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Preparation of Rocky Mountain Spotted Fever Vaccine Suitable for Human Immunization

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Rocky Mountain spotted fever vaccine was produced from rickettsiae grown in chicken embryo cells in roller bottle cultures. The rickettsiae were concentrated and purified by passage through a sucrose gradient and inactivated with formalin. This vaccine satisfactorily passed preinactivation and final container testing and is believed to be superior to the presently available yolk sac vaccine.

Rocky Mountain spotted fever (RMSF) is an acute febrile illness caused by *Rickettsia rickettsii*. The disease is naturally transmitted by the bite of ticks; however, laboratory workers have been infected by an aerosol exposure (1, 7). Clinical illness is characterized by abrupt onset of headache, chills, and fever. A characteristic exanthem appears on the extremities and the trunk on approximately day 4 of illness. In untreated cases the average mortality rate is approximately 20%, but in people over 40 it approaches 50%. The tetracycline group of antibiotics and chloramphenicol are rickettsiostatic and effective in the treatment of RMSF if therapy commences during week 1 of illness (14).

The first vaccine for RMSF was a phenolized suspension of infected ticks (13). This was replaced by the Cox-type vaccine prepared from rickettsiae grown in egg yolk sacs of fertile chicken eggs and killed with formaldehyde (2). Accumulated experience indicates that neither vaccine confers a satisfactory level of immunity in man, and there are reports of disease occurring in vaccinated laboratory workers (1, 7). The most conclusive evidence concerning the efficacy of available RMSF vaccines was supplied by DuPont et al. (3) with volunteers. Neither the current yolk sac vaccine nor the original tick vaccine (which is not commercially available) conferred protection in humans. Both vaccines commonly induced local erythema and tenderness at the site of inoculation, predominantly after the second or third dose of vaccine. The currently available yolk sac vaccine leaves much to be desired both in terms of efficacy and purity. We report here the production and testing of a RMSF vaccine for human immunization prepared from rickettsiae grown in chicken embryo cell (CEC) culture.

MATERIALS AND METHODS

Vaccine seed preparation. The Sheila Smith (SS) strain of *R. rickettsii* was used for vaccine preparation. This strain was received from Charles Wisseman (University of Maryland). It had been isolated from a human case of RMSF and passaged an unknown number of times in guinea pigs and in chicken eggs. This seed stock was passaged four times in duck egg yolk sacs, then two times in leukosis-free chicken egg yolk sacs to obviate potential contamination with avian leukosis viruses. Appropriate testing showed that this Master Seed (SSR-2cys) was leukosis virus free. The Master Seed was passaged six additional times in leukosis-free chicken egg yolk sacs to prepare the Working Seed (SSR-8cys).

Cell culture. CEC were prepared from 9-day-old embryonated eggs. Roller bottles (1 liter) were seeded using 5.0×10^8 cells per bottle. One hundred fifty milliliters of medium 199 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (without antibiotics) were used per bottle. Cells were placed on a roller apparatus set at 0.4 rpm and incubated at 36 C for approximately 24 h.

Experimental animals. Guinea pigs (Hartley strain) were obtained from Buckberg Lab Animals, Tompkins Cove, N.Y. Mice (Swiss) were obtained from Microbiological Associates, Inc., Walkersville, Md. Eggs were obtained from SPAFAS, Inc., Norwich, Conn. Rabbits (New Zealand white) were obtained from Bunnyville Farm, Littlestown, Pa.

