group and the Lyophilization of *Klebsiella* Cultures

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Capsular antisera against the 72 types of *Klebsiella* have been prepared in rabbits by using an acetone-dried vaccine. It was shown that encapsulated cells dried with acetone and ground to a fine powder with a mortar and pestle retain their capsules. Antisera obtained from rabbits vaccinated with these vaccines had quellung titers ranging from 1:16 to 1:64. The dried vaccine was stable after storage for over 2 years at room temperature. Encapsulated cultures lyophilized in the presence of 5% sucrose, 5% sodium glutamate, and 5% polyvinylpyrrolidone remained viable and retained their capsules after storage for 10 months at room temperature.

At the present time, 72 capsular types of the *Klebsiella* group have been identified on the basis of the quellung (Neufeld) reaction by investigators using antiserum specific for each type (1, 2, 5). In the past, antisera against these organisms have been prepared by using fresh formalized cultures as vaccines (4). This type vaccine has two disadvantages. (i) A fresh vaccine must be prepared for each inoculation. (ii) Stock cultures of some strains tend to become mixtures of encapsulated and nonencapsulated cells or lose their capsules, and other strains have a tendency to form large capsules or to become highly mucoid. When antisera against a large number of types have to be prepared, these disadvantages become very important with regard to time and the quality of antisera obtained.

Edwards and Fife (1) described the preparation of a dried alcohol-treated vaccine that produced good antisera against the *Klebsiella* group. However, they used a formalized broth vaccine for preparing most of their antisera.

Antiseras against many of the *Klebsiella* types also contain antibodies against heterologous types and must be absorbed to remove these crosses. Since the heterologous cells used for absorption must also be encapsulated, problems of maintaining stock cultures and selecting colonies are encountered that are similar to those involved in the preparation of a vaccine. This same situation is also encountered in the preparation of antigens used for evaluating antisera since freshly prepared encapsulated cultures must be used (1). The availability of lyophilized stock cultures in which most of the cells retain their capsules would help resolve these latter problems; this would be particularly applicable to authenticated stock cultures used for the preparation and evaluation of capsular antisera.

**MATERIALS AND METHODS**

_Cultures._ Authenticated cultures of the 72 serotypes of *Klebsiella* were obtained from W. H. Ewing and Mary A. Fife of the Enteric Unit, Laboratory Division, National Communicable Disease Center (NCDC).

_Preparation of vaccines._ Stock cultures maintained on blood-agar base medium were transferred to Worfel-Ferguson (6) agar plates and incubated overnight at 37 C. Isolated colonies were checked for encapsulated cells in a wet mount containing India ink; if found satisfactory, the colonies were transferred to Worfel-Ferguson agar slants and incubated overnight at 37 C. These latter cultures were again checked for encapsulated cells and also for type specificity by using homologous antiserum. Encapsulated mucoid cultures were not used because animals vaccinated with dried antigens of these cultures did not give a satisfactory serological response. When preparing the vaccine, we suspended the growth from a Worfel-Ferguson agar slant in 10 ml of sterile 0.85% NaCl and then transferred 3 ml to each of several large petri plates containing 1.5% tryptose (DiBio), 1.5% Trypticase (BBL), 0.3% yeast extract, and 2% agar. The growth from overnight cultures was harvested with 0.5% formalized water, and to
this suspension an equal volume of acetone was added. The cells were sedimented at 13,000 × g for 30 min, and the supernatant was discarded. The sediment was washed three times with acetone to ensure the removal of all of the water. Finally the cells were resuspended as a thick slurry in acetone and air-dried at room temperature. After the cells were dried, they were ground to a fine powder in a sterile mortar and pestle and stored at room temperature in screw-capped tubes.

Vaccination schedule. New Zealand white rabbits were inoculated intravenously (iv) with weighed amounts of dried vaccine suspended in 1.0 ml of 0.85% NaCl. The amount of vaccine given for each inoculation and the schedule of inoculations are given in Table 1. We test-bleed the rabbits 5 to 7 days after the last inoculation and then used freshly prepared encapsulated cells to determine the quellung titer. If quellung reactions were obtained at serum dilutions of 1:8 or higher, the rabbits were exsanguinated. Rabbis from which we failed to obtain a satisfactory titer were rested for 4 weeks and the inoculation series was repeated.

Evaluation of antisera. Antiserum against each capsular type was evaluated by the slide agglutination test and by the quellung reaction. Antigen for the slide agglutination test was prepared by harvesting the growth from an overnight Worfel-Ferguson agar slant with 0.5% formalized saline and adjusting the density to a no. 3 McFarland standard. For the quellung reaction, the slide antigen was diluted with 0.5% formalized saline to contain 10 to 20 organisms per oil immersion field. Fresh antisera were prepared each day. Both the quellung and agglutination tests were carried out by mixing one drop of antigen suspension with one drop of antiserum. As a control, one drop of saline was mixed with one drop of antigen suspension. Each antisera was tested undiluted by the slide test and by the quellung reaction against antisera of all 72 capsular types. Serial twofold dilutions of the antisera were then tested by the quellung reaction against each antigen reacting with the undiluted antisera to determine the homologous and heterologous titer.

