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The Conglutination Phenomenon

XIII. *In Vivo* Interactions of Conglutinin and Experimental Bacterial Infection

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Summary. Some interrelations between the conglutinating activity of the serum of an animal and experimental bacterial infection were investigated. The passive transfer of conglutinating activity was demonstrated. The level of this activity reaches a peak within 2 days after subcutaneous injection, then declines until no significant titres are demonstrable in 12 to 14 days. It is shown that infection of animals with *Salmonella typhimurium* causes a rapid reduction in the conglutinating activity of the serum immediately after challenge. Evidence is presented which indicates that the previous injection of conglutinin preparations enhances bactericidal activity of the mouse against *Salm. typhimurium*.

INTRODUCTION

AN increased conglutinating activity of the serum during the course of infectious diseases has been reported to occur in man by Marks and Coombs (1957), and in the rabbit, guinea pig and mouse by Ingram, Barber, Mclean, Soltys and Coombs (1959). The production of immuno-conglutinin during infections may be one of the physiological reactions of the body in the battle against an invading parasite, and immuno-conglutinin may aid the host in the elimination of infectious agents.

Coombs and Coombs (1953) advanced the theory that complement adsorbed on an immune aggregate, or on a surface, assumed a configuration which it did not possess in solution, that this changed surface configuration was a common characteristic of complement regardless of the species of origin, and that this changed or unfolded configuration was the stimulus for immuno-conglutinin production. Streng (1930) and Coombs and Coombs (1953) have differentiated immuno-conglutinin from specific bacterial agglutinins by absorption experiments. The great variety of agents which stimulate the production of immuno-conglutinin (Wartiovaara, 1932; Coombs and Coombs, 1953; Marks and Coombs, 1957; Ingram *et al.*, 1959) indicates that immuno-conglutinin is not related to any specific antigenic component of the stimulating organism or substance. The non-specific nature of immuno-conglutinin, with regard to the injected antigen, suggests that if immuno-conglutinin aids the host in the elimination of the organism which has stimulated its production it may exert a similar effect on heterologous infectious organisms.

In the experiments reported in this paper some *in vivo* interrelations of the conglutinating activity of the serum and bacterial infections were investigated.

EXPERIMENTAL METHODS

The conglutinin preparation was derived from fresh normal bovine serum. The serum was dialysed against distilled water for 48 hours and the precipitate separated by centrifugation. The supernatant fluid was discarded. The euglobulin precipitate was redissolved in saline to the original volume of serum and heated at 56° C. for 30 minutes. This preparation had a conglutinin titre of 1 in 640.

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Immuno-conglutinin serum was produced in rabbits by the intravenous injection of kaolin coated with active horse serum as described by Coombs and Coombs (1953). The serum had a conglutinating titre of 1 in 8000.

The mice were descended from strain FF, but inbreeding was not practised in the colony. Young-adult mice were used and in each experiment animals of the same sex, age and size were used in the treated and control groups. The mice were bled by the method described by Bullen and Batty (1956). All sera and serum preparations were stored at −20° C. The sera in any series were stored until the entire series had been collected and were titrated as individual samples at one time by the alexinated cell technique IIa as described by Coombs and Coombs (1953) using a total volume of 0·5 ml. per tube. All mouse sera were titrated in doubling dilutions from 1 in 2·5 and any serum which gave no reaction at this dilution was considered to have no conglutinating activity.

The conglutinin preparations were injected subcutaneously into the dorsal cervical region of mice. Where applicable, mice were infected by the intraperitoneal inoculation of 2×10^5 viable *Salm. typhimurium* (20 LD50) 24 hours after the injection of the conglutinin preparations.

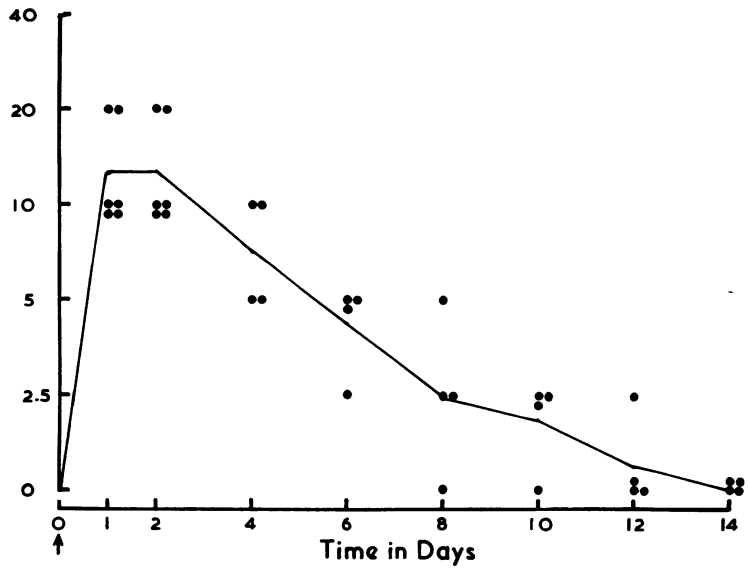

FIG. 1. The rise and fall of conglutinating activity in the serum of mice after subcutaneous injection of bovine conglutinin.
Each dot represents the titre in one mouse. The line joins the average titres at each test period. Each mouse was injected with 0·25 ml. of bovine euglobulin on day 0.

