1. Wissler, R. W., Frazier, L. E., Soules, K., Barker, P., Bristow III, E. C., *A.M.A. Arch. Path.*, 1956, v62, 62.

2. Wissler, R. W., Fitch, F. W., La Via, M. F., Gunderson, C. H., *J. Cell. and Comp. Physiol.*, 1957, Supp. 1, 50.

3. La Via, M. F., Uriu, S., Ferguson, L. A., *J. Immunol.*, 1960, v84, 48.

4. Cannon, P. R., Chase, W. E., Wissler, R. W., *ibid.*, 1943, v47, 133.

5. La Via, M. F., Barker, P. A., Wissler, R. W., *J. Lab. and Clin. Med.*, 1956, v48, 237.

6. Taliaferro, W. H., Taliaferro, L. G., *J. Immunol.*, 1951, v66, 181.

7. Kass, E. H., Finland, M., *Ann. Rev. Microbiol.*, 1953, v7, 361.

8. Spurr, C. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 259.

9. Schwartz, R., Stack, J., Dameshek, W., *ibid.*, 1958, v99, 164.

10. Uphoff, D. E., *ibid.*, 1958, v99, 651.

11. Dutton, R. W., Dutton, A. H., George, M., *Nature*, 1958, v182, 1377.

12. Schwartz, R., Eisner, A., Dameshek, W., *J. Clin. Invest.*, 1959, v38, 1394.

13. Berenbaum, M. C., *Nature*, 1960, v185, 167.

14. Sterzl, J., *Nature*, 1960, v185, 256.

15. Dutton, R. W., *Biochem. J.*, 1960, v74, 24P.

16. Sterzl, J., *Folia Microbiol.*, 1959, v4, 91.

17. Taliaferro, W. H., Talmage, D. W., *J. Infect. Dis.*, 1955, v97, 88.

18. Elion, G. B., Hitchings, G. H. in *Ciba Foundation Symposium on Chemistry and Biology of Purines*, Little, Brown and Co., Boston, 1957.

19. Dittmer, K., Frazier, B. E., Wissler, R. W., Cannon, P. R., *Science*, 1950, v111, 94.

Received May 20, 1960. P.S.E.B.M., 1960, v104.

Immunization Studies with Living Vaccine of *Salmonella typhimurium*. (25909)

L. S. BARON AND S. B. FORMAL

Division of Immunology, Walter Reed Army Inst., Washington, D. C.

Production of artificial immunity to Salmonella infections in both man and animals is a problem which for the most part remains unresolved. Only recently has a properly controlled field study shown that typhoid vaccination confers some level of protection against infection in man(1). In laboratory animals, some information indicates that recovery from the disease yields best immunity to reinfection. In the past, such results were explained on basis of selection of innately resistant animals initially present(2). However, Hobson(3) recently showed that mice infected with relatively avirulent strain of *Salmonella typhimurium* developed a resistance to reinfection with virulent strains without appreciable deaths occurring from initial infection. Studies using killed *S. typhimurium* vaccines to immunize against mouse typhoid demonstrated that these vaccines offer only low level of protection against infection under natural conditions(4) and are only somewhat more satisfactory against challenge by intraperitoneal route(5). In fowl typhoid, where recent studies have indicated that naturally recovered chickens are immune to reinfection with *Salmonella gallinarum*(6), Smith(7) showed that dead vaccines had little or no protective effect while good immunity was produced by live vaccines. In view of these findings, and since our previous attempts to protect against mouse typhoid with killed vaccines have been unsuccessful, a living avirulent mutant of *S. typhimurium* was sought for further investigation. Isolation of streptomycin-dependent bacterial strains by Miller and Bohnhoff(8) suggested a technic which could be employed to obtain an avirulent mutant of *S. typhimurium*. Herzberg and Elberg(9,10) reported isolation and characteristics of such mutant of *Brucella melitensis* and on use of this variant in immunization studies. This communication gives results in which living vaccines were employed to protect mice against intraperitoneal challenge with a virulent culture of *S. typhimurium*.

