Immunization of Mice against Infection with Salmonella blegdam. Especially Protection of Mice from Typhoid Infection by Immunization with Living Rough Variants of Salmonella.*

By

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It is generally known that immunization with *killed* bacilli has a protective effect on *bacteriemia*. But typhoid fever, paratyphoid fever and anthrax, which are also accompanied with *bacteriemia*, have been considered to be exceptional cases. For anthrax effective immunity could not be introduced with *killed* vaccine, though Louis Pasteur[1] invented a preventive inoculation method in 1881, until recently low virulent *living* organism inoculations have been considered to be the only effective method, as far as anthrax is concerned. Recently Cromartie, Watson, Bloom and others[2] have proved why immunization with living bacilli is the only preventive measure for anthrax. Based on their findings, it is now possible to provide protection with vaccines containing no *living* organisms.

Thus one of the exceptional cases has been removed, leaving mechanism of immunity of typhoid diseases as the remaining problem. In typhoid and paratyphoid fever, it is statistically known that preventive inoculation with *killed* bacilli has lowered morbidity and lethality. But the immunity resulting from this vaccination is not as strong and effective as in immunity after recovery Moreover its effectiveness is not completely supported, either experimentally or theoretically. This subject has been studied at a high level in our country, particulary by Kobayashi and his collaborators[3]. According to their opinion, the protective power against typhoid and paratyphoid infection can be established by vaccination with *living* bacilli, whether in smooth or in rough type, but not with *killed* vaccine. However, Hazato and his collaborators[4] have reported that intravenous injections of *killed* bacilli have given satisfactory protection to mice against the infection of Salmonella enteritidis equal to those of *living* bacilli. Hosoya and others[5] have recently stated that immunization with their so-called T.T.T. or T.A.T. vaccine, which contains no *living* bacilli, has protective effect on infection of Salmonella enteritidis of mice and Salmonella abortus equi of guinea-pigs. Ominaga[6] has denied

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1) Pasteur, L.: C. R. Acad. Sci., 92, 429 (1881); Ibid., 92, 666 (1881).

2) Cromartie, W. J., Bloom, W. L. & Watson, D. W.; J. inf. Dis., 80, 1 (1947).
Cromartie, W. J., Bloom, W. L., Watson, D. W. & Heckly, R. J.: Ibid., 80, 14 (1947).
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Heckly, R. J. & Goldwasser, E.: Ibid., 84, 92 (1949).

3) Kobayashi, R.: Shinryo-Taiken, 2, 1 (1933); Rinsho no Nihon, 12, 50 (1938~1939); Nihon-Igaku-oyobi-Kenko-Hoken, 3240~3252, 1745, 1803, 1871, 1929 (1941); Ibid., 3360~3361, 3, 61 (1944).
Ushiba, D.: Nihon-Igaku, 3421, 97 (1948).

4) Hazato, H., Ota, M. & Ogawa, T.: Jap. J. Bact., 1, 52 (1944).

5) Hosoya, S.: Kiso-to-Rinsho, 2, 33 (1948).

6) Ominaga, Y.: Jap. J. Bact., 3, 18 (1948).

the opinion of Hazato and others. In the report of Hosoya and others it is pointed out that the invasion of bacillus into animal body is suppressed. If this is a fact, a similar suppression should be observed in the case of animals immunized with *living* bacilli. However it is regrettable to note that this has not yet been confirmed.

Concerning the immunity in typhoid infections there remain many important problems to be studied from theoretical and practical standpoints. Our observations, incorporated in this paper, deal mainly with vaccination with *living* rough bacilli and its possibilities as a protective measure to mice against infection of S. blegdam.

Materials and Methods.

1) *Challenge strain*: Bl_3S strain is a smooth type of Bl_3 strain which is one of two strains Bl_1 and Bl_3 which Uetake and others[7] isolated from a patient named Yasuhara in 1945 and identified as Salmonella blegdam. In 5 to 12 days after oral administration of 2 mg. of Bl_3 strain, mice succumb to secondary septicaemia followed by typhoid disease.

