

# STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

## III. ACTIVE IMMUNIZATION WITH R FORMS

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In previous papers (1), (2) studies on the dissociation of the so-called hog cholera bacillus were presented. It was found that at least 4 distinct forms of the bacillus existed—the “normal” or MS strain and its 3 variants, MR, NS and NR. From immunological tests it was apparent that the symbols MS, MR, NS and NR corresponded to the symbols OH, ØH, O and Ø, respectively, used by previous investigators. The virulence of the S forms, as in other bacterial species, was greater than that of the R forms. Both MS and NS forms were virulent for hamsters and rabbits. The NR form, however, usually gave rise to fever or abscess formation and occasionally caused the death of these animals. In attempts to induce reversion from one form to another, it was found that the MR form, which, as such, consistently lacked virulence, was under certain conditions the most unstable of the forms. It reverted either to its prototype, the virulent MS form, or dissociated further into the NR form (2). This observation was of particular interest and will be referred to presently.

It is of importance to determine for practical purposes whether the killed, or especially the living avirulent R forms, are of value as prophylactic vaccines. It is also desirable to determine which of the antigenic components, the H, O or the Ø, separately or in combination, are responsible for the stimulation of immunity. In this paper studies are reported which were made to determine the immunizing power of the avirulent forms of the hog cholera bacillus isolated in this laboratory.

Many studies have already been made in regard to the virulence or avirulence of bacteria used as antigens. Rowland (3), working with plague bacilli, Weber (4), with typhoid bacilli, and Griffith (5), with pneumococci, all agreed that avirulent forms of the respective bacteria were unsuitable for the production of efficient vaccines or antisera. The Army Council (6), however, reported that virulence is not an essential characteristic of organisms used for the production of vaccine. Tillett (7) also found that some degree of immunity against S strains could be established in rabbits with killed R Pneumococci, although antiserum produced by the injection of R forms into rabbits was valueless in passive protection of mice.

Other investigations have shown that animals inoculated with the living avirulent form were resistant to the virulent form of the same organism. Manninger (8) found this to be true with *B. avisepticus* in mice, De Kruif (9) with the *B. leipsepticum*, and Cowan (10) with the streptococcus in mice. With considerable difficulty, White (11) succeeded in immunizing rabbits against the hog cholera bacillus by vaccinating them with killed S and R organisms as well as with living R forms alone.

Much attention has also been devoted to the comparative antigenic power of the various antigenic components present in bacterial vaccines. Felix and Oltzki (12) and Arkwright (13) believed that the "O" antigen was chiefly responsible for the stimulation of immunity, and according to the latter author the presence of the H or  $\phi$  (rough O) element was unimportant. Springut (14) working with *B. typhi murium*, however, concluded that animals immunized with both H and O components have a higher degree of protection than those immunized with O antigen alone. Similar observations were made by Ibrahim and Schütze (15) who showed that vaccines containing both H and O elements were most effective. "O" vaccines stimulated slight protection and vaccines containing H or  $\phi$  alone or together were without value.

### *Method*

The hamsters (*Cricetulus griseus*) and rabbits used in these experiments were all derived from the same source and were kept under similar conditions for at least a month before use. In all experiments animals of similar weight were used. Before use the blood of each rabbit was tested and found free from agglutinins for hog cholera bacilli and for rabbit typhoid bacilli.

*Vaccine.*—24 hour old agar slant cultures of the 4 forms MS, MR, NS and NR of the hog cholera bacillus were suspended in 0.1 per cent sodium chloride solution and standardized by counting and diluting. The organisms were killed by heating to 56°C. for 30 minutes. Living vaccines were prepared by suspending the growth from a 24 hour old broth culture in 0.1 per cent saline solution.

*Immunization of Animals.*—Hamsters were immunized by injecting vaccine subcutaneously on one side of the dorsal surface once or several times at 7 day intervals. When the animals were tested for immunity, the test organisms were

inoculated subcutaneously on the opposite side of back 7 to 24 days after the last injection. Rabbits were immunized in the same way except that the ventral surface was used. For immunization and for immunity tests the desired amount of vaccine or test organisms were suspended in 1 cc. of 0.1 per cent saline solution before injection.