RESULTS

(A) PASSIVE TRANSFER OF CONGLUTINATING ACTIVITY

Normal mice of the strain used in these experiments have no, or very low, conglutinating activity in their serum (Ingram *et al.*, 1959; Coombs, 1954). Eight mice were injected with 0·25 ml. of the bovine conglutinin preparation. Each animal was bled immediately before injection and at 2 or 4 day intervals for 14 days. The results of the titration of the serum samples are illustrated in Fig. 1. The peak titre of conglutinating activity averaged

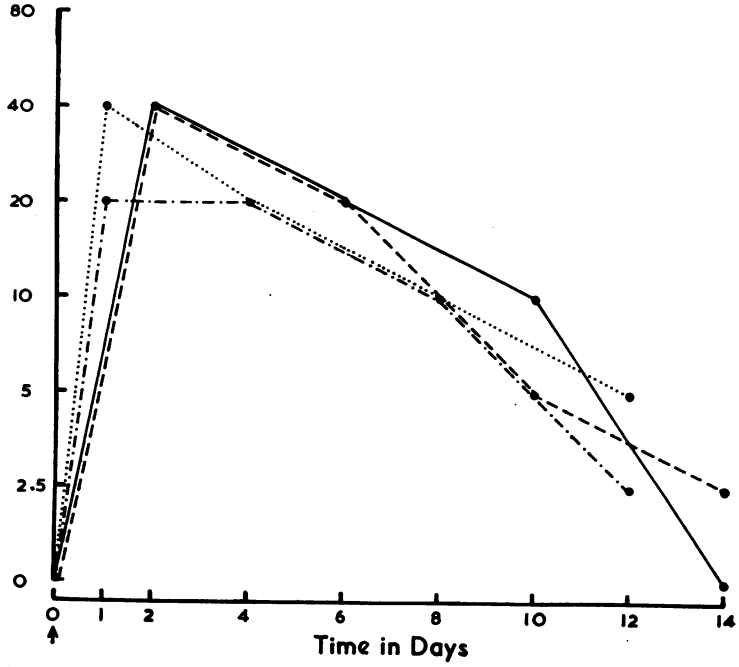

FIG. 2. The rise and fall of conglutinating activity in the serum of mice after subcutaneous injection of immuno-conglutinin serum.
Each line traces the titres found in a single mouse. Each mouse was injected on day 0 with 0·5 ml. of rabbit serum containing immuno-conglutinin.

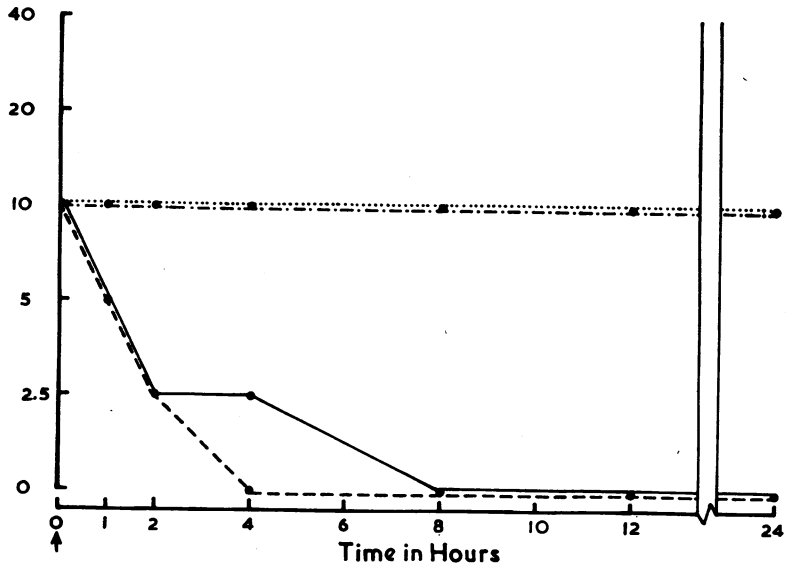

FIG. 3. Fall of conglutinating activity in the serum of mice injected with bovine conglutinin and subsequently infected with *Salmonella typhimurium*.
Each mouse was injected subcutaneously with 0·25 ml. of bovine euglobulin 24 hours before challenge.

