Modification of *Salmonella typhimurium* Infection of Embryonated Eggs by Antiserum, Adult Splenic Tissue and Combinations of these Agents.* (28047)

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Numerous investigators have observed that chick embryos are unable to respond to antigenic stimulus by the production of specific antibody(1-8).

As long ago as 1906, Levaditi(9) observed that embryonated eggs derived from immunized mother hens were less susceptible to challenge by spirilla which were otherwise lethal. Buxton(10) demonstrated the transference of bacterial antibodies from the mother hen to the egg, and Watanabe and his co-workers(11) have demonstrated that such passive transfer of antibody leads to increased resistance of the embryos to challenge by *Salmonella pullorum*. Thus, it appears that although the embryonated egg is unable to produce antibody in response to antigenic stimulus, it is able to make use of antibody when the latter is incorporated by the mother hen.

To our knowledge, the study of antibody mechanisms of resistance to infectious challenge in embryonated eggs has been limited to the neutralization approach (reviewed by Smadel, 12), whereby an infectious agent is mixed with diluted antiserum, allowed to react for a time, then injected into eggs by various routes. Apparently, this technic depends on the direct virucidal or bactericidal activity of the antiserum on the infectious agent, the host animal being used merely to titrate the infectious agent.

Sibal and Olson(13) showed that antibody may be produced in the embryonated egg by transfer of spleen cells or leukocyte suspension from an immunized adult donor, without further contact with antigen. These results were confirmed by Isacson(14).

In these experiments antibody production has been evaluated by serological methods. Various workers have noted phagocytosis in embryonic tissue and convincing evidence has left no doubt that the phagocytic activity begins early in embryonic development (reviewed by Kent, 15).

During research directed toward the evaluation of reticuloendothelial - stimulating agents(16), we observed that injection of antiserum into embryonated eggs prior to challenge modified their susceptibility to *S. typhimurium*, and that implantation of splenic tissue from adult normal or immunized donors also modified susceptibility. Furthermore, combinations of spleen tissue and antiserum induced further modifications. This paper describes the alterations in susceptibility observed.

Materials and methods. Immunization of adult White Leghorn hens, weighing 2 to 3 kg, obtained from a commercial hatchery, was accomplished by repeated injections of an alcohol-killed vaccine, prepared from the stock *Salmonella typhimurium* used for challenge (agglutination titre anti "O" 1:640 to 1:1280). The donor hens (normal or immune, in this case 3 days after the final stimulation) were killed by decapitation. Immediately thereafter the abdomen was opened and the spleen removed aseptically. The excised spleen was then rinsed with sterile Medium 180 (Capell†), cut into 5-6 large fragments, rinsed again, and minced with scissors in sterile Medium 180 until an adequate number of approximately 1 mm fragments were obtained. Tissue fragments were introduced onto the chorioallantoic membrane (CAM) of 8-day-old embryonated White Leghorn eggs, obtained from the same commercial hatchery, with sterile forceps. The eggs were incubated at 35°C in a humidified incubator and were observed daily for mortality. Vascularization of the implant on the CAM is readily observed in 80 to 90% of the eggs at 3-4 days and only eggs showing definite

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TABLE I. Fifty Percent Survival Time (Days) of Embryonated Eggs Challenged with *Salmonella typhimurium* 20-40 Eggs Per Trial.

Treatment	SD_{50}*	95% confidence limits*	Trials
None	1.56	1.28-1.91	3
Normal hen's serum	1.70	1.43-2.02	2
Antiserum	2.67	1.99-3.60	3
Implant normal spleen	2.00	1.72-2.45	2
" " " plus antiserum	3.06	2.12-4.25	2
Implant immune spleen	2.13	1.27-2.94	3
" " " plus antiserum	4.43	2.55-7.28	3

* Average from trials shown; computed by method of Litchfield(24).

TABLE II. Average Gain in 50% Survival Time (Days).

Treatment	Avg gain in SD_{50}	Rank
Implant immune spleen plus antiserum	2.53	1
Implant normal spleen plus antiserum	1.61	2
Antiserum	1.12	3
Implant normal spleen	.54	4
Implant immune spleen	.49	5
Normal serum	.25	6

"takes" were included in the experimental results.

Antisera against *Salmonella typhimurium* were produced in rabbits or White Leghorn hens and evaluated by agglutination titre (anti "O" 1:640 to 1:1280) after heating at 56°C for 30 minutes. Sera were always inactivated before being injected into eggs.

Salmonella typhimurium, strain SR-11 (ST), was the challenge organism used. The challenge dose consisted of 9 to 50 viable cells, equivalent to 9-50 lethal doses.

Spleen implants were always made when the eggs were 8 days old. Antiserum was administered 90 minutes before challenge. Eggs were challenged when they were from 13 to 15 days of age.

All injections were made on the CAM, the same site being used when repeated injections were required. The dose of inoculum used was delivered in 0.1 ml amount.

