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The Demonstration, and A Suggested Immune Role, of Mouse Antibodies Against Salmonella enteritidis Endotoxins

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Mouse immunity or resistance to **Salmonella enteritidis** infection is a complex mosaic of many facets. This report is concerned with that aspect which involves mouse reaction to the endotoxins of the bacteria.

It is a generally held view that protective antibodies against infections by the Gram-negative enteric bacteria, **Salmonella,** are those specific for the Vi, when present, and O (endotoxin) antigens. Antibodies formed against the H (flagellar) antigens are held to be non-protective. Although these views specifically relate to clinical salmonellosis in man, classically typhoid fever, the same is reported for chimpanzees (20) and, by implication, thus would seem to apply to other mammals. Inoculation of mice with **S. enteritidis** was, therefore, expected to result in the development of protective antibodies against the O antigens of the organism (Vi is absent). However, when agglutination tests were conducted with sera from immunized NAMRU mice, titers were either non-existent or very low (14, 15) compared to titers of 1:320 or more in human typhoid fever. Lockhart and Paulissen (9), therefore, performed agglutination tests under varying conditions to improve the test sensitivity. They found titers up to 1:320 against the H antigens but still only to 1:40 against the O antigens in the mouse antisera. The phenomenon is not peculiar to this strain of mice. Hobson (7) reported agglutinin titers of up to 1:80 in an unidentified strain of mice immunized with **Salmonella typhimurium.** Morello **et al** (10) were able to detect O agglutinin titers to 1:320 in pooled sera from CD-1 mice hyperimmunized against **S. typhimurium** but, in a group of five individual mice infected with the organism, one had a titer of 1:80 while the rest had 1:40 or less. Hashimoto **et al** (6) reported that ddN strain mice produced O agglutinin titers of 1:160 one month following immunization with **S. enteritidis.**

Despite these comparatively low titers, mice do develop protective serum factors of some sort following immunization with **Salmonella** organisms. This was shown by Lockhart and Paulissen (9) who reported that survival time of mice in experimental salmonellosis was extended from 1.6 days to 13.6 days when the animals were passively immunized with mouse antisera before being challenged. Also, Lieberman **et al** (8) reported that serum from a mouse injected with a **S. enteritidis**-paraffin oil-adjuvant mixture gave passive protection to 50% of challenged mice when inoculated with 0.01 ml./ mouse antiserum.

These several observations led to the speculation that either O agglutinins were unimportant in mouse immunity to **Salmonella,** or that, simply, they were not being adequately measured, i.e. a more sensitive test for them was needed. Actually, O agglutinins may be unimportant. Hobson (7) for example, concluded from his work that the anti-O antibody of mice against **S. typhimurium** was of doubtful benefit. Also, Gorer and Schutze (5) could not correlate anti-O agglutinins in mice with resistance to **S. enteritidis.** Still, it seemed to us that the agglutinins, even of low titer, should not be considered lightly. Their very presence in mouse sera after exposure to the bacteria seemed to implicate them in some sort of functional role in immunity. Thus we decided to try to develop a more sensitive test for mouse serum antibodies. One method for enhancing the sensitivity of an agglutination test is to coat erythrocytes with bacterial antigens (3). After a number of trials, a successful procedure was developed by which agglutination of sheep cells coated with **S. enteritidis** endotoxin was accomplished, and a high titer of mouse "agglutinins" was demonstrated.

The endotoxins of **Salmonella** also have pharmacological effects on the mammalian hosts. Among various organs and tissues, they involve the liver which appears to have a major role in their detoxification as well as in clearance of toxic bacteria from the blood (1, 17). With the demonstration of endotoxin antibodies it was decided to see if mouse antisera in some way mediated the effects of the bacterial endotoxins of the animal. It was found that an increase in the respiration of liver tissue occurred in animals given large numbers of bacteria (endotoxin). This increase, however, was considerably lessened when mouse antisera were employed in counteracting the stimulatory effects.

MATERIALS AND METHODS

Experimental Animals: Ten-to-sixteen week old NAMRU (4) mice were used throughout this work. The animals were housed in one-gallon glass jars and supplied Purina Laboratory Chow and water **ad libitum.** A six-to-eight week old rabbit housed in a standard wire cage was used to obtain serum for use as a control. Commercial rabbit food (Farmer's CO-OP) and water were supplied **ad libitum.**

Test Organism: Salmonella enteritidis (I, IX, XII . . . g, m) was obtained from the culture collection of the Department of Botany and Bacteriology, University of Arkansas and was originally identified as number 64 of the Agricultural Experimental Station of the University of Kentucky (14). The culture was maintained lyophilized on beads in individual ampoules in order to minimize virulence, or other, changes. When cultures were required, the beads were plated directly upon tryptose agar or heart infusion agar plates and held 24 hours at 37 C.
Vaccine: The heat-killed vaccine was prepared essentially as described heretofore (14), and contained approximately 2.2 x 10^9 bacteria/ml.

