mones and their specific antibodies should make an interesting subject for further investigation.

*Summary.* Studies assessed the extent to which insulin-$^{131}$I and unlabeled insulin exchanged in antiserum from immunized guinea pigs. Comparisons of patterns of radioactivity obtained after agar gel electrophoresis of antiserum containing labeled and stable insulin, added in reversed sequences, revealed almost no exchange between insulin-$^{131}$I and crystalline insulin.

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## Passive Hemolytic Antibody Response of Primed, Partially Resistant Mice to Heat-Killed *Salmonella typhimurium* Vaccine* (33027)

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There is considerable literature concerning the response to *Salmonella typhimurium* of mice in terms of increased resistance and antibody formation following administration of various types of immunizing agents. In some cases, attempts have been made to correlate these two parameters. Kenny and Herzberg (1) were able to demonstrate that the rise in bactericidal antibody corresponded to development of resistance against challenge with viable organisms. They also indicated that appreciable levels of resistance and antibody formation occurred whether the immunizing agent was a killed vaccine, or a live avirulent one. Other workers have felt that inactivated vaccines are of relatively little value in enhancing resistance as compared to live organisms (2,5).

The present study suggests that the efficacy of a heat-killed vaccine depends to a considerable extent on the spacing of priming dose and challenge, and that circulating antibodies formed against the "O" antigen (lipopolysaccharide) do not correlate particularly well with the resistance induced.

*Materials and Methods. Bacterial cultures.* *S. typhimurium*, strain SR-11[1] was routinely maintained by subculture on Trypticase soy agar[2] (TSA). The subcultures were held at 5°C. New stock cultures were revived from lyophilized suspensions at approximately 6-month intervals.

Viable bacterial suspensions for challenging mice were prepared by growing the organisms on TSA for 16–18 hours at 37°C, and then suspending the growth in 0.85% sodium chloride solution. The optical densities of the suspensions were adjusted to a reading of 440 using the blue (no. 47) filter on the Klett photoelectric colorimeter. Tenfold serial dilutions were prepared and were counted by the method of Miles *et al.* (6) using calibrated 18-gauge dropping needles. The counts of such suspensions remain constant within the limits of error of the counting method for at least 3 hours. Counts of the Klett-adjusted suspensions varied between 5.22 to 5.89 $\times$ $10^9$ viable cells/ml.

*Determination of the 50% lethal dose* ($LD_{50}$). Graded doses of the suspension were inoculated in 0.2-ml quantities intraperitoneally into mice. Deaths among the animals

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[1] Kindly furnished by Dr. R. E. Chamberlain, Smith Kline and French Laboratories, Philadelphia, Pennsylvania.

[2] Difco Laboratories, Detroit, Michigan.

were recorded daily for 20 days after challenge. Dead animals were removed and frequently checked by autopsy and culture for infection with *S. typhimurium*. Results were analyzed by the method of Shortley and Wilkins (7). This method is based on the assumption that the individual organisms constituting a challenge dose act independently. Using this assumption, the authors asserted that the probability of response $R$ of a host to a dose of organisms, $d$, is given by:

$$R = 1 - e^{-0.69(d/d_0)}$$

where $0.69 = \log_e 2$; $e$ = base of natural logarithms; and $d_0$ is the median effective dose. The term $LD_{50}$ is more familiar and is used in place of $d_0$ throughout this paper. The estimation of the $LD_{50}$ by graphic methods involves plotting the $\log_{10}$ of the dose against the so-called Weibull transformation of the percentage response, $\log_{10}[-\log_e(1 - R)]$. We used a program designed to calculate a best-fitting regression line by the method of least squares,[3] employing an IBM 1620 computer.

*Preparation and evaluation of vaccines.* Vaccines used for the experiments reported herein have been exclusively of the "O" type (alcohol-treated, heat-killed) prepared by methods outlined by Burrell and Mascoli (8) from TSA-grown salmonellae. Fifty percent effective doses ($ED_{50}$) at different time intervals before challenge or secondary stimulation were determined by inoculating graded doses i.p. The animals so immunized were challenged with 100 $LD_{50}$ of viable cells, prepared as described above. The $ED_{50}$ values were calculated in a manner similar to that used for the $LD_{50}$.

