New Assay of Protective Activity of Rocky Mountain Spotted Fever Vaccines

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Areas under the fever curves of guinea pigs inoculated with Rocky Mountain spotted fever vaccine over a restricted dose range and infected with a standardized dose of *Rickettsia rickettsii* varied linearly with $\log_{10}$ dose of vaccine. A calculator was programmed to plot fever curves and calculate the vaccine dose that reduced the fever of infected animals by 50%.

The official procedure for the bioassay of Rocky Mountain spotted fever vaccine dates back to 1945 (7). According to that procedure an acceptable vaccine, when inoculated subcutaneously, must protect at least two-thirds of the guinea pigs against fever (39.6°C) after intraperitoneal (i.p.) challenge 10 days later with fresh citrated heart's blood drawn from an infected guinea pig. This same challenge dose must kill at least one-half of the control guinea pigs. Since this test does not readily permit quantitative comparisons of acceptable vaccines, we devised a test to determine the protective activity of spotted fever vaccines in guinea pigs. Results obtained with our test were published previously (1), but data illustrating the basis for and reliability of the assay have not been reported. Vaccine potency may also be estimated in mice (4). However, the mouse test has not yet gained official acceptance, so our efforts have been directed toward improvement of the guinea pig test.

For our test, male Hartley guinea pigs in groups of six to eight, weighing 400 to 450 g, were inoculated i.p. with 1.0-ml volumes of graded doses of the vaccine. Vaccine was given i.p. rather than subcutaneously in our study because (i) i.p. inoculations are faster and (ii) fourfold less antigen was required to achieve the same level of protection. Eight to 10 uninoculated animals were held as controls. After 10 days all animals were inoculated i.p. with 10,000 guinea pig 50% infectious doses of the R strain of *Rickettsia rickettsii* (3) in 1.0 ml of snyder I solution (6) held at 0°C. *Rickettsiae* for challenge were prepared from infected yolk sacs (8) blended for 2 min with 4 volumes of Snyder I solution and then centrifuged at $500 \times g$ for 5 min at 4°C; the supernatant fluid was sealed in glass ampoules, shell-frozen in a dry ice-alcohol mixture, and stored at -65°C. After adminis-

tration of the challenge dose, rectal temperatures were taken daily with a telethermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) for 12 days or until the animal's temperature dropped below 39.6°C, whichever was longer.

Areas under the fever curves above 39.6°C were determined with a model 9100A Hewlett-Packard calculator programmed to measure area by the trapezoidal rule. As the temperatures were entered into the calculator, the degrees of fever were plotted by a Hewlett-Packard 9125B calculator plotter on a scale of 1 inch (2.54 cm) on the ordinate per degree centigrade and 0.5 inch (1.27 cm) on the abscissa per day. One unit of fever is 1 day by 1 degree or 0.5 inch² (3.23 cm²) of area. In five experiments the average number of fever units for groups of normal control animals was 6.411 (standard deviation $\pm$ 0.680 and range, 5.315 to 7.086). Fewer than 5% of the 429 animals tested thus far have died; animals that died prematurely were eliminated from consideration.

In initial experiments to determine the nature of the guinea pig's response, animals were inoculated with either of two kinds of spotted fever vaccine, one (Sheila Smith strain) obtained commercially and the other (R strain) prepared at the Rocky Mountain Laboratory by sucrose density gradient zonal centrifugation (2). (A previous study [1] indicated that in comparisons of vaccines, similar results were obtained when the experimental animals were challenged with either the R strain or the Sheila Smith strain of *R. rickettsii*.) The results in Fig. 1 demonstrate that the S-shaped curve characteristic of many biological systems also denoted the response for our system. Since both curves for the dissimilar vaccines had regions that varied linearly with a vaccine dose over approximately a 2-log range, it seemed proba-

FIG. 1. Fever responses of groups of six guinea pigs inoculated intraperitoneally with graded doses of Rocky Mountain spotted fever vaccine and challenged 10 days later with 10,000 guinea pig 50% infectious doses of *Rickettsia rickettsii*. (A) Commercial vaccine; (B) vaccine prepared by sucrose density gradient zonal centrifugation. Standard error of the mean is shown for each group.

ble that vaccines could be compared on the basis of the dose at the midpoint of the linear portion. This value, the 50% protective dose (PD_{50}), was defined as the vaccine dose at which the ordinate on the curve was one-half the average number of fever units of the controls.