Mouse antisera: Each mouse received three intraperitoneal injections of 0.2 ml vaccine on alternate days. Fifteen-to-sixteen days following the final injections, the mice were sacrificed and exsanguinated by severing major blood vessels in the thoracic cavity. The pooled blood was collected in sterile tubes, held at room temperature two hours, ringed with a wooden splint and refrigerated overnight. The serum was removed with a capillary pipette the following day and stored in screw-capped tubes at approximately -20 C. Non-immunized mice were employed to collect normal serum.
Rabbit antiserum: Two days before starting the immunization of the rabbit, it was bled for the collection of the normal control serum. The rabbit received six injections of vaccine on alternate days as follows: (1) 0.5 ml subcutaneously, (2) 1.0 ml intraperitoneally, (3) 2.0 ml intraperitoneally, (4) 3.0 ml intraperitoneally, (5) 5.0 ml intraperitoneally and (6) 0.2 ml intravenously. The serum was collected 14 days after last injection and was processed and preserved by the same procedure used for mouse sera above.

Tube agglutination tests: Standard procedures were used in tube agglutination tests to detect and measure agglutinin titers in rabbit and mouse antisera, using saline as a diluent.

Endotoxin preparation: After three unsuccessful methods, one suggested by Oakley (13) and Thomas (19), a second after Neter **et al.** (12) a third after Ribi **et al,** (16) were tried, a fourth was found to work. It was essentially the same as the third mentioned but with an additional step of heating as suggested by Neter **el al.** (12). The procedure is as follows: Several Roux bottles containing 50 ml heart infusion agar were inoculated with 2 ml each of a 24-hour culture of **S. enteritidis** in heart infusion broth. After incubation, 20 ml cold sterile saline were added to each bottle for harvesting the organisms. The cell suspension was washed three times in sterile saline, diluted to a net reading of 770 on the Klett-Summerson colorimeter with a no. 54 filter, and cooled to 6-12 C. Two volumes of pre-cooled diethyl ether were added to the cell suspension contained in a separatory funnel and shaken gently for six consecutive ten second intervals. The bacteria were left overnight in the funnel at 6-12 C. The aqueous phase was drawn off, and an equal volume of ether added, shaken and allowed to

stand overnight as before. The aqueous phase was again drawn off and residual ether was removed by bubbling air through the remaining material. The organisms were removed by centrifugation at 3800 rpm for 70 minutes and then discarded. The supernatant was dialyzed five days in daily changes of distilled water at 4-5 C. Sodium chloride was added to a concentration of 0.85%. The endotoxin was precipitated by the addition of cold absolute ethanol to a total volume of 68%. It was added slowly with continuous stirring and the suspension was allowed to stand overnight. The precipitate was collected by centrifugation at 4000 rpm for 70 minutes, the supernatant discarded and the precipitate dissolved in distilled water. This was transferred to a dialysis bag and dialysis against water was continued 36 hours in the cold, reprecipitated and collected as before. The precipitate was dissolved in a minimal quantity of distilled water. One-ml portions were pipetted into 10 x 100 mm soft glass tubes and dried under vaccum. The sealed tubes were stored at 4-6 C. When needed for hemagglutination tests, a vial containing endotoxin was dissolved in sterile saline and mixed thoroughly to a total volume of 12.5 ml in a round-bottom flask fitted with a reflux condenser. This was heated to a gentle boil for one hour.

Coated Sheep erythrocytes: Ten ml of defibrinated sheep blood were centrifuged at 2000 rpm for ten minutes after which the liquid part was discarded. The erythrocytes were then resuspended in sterile saline to the original volume. Washing in this manner was done three times. The final washing was carried out at 1000 rpm for 8 mins. after which saline was added to make a 10% suspension of cells which was stored at 4-6 C until use. Coating of the cells after Fulthorpe (3), was accomplished by incubating them with an equal volume of the endotoxin preparation for two hours in a 37 C water bath with frequent shaking. They were then washed 3 times in 12 ml saline to remove free or unabsored endotoxin and finally saline was added to make a 10% suspension. Two drops (0.033 ml) of this sensitized erythrocyte suspension were added to each tube containing the serum dilutions from a 19 gauge needle which delivers 60 drops per ml. The cells were evenly distributed by shaking, incubated 30 minutes in a 37 C water bath and stored overnight at 4-6 C.