2) *Other Salmonella strains* utilized for immunization: All strains were received from the Japanese Branch of the International Salmonella Committee. Rough variant of each strain was isolated from the culture in broth containing the respective homologous immune serum which was produced in rabbits by inoculation of smooth organisms of a corresponding strain.

a) *Bl_1R_3 strain* and *Bl_1R_4 strain* were isolated from Bl_1S strain. Both strains have the properties of rough organisms, judging from the following: colonial morphology, character of growth in broth, virulence, salt sensitiveness, copper sulphate test, Millon's test, trypaflavine test, agglutination reaction by the antiserum against S. typhi R_2 strain which has all the characteristics of rough strain. But in absorption test, they absorbed all the antibodies against the original smooth organisms. Moreover, rabbit antisera against these rough variants contain antibodies reactive to O antigens of the original smooth strain. This suggests that these rough variants still have traces of smooth somatic antigens.

b) *$64R_1$ strain* was isolated from S. enteritidis 1891, *$65R_1$ strain* from S. dublin 215, *$76R_2$ strain* from S. london 1446 and *$84R_2$ strain* from S. newington C_2. Judging from the above mentioned tests, these have been determined as R variants. Moreover each variant did not absorb O antibodies from antiserum against the respective original smooth organisms.

3) *Protection test*: Mice used were from 12 to 15 gm. in weight. The methods of immunization will be described in each experimental part. After a fortnight following the last immunization, we challenged the mice by oral administration of 2 mg. of Bl_3S strain, and after that continued observations for a month. All dead mice were dissected, and from the blood specimens and organs, cultural examinations were made to determine whether they died from infection or not. The survivors were killed thirty days after challenge, and direct and bile cultures were made from blood specimens and various organs to recover the challenging or the immunizing organisms. Organisms recovered were invariably identified as to type and variant type.

Experimental Results.

A. *Immunization with Bl_1R_3 strain.*

Our primary aim was to determine whether immunization with living rough organisms of S. blegdam gave mice protection. As previously mentioned, it is extremely difficult to obtain rough organisms which are completely devoid of smooth somatic antigens. As a result we were obliged to employ Bl_1R_3 strain. This strain, as above mentioned, has various properties of rough organisms, which has but few O antigens of smooth organisms remaining. As controls we observed non-treated group together with formalized vaccine of Bl_3S strain group and living S. typhi R_2 strain group.

7) Uetake, H., Matsumiya, H., Odani, T. & Kogo, T.: New Clinic, 2, 147 (1948).

The mice of living Bl_1R_3 group were given one intraperitoneal injection with 10^{-3} mg. of this strain grown on agar slant at 37°C for 18 hours and those of living S. typhi R_2 group were given one 2×10^{-3} mg. injection intraperitonealy.

The mice of formolized Bl_3S vaccine group were intraperitonealy inoculated four times at one week intervals with increasing doses of 0.05, 0.1, 0.2 and 0.3 mg. Fifteen days after the last injection, all mice were challenged orally with 2 mg. of Bl_3S strain and observed for a month. The results are shown in table 1.

Table 1

Immunized with	Results
living Bl_1R_3 strain	●8 ○ ○ ○ ○ ○
killed Bl_3S strain	●8 ●10 ●11 ●13 ●16 ●17 ●24
living S. typhi R_2 strain	●7 ●10 ●11 ●12 ●12 ●17 ●25
non-treated control	●5 ●5 ●6 ●6 ●7 ●7 ●8 ●8 ●10 ●10

Note: ○ survivors; ● dead mice; Numerals flanking black marks indicate survival days from challenge to death.

In the Bl_1R_3 group only one mouse died and the other five survived, whereas all mice of the other groups succumbed. In all of the dead mice, the challenge bacilli were recovered from the heart blood specimens and the other organs, indicating the occurrence of secondary septicaemia.

Comparing the lethality of each group the thirtieth day after the challenge by direct calculation of probabilities, the difference should be regarded as significant between the living Bl_1R_3 group and the killed Bl_3S or living S. typhi R_2 group at the level of significance of 2.85%, and between living Bl_1R_3 group and non-treated control group at that of 0.137%.

