Role of Divalent Cations in Bactericidal Action of Normal Bovine Serum to Brucella abortus strain 19

Tohoku Journal of Agricultural Research
Vol. 26, No. 2, December 1975
Printed in Japan

Role of Divalent Cations in Bactericidal Action of Normal Bovine Serum to *Brucella abortus* strain 19

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(Received, July 5, 1975)

Summary

The effects of cations on the bactericidal activity of bovine serum to *Brucella abortus* strain 19 were studied using a photometric method, by which the titration of the activity was done rapidly and simplified. The anti-st. 19 activity reduced remarkably when bovine sera were dialyzed against various buffers or treated with EDTA. The reduced activity returned to a normal level by addition of Mg^{2+}, Cu^{2+}, Co^{2+} or Cd^{2+}. Since the latter three divalent cations were toxic themsevles to *Br. abortus* st. 19, it was thought that only Mg^{2+} played a part in the bactericidal action. This fact suggests that one of the non-permeable agents is the complement.

In our previous papers (1, 2), it was observed that avirulent *Brucella abortus*, strain 19, was very sensitive to the bactericidal action of normal sera from cattle, guinea-pig and so on but virulent strain 544 was not. Now, identification of the bactericidal factor in bovine serum has been investigated, in which it was found that when bovine serum was dialyzed against PBS, the activity of the serum remarkably reduced though the outer fluid did not show this activity. This indicates that both permeable and non-permeable agents participate in the serum bactericidal action to st. 19. So in this paper, the role of divalent cations as the permeable agent was studied.

Materials

Suspensions of *Br. abortus* strain 19 were prepared with Tris-HCl buffer (pH 7.2) according to the methods described in the previous papers (1, 2). Bovine serum was obtained from clotted blood by centrifugation at 4°C and immediately filtrated with Sartorius Membranefilter (pore size 0.45 μ) and then frozen at -20°C until the experiments were performed.

A part of this article was orally presented in the 76th Meeting of the Japanese Society of Veterinary Science held in August 1973 in Kagoshima.

Experiments and Results

Photometric assay for bactericidal activity against st. 19

Firstly, to establish a photometric method for titration of anti-st. 19 activity, a correlation between the initial numbers of inocula and their growth rates in a broth culture was observed.

Test tubes containing 10 ml of Tripticase Soy Broth (BBL) were inoculated with 0.1 ml of the bacterial suspensions involving 10^5, 10^6, 10^7, and 10^8 cells of st. 19, respectively. They were incubated at 37°C for 66 hrs and their optical densities (O.D.) were successively determined at some intervals with a spectrophotometer (Hitachi, FPW-4) set at 660 mμ.

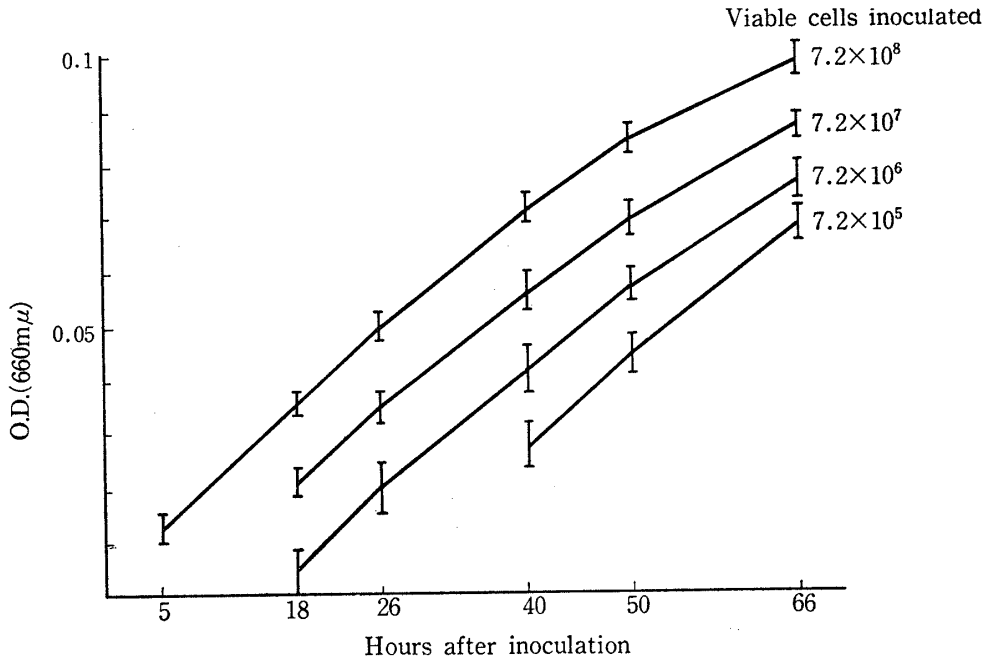

FIG. 1. Growth curve of *Brucella abortus* strain 19.
10 ml of Trypticase Soy Broth was inoculated with 0.1 ml of suspension of *Br. abortus* st. 19.

