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# Action of Bacterial Toxins on Tumors. VI. Protection Against Tumor Hemorrhage Following Heterologous Immunization.

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That active protection against tumor hemorrhage caused by injection of bacterial products can be induced by immunization with the homologous endotoxin has recently been demonstrated by us.[1] But that animals may be immunized with the endotoxin of one gram-negative bacterium and also exhibit a cross-immunity to the endotoxins of unrelated gram-negative organisms has, to our knowledge, not been previously reported.

During the course of this series of studies it has been observed occasionally that mice whose earlier history had included injections of the endotoxin of a gram-negative bacterium would, when used for work with implanted tumors, exhibit anomalous resistance to tumor hemorrhage following the injection of endotoxins of gram-negative species immunologically different from those used for the earlier injections. The present study represents an effort to test whether this anomalous protection against toxin-induced tumor hemorrhage is referrable to a cross-immunity mechanism.

*Methods.* (1) *Source of immunizing materials.* Three organisms, 2 closely related and the third taxonomically remote from the other two, were chosen for these experiments. They were *Shigella paradysenteriæ* Flexner, *Salmonella typhimurium* and *Rhodospirillum rubrum.* These organisms were grown in large quantities in suitable liquid culture media.[2] The cultures were killed by adding phenol to 2%, reduced in volume by pervaporation and precipitated from 66% acetone. Aqueous solutions of these precipitates, injected intraperitoneally, were used throughout the tests. Constant care was taken to prevent contamination, each culture flask being streaked on plates before killing to confirm its purity. The parent cultures were checked for purity by agglutination tests against standard typing sera. These and other precautions to prevent contaminations of cultures are described in detail elsewhere.[2]

(2) *Immunization.* Nine separate groups of white Rockland male mice weighing 20-22 g were subjected to the following schedule of immunization: ¼ LD50 was injected on the first day; ½ LD50 on the third day; 1 LD50 on the fifth day; 1 LD50 on the sixth day; and 2 LD50 on the eighth day. This schedule of immunization was employed for each of the 3 endotoxin preparations. During the course of this immunization between ten and thirty per cent of the animals were lost, presumably because of primary toxicity of the injected material.

(3) *Tumors.* On the eighth day of the immunization schedule bits of Mouse Sarcoma 180 tissue were implanted under the axillary skin according to a standardized trocar implantation technic.[3] The tumor implant was allowed to grow for 7 days, by which time it had become well vascularized and had developed into a spherical mass about 1 cm in diameter. At this time a test injection of endotoxin-containing material was made. Following injection of the endotoxin-containing materials, hemorrhage in similar tumors carried by normal (non-immunized) mice reaches its maximum at 5 to 8 hours. The experimental animals were usually sacrificed about 12 hours after injection and the tumors dissected. The degree of tumor hemorrhage was graded as slight (1), considerable (2), and marked (3). Normal tumor tissue is pink; hemorrhagic tumor tissue is deep red to deep purple.

*Results and Discussion.* Previous experiments[1] have indicated that in normal non-

[1] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 48.

[2] Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, in press.

[3] Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

TABLE I.

Toxin-produced Tumor Hemorrhage in Homologously and Heterologously Immunized Mice.

| Toxin used for immunization | Toxin used for tumor hemorrhage production | Amount injected and degree of hemorrhage* | | | |
|---|---|---|---|---|---|
| | | 1/40 LD50 | 1/4 LD50 | 1 LD50 | 4 LD50† |
| *Shigella* | *Shigella* | 0 0 | 0 0 0 | 0 0 0 | 3 3 3 |
| " | *Salmonella* | 0 0 0 0 0 | 0 0 0 0 0 0 2 1 1 1 1 | 0 0 0 0 0 0 0 0 0 2 1 2 1 2 | 2 2 3 3 3 3 |
| " | *Rhodospirillum* | 0 0 | 0 0 0 0 0 0 0 1 2 1 2 | 0 0 0 0 1 2 2 | 0 0 1 1 1 2 2 |
| *Salmonella* | Salmonella | 0 0 0 0 0 0 | 0 0 0 0 0 0 | 0 0 0 0 0 0 0 0 0 1 1 2 2 2 | 0 0 2 2 2 2 2 |
| " | *Shigella* | 0 0 | 0 0 1 2 | | 2 3 |
| " | *Rhodospirillum* | 0 0 | 0 0 1 1 | 0 0 0 2 2 1 | 3 3 3 |
| *Rhodospirillum* | " | 0 0 0 0 | 0 0 0 0 | 0 0 0 0 0 1 1 2 3 | 1 2 3 |
| " | *Salmonella* | 0 0 0 0 | 0 0 0 0 1 1 2 2 | 0 0 0 0 0 0 0 1 1 2 2 2 2 2 3 | 0 2 2 2 2 3 3 |
| " | *Shigella* | 0 0 | 1 3 | 2 3 | 3 3 |
| Non-immunized | " | 1 2 2 2 3 3 3 3 | 2 2 2 3 3 3 | 2 2 2 3 3 | 2 2 3 3 3 3 |
| " | *Salmonella* | 0 2 2 2 3 3 3 3 3 3 3 3 | 1 1 2 2 3 3 | 2 2 2 3 3 3 3 | |
| " | *Rhodospirillum* | | 0 0 1 2 2 2 | 1 1 1 2 2 3 3 3 | |

* Each designation below represents one animal. 0 = no hemorrhage; 1 = slight hemorrhage; 2 = considerab hemorrhage; 3 = marked hemorrhage.

