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Research Article

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Bacterial Interference in Chick Embryos *

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Summary. Chick embryos infected intra-allantoically with nonvirulent staphylococci are protected from death due to infection with virulent staphylococci. The protection is associated with a delay in growth of the challenge strain and a delay in the production of toxic substances in the allantoic fluid. The protection is influenced by the number of bacteria in the protecting and challenge inocula and by the interval between the administration of the protecting and challenge strains. Protection cannot be transferred by administration of sterile filtrates of allantoic fluid in which the protecting strain has grown.

Introduction

We have previously demonstrated that a strain of *Staphylococcus aureus* implanted on the nasal mucosa or umbilicus protects newborn infants from colonization and disease caused by epidemic strains of staphylococci (1). In the present studies, we have shown that the growth of a nonvirulent staphylococcus in the allantoic cavity of chick embryos can protect the embryos from death due to subsequent infection with virulent staphylococci. We have investigated the factors that influence the protective effect.

Methods

Materials

Chick embryos. Chick embryos were used in these studies because lethal staphylococcal infection can be produced by the intra-allantoic injection of as few as 10 colony-forming units (cfu) of certain strains of staphylococci (2). Growth of staphylococci and production of toxic substances can be conveniently studied in the easily accessible allantoic fluid. Chick embryos [1] were kept at 35 to 37° C in an incubator with 60% relative humidity and were rotated every 12 hours.

Staphylococci

Protecting or interfering strain. Strain W was chosen for the protecting strain because of its low virulence for chick embryos. This strain grows on agar in white colonies, does not produce coagulase, and does not kill mice injected intravenously with 10^9 cfu. Several other coagulase-negative strains and certain laboratory strains of coagulase-positive staphylococci were also found to have low virulence for chick embryos and could also serve as the protecting strain. The coagulase-positive strain 502A used by us (1) as an interfering strain in newborn infants was not satisfactory as a protecting strain in chick embryos because of the high mortality resulting from infections with this organism.

Challenge strain. Several strains of coagulase-positive staphylococci were capable of killing chick embryos, but none of the 14 strains tested produced death as rapidly and consistently as strain 502A; therefore, in most of the experiments, 502A was utilized as the challenge strain. This strain was initially isolated from the nose of an asymptomatic person and has been described in detail (3). It grows in yellow colonies; is coagulase positive when tested by the tube or slide method; produces lysis of rabbit, sheep, and human erythrocytes; and kills 75% of mice within 6 days after intravenous injection of 5×10^8 cfu. Growth of this strain is inhibited by 0.05 μg per ml of penicillin G (benzyl penicillin), but not by 25 μg per ml of tetracycline (4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacene-carboxamide).

All strains were lyophilized and stored at room temperature. Staphylococci were grown in trypticase soy broth for 18 to 20 hours, and appropriate dilutions of the

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[1] Shamrock Farms, North Brunswick, N. J.

TABLE I

Protection against lethal effect of strain 502A by prior inoculation of strain W

	Group A	Group B	Group C	Group D
Day −1	0.1 ml broth ia*	0.1 ml W ia	0.1 ml W ia	0.1 ml broth ia
Day 0	0.1 ml 502A ia	0.1 ml broth ia	0.1 ml 502A ia	0.1 ml broth ia
Day 1	154/208(74)†	149/174(86)	222/252(88)	87/92(95)
2	78/208(38)	140/174(81)	211/252(84)	82/92(89)
3	39/208(19)	130/174(75)	195/252(77)	79/92(86)
4	22/208(11)	104/140(74)	156/218(72)	79/92(86)
5	15/208(7)	116/174(67)	179/252(71)	79/92(86)
6	13/144(9)	93/119(78)	128/195(66)	50/57(88)
7	12/130(9)	72/96(75)	96/170(57)	36/43(84)
8	12/130(9)	70/96(73)	70/141(51)	34/43(79)
9	12/130(9)	55/78(71)	46/117(39)	29/38(76)
10	9/118(8)	43/68(63)	26/98(27)	27/38(71)
11	3/56(5)	20/36(67)	13/48(27)	24/38(63)

* ia = intra-allantoic injection.