Materials and methods. Cultures. An antigenically complete culture of *S. typhimu-*

rium (1,4,5,12;i,1,2) designated as strain 10, was obtained from culture collection of Walter Reed Army Inst. of Research. Within 7 to 14 days intraperitoneal injection of as few as 10^2 cells of this strain resulted in death of 90-100% of mice of the Bagg strain. This culture and a streptomycin-dependent (S^d) mutant derived from it were employed in initial experiments. The S^d mutant was obtained by plating approximately 5 x 10^{10} cells of strain 10 on nutrient agar containing 0.6 mg/ml of streptomycin sulfate (Pfizer). A colony was observed after 5 days incubation at 37°C which, on further testing, proved a streptomycin-dependent, but otherwise unchanged, derivative of parent culture. The cultures were grown on nutrient agar (Difco), or with S^d variant of strain 10, on nutrient agar supplemented with 0.6 mg of streptomycin/ml. *Immunization tests.* Living vaccines were prepared from washed cells of 16 hour cultures standardized turbidimetrically to contain 10^9 bacteria/ml. Killed vaccines were obtained by heating aliquots of living preparation at 56°C for 1 hour. Mice were immunized with 0.5 ml of appropriate vaccine intraperitoneally and challenged by same route with 5 x 10^7 cells of *S. typhimurium* strain 10 eighteen days later. This high challenge dose was chosen to provide a stringent test for the vaccine thereby eliminating evidence of low level immunity previously reported(5).

Results. Preliminary experiments demonstrated that some mice injected intraperitoneally with 10^7 washed S^d cells of *S. typhimurium* died in 3 weeks following inoculation. Post-mortem cultures revealed presence of streptomycin independent *S. typhimurium*. *In vitro* tests were then carried out to determine frequency of reversion of the S^d strain to streptomycin independence. When 10^8 washed S^d cells were plated on nutrient agar lacking streptomycin, approximately 4-5 colonies appeared in 24 hours. These colonies were independent of and sensitive to streptomycin and were in sharp contrast to the slow residual growth of the cell population plated, occurring probably due to presence of intracellular streptomycin not removed by the washing process. As a result of this residual growth, further reversions to streptomycin independence were observed during the next few days of incubation. In view of the high virulence ($LD_{90-100} = 10^2$ or fewer cells injected intraperitoneally) of the strain of *S. typhimurium* employed, the presence of even one reverted cell is undesirable.

It is known that purine-requiring mutants of *S. typhosa* and *Klebsiella pneumoniae* do not multiply in the peritoneum of mice due to absence of purines in this region(11,12,13). Thus, to minimize or effectively eliminate reversions from avirulent to virulent state *in vivo*, a streptomycin-dependent, purine-requiring mutant of *S. typhimurium* was isolated by subjecting the S^d strain to ultraviolet irradiation followed by penicillin technic of Lederberg and Zinder(14). Studies to determine frequency of back mutation of this 'double mutant' to the purine-streptomycin independent state indicated that such double reversions could not be detected by either *in vitro* or *in vivo* procedures.

A bacteriologic study of the fate of this double mutant strain following intraperitoneal injection into mice was then undertaken. The number of organisms of this strain which could be administered without causing deaths in mice was of the order of 5 x 10^8 bacteria. Blood, liver, spleen and peritoneal contents of groups of 10 mice were examined by streaking from these areas on plates of nutrient agar containing streptomycin at daily intervals following inoculation of the cells. The results indicated a clearance of organisms within 72 hours, such that no instances of positive blood or spleen cultures were detected with the method after 72 hours. At this time the peritoneal fluid was also free of organisms. When bacterial counts were made on individual whole mice at periods after immunization using Waring blender technic(15), a decrease in total number of organisms from original inoculum of 5 x 10^8 cells to 1 x 10^5 cells occurred at the end of 7 days with a further decrease to about 5 x 10^4 cells at the end of 18 days. This double mutant strain was then employed in immunization experiments.

Groups of mice were immunized with a single injection of either the double mutant, a

TABLE I. Comparison of Protection Afforded Mice against Intraperitoneal Challenge by Heat-Killed and Living Vaccines of *S. typhimurium*.