Preparation of liver slices: For respiration studies mouse liver slices were processed as follows: The mice were injected intravenously with 0.2 ml containing approximately 2.2 x 10^9 heat-killed bacteria. To facilitate the injection, the mouse's tail was held 3-4 min. in a beaker of warm water. The controls received 0.2 ml saline. Thirty minutes afterward all mice were sacrificed by cervical dislocation, the livers were excised and placed in petri plates filled with chilled Krebs-Ringer phosphate solution pH 7.4. The lobes of each liver were separated and the gall bladder was removed and discarded. The liver was sliced with a pre-cooled Stadia-Riggs microtome which produced slices 0.5 mm in thickness. Each slice was maintained in ice-cold Krebs-Ringer phosphate solution, pH 7.4, until just prior to gassing the Warburg flasks at which time it was placed in a flask.

Measurement of respiratory rates: Carbon dioxide production by liver slices was determined by the direct method with the Warburg respirometer. The Warburg flasks were charged with 2.5 ml of Krebs-Ringer phosphate solution, pH 7.4, in the main chamber, 0.5 ml of 01. M glucose in Krebs-Ringer phosphate solution in the sidearm and in the center well either 0.2 ml 20% sodium hydroxide for carbon dioxide absorption or 0.2 ml distilled water to preclude carbon dioxide absorption. Liver slices from each mouse were placed in the main compartments of duplicate flasks. All flasks were gassed five minutes with 100% oxygen before being placed on the 37 C water bath of the Warburg. Five minutes were allowed for temperature equilibration before closing the respirometer and taking readings resulting from endogenous respiration. These continued for ten minutes until zero times when the substrate was tipped into the main compartment. The tests were allowed to continue one hour with readings made at 15 minute intervals. A dry-weight determination of each slice was made after rinsing with distilled water, drying at 50 C for 24 hours and storing in a desiccator for 8-12 hours.

RESULTS AND DISCUSSION

The initial experiments in this work were undertaken to substantiate the development of only low titers of bacterial agglutinins in mice against **S. enteritidis.** We immunized mice with **S. enteritidis** according to a schedule known to produce immunity (14). A rabbit was also immunized in order to obtain antiserum which would be used to check the serological test procedures. In Table I are found results which show that agglutinins were indeed found to be wanting in mouse antisera, but reached a titer of 1:2560 in the rabbit antiserum. We proceeded to attempt the more sensitive test. Methods have been developed whereby soluble antigens, like endotoxins, are absorbed onto relatively large particles like charcoal, alumina, or erythrocytes, so that when mixed with specific antisera an "agglutination" of the particles takes place. Because the particles are large, a readily visible reaction occurs. Our first attempt at performing this kind of test was unsuccessful. The culture filtrate was used as suggested by Oakley (13) and Thomas (19) but did not produce the desired results because the preparation hemolyzed the cells. A second method of endotoxin preparation by Neter **et al.** (12) also failed to work. In Table I it can be seen, using this method, that although the rabbit antiserum titer was enhanced from 1:2560 to 1:5120, no "agglutinins" were demonstrated in the antisera of mice. The third method after Ribi **et al. (16)** involving extraction of the endotoxin with aqueous ether also did not work in that no hemagglutinating antibodies were demonstrated in the mouse sera but, furthermore, the antibody titer of the rabbit serum was also not detectable (Table I). Since heating has been

reported to help in hemagglutination tests (12) we heated our endotoxin to 100 C for 1 hour. When the endotoxin, prepared by the method of Ribi, **et al.** (16) was heated before coating the sheep erythrocytes, titers of hemagglutinins of 1:1280 for mouse antisera and 1:40960 for the rabbit antiserum were obtained (Table I). Thus it is seen that mice do develop antibodies against the **S. enteritidis** endotoxin and can be measured by this test. The test proved to be sixteen times more sensitive than the bacterial agglutination test; an "increase" in titer of the rabbit serum from 1:2560 to 1:40960. Fulthorpe (3) found the hemagglutination test to be about eight times more sensitive than the bacterial agglutination tests, also using rabbit antisera. Both Neter **et al.** (11) and Morello **et al.** (10), however, found the bacterial agglutination test titers equal to the hemagglutinin titers using coated erythrocytes.