† The first number refers to survivors, the second to the total number of embryos, and the number in parentheses to the per cent survival.

bacteria for injection were made in 0.9% saline or in distilled water. Injections were made within 30 minutes after dilution. During this period there was no killing of staphylococci in saline or distilled water.

Culture media. Trypticase soy broth and trypticase soy agar [2] were used in all experiments. Bacterial filters [3] were used as indicated.

Injection of embryos

Allantoic inoculations were made by the technique described by Beveridge and Burnet (4). Intravenous injections were made into allantoic veins after a small area of overlying shell had been removed. After injection, the window in the shell was covered with Scotch tape. Inoculations onto the chorioallantoic membrane were made by the method described by Scott (5). The volume injected was 0.1 ml unless otherwise stated.

Enumeration of bacteria in allantoic fluid

To remove allantoic fluid we first cooled the embryos at 4° C for 2 hours. The shell over the air sac was removed, the membranes were punctured, and the allantoic fluid was pipetted. Grossly bloody fluids or fluids containing yolk were discarded. Tenfold dilutions of allantoic fluid in sterile distilled water were spread onto trypticase soy agar plates. When both strain 502A and strain W were present, the dilutions of the fluid were also plated onto trypticase soy agar containing 5 μg per ml of tetracycline. This concentration of tetracycline suppressed the growth of strain W, but allowed full growth of strain 502A.

Determination of hemolytic activity

One-half ml of twofold dilutions of allantoic fluid to be tested for hemolytic activity was mixed with 0.25 ml of a 2% suspension of washed rabbit red cells in 0.9% saline. The mixtures were incubated for 1 hour at 37° C and then refrigerated at 4° C overnight. The amount of hemolysin was expressed in units that represented the reciprocal of the greatest dilution of allantoic fluid that resulted in complete hemolysis.

[2] Difco Corp., Detroit, Mich.

[3] Millipore Filter Corp., Bedford, Mass.

Results

Protection of chick embryos from lethal staphylococcal infection

Groups of 10–day–old chick embryos were injected intra-allantoically with strain W (10^5 to 10^6 cfu) or with 0.1 ml of a 10^{-2} dilution of trypticase soy broth. After 24 hours of incubation, the same groups of embryos were injected intra-allantoically with strain 502A (10^3 cfu) or with a 10^{-5} dilution of broth. The embryos were incubated and examined for death.

The results of seven separate experiments are summarized in Table I. Infection of the embryos with strain 502A resulted in a lethal infection, so that by the third day after the second injection there was only 19% survival. In contrast, injection of strain W resulted in a less lethal infection, so that by the third day survival was 75%, and at day 11, 67%.

Injection of strain W before introduction of strain 502A decreased the lethality of 502A. In the embryos with double infection, the survival at day 3 was 77%, compared to 19% survival in the embryos infected with 502A alone. On days 1 through 5, the survival of the doubly infected group approximated that of the embryos infected

TABLE II

*Concentration of strain 502A and hemolysin in allantoic fluids from chick embryos infected with 502A and with a combination of strains W and 502A**

	Day 1 after challenge	Day 2	Day 4	Day 5
Group 1	6.5×10^8 (2)†	8.1×10^8 (32)	6.0×10^8 (32)	No survivors
	4.6×10^8 (32)	1.7×10^6 (0)	3.0×10^9 (32)	
	3.1×10^8 (64)	5.6×10^8 (32)	7.9×10^4 (0)	
	1.3×10^9 (128)	1.3×10^9 (128)	2.0×10^7 (64)	
	3.2×10^7 (0)	1.1×10^9 (128)		
		9.8×10^8 (128)		
		5.8×10^8 (128)		
Group 2	2.5×10^5 (0)	3.8×10^7 (16)	3.3×10^8 (64)	4.5×10^8 (16)
	7.1×10^6 (0)	1.1×10^7 (16)	1.9×10^8 (32)	5.1×10^8 (32)
	7.9×10^5 (0)	8.3×10^8 (2)	1.5×10^8 (32)	1.0×10^9 (128)
	2.1×10^4 (0)	2.3×10^7 (16)	3.3×10^8 (16)	1.7×10^8 (128)
	7.9×10^1 (0)	1.1×10^6 (0)	7.8×10^8 (2)	1.4×10^9 (64)
	9.1×10^5 (0)	9.8×10^7 (0)		
	1.3×10^5 (0)	7.2×10^8 (16)		
		9.6×10^5 (0)		
		8.3×10^5 (0)		
		2.5×10^4 (0)		

* In group 1, the first injection was broth and the second, strain 502A; in group 2, the first injection was strain W and the second, 502A.