As shown in Fig. 1, the O.D. values rose linearly during the incubation and were proportional to the numbers of inoculated cells. The facts indicate that the number of viable cells in an inoculum can be counted indirectly by the photometric method.

In the next experiment, 10^8 viable cells of st. 19 suspended in 0.1 ml of Tris-HCl buffer were mixed in the 0.5 ml of 2-fold serially diluted bovine sera to be kept for 3 hrs at 37°C for sensitization by the bactericidal factor, and then 0.1 ml of these mixtures were inoculated into 10 ml of Trypticase Soy Broth. They were incubated at 37°C and their O.D. values were observed at the 20th and 30th hrs of inoculation. As controls, 0.1 ml of the same suspension of st. 19 and its 10-fold

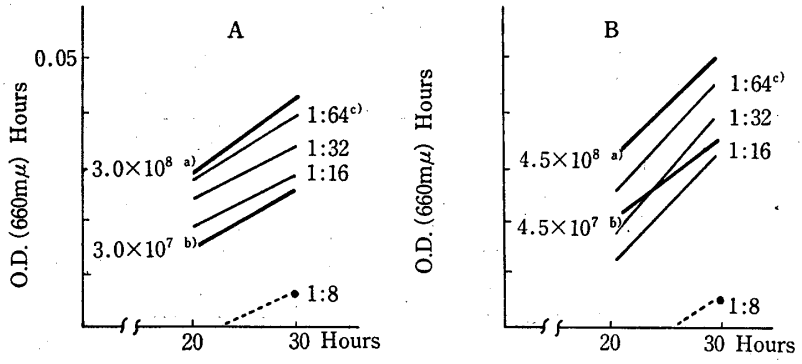

FIG. 2. Method of measurement of bactericidal titer.
a) Number of viable cells at inoculation.
b) Number of viable cells when 90% of inoculated cells were killed during sensitization (Control).
c) Dilution of serum.
Bactericidal titer is expressed as the reciprocal of highest dilution in which the O.D. value do not exceed the control O.D. value. For example, titer of A is 8, titer of B is 16.

dilution were mxied to 0.5 ml of Tris-HCl buffer respectively. Then, 0.1 ml of each of them was inoculated into the Trypticase Soy Broth and the O.D. values were observed the same as above.

The results, as shown in Fig. 2, indicate that the O.D. values were correlative to the ratio of dilution of serum. In higher dilution, the O.D. values neared the value of the viable cells before sensitization and in lower dilution, the value became less than that of one-tenth of the cells. This means that in such a low dilution, more than 90 per cent of cells added in the diluted sera were killed during the sensitization.

From these results, the titer of the anti-st. 19 activity of variously treated sera in the following experiments was expressed as the reciprocal of the highest dilution of them in which both O.D. values detected at 20th and 30th hrs of the incubation did not exceed the values of one tenth of the non-sensitized cells.

Effects of dialysis on anti-st. 19 activity

Ten ml of bovine serum was dialyzed against 500 ml of distilled water, physiological saline, PBS (pH 7.2), 1/15 M phosphate buffer (pH 7.2) or Tris-HCl buffer (pH 7.2) at 4°C overnight. Six tenth ml of dialyzed or non-dialyzed sera was added to 0.4 ml of Tris-HCl buffer and diluted serially to examine their bactericidal titers. The results are shown in Table 1. Every titer of dialyzed sera was less than 2, though it was 8 before dialysis.

Next, 0.1 ml of 0.02 M solution of $MgCl_2$, $CaCl_2$, $MnCl_2$, $CuCl_2$, $CdCl_2$ or $CoCl_2$ and 0.3 ml of Tris-HCl buffer were added to 0.6 ml of serum dialyzed against Tris-HCl buffer. Then the bactericidal titers of these mixtures were determined. As the results, the reduced bactericidal titer of the dialyzed serum returned to the

TABLE 1. *Anti-st. 19 Titer of Dialyzed Bovine Sera*

Outer fluids	Titer
Distilled water	-
Physiological saline	2
1/15M phosphate buffer	2
PBS	2
Tris-HCl buffer	2
Control (before dialysis)	8

Ten ml of bovine serum was dialyzed against 500 ml of each outer fluid.

TABLE 2. *Effects of Divalent Cations on Anti-st. 19 Titer of Dialyzed Bovine Serum*

Pretreatment of serum	Added cation (final conc. 0.002M)	Titer
Dialyzed[a)]	$MgCl_2$	8
	$CaCl_2$	2
	$MnCl_2$	2
	$CuCl_2$	16
	$CdCl_2$	8
	$CoCl_2$	8
	—	2
Non-treated	—	8

a) Dialyzed against Tris-HCl buffer (pH 7.2).

