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Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium

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ABSTRACT

CHAMBERLAIN, ROBERT E. (The National Drug Co., Swiftwater, Pa.). Evaluation of live tularemia vaccine prepared in a chemically defined medium. Appl. Microbiol. **13**:232–235. 1965.—A chemically defined medium was prepared which adequately supported growth of a vaccine strain of *Pasteurella tularensis*. This medium differed from those previously described in: (i) concentration of components, (ii) a requirement for calcium pantothenate to obtain increased growth, and (iii) a low initial *p*H. Varying the concentration of individual components up to 10 times the standard amount did not increase the viable population or affect dissociation. The vaccine strain grown in this chemically defined medium, although lower in viable population, appears to retain its identity and to be equal in potency to that prepared by the conventional method. This preliminary study indicates the potential utility of this medium as a basis for controlled studies of a live bacterial vaccine in terms of growth characteristics, dissociation, virulence, and immunogenicity.

Recently, Eigelsbach and Downs (1961) produced a live tularemia vaccine, which has proved effective in both animals and man. The method of preparation, however, employs three distinct types of media prepared from natural products and six serial transfers. Because of the variations in lots of media and their potential effect upon dissociation, the preparation of the final product requires strict control.

In the past few years, certain chemically defined media (Mager, Traub, and Grossowicz, 1954; Traub, Mager, and Grossowicz, 1955; Nagle, Anderson, and Gary, 1960) have been successfully employed for the growth of various strains of *Pasteurella tularensis*. Use of a medium of this type facilitates controlled study of the factors affecting growth, dissociation, virulence, and immunogenicity. The results obtained in a preliminary study of this possibility are presented.

MATERIALS AND METHODS

Strains. Lyophilized stock cultures for use in this study were prepared from the live vaccine strain (LVS) kindly supplied by H. T. Eigelsbach. For challenge studies, *P. tularensis* strain SCHU S4 was employed. The LD_{50} was determined by the method of Reed and Muench (1938).

Preparation of media. The standard vaccine media, peptone-cysteine-agar (PCA) and modified casein partial hydrolysate (MCPH), and the gelatin-saline diluent were prepared according to Eigelsbach and Downs (1961). The components of the chemically defined medium (CDM) as finally adopted are listed in Table 1. All components except KH_2PO_4 and K_2HPO_4 were prepared as individual solutions. The *p*H of the complete medium was adjusted to 6.2 to 6.4 with 3 N HCl and sterilized by filtration through an ultrafine sintered-glass filter. CDM agar was prepared by adding agar to CDM liquid to give a final agar concentration of 1%.

Preparation of vaccine. Vaccine for the following studies was prepared simultaneously in both complex and chemically defined media according to the method of Eigelsbach and Downs (1961).

A lyophilized stock culture was first reconstituted with distilled water. Streak plates were prepared on both PCA and CDM. After 4 days of incubation at 37 C, colonial morphology (Eigelsbach and Downs, 1961) was recorded, and growth was transferred to PCA and CDM slants. Slants were incubated for 24 hr, and transfers were made to a second set of slants. Growth from the second slants was harvested (24 hr later) in 10 ml of gelatin-saline, and 5-ml quantities were transferred to 250-ml Erlenmeyer flasks containing 25 ml of MCPH or CDM liquid media. The flasks were incubated with continuous shaking for 18 hr. Quantities (20 ml) of the first liquid cultures were then transferred to 1-liter flasks containing 200 ml of either medium. These were incubated with shaking for 18 hr, and 25-ml quantities were transferred to 2-liter flasks containing 300 ml of either medium. These were also incubated for 18 hr with continuous shaking. The third liquid cultures were used in the subsequent studies. Viable counts were performed according to Downs et al. (1947).

Mouse virulence and immunogenicity. The virulence in mice of vaccine prepared in MCPH

and in CDM was compared by the following method.

Female CFW mice (18 to 20 g) were injected subcutaneously in the inner aspect of the hind leg with serial 10-fold dilutions of vaccine over a range of 10^2, 10^4, 10^6, and 10^8 viable organisms per 0.2-ml dose. Sixty mice were injected with each dilution, and 60 additional mice received diluent only (gelatin-saline) as control. The number of deaths was recorded daily for a 15-day period. All dead mice were autopsied, their gross pathology was observed, and segments of spleen and liver were removed and streaked on GCB plates. At the end of the observation period, the per cent virulence was calculated for each dose level and the average virulence was determined. The survivors from each dose level were challenged subcutaneously with *P. tularensis* SCHU S4. The challenged mice were observed for an additional 20 days, after which the per cent protection was determined for each challenge dose level.

