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Comparative studies on *Salmonella typhi* grown *in vivo* and *in vitro*

II. The effect of extracts from normal and infected organs on the bactericidal serum action on strains grown *in vivo* and *in vitro*

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In previous experiments of Olitzki & Godinger (1963) it was shown that *Salmonella typhi* strain Ty 2 grown *in vivo* was more virulent for mice than its corresponding culture grown *in vitro*. Extracts of infected organs acted as infection-promoting substances, while extracts from normal organs exerted similar but weaker effects. The experiments described below were carried out in order to examine whether virulent *S. typhi* grown *in vivo* is less sensitive to the bactericidal action of immune sera than the *in vitro* grown, and whether organs of normal and infected mice exert modifying effects on the bactericidal serum action, or are, in this respect, inert.

MATERIALS AND METHODS

Bacterial inocula

S. typhi strains O 901 and Ty 2, were employed. The bacteria were daily subcultured on nutrient agar and on the day of the experiment washed off from the agar slant with 5 ml. of brain-heart infusion (Difco) and pre-incubated for 1 hr. In other experiments they were washed off with 5 ml. of salt solution which contained 0·85 % of sodium chloride and 0·063 % of magnesium chloride, and used without pre-incubation. Then the number of bacteria (about 10^9/ml.) was determined photometrically and by plating. Suitable dilutions of the suspension were made in order to obtain an inoculum of 10^4 bacteria per test tube. The *in vivo*-grown bacteria were taken from spleens of infected mice, separated from the tissue as described by Olitzki & Godinger (1963), and the suitable inoculum determined by the same method as employed with the *in vivo*-grown bacteria.

Preparation of immune sera

The antisera employed were the same as used by Olitzki & Godinger (1963).

Tissue extracts

These extracts were prepared from organs of normal and infected mice according to the method described by Olitzki & Godinger (1963). 0·5 ml. of organ extract per 2·0 ml. of the total volume were added to each tube in order to examine the effect of the organ extract on the bactericidal serum action.

The bactericidal test

This test was performed according to the technique described by Felix & Olitzki (1926). Modifications of this technique are mentioned. The incubation times of the tubes which contained the bacteria exposed to immune serum and complement and of the corresponding control tubes were varied according to the conditions of the individual experiments as described below. While in the experiments of Felix & Olitzki (1926) all test tubes contained constantly 10% broth, we varied this broth concentration according to the conditions of the individual experiments.

RESULTS

The experiment presented in Table 1 shows that the bactericidal action of an anti O 901 immune serum on the homologous strain is highly enhanced by the presence of 0·1 ml./2·0 ml. broth in each test tube. This nutrient enhanced the growth of the bacteria which were not exposed to the bactericidal action of immune serum and complement but, on the other hand, it enhanced the bactericidal action when immune serum and complement were present. The experiment presented in Table 2 also shows clearly that multiplying bacteria are more sensitive to the bactericidal immune serum-complement action than non-multiplying bacteria. Table 3 shows that there exists a quantitative relationship between broth concentration, ability to multiply in it and sensitivity to the bactericidal serum action. The latter increases with the broth concentration and the improvement of the growth condition.

Table 1. *Observations on the influence of nutrient broth on the serum bactericidal action at different exposure times*

(Broth concentration, 5%. Dilution of anti-O 901-immune serum, 10^{-3}. Dilution of complement, 10^{-1}. Strain O 901 grown *in vitro*)

	Bacterial count (thousands)			
	In the presence of broth		Without broth	
Time of exposure (min.)	With antibody and complement	Without antibody and complement	With antibody and complement	Without antibody and complement
10	0·80	16·4	2·2	16·4
20	0·20	26·4	0·90	17·8
30	0·40	—	0·80	—
40	0·04	28·0	0·12	18·0
50	0	—	0·02	—
60	0	32·0	0·02	16·8
120	0	43·0	0·02	19·0
0	—	—	—	22·4

The addition of glucose and ammonium sulphate to the test tube enhanced the bactericidal serum action, although these nutrients did not effect an immediate multiplication of the micro-organisms. As shown in Table 3, the growth-enhancing

activity of the two together was approximately equivalent to a 0·025 % broth concentration. The enhancement of the bactericidal serum action in the presence of 1·0–2·0 % glucose and 0·5–1·0 % ammonium sulphate was well marked. These

Table 2. *The influence of two broth concentrations on the bactericidal action of different serum and complement dilutions*

(Strain O 901 grown *in vitro*)

