IMMUNOGENIC SUBSTANCES IN CULTURE FILTRATES AND LYSATES OF PASTEURELLA TULARENSIS

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ABSTRACT

HATCH, MELVIN T. (University of Utah, Salt Lake City), and PAUL S. NICHOLES. Immunogenic substances in culture filtrates and lysates of Pasteurella tularensis. J. Bacteriol. 88:566–573. 1964.—Culture filtrates and lysates of Pasteurella tularensis were tested for immunogenicity in mice subsequently infected with either strain 425 or 425 F4G. The efficacy of the vaccines varied with dosage and was significantly dependent upon methods of preparation. The optimal procedures for the production of an immunologically potent vaccine included: (i) aging the cultures after growth under partial anerobiosis at 37 C, and (ii) inactivating the cells with phenol or formaldehyde. An unusual “survival phenomenon” was suggested when mice were administered large doses of cell-free vaccines and subsequently large doses of moderately virulent P. tularensis. The data indicated that the filtrates and lysates elicited an immune response sufficient to protect against an active infection with strains 425 or 425 F4G, and that the challenge dose per se stimulated an enhanced immunity. Furthermore, this survival phenomenon was demonstrable when immunized mice were subsequently given massive doses of strain 425 and challenged with approximately 1,000 LD50 of the fully virulent strain Schu. On the basis of our data, we have hypothesized that the protective antigens were released into the suspending medium as a result of alterations in the permeability of cells undergoing either complete or partial enzymatic degradation. We believe that the envelope antigens were released from the cell by mechanisms analogous to those causing leakage of intracellular constituents in cells maintained at an incubation temperature in an unfavorable growth environment.

Previous reports indicated that killed suspensions and lysates of Pasteurella tularensis were practically ineffective when employed as vaccines in animals highly susceptible to tularemia, and that live vaccines were a superior prophylactic agent (Eigelsbach, Downs, and Herring, 1958; Eigelsbach and Downs, 1961; Obu'ev and Rudnev, 1960). Few studies have been reported, however, concerning the immunogenicity of soluble antigens produced during cultivation in vitro. Coriell, Downs, and Clapp (1947) first reported that a broth filtrate of P. tularensis was incapable of evoking an immune response in mice subsequently infected with a fully virulent strain. On the other hand, preliminary studies from this laboratory (Nicholes et al., unpublished data) indicated that immunogens were released into the liquid medium by tularemia organisms. The immunogenicity of the filtrates was evidenced by a significant increase in longevity of immunized animals challenged with P. tularensis strain 425. It was also shown that mice treated with concentrated filtrates were more resistant to subsequent infection with the fully virulent strain Schu than were mice treated with unconcentrated filtrates. Recently, others reported on the immunogenic activity of P. tularensis filtrates (Pannell, 1958; Pannell and Cordle, 1962).

The present studies were extended to: (i) develop a technique for inducing a maximal release of the protective antigens into the culture medium, and (ii) demonstrate the extent of the immunogenicity and antigenicity of the soluble antigens in culture filtrates by in vivo and in vitro methods.

MATERIALS AND METHODS

Strains of P. tularensis. Soluble antigens were obtained from filtrates and lysates of the fully

1 This report was taken from a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree at the University of Utah, 1961. Presented in part at the Semiannual Meeting of the Intermountain Branch of the American Society for Microbiology, April, 1958.

2 Present address: Naval Biological Laboratory, School of Public Health, University of California, Berkeley.
virulent strain Schu, strain Jap H, and a variant strain, Jap4. Mice inoculated with the cell-free solutions were challenged with strain 425 or 425 4FG. In some experiments, immunized animals surviving sublethal infection with the challenge strains were reimmunized with the Schu strain.

Media. The liquid medium employed for growing *P. tularensis* consisted of either modified casein-partial hydrolysate (MCPH) broth (Mills et al., 1949) or a chemically defined medium formulated by the late Lee Foshay (see Hatch, 1961). The solid medium was glucose-cysteine-blood-agar (GCBA).