Lyophilization of stock cultures. Initially studies were made on the viability of lyophilized cultures of capsular types 2 and 10 which had been suspended in one of the following media before lyophilization: (i) double strength nutrient broth (NB); (ii) 10% skim milk (SM); and (iii) a mixture of 5% sucrose, 5% sodium glutamate, and 5% polyvinylpyrrolidone (PVP). Encapsulated cultures were grown overnight on Worfel-Ferguson agar slants, suspended in the desired lyophilizing medium, and dispersed in 0.2-ml amounts into lyophil vials. A chamber type freeze-dryer equipped with an adjustable stopping plate and a controlled shelf temperature was used. The temperature of the shelf was lowered to −50°C, and the lyophil vials were placed directly on the shelf for 2 hr to ensure complete freezing of the culture. Lyophilization was carried out under a vacuum of 30 µ of mercury and a shelf temperature of 0°C for 18 to 24 hr. When lyophilization was complete, the vials were sealed under vacuum with butyl rubber stoppers previously placed in the mouth of each vial. Lyophilized cultures were stored at room temperature.

Viability studies on the lyophilized cultures consisted of plate counts of serial 10-fold dilutions on Worfel-Ferguson agar plates. The presence or absence of encapsulated cells was also determined microscopically in a wet mount with India ink.

RESULTS

India ink preparations of acetone-dried vaccines resuspended in 0.85% NaCl showed that the cells retain their capsules although they are smaller than the capsules observed in the original culture (Fig. 1). The decrease in size of the capsule of a dried vaccine is an advantage since cells having large capsules do not make as good a vaccine as cells having a medium size capsule (1) and therefore colony selection for capsular size is not necessary.

All of the rabbits vaccinated with the dried vaccine gave a satisfactory serological response. The homologous quellung titers for the individual capsular types varied from 1:16 to 1:64. Antiserum against 45 of the types had heterologous quellung titers of 1:8 or less. Antiserum against six types had heterologous titers of 1:16 to 1:32, whereas 20 of the antiserum had no demonstrable crosses. These results are comparable to those obtained by Edwards and Ewing (3) with Formalin-killed vaccines. Rabbits vaccinated with 6 of the 72 vaccines failed to give a satisfactory quellung titer after the first series of inoculations. All of the latter rabbits gave a satisfactory response when the inoculation schedule was repeated after a rest period of 4 weeks.

Stability of acetone-dried vaccines. Acetone-dried vaccines of three capsular types (types 2, 7, and 10) stored at room temperature for over 2 years were used to prepare a second lot of antisera. The same vaccination schedule previously used was followed. All of the rabbits gave a satis-

### Table 1. Vaccination schedule for the preparation of capsular antisera in rabbits against the Klebsiella group by using acetone-dried vaccines

| Day of inoculation | Ant of vaccine per inoculation |
|--------------------|-------------------------------|
| 0                  | 0.5 mg                        |
| 3                  | 1.0 mg                        |
| 7                  | 2.0 mg                        |
| 10                 | 3.0 mg                        |
| 14                 | 4.0 mg                        |
| 17                 | 5.0 mg                        |

* Desired weight of vaccine suspended in 1.0 ml of 0.85% NaCl. All inoculations given iv.
factory serological response after the first series of inoculations. A comparison of the first and second lots of antisera prepared with these vaccines is shown in Table 2. The homologous quellung titers of the two lots of antisera prepared with each of the vaccines are comparable. The heterologous quellung titers appear to be somewhat lower in the second lot of antiserum than in the first lot. Thus, it would appear that acetone-dried vaccines of encapsulated *Klebsiella* are as stable as the modified Roschka vaccine used by Edwards and Ewing (3) for preparing antisera against the somatic antigens of the Enteric group.

**Viability of lyophilized cultures of the Klebsiella group.** Viability counts on lyophilized cultures of capsular types 2 and 10 suspended in three different media are shown in Table 3. There was a rapid drop in the number of viable cells of both types suspended in NB and SM. After 110 days of storage in the latter media, cultures of both types were nonviable. The viable count of cultures suspended in the PVP medium dropped over 2
logs in the first 24 hr after lyophilization but remained essentially unchanged over the next 10 months. Approximately 75\% or more of the isolated colonies from cultures lyophilized in PVP contained encapsulated cells. Because of these results, the other 70 capsular types were suspended in PVP for lyophilization. A spot check on the viability of the latter cultures has yielded the same results as those obtained with capsular types 2 and 10.