Two experiments that illustrate how this method was applied and that also indicate the reliability of the method are presented in Fig. 2. In experiment 1 (Fig. 2A), one Rocky Mountain Laboratory vaccine was diluted in M/15 phosphate-buffered saline at pH 7.2 in fourfold steps at two separate times on the same day and inoculated into guinea pigs (treatment A and B). After 10 days, two ampoules of the rickettsial challenge stock were diluted separately and one preparation was used to inoculate one series of animals (A), and the other was used for the second series (B) and for eight control animals. The same procedure was followed for experiment 2 (Fig. 2B), except that (i) a different but similarly prepared Rocky Mountain Laboratory vaccine was used, and (ii) there were two sets of control animals, one for each series. After the fever data were obtained, the means for each group of animals in each series were entered in the calculator which was programmed to calculate and plot the least squares

regression line relating fever units (y) to $\log_{10}$ vaccine dose (x). (For these experiments all values of y were plotted. In practice, if fourfold dilutions of vaccine were tested, only those three or four values of y that bracket one-half of the fever units of the controls should be plotted.) Then the mean fever units for the control group was used to calculate the PD_{50} . PD_{50} values (95% confidence limits) were for experiment 1A: 0.0109 μ g (0.0050 to 0.0204), experiment 1B: 0.0084 μ g (0.0039 to 0.0183), experiment 2A:

FIG. 2. Reproducibility of dose-response curves in guinea pigs given graded doses of gradient-purified Rocky Mountain spotted fever vaccine in duplicate experiments. Different vaccines were used for each experiment, but animals in treatment groups A and B of each experiment received the same calculated doses of the same vaccine diluted on two separate occasions. The horizontal lines, which represent one-half of the average number of fever units exhibited by the control animals, strike the response curves at the PD_{50} dose levels indicated by vertical lines. Standard error of the mean is shown for each group.

0.0035 μg (0.0015 to 0.0079), and experiment 2B: 0.0091 μg (0.0014 to 0.0570).

Data from experiments 1 and 2 were subjected to analysis of variance to help evaluate the significance of differences between treatment groups (5). The probability that differences in fever responses for treatments A and B could occur by chance alone was 0.45 for experiment 1 and 0.10 for experiment 2. Differences in parallelism of the slopes for treatments A and B could occur by chance at a probability of 0.75 for experiment 1 and 0.10 for experiment 2. Differences in PD_{50} values for series A and B could occur by chance with a probability of 0.5 for experiment 1 and 0.2 for experiment 2. Agreement, therefore, between trials in experiment 1 was good. Differences observed in experiment 2 were greater but were not significant at the 0.05 level, the standard usually applied to data from biological experimentation. When one considers the many opportunities for variation in this kind of an experiment, differences of the magnitude observed in experiment 2 are not unexpected.

Even though the vaccines and challenge rickettsiae were derived from yolk sacs and administered by the same route, it is unlikely that a host immune response to yolk sac antigens contributed significantly to the degree of resistance observed in these experiments. The vaccine with little or no yolk sac antigen, as determined by the complement fixation test (2), induced a much higher level of resistance in guinea pigs than did the commercial vaccine with a considerable amount of yolk sac antigen. If yolk sac antigen had participated in the response, the opposite result would have been obtained.

There are several reasons why this assay procedure is superior to that promulgated in 1945. First, in contrast to the "official" method, the protective activity of the vaccines may be obtained by our method. Thus, one can readily compare different vaccines or different methods of preparation of vaccines. Bioassays based on the average number of days of fever experi-

enced by test animals have been described (4), but, in our opinion, such data are less informative than those relating to the total amount of fever. Our results show that fever areas can vary two-fold from animal to animal even though the number of days of fever may be the same. Second, data obtained from these assays may be analyzed by standard statistical procedures; the calculated standard errors and confidence intervals adequately describe the reliability of the estimated PD_{50} values. Finally, rickettsial challenge material can be prepared in large quantities and standardized so that numerous vaccines can be tested in guinea pigs challenged in a reproducible way. For these reasons we believe that the official procedure for the assay of Rocky Mountain spotted fever vaccines can be improved by adopting some of the techniques described above.

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