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Research Article

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Interference required infection with viable bacteria. The onset of interference appeared within a few minutes after injection of the interfering strain, but was not maximal until 24 hours had elapsed between injection of the interfering and challenge strains. The protection afforded by the production of interference could not be overcome by increased inoculum size of the challenge strain and extended even to challenge with 10^9 bacteria.

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Bacterial Interference Induced in Embryonated Eggs by Staphylococci *

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Summary. Studies of experimental infections in embryonated eggs demonstrated that prior allantoic infection with avirulent staphylococci afforded significant protection against subsequent challenge with virulent strains. All strains of coagulase–positive and coagulase–negative staphylococci tested that were relatively avirulent for embryonated eggs were capable of producing interference. The interference induced afforded protection not only against challenge with virulent staphylococci, but also against *Diplococcus pneumoniae, Salmonella typhimurium, Escherichia coli, Proteus mirabilis,* and one strain of influenza virus (A_2J 305). Prior allantoic infection with avirulent staphylococci also protected against intravenous as well as allantoic infection with challenge strains.

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Introduction

Clinical and laboratory observations have suggested that normal flora of both man and experimental animals exert a protective effect against bacterial superinfection. Inhibition of the gastrointestinal flora by broad spectrum antibiotics predisposes to the development of staphylococcal enterocolitis (1, 2). Similarly, prior antibiotic therapy also allows gastrointestinal infection of previously resistant experimental animals with *Salmonella* (3) and *Shigella* (4). In addition, Shinefield and associates have recently demonstrated that artificial colonization of the nares and umbilici of newborn infants prevents subsequent colonization and infection with epidemic strains of staphylococci (5). Somewhat similar observations of interference between strains of virus led to the identification of interferon (6–8). Bacterial

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interference has not been extensively investigated, however, despite its clinical importance, and the mechanism of this phenomenon has yet to be defined. A preliminary study which demonstrated that prior infection with avirulent staphylococci protected embryonated eggs against subsequent challenge with virulent staphylococci (9) provided a suitable experimental system for investigations of bacterial interference. The present report describes extension of these studies and investigation of factors influencing bacterial interference.

Methods

Bacterial strains

Interfering strains. Four strains of coagulase-positive *Staphylococcus aureus* and six strains of coagulase-negative *Staphylococcus epidermidis,* previously shown to be avirulent for embryonated eggs (10), were studied for their ability to protect against subsequent challenge with egg virulent strains. One strain each of coagulase-positive staphylococcus K and coagulase-negative staphylococcus P was utilized in most of the studies of interference and determinations of bacterial growth. These strains grow as porcelain white colonies on agar; this allowed their differentiation from the yellow-pigmented colonies of the challenge strains.

Challenge strains. Three strains of coagulase-positive *S. aureus,* G, OB, and W, were used primarily as the challenge strains, although other coagulase-positive staphylococci were occasionally studied. These strains produce a golden yellow pigment, which allowed their differentiation from the interfering strains, and all produce fatality rates ranging from 80 to 100% in embryonated eggs (10).

Additional bacterial species were obtained from clinical specimens submitted to the University Hospital diagnostic bacteriology laboratory for use as challenge strains. Isolates of *Streptococcus pyogenes, D. pneumoniae, S. typhimurium, E. coli, P. mirabilis,* and *Pseudomonas aeruginosa* were tested for their lethality for embryonated eggs, and virulent strains were subsequently used as challenge organisms. All strains were grown for 18 hours in trypticase soy broth and were serially diluted in peptone saline to provide an inoculum of 100 colony-forming units (cfu) for injection into embryonated eggs. This inoculum size was routinely used for both the interfering and challenge strains, except in studies of the relationship of the dose of the challenge strain to interference.

In addition, two strains of influenza virus, A_2J 305 and AF 57, were obtained.[1] Strain A_2J 305 proved to be virulent for embryonated eggs after allantoic injection and was maintained by serial egg passage. Infected allantoic fluid was used as the inoculum for challenge experiments.

