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Bactericidal Activity of Two Factors in Normal Bovine Serum for *Brucella abortus* Strain 19.

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Summary

Normal bovine serum has a bactericidal activity for avirulent *Brucella abortus*, strain 19, but not for virulent one, strain 544. This bactericidal activity, we have called anti-st. 19 activity, depends on two factors contained in an albumin and a gloublin fractions of the serum. The globulin factor combines directly to the bacterial cells and the albumin factor secondly acts on the cells through a medium of the gloublin factor combining to them, and consequently the area of cell wall and cell membrane of strain 19 becomes abnormal to lose the life. This serial reaction seems to be an alterante complement fixation reaction from the characteristics of the two factors. No cellular damage of st. 544 was found though the two factors combined to the cells. The matter may depend on some differences of cell component or structure between st. 19 and st. 544.

In our previous papers (1–3) it was shown that there were two agents in normal bovine serum which were bactericidal for avirulent *Brucella abortus*, strain 19, but not for virulent one, strain 544. It was also shown that one was contained in a globulin fraction salted out at between 0 and 33 per cent saturation of ammonium sulfate and another was in an albumin fraction obtained between 50 and 100 per cent saturation, and that they acted cooperatively each other on st. 19 under the existence of Mg^{++} but each of them alone did not act on the cells. The agent in the globulin fraction was heat-stable, acid-labile and removable by an absorption with *Br. abortus*, though that in the albumin fraction was very heat-labile, acid-stable and not removable by the absorption (4). The anti-st. 19 activity of the mixture of these agents was temperature dependent and the optimal temperature was about 37°C. These agents were most highly contained in the subfractions of the globulin and albumin fractions salted out at from 25 to 33 per cent saturation of ammonium sulfate and at from 50 to 60 per cent saturation, respectively (3), and we called these two subfractions the globulin factor and albumin factor.

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In this paper, the mechanism of the anti-st. 19 action of these two factors was studied.

Materials and methods

Bacterial cells: Two strains, 19 and 544, of *Br. abortus* were prepared according to the method described in the previous paper (1–3).

Albumin and gloublin factors: The two factors were obtained from bovine serum by the method described earlier (3), and their protein concentrations were adjusted to 10 mg per ml with Mg-Tris buffer.

Sensitization of globulin and albumin factors for Br. abortus: Three different methods were used for the sensitization. In the first method, 2.5 ml of bacterial suspension containing 10^3 viable cells of *Br. abortus* st. 19 was added to 5 ml of the globulin factor to be sensitized for 3 hours at 37°C, then centrifuged at 4°C for 10 minutes at 6000 g. The precipitated bacterial cells were resuspended in 5 ml of the albumin factor to be sensitized for 3 hours at 37°C.

In the second method, strain 19 was previously sensitized with the albumin factor and then with the globulin factor, according to the above method. In the thrid method, st. 19 was sensitized for 3 hours at 37°C with a mxiture consisting of equal volume of the globulin and albumin factors.

Thus, the sensitized bacterial suspensions were inoculated in Tripticase Soy Agar plate and colonies were counted. The anti-st. 19 activity was determined by the bactericidal rate in the course of the sensitization. In addition, according to the purposes of the experiments the temperature and duration of the senstization were varied.

Absorption test: Cells of st. 19 were sensitized by the globulin factor at 4°C for 1 hour and washed with Mg-Tris buffer by the method described in the previous paper (1). This treated cells were suspended with the albumin factor and kept at 4°C for 1 hour then centrifuged to get the supernatant, of which the residual anti-st. 19 activity was measured as follows. The supernatant was two-fold serially diluted with Mg-Tris buffer. 0.2 ml of each dilution was added into 0.2 ml of the globulin factor and inoculated with 0.1 ml of st. 19 suspension. After incubation for 3 hours at 37°C, the bactericidal rate was detected. As a control, the albumin factor was absorbed with intact st. 19 which had not been sensitized by the gloublin factor.

Fluorescent antibody technique: From antisera of rabbits immunized with the globulin or albumin factor, γ-globulins were obtained with salting out technique with ammonium sulfate and conjugated with fluorescent isothiocyanate. Viable cells of st. 19 and st. 544 sensitized or not sensitized with the globulin factor for 3 hours at 37°C were centrifuged, washed, smeared on slide glass and stained by fluorescent antibody against the globulin factor. Also the cells sensitized with or without the globulin factor were sensitized with the albumin factor and stained by

fluorescent antibody against the ablumin factor.