Preparation of culture filtrates. *P. tularensis* was initially grown on GCBA slants at 37 C for 24 to 48 hr. The cells were then inoculated into low-form, wide-bottom flasks (no. 29172-1; Braun-Knecht-Heimann Co., Division of Van Waters and Rogers, Inc., Brisbane, Calif.) containing MCPH broth or the defined medium. All cultures were incubated at 37 C on a reciprocal shaking at 24 to 168 hr.

The bacterial suspensions were treated with 0.05 to 0.9% phenol, centrifuged, and filtered through a Seitz filter to remove cells. In some experiments, cultures were stored in stoppered flasks under static conditions at 37 C for 7 to 12 days to achieve partial anaerobiosis; these suspensions, with or without disinfectant, were then treated with phenol to a final concentration of either 0.1 or 0.9% and then were filtered. In other experiments, freshly harvested cultures were immediately treated with 0.1 or 0.5% formaldehyde. Filtrates were dialyzed against either running tap water or several changes of distilled water for 48 hr. Some filtrates were concentrated by pervaporation at 25 C to about 5% of the original volume. Others were lyophilized after a preliminary concentration in a flash evaporator (model no. FE-2C; Laboratory Glass and Instrument Corp., New York, N.Y.).

Preparation of lysates. Lysates were prepared from cell populations obtained by the conditions of growth used for the filtrates. After the growth period, cells were lysed with sodium deoxycholate at a final concentration of 1% and the lysates were either dialyzed immediately or held under partial anaerobiosis for 72 hr before dialysis. In some experiments, a precipitate formed in the filtrates and lysates after dialysis. These preparations were separated into supernatant and precipitate fractions and were lyophilized.

Animals. The two strains of mice used were: (i) *Mus musculus* received from the Rocky Mountain Laboratory, and (ii) CFW obtained by random selection from the University of Utah animal colony. Male and female mice, 6 to 8 weeks old, were used. Humoral antibody was produced in domesticated rabbits by intravenous injection of culture filtrates and killed suspensions of whole cells. Antitularensese sera were also produced in goats and in one adult badger. These animals had recovered from tularemia infection, and were further immunized by numerous injections of fully virulent Schu organisms prior to bleeding. All sera were maintained at −70 C until used.

Vaccination of mice with cell-free concentrates. Three injections of 0.25 ml (a total dose of 15 mg, dry weight) were administered to each animal subcutaneously in the inguinal region at 48-hr intervals either with the concentrated filtrates or with suspensions reconstituted from the lyophilized preparations. Dried vaccines, unless otherwise noted, were always reconstituted either with distilled water or with normal saline to a concentration of 20 mg/ml (dry weight).

Challenge. The challenge inoculum was prepared from suspensions of *P. tularensis* in 0.85% NaCl solution containing 1% gelatin. Suspensions were diluted to contain the desired cell number per milliliter by turbidimetric techniques with the use of a Klett-Summerson photoelectric colorimeter and a blue filter. The actual dosage administered to each animal was based on conventional methods for assay of viability. Suspensions were decimally diluted, and 0.25 ml of the appropriate dilutions were inoculated subcutaneously in the nuchal region of immunized and untreated control animals. All animals were observed daily for 13 days, at which time the experiments were terminated.

Statistical analysis. The LD₉₀ end points were determined by the method of Reed and Muench (1938), with the standard error calculated by the method of Pizzi (1950). Statistical evaluation of the efficacy of varying vaccine dosages was made by the analysis of variance method with arc sine transformation (Snedecor, 1946). The efficacy of the cell-free vaccines was assessed by chi-square analyses.

Results

Immunogenic potency of *P. tularensis* filtrates. Immunogenic substances were contained in the
Wide differences existed in the immunogenic potency of various filtrates that had been prepared and tested over a period of several months. For example, the LD₅₀ ranged from 7,400 ± 306 to >3,540,000. Thus, it was postulated that two conditions were essential for the production of a potent vaccine: (i) exposure of the organism to phenol or formaldehyde, and (ii) storage under partial anaerobiosis at 37 C after active growth.

