the seventh day post-ovulation the deciduomata may be smaller, equal to or larger than the normal conceptual swelling. They may increase in mass on the following day but necrosis is usually evident by the ninth day. Relatively little is known as yet of the life span of such deciduomata in the hamster.

The function of the glycogen may be to provide a rapidly available source of energy for the developing embryo and/or accessory structures, but no evidence on this point was obtained in the present study.

Whether the appearance of glycogen is due to a maternal or fetal stimulus is not certain. Since it appears in decidualization of pseudopregnancy when no fetal tissue is present a maternal origin seems most likely. Its accumulation may very well be triggered by the sequence of events associated with early gestation. These phenomena are common in pregnancy and pseudopregnancy, when the latter is accompanied by a stimulus sufficient to produce decidualization (*e.g.*, air injection), since simple pseudopregnancy without this additional stimulus results in no comparable opacity formation.

1. Orsini, M. W., *J. Reprod. Fertil.*, 1962, v3, 283.
2. ———, *ibid.*, 1962, v3, 288.
3. ———, in *Symposium on Delayed Implantation in Mammals,* A. C. Enders, ed., Univ. of Chicago Press, 1963, in press.
4. Seifter, S., Dayton, S., Novic, B., Muntwyler, E., *Arch. Biochem., Biophys.*, 1950, v25, 191.
5. Carroll, N. V., Longley, R. W., Rce, J. H., *J. Biol. Chem.*, 1956, v220, 583.
6. *Methods of Analysis of the Association of Official Agricultural Chemists,* 9th Ed., Assn. of Official Agri. Chemists, 1960, p643.
7. Chen, P. S., Jr., Toribara, T. Y., Warner, H., *Anal. Chem.*, 1956, v28, 1756.
8. Bunce, G. E., Ph.D. Thesis, Univ. of Wisconsin, 1960.
9. Orsini, M. W., Slautterback, D. B., unpublished experiments.
10. Revel, J. P., Napolitano, L., Fawcett, D. W., *J. Biophys. and Biochem. Cytol.*, 1960, v8, 575.

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## EDTA, Antiserum, and Restim as Factors in Infection of Mice with *S. typhimurium.* (28337)

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We presented evidence(1) that stimulation of the reticuloendothelial system (RES) by means of a yeast cell wall preparation (restim) did not result in enhanced resistance of mice to *Salmonella typhimurium* unless specific antibody were also present. The evidence suggested that some other activity of the RES, *e.g.*, intracellular digestion, must be involved in enhancement of resistance brought about by restim.

Elberg(2) reported that brucellae were made more susceptible to intracellular digestion by treatment with glycine. Repaske(3, 4) reported that Gram-negative bacilli which are normally insensitive to lysozyme were lysed at a rapid rate if they were pre-treated with versene (EDTA) in Tris buffer. In our hands, glycine has been ineffective in enhancing resistance of mice to *S. typhimurium* infection; however, EDTA has shown a significant effect when mice were pre-treated with restim. This report also shows that the blood of restim-treated mice has a higher content of lysozyme than that of normal mice.

*Methods and materials. Salmonella typhimurium* strain SR-11 was used as indicated in the previous report(1).

*Mice* of 20-25 g body weight were obtained from commercial sources and handled as previously reported.

*Opsonization of bacterial cells.* Rabbit anti-"O" serum against *Salmonella typhimurium,* having an "O" agglutination titer of 1:320, was heated at 56°C for 40 minutes to inactivate complement. To 9 ml of

1:100 heated antiserum, 1 ml of a suspension of bacteria containing approximately $10^6$ cells per ml, prepared by suspending the growth from a 24 hr culture on Trypticase Soy Agar (Difco) was added. Another 1 ml of bacterial suspension was added to 9 ml of 0.85% sterile NaCl. Suspensions were kept in an ice bath during preparation. Both diluted suspensions were incubated at 37°C for 20 minutes, following which they were washed 3 times in cold sterile saline, using a refrigerated centrifuge at 5°C. After the final centrifugation, the cells were resuspended in saline to the original volume. The final suspensions were further diluted 10-fold and used for challenging mice by the intraperitoneal route. Viable counts conducted by the method of Miles, Misra, and Irwin(5) indicated that the challenge doses (0.1 ml of suspensions) were 1,900 cells for the non-opsonized and 900 cells for the opsonized cells.

Experiments too lengthy to be reported here indicate that the observed differences are due to agglutination of the opsonized cells and not to bactericidal effects of the serum. This is in agreement with results reported elsewhere(6) for inactivated specific antiserum to Gram-negative bacilli.