† The first number represents the concentration of strain 502A in colony-forming units per milliliter, and the number in parentheses represents the hemolysin titer.

with strain W alone. After that time, survival decreased but was still greater than survival of embryos infected only with 502A.

The protection was reproducible and was demonstrated in all of the seven experiments. In the individual experiments, survival on the fifth day in the doubly infected group ranged from 30 to 75%, whereas in the embryos infected with 502A alone survival ranged from 0 to 16% on the fifth day after challenge.

The following experiment was performed to investigate the influence of infection with the protecting strain on the growth and toxin production of the challenge strain.

Groups of 10-day-old chick embryos were inoculated as in the previous experiment. At different times after the second injection the allantoic fluid was removed, and the concentrations of the protecting strain W and the challenge strain 502A were determined. The strains could be differentiated by pigment production and ability to grow on agar containing tetracycline. Bacteria were then removed from the allantoic fluids by filtration through a Millipore filter (0.45-μ pore size), and the sterile filtrate was tested for its ability to lyse rabbit red cells. The toxicity of the fluid was tested by intravenous injection of 0.1 ml into 14-day-old chick embryos.

In the group infected with 502A alone the bacterial concentration ranged from 3.2×10^7 to 1.3×10^9 cfu per ml and the hemolysin titers from 0 to 128 (Table II), whereas on day 1, in the embryos infected with both strain W and strain 502A, the bacterial concentration of 502A ranged from 7.9×10^1 to 7.1×10^6 cfu per ml, and there was no detectable hemolysin in any of the embryos. By the second day, the bacterial concentration of strain 502A was higher in the group infected with it alone than in the group infected with both it and strain W (p less than 0.05).[4] At this time, six of the seven embryos infected with 502A alone had hemolysin titers of 32 or more, whereas all of the ten embryos infected with strain W before challenge with strain 502A had hemolysin titers of 16 or less. By the fourth day after challenge, the concentrations of bacteria and toxin in doubly infected embryos approximated those found in embryos infected with 502A alone. Filtrates of fluids from both groups obtained 2 days after the second injection were injected intravenously into 14-day-old chick embryos. The fluids from the embryos infected with strain 502A killed all of the 24 injected embryos, but fluids from the doubly infected group killed none of the 24 injected.

[4] Determined by using chi-square with Yates correction.

Strain W had grown to a concentration of about 10^8 cfu per ml at the time of the challenge infection with strain 502A and did not change during the course of the experiment. The concentration of strain W in embryos infected with it alone did not differ from that in the doubly infected embryos. No hemolysin or toxin could be detected in the allantoic fluid of embryos infected with strain W alone.

Factors that influenced the protection of chick embryos

1) Influence of viability of the protecting strain. Groups of 10–day-old chick embryos were injected intra-allantoically with either living strain W (10^6 cfu) or a concentrated suspension that had been autoclaved for 30 minutes (10^9 cfu before autoclaving). Twenty-four hours later, both groups of embryos were injected with 10^3 cfu of 502A and were examined daily for survival.

In the group of embryos previously injected with living strain W, there was 79% survival at 5 days after the second injection, compared with 10% survival in the group that had previously received the heat–killed staphylococci of strain W. Dead staphylococci afforded no more protection than the injection of sterile broth before challenge.

2) Influence of the number of bacteria on protection. The protective effect could be influenced by varying the number of either of the strains.

a) Influence of number of strain W. Groups of 10–day–old chick embryos were injected with 0.1 ml of broth or with 0.1 ml of tenfold dilutions of a broth culutre of strain W containing from 2.5×10^1 to 2.5×10^8 cfu. Twenty-four hours later, each of the groups of embryos was challenged with 5×10^2 cfu of strain 502A and observed daily for survival.