———— Mouse 1 } challenged by intraperitoneal inoculation of 2×10^5
– – – – – – – Mouse 2 } viable organisms in 0·5 ml. saline at 0 hour.
· · · · · · · · · Mouse 3 } injected intraperitoneally with 0·5 ml. sterile saline at
—·—·—·— Mouse 4 } 0 hour.

1 in 12·6 and occurred on day 1 and 2. This peak was followed by a steady decline until all activity had disappeared by day 12 or 14.

Four mice were injected with 0·5 ml. of the rabbit immuno-conglutinin serum and bled as above. Fig. 2 shows the titres of conglutinating activity of the sera from these animals. The peak titre averaged 1 in 33·5 on day 1 and 2 and the level declined similarly to that of the bovine-conglutinin-treated animals.

(B) THE EFFECT OF INFECTION ON THE LEVEL OF PASSIVELY TRANSFERRED CONGLUTININ

Four mice were injected subcutaneously with 0·25 ml. of the bovine conglutinin preparation. One day later 2 of them were infected with a suspension of *Salm. typhimurium* in

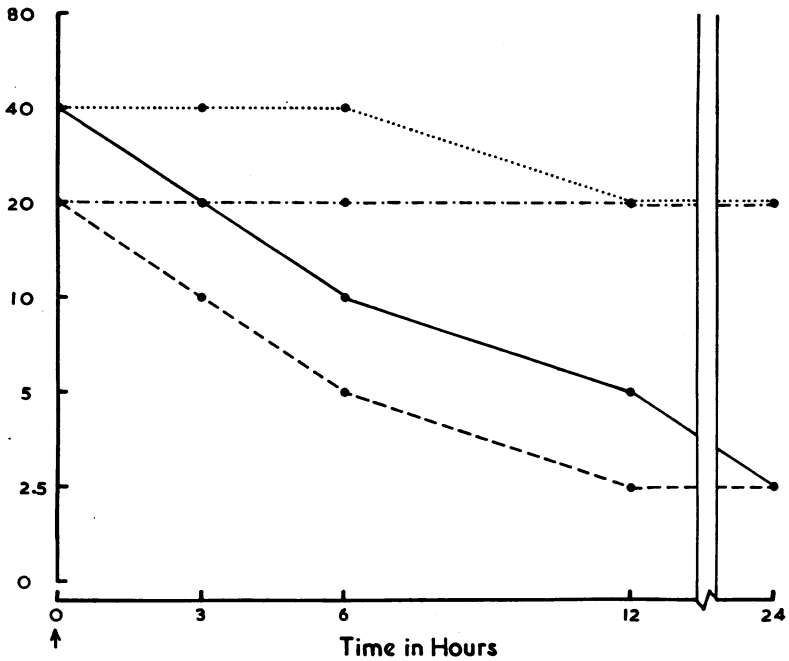

FIG. 4. Fall of conglutinating activity in the serum of mice injected with immuno-conglutinin serum and subsequently infected with *Salmonella typhimurium*.
Each mouse was injected subcutaneously with 0·5 ml. of rabbit immuno-conglutinin serum 24 hours before challenge.
———— Mouse 5 } challenged by intraperitoneal inoculation of 2×10^5 viable
– – – – – – – Mouse 6 } organisms in 0·5 ml. saline at 0 hour.
····· Mouse 7 } injected intraperitoneally with 0·5 ml. sterile saline at 0
– · – · – · – Mouse 8 } hour.

saline and the other 2 injected with an equal volume of sterile saline. Each animal was bled immediately before challenge and at 1, 2, 4, 8, 12 and 24 hours after challenge. As shown in Fig. 3, the conglutinating activity of the serum of the infected mice fell rapidly and no activity was demonstrated in the serum of these animals after 4 hours. The conglutinating activity of the serum of the control mice was not reduced throughout the period of the test.

The same procedure was followed using 4 mice injected with 0·5 ml. of rabbit immuno-conglutinin serum and the results are presented in Fig. 4. To determine whether the

decrease in activity was due to infection or was merely caused by the injection of the foreign protein, this experiment was repeated using, as controls, mice injected with immuno-conglutinin serum and injected with 2×10^5 killed *Salm. typhimurium*. The results are illustrated in Fig. 5. In all cases, a marked decrease of conglutinating activity occurred in the infected mice, while the conglutinating activity of the controls either remained at the original level or decreased one dilution during the test period.