To test these postulates and to investigate the chemical and physical manipulations necessary for the production of an immunologically potent concentrate, other cell-free vaccines were prepared by several different methods, and attempts were made to determine the effects of: (i) varying the quantity and sequence for the addition of phenol or formaldehyde, (ii) varying the growth period, (iii) aging the cultures under partial anaerobiosis followed by the addition of phenol, and (iv) lysing the cells with sodium deoxycholate. The results of this experiment are pre-

### Table 1. Immune response of mice inoculated with concentrated filtrates of Pasteurella tularensis and challenged with the moderately virulent strain 485

<table>
<thead>
<tr>
<th>Challenge dose in 0.25 ml ± 5.5%</th>
<th>Animal groups* (dead/total)</th>
<th>Challenge dose in 0.25 ml ± 5.5%</th>
<th>Animal groups* (dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>30,000</td>
<td>0/8</td>
<td>1/8</td>
<td>7/8</td>
</tr>
<tr>
<td>3,000</td>
<td>0/8</td>
<td>2/8</td>
<td>5/8</td>
</tr>
<tr>
<td>300</td>
<td>0/8</td>
<td>1/8</td>
<td>5/8</td>
</tr>
<tr>
<td>30</td>
<td>0/8</td>
<td>2/8</td>
<td>6/8</td>
</tr>
<tr>
<td>3</td>
<td>0/8</td>
<td>1/8</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>0.102</td>
<td>0/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>

* Group 1: mice were immunized with filtrate from Schu cells grown in modified casein-partial hydrolysate (MCPH); the filtrate was concentrated to about 6% of the original volume by pervaporation. Group 2: mice were immunized with filtrate from Jap H cells grown in MCPH; the filtrate was concentrated to about 5% of the original volume by pervaporation. Group 3: placebo-treated controls.

concentrated filtrates (Table 1). Furthermore, Schu and Jap H cells were equally capable of releasing immunogens during in vitro cultivation, although either the former yielded more antigens per se or each molecule had enhanced immunogenicity. With the micro-Kjeldahl method, 0.932 mg of nitrogen per 10-mg sample (dry weight) of the filtrates from Schu cells, and 0.644 mg of nitrogen per 10-mg sample (dry weight) of Jap H filtrates, were found. Although the data seem to indicate some correlation between the protective substances and the total nitrogen concentration, further data (to be published) will show a greater immune response with preparations containing less nitrogen than shown above. The efficacy of a cell-free vaccine was not diminished by selective removal of certain nitrogenous substances. The methods employed for purification of the filtrate antigens were reported by Biehler (1957) and by Hatch, Biehler, and Nicholes (1959). Protective substances were present in the filtrates when the cells were inactivated by the addition of formaldehyde as well as by phenol (Table 2). The antigens were produced in both MCPH and defined growth media.

### Table 2. Immune response of mice inoculated with concentrated filtrates of Pasteurella tularensis and challenged with the moderately virulent strain 485

<table>
<thead>
<tr>
<th>Challenge dose in 0.25 ml ± 1.5%</th>
<th>Animal groups* (dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>3,540,000</td>
<td>1/5</td>
</tr>
<tr>
<td>354,000</td>
<td>3/5</td>
</tr>
<tr>
<td>35,400</td>
<td>3/5</td>
</tr>
<tr>
<td>3,540</td>
<td>0/5</td>
</tr>
<tr>
<td>354</td>
<td>0/5</td>
</tr>
<tr>
<td>3.54</td>
<td>0/5</td>
</tr>
<tr>
<td>0.354</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Mice were immunized with filtrates of strain Schu. The filtrates were concentrated in a flash evaporator, lyophilized, and reconstituted in distilled water at a concentration of 20 mg/ml (dry weight). Group 1: cells were grown in modified casein-partial hydrolysate (MCPH) and inactivated with phenol. Group 2: cells were grown in MCPH and inactivated with formaldehyde. Group 3: cells were grown in a chemically defined medium and inactivated with formaldehyde.
† Mice were immunized with MCPH medium, concentrated in the flash evaporator, lyophilized, and reconstituted in distilled water at a concentration of 20 mg/ml (dry weight).
‡ Untreated control mice.
Table 3. Immune response in mice inoculated with cell-free vaccines prepared by different methods and challenged with Pasteurella tularensis strain 455 P49*