On day 2 after the second injection, the percentage of surviving embryos was proportional to the inoculum of strain W over the range from 2.5×10^1 to 2.5×10^5 cfu, with maximal survival (96%) in the group that had received 2.5×10^5 cfu (Figure 1). In groups that had received inocula larger than 2.5×10^5 cfu, fewer embryos survived than in groups receiving the smaller inocula. Fifty control embryos inoculated with 10^6 cfu or more strain W (and not challenged with strain 502A) had 60 to 70% survival at day 2. Fifty control embryos that received 10^5 cfu or fewer strain W had 100% survival at day 2. Protection could still be demonstrated at day 11 in the groups that had received 10^5 or 10^6 cfu of strain W.

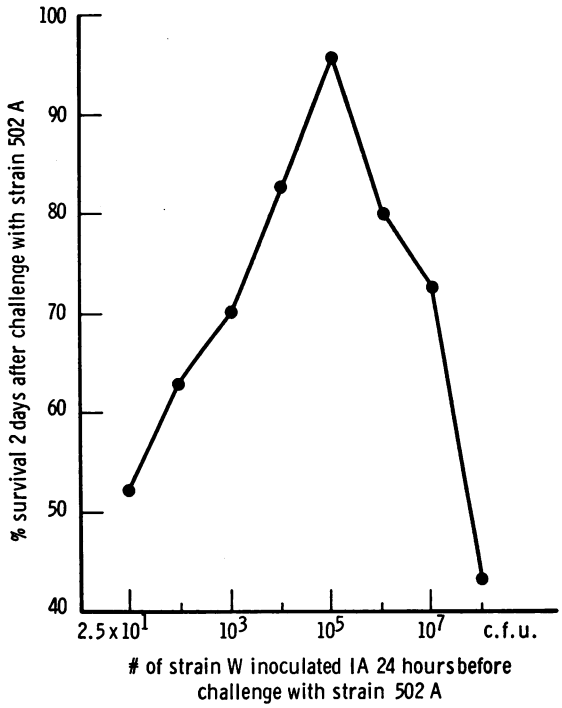

FIG. 1. INFLUENCE OF THE NUMBER OF STRAIN W ON THE SURVIVAL OF CHICK EMBRYOS INFECTED WITH STRAINS W AND 502A. Each points represents per cent survival in a group of 30 embryos. CFU = colony-forming units; IA = intra-allantoically.

b) Influence of number of strain 502A. Groups of 10–day–old chick embryos were injected with either 1×10^6 cfu of strain W or broth. Twenty-four hours later, the groups of embryos were injected with tenfold dilutions of a broth culture of strain 502A containing from 2×10^1 to 2×10^8 cfu. The embryos were examined daily for survival.

On the second day after the challenge injection, a protective effect of prior infection with strain W was evident, even in groups that had been challenged with large inocula of strain 502A (Figure 2), although the degree of protection was small in the embryos challenged with 10^8 cfu of strain 502A. Five days after challenge, protection was not evident in the embryos that had received 10^4 cfu or more of strain 502A, but protection could be demonstrated for as long as 11

days in the embryos challenged with 10^3 or fewer organisms.

3) Influence of interval between injections of the two staphylococcal strains. Groups of 10–day–old chick embryos were injected intra-allantoically with 0.1 ml of strain W (10^6 cfu) at various times before and 5 hours after injection of 1×10^2 cfu of strain 502A. The embryos were examined daily for survival.