*Mice.* Swiss albino mice weighing 20–25 gm at the time of receipt were obtained from a single producer. The mice were housed 5 to a cage in polypropylene (autoclavable) cages and fed Purina laboratory chow and water *ad libitum*. Mouse-to-mouse transmission of *S. typhimurium* infection does not occur under these conditions (9). When the animals were sacrificed, blood was obtained by cardiac puncture; otherwise, by retro-orbital puncture.

Base-line sera of these mice did not contain any antibodies against *S. typhimurium* detectable by passive hemagglutination, passive hemolysis, bactericidal antibody assay, or Coombs-type passive hemagglutination tests.

*Serology.* Sheep red blood cells (SRBC) were modified by the method of Neter *et al.* (10) in a solution of *S. typhimurium* lipopolysaccharide (LPS)[4] in phosphate-buffered saline at pH 7.2. The LPS was of the type which is extracted from bacterial cells by the Westphal method. Preliminary titration with known positive serum indicated that the optimum concentration for modification of SRBC was 80 μg/ml. The LPS was boiled for 1 hour before use in the modification procedure. Complement used for passive hemolysis was obtained as lyophilized guinea pig serum from the Colorado Serum Co., Denver, Colorado. After reconstitution with distilled water it was absorbed routinely with unmodified and modified SRBC. The end point of titrations was considered to be the lowest dilution which showed distinct hemolysis after centrifugation at 500*g* for 5 min.

The passive hemolysis technique was adapted for the microtiter procedure (11), using equipment manufactured by Cooke Engineering Company, Falls Church, Virginia. Unmodified cells were used as controls with each serum. In occasional instances, antibodies in mouse sera against SRBC were noted. Such sera were absorbed with unmodified SRBC and the tests repeated.

*Results.* The determination of 50% effective dose ($ED_{50}$) of *S. typhimurium* "O" vaccine at various time intervals before challenge: The single dose of *S. typhimurium* "O" vaccine which would be required to protect 50% of the mice against a challenge of 100 $LD_{50}$ of viable organisms was determined at different time intervals between immunization (priming) and challenge. Each $ED_{50}$ determination was carried out using freshly prepared graded doses of vaccine ("Materials and Methods") on 50–60 mice; 10 mice were used for each dose.

[3] Prepared by Mrs. Joan Miller and Frederic G. Ransom.

[4] Obtained from Difco Laboratories, Detroit, Michigan.

TABLE I. Mean Effective Doses of *S. typhimurium* Vaccine at Various Prechallenge Time Intervals.

| $ED_{50}$ at: (days) | No. of organisms ($\times 10^4$) |
|---|---|
| 30 | 68 |
| 21 | 288 |
| 15 | 7 |

Table I shows that this quantity of vaccine, as measured by the bacterial cell content, varied considerably. Thus, a relatively high dose ($288 \times 10^4$ bacteria) was required to produce resistance in 50% of the mice when the vaccine was given 21 days before challenge. The $ED_{50}$ at 30 days was intermediate between these two values ($68 \times 10^4$ bacteria).

The effect of $ED_{50}$ priming doses on circulating lipopolysaccharide antibodies following secondary stimulation: When animals are challenged with viable organisms, their responses may be very complex. The viable challenge does not remain static, but may either multiply or decrease, changing the total mass of antigenic material. Further, the administered organism may move into various anatomical loci in successive waves, so that the situation comes to resemble a series of repeated antigenic stimuli. In order to simplify the situation for the purpose of studying antibody response of the primed animals following challenge, the equivalent of the standard challenge dose (100 $LD_{50}$) was given in heat-killed organisms ($1.64 \times 10^7$ bacteria).

*Absence of antibodies at the time of challenge.* A group of 52 mice was subdivided into subgroups of 13 each. These were treated according to schedules corresponding to 3 $ED_{50}$ doses at various selected time intervals and one unimmunized control group, as shown in Table I.