Results are summarized in Tables I and II. In the experiments shown, the 50% survival time in days (SD_{50}) of embryonated eggs, challenged with *ST* was 1.56. Eggs receiving antiserum, having an anti "O" titre of 1:640 or more, administered on the CAM's before challenge, responded in a variable manner. They experienced increased survival time (SD_{50} – 2.67), and average gain in 50% survival time was 1.12 days. When the serum used was from an unimmunized adult fowl, the gain in survival time or in survivorship was not significant (SD_{50} – 1.70; average gain in SD_{50} – 0.25).

Chick embryos implanted with splenic tissue from a normal unimmunized adult fowl or from an adult fowl donor which had previously been immunized, showed a slight enhancement of resistance (SD_{50} – 2.00 and 2.13 respectively; average gain in SD_{50} – 0.54 and 0.49 respectively).

Increased resistance became truly significant when a combination of spleen and antiserum was used. When the splenic tissue was taken from an immunized adult fowl, the effect was greater (Table I).

The average gain in 50% survival time in days permits us to rank the various categories from greatest to least enhanced resistance (Table II).

Discussion. These experiments demonstrate that resistance to infection of embryonated eggs by *ST* may be qualitatively modified by antiserum, by implantation of splenic tissue from normal or from immune adult fowl, or by combinations of these. The optimum category (rank 1, Table II) is related to both the presence of antiserum and tissue with a history of previous exposure to the antigen. The second category (rank 2, Table II) is that produced by the presence of antiserum and immunologically competent tissue which has not been previously stimulated with antigens of the challenge organisms. The chief determinant in each case was antiserum, since the absence of the latter reduced the difference between these categories (ranks 4 and 5, Table II).

Known mechanisms which may be invoked to explain these phenomena include (1) enhanced phagocytic capacity of the reticuloendothelial system; (2) increase in ability of RES cells to destroy captured organisms in-

tracellularly; (3) direct bactericidal effect of antiserum.

It is known(17) that Gram negative bacilli are slowly phagocytosed unless opsonized by antiserum, and that opsonins, in addition to promoting phagocytosis, may also accelerate the intracellular destruction of ingested bacteria. Antiserum alone does enhance resistance (Table I), and this fact may be related to a combination of increased clearing power and increased intracellular destruction of the challenge organisms. The observation that normal splenic tissue, and especially splenic tissue from a previously immunized adult donor, markedly enhances these functions could be explained by the addition of cells of phagocytic capacity. However, the statistical difference between results obtained with normal and previously immunized cells can only be explained if one assumes, as pointed out by Mackaness(18) and Miya *et al.*(19,20) that the cells themselves carry some additional competence as a result of the immunization process. On the other hand, this additional competence should be expressed in the absence of antibody. This does not appear to be the case (compare again ranks 4 and 5, Table II). Evidence from this laboratory(16) indicates that some activity of circulating antibody other than phagocytosis is operant in mice challenged with *ST*. The nature of this activity, both in eggs and mice, is being investigated.

It is unlikely that heat-inactivated antiserum acts directly upon the challenge organisms, because such bactericidal action is known to be complement-dependent(21,22). The absence of C′ in the embryonated chicken egg is well known(5,6). The only conceivable source of C′ in these experiments would be in the spleen explants from adult donors, but the dilution (at least 1 in 5,000) would be beyond the usually accepted range of C′ activity. Synthesis of C′ after transfer to the embryonated egg is a possibility which remains to be investigated. The evidence so far suggests that the action is indirect and host-mediated.

Procedures similar to those described may prove useful for study as a model of the host-parasite relationship with a balance very much against the host.

Summary. Embryonated chicken eggs showed increased resistance to infection with lethal doses of *S. typhimurium* as a result of treatment with specific antiserum, or by implantation of splenic tissue from adult chickens, or by combinations of these treatments. The treatments studied, in order of decreasing effectiveness, were: implantation of spleen tissue from immunized donor on 8th day, together with inoculation of antiserum on the day of challenge (13th or 15th day); implantation of spleen tissue from non-immunized donor together with antiserum treatment; antiserum alone; implantation of spleen tissue alone, either from normal or immunized donor; normal serum.

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1. Beveridge, W. I. B., Burnet, F. M., *Spec. Rep. Ser. Med. Res. Coun.*, London, No. 256, 1946.