[1] Through the courtesy of Dr. Rodolfo Jao of the Thorndike Memorial Laboratory, Boston City Hospital.

Experimental infections

Ten-day-old embryonated eggs were infected intraallantoically, incubated, and fatality rates determined as before (10, 11). In intravenous challenges, 0.05-ml vol were injected into allantoic veins after a small section of overlying shell had been removed and the defect closed with a drop of melted paraffin.

Studies of bacterial interference were carried out in lots of 80 10-day-old embryonated eggs. Two groups of 20 10-day-old eggs (A and B) were injected with 100 cfu of the interfering strain in 0.1 ml peptone saline. Two additional groups of 20 eggs (C and D) were injected with a similar volume of sterile peptone saline simultaneously. Two days later, lots A and C were injected with 100 cfu of the virulent challenge strain, and lots B and D were injected with 0.1 ml of peptone saline. Group D served as controls of the trauma of the procedures, group C was a control of the lethality of the challenge strain, group B was a control of the lethality of the interfering strain, and group A served to demonstrate whether interference had resulted from injection of the protective strain. Cumulative fatalities 7 days after injection of the challenge strains were used for all determinations of interference, except where indicated. No attempt was made to correct for fatalities resulting from infection with the interfering strain alone in the results reported.

Bacterial enumeration

Allantoic fluid was harvested by aspiration with a 20-gauge needle and syringe after the eggs had been opened over the air sac. After complete removal of allantoic fluid, the amnion was washed with 70% alcohol, and the amniotic fluid was aspirated. The embryos were removed with sterile forceps and immersed in iodine and then alcohol, flamed, and washed three times in sterile saline. The embryos were then homogenized in 5 ml of peptone saline in a Waring blendor. Serial tenfold dilutions of 1-ml samples each of allantoic and amniotic fluids and the homogenized embryo were made and plated onto trypticase soy agar. Differentiation between the interfering strain and the challenge strain was based on colonial pigmentation. Studies utilizing antibiotics incorporated into the agar to inhibit either the interfering or protective strain afforded no improvement over results of enumerations based on pigment differences and were not continued.

Growth curves in allantoic fluid

Allantoic fluid was harvested 2 days after injection of an interfering strain or peptone saline as described. This fluid was sterilized by filtration through a 0.45-μ Millipore filter and inoculated with 10^2 cfu of challenge strains of bacteria. Samples were obtained immediately after inoculation and 6 and 24 hours later for bacterial counts.

Whole blood bactericidal activity

Two days after injection of either peptone saline or an interfering strain, blood was aspirated from a vein in the allantoic membrane with a tuberculin syringe and a

27-gauge needle. Blood samples from lots of 20 eggs were pooled, rapidly defibrinated, and dispensed in 0.9-ml portions. Suspensions of the challenge strains containing approximately 1,000 bacteria in 0.1 ml were added to capped sterile tubes containing the blood specimens. After thorough mixing, a 0.1-ml sample was taken for bacterial counts. Tubes were placed on a rotator and incubated at 37° C for 3 hours; then samples were obtained for serial dilution and plating.

Results

Protection against lethal infection by avirulent staphylococci

Studies of interference were performed in 1,274 embryonated eggs. The lethality after injection of a poorly virulent or interfering strain followed by saline was 21%. The fatality rate after the injection of saline followed 2 days later by challenge with strains W, OB, and G of virulent coagulase-positive *S. aureus* was 80%. In contrast, when injection of the interfering strain preceded challenge with virulent staphylococci, the fatality rate 7 days later was reduced to 32%. The protection afforded by injection of an interfering strain before challenge with a virulent strain was highly significant ($x^2 = 212$; $p < 0.001$) when compared with the lethality of the challenge strain after saline. The fatality rate observed after injection of the interfering strain followed by the challenge strain was also greater ($x^2 = 12$; $p < 0.001$) than that observed with injection of the interfering strain alone. However, the fatality rate in excess of that of the interfering strain alone was only 11%. When this is compared with the 80% fatality rate in eggs injected with saline before challenge, it is apparent that protection afforded by prior injection of an interfering strain is of considerable magnitude. Variations among individual experiments did not exceed 20%, and significant protection ($p < 0.05$) from interference was observed in each of the more than 20 experiments.