Electron microscopy: Cells of st. 19 and st. 544 were sensitized with the globulin and albumin factors at 37°C for 3 hours and centrifuged for 10 minutes at 6000 g. The pellets of the sensitized and intact cells were made with heat-inactivated bovine serum. These pellets were treated with an ice-cold solutions of glutalaldehyde and osmiumtetroxide, then dehydrated with aceton. Epon mixtures were used for embedding and ultrathin sections were prepared. The sections were post-stained in uranylacetate and lead citrate, and viewed in an electron microscope.

Results

The order of the gloublin and albumin factors to sensitized Br. aboruts st. 19: To know the mechanism of the anti-st. 19 action of the gloublin and albumin factors, the order of both factors in the sensitization for st. 19 was examined. When st. 19 previously sensitized with the globulin factor was sensitized with the albumin factor, the anti-st. 19 activity was as high as the control in which st. 19 was sensitized with the mixture of the two factors, as shown in Table 1. On the contrary, when st. 19 was sensitized firstly with the albumin factor and secondly sensitized with the gloublin factor, the anti-st. 19 activity was almost negligible. From the results, it became clear that the globulin factor firstly acted on st. 19 and albumin factor did next, and consequently the bactericidal phenomenon occured.

TABLE 1. *Effects of the Order of Sensitization of the Globulin and Albumin Factors on the Anti-st. 19 Activity*

Order of sensitization	Anti-st. 19 activity[1)]	Relative activity
Gloublin factor→Albumin factor	89	97
Albumin factor→Globulin factor	13	14
Gloublin factor+Albumin factor	92	100

1) Bactericidal rate (%) of *Br. abortus* st. 19 after sensitization.

Absorption of the albumin factor with st. 19 sensitized by the gloublin factor: The albumin factor acted on only the brucella organisms previously sensitized with the globulin factor as above mentioned, though the activity of the albumin fraction was not affected by the absorption with intact st. 19 (3). So that the albumin factor may combine to the cells sensitized by the globulin factor but not to the intact cells. To solve this assumption, an absorption test of the albumin factor was done using st. 19 previously sensitized with or without the globulin factor. The results are shown in Table 2, in which it is seen that the residual activity of the albumin factor disappered at 1:32 of dilution in the case of the absorption with sensitized cell but it appeared even at 1:64 of dilution of the supernatant absorbed with the non-sensitzed cells. So it is said that the albumin factor was more

absrobed by the cells sensitized with the globulin factor than by the intact cells. This suggests the albumin factor combines indirectly to st. 19 through a mediator of the globulin factor. Next experiment was carried out to solve this assumption.

TABLE 2. *Effects of Absorption of the Albumin Factor with st. 19 Sensitized with the Globulin Factor on the Anti-st. 19 Activity*

Dilution of residual albumin factor after absorption	Absorbed with	Anti-st. 19 activity[1)]
1:32	Non-sensitized st. 19	26
	Globulin factor-sensitized st. 19	0
1:64	Non-sensitized st. 19	20
	Gloublin factor-sensitized st. 19	0

1) Bactericidal rate (%) of *B.r abortus* st. 19 after sensitization with the intact globulin factor and with the residual albumin factor after absorption.

Observation of the sensitized cells with fluorescent antibody: As shown in Table 3, cells of st. 19 sensitized with the globulin factor were well stained by fluorescent antibody against the globulin factor, and also those which were sensitized with the globulin and albumin factors were well stained by the fluorescent antibody to the albumin factor. However, the cells sensitized with the albumin factor alone were not stained by this antibody. These facts show that the globulin factor combined to st. 19 directly and the albumin factor combined indirectly to st. 19 through a medium of the globulin factor. Simillar results were obtained in the case of st. 544.

TABLE 3. *Microscopic Findings of Sensitized Br. abortus st. 19 and st. 544 Stained with Fluorescent Antibody*

Strains used	Sensitized with	Stained by fluorescent antibody against	Results of staining
St. 19	None	Gloublin factor	Negative
	Gloublin fctor	Gloublin factor	Positive
	Globulin and albumin factor	Albumin factor	Positive
	Albumin factor	Albumin factor	Negative
St. 544	None	Gloublin factor	Negative
	Gloublin factor	Gloublin factor	Positive
	Gloublin and albumin factor	Albumin factor	Positive
	Albumin factor	Albumin factor	Negative

Incubation time required for anti-st. 19 action: Cell suspension of st. 19 was added to the globulin factor, incubated for varying times between 5 to 180 minutes at 37°C and then centrifuged. The precipitated cells were resuspended with the albumin factor and incubated for 3 hours at 37°C. As the results, the anti-st. 19 activity exhibited the maximum in 5 minutes of the globulin-sensitiza-

tion. On the contrary, when the cells previously sensitized with the globulin factor for 3 hours were incubated in the albumin factor for varying times, the activity increased with time of incubation and did not approximate to the maximum until 2 hours (see Figure 1). So, relatively long time is necessary for sensitization by the albumin factor, but not by the gloublin factor.