Secondly, comparing the survival days, the calculated values are as described in table 2. The average survival days of the populations are 14.1 ± 5.10 days (from 9.0 to 19.2 days), 13.4 ± 5.53 days (from 7.87 to 18.97 days) and 7.2 ± 1.295 days (from 5.905 to 8.495 days) at the level of significance of 5%, respectively in killed Bl_3S, living S. typhi R_2 and control group. Thus the difference between the killed Bl_3S and control group should be regarded as significant. In other words, it suggests that the vaccination, even with killed smooth baccilli, may prolong the survival period of mice. But at the same level our observed difference should not be regarded as significant between killed Bl_3S and living S. typhi R_2 group or between living S. typhi R_2 and the control group.

Table 2 *Comparison of mean survival time of dead mice*

Immunized with	killed Bl_3S (days)	living S. typhi R_2 (days)	none (control) (days)
sample mean ($\bar{x}$)	14.1	13.4	7.2
unbiased estimator of population variance (μ^2)	30.55	35.84	3.29
μ	5.52	5.98	1.8038
confidence interval of population mean (m)*	14.1 ± 5.10 ($9.0\leq m\leq19.2$)	13.4 ± 5.53 ($7.87\leq m\leq18.97$)	7.2 ± 1.295 ($5.905\leq m\leq8.495$)

* confidence coefficient of 95%

In short, from the above-mentioned experimental results, it can be said that the vaccination with living Bl_1R_3 strain gives mice protective power with comparative case, whereas with Bl_3S vaccine the survival time of mice can only be prolonged but for a short period. Here, it would give rise

to discussion, that Bl_1R strain has, though a little, O antigens of smooth organisms. No one can positively state that the antibodies against smooth somatic antigens have nothing to do with immunity, for even this amount of smooth O antigens can stimulate the production of antibodies against these antigens. Moreover on the other hand, it was extremely difficult to obtain a perfect rough variant from Bl S strain. Our next step was to immunize mice with rough variants of heterologous Salmonella.

B. *Immunization with typical rough organisms.*

As rough variants, we obtained *64R_1 strain*, *65R_1 strain*, *84R_2 strain* and *76R_2 strain*, respectively from S. enteritidis 1891, S. dublin 215, S. newington C_2 and S. london 1446 strain. The above rough strains do not absorb the O antibodies in the antiserum against their respective original smooth strains. But they still have H antigens (i.e. H form). Beside the above *S. typhi R_2*, *Bl_1R_4 strain* and formalized vaccine of Bl_3S strain were used as a comparison.

Immunization: In the groups vaccinated with rough strain, living organisms were inoculated intraperitoneally one or two times at intervals of one week. The vaccination with $64R_1$ or $65R_1$ strain consist of two doses of 10^{-4} and 10^{-3} mg., that with $84R_2$, $76R_2$ or Bl_1R_4 strain consists of one dose of 10^{-3} mg., and that with S. typhi R_2 consists of two doses of 10^{-3} and 10^{-2} mg. Formalized Bl_3S vaccine was injected intravenously at one week intervals five times, 0.05, 0.06, 0.06, 0.1 and 0.2 mg. Fourteen days after the last injection, all mice were given 2 mg. of Bl_3S strain per os.

The results are shown in table 3.

Table 3

Immunized with	Results
living $64R_1$	●15 ●18 ●26 ○ ○ ○ ○ ○ ○ ○ ○ ○
living $65R_1$	●10 ●12 ●18 ●19 ●22 ○ ○ ○ ○ ○ ○ ○ ○ ○ ○
living $76R_2$	●12 ●14 ●16 ●17 ●18 ●18 ●18 ●24 ●24 ●28 ●37 ○ ○ ○ ○
living $84R_2$	●10 ●11 ●13 ●15 ●24 ●27 ○ ○ ○ ○ ○ ○ ○ ○
living Bl_1R_4	●11 ●16 ●31 ●31 ○ ○ ○ ○ ○ ○
living S. typhi R_2	●6 ●10 ●14 ●14 ●16 ●16 ●18 ●26 ●35 ○ ○ ○ ○
killed Bl_3S	●12 ●12 ●14 ●18 ●18 ●18 ●19 ●24 ●35 ○
none (control)	●10 ●10 ●12 ●13 ●18 ●20 ●20 ●2 ●23 ●25 ○

Notes are same as in table 1.