Protection against other challenge strains

Similar protection was obtained in this system against challenge with five other strains of virulent coagulase-positive staphylococci, in addition to the protection afforded against challenge with strains W, OB, and G. The protective effect of prior infection with interfering strains P or K against lethal infection by other bacterial species was also investigated. Table I illustrates the fatality rates observed with challenge strains of *D. pneumoniae, S. pyogenes, S. typhimurium, E. coli, P. mirabilis,* and *P. aeruginosa* alone and after the injection of an interfering strain. Significant interference with the lethality of *D. pneumoniae, S. typhimurium, E. coli,* and *P. mirabilis* was observed. Prior injection of an interfering strain also led to a reduction in the lethality from challenge with *S. pyogenes,* but the differences were not significant. Similarly, although fatality rates 7 days postchallenge were identical after challenge with *P. aeruginosa,* prior injection of an interfering strain materially prolonged the survival time of such embryos.

TABLE I

Interference with lethality of other bacteria and influenza virus

Challenge strain	Alone	After interfering strain	p
D. pneumoniae	31/48 (65%)*	9/83 (11%)	<0.001
S. pyogenes	12/20 (60%)	6/20 (30%)	<0.05
S. typhimurium	29/29 (100%)	19/38 (50%)	<0.001
E. coli	20/20 (100%)	2/20 (10%)	<0.001
P. mirabilis	12/20 (60%)	2/20 (10%)	<0.005
P. aeruginosa	19/19 (100%)	19/19 (100%)	
Influenza virus (A_2J 305)	71/100 (71%)	60/200 (30%)	<0.001

* Fatalities/number of eggs challenged (per cent fatalities).

In addition to the protection afforded against challenge with various bacterial species, prior infection with avirulent staphylococci afforded significant protection against challenge with influenza virus. Seventy-one, or 71%, of 100 embryonated eggs challenged with influenza strain A_2J 305 2 days after saline injection died, whereas only 60 of 200 (30%) succumbed to infection with influenza virus when this followed injection of *avirulent staphylococci* ($x^2 = 45$; $p < 0.001$). Thus, prior infection with avirulent staphylococci afforded significant protection against subsequent infection with one strain of virus as well as several species of bacteria.

Protection afforded by other interfering strains

Figure 1 illustrates the protection observed when three additional strains of avirulent coagulase-positive staphylococci and five strains of avirulent coagulase-negative staphylococci were used as interfering strains. The diagonally hatched bars represent the lethality from injection of the

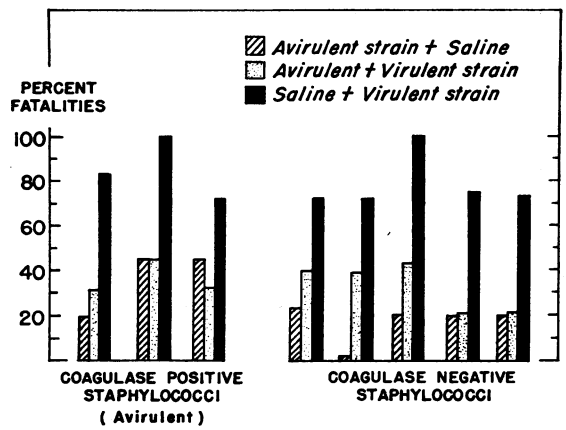

FIG. 1. INTERFERENCE INDUCED BY ADDITIONAL STRAINS OF AVIRULENT COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI. Each bar represents fatality rates in lots of 20 or more embryonated eggs. Fatality rates in eggs injected with avirulent staphylococci before challenge with virulent staphylococci were significantly less ($p < 0.05$) than in controls injected with saline before challenge in each of the eight experiments.

avirulent strain, the closed bars depict fatality rates after injection of the virulent challenge strain, and the stippled bars illustrate the fatality rates after injection of avirulent strains followed by challenge with virulent staphylococci. In each instance, significant protection ($x^2 > 3.8$, $p < 0.05$) against lethality from the challenge strain was afforded by prior infection with an avirulent strain.