**DISCUSSION**

To our knowledge this is the first report on the use of acetone-dried vaccine for preparing capsular antisera against the *Klebsiella* group. Edwards and Fife (1) used a dried *Klebsiella* vaccine prepared with absolute alcohol and found it stimulated a serological response in rabbits comparable to that of a Formalin-killed vaccine. However, they felt the dried vaccine was more difficult to prepare and therefore used the Formalin-killed vaccine for preparing their antiserum. We also found the alcohol-treated vaccine somewhat difficult to prepare because the more viscous cultures were difficult to sediment during centrifugation and washing. This problem was not encountered with the acetone-treated cells.

The major advantages of the acetone-dried vaccine are the elimination of the necessity of preparing a fresh vaccine for each animal inoculation and the stability of the vaccine during storage. It can also be used as a slide agglutinating antigen for determining the agglutinin titer of sera from test bleedings; however, since this titer does not always correlate with the quellung titer, we did not use it routinely. Quellung reactions could be observed with the dried antigen after it was resuspended in saline but they were more difficult to interpret than those in fresh formalized cells; the latter, therefore, were used for this purpose.

The stability of the dried vaccine should be particularly valuable to laboratories that find it necessary to prepare new lots of antiserum from

**TABLE 2. Comparative quellung titers of *Klebsiella* antisera prepared with aged (2 years) and unaged acetone-dried vaccines**

| Antigen type | Type 2 antisera | Type 7 antisera | Type 10 antisera |
|--------------|-----------------|-----------------|------------------|
|              | Lot 1b          | Lot 2b          | Lot 1            | Lot 2            |
| 2            | 64a             | 64              | U                | 4                |
| 5            |                 |                 | 8                | 4                |
| 6            | 2               | 2               | 8                | 4                |
| 7            | 2               | 2               | 8                | 4                |
| 8            |                 |                 | 8                | 4                |
| 10           |                 |                 | 8                | 4                |
| 13           | U               |                 | U                | 2                |
| 20           | U               |                 | U                | 2                |
| 25           |                 | U               |                 | 2                |
| 26           |                 | U               |                 | 2                |
| 27           |                 |                 |                 | 2                |
| 30           |                 |                 |                 | 2                |
| 45           | 2               |                 |                 | 4                |
| 51           |                 | U               |                 |                 |
| 54           |                 | 4               |                 |                 |
| 56           |                 | 4               |                 |                 |
| 61           |                 |                 | U               | 2                |
| 69           | 8               |                 | U               | 2                |

* Types not listed were negative in the quellung reaction.
* Lot 2 antisera of each type was prepared with the acetone-dried vaccine used to prepare lot 1 after storage for 2 years at room temperature.
* Reciprocal of the highest serum dilution giving a positive quellung reaction. U, positive quellung reaction with undiluted serum only.

**TABLE 3. Viability of lyophilized *Klebsiella* cultures**

| Time (days) | Viable plate counta |
|-------------|----------------------|
|             | Type 2               | Type 10               |
|             | NBb                  | SM                    | PVP                  | NB                  | SM                    | PVP                  |
| 0           | $130 \times 10^8$    | $200 \times 10^4$    | $270 \times 10^9$    | $10^{10}$           | $500 \times 10^{10}$ | $130 \times 10^{10}$ |
| 1           | $50 \times 10^7$     | $30 \times 10^7$     | $68 \times 10^7$     | $<10^5$             | $<10^5$              | $80 \times 10^9$     |
| 14          | $56 \times 10^4$     | $10^4$                | $60 \times 10^7$     | $<10^4$             | $<10^4$              | $28 \times 10^4$     |
| 20          | $<10^4$              | $<10^4$              | $85 \times 10^7$     | $<10^4$             | $<10^4$              | $90 \times 10^4$     |
| 110         | NDc                  | ND                    | $46 \times 10^7$     | ND                  | ND                    | $100 \times 10^4$    |
| 308         | ND                   | ND                    | $58 \times 10^7$     | ND                  | ND                    | $81 \times 10^4$     |

* Average of duplicate plate counts on Worfel-Ferguson agar (6).
* NB, double strength nutrient broth; SM, 10% powdered skim milk; PVP, mixture of 5% sucrose, 5% sodium glutamate, and 5% polyvinylpyrrolidone.
* ND, not done.
time to time. One would expect this type of vaccine to come as near as possible to giving serological reproducibility from lot to lot of antisera, considering the variations of serological response obtained with individual animals.

The usefulness of acetone-dried vaccines for preparing antisera against other encapsulated organisms is currently under investigation. To date, dried preparations of Bacillus anthracis, Pasteurella pestis, and Streptococcus pneumoniae cells have been prepared. Morphologically all of these organisms retained their capsules after they were dried and resuspended in saline.

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