TABLE 3. *Effects of EDTA on Anti-st. 19 Activity of Bovine Serum*

Final concentration of EDTA added	Titer
0.008M	-
0.006	-
0.004	-
0.002	8
0.001	8
0.0008	8
—	8

level of normal serum by the addition of $MgCl_2$, $CuCl_2$, $CdCl_2$ or $CoCl_2$ (Table 2).

Effect of EDTA on anti-st. 19 activity on bovine serum.

One tenth of the EDTA solutions varying from 0.008 M to 0.08 M and 0.3 ml of Tris-HCl buffer were added to each 0.6 ml of normal serum and kept at 37°C for 30 min. for the chelation of metal ions in the serum. The bactericidal titers of these mixtures became negative by addition of over 0.004 M EDTA at final concentration though they were not affected at the concentrations of under 0.002

TABLE 4. *Effects of Diavlent Cations on Bovine Serum Treated with 0.004M EDTA*

Added cation[b] (final conc.)	Titer[a]					
	$MgCl_2$	$CaCl_2$	$CuCl_2$	$CoCl_2$	$MnCl_2$	$CdCl_2$
0.004M	8	8	16	8	8	16
0.002	8	8	8	8	8	8
0.001	-	-	-	-	-	-
0.0005	-	-	-	-	-	-

a) Normal serum 8, EDTA-treated serum, negative.
b) Added to EDTA-treated serum.

TABLE 5. *Effects of Divalent Cations on Heat-inactivated Bovine Serum*

Pretreatment of serum	Added cations (final conc. 0.002M)	Titer
Inactivated at 56°C 30 min.	$MgCl_2$	-
	$CaCl_2$	-
	$MnCl_2$	-
	$CuCl_2$	16
	$CdCl_2$	16
	$CoCl_2$	8
	—	-
Non-treated	—	8

M, as shown in Table 3.

In the next experiment, 0.1 ml of 0.04 M EDTA solution and 0.2 ml of Tris-HCl buffer were added to each 0.6 ml of normal serum and kept for 30 min. at 37°C. Then, 0.1 ml of the solutions from each of the six salts, described above, varying from 0.005 M to 0.04 M of their concentrations were added into the EDTA treated sera and kept again for an additional 30 min. at 37°C. Then the bactericidal titers of these treated sera were determined. From the results, it was known that the bactericidal activity, which disappeared by addition of EDTA, was restored by the addition of 0.002M (final concentration) of each salt, as shown in Table 4.

Toxic effect of divalent cations on st. 19

One tenth ml of 0.02 M salt solutions used in the above experiments and 0.3 ml of Tris-HCl buffer were added to 0.6 ml of heat-inactivated serum which lost its anti-st. 19 activity, and the bactericidal titers were tested. The results show that the addition of $MgCl_2$, $CaCl_2$ and $MnCl_2$ did not have any effect but $CuCl_2$, $CoCl_2$ and $CdCl_2$ had a high toxic effect on st. 19 (Table 5).

Discussion

Muschel (3) reproted a rapid photometric assay method of bactericidal activity against *Salmonella typhosa* instead of the conventional plate count assay. A modification of this method was applied for the titration of bactericidal activity of bovine serum to *Br. abortus* st. 19. Thus, the determination of the activity became easier. When bovine sera being bactericidal to st. 19 were dialyzed against various buffers, bactericidal titers reduced significantly. And the reduced titers were restored by addition of Mg^{2+}, Cu^{2+}, Cd^{2+} or Co^{2+} to these sera. So it is thought that the reduction of the activity by the dialysis was due to the removal of these ions.

The other hand bactericidal activity of normal serum disappeared by chelation of the divalent cations. And the reduced activity of EDTA-treated sera was restored also by the addition of Mg^{2+}, Ca^{2+}, Mn^{2+} Cu^{2+}, Cd^{2+} or Co^{2+}. The results agreed with the case of dialyzed serum, except for Ca^{2+} and Mn^{2+}.

But the fact that Cu^{2+}, Co^{2+} and Cd^{2+} were toxic shows that the effects of the addition of them on the dialyzed or EDTA-treated sera were due to the toxicity of these divalent cations themselves and not due to the activation of the anti-st. 19 factor by them.

Mg^{2+}, Ca^{2+} and Mn^{2+} recovered the anti-st. 19 activity in the EDTA-treated sera, though only Mg^{2+} recovered the activity in the dialyzed sera. Since Mg is less stable than Ca and Mn in metal complexes with EDTA (4), Mg ions chelated in EDTA treated serum are replaced by the addition of Ca^{2+} or Mn^{2+} and the Mg^{2+} becomes free again. This released Mg^{2+} will take part in the bactericidal action.

In a previous paper, it was thought that the agents relating to the anti-st. 19 action were normal antibody and complement. Participation of Mg^{2+} in this phenomenon suggests that one of the non-permeable agents is complement.

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