Since antibodies to **S. enteritidis** endotoxins were found in the mouse, it was wondered if they had any protective value. It is known that both clearance of bacteria from the blood and the endotoxin action by bacteria implicates the liver of mammals (1, 17). One approach to determine liver involvement is to test the tissue for increased respiration as a reaction to the toxin. This was suggested by the work of Woods, **et al.** (21) who observed a slight elevation, above the control level, in the respiratory rates of tissues from mice previously injected intravenously with a sublethal dose (20 ug) of **Serratia marcescens** endotoxin. In our initial trials, liver slices were taken from mice fasted six hours before sacrifice but they showed essentially no change when exposed to the bacteria. When mice were fasted 24 hours, however, the liver tissues were found to show increased respiration to a ratio of 3.17:1 in one experiment and to 2.44:1 in a repeat experiment (Table II). This measurable stimulation provided a basis for testing whether antibodies to **S. enteritidis** may moderate the liver's response. This was done in two ways. By the first procedure, mice were passively immunized with 0.1 ml. mouse antisera against **S. enteritidis** 12 hours before being inoculated with the bacteria (endotoxin). The liver tissues were removed 30 minutes later. The ratio of respiration was found to be 1.39:1 (Table II). By the second method, the bacteria were incubated 30 min. at 37 C with 1:15 dilution of the mouse antisera before injection into the mice, the ratio of respiration of liver slices became 1.58:1. In both cases, then, the stimulation of respiration was considerably modified. If it is assumed that such a modifying influence ameliorates the taxing effect of the toxin on the animal, it can be construed as beneficial to the mice in dealing with the toxin action. The mechanism seems to be related less to possible antiserum action on the liver than to the modification of the toxin by the antibodies in a way that increased respiration of the liver slices did not occur. This seems all the more likely when it was seen that bacteria, incubated with antisera before injection, also failed to stimulate liver slices. Just what role antibodies play in an animal's resistance to endotoxin is not exactly known (18). Freedman (2) showed that tolerance to endotoxin can be passively transferred in plasma or serum from animals conditioned to be endotoxin-tolerant by repeated endotoxin injections. This suggests an antibody role but when he mixed the plasma with endotoxin before injection together, protection was not obtained, thus raising the question whether an antibody was involved. In the present work, mixture of the antisera with the bacteria (endotoxin) before injection "reduced" liver stimulation just as passive inoculation with antisera before injection of the bacteria. Here it seems an antibody is involved and suggests it has some kind of neutralizing effect on the endotoxin so that the liver's respiration is not elevated.

Table I

Demonstration of Mouse and Rabbit Serum Antibodies Against **Salmonella enteritidis** (Reciprocals of Titers)

Experimental Animals	Immunized With Heat-Killed **S. enteritidis**	Standard Bacterial Tube Agglutination Tests	Hemagglutination Tests Using Sheep Red Blood Cells Coated With **S. enteritidis** Endotoxin (Fulthorpe's Method) Extracted From Bacteria By:		
			Heating The Bacteria In Saline At 100C One Hour	Aqueous Ether	Aqueous Ether And Heating 100C One Hour
Mice	Yes	0	0	0	1 280
Mice	No	0	0	0	0
Rabbit	Yes	2 560	5 120	0	40 960
Rabbit	No	0	0	0	0

Table II

Modifying Effects of Mouse Antisera Upon Stimulation of Liver Tissue Respiration by **Salmonella enteritidis** Administered After 24 Hours Fasting

Experiment No.	Pretreatment	$Q^{O_2}_{CO_2}$ Values (μ CO_2/mg Dry Wt. Liver/Hour in 100% O_2 Atmosphere) of Liver Slices Obtained From Mice 30 Min. After Inoculation With:		$Q^{O_2}_{CO_2}$ Ratio Between Bacteria-Inoculated And Saline-Inoculated (Control) Mice
		2.2 x 10^8 Heat-Killed **S. enteritidis** in 0.2 ml. Saline	Saline — 0.2 ml.	
I	None	8.46	2.67	3.17:1
II	None	2.29	0.94	2.44:1
III	Mice Were Given 0.1 ml. Mouse Antisera I. p. 12 Hours Before Inoculation	2.16	1.55	1.39:1
IV	Before Inoculation, Bacterial Suspension Was Incubated 30 Min. At 37°C In An Equal Volume Of 1:5 Dilution Of Mouse Antisera	3.28	1.07	1.58:1

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