*EDTA treatment of bacterial cells.* Ethylene diaminetetraacetate disodium* (EDTA) was made up in .05 M concentration in .05 M Tris-hydroxy aminomethane (THAM, Fischer reagent), which was then adjusted to pH 7.5 with N/1 HCl and sterilized at 15 lbs for 15 minutes in the autoclave. For these experiments, a control solution omitting EDTA was also made up. Cells were exposed to these solutions for 45 minutes at room temperature without significant decrease in count, as established by preliminary experiments, and were then washed twice, standardized to a Klett reading of 294 using the blue (No. 42) filter (approx. $2 \times 10^9$ cells/ml) and further diluted 1,000-fold. The final challenge doses were $1\text{-}2 \times 10^5$ cells per mouse.

*Restim,* a preparation of yeast cell walls (1), was given intravenously, 0.1 mg suspended in 0.1 ml 5% glucose, 48 hours before challenge, for the experiments using opsonized cells. For experiments on EDTA treated cells, 1.0 mg dosage was used 72 hours before challenge.

No attempt has been made to compare these two protocols, either of which is effective. The second protocol was simply found to yield more convenient time intervals for consideration of week-ends.

Assays of lysozyme in mouse blood were made by the method of Smolelis and Hartsell(7) using commercially available reagents (Digestive Ferments Co., Detroit, Mich.).

*Results. Effect of pre-treatment of mice with restim prior to challenge with opsonized cells.* We have previously established that (1) normal mice pre-treated with restim prior to challenge with conventional *S. typhimurium* cells experience no increase either in 50% survival time nor in 20-day survivorship; (2) immunized mice, pre-treated with restim, experience greater survival time and in some cases greater survivorship than immunized controls. The following experiment extended these findings. Groups of 20 mice were pre-treated with 0.1 mg restim in 0.1 ml 5% glucose, or with 0.1 ml of glucose solution only. Two days later, the mice were challenged either with opsonized bacterial cells (see Methods and Materials) or with conventional bacterial cells. The results, shown in Table I, indicate that an increase of survival time and survivorship occurs in mice receiving opsonized cells when they have been pre-treated with restim. Our working hypothesis assumes that some function of antibody involving the portion which is sufficient just to coat the bacterial cells is enhanced by restim.

*Effect of pre-treatment of mice with restim prior to challenge with EDTA-treated cells.* An experiment was next conducted in which the cells were treated with EDTA instead of with antiserum (See Methods and Materials). As shown in Table II, an increase in survival time and survivorship was demonstrated in mice receiving EDTA cells as compared to those receiving untreated cells. Further enhancement of this resistance

* Versenates, Inc., Framingham, Mass.

TABLE I. Effect of Pre-treatment of Mice with Restim Prior to Challenge with Opsonized *S. typhimurium* Cells.

| Pre-treatment | Type of challenge | $SD_{50}$* | Survivors/total |
|---|---|---|---|
| Restim, 0.1 mg i.v., 48 hr before challenge | Opsonized bacteria 1 | 19.5 (17.4 -21.8) | 12/20 |
| 5% glucose, as above | " " | 15.5 (13.9 -17.2) | 3/20 |
| Restim, 0.1 mg i.v., 48 hr before challenge | Non-opsonized bacteria | 9.2 ( 6.75-12.5) | 2/10 |
| 5% glucose, as above | " " " | 10.5 ( 8.6 -12.8) | 2/20 |

* Determined by method of Litchfield(16). Figures in parentheses are 95% confidence limits.

TABLE II. Effect of Versene Treatment of *S. typhimurium* Cells on Response to Challenge of Restim-treated mice.

| Pre-treatment | Type of challenge | $SD_{50}$ | Survivors/total |
|---|---|---|---|
| 1 mg restim i.v., 72 hr before challenge | EDTA-treated bacteria | 22.2 (11.0-44.8 ) | 11/20 |
| 5% glucose, as above | " " " | 8.0 ( 4.0-15.9 ) | 5/19 |
| *Idem* | Buffer-treated " | 4.9 ( 3.9- 6.08) | 1/19 |

was observed in restim pre-treated mice.

*Possible role of lysozyme.* The next experiment was simply an assay of lysozyme in the blood of mice which had been treated with restim compared to normal controls. Blood from 6 mice in each group was collected by bleeding to death from the subclavian artery. The blood was pooled and assayed as indicated under Methods and Materials. Control mice had no detectable blood lysozyme, a result in accord with the findings of Meier and Hoag(8). The restim-treated mice had blood levels of 21.25 μg/ml. The above cited authors have noted considerable variation in blood lysozyme levels of various strains of mice, but the highest value reported by them was well below (6.0 μg/ml) that observed in these restim-treated mice. The activity does not involve complement, since sera used for opsonization were inactivated. The possibility that complement from the mouse might play a role is remote, since mice are known to be deficient in complement(9).