Cultures on China blue rosolic acid agar plates were made from the spleens of all animals dying during immunization or after the immunity tests. The organisms recovered were identified in the usual way.

After the test inoculation all animals were observed for 30 days. The temperature of the rabbits was taken daily. At the end of 30 days all of the survivors were killed and in most cases cultures of the spleen were made.

#### EXPERIMENTAL

*Vaccination with Killed Organisms.*—Preliminary experiments indicated that the injection of killed organisms did not confer immunity against virulent hog cholera bacilli in rabbits. Further efforts were then made to immunize hamsters by the same method.

40 hamsters were divided into 4 groups of 10 each. Each group was immunized with one of the 4 forms MS, MR, NS and NR by injecting 4 increasing doses (40, 100, 500 and 1000 million) of heat killed bacilli at 7 day intervals. 10 days after the last injection, all the hamsters, together with 10 unvaccinated control animals, were inoculated subcutaneously with .0001 cc. of a culture of virulent MS bacilli.

*Results.*—Vaccines consisting of heat killed MS, MR and NR forms were without effect. 8 of the hamsters vaccinated with the MS form, 9 with the MR form and 10 with the NR form died within 2 weeks. 9 of the controls died. The heat killed NS vaccine, however, appeared to confer some degree of immunity since only 5 of the 10 hamsters vaccinated with this form succumbed.

*Vaccination with Living MR and NR Forms.*—

Two groups of 15 hamsters each were given single subcutaneous injections of .01 cc. of living broth culture of the MR and the NR forms respectively. One hamster from each group died. No growth was obtained from the spleen of the one vaccinated with the MR form, but the NR form was recovered from the animal which was inoculated with that form. 11 of the 14 animals which survived injection of the NR form developed an abscess at the site of inoculation.

Two weeks after vaccination all of the hamsters together with 10 unvaccinated ones were given .0001 cc. of the living MS form subcutaneously. The results are shown in Table I.

*Results.*—More than 50 per cent of the vaccinated animals survived while all of the control animals died within 10 days. It appears therefore that the living organisms of both MR and NR forms confer active immunity.

The experiment was repeated by immunizing 20 hamsters with a smaller dose (.005 cc.) of living NR bacilli and repeating the injection 20 days later. 2 animals died after the second injection and NR bacilli were recovered from the spleen. The remaining 18 animals and 10 controls were inoculated with .001 cc. and .0001 cc. respectively of a broth culture of the MR form 24 days after the second vaccination.

TABLE I

*Immunization of Hamsters with 1 Dose of .01 Cc. Broth Culture of Living MR and NR Hog Cholera Bacilli*

The animals were inoculated subcutaneously with .0001 cc. of 24 hour broth culture of MS form of hog cholera bacillus 2 weeks later.

Immunized with	No. used for test	No. died in days after test-dose				Percentage of survivors after 30 days
		1-5	6-10	11-15	16-20	
MR	14	1	2	1	2	57
NR	14	0	1	4		64
Unvaccinated (control)	10	3	7			0

*Results.*—9 of the 10 controls died while 14 out of 18 or 77 per cent of the vaccinated animals survived. All of the animals which recovered were killed after 30 days and spleen culture were made. The MS form was recovered from the 1 control animal and from 8 of the 14 vaccinated hamsters. No growth was obtained from the remaining 6.

*Cross Immunity with B. paratyphosus "C."*—It has already been shown by TenBroeck (16) and confirmed by Schütze (17) that a cross immunity exists between the hog cholera bacillus and *B. paratyphosus* "C" (Hirschfeld). Experiments were then made to determine the immunity of hamsters to *B. paratyphosus* "C" after vaccination with the living R forms of hog cholera bacilli.