(c) THE EFFECT OF INFECTION ON THE LEVEL OF ACTIVELY STIMULATED CONGLUTININ

Three rabbits were stimulated to produce immuno-conglutinin by the intraperitoneal inoculation of 6×10^9 killed *Listeria monocytogenes*. Seven days later, when the immuno-

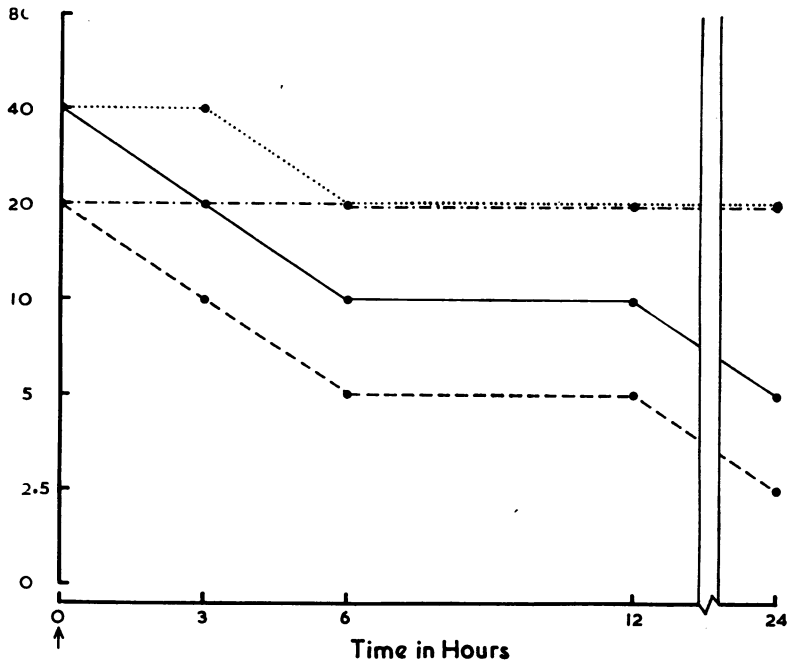

FIG. 5. Fall of conglutinating activity in the serum of mice injected with immuno-conglutinin serum and subsequently inoculated with viable or killed *Salmonella typhimurium*.
Each mouse was injected subcutaneously with 0·5 ml. of rabbit immuno-conglutinin serum 24 hours before challenge.
———— Mouse 9 } challenged by intraperitoneal inoculation of 2×10^5 viable
– – – – – – Mouse 10 } organisms in 0·5 ml. saline at 0 hour.
· · · · · · · · · Mouse 11 } challenged by intraperitoneal inoculation of 2×10^5 killed
– · – · – · – · Mouse 12 } organisms in 0·5 ml. saline at 0 hour.

conglutinin titre was near its peak, the animals were infected by mixing 6×10^{10} viable *Salm. typhimurium* with the food of each animal. This feeding was repeated the next day. One control rabbit was injected with *List. monocytogenes* but was not infected, although it was bled and tested in parallel with the infected animals. The course of the infection was followed by taking the body temperature, faecal cultures and blood samples at 2 or 3 day intervals as described by Ingram *et al.* (1959).

The reactions of rabbit 694 following injection and subsequent infection are shown in

Fig. 6. Temperatures above normal were recorded from day 3 to 9. The rabbit was sick on day 3 but was recovering on day 4. Therefore *Salmonella* was again given in its food on day 5. This third feeding caused a severe illness which lasted until day 9.

The production of antibody to *List. monocytogenes* followed a typical curve. Following infection with *Salm. typhimurium* the 'normal' antibody to this organism disappeared by day 2, but specific immune antibody was detected by day 6 and reached a peak on day 10. The complement titre of the serum decreased immediately after injection of *Listeria*, then