*Discussion.* Boehme and Dubos(10) remarked that phagocytosis with consequent removal of bacteria from the blood may be but one factor in resistance to disease. Brumfitt and Glynn(11) have called attention to the fact that intracellular destruction of *Micrococcus lysodeikticus* strains is related to lysozyme sensitivity, and that this factor operates independently with respect to phagocytosis. Elberg (loc. cit.), Ralston *et al.* (12) and Repaske (loc. cit.) have shown that lysozyme, an enzyme originally believed to be active only on certain species(13), is capable of attacking Gram-negative bacilli when the surfaces are altered by chelating agents. The data showing similarity in the effect of opsonization on bacterial cells permits us to postulate a further function of antibody in conditioning the cells for the action of lysozyme-like enzymes.

Results reported here indicate that an important function of restim in increasing resistance to infection, in addition to enhancing phagocytic activity, is to increase available lysozyme. This increase so far has been demonstrated only in the blood stream. Further work is in progress to determine whether the lysozyme originates from an overall body increase or from excretion or release from tissue cells and polymorphonuclear leukocytes, as reported by Kerby(14) for endotoxin, and Janicki(15) in mycobacterial sensitization.

*Summary.* Mice treated with a RES-stimulating agent (restim) were less susceptible than control mice to subsequent infection with opsonized *S. typhimurium* cells. A similar enhancement of resistance was observed when restim-treated mice were challenged with bacterial cells exposed to EDTA

rather than antibody. Preliminary results indicate an increase in lysozyme in the blood of restim-treated mice.

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1. Ransom, J. P., Pasternak, V. Z., Heller, J. H., *J. Bact.*, 1962, v84, 466.
2. Elberg, S. S., *Bact. Rev.*, 1960, 24, 67.
3. Repaske, R., *Biochim. et Biophys. Acta*, 1956, v22, 189.
4. ———, *ibid.*, 1958, v30, 225.
5. Miles, A. A., Misra, S. S., Irwin, J. O., *J. Hyg.* (Cambridge), 1938, v38, 752.
6. Muschel, L. H., Treffers, H. P., *J. Immunol.*, 1956, v76, 1.
7. Smolelis, A. N., Hartsell, S. E., *J. Bact.*, 1949, v58, 731.
8. Meier, H., Hoag, W. G., *ibid.*, 1962, v83, 689.
9. Muschel, L. H., Muto, T., *Science*, 1956, v123, 62.
10. Boehme, D., Dubos, R. G., *J. Exp. Med.*, 1958, v107, 523.
11. Brumfitt, W., Glynn, A. A., *Brit. J. Exp. Path.*, 1961, v42, 408.
12. Ralston, D. J., Baer, B. S., Elberg, S. S., *J. Bact.*, 1961, v82, 342.
13. Salton, M. R. J., *Bact. Rev.*, 1957, v21, 82.
14. Kerby, Grace P., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 381.
15. Janicki, B. W., Patnode, R. A., *Am. Rev. Resp. Dis.*, 1961, v83, 872.
16. Litchfield, J. T., Jr., *J. Pharmacol. and Exp. Ther.*, 1949, v97, 399.

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## Chlortetracycline and Bone Demineralization in the Rachitic Rat.* (28338)

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Certain antibiotics have been found to produce unexpected growth responses in experimental animals. Attempts have been made to explain these unusual responses in a number of ways. One of the more common theories suggests that the antibiotic stimulates an increased synthesis of essential nutrients in the digestive tract of the test animal. Another theory suggests that the antibiotic favorably affects absorption of essential nutrients from the digestive tract. However, there is evidence that neither of these theories adequately explain some of the observations made in this area of investigation. It appears that studies in the realm of mineral metabolism offer possibilities of throwing further light on this phenomenon. This is because mineral elements are not expected to be synthesized in the digestive tract but possibly they could be rendered more available by the presence of certain antibiotics in the diet. The differences in metabolic availability of some minerals in the presence or absence of antibiotics may be rather easily measured.

Migicovsky *et al.*(4) reported that an increase in calcium absorption resulted when penicillin was incorporated into a low-calcium diet fed to growing chicks. Ross *et al.*(6) reported that inclusion of penicillin in the diets of growing chicks resulted also in a higher bone-ash value. Rusoff *et al.*(7) reported that administering Aureomycin to young calves (either orally or parenterally) resulted in greater skeletal growth and an increase in bone size. Murray *et al.*(5) found that incorporation of Aureomycin into rachitogenic diet resulted in higher "line test" values for bones from rachitic rats receiving sub-optimal doses of Vit. D. On the other hand, Hartsook(3) reported that incorporation of Aureomycin into purified diets adequate in all known dietary essentials except calcium resulted in no increase in body-calcium retention by growing rats. It is to be noted that the diets employed by this in-

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