Protection on the fifth day after challenge was proportional to the interval between the injection of strains W and 502A (Figure 3). The survival of embryos was about 40% if strain W was injected 45 hours before challenge with strain 502A and about 35% if the injections were spaced 20

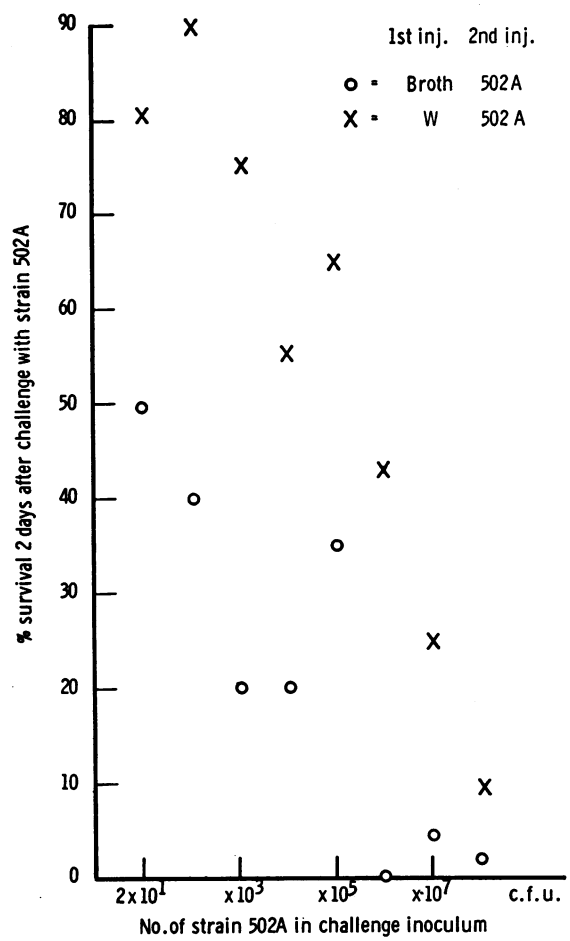

FIG. 2. INFLUENCE OF THE NUMBER OF THE CHALLENGE STRAIN 502A ON THE SURVIVAL OF CHICK EMBRYOS INFECTED WITH STRAIN W. Control embryos were injected with broth and then graded doses of strain 502A. Each point represents per cent survival in a group of 20 embryos.

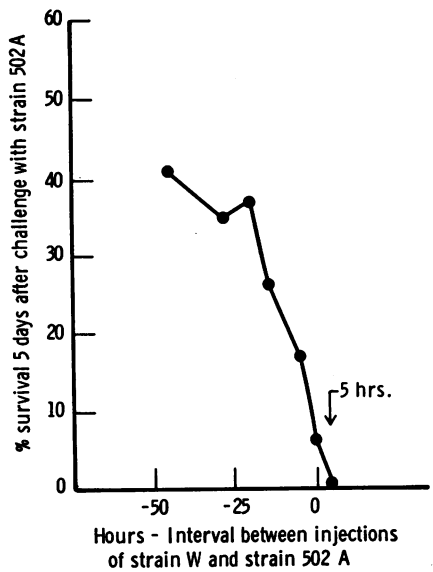

FIG. 3. INFLUENCE OF THE INTERVAL OF TIME BETWEEN THE INJECTIONS OF STRAINS W AND 502A ON SURVIVAL OF CHICK EMBRYOS. Each point represents per cent survival in a group of 35 embryos.

to 27 hours apart. Administration of the two strains simultaneously did not result in protection, nor was there any protection if strain W was administered 5 hours after strain 502A.

4) Influence of the route of administration of strain 502A on the protective effect of intra-allantoic infection with strain W. Ten–day–old chick embryos were injected intra-allantoically with either 0.1 ml of strain W (10^4 cfu) or broth. The next day the chorioallantoic membrane (CAM) was lowered, and 0.2 ml of a dilution of strain 502A (10^2 cfu) was placed on the depressed membrane.

In another series of experiments, 13–day–old embryos were injected intra-allantoically with 10^6 cfu of strain W, and the next day the embryos were injected intravenously with 0.1 ml of a dilution of strain 502A containing 10^3 cfu.

Strain W growing in the allantoic fluid did not significantly protect the embryos from death due to 502A given intravenously or placed on the CAM.

Attempts to transfer protection to other chick embryos

Allantoic fluid from normal uninfected 10–day–old chick embryos and allantoic fluid from embryos

infected with strain W were passed through a Millipore filter (0.45 μ). Two–ml samples of each of the sterile fluids were injected intra-allantoically into other embryos 24 hours before and 30 minutes before injection of 10^3 cfu of strain 502A. Another group of embryos received 2 ml of saline 24 hours and 30 minutes before the same challenge. A fourth group of embryos received allantoic fluid 24 hours and 30 minutes before administration of sterile diluted broth. The embryos were examined for survival 48 hours after challenge. The results are presented in Table III. No protection was afforded by the injection of saline or of allantoic fluid from either uninfected or infected embryos. The administration of 4 ml of allantoic fluid without subsequent bacterial challenge resulted in 73% survival.