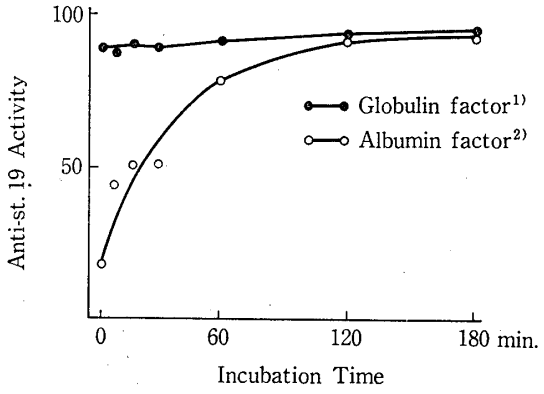

FIGURE 1. Incubation time required for maximum Anti-st. 19 Action of Globulin and Albumin Factors

1) St. 19 was incubated for corresponding time with the globulin factor and then sensitized for 180 minutes with the albumin factor.
2) St. 19 was sensitized with the globulin factor for 180 minutes and then incubated for corresponding time with the albumin factor.

Effect of temperature on the anti-st. 19 action: The cell suspension of st. 19 was added to the globulin factor and incubated for 3 hours at various temperature. Then the washed cells obtained by centrifugation were resuspended in the albumin factor, kept for 3 hours at 37°C and the anti-st. 19 activity was determined. From the results, it was known that the sensitization by the globulin factor was done enough at every temperatures from 5 to 37°C. On the other hand, when the cells previously sensitized enough by the globulin factor at 37°C for 3 hours were sensitized by the albumin factor at various temperatures, the anti-st. 19 activity was scarcely seen at lower than 10°C and increased with the rise of temperature, 15°C to 37°C (see Figure 2).

These results show that the action of the albumin factor is temperature-dependent but that of the gloublin factor is not.

Morphological changes observed by electron microscope: Ultrathin sections of strain 19 and 544 sensitized with or without the globulin and albumin factors were observed (Plate 1). The outward form of the cells in st. 19 sensitized with both factors was irregular and not circular. The border between cell wall and cell membrane was not distinguishable, of which area swelled and gave very low electron density. Cells of non-sensitized st. 19, however, had smooth and circular outside, of which cell wall and cell membrane were distinct each other.

In the case of st. 544 no differences were observed between sensitized and non-sensitized cells.

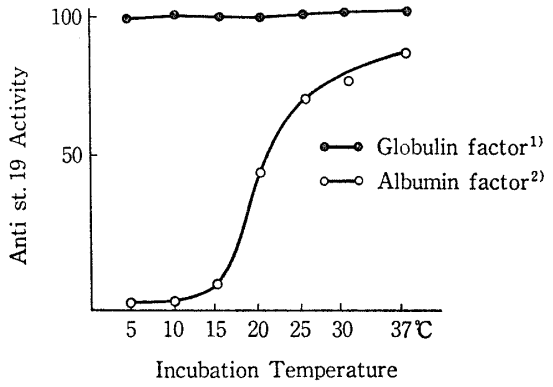

FIGURE 2. Optimal temperature of Anti-st. 19 Action of Globulin and Albumin Factor
1) St. 19 was incubated at corresponding temperature with the globulin factor and then sensitized at 37°C with the albumin factor.
2) St. 19 was sensitized at 37°C with the globulin factor and then incubated at corresponding temperature with albumin factor.

Discussion

In our previous paper (4), we suggested that the anti-st. 19 action of normal bovine serum was a type of complement fixation reaction judging from the characteristics of the active agents in albumin and globulin fractions of the serum, and in this paper it was shown that each of the globulin and albumin factors combined to the cells of st. 19, not simultaneously but individually in a definite system. That is, the globulin factor combined directly to the cell and the albumin factor indirectly acted on through a medium of the gloublin factor (Table 1–3). These facts indicate that the system is the complement fixation reaction. The activity of globulin factor was lost by the treatment of 2-Mercaptoethanol (6), so the factor is IgM.

Since there were small amount of C_2 and no detectable C_4 of the complement component in bovine serum (5), and Ca^{++} was not required in the anti-st. 19 action (2), this action will follow an alternate pathway in which these components are not necessary. As the results, morphological damages would occur in the area of the cell wall and cell membrane of st. 19. However, in the cells of sensitized st. 544, no detectable changes were observed (Plate 1) though they combined both the albumin and gloublin factors. These facts suggest that there are some differences of cell component or structure between avirulent strain 19 and virulent strain 544.