Complement dilutions	Bacteria suspended in	No. bacteria after 2 hr. incubation: At antibody dilutions 10^{-2}	10^{-3}	10^{-4}	With complement only
1/10	Saline-$MgCl_2$ solution	0·56	0·80	1·20	5·20
	5 % broth added	0·02	0	0·02	0·40
	10 % broth added	0·02	0	0	0·40
1/15	Saline-$MgCl_2$ solution	0·48	1·40	1·60	7·20
	5 % broth added	0·02	0·04	0·04	1·60
	10 % broth added	0	0	0·04	0·40
Controls without complement	Saline-$MgCl_2$ solution	2·2	8·6	8·2	—
	5 % broth added	2·4	7·5	13·0	—
	10 % broth added	3·2	11·0	9·2	—

At 0 hr. inoculum, 27·0; after 2 hr. in saline-$MgCl_2$, 29·0; after 2 hr. in 5 % broth, 76·0; after 2 hr. in 10 % broth, 79·0.

Table 3. *The influence of different nutrients and varying broth dilutions on the bactericidal action of anti O-immune serum (dilution* 10^{-3}*) and complement (dilution* 1/20)

(Exposure 2 hr. at 37° C. *S. typhi* O 901)

Nutrients present and their concentrations %	Bacterial count (thousands) after exposure to the suspension fluid: With O-antibody and complement	Without antibody and complement	With antibody only	With complement only
Broth 2·5	0·04	61·0	—	—
1·0	0·19	51·9	—	—
0·05	0·37	48·8	—	—
0·025	0·48	30·4	—	—
Glucose 2 % + $(NH_4)_2SO_4$ 1 %	0·10	29·2	—	—
Glucose 1 % + $(NH_4)_2SO_4$ 0·5 %	0·13	25·9	—	—
Glucose 0·5 % + $(NH_4)_2SO_4$ 0·2 %	0·26	27·0	—	—
Saline $MgCl_2$ solution	0·56	27·5	4·4	2·1
Saline $MgCl_2$ solution 0 hr.	—	22·4	—	—

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nutrients effected also a slow multiplication of the micro-organisms which became visible after an incubation at 37° C. for 18 hr. Experiments with short incubation times at 37° C., as that presented in Table 4, showed that the bactericidal serum action started after only a few minutes contact between the micro-organisms and the antibody-complement system. During this short period no increase of the bacterial count was observed in the control tubes, which contained the bacterial suspension without antibody or complement. When bacteria pre-incubated for 1 hr. in brain-heart infusion were transferred into the salt solution there was even some decrease of the bacterial count. These experiments proved that the bactericidal action of the serum was not necessarily associated with the real multiplication of the micro-organisms, but rather with the morphological and biochemical changes which took place during the lag phase, e.g. the increase of the average body length which preceded the cell division and had been described by Henrici (1923). The relationship between sensitivity to bactericidal serum action and increase in length during the lag phase is demonstrated in Table 4. It shows that the sudden increase in length during the incubation time is correlated with the sensitivity to bactericidal action which starts immediately on exposure to antibody and complement, before real cell divisions have taken place, since according to the bacterial counts in the control tubes the inoculum became doubled only after a 2 hr. incubation, meaning that one cell division was completed only after 2 hr. Therefore we concluded that the presence of nutrients may modify the bactericidal test in two ways: (1) it may speed up the increase of the bacterial count in the control tubes which do not contain antibody and complement; (2) it may enhance the bactericidal power of the antibody-complement system. It follows that experiments with normal and infected organ extracts may be influenced by their nutritional values and that the results obtained with them should be evaluated accordingly.

Table 4. *The morphological changes which occur after the transfer of* S. typhi *strain* O 901 *from a 24 hr. culture into two suspension fluids and the correlation of these changes with the sensitivity to bactericidal action*

(Dilution of anti-O immune serum 10^{-3}, dilution of complement 10^{-1}.)

		Changes observed after inoculation									
		Into broth after an incubation of (min.)					Into $NaCl$-$MgCl_2$ solution after an incubation (min.)				
Observed changes	Control before incubation	5	10	20	30	120	5	10	20	30	120
Average length (μ)	2·87	3·34	3·50	3·89	2·95	—	3·16	3·16	3·29	3·46	—
% of bacteria:											
shorter than 3·5 μ	79	58	60	42	40	—	77	77	64	57	—
3·5 μ or longer	21	42	40	58	60	—	23	23	36	43	—
Bacterial count/1000 with amboceptor and complement	17·5	1·2	0·7	—	0·07	0	2·4	1·4	—	0·6	0·4
Control without amboceptor and complement	17·5	—	—	—	—	35·0	—	—	—	—	—

Table 5 shows the effect of organ extracts of normal and infected mice on the bactericidal serum action on strain O 901. The control experiments without the bactericidal system showed that both the normal and the infected organ extracts

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exerted a marked growth-promoting activity when added to the bacterial inoculum in saline or in broth.