The first notable fact on scanning this table is that the survival time of the dead mice seems longer than those in the first experiment. Comparing the mean survival days of the nontreated control groups in both experiments, those of the populations in the experiment A and B are respectively 7.2 ± 1.8501 (from 5.3499 to 9.0501) days and 17.2 ± 5.6239 (from 11.5761 to 22.8239) days at the level of significance of 1%. Thus it can be accepted at this level of significance that the survival time of dead mice in the experiment B is longer than that in the experiment A. The mice used in experiment A are of English stock, and in experiment B of German stock.

It is possible that the difference that has been observed may be due to the difference in stock.

As the average survival days have become longer we extended the observation period. At 50 days after the challenge we compared all groups to ascertain any difference that might exsist between the respective death rates of all groups. By direct calculation of probabilities P-values as shown in table 4 were calculated. P is the sum of the probabilities of the set of frequencies observed and the two possible more extreme sets of frequencies which might have been observed.

Thus P-values compared with the control group are 0.0029, 0.0052, 0.0358 and 0.023 respectively in $64R_1$, $65R_1$, $84R_2$, and Bl_1R_4 group. At the level of significance of 1% in the former two

Table 4 *P* between two groups*

	living $65R_1$	living $76R_2$	living $84R_2$	living Bl_1R_4	living S. typhi R_2	killed Bl_3S	none (control)
living $64R_1$	>0.2729	0.0212	>0.2113	>0.2704	0.0499	0.0037	0.0029
living $65R_1$		>0.0562	>0.2606	>0.3090	>0.0533	0.0118	0.0052
living $76R_2$			>0.0790	>0.0877	>0.3133		>0.2 ···
living $84R_2$				>0.3221	>0.1233	0.042	0.0358
living Bl R_4					>0.1346	0.0321	0.023
living S. typhi R_2							>0.1845

Note *: P indicates the sum of the probabilities of the set of frequencies observed and the two possible more extreme sets of frequencies which might have been observed.

groups and 4% in the latter two, it can be accepted that there is a definite correlation between vaccination and reduction of the lethality. But P-values more than 0.1854 in S. typhi R_2 group and more than 0.2 in $76R_2$ group, so it can not be said that these vaccines have any lethality reducing effect. The effect of Bl_3S vaccine is, at sight, smaller than that of $76R_2$ strain. In other words killed smooth organisms have no effect to reduce lethality even by intravenous inoculations.

Comparing the groups immunized with living rough organisms and killed Bl_3S vaccine group by direct calculation of probabilities, P-values of $64R_1$, $65R_1$, Bl_1R_4 and $84R_2$ group are, as shown in table 4, 0.0037, 0.0118, 0.042 and 0.0321. At the level of significance of 5%, the difference should be regarded as significant between the living vaccine group and the killed vaccine group, while it should not be regarded as significant at the same level between $76R_2$ or S. typhi R_2 group and Bl_3S vaccine group.

Furthermore, comparing the groups vaccinated with living rough organisms, as shown in table 4, generally speaking the difference between each should not be regarded as significant at the level of significances of 5%. Only when comparing of $64R_1$ group with $76R_2$ or S. typhi R_2 group, P is so small (0.0212 or 0.0499), that these differences should be regarded as significant. In other words, it cannot be decided from these experiments, which of $64R_1$, $65R_1$, $84R_2$ and Bl_1R_4 strain has the highest immunizing effect.

Contrary to the former, in the present experiment it is suggested that mice of German stock are generally more resistant to S. blegdam infection that those of English stock, as the survival period was longer and one out of eleven control mice survived. Therefore to confirm the above results, and to prove further that it was not by a mere chance of low virulence of the infecting bacilli, and also to prove that the mice had actually survived the infection and had not survived due to some unforseen coincidence, we repeated the challenge to all survivors by oral administration of 2 mg. of Bl_3S strain 50 days after the first challenge. As a result thirty days after the second challenge none were dead by infection. At that time all mice were killed and cultural examinations were performed on each organ. The results are shown in table 5.

It is obvious from this table that though all mice had resisted the reinfection, we could still recover the challenge strain from livers, spleens or mesenterial lymphatic glands of the majority of mice even thirty days after the second challenge. Only four out of fourty mice failed to show challenge organisms. In all other mice the challenge bacilli were recovered by direct cultivation or by enriching in bile media. This indicates that the mice had resisted the challenge bacilli which had positively invaded the body. This suggests the following.