20 hamsters were inoculated subcutaneously with .01 cc. of a living broth culture of the MR form. Later, 2 doses, .005 cc. and .01 cc. of a living broth culture of

NR bacilli were given subcutaneously, 7 days intervening between each injection. None of the animals died during the immunization period. The immunity was tested 14 days later by injecting half of the hamsters with virulent hog cholera bacilli and the other half with *B. paratyphosus* "C." The results are shown in Table II.

*Results.*—It is evident that hamsters vaccinated with R forms of hog cholera bacilli are immune to *B. paratyphosus* "C" as well as to the MS form of the homologous race.

*Vaccination with R. Paratyphoid Bacilli.*—An experiment the reverse of the preceding was next carried out.

TABLE II

*Hamsters Immunized Subcutaneously with 3 Doses of Living MR and NR Forms of Hog Cholera Bacilli: MR .01 Cc., NR .005 Cc. and .01 Cc. at 7 Day Intervals*

The animals were inoculated subcutaneously with MS form of *B. paratyphosus* "C" or MS hog cholera bacillus 14 days after the last immunizing dose.

Immunized with	No. used	Test-dose	No. died	Percentage of survivors after 30 days
Hog cholera bacillus MR and NR	10	<i>B. para.</i> "C", MS, .001 cc.	0	100
Unvaccinated (control)	10	<i>B. para.</i> "C", MS, .001 cc.	8	20
Hog cholera bacillus MR and NR	10	Hog cholera bacillus MS, .0001 cc.	2	80
Unvaccinated (control)	8	Hog cholera bacillus MS, .0001 cc.	8	0

A motile rough (MR) variant was derived from the "normal" MS paratyphoid "C" bacillus. The MR form was found to be avirulent for hamsters. 20 hamsters were then immunized with this form by injecting subcutaneously at weekly intervals one dose of 400 million killed bacilli followed by .0001 cc. and .002 cc. of the living broth culture. None of the animals died during immunization. 10 days after the last dose, 10 of the hamsters were inoculated with MS hog cholera bacilli and 10 with MS paratyphoid "C" bacilli. 20 unvaccinated control hamsters divided into 2 groups of 10 each were also inoculated with MS hog cholera and paratyphoid "C" bacilli respectively.

*Results.*—The results after 30 days were practically the same as those of the preceding experiment. Vaccination with MR paraty-

phoid "C" bacilli conferred immunity against both virulent hog cholera bacilli and the MS form of the homologous race. 80 per cent of the vaccinated animals survived the injection of hog cholera bacilli while only 10 per cent of the controls did. All of the vaccinated animals survived inoculation with MS paratyphoid "C" bacilli and all of the controls died.

The foregoing experiments are summarized in Table III. The results of the vaccination of hamsters with the living R form of both

TABLE III  
*Summary of Immunization Experiments in Hamsters with Living R Culture against S Hog Cholera and Paratyphoid "C" Bacilli*

Immunized with	Tested with	No. used	No. died	Percentage of survivors after 30 days
Hog cholera bacillus, MR	Hog cholera bacillus, MS	14	6	57
"    NR	"    "	14	5	64
"    NR	"    "	18	4	77
"    MR & NR	"    "	10	2	80
"    MR & NR	B. para. "C" MS	10	0	100
B. para. "C" MR	"    "	10	0	100
"	Hog cholera bacillus, MS	10	2	80
Total .....	Hog cholera bacillus, MS or B. para. "C," MS	86	19	78
Unvaccinated (control)	Hog cholera bacillus, MS or B. para. "C," MS	68	63	7

hog cholera and paratyphoid "C" bacilli are striking. Of the 86 animals vaccinated with living R forms, 67 survived the subsequent inoculation of virulent S forms of bacilli, either of the homologous or the heterologous race. Only a small number (7 per cent of 68 animals) of the unvaccinated control hamsters survived although in many instances they received much smaller doses of virulent bacilli than the vaccinated animals.

*Experiments with Rabbits.*—As mentioned previously, vaccination of rabbits with killed hog cholera bacilli was unsuccessful.