As expected, all organ extracts from normal mice enhanced the bactericidal serum action both in saline and in broth. The extracts of organs from infected mice also promoted bacterial growth and, therefore, they enhanced also the bactericidal serum action when added to a bacterial suspension exposed to immune serum and complement in a salt solution. However, when extracts of infected organs were added to bacteria suspended in broth which itself constituted a powerful nutrient then the bactericidal action was lowered probably by soluble antigens present in infected liver, lungs and kidneys.

Table 5. *The influence of organ extracts from normal and infected mice on the growth of strain* O 901 *grown* in vitro *and the bactericidal serum action on it, anti-O immune serum dilution* 10^{-3}, *complement dilution* 1/20, *incubation* 2 *hr. at* 37° C.

	Bacterial count (thousands) in the presence of							
	Amboceptor and complement with addition of				Without amboceptor and complement with addition of			
	Infected organ extracts		Normal organ extracts		Infected organ extracts		Normal organ extracts	
Extracts prepared from	Salt solution	Broth 5 %	Salt solution	Broth 5 %	Salt solution	Broth 5 %	Salt solution	Broth 5 %
Heart	1·50	0·10	0·60	0·04	90·0	190·0	77·0	206·0
Lung	5·60	1·20	3·70	0·32	88·0	160·0	72·0	190·0
Lymphatic glands	0·06	0	0·20	0·04	110·0	150·0	48·0	140·0
Kidneys	1·30	0·12	0·36	0·04	120·0	220·0	72·0	154·0
Spleen	0·04	0·04	0·56	0·04	69·0	210·0	56·0	180·0
Liver	8·00	4·00	0·36	0·24	44·0	160·0	54·0	200·0
Peritoneal fluid	2·30	0·06	0·88	0·04	48·0	190·0	78·0	300·0
Brain	0·16	0·08	1·50	0·12	76·0	260·0	68·0	330·0
Controls without organ extract:								
0 hr.	—	—	18·0	18·0	—	--	18·0	18·0
2 hr.	—	—	12·0	0·72	—	—	26·0	120·0

If we compare in Table 5 the bactericidal effects exerted in saline and in broth, in the presence of normal extracts from heart, lungs, kidneys and liver, with that exerted in the presence of the respective extracts from infected organs, then we find that the bactericidal effect in the presence of infected organs was always lower than that observed in the presence of normal organ extracts. We repeated three times the experiments with normal and infected heart, kidneys and liver extracts and obtained the results summarized in Table 6. The results obtained with several extracts show that the majority of organ extracts of mice infected

with strain Ty 2 may exert an inhibiting effect on the bactericidal serum action on strain O 901.

Experiments with *S. typhi* strain Ty 2, grown *in vitro*, showed that this strain was less sensitive to the bactericidal action of an anti-O 901-immune serum than O 901 itself, but this resistance could be broken when 5 % broth was added and complement dilutions of 1/10 and 1/6 employed, as shown in Table 7.

Table 6. *The influence of organ extracts on the bactericidal serum action on strain* O 901 *grown* in vitro

(Incubation time 2 hr. at 37° C. Other experimental conditions as in Table 5.)

		Bacterial count (thousands) in the presence of amboceptor and complement with addition of			
		Infected organ extracts		Normal organ extracts	
Expt.	Organs	Saline-$MgCl_2$	Broth 5 %	Saline-$MgCl_2$	Broth 5 %
1	Heart	15·0	—	1·1	—
	Kidneys	8·6	—	1·4	—
	Liver	7·3	—	8·5	—
	Control without extract	16·0	—	—	—
2	Heart	16·0	—	0·5	—
	Kidneys	11·0	—	0·5	—
	Liver	16·0	—	4·4	—
	Control without extract	14·0	—	—	—
3	Heart	13·3	7·8	0·9	0·4
	Kidneys	13·8	3·6	0·5	0·08
	Liver	20·9	14·8	13·0	7·9
	Control without extract	16·0	0·3	—	—

Table 7. *The bactericidal power of anti-*O 901 *immune serum on Ty* 2 *grown* in vitro

(Incubation period 2 hr. Immune serum dilution 10^{-3}. Three dilutions of complement.)