2. Burnet, F. M., *Austr. J. Exp. Biol. Med. Sci.*, 1941, v19, 291.

3. Burnet, F. M., Stone, J. D., Edney, M., *ibid.*, 1950, v22, 291.

4. Grasset, F. G., *C. R. Soc. Biol., Paris,* 1929, v101, 1102.

5. Polk, A., Buddingh, G. J., Goodpasture, E. W., *Am. J. Pathol.,* 1938, v14, 71.

6. Sherman, H. W., *J. Inf. Dis.,* 1919, v24, 1; v25, 256.

7. Weinberg, M., Guelin, A., *C. R. Soc. Biol., Paris,* 1936, v122, 1229.

8. Buxton, A., *J. Gen. Microbiol.,* 1954, v10, 398.

9. Levaditi, C., *Ann. Inst. Pasteur,* 1906, v20, 924.

10. Buxton, A., *J. Gen. Microbiol.,* 1952, v7, 268.

11. Watanabe, S., Nagai, T., Hashimoto, K., Kume, T., Sakazaki, R., *Bull. Nat. Inst. Animal Hlth.,* Tokyo. 1960, v39, 37.

12. Smadel, J. E., *Viral and Rickettsial Infections of Man,* J. B. Lippincott, Phila., 1948, Chapt. 3.

13. Sibal, L. R., Olson, V. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 575.

14. Isacson, P., *Yale J. Biol. and Med.,* 1959, v32, 209.

15. Kent, R., *J. Embryol. Exp. Morph.,* 1961, v9, 128.

16. Ransom, J. P., Bliznakov, E., Pasternak, V. Z., Heller, J. H.,, *C. R. Soc. Biol.,* Paris, 1962, v156, 1022.

17. Howard, J. G., *Scot. Med. J.,* 1961, v6, 60.

18. Mackaness, G., *J. Exp. Med.,* 1960, v112, 35.

19. Miya, F., Marcus, S., Perkins, E. H., *J. Immunol.,* 1961, v86, 526.

20. Miya, F., Marcus, S., *ibid.*, 1961, v86, 652.
21. Muschel, L. H., *Ann. N. Y. Acad. Sci.*, 1960, v88, 1265.
22. Landy, M., Michael, J. G., Whitby, J. L., *J. Bact.*, 1962, v83, 631.
23. Muschel, L. H., Treffers, H. P., *J. Immunol.*, 1956, v76, 1.
24. Litchfield, J. T., Jr., *J. Pharm. Exp. Ther.*, 1949, v97, 399.

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Nucleic Acid Content of Mammary Glands of Pregnant Rats.*† (28048)

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Deoxyribonucleic acid (DNA) content as a measure of cell numbers and ribonucleic acid (RNA) content as an estimate of protein synthesis were first applied to mammary tissue by Kirkham and Turner(1). Since DNA content per mammary cell nucleus was constant in glands of pregnant and of lactating rats(2), DNA content of mammary tissue may be used directly as an index of development. Histological studies of the mammary gland of the rat have indicated that the gland reached its maximum growth, so far as the number of epithelial cells was concerned, by the thirteenth day of pregnancy(3) and that evidence of cellular proliferation was extremely rare in the second half of pregnancy (4). Results of a cytological study(5) and experiments based on colchicine technic(6) and DNA content (7,8) however, have indicated that mitosis continued throughout pregnancy. A very limited number of RNA values have been reported for rat mammary glands during pregnancy(1,7).

DNA and RNA content of mammary glands of the hooded Norway rat was determined at 7 stages of pregnancy. Protein synthetic activity per cell was estimated from the RNA/DNA ratio.

Methods. Six abdominal-inguinal mammary glands were obtained from rats pregnant 0, 4, 8, 12, 14, 18, and 20 days (12 animals per stage). Adult virgin female rats, sacrificed at a weight (approximately 170 g) corresponding to that of other female rats when first placed in breeding cages, represented day zero of pregnancy. Day one of pregnancy was the day sperm were noted in vaginal smears. DNA and RNA content was determined as previously described(2). The pattern of mammary gland development was obtained from mg of DNA per 100 g of body weight(9) and from total mg of DNA in the 6 glands. Amount of protein synthesis was estimated from total mg of RNA and the ratio of total RNA and total DNA estimated protein syntheic activity per cell.

Results and discussion. Six abdominal-inguinal mammary glands contained 2.77, 2.80, 3.33, 4.47, 5.69, 7.36, and 7.89 mg of DNA per 100 g of body weight as stage of pregnancy advanced. Thus, little mammary gland growth occurred during the first 4 days of pregnancy; by the eighth day, however, mammary development increased 20.2% over virgin controls (day zero). Glands continued to grow throughout pregnancy and at the 20th day the number of cells had increased 184.8% over virgin controls. Rate of cellular proliferation was greatest between days 12 and 14. Total mg of DNA showed a somewhat similar pattern. Averages obtained for the 7 stages of pregnancy were 4.76, 4.85, 6.03, 8.86, 11.50, 15.97, and 17.19 mg. Total DNA on days 8 and 20 increased 26.7 and 261.1%, respectively, over virgin controls. These values are greater than those observed when body weights were standardized (Table I). These results indicate that mammary hyperplasia occurs throughout pregnancy.

Advancing pregnancy caused total RNA to increase, as follows: 3.06, 3.08, 4.04, 7.00, 8.87, 13.94, and 15.44 mg. On the eighth

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