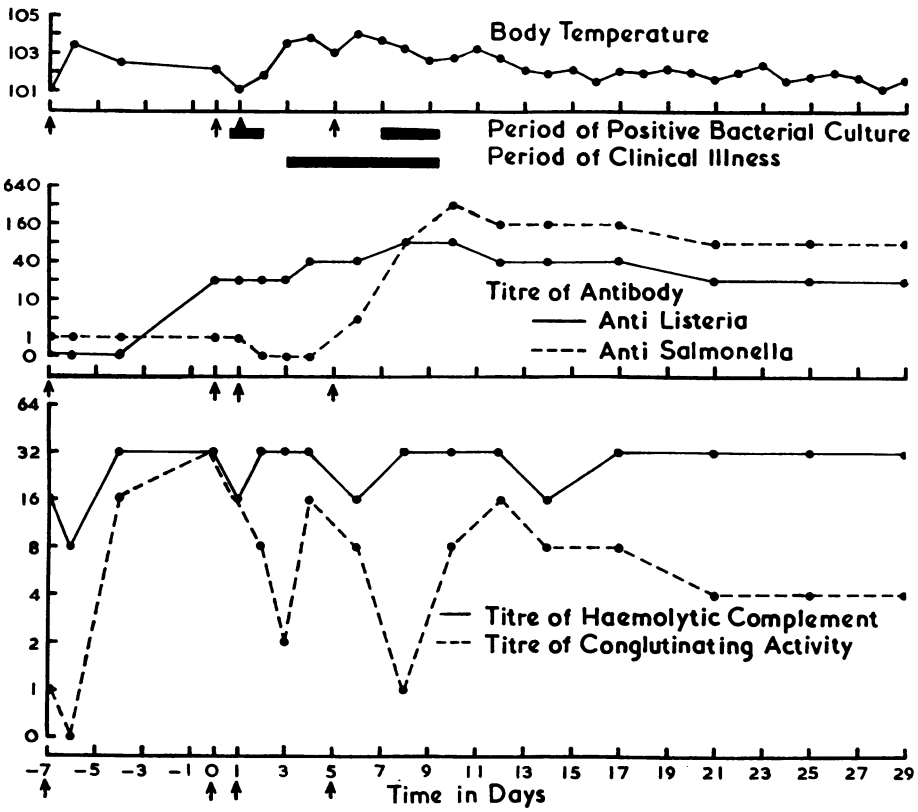

FIG. 6. Reactions of rabbit 694 following injection of killed *Listeria monocytogenes* and subsequent infection with *Salmonella typhimurium*.
A normal rabbit injected intraperitoneally with 6×10^9 killed *List. monocytogenes* on day-7 and infected by mixing 6×10^{10} viable *Salm. typhimurium* with the feed on day 0, day 1 and day 5.

rose to a level above the preinoculation samples. After infection and reinfection decreased complement titres were recorded with subsequent rises to the preinfection level.

Conglutinating activity was present in the neat serum before *Listeria* injection. By 12 hours after injection this activity had disappeared. A titre of 1 in 32 was detected on the day of infection. After infection with *Salmonella* a sharp drop occurred by day 3, but by day 4 the activity was again detectable at a titre of 1 in 16. After reinfection on day 5 another drop in activity was recorded and this, in turn, was followed by an increase to 1 in 16 and then a more gradual decline.

The sharp drop in conglutinating activity immediately following infection was a consistent finding in all animals. Two rabbits which had relapses in *Salmonella* infection, on day 9 and day 13, showed a second drop in conglutinating activity which was similar in all respects to the case illustrated here to coincide with reinfection of the animal. In both relapse cases the evidence of (1) a second period of positive bacterial cultures following a time when no *Salmonella* were isolated, (2) a second rise in the titre of antibody to *Salmonella*, and (3) a period of increased body temperature indicated that a second challenge had occurred during these relapses. In all cases a sharp drop in immuno-conglutinin titre occurred at the time of the relapse.

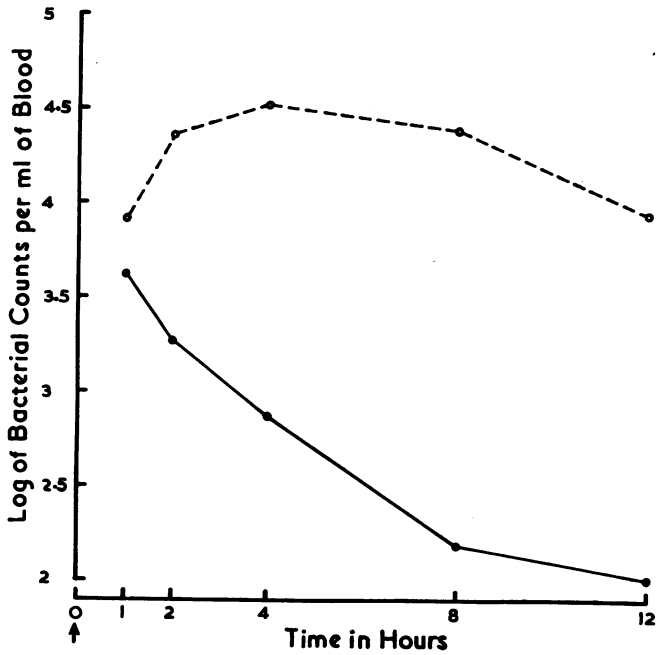

FIG. 7. Clearance of *Salmonella typhimurium* from the blood stream of mice previously injected with bovine conglutinin.
●——● Geometric means of bacterial counts of 6 'treated' mice injected subcutaneously with bovine euglobulin 24 hours before challenge.
O - - - - O Geometric means of bacterial counts of 6 'control' mice injected subcutaneously with sterile saline 24 hours before challenge.
All mice challenged at 0 hour by intraperitoneal inoculation of 2×10^5 *Salm. typhimurium*.