† Some of the mice indicated below succumbed to the lethal effects of the toxic material, but hemorrhage w evident before death.

The amount of dry toxic material necessary to produce death within 24 hours of 50% of non-immunized mi injected intraperitoneally with the aqueous solution was: *Shigella*, 8.0 mg; *Salmonella*, 2.2 mg; *Rhodospirillum*, 6.0 m

immunized animals, and using relatively unpurified preparations obtained from gram-negative organisms, 1/200 of an LD50 is sufficient to induce hemorrhage in tumors. It was found that tumor hemorrhage in immunized mice could not be induced regularly with a dosage lower than one LD50. To test degree of immunity in the animals of the present experiment, 4 arbitrary dosage levels were therefore selected: 1/40, 1/4, 1, and 4 LD50. In non-immunized animals these dosages of the 3 toxic materials gave regular and uniform hemorrhage (see Table I).* On the other hand, among the immunized animals, there was no tumor-hemorrhage whatever following injection with 1/40 LD50, very little with 1/4 LD50, some with 1 LD50, but uniform tumor hemorrhage occurred with 4 LD50. The results (Table I) indicate that cross-protection appears to exist at about the same level as the homologous protection for each of the 3 organisms.

Tumor hemorrhage is a sensitive indicator of the type of vascular damage produced by the endotoxins of gram-negative bacteria. It is generally considered that the lethal effect of these endotoxins results from a similar but generalized vascular damage.

* With the exception of *Rhodospirillum* which was not tested in a dose of 1/40 LD50 in controls.

Boivin and Mesrobeanu,[4] using lethality as an index, failed to obtain cross-protection with the purified toxic O antigens of species of *Pseudomonas, Shigella,* and *Salmonella.* We have repeated our cross-immunity experiments using lethality instead of tumor hemorrhage as a criterion, and we have observed definite cross-protection. A detailed discussion of this apparent contradiction, and the problems posed by the cross-protection reported among heterologous gram-negative organisms, is being published elsewhere.[2]

Our previous work with a large and highly heterogeneous assortment of gram-negative bacteria led to the postulation of the existence of a common toxic factor as the incitant of tumor hemorrhage among substantially all gram-negative bacteria.[3,5] The immunochemical work of Morgan and Partridge[6] and of the Boivin school had indicated that for the gram-negative bacteria studied, most of the toxicity is attributable to a portion of the O

[4] Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1937, **125**, 273; 1938, **127**, 752; **128**, 835; *Rev. d'Immunol.*, 1938, **4**, 40; 1938, **4**, 469.

[5] Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

[6] Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, 1941, **35**, 1140; *Brit. J. Exp. Path.*, 1942, **23**, 151.

antigen, and that this portion is antigenic. This would lead one to anticipate the possibility of securing, under appropriate conditions, a relative cross-immunity to vascular damage with gram-negative organisms generally. The organisms used in the experiments reported here were selected as a test of this hypothesis, and the results observed support our earlier conclusions.

*Summary.* Mice immunized with endotoxin preparations of *Shigella paradysenteriæ* Flexner, *Salmonella typhimurium* and *Rhodospirillum rubrum* were found to have been protected against the induction of hemorrhage in implanted tumors.

Protection by immunization was found to be about as effective against the induction of tumor hemorrhage when the heterologous organisms were used as when the homologous were used. This finding supports our earlier hypothesis that a common antigenic toxic component is characteristic of gram-negative bacteria generally.

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## A Rapid Method for Estimation of Penicillin

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In connection with chemical studies on penicillin and, more particularly, with efficient large scale production of the antibiotic substance, there has been an urgent need for a rapid method for the estimation of the substance. It had been hoped that the antibioluminescent activity of other antibiotic agents might be found to be a property of penicillin since use of this property gave a method for rapid titration of potency; such however did not prove to be the case.[1,2] Methods of titration requiring 3 to 6 hours delay are known and employed,[3], but with the rapid rise in activity seen in the more recent methods of penicillin production, this time interval is still too long for efficient production.

A report from England[4] stated that the group at the Wellcome Physiological Research Laboratories had devised a new method of testing for penicillin based on the hemolysis of red blood cells by hemolytic streptococci. This test was complete within 3 to 4 hours, *i.e.*, was not much faster than tests already in use. Rammelkamp[5] had already described an 18-hour test which was based on the hemolysis of red cells by hemolytic streptococci. In connection with studies on *Aspergillus flavus* in which the hemolytic streptococcus was used as the test organism[6] the present authors had occasion to note the very rapid appearance of hemolysis which occurred if conditions were optimal. This suggested that a method of titrating penicillin or other antibiotic substances could be devised with conditions so arranged that a reading could be made in as little as 60 to 90 minutes.

Of the strains of hemolytic streptococci tested, strain C-203 has produced detectable amounts of hemolysin most rapidly. Up to the present time no other group of bacteria has proven more satisfactory than *Streptococcus pyogenes*. Since penicillin acts only by

1 Rake, G., McKee, C. M., and Jones, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 273.

2 Rake, G., Jones, H., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 136.

3 McKee, C. M., unpublished data; Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1943, **46**, 187; Foster, J. W., and Wilker, B. L., *J. Bact.*, 1943, **46**, 377.

4 Wilson, U., *Nature*, 1943, **152**, 475.

5 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

6 Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, 1943, **45**, 461.