TABLE IV  
*Immunization of Rabbits with Killed S or with Living R Hog Cholera Bacilli against the Virulent MS Form*  
 S = survival, D = death, F = fever, 40-41°C.

Rabbit No.	Immunization				Test		Result
	Variant	Treatment	Dose	Route	Days after last injection	Dose	
1	NS	Heated 100°C., 60'	7 doses, 50-500 million at 7 day interval	I. V.	7	.0001	D. in 11 days
2	NS	Heated 56°C., 30'	"	"	"	"	D. in 10 days
3	NR	"	"	"	"	"	D. in 8 days
4	MS	"	"	"	"	"	D. in 8 days
5	MR	Living	1 dose, 0.1 cc.	Subc.	14	.1	D. in 5 days
6	NR	"	"	"	"	"	S., F. 19 days
7	MR	"	"	"	20	.001	S., F. 9 days
8	NR	"	"	"	"	"	S., F. 9 days
9	MR & NR	"	3 doses, MR .05 cc., NR .025 cc. and .1 cc. at 7 day intervals	"	14	"	S., F. 12 days
10	"	"	"	"	"	"	S.
11	"	"	"	"	"	"	S., F. 15 days
12	Unvaccinated (control)					.1	D. in 7 days
13	"					.001	D. in 4 days
14	"					.0001	D. in 6 days
15	"					.000001	D. in 7 days

4 animals were given 7 intravenous injections at 7 day intervals of large doses (50 million to 500 million) of the heat killed bacilli. All succumbed within 8 to 11 days after the inoculation of .0001 cc. of the MS form.

7 rabbits were then inoculated subcutaneously with 1 or more doses of living R cultures as indicated in Table IV. Some of the animals developed fever of 40°C. lasting from 1 to 5 days after the first dose and lost weight. No abscess formation was observed. 2 or 3 weeks after the last injection, they were inoculated subcutaneously with the MS form.

As shown in Table IV only 1 of the animals died after receiving a large dose, .1 cc., of a broth culture. All of the controls, including the one which received only .000001 cc. of the culture, died within 7 days. Most of the survivors, however, developed fever 2 to 4 days after the inoculation which lasted from 9 to 19 days, and lost weight. With the disappearance of fever they regained weight and recovered.

#### DISCUSSION

It has been demonstrated that the vaccination of hamsters and rabbits with living R forms of hog cholera bacilli and paratyphoid "C" bacilli elicits a definite immunity against the virulent S forms of both kinds of bacilli. Further work is, of course, necessary to determine the practical value of using living R forms of various bacteria as prophylactic vaccines. It should be borne in mind that in the case of the hog cholera bacillus, the NR cultures are not absolutely innocuous. NR bacilli occasionally cause death and frequently give rise to abscess formation in hamsters. Both NR and MR bacilli cause illness and fever in rabbits. Furthermore, as alluded to before, it was shown in a previous paper (2) that MR bacilli may revert to the virulent MS form *in vitro* and *in vivo*. The reversion to the MS form *in vivo* occurred after large doses had been injected intraperitoneally. In the present investigation the MR forms were injected subcutaneously and no reversion was observed.

In regard to the comparative immunizing power of the various antigenic components in the vaccine, the results of these experiments are at variance with those of certain other investigations. It was found that immunity was stimulated by both ØH (MR) and Ø (NR) forms of the living bacilli.

#### SUMMARY

1. Heat killed organisms of the MS (OH), MR (ØH), NS (O) or NR (Ø) forms of hog cholera bacilli failed to confer immunity, at



least to any considerable degree, in hamsters or rabbits against virulent hog cholera bacilli.

2. Hamsters and rabbits were successfully immunized against virulent MS bacilli by vaccination with living MR (ØH) and NR (Ø) forms.

3. Hamsters were immunized against MS hog cholera bacilli with living R paratyphoid "C" bacilli and conversely against MS paratyphoid "C" bacilli with living R hog cholera bacilli.

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