Guinea pig virulence and skin reaction. The virulence of the vaccines prepared by the two methods was determined by subcutaneous injection of 350-g Hartley strain guinea pigs with 10^7, 10^8, and 10^9 viable organisms per 0.2-ml volume from third broth cultures. The pigs were observed for 15 days after injection.

Skin tests were performed by placing two drops of a 1:100 dilution of the third broth culture on the shaved surface of the thigh and lightly puncturing the skin through the vaccine 60 times. The pigs were observed over a 15-day period for typical reaction involving inflammation, followed by pustule formation, scab formation, and sloughing.

TABLE 1. *Composition of chemically defined medium**

Ingredient	Amt (mg/100 ml)
L-Arginine (free base)	40
L-Aspartic acid	40
L-Cysteine HCl	20
L-Histidine (free base)	20
DL-Isoleucine	40
L-Leucine (methionine-free)	40
L-Lysine (mono HCl)	40
DL-Methionine	40
L-Proline (hydroxy-L-proline-free)	200
DL-Serine	40
DL-Threonine (allo-free)	200
L-Tyrosine	40
DL-Valine	40
Spermine phosphate	4
Thiamine HCl	0.4
DL-Calcium pantothenate	0.2
Glucose	400
NaCl	1,000
$MgSO_4 \cdot 7H_2O$	13.5
$FeSO_4 \cdot 7H_2O$	0.2
KH_2PO_4	100
K_2HPO_4	100

* All ingredients except cysteine HCl, glucose, and salts were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; cysteine HCl was obtained from Merck & Co., Inc., Rahway, N.J., and glucose and salts were obtained from J. T. Baker Co., Phillipsburg, N.J., as reagent grade.

RESULTS

Growth. In preliminary tests with "basal medium B" (Traub et al., 1955), poor or erratic growth of the vaccine strain was obtained. It was later established that growth could be obtained consistently, provided the *p*H of the medium was reduced from 6.8 to 6.2–6.4. Further tests indicated that greater growth could be obtained if Ca pantothenate was added, and the concentration of each component was increased to twice the original amount (Table 1). This medium routinely produced a viable population of 15×10^9 to 20×10^9 organisms per milliliter compared with 30×10^9 to 35×10^9 organisms per milliliter in the complex medium. Alteration of inoculum size, medium volume, or incubation time did not increase growth.

With the above defined medium as a standard, an attempt was made to determine the optimal concentration of each ingredient by adding various concentrations of each component in turn to the standard medium containing all other ingredients at the standard concentration. The concentrations used ranged from 0.25 to 10 times the standard. No increase in growth over that of the standard was obtained with any of the concentrations employed. Certain ingredients (NaCl, K salts, arginine, aspartic acid, methionine, and proline) were found to be inhibitory at the higher concentrations. Comparison tests of the standard medium with that of Nagle et al. (1960) gave equivalent growth.

Morphology. The vaccine strain employed in this study generally contained two distinct colonial types, i.e., "blue" (immunogenic) and "gray" (nonimmunogenic). The "gray" type usually constituted less than 1% of the total population. Dissociation of the culture toward an increasing proportion of the nonimmunogenic type is often a problem during serial transfer, unless continuous quality control is exercised. This is due, among other things, to variations in the complex undefined media employed. With this in mind, it seemed of interest to determine whether changes in the proportions of the two colonial types during serial transfer could be correlated with varying concentrations of individual ingredients in the chemically defined medium. This study was done in conjunction with the tests for optimal growth discussed in the previous section.

The results indicated no significant changes in the proportions of "blue" or "gray" types with any of the ingredients tested. Preliminary tests in which 10 serial transfers were made in the standard chemically defined medium did show, however, slightly less variation than similar tests with MCPH media prepared with different lots of ingredients.

Virulence. One of the characteristics of live tularemia vaccine is that it shows some virulence in mice. Therefore, a study was made with several lots of vaccine prepared in both complex and CDM media to compare mouse virulence (Table 2).

The results indicate no significant difference in virulence between lots of vaccine prepared in either medium. Similar tests in guinea pigs, performed with higher concentrations (10^7, 10^8, and 10^9 organisms per milliliter) of the same vaccine lots, also showed no difference.