At the expiration of the time interval for each schedule, blood was taken for antibody determination by passive hemolysis ("Materials and Methods"). All animals were devoid of detectable antibodies.

*Development of circulating antibodies following secondary stimulation.* Forty mice which had received priming doses corresponding to the $ED_{50}$ at 15, 21, and 30 days, respectively, as indicated in the preceding paragraph were challenged with the equivalent of 100 $LD_{50}$ in killed organisms ($1.6 \times 10^7$ bacteria). The 40 animals were divided into groups of 10 each for each $ED_{50}$–time interval set and one control group which had not previously been immunized. They were bled by retro-orbital puncture at 3, 5, 10, 15, and 20 days after the secondary challenge.

All sera were examined for hemolyzing antibodies. The mean total serum titers, expressed as $1 + \log_2$ of the reciprocal of the end-point dilution, are shown in Fig. 1.

One might assume that the comparable status of resistance of all of these groups toward the viable challenge agent might be reflected in similar patterns of antibody response. This was not the case; the patterns elicited by the second dose of antigen varied over a wide range. Comparison of the immunized animals with the controls (Fig. 1) suggests that the chief advantage of all groups of partially resistant animals lies in the more prompt secondary response, so that within 3 days after challenge, at the time when high mortality begins in fully susceptible animals, considerable levels of circulating antibody were available. Although wide differences occurred among the groups, the early high

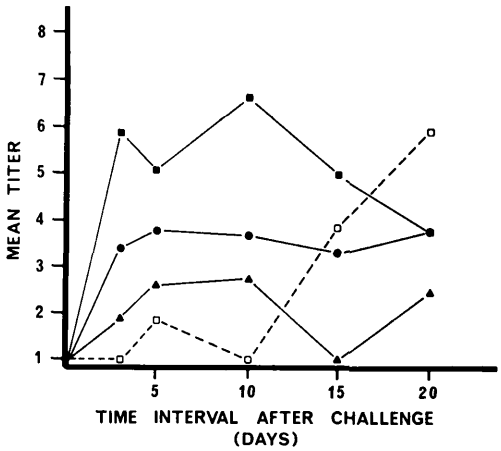


FIG. 1. Response of primed mice to a secondary challenge as measured by passive hemolysis. (■), Priming dose, $288 \times 10^4$ bacteria 21 days before secondary challenge; (●), Priming dose, $68 \times 10^4$ bacteria 30 days before secondary challenge; (▲), Priming dose, $7 \times 10^4$ bacteria 15 days before secondary challenge; and (□) Control, no priming dose.

levels such as that reached by the $ED_{50}$[21] group apparently conferred no particular advantage, since these animals would presumably have been no more resistant than those belonging to the other $ED_{50}$ classes had a viable challenge agent been administered.

The control group which received a 100 $LD_{50}$ equivalent dose without previous immunization, made an excellent primary response rather late in the day, apparently after the issue of death or survival would have been decided in the case of a viable challenge.

Moderate to high levels of hemolytic antibody persisted throughout the period 3–10 days after secondary stimulation, followed by a decrease during the period 10–15 days. At this time, a secondary rise occurred in the groups which had received primary immunization at 15 and 30 days before secondary stimulation.

Groups of 3 mice for each $ED_{50}$–time interval set which received no secondary immunization did not at any time show positive serology, nor did a group of mice which received no immunization at all ("environmental" controls).

*Discussion.* Space does not permit a full discussion of the experimental approaches which have been tried in studying the immune reactions of mice to *S. typhimurium.* A wide variety of antigens, challenge doses, routes of immunization and challenge, and immunization schedules have been used. In general it is agreed that the somatic "O" antigens (lipopolysaccharide) are of prime importance in immunization against salmonelloses (12). The belief has also been frequently expressed that live avirulent vaccines are more effective than killed vaccines (3,4).