Vaccine	Deaths/Total*
Heat-killed *S. typhimurium*, wild type	22/48
Heat-killed *S. typhimurium*, double mutant (S^d Pur^-)	20/49
Live *S. typhimurium*, double mutant (S^d Pur^-)	20/50
Heat-killed *S. flexneri*	47/47
No vaccine	49/49

* Challenge dose = 5×10^7 *S. typhimurium* administered intraper. 18 days after immunization with 5×10^8 cells of the vaccines.

heat-killed preparation of these same cells, or a heat-killed *Shigella flexneri* vaccine. The *S. flexneri* vaccine was included as a control against possible development of a non-specific immunity similar to that described by Landy and Pillemer(16). Immunized mice and untreated controls were challenged 18 days after immunization with 5 x 10^7 cells of the virulent, wild type strain of *S. typhimurium*. The results showed that when this high challenge dose was employed, the living vaccine was not superior to heat-killed vaccine in protecting mice. A typical protocol is presented in Table I.

Discussion. These studies confirm observations that heat-killed vaccines of *S. typhimurium* do not confer a high level of protection on mice against intraperitoneal challenge. An attempt was made to determine if living vaccine would yield better results. However, when a streptomycin-dependent mutant of a highly virulent strain was used as living vaccine, it proved unsafe; the combination of a relatively high reversion rate to streptomycin independence and high virulence of the streptomycin independent form was sufficient fatally to infect an appreciable number of mice receiving this preparation. To prevent reversion to the virulent state, addition of a purine requirement to the streptomycin-dependent strain was accomplished yielding a culture which was safe for use in mice as a living vaccine. Nevertheless, this immunizing agent did not prove to be significantly better than heat-killed vaccine in protecting animals against challenge doses administered by the intraperitoneal route.

Summary. A streptomycin-dependent mutant was isolated from a virulent strain of *S. typhimurium* for evaluation as a living vaccine in mice. *In vivo* and *in vitro* studies indicated that this strain was unsafe as an immunizing agent due to reversions to streptomycin independence. A mutant of the streptomycin-dependent strain was obtained requiring purine, a nutritional factor absent in mouse peritoneum. Reversions of this double mutant strain to the purine-streptomycin-independent state could not be detected. The results of experiments comparing this living vaccine with heat-killed preparation indicated that the living vaccine was not superior in protecting mice.

1. Cvjetanovic, B. B., *Am. J. Pub. Hlth.*, 1957, v47, 578.
2. Gowen, J. W., *Am. J. Hum. Genet.*, 1952, v4, 285.
3. Hobson, D., *J. Hyg., Camb.*, 1957, v55, 334.
4. Greenwood, M., Topley, W. W. C., Wilson, J., *ibid.*, 1931, v31, 257.
5. MacLeod, D. R. E., *ibid.*, 1954, v52, 9.
6. Smith, H. W., *J. Comp. Path.*, 1955, v65, 37.
7. ———, *J. Hyg., Camb.*, 1955, v54, 419.
8. Miller, C. P., Bohnhoff, M., *Science*, 1947, v105, 620.
9. Herzberg, M., Elberg, S., *J. Bact.*, 1953, v66, 585.
10. ———, *ibid.*, 1954, v69, 432.
11. Bacon, G. A., Burrows, T. W., Yates, M., *Brit. J. Exp. Pathol.*, 1951, v32, 85.
12. Garber, E. D., Hackett, A. J., Franklin, R., *Proc. Nat. Acad. Sci.*, 1952, v38, 693.
13. Formal, S. B., Baron, L. S., Spilman, W., *J. Bact.*, 1954, v68, 117.
14. Lederberg, J., Zinder, N., *J. Am. Chem. Soc.*, 1948, v70, 4267.
15. Berry, L. J., *Ann. N. Y. Acad. Sci.*, 1955, v62, 327.
16. Landy, M., Pillemer, L., *J. Exp. Med.*, 1956, v104, 383.

Received May 23, 1960. P.S.E.B.M., 1960, v104.