Vaccine preparation. The growth medium was removed and each bottle was inoculated with 15 ml of 10% yolk sac (SSR-8cys) rickettsial suspension in sucrose phosphate glutamate buffer (sucrose, 74.6 g/liter; KH_2PO_4, 0.52 g/liter; Na_2HPO_4, 1.22 g/liter; L-glutamate, 0.72 g/liter; pH 7.2) (6). Exposure to the inoculum was accomplished (multiplicity of infection about one rickettsia per six cells) while the bottles revolved at 0.2 rpm for 1 h at room temperature. The residual inoculum was removed and each cell culture was fed with 150 ml of Eagle minimal essential

medium supplemented with 0.5% human serum albumin. During incubation at 34 C, bottles were rolled at 0.4 rpm; medium was changed at 24 and 96 h postinoculation. At 96 h the medium was replaced with Eagle minimal essential medium prepared with pyrogen-free water, and at 120 h the Eagle minimal essential medium was replaced with pyrogen-free sucrose phosphate glutamate buffer (55 ml per bottle). The monolayers were then frozen and thawed, and the resultant rickettsiae and cell debris were stored as separate pools at −70 C until further processing. Preliminary sterility tests on each pool were performed using Trypticase soy broth and blood agar. After sterility testing and before formalin inactivation, rickettsiae-cell debris pools were thawed, passed through a 21-gauge needle several times to disperse clumps, and centrifuged for 15 min at 150 × *g* to remove large cellular debris. The pools were combined and an appropriate volume was removed for preinactivation testing. Formalin (Formaldehyde, 37% USP) was added to the clarified bulk rickettsial pool to a final concentration of 0.1%. The mixture was stirred at room temperature for 48 h and then stored at 4 C until further processed. Medium from an appropriate number of uninfected CEC roller bottles was pooled and stored at −70 C until appropriate testing (5) was performed.

Formalin-treated rickettsiae were separated from cellular debris by passage through a sucrose gradient in a J-1 rotor with the model RK centrifuge (Electronucleonics, Inc.). While the rotor filled with sterile water remained stationary, 400 ml of 10% sucrose with 0.1% formalin followed by 400 ml of 63% sucrose with 0.1% formalin was pumped through the bottom port. The rotor was slowly accelerated to 5,000 × *g* to orient the gradient and then accelerated to 75,000 × *g*. The vaccine was fed onto the gradient from the top at a flow rate of 66 ml/min. The vaccine was centrifuged for 7 h and the rotor was stopped without braking during which time the gradient reoriented. Sixty-five percent sucrose with 0.1% formalin was pumped into the bottom port and 30-ml samples were collected from the top port under ultraviolet light to maintain sterility. Fractions containing whole rickettsiae as determined by light microscropy and optical density measurements were combined; sucrose phosphate glutamate buffer (prepared in pyrogen-free water with 0.025% human serum albumin and 0.1% formalin) was slowly added to bring the volume to one-half that of the original sucrose phosphate glutamate harvest. Free formaldehyde, determined by the National Institutes of Health Fuchsin-sulfurous acid photometric method (10), was neutralized with sterile 35% sodium bisulfite. Total protein was measured by the Lowry method (9).

After neutralization of formalin, 2.7-ml aliquots of vaccine were added to 15-ml vaccine vials. Stoppers were partially inserted, and the vials were placed on a shelf refrigerated at −45 C for 2 h in a cabinet freeze-dryer. After attainment of a satisfactory vacuum, heat was applied to the vials, and freeze drying proceeded for 60 h. At the termination of this cycle, vials were stoppered under vacuum, removed from the cabinet, capped with aluminim seals, tested for vacuum, and stored at −20 C.

Testing on bulk-clarified rickettsial pool (before inactivation). General sterility tests for *Mycobacterium* spp. were conducted in accordance with U.S. Government regulations (5) as were tests for *Mycoplasma*. Tests for avian leukosis viruses were conducted by the Avian Leukosis Unit, Laboratory of Virology and Rickettsiology, Division of Biologics Standards, National Institutes of Health.

The following tests for the presence of adventitious agents were performed. Twenty adult mice were inoculated intracerebrally with 0.03 ml of each of the test samples, and the 20 different mice were inoculated intraperitoneally (i.p.) with 0.5 ml of each of the test samples. These mice were observed for 21 days for death or signs of illness.