Immunogenicity. A criterion of vaccine effectiveness is the ability of vaccinated mice to survive challenge with at least 10^3 LD_{50} of *P. tularensis* SCHU S4. Accordingly, the survivors of the mouse virulence tests were challenged with the standard dose of SCHU S4. The results obtained 20 days after challenge are presented in Table 3.

TABLE 2. *Comparative mouse virulence of vaccines prepared in complex and chemically defined media*

Vaccine dose (no. of viable cells per 0.2 ml)	Mortality (per cent)	
	CDM	Complex medium
10^2	5*	4*
10^4	10	6
10^6	12	6
10^8	47	53
Controls	0	0

* Average of five separate tests.

TABLE 3. *Comparative immunogenicity of vaccines prepared in complex and chemically defined media*

Vaccine dose (no. of viable cells per 0.2 ml)	Survival* (per cent)	
	CDM	Complex medium
10^2	96†	96†
10^4	99	96
10^6	97	93
10^8	100	79
Controls	0	0

* Challenge dose = 10^3 LD_{50}.
† Average of five separate tests.

TABLE 4. *Comparative immunogenicity of vaccine prepared in complex and chemically defined media against large challenge doses*

Vaccine dose (no. of viable cells)	Challenge dose (LD_{50})	Survival (per cent)	
		CDM	Complex medium
10^2	10^5	90	95
10^2	10^7	83	85
10^4	10^5	95	92
10^4	10^7	84	82
10^6	10^5	96	94
10^6	10^7	80	85
10^8	10^5	100	79
10^8	10^7	90	90
Controls	10^5	0	0
	10^7	0	0

With the exception of the group vaccinated with 10^8 organisms, there was no appreciable difference in immunogenicity between lots of vaccine. The difference noted at the highest vaccine dose is unexplained.

In addition to the above, duplicate tests were performed in which vaccinated mice were challenged with larger amounts of strain SCHU S4 (Table 4).

It is evident that no significant difference in immunogenicity exists between vaccines prepared in either medium, even when the challenge dose is 10,000-fold higher than that normally used.

Guinea pig skin reaction. An additional test employed for the evaluation of live tularemia vaccine involves the production of a skin reaction in guinea pigs. The usual reaction proceeds as follows. After 1 to 2 days erythema appears. After 3 to 8 days vesicles and pustules appear, and after 8 to 12 days the site appears dry and scab formation begins. Erythema disappears after 12 to 15 days, and the scab sloughs, leaving slight scar.

The vaccines prepared in both media produced the typical skin reaction with the same intensity and duration.

DISCUSSION

Although the chemically defined medium produces a lower viable population, it is otherwise comparable to the complex undefined media used in the production of live tularemia vaccine. The components are essentially the same, except for optimal concentration, as those previously described by Traub et al. (1955).

The concentration of individual ingredients may be varied within rather wide limits without any apparent effect upon frequency of dissociation. As expected, however, multiplication is inhibited in the presence of higher concentration of some components. The conditions affecting the proportions of dissociants, as well as those responsible for greater multiplication in undefined media, remain to be determined.

In contrast to the results of previous studies, where both virulent strains and those of reduced virulence grew at *p*H 7.0, the vaccine strain grew best when the *p*H of CDM was adjusted to 6.2 to 6.4 prior to inoculation. Interestingly, the *p*H always increased to 7.0 to 7.2 during the 24-hr growth period.

Growth of the vaccine strain in CDM, as measured turbidimetrically, was comparable to that obtained with the medium suggested by Nagle et al. (1960) for strains of reduced virulence. The addition of uracil, adenine, and guanine to CDM did not enhance growth.

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LITERATURE CITED

EIGELSBACH, H. T., AND C. M. DOWNS. 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J. Immunol. **87**:415–425.

MAGER, J., A. TRAUB, AND N. GROSSOWICZ. 1954. Cultivation of *Pasteurella tularensis* in chemically defined media: effect of buffers and spermine. Nature **174**:747–748.

TRAUB, A., J. MAGER, AND N. GROSSOWICZ. 1955. Studies on the nutrition of *Pasteurella tularensis*. J. Bacteriol. **70**:60–69.

NAGLE, S. C., R. E. ANDERSON, AND N. D. GARY. 1960. Chemically defined medium for the growth of *Pasteurella tularensis*. J. Bacteriol. **79**:566–571.

DOWNS, C. M., L. L. CORIELL, S. S. CHAPMAN, AND A. KLAUBER. 1947. The cultivation of *Bacterium tularense* in embryonated eggs. J. Bacteriol. **53**:89–100.

REED, L. J., AND H. MUENCH. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.