	Bacterial count (thousands) after 2 hr. incubation with complement diluted		
Suspension fluid	1/6·7	1/10	1/20
Salt solution	100·0	110·0	122·0
5 % broth	1·7	9·6	89·0
Controls:			
Salt solution 0 hr.	—	—	120·0
Salt solution 2 hr.	—	—	160·0
Broth 5 % 2 hr.	—	—	210·0

There were no differences between the sensitivities of the *in vitro*- and the *in vivo*-grown strain Ty 2. The latter, when suspended in the salt solution, was resistant to *S. ballerup* immune serum which contained the Vi-antibody and to anti-O 901 immune serum which contained the O-antibody, but became sensitive

as soon as 5 % broth was added. In order to get a bactericidal effect, complement dilutions of 1/5 and 1/10 were employed, as demonstrated in Table 8.

Table 9 shows that all normal organ extracts added to saline or to broth promoted the multiplication of Ty 2 grown *in vivo*. The majority of the organ extracts from infected mice exerted similar growth promoting effects.

Table 8. *The bactericidal effect of anti-*S. ballerup *and anti-*O 901 *immune sera on strain Ty* 2 *grown* in vivo

Incubation period 2 hr.

		Bacterial count (thousands) after 2 hr. incubation with				
		Antibody and complement, diluted		Complement only, diluted		
Immune serum	Suspension fluid	1/5	1/10	1/5	1/10	Antibody only
S. ballerup 10^{-2}	Salt solution	1·8	1·9	2·1	1·9	1·7
	5 % broth	0·2	0·3	0·2	0·3	2·6
S. ballerup 10^{-3}	Salt solution	1·3	2·0	2·1	1·9	1·0
	5% broth	0·08	0·08	0·2	0·3	2·4
S. typhi O 901 10^{-3}	Salt solution	2·9	3·2	1·3	1·9	3·0
	5 % broth	0·2	0·08	0·1	0·3	1·0

Controls without immune serum and complement: 0 hr., 2·3; 2 hr. salt solution, 2·8; 2 hr. broth, 4·4.

The bactericidal action was enhanced by almost all normal organ extracts independently of whether the bacteria were suspended in the salt solution or in broth. Only in one experiment with a 4 hr. incubation time did the normal liver and the normal lung extracts effect some inhibition of bactericidal action. In the experiment carried out with a 4 hr. incubation time, anti-bactericidal effects were exerted by extracts from infected heart, lung, lymphatic glands, kidneys, liver and peritoneal fluid. These effects appeared when the extracts were added either to the salt solution or to the 5 % broth solution.

In the second experiment presented in Table 9, done with a 2·0 hr. incubation, these effects of infected organ extracts were less marked. However, higher bacterial counts in comparison with normal extracts were observed in the presence of infected extract from the heart, lungs, lymphatic glands, kidneys, liver and peritoneal fluid.

DISCUSSION

The experiments described have shown that the avirulent *S. typhi* strain O 901 and the virulent strain Ty 2, grown *in vitro* or *in vivo*, are affected by bactericidal immune sera, if the conditions of the suspension fluid permit the multiplication of the micro-organisms or at least their elongation which precedes cell division. These results are in accordance with the observations made by Adler & Olitzki (1955)

Table 9. *The influence of organ extracts from normal and infected mice on the growth of strain Ty 2 grown* in vivo *and the bactericidal serum action on it*

(Immune serum dilution 10^{-3}. Complement dilution 10^{-1}.)

		Bacterial count (thousands)							
		In the presence of antibody and complement with addition of				Without antibody and complement with addition of			
		Infected organ extracts		Normal organ extracts		Infected organ extracts		Normal organ extracts	
Incubation period and immune sera employed	Extracts prepared from	Salt solution	Broth 5%	Salt solution	Broth 5%	Salt solution	Broth 5%	Salt solution	Broth 5%
4·0 hr. anti-O 901 immune serum	Heart	16·0	2·0	0·6	6·5	160·0	200·0	240·0	329·0
	Lung	40·0	28·0	24·0	2·9	180·0	240·0	120·0	190·0
	Lymphatic glands	20·0	28·0	0·1	0·2	240·0	300·0	104·0	290·0
	Kidneys	8·9	41·0	0·7	0·3	240·0	245·0	168·0	290·0
	Spleen	2·4	0·4	5·0	0·9	180·0	200·0	210·0	160·0
	Liver	13·0	8·8	38·0	20·0	124·0	230·0	88·0	300·0
	Peritoneal fluid	32·0	18·0	9·6	0·3	24·0	180·0	192·0	300·0
	Brain	2·0	0·8	1·3	0·8	180·0	320·0	190·0	340·0
	Control without extract								
	0 hr.	—	—	17·2	17·2	—	—	17·2	17·2
	2 hr.	—	—	39·0	0·8	—	—	30·0	160·0
2·0 hr. anti-O 901 immune serum	Heart	2·4	0·2	0·2	0·02	8·8	12·0	8·6	10·8
	Lung	3·0	1·0	0·9	0·3	8·4	7·6	6·4	13·6
	Lymphatic glands	4·2	5·2	0·2	0·08	5·6	7·6	6·4	8·4
	Kidneys	1·3	1·2	0·2	0·08	8·0	6·8	7·2	9·2
	Spleen	0·2	0·02	0·2	0·1	8·4	8·8	7·0	13·0
	Liver	0·9	0·8	0·5	0·1	5·2	6·0	6·0	10·0
	Peritoneal fluid	2·9	2·1	0·6	0·08	8·4	8·0	10·0	8·0
	Brain	0·08	0·04	0·2	0·06	7·2	8·0	9·2	8·0
	Control without extract								
	0 hr.	—	—	1·2	1·2	—	—	1·2	1·2
	2 hr.	—	—	2·9	0·2	—	—	1·9	5·1