(D) THE EFFECT OF CONGLUTININ ON BLOOD CLEARANCE OF BACTERIA

Animals whose serum had conglutinating activity showed a rapid and marked reduction in titre following infection or during a relapse in an existing infection. Because the level decreased within hours after infection it is apparent that if conglutinin or immuno-conglutinin were influencing the course of the infection it must be active during this period.

Intraperitoneal inoculation of *Salm. typhimurium* into a normal mouse results in a rapidly developing septicaemia which persists for over 24 hours. Mice were injected with 0·125 ml.

of the bovine conglutinin preparation, or rabbit immuno-conglutinin serum, and control mice were injected with the same volume of sterile saline or normal rabbit serum, respectively. All mice were challenged 24 hours later with *Salm. typhimurium* intraperitoneally. Each animal was bled at intervals up to 24 hours post-infection. The method of bleeding and culturing was described by Berry and Mitchell (1954). The blood samples obtained by amputating the end of the tail were taken into a r.b.c. counting pipette, diluted with sterile saline containing versene and plated on Leifson's agar. The plates were counted after overnight incubation at 37° C. and the number of bacteria per ml. of blood were calculated.

The data obtained from 6 conglutinin treated and 6 control mice are presented in Fig. 7, which shows the mean of the logs of the calculated number of bacteria per ml. of blood. Mice which had been injected with the conglutinin preparation cleared the organisms from the blood stream much better than the controls.

In other experiments mice treated with rabbit serum containing immuno-conglutinin completely cleared the organisms from the blood stream within 4 hours, whereas control mice injected with normal rabbit serum had a septicaemia for at least 24 hours after infection (Table 1).

TABLE 1

ENHANCED BLOOD CLEARANCE OF *Salmonella typhimurium* IN MICE INJECTED WITH IMMUNO-CONGLUTININ SERUM

Time after infection	*Treated animals*		*Control animals*	
	1	*2*	*3*	*4*
1 hour	40	0	6880	7872
2 hours	537	0	39,205	15,897
4 hours	0	0	4296	15,682
6 hours	0	0	2148	60,579
9 hours	0	0	859	31,364
12 hours	0	0	215	7304
24 hours	0	0	82	24,096

Numbers are bacterial counts per ml. of blood.
Treated animals were injected subcutaneously with 0·125 ml. of rabbit immuno-conglutinin serum 24 hours before challenge.
Control animals were injected subcutaneously with 0·125 ml. of normal rabbit serum 24 hours before challenge.
Each mouse was challenged with 2×10^6 *Salm. typhimurium* intraperitoneally.

These experiments suggested that the conglutinin and immuno-conglutinin preparations had demonstrable *in vivo* activity in that they enabled the mouse to clear *Salm. typhimurium* from the blood stream more efficiently. Both of the conglutinin preparations were tested for antibodies to *Salm. typhimurium* and neither had any significant *Salmonella* agglutinins.

(E) THE EFFECT OF IMMUNO-CONGLUTININ ON *Salmonella typhimurium* INFECTION IN MICE

It was found that the number of circulating *Salm. typhimurium* in the blood stream was reduced relatively rapidly in conglutinin-treated mice, but the fate of the organisms was not known. The bacteria may have been destroyed in the blood stream or they may have

been removed from the circulating blood by the action of the reticulo-endothelial system. The liver and spleen are the organs most active in the clearance of particles from the blood. Therefore, the livers and spleens of treated and control mice were cultured at intervals following challenge to trace the course of the early stages of the infection.

Treated mice were injected subcutaneously with 0·125 ml. of rabbit immuno-conglutinin serum and control mice, with 0·125 ml. of normal rabbit serum. The standard intraperitoneal challenge of *Salm. typhimurium* was given 24 hours later. At intervals varying from 15 minutes to 8 hours after infection one mouse from the treated group and one from the control group were killed. The livers and spleens were removed aseptically, weighed, homogenized in glass tissue grinders and viable bacterial counts were made from these homogenates by plate counts on 10-fold dilutions.