<table>
<thead>
<tr>
<th>Immunizing agent</th>
<th>Method of preparation</th>
<th>Animal response (dead/total)</th>
<th>Chi-square†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active growth at 37°C</td>
<td>Treatment of cultures†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>0.5% phenol added after active growth</td>
<td>21/22</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B1</td>
<td>0.5% formaldehyde added after active growth</td>
<td>22/22</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C1</td>
<td>0.5% phenol added after active growth, 12 days of storage at 37°C under partial anaerobiosis; phenol added to 0.9% final concentration</td>
<td>10/22</td>
<td>13.86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D1</td>
<td>0.9% phenol added after active growth, 7 days of storage at 37°C under partial anaerobiosis; phenol added to 0.1% final concentration; a filtrate fraction</td>
<td>14/22</td>
<td>7.48</td>
<td>0.01-0.005</td>
</tr>
<tr>
<td>E1</td>
<td>Precipitate fraction of E1</td>
<td>19/22</td>
<td>1.44</td>
<td>0.25-0.20</td>
</tr>
<tr>
<td>E2</td>
<td>Precipitate fraction of E1</td>
<td>168</td>
<td>7/22</td>
<td>23.82</td>
</tr>
<tr>
<td>F2</td>
<td>Precipitate fraction of F1</td>
<td>72</td>
<td>4/22</td>
<td>27.18</td>
</tr>
<tr>
<td>F1</td>
<td>Cells lysed with sodium deoxycholate added at final concentration of 1% after storage under partial anaerobiosis at 37°C for 7 days; a filtrate fraction</td>
<td>72</td>
<td>17/22</td>
<td>3.62</td>
</tr>
<tr>
<td>G1</td>
<td>Foshay-type whole organism vaccine</td>
<td>Untreated controls</td>
<td>21/22</td>
<td>—</td>
</tr>
</tbody>
</table>

* The challenge dose in 0.25 ml contained 5.3 × 10⁴ ± 1.8% cells.
† Mice were immunized with cell-free solutions from Schu cells grown in modified casein-partial hydrolysate medium.
‡ Chi-square values were determined from vaccine vs. untreated controls.

sented in Table 3. Only four cell-free concentrates elicited an immune response at or below the 0.05 level of significance. The superior efficacy of the preparations which were allowed to incubate at 37°C after a period of active growth suggested to us that immunogens were either released or produced during storage under partial anaerobiosis. The release may involve either complete lysis of the cells or partial dissolution of the cell wall, because the data indicated that lysates of Pasteurella tularensis contained greater immunogenic activity than did the filtrates. Further, it is evident that, in preparations wherein a precipitate formed during dialysis, the protective antigens were contained in the precipitate fractions. Appreciable purification was effected by such precipitation.

A study was made to determine whether the highly immunogenic and moderately avirulent variant Jap4 (Moody and Downs, 1955) would release protective substances into liquid medium after active growth and storage under partial anaerobiosis. A fraction from Jap4 filtrates was precipitated during concentration in a flash evaporator, as noted above. The supernatant fluid and precipitate were tested separately for immunogenicity. Data presented in Table 4 are consistent with the previous findings, and indicate that protective immunogens were released into the filtrates by Jap4 cells as well as by cells of Jap H and Schu. The data suggest that the precipitated antigens were subsequently soluble in water. We consider it likely that the antigens were sorbed onto nonspecific medium constituents that precipitated.

There were no significant differences in protection afforded by the precipitate H2 (Table 4) and the antigens extracted from the precipitate fraction H3 (P value, 0.75 to 0.70). There were significant differences, however, in protection...
TABLE 4. Immune response in mice inoculated with filtrate fractions of Pasteurella tularensis strain Jap4 and with a lysate from strain Schu and challenged with strain 425 F4G*

<table>
<thead>
<tr>
<th>Immunizing agent</th>
<th>Animal response (dead/total)</th>
<th>Chi-square†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>38/70</td>
<td>41.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>H2</td>
<td>15/70</td>
<td>89.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>H3</td>
<td>17/70</td>
<td>85.2</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>F2</td>
<td>7/70</td>
<td>114.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>C</td>
<td>70/70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The challenge dose in 0.25 ml contained 4.38 X 10^4 ± 1.5% cells.
† H1: fraction prepared from Jap4 cells grown in modified casein-partial hydrolysate for 110 hr; cultures were allowed to remain under partial anaerobiosis for 7 days, followed by addition of 0.1% phenol; a filtrate fraction. H2: precipitate fraction of H1. H3: a portion of fraction H2, antigen-extracted with water; filtrate fraction. F2: see Table 3. C: untreated controls.
‡ Chi-square values were determined from vaccines vs. control data.