who observed that strain O 901 was not attacked by active rabbit serum diluted 1/5, when the calcium and magnesium ions were removed by ethylene diamine tetra-acetate (EDTA). In all control test tubes which contained 5% broth, inactivated immune serum and EDTA, incubated for 3 hr. at 37° C., the bacterial count was exactly the same as that of the inoculum at the beginning of the experiment. This observation proved that in absence of calcium and magnesium ions bacterial growth was stopped. Thus, the addition of EDTA inhibited both the multiplication of the bacteria and also the bactericidal action of the active serum which in the absence of EDTA was able to reduce the bacterial count within 3 hr. from 10^4 to about 10. The experiments described above confirm generally the results of the bactericidal tests obtained by Felix & Olitzki (1926), but, on the other hand, they show that the resistance of *S. typhi* to the bactericidal serum action is determined not only by the antigenic structure, e.g. presence of the Vi-antigen, but also by growth phase and the nutritional conditions of the suspension fluid during contact with the antibody-complement system. The nutrients which enhance the bactericidal action need not lead to an immediate stimulation of cell division and rise of the viable count, but may activate the processes which take place during the lag phase and which manifest themselves by an increase in cell length (Henrici, 1923), by an increase of total nitrogen (Herschey, 1938), by an initial drop and a second rise of ribonucleic acid (Wade, 1952) and by an increase of oxygen uptake (Martin, 1932). It is possible that the fixation of the antibody-complement system by the bacterial cell interferes with one of these preparatory processes which take place during the lag phase. Since resting cells of strain Ty 2 kept in the absence of any nutrient are resistant to the bactericidal serum action, it becomes clear that in the course of the *in vivo* multiplications the tissue fluids may exert a twofold effect: they may supply the necessary nutrients for the multiplication of the invader, but by the same supply of nutrients they may enhance the bactericidal serum action *in vivo* and speed up the elimination of the invader. On the other hand, it may happen that an environment poor in nutrients may prevent the multiplication of the invader, in which case its elimination by the bactericidal serum action may also be inhibited by the lack of the suitable phase which makes it sensitive to bactericidal serum action. In such a case a resting, stationary phase of the infection process may appear.

An absolute resistance of *in vivo*-grown bacteria to bactericidal serum action was not observed, and if in some experiments the *in vivo*-grown strain Ty 2 was resistant, this resistance could be broken by addition of nutrients as broth and/or organ extracts. On the other hand, it became evident that in the course of the experimental typhoid infection of the white mouse with strain Ty 2 infection-promoting substances were produced. The activity of these substances was demonstrated by their ability to lower the minimal lethal dose (Olitzki & Godinger, 1963) and, in the experiments described above, by their anti-bactericidal action. These substances seem to be partly identical with the known agglutinogens and partly identical with soluble antigens demonstrated in infected organs by the aid of the agar gel precipitation by Olitzki & Godinger (1963).

SUMMARY

The sensitivity of *in vitro*- and *in vivo*-grown strains of *S. typhi* was determined not only by their antigenic structure, but also by their growth phase. An increased vulnerability to the antibody-complement system has been found in cells during the lag and the early exponential phase, while non-multiplying cells devoid of any nutrient were almost invulnerable to the antibody-complement system. Extracts of organs from normal and infected animals may promote the growth of *S. typhi*, and, therefore, increase its vulnerability to the antibody-complement system. The majority of extracts from organs of mice infected with strain Ty 2 inhibited markedly the bactericidal action of serum on this strain and, to a lower extent, on strain O 901.

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