1) The resistance of mice is obviously due to immunity.

2) This immunity neither is able to suppress the invasion of challenge bacilli into the body, nor has a bactericidal effect on invading bacilli in the body.

Table 5

Immunized with	Mous No.	Cultivated from: Heart blood	Liver	Spleen	Mesent. gland
living $64R_1$	6404	−	+	+	−
	5	−	⊕	+	
	6	−	−	$+_1$	
	7				
	8	−	⊖	$+_4$	
	9				
	10	−	⊕	⊖	
	11	−	⊕	⊕	$+_6$
	12	−	⊕	⊕	$+_1$
living $65R_1$	6506	−	⊖	⊕	+
	7	−	⊖	⊖	−
	8	−	$+_6$	⊕	$+_4$
	9	−	⊖	⊖	−
	10	−	⊕	⊕	⊕
	11	−	⊖	⊖	⊕
	12	−	$+_3$	$+_1$	$+_1$
	13	−	⊕	⊖	⊕
	14	−	⊕	⊕	$+_3$
	15	−	$+_1$	$+_1$	$+_1$
living $76R_2$	7612	−	⊕	⊕	$+_4$
	13	−	⊕	⊖	$+_1$
	14	−	⊖	⊖	⊖
	15	−	$+_1$	$+_2$	$+_1$
living $84R_2$	8407				
	8	−	⊕	⊕	⊖
	9	−	⊕	⊕	$+_1$
	10	−	X_1	⊖	X_1
	11	−	⊖	⊖	$+_1$
	12	−	⊖	⊖	⊖
	13	−	⊕	⊕	$+_3$
	14	−	⊖	$+_1$	X_2
living Bl_1R_4	1121	−	⊕	$+_2$	⊖
	22	−	$+_2$	$+_3$	$+_2$
	23	−	⊖	$+_4$	$+_2$
	24	−	⊕	⊕	⊖
	25	−	$+_1$	$+_4$	$+_8$
	26	−	⊖	⊖	⊖
living S. typhi R_2	6316	−	⊕	$+_1$	⊖
	17	−	⊕	⊕	$+_4$
	18	−	⊕	⊕	$+_2$
	19	−	⊕	⊕	⊖
killed Bl_3S	520	−	⊕	$+_3$	$+_5$
none (control)	131	−	⊕	$+_4$	$+_1$

+ indicates recovery of challenge organisms by direct cultivation;
⊕ indicates recovery of challenge organisms after enrichment in bile medium;
Numerals on right of + indicates unmber of colonies;
− indicates negative cultivation;
⊖ indicates absence of challenge organisms after enrichment in bile medium;
X indicates recovery of immunizing bacilli.

In other words, regardless of the mice stock, when rough living organisms are used to immunize mice against typhoid infection it serves as an effective protective measure. Basicly the results are identical with that of Experiment A. Another important fact is that the immunizations with living rough variants isolated from heterologous S. enteritidis, S. dublin, S. newington etc., also provide protection to mice against infections of S. blegdam.

C. *Reexamination of experiment B and the vaccine consisting of killed organisms.*

As the mice stock used in Experiment B differed from that of Experiment A, we conducted a reinvestigation by using the same stock of mice as in A to reconfirm a part of Experiment B and at the same time to confirm the effect of killed rough organisms, mixed vaccine of killed smooth and killed rough organisms and antiserum of rabbit immunized with living rough organisms. Of the living rough strains, *$84R_2$* and *$76R_2$ strains* were reexamined, as in these two groups in experiment B, the number of survivors was relatively small.

Immunization: 10^{-3} mg. of $84R_2$ or $76R_2$ strain, in living state, was inoculated intraperitoneally twice at intervals of 9 days. Both strains were grown on agar at 37°C. for 18 hours. Formalized

vaccine of Bl_1R_4 strain was injected four times at intervals of 7 days intraperitoneally with doses of 0.1, 0.3, 0.8 and 1.2 mg. Mixed vaccine was injected in the same way with doses of 0.1 mg. of formalized Bl_1R_4 vaccine and 0.05 mg. of formalized Bl_3S vaccine, 0.2 and 0.1, 0.4 and 0.3, 0.8 and 0.4. In one group of mice, 0.5 ml of rabbit antiserum against living Bl_1R_3 strain was injected intraperitoneally. O agglutinine titer of this antiserum is 1 : 1280 and ϕ agglutinine titer is 1 : 2560. Thus it includes, as reported by Obara and others[8], both O antibodies and ϕ antibodies.