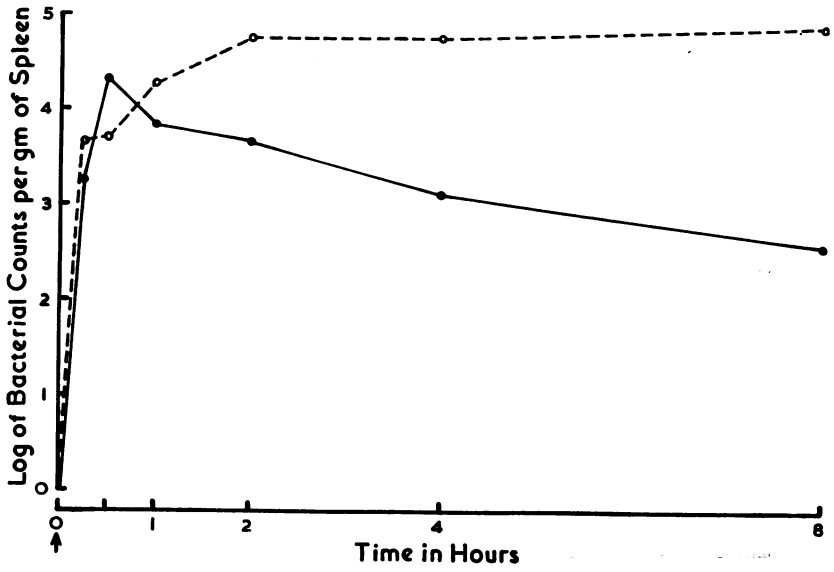

FIG. 8. Numbers of *Salmonella typhimurium* in the livers of mice previously injected with normal serum or immuno-conglutinin serum.
●———● Geometric means of bacterial counts of mice injected subcutaneously with rabbit immuno-conglutinin serum 24 hours before challenge.
O - - - - O Geometric means of bacterial counts of mice injected subcutaneously with normal rabbit serum 24 hours before challenge.
All mice challenged at 0 hour by intraperitoneal inoculation of 2×10^5 *Salm. typhimurium*.

The bacterial counts calculated in two experiments are presented in Table 2. These results are typical of all experiments performed. Fig. 8 shows the bacterial counts plotted in logs per gm. of liver from all (6 treated and 6 control for each period) mice tested. At 15 minutes after infection the average bacterial count per gm. of liver of treated mice was 590, while that of the controls was 1969. By 30 minutes, the average count of the treated group was 3601 as compared with 1379 in the control group. From this peak the numbers in treated mice declined until at 8 hours the average was 84. During this same period the numbers increased in the control mice to an average of 24,979. The results of the bacterial counts of the spleens of these animals, illustrated in Fig. 9, were very similar in all respects to the counts of the livers.

TABLE 2

THE EFFECT OF INJECTION OF IMMUNO-CONGLUTININ SERUM ON BACTERIAL COUNT ON LIVER AND SPLEEN OF MICE INFECTED WITH *Salmonella typhimurium*

	Time after infection	*Liver*		*Spleen*	
		Treated	*Control*	*Treated*	*Control*
Estimated number of organisms per organ	15 minutes	1318	6946	583	1197
	30 minutes	6537	3004	1571	629
	1 hour	2801	14,105	509	8034
	2 hours	3643	4248	1986	2040
	4 hours	241	128,182	84	35,693
	8 hours	56	3122	116	2537
Estimated number of organisms per gm. of organ	15 minutes	869	4081	7676	12,221
	30 minutes	4180	1980	21,816	7676
	1 hour	1529	9416	4141	82,820
	2 hours	2640	2706	30,098	22,422
	4 hours	187	102,300	1111	375,720
	8 hours	44	2904	1616	27,826

Treated animals were injected subcutaneously with 0·125 ml. of rabbit immuno-conglutinin serum 24 hours before challenge.
Control animals were injected subcutaneously with 0·125 ml. of normal rabbit serum 24 hours before challenge.
Each mouse was challenged with 2×10^5 *Salm. typhimurium* intraperitoneally