The sensitization by the albumin factor was temperature dependent and was progressive with time. However it is not clear whether the fixation of the albumin factor on the cells requires suitable temperature and relatively long time or the lethal cellular damage, which will occur after the fixation, requires them.

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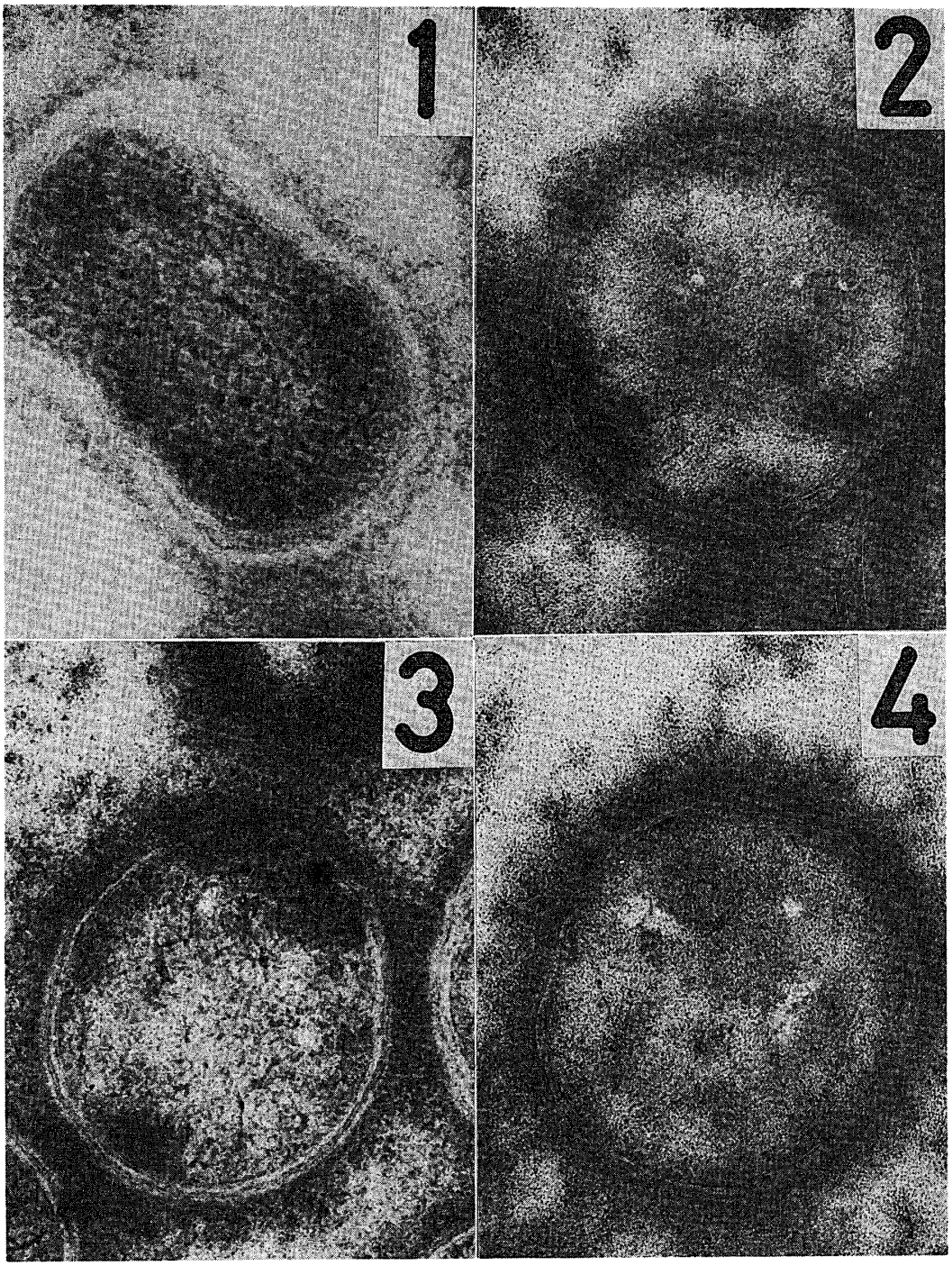

PLATE 1

1. Sensitized *Br. abortus* st. 19 (× 60000)
2. Non-sensitized *Br. abortus* st. 19 (× 60000)
3. Sensitized *Br. abortus* st. 544 (× 60000)
4. Non-sensitized *Br. abortus* st. 544 (× 60000)