Elicited by the filtrate fraction H1 vs. the extracted antigens in H3 (P value, <0.001), and differences between the precipitate fraction H2 and the lysate F2 only approached significance (P value, 0.10 to 0.05).

Immunization with P. tularensis vaccines and sublethal infection with strain 425. Mice immunized with Fosbary vaccine, with culture filtrates, and with lysates frequently survived massive doses of strains 425 and 425 F4G. These surviving animals were rechallenged with the fully virulent strain Schu to investigate and compare the extent of the protection afforded by the combination of immunization with these vaccines and sublethal infection with the above organisms. The results of three experiments are presented in Table 6. The greatest differences in immunogenicity among the vaccines were obtained when large doses of P. tularensis strains 425 and Schu were employed. The immune response was correlated with the type of vaccine administered and the numbers of strain 425 cells given. This unusual "survival phenomenon" is similar in some respects to that described by Ginsburg (1947), who observed an enhanced survival in guinea pigs injected with suspensions containing large numbers of living attenuated P. tularensis cells and small numbers of the fully virulent cells. Apparently, survival of mice and guinea pigs is dependent upon the host receiving a massive quantity of tularemia antigen and fully virulent cells.

TABLE 5. Immune response in mice administered filtrate B1 (Table 3) in varying dosages and challenged with Pasteurella tularensis strain 425 F4G

<table>
<thead>
<tr>
<th>Total immunogen given each animal</th>
<th>Organisms received (±1.9%)</th>
<th>Animal response (dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>3,800,000</td>
<td>12/24</td>
</tr>
<tr>
<td>38,000</td>
<td>18/24</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3,800,000</td>
<td>15/24</td>
</tr>
<tr>
<td>38,000</td>
<td>19/24</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3,800,000</td>
<td>20/24</td>
</tr>
<tr>
<td>38,000</td>
<td>22/24</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>16/24</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>3,800,000</td>
<td>22/24</td>
</tr>
<tr>
<td>38,000</td>
<td>16/24</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>18/24</td>
<td></td>
</tr>
<tr>
<td>3.75</td>
<td>3,800,000</td>
<td>23/24</td>
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<tr>
<td>38,000</td>
<td>23/24</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>21/24</td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>3,800,000</td>
<td>24/24</td>
</tr>
<tr>
<td>38,000</td>
<td>24/24</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>24/24</td>
<td></td>
</tr>
</tbody>
</table>
**Discussion**

Immunogenic substances in filtrates of *P. tularensis* were demonstrated by Pannell (1958), Pannell and Cordle (1962), and these data. The origin and mode of release of these substances are obscure; however, these combined experiments indicated a superior immunogenicity of aged cultures, and further scrutiny of the events occurring during aging would be invaluable.

The release of intracellular substances by microbial cells in unfavorable growth environments was reported (Mandelstam, 1958; Postgate and Hunter, 1962; Herbst and Doctor, 1959). These substances were thought to be fragments of nucleic acid and proteins which were released by bacteria metabolically active in adaptive enzyme synthesis (Strange, 1961). The increase in antigenicity of aged filtrates suggested a correlation with the above phenomenon. Because low levels of disinfectants are incapable of completely inactivating all vital processes of cells, including enzymatic activities (Roberts and Rahn, 1946), aging of cultures containing sublethal concentrations of phenol and of formaldehyde merely altered the physiological activities of the microbial population. Consequently, cytolytic damage and excessive leakage of cell constituents occurred in the bacterial suspensions treated with phenol (Gale and Taylor, 1947).