TABLE III

*Survival of embryos after administration of bacteria–free filtrates of allantoic fluid before challenge with strain 502A**

Group A	Group B	Group C	Group D
1/20†	1/24	0/22	19/26

* Group A received filtered allantoic fluid from uninfected embryos and was challenged with 502A. Group B received filtered allantoic fluid from embryos infected with strain W and was challenged with 502A. Group C received saline and was challenged with 502A. Group D received allantoic fluid from embryos infected with strain W and was not challenged with bacteria.

† The first number refers to survivors and the second to the total number of embryos.

Discussion

Intra-allantoic infection of chick embryos with nonvirulent staphylococci protected them from death due to subsequent infection with virulent staphylococci. The protection was associated with initial suppression of growth of the virulent challenge strain and a delay in production of toxic and hemolytic substances. It has been shown (6) that death of chick embryos infected intra-allantoically with strain 502A is due to the production of toxin and that any procedure which results in a decrease in the concentration of toxin in the allantoic fluid is associated with increased survival of the embryos.

During the first 2 days after challenge of the embryos, the concentration of the challenge strain was less than 10% of the concentration attained in unprotected embryos after 1 day, and toxin titers in protected embryos were lower than in unprotected embryos. At 4 days after challenge, the concentration of the virulent strain approached that seen in unprotected embryos, and significant amounts of toxic and hemolytic substances were present in the allantoic fluid. The protection observed at 4 days after challenge was probably due to the increased resistance of older embryos to staphylococcal infections. The susceptibility of chick embryos to the lethal effects of staphylococcal infections decreases with increasing age (2). Older embryos are more resistant to the lethal effects of infection with strain 502A, as well as to the lethal effects of crude toxin prepared from this strain. Survival at 2 days after intra-allantoic infection of 11–day–old embryos with strain 502A was 18%, but it was 41% in embryos challenged when they were 14 days old. None of ten 11–day–old embryos survived challenge with 0.1 ml crude 502A toxin, but 12 of 17 15–day-old embryos survived the administration of the same amount of toxin (6).

McCabe (7) has also demonstrated that infection of chick embryos with coagulase–negative staphylococci results in protection of the embryos from the lethal effects of infection with virulent coagulase–positive staphylococci. He has reported that protection is associated with a decrease in growth of some virulent strains; however, he observed protection against one virulent staphylococcus, despite growth of this strain to high titer in the allantoic cavity.

It is unlikely that the protection induced by intra-allantoic infection with strain W resulted from an increase in the host defenses of the embryos. Intra-allantoic infection with the protecting strain did not prevent death due to staphylococcal infections initiated by the intravenous and chorioallantoic routes. In addition, infection with the protecting strain did not alter the susceptibility of embyros to the lethal effects of partially purified staphylococcal toxins administered intravenously or intra-allantoically (8).

The mechanism by which strain W interfered with the growth of strain 502A is not clear from these investigations. The necessity for large numbers of viable cells of the protecting strain and for an interval of several hours between the protecting and challenge injections suggests that the protecting strain produced an inhibitor of bacterial

growth or utilized a nutrient substance required for optimal growth of the challenge strain. The failure to transfer protection to other embryos by the administration of large amounts of sterile allantoic fluid in which strain W had grown seems to exclude the presence of a potent antibacterial substance. However, the presence of an inhibitor that can be destroyed in the allantoic fluid of recipient embryos cannot be excluded.

Studies *in vitro* in trypticase soy broth (9) have shown that interference with the growth of strain 502A by strain W can be reversed by the addition of small amounts of nicotinamide to mixed cultures. However, addition of large amounts of nicotinamide to the allantoic cavity of chick embryos did not stimulate the growth of the challenge strain or reverse the phenomenon of protection.

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