Factors influencing protection

Viability of interfering bacteria. The effect of prior injection of nonviable interfering strains on subsequent challenge with virulent strains was assessed. Two 20-ml overnight broth cultures each of interfering strains P and K were concentrated by centrifugation, the supernatant was removed, and the bacteria were resuspended in 2 ml of peptone saline to a concentration of 10^{10} bacteria per ml. One specimen of each strain was sterilized by the addition of aqueous benzalkonium chloride [2] to a concentration of 1:1,000, whereas the second was sterilized by autoclaving at 121° for 15 minutes and injected intra-allantoically. Two days later, eggs were challenged with 10^2 cfu of virulent staphylococci. Four of 40 eggs injected with heat-killed bacteria and one of 40 eggs injected with benzalkonium chloride-killed bacteria survived challenge with virulent staphylococci. There were four survivors among the 80 control eggs injected with saline before challenge, indicating that killed bacteria were incapable of inducing interference.

Temporal relations of interference. The effect of variations in the time intervals between the injection of avirulent staphylococci and challenge with virulent staphylococci on interference was evaluated. Groups of 20 or more eggs were injected with 100 cfu of protective strain K or saline and then were challenged immediately and 2, 6, 8, 12, 24, 48, 72, 140, and 168 hours later with 100 cfu of strain G. Lethality was assessed 3 days after injection of the challenge strain instead of the usual 7 days. The number of survivors per number of eggs injected and the survival rate in eggs injected with saline or an interfering strain at various time intervals between injections of the interfering and the challenge strains are shown in Table II. Since the survival rate after challenge was greater in older eggs, this was corrected by calculating the increase in survival rate afforded by prior injection of an interfering strain over that observed with saline injection before challenge, as is shown in the last column. Significant protection was observed even in eggs challenged immediately after injection of the interfering strain, and protection was maximal when the interval between injection of the interfering and the challenge strains was 24 hours. Protection tended to decrease slightly thereafter, but it persisted even when the interval between injection of the two strains was as long as 168 hours.

Variable inoculum size of the challenge strain.

TABLE II

Relation to interference of time interval between injection of interfering and challenge strains

Hours after protective strain	Challenge strain alone	Protective strain followed by challenge strain	Increase in survival rate
			%
0	6/80 (8%)*	22/80 (28%)	20
2	6/80 (8%)	29/80 (36%)	28
6	6/80 (8%)	35/80 (44%)	36
12	6/80 (8%)	26/40 (65%)	57
24	5/49 (10%)	50/66 (76%)	66
48	19/56 (32%)	52/61 (85%)	53
72	36/94 (38%)	83/96 (86%)	48
140	36/94 (38%)	30/34 (88%)	50
168	36/94 (38%)	30/39 (78%)	40

* Number of survivors/number challenged (per cent survivors).

[2] Zephiran chloride, Winthrop Lab. New York, N. Y.

TABLE III

Effect of inoculum size of challenge strain on interference

Inoculum size of challenge strain	Embryonated eggs injected 2 days earlier		Increase in survival rate
	With saline	With interfering strain	
			%
10^1	5/40 (13%)*	48/60 (80%)	67
10^2	6/40 (15%)	48/60 (80%)	65
10^3	6/60 (10%)	58/73 (80%)	70
10^5	6/58 (11%)	56/77 (73%)	62
10^7	0/57 (0%)	56/79 (71%)	71
10^8	0/40 (0%)	26/37 (70%)	70
10^9	0/40 (0%)	55/80 (69%)	69

* Number of survivors/number injected (per cent survivors).