Cells treated with sublethal amounts of formaldehyde were in an imbalanced condition of growth resulting from an inhibition of cell division and cytoplasmic synthesis (Neely, 1963a, b). Since the immunizing capabilities of the tularensia pathogen reside only in the "soluble" components associated with the intact bacterium (Ormsbee, Bell, and Larson, 1955) and exist as "surface-somatic" and "somatic" antigen complexes (Olsuf'ev and Emel'ianova, 1987), we postulate that the filtrate antigens were released from cells in an imbalanced condition of growth by either complete or partial enzymatic degradation of cell-wall constituents. The observation that the immunogenicity of aged lysates and of cultures prepared after storage was superior to preparations obtained immediately after active growth substantiates this hypothesis.

Some intracellular synthesis and continuous release of protective antigens could have occurred during the aging period. That the immunogens were extracellularly synthesized is also possible, because in vitro synthesis of proteins had been demonstrated in cellular systems (Zamecnik and Keller, 1954; Siekevitz, 1952; Schweet, Lamfrom, and Allen, 1958). But, it is unlikely that the ribosomal ribonucleic acid is capable of penetrating the cell membrane and leaking out in sufficient quantities without a concomitant loss of infectivity and viability. We found that cell suspensions containing sublethal concentrations of phenol were, nonetheless, still capable of producing clinical infection in a susceptible host.

Ormsbee et al. (1955) showed that the protection afforded mice by *P. tularensis* vaccines was proportional to the antigen content. In our study, the efficacy of filtrates and lysates generally varied with the immunizing dosages (Table 5).

However, if the data in Table 5 are carefully scrutinized, an unusual survival phenomenon emerges. There was a difference of two survivors between the lots of mice given a total vaccine dose of 15 mg and challenged with 10^6 and 10^6 cells. When the same challenge doses were employed but the vaccine dosage was 30 mg, the number of survivors differed by four animals, and

<table>
<thead>
<tr>
<th>Immunizing agent*</th>
<th>Initial challenge dose of <em>P. tularensis</em> strain 425</th>
<th>Rechallenge dose of <em>P. tularensis</em> strain Schu</th>
<th>Animal response (dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>1,580,000</td>
<td>124</td>
<td>8/20</td>
</tr>
<tr>
<td>V2</td>
<td>2,800,000</td>
<td>1,360</td>
<td>5/20</td>
</tr>
<tr>
<td>V3</td>
<td>2,800,000</td>
<td>1,360</td>
<td>4/19</td>
</tr>
<tr>
<td>A1</td>
<td>1,580,000</td>
<td>124</td>
<td>10/20</td>
</tr>
<tr>
<td>F2</td>
<td>1,580,000</td>
<td>124</td>
<td>7/20</td>
</tr>
<tr>
<td>F1</td>
<td>4,380,000</td>
<td>418</td>
<td>8/20</td>
</tr>
<tr>
<td>C</td>
<td>4,380,000</td>
<td>1,180</td>
<td>1/30</td>
</tr>
</tbody>
</table>

* V1: Foshay-type vaccine recently prepared in our laboratory. V2: vaccine obtained from Dr. Foshay. The vaccine was lyophilized and stored at room temperature for 1 year. For reconstitution procedure see text. V3: vaccine recently prepared and obtained from Dr. Foshay. A1: see Table 3. F2: see Table 3. C: untreated controls.
by six animals when a 60-mg vaccine dosage was administered. These differences were not significant on a statistical basis; nonetheless, the data suggested to us that the concentrated filtrates elicited an immune response sufficient to afford protection in animals subsequently infected with large doses of moderately virulent organisms, and the massive challenge dose per se stimulated an enhanced immune response.

Furthermore, data in Table 6 indicated that mice immunized with massive doses of moderately virulent organisms demonstrated this survival phenomenon when subsequently reinfected with the fully virulent strain Schu. In the lysate-treated animals, this combined protection apparently elicited an immune response which approached the efficacy of live vaccines (Emel'ianova, 1957; Olsuf'ev and Rudnev, 1960). Thus, a high level of resistance may be obtained by vaccinating first with a potent nonliving vaccine and then with a living strain possessing low virulence but high immunogenicity.

Acknowledgment

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