In all groups, mice were challenged per os with 2 mg. of Bl_3S strain grown on agar at 37°C. for 18 hours. In the groups of active immunization the challenge was made two weeks after the final immunization, and in the passive immunization on the same day as the injection of serum.

The results are shown in table 6.

Table 6

Immunized with	Results
living $84R_2$	●7 ●8 ●9 ●9 ●10 ●10 ●10 ●11 ●11 ●15 ○ ○ ○ ○
living $76R_2$	●6 ●7 ●7 ●7 ●7 ●8 ●8 ●9 ●9 ●9 ●11 ●14 ●20 ○ ○
killed Bl_1R_4	●9 ●10 ●10 ●10 ●10 ●11 ●11 ●11 ●13 ●13
killed Bl_3S + killed Bl_1R_4	●5 ●6 ●6 ●7 ●9 ●10 ●10 ●11 ●12 ●14
Immune serum against living Bl_1R_3	●6 ●9 ●9 ●9 ●10 ●10 ●10 ●10 ●13 ●13
none (control)	●5 ●6 ●6 ●6 ●6 ●7 ●7 ●7 ●7 ●8 ●8 ●8 ●8 ●8 ●9 ●9 ●9 ●10 ●11 ●11

Notes are same as in table 1.

Table 7 *Comparison of mean survival time of dead mice*

Immunized with		living $76R_2$	killed Bl_1R_4	killed Bl_3S + killed Bl_1R_4
sample mean	($\bar{x}$)	9.21 days	10.8 days	9 days
unbiased estimator of population variance	(μ^2)	13.96	1.73	8.66
	μ	3.7363	1.3156	2.9428
confidence interval of population mean		9.21±2.1557 days	10.8±0.941 days	9±2.105 days
(confidence coefficient of 95 per cent.)		(7.0543≤m≤11.3657)	(9.859≤m≤11.741)	(6.895≤m≤11.105)
ibid.			10.8±1.355 days	
(confidence coefficient of 99 per cent.)			(9.445≤m≤12.155)	

Immunized with		Immune serum against Bl_1R_3	none (control)
sample mean	($\bar{x}$)	9.9 days	7.8 days
unbiased estimator of population variance	(μ^2)	4.1	2.8
	μ	2.0248	1.6733
confidence interval of population mean		9.9±1.447 days	7.8±0.783 days
(confidence coefficient of 95 per cent.)		(8.453≤m≤11.347)	(7.017≤m≤8.583)
ibid.			7.8±1.070 days
(confidence coefficiente of 99 per cent.)			(6.73≤m≤8.87)

Notes are same as in table 2.

Comparing the lethality there is a significant difference only between the $84R_2$ group and the control group, i.e. $P \fallingdotseq 0.02158$ (direct calculation of probabilities). But the difference should not be accepted as significant at the level of significant of 5% between $76R_2$ group, killed Bl_1R_4 group, mixed vaccine group or antiserum group and the control group.

Then, comparing the mean survival days of the populations, which are shown in table 7, the difference should be regarded as significant at the level of significance of 1% between the killed Bl_1R_4 group and the control group, though no significant difference should be accepted at the level of 5% between any other treated group and the control group, and no significant difference should be accepted at the level of significance of 5% between any two treated groups.

The mean survival days of the population of the control group is 7.8 ± 1.070 (from 6.730 to 8.870) days at the level of significance of 1%, while that of killed Bl_1R_4 vaccine group is 10.8 ± 1.355 (from 9.445 to 12.155) days. Therefore the difference between these two groups should be regarded as significant at this level. However, as the Bl_1R_4 strain has traces of O antigens of smooth organisms, it can not be determined whether the prolongation of the survival time of mice is due to smooth O antigens or to ϕ antigens.

In short, it may be said that the results are almost the same as those in experiment B regardless of the difference in the stock of the mice.