The present work indicates that single moderate doses of killed vaccines are effective in producing infection immunity provided that the time interval to challenge is optimally matched to the priming dose. The data do not clearly reveal the reasons for the increased requirement for antigen when mice were primed at 21 days as compared to 15 days (Table I) nor why the antigen requirement for 30 day priming should have been intermediate between these two. A clue is provided by the finding of Jenkin and Karnovsky (13) that the magnitude of the secondary response is dependent on the antigen–antibody ratio at the time of challenge, although as discussed below, the antibody component of this ratio would have to be at a less than detectable level in this system.

The magnitude of the antibody response (Fig. 1) was in the same order as the antigen requirement, e.g., the 21-day priming dose gave the highest response, the 15-day priming dose the lowest response, and the 30-day priming dose was intermediate between these two. These differences were apparently not significant for resistance to viable challenge, since all three groups of animals shared similar levels of survivorship. The other feature shared by all three groups was the lack of detectable antibody against LPS at the time of challenge. In conclusion, the kinetics of the secondary response appear to involve a relationship between antigen priming dose, the interval between priming and challenge, and perhaps an as yet undefined interaction between antigen and antibody at the cellular level.

*Summary.* Mean effective doses (50% protection) of *S. typhimurium* heat-killed, alcohol-treated bacterial vaccine depended upon the interval before challenge at which the single dose was given. Circulating antibody response against the lipopolysaccharide component of the organism appeared to be related to the magnitude of the mean effective dose, but not to the partial immunity developed. Killed vaccine was an effective immunizing agent if given at an optimal time interval before challenge.

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# The Interaction* between K Depletion and $CO_2$ Inhalation on Intracellular Bicarbonate Content (33028)

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In recent work from our laboratory (2) the plasma $pCO_2$ of control and K-deficient rats was altered between 20 and 90 mm Hg with a respirator and the intracellular pH ($pH_i$) of skeletal muscle measured by 5,5-dimethyl-2,-4-oxazolidinedione (DMO) distribution. Intracellular bicarbonate content ($[HCO_3^-]_i$ at the various pressures of $CO_2$ was calculated by substitution of $pH_i$ values into the Henderson–Hasselbalch equation. The $[HCO_3^-]_i$ of low-K muscle (together with $pH_i$) was found generally to be lower than that of normal muscle.

An alternative method of obtaining $[HCO_3^-]_i$ is by derivation from the total $CO_2$ content of muscle. The $CO_2$-derived $[HCO_3^-]_i$ values for potassium deficient muscle have been reported from several laboratories. The data of one report (3) suggest that low-K $[HCO_3^-]_i$ is reduced to less than half the $[HCO_3^-]_i$ of normal muscle. This marked reduction is at variance with the slight reduction reported from another laboratory (4). More recently, Hudson and Relman (5), using a modified $CO_2$ method, found no significant change in the $[HCO_3^-]_i$ of muscle from potassium depleted rats. In view of these differences, it was decided to determine the $CO_2$-derived $[HCO_3^-]_i$ of our low-K rat muscle, the object being to see if the $[HCO_3^-]_i$ values so obtained would agree with, or at least parallel, values calculated from DMO determined $pH_i$.

Our recent work (2) showed also that the reduction in low-K $[HCO_3^-]_i$ (calculated from DMO derived $pH_i$) was not constant at all levels of $pCO_2$. As $pCO_2$ was increased, less $HCO_3^-$ was generated in low-K than in normal muscle. The different rate of bicarbonate increase caused the slope representing low-K muscle in a pH–bicarbonate diagram [see Fig. 2 in Ref. (2)] to be significantly less than the slope representing control muscle. It was concluded from this slope difference that the *in vivo* buffer capacity of low-K muscle is less than that of normal muscle. The present experiments were designed to determine whether a similar, unequal intracellular bicarbonate increase would occur when deriving $[HCO_3^-]_i$ from tissue $CO_2$ content.

*Methods.* The present investigation consisted of four series of experiments with control or low-K male rats (Wistar strain) weighing 280–300 gm. The control and low-K regimens previously described (6) were

* The term interaction is used here in its statistical meaning. That is, to indicate the additivity (or lack of it) of the effect of two stresses, K depletion and $CO_2$ inhalation, on extracellular and intracellular bicarbonate content. See Snedecor (1) for discussion of the factorial experimental design.