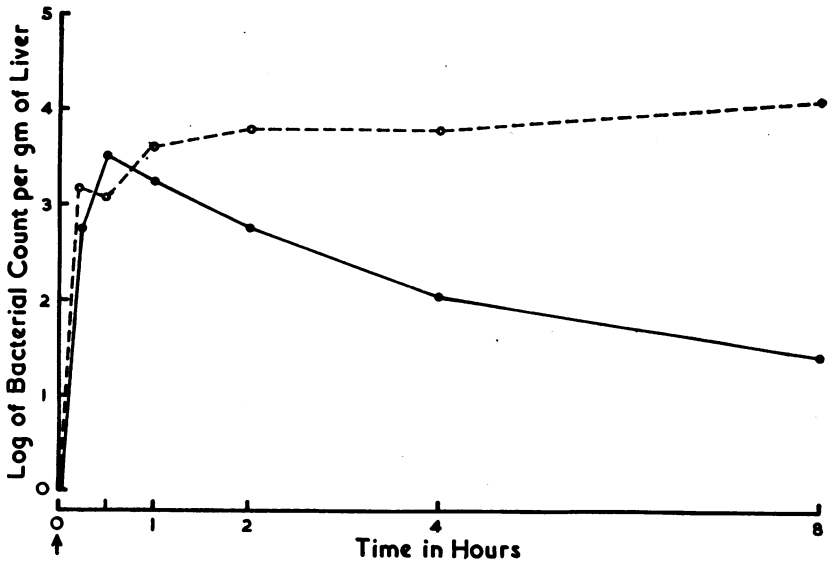

FIG. 9. Numbers of *Salmonella typhimurium* in the spleens of mice previously injected with normal serum or immuno-conglutinin serum.
●———● Geometric means of bacterial counts of mice injected subcutaneously with rabbit immuno-conglutinin serum 24 hours before challenge.
O - - - - O Geometric means of bacterial counts of mice injected subcutaneously with normal rabbit serum 24 hours before challenge.
All mice challenged at 0 hour by intraperitoneal inoculation of 2×10^5 *Salm. typhimurium*.

DISCUSSION

The injection of the conglutinin preparations transferred conglutinating activity to the serum of the recipient. The period and degree of activity were similar to those which

would be expected with passively transferred antibody. The rabbit immuno-conglutinin serum which had a higher titre than the bovine euglobulin preparation transferred a greater activity to the serum of the recipient mouse. These experiments illustrated the rise and fall of conglutinating activity in the serum of the mouse following the subcutaneous injection of bovine euglobulin or rabbit serum containing immuno-conglutinin.

Mice which had a passively acquired conglutinating activity showed a rapid and marked decline in this activity immediately following infection. Because mice challenged with killed organisms did not show a drop in titre, the decreasing levels in infected mice was not due to the injection of bacteria but was probably caused by the interaction of the viable bacteria and the defence mechanisms of the mouse. These findings indicate that conglutinin is utilized or destroyed *in vivo* in the struggle between the host and an invading organism.

In rabbits which had actively stimulated immuno-conglutinin, a significant decrease in conglutinating activity occurred following infection or reinfection. Fig. 6 illustrates the reactions of a rabbit which was infected on day 0 and 1 and was reinfected on day 5. It demonstrates the decreasing conglutinating activity of the serum during these periods of challenge. In 2 other rabbits, a second temperature peak following a slight reduction, a second period of diarrhoea, a second period of positive bacterial cultures and a two-phase antibody response indicated a relapse in the infection. The levels of immuno-conglutinin in both these animals decreased immediately after infection. This initial drop was followed by increasing levels and the increase was interrupted by a second decline coinciding with the time of the relapse. These data give much greater significance to the disappearance of low titre conglutinin which has been previously reported by Ingram *et al.* (1959) to occur in normal rabbits exposed to bacterial infections. The evidence indicates that immuno-conglutinin is not only stimulated by infection but it is also involved in the reactions of the animal to infection. The pattern of variation of immuno-conglutinin levels during relapses suggests that it is possible to trace the course of a disease and to detect repeated challenges without knowing the specific infecting organism. This may be of importance in diseases of unknown aetiology or in diseases where the detection of specific antibody is difficult or impossible.

Both the conglutinin and immuno-conglutinin preparations markedly increased the ability of mice to clear *Salm. typhimurium* from the blood stream. The big difference in the 8-hour bacterial counts from the organs of treated and control mice demonstrates that the immuno-conglutinin serum was aiding the treated mice in the destruction of the invading bacteria. Blood clearance experiments showed that 4 mice treated with immuno-conglutinin serum had sterile circulating blood at this time after infection. Therefore, the organisms which had been held in the liver and spleen probably were not being removed to another site in the body. At 8 hours post-infection the control mice had a large number of organisms in both the liver and spleen and in the circulating blood. It would appear that the bacteria were multiplying in the control mice while the numbers were being greatly reduced in the treated mice.

Blood clearance tests and bacterial counts of the livers and spleens have shown that the conglutinin and immuno-conglutinin preparations have a marked effect on the early stages of *Salm. typhimurium* infection in mice. At this same time, decreasing levels of conglutinating activity are demonstrable in infected mice. Therefore, it is reasonable to suppose that these 2 phenomena are associated and that the factor causing the conglutinating activity may also be causing the anti-bacterial effect. The utilization of this

substance for anti-bacterial action would cause the reduction of the conglutinating activity of the serum. The effect of conglutinin and immuno-conglutinin on the resistance of mice to the fatal effects of bacterial infection will be reported in a subsequent paper.

ACKNOWLEDGMENTS

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