The challenge organisms were recovered from livers, spleens, mesenterial lymphatic glands or kindneys of the survivors which were killed thirty days after the challenge.

IV. Summary and Discussion.

In carrying out our investigations of the mechanisms of immunity in typhoid disease using S. blegdam, we conducted our primary experiments to confirm that vaccination with living rough organisms gave mice protective powers against typhoid infection as reported by Kobayashi and others[3]. We cultivated Bl_1S and Bl_3S strains (both S. blegdam) in broth containing homologous antiserum to obtain rough variants. But we were unable to isolate a completely rough variant devoid of smooth somatic antigens. Therefore we were compelled to use Bl_1R_3 strain, which had almost completely changed to R type to immunize mice in an effort to prove effectiveness against infection. The results are as shown in experiment A. Thus mice of living Bl_1R_3 group were highly resistant to the infection, while all mice of killed Bl_3S and living S. typhi R_2 gruop, used as control groups, were dead. But this Bl_1R_3 strain is not completely rough. Therefore, as reported by Obara and others[8], we must not ignore the influence of the remaining smooth antigens. Thus we employed compeltely rough variants of other Salmonella bacilli in our experiments, cofident that to a certain extent, cross immunity could be expected, as a result of our perusal of literatures.[4),11)] For this purpose, we used $64R_1$, $65R_1$, $84R_2$, $76R_2$ and S. typhi R_2 strain.

While vaccination with living $64R_1$, $65R_1$ or $84R_2$ strain brought a protective effect on mice subjected to oral infection of S. blegdam, a significant difference was not found between the $76R_2$ or S. typhi R_2 group and the non-treated control group. In the latter two groups even a prolongation of survival time could not be noticed in our experiments.

In any event, however, it was found that typboid infection could be protected by vaccination with living bacilli of completely rough type and moreover with living rough organisms of heterologous

8) Obara, T., Tamura, S. & Matsumiya, H.: Hokkaido-Joshi-Igaku-Senmongakko-kiyo, 2, 71 (1950).
9) Sato, R.: Musokan-kenteicho, (1947).
10) Sato, K.: Heikinchi-to-Hyakubunritsu, (1948).
11) Tenbroeck, C.: J. Exp. Med., 28, 759 (1918): Ibid., 32, 19 (1920).
Ando, K.: Saikingaku-Zasshi, 128 (1934).
Shudo, S.: Ibid., 303 (1934); Ibid., 495 (1938).
Ando, K., Ochi, Y. & Shudo, S.: Ibid., 757 (1934).

Salmonellas. This fact supports, thoughly, the theory of Kobayashi and others. And it indicates, in other words, that in the immunity of mice to typhoid infection of S. blegdam, so-called smooth somatic antigens do not play an important role, though these antigens indicated in Kauffmann-White's table are important in diagnostic typing of Salmonella.

Survivors of living $64R_1$, $65R_1$, $84R_2$ or Bl_1R_4 group were killed thirty days after the challenge to be cultured. In most cases, the bacilli were recovered from livers, spleens, mesenterial lymphatic glands or kidneys. This fact suggests, that "this immunity has neither the suppressive effect on invasion of the infecting bacilli into the body nor the bactericidal effect on the invading bacilli", but that "the multiplication of the invading bacilli is inhibited". This interpretation supports the opinion of Kobayashi and others. On the other hand, however, this observation seems to be somewhat different from that obtained from guinea pigs. In guinea pigs immunized with living rough organisms, the challenge organisms were seldom recovered from various organs fifteen days after the oral challenge while they were recovered in the non-treated control group and in the group immunized with killed smooth organisms[12]. And in these cases it is confirmed that the invasion of the challenge bacilli into the body is not suppressed and the invading bacilli are killed relatively rapidly. And therefore it is obvious that the mechanism of immunity in guinea pigs is different from that in mice.

Even among the same rough variants of Salmonella there are strains such as $76R_2$ and S. typhi R_2 which even in their original smooth type fail to establish typhoid in mice. The same bacilli in rough type also fail to produce a sufficient amount of resistance in mice that could be considered significant when compared with the control group. However, even in the case of Salmonella that does not cause typhoid, it would be still impossible to state that the said Salmonella does not produce immunity against typhoid until further experiments have been conducted.