Twenty suckling mice were inoculated intracerebrally (0.01 ml) and 20 were inoculated i.p. (0.1 ml) with each of the test samples. These mice were observed for 14 days for death or signs of illness.

Samples to be tested in cell culture were diluted with an equal volume of Eagle minimal essential medium oxytetracycline, 50 mg/ml; 1 ml of the diluted test material was added per 3 cm^2 of confluent monolayers. Chicken and duck embryo cells, VERO cells, and WI-38 cells were used for this test. Monolayers were observed daily for cytopathic effect. On day 7 one-half of the medium from one-half of the flasks was removed and placed on fresh cells. After an adsorption period of 1 h, the old medium was replaced with fresh medium and the flasks were incubated for 7 additional days. Each flask was observed for a total of 14 days after inoculation at which time each was tested for hemadsorption using fresh guinea pig erythrocytes (11).

Thirty 5-day-old embryonated chicken eggs were inoculated with 0.3 ml of sample by the yolk sac route. The suspending fluid contained oxytetracycline (2.5 mg/ml) to inhibit replication of rickettsiae. In addition, 30 9-day-old embryonated eggs were inoculated with 0.3 ml of sample (with tetracycline) by the allantoic route. An equal number of embryonated duck eggs were inoculated by both the allantoic and yolk sac routes as described for chicken eggs. The suspending fluid contained 5.0 mg of oxytetracycline per ml. For yolk-sac inoculation, 9-day-old embryonated duck eggs were used; for allantoic-sac inoculation, 15-day-old embryonated duck eggs were used. All eggs were candled daily. The test was considered satisfactory if all embryos were viable 10 days after inoculation.

Final container testing. General sterility and safety testing was performed as requried by federal regulations (5).

Identity tests were considered satisfactory when vaccinated guinea pigs showed protection against the SS strain of RMSF obtained from the American Type Culture Collection and the Bitter Root strain of RMSF after i.p. challenge with approximately 10^6 plaque-forming units. Guinea pig temperatures were monitored for 10 days.

Potency assays were conducted in 250 to 300 g of

male guinea pigs. Guinea pigs were inoculated i.p. with 0.5 ml of undiluted vaccine or a 1:5, 1:10, 1:100, or 1:500 dilution. At 14 days postvaccination each guinea pig was challenged i.p. with a lethal dose of rickettsiae (ca. 10^6 organisms). Temperatures and deaths were recorded for 10 days after challenge.

Rickettsiae were quantitated by the direct counting method described by Silberman and Fiset (12).

RESULTS AND DISCUSSION

Before vaccine preparation, experiments were performed to determine if more than one band with immunogenic properties could be recovered from preliminary sucrose gradient runs. It was a consistent finding that only one band, composed of intact rickettsiae (density 1.25 g/ml), was immunogenic for guinea pigs (R. Kenyon, unpublished data).

No evidence of bacterial or fungal contamination was detected in the bulk vaccine before inactivation with formalin or in the uninfected control medium (Table 1). In addition, no leukosis or other adventitious viruses were detected.

When tested for purity, the final vaccine contained 0.1% residual moisture, considerably less than an upper limit of 1% designated by federal regulations (5).

The identity test was performed to ensure that the final product was an RMSF vaccine. Guinea pigs inoculated with two 0.5-ml doses of vaccine administered 14 days apart were protected against both the Bitter Root and the SS strains (American Type Culture Collection) (ATCC) of *R. rickettsii*, indicating that the vaccine confers protection against other typical RMSF strains in addition to the vaccine seed strain (Table 2).

The potency assay (Table 3) demonstrated that protection against lethal infection was effected by one immunization with a 1:100 dilution of vaccine but not with a 1:500 dilution. From these data a 50% guinea pig protective dose (protection from death) was calculated by probit analysis (4) to be 0.5 ml of a 1:395 dilution of vaccine.

All final container testing is summarized in Table 4. The final product contained 0.37 mg of protein and 6.5×10^7 rickettsiae per recommended human dose. Safety, potency, purity, and identity tests were satisfactory.