Comparing the lethality of $64R_1$, $65R_1$, $84R_2$ and Bl_1R_4 groups, the differences between each other could not be considered significant, as indicated in table 4.

Though the group vaccinated with killed smooth organisms (Experiment A) or the killed rough organisms (Experiment C) showed a longer survival time than the control group, none of these groups or mixed vaccine group showed a reduce in lethality. To deny the effect of killed vaccine, it is absolutely necessary that the conditions under which the vaccine was prepared and the methods of immunization were of the best. Though it would be difficult to insist that the vaccine used by us and its administration were impecable, the intensity of our immunization was sufficient to cause the death of several or more mice during the immunization process. It may be said that the above-described experiments indicate that the antibodies against the surface antigens of smooth or rough organisms have no essential significance in the immunity of the typhoid diseases. These results are quite similar to the previous reports of Kobayashi and his collaborators, whose theory is supported by our experiments.

However, as another point, we cannot ignore the reports of Hazato and others[4] and that of Hosoya[5]. Hazato and others said that the intravenous injection of killed vaccine protected mice from the infection of Gärtner's bacillus to a degree not lower than that of immunization with living rough organisms. And they have claimed that its mechanism lies in the production of typhoid granuloma. Ominaga[6] has made a report opposing and denying the above opinion and explanation. The author and his associates have also been unable to obtain satisfactory results by intravenous injections. (Experiment B). Hosoya reports that by using his so-called T.T.T. vaccine or T.A.T. vaccine, he was able to immunize guinea pigs against the infection of S. abortus equi and mice against Gärtner's bacillus. It also should be noticed that he reports that the invasion of challenge bacilli into the animal body can be suppressed. If this were the case, the two different mechanisms of immunity, the suppression of invasion of the infecting bacilli into the animal body

12) not yet published.

and the suppression of multiplication of the invading bacilli in the body, should be observed simultaneously when immunized with living smooth organisms. But such a phenomenon has not yet been described. Before the opinion of Hosoya and others can be accepted, experiments based upon the above-mentioned point of view must be conducted. Concerning the above the author and his associates are at present conducting experiments to be reported at a later date.

Vaccination with killed rough organisms induced a prolongation of survival time of mice. As previously noted, however, it is not obvious whether this effect is due to the ϕ antibodies or to the O antibodies, for the latter may have been produced in response to the stimulus of the remaining smooth O antigens of rough organisms.

In any event, as the effect of the killed rough organisms or living 76R_2 or S. typhi R_2 strain in the protection test is not recognized, the antibodies against the surface antigens of rough bacilli could be considered as unimportant in regard to protection. We suspect that the protective effect may be due to the living state of the bacilli. Therefore, we attempted to immunize mice passively with the antiserum against living rough organisms. But this passive immunization did not induce any protective effect. However, there is a possibility that this failure may be due to the rabbit antiserum. Furthermore, in view of the recent reports on immunization of anthrax, a premature conclusion would be dangerous.

In short, in the prevention of typhoid against mice it is possible to produce a protective power by utilizing rough organisms which have lost specific surface O antigens of smooth organisms, provided that they are in a living state. Moreover protective power may be induced by utilization of a rough variant of heterologous Salmonella, also in a living state. It is notable that though the said immunity may neither have a suppressive effect on invasion of challenge bacilli nor have a bactericidal effect, it has a supressive effect on its multiplication. Our above findings coincide and completely support the opinions of Kobayashi and others[3].

(Receivead Feb. 20 1951)

Conclusion.

1. The mice infected with S. blegdam per os succumb, 5 to 12 days after ingestion, due to secondary septicaemia following typhoid infection.

2. Infection of mice was prevented by immunization with living, almost completely rough variant of S. blegdam, or living completely rough variant of S. enteritidis, S. dublin, or S. newington, but not with that of S. london 1446 or S. typhi R_2.

3. The mice vaccinated with killed smooth or rough organisms of S. blegdam survived longer than those of the control group. But the detailed mechanism of the latter is not clear.

4. In immunization with various living rough organisms, neither was the invasion of infecting bacilli into mice inhibited, nor were the invading bacilli killed rapidly. But it may be accepted that in the animal body the multiplication of bacilli was suppressed.