Growth conditions, processing, and characteristics of the CEC vaccine preparation are

TABLE 1. *Preinactivation testing*

Test	Vaccine[a] results showing no evidence of:
General sterility	Contamination
Mycobacterium spp.	Contamination
Mycoplasma spp.	Contamination
Avian leukosis viruses	Contamination
Adventitious viruses adult mice	Adventitis viruses
Suckling mice	Adventitious viruses
Cell culture	
CEC	Adventitious viruses
DEC	Adventitious viruses
VERO	Adventitious viruses
WI-38	Adventitious viruses
Embryonated chicken eggs	Adventitious viruses
Embryonated duck eggs	Adventitious viruses

[a] Control cell medium was also tested and showed results identical to the vaccine.

TABLE 2. *Identity test in guinea pigs of RMSF vaccine*

Group	Avg no. of °F (°C) days[a]	Deaths/ total tested
Vaccinated, challenged with Bitter Root strain of *R. rickettsii*	0.4 (18)	0/5
Nonvaccinated, challenged with Bitter Root strain of *R. rickettsii*	5.7 (15)[b]	0/5
Vaccinated, challenged with SS strain of *R. rickettsii* from ATCC	0 (32)[b]	0/6
Nonvaccinated, challenged with SS strain of *R rickettsii* from ATCC	12.7 (11)[b]	2/6

[a] Average number of degree days = sum of number of degrees above 103.8 F (ca. 40 C) in any one group per total number guinea pigs in that group.

[b] Approximate temperature.

TABLE 3. *Survival of vaccinated guinea pigs after challenge with SS strain rickettsiae*[a]

Vacine	Survivors/total
Undiluted	4/4
1:5	4/4
1:10	4/4
1:100	4/4
1:500	1/4
Nonimmunized controls	0/4

[a] Guinea pigs were vaccinated i.p. with a single dose (0.5 ml) of the appropriate dilution of vaccine and challenged i.p. on day 14 with about 10^6 SS strain rickettsiae.

TABLE 4. *Summary of final container testing on RMSF vaccine*

Test	Result
Sterility	No evidence of contamination
Safety	No temperature rise in guinea pigs; no evidence of disease in guinea pigs or mice
Purity	Residual moisture, 0.105%
Potency	50% Guinea pig protective dose, 0.5 ml of 1:395 dilution of vaccine
Identity	Protection against SS and Bitter Root strains
Nitrogen content	0.37 mg/recommended human dose[a]
Rickettsial quantitation	6.6×10^7/recommended human dose

[a] An amount of 0.15 mg is contributed by human serum albumin in suspending fluid.

markedly different from those of the present commercially available RMSF vaccine (Lederle). The commercial product is prepared by a method described in 1939 (2): after infected yolk sacs are harvested and blended in a buffered saline solution, the rickettsiae are inactivated with formalin and an ether extraction is performed to reduce the content of egg proteins. Intact rickettsiae cannot be observed by light microscopy and there is no attempt to separate the rickettsiae from the contaminating egg proteins by physical methods. A single human dose contains 5.3 mg of protein. Information is not available concerning the final dilution of the original infected egg yolk sac harvest, or concerning the effect of ether extraction on immunogenicity. Undiluted yolk sac vaccine confers protection to guinea pigs, but a dilution of 1:10 is essentially ineffective (8). The yolk sac vaccine fails to prevent human illness but does have the beneficial effect of lengthening the incubation period and decreasing the incidence of relapse; no correlation between protection from the disease and presence of Weil-Felix or complement fixation antibodies has been detected (3).

The accumulated evidence indicated that an improved RMSF vaccine is highly desirable. As demonstrated in this paper, our CEC vaccine is efficacious in guinea pigs. A study is under way to study the efficacy of the CEC and Lederle vaccines in rhesus monkeys. After the required approval is obtained our CEC vaccine will be tested in humans.

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