The effect of differing inoculum sizes of the two challenge strains on the protection afforded by an interfering strain was compared with the fatality rates in eggs previously injected with saline. The results are illustrated in Table III. Significant protection was afforded by the interfering strain against inocula of from 10^1 to 10^9 cfu of virulent staphylococci. The magnitude of protection afforded did not vary significantly with the different size inocula of the challenge strain.

Protection against intravenous challenge. The protective effect of prior infection with avirulent staphylococci against intravenous rather than intra-allantoic challenge with virulent staphylococci was assessed. Two days after intra-allantoic injection of saline or 10^2 avirulent staphylococci, groups of 20 eggs were challenged intravenously with 1×10^4 virulent staphylococci. A summary of several such experiments is in Table IV. The fatality rate in the 188 control eggs injected with saline then challenged intravenously with virulent staphylococci was 60%. Intravenous challenge with virulent staphylococci after prior injection of avirulent strains was significantly less lethal ($x^2 = 25$; $p < 0.001$), killing only 117, or 37%, of 317 embryonated eggs. Thus, intra-allantoic injection of a nonvirulent strain afforded significant protection against intravenous as well as intra-allantoic challenge with virulent staphylococci. The degree of protection afforded against intravenous challenge was not so great, however, as against allantoic challenge. Intravenous administration of both challenge strains was considerably less lethal than intra-allantoic challenge with similar sized inocula.

Assays for antibiotic and bacteriocin production

Assays for antibiotic or bacteriocin production by interfering strains were made to exclude these as possible mechanisms of interference. Broth cultures and allantoic fluid from infected eggs were obtained at varying intervals after injection of interfering strains P and K. After Millipore filtration, inhibitory activity against challenge strains G, OB, and W was assayed by the agar diffusion technique described by Grove and Randall (12). No antibiotic production by the interfering strains was detectable. Similarly, bacteriocin production utilizing the technique of Fredericq (13) failed to demonstrate bacteriocin activity by interfering strains P or K against challenge strains G, OB, and W. Additional assays for bacteriocin activity were performed.[3] Slight bacteriocin activity was detected with strain P against challenge strains G and W, but no bacteriocin activity of strain K was detectable.

Bacterial growth in allantoic fluid in vitro

The possibility that the protective effect induced by prior injection of avirulent staphylococci might result from growth inhibition of the challenge strain was assessed by comparison of the growth of challenge strains in control and infected allantoic fluid *in vitro* and *in vivo*. Groups of 10–day–old embryonated eggs were injected with saline or interfering strains P and K, and the allantoic fluid was harvested 2 days later. The infected and uninfected fluids were filtered through 0.45–μ Millipore filters and 5 ml inoculated with 10^2 cfu of challenge strain OB or G. Samples of allantoic fluid were taken immediately and 6 and 24 hours after addition of strain OB or G, and the number of bacteria was determined. The growth of the two challenge strains in harvested allantoic fluid that had been infected 48 hours earlier and

TABLE IV

Interference against intravenous challenge

	Fatalities	
Saline followed by virulent staphylococci iv	112/188 (60%)*	$x^2 = 25$; $p < 0.001$
Avirulent staphylococci followed by virulent staphylococci iv	117/317 (37%)	

* Number of deaths/number challenged (per cent deaths).

[3] Done through the courtesy of Dr. A. I. Braude, University of Pittsburgh School of Medicine.

TABLE V

Growth curves of challenge strains in normal allantoic fluid and allantoic fluid previously infected in vivo

Allantoic fluid	Challenge strain	Number of bacteria (challenge strain) Hours after inoculation 0	6	24
Uninfected	OB	2×10^2	4×10^4	6×10^8
Infected with strain P	OB	2×10^2	3×10^4	2×10^8
Infected with strain K	OB	4×10^2	1×10^3	1×10^6
Uninfected	G	3×10^2	3×10^4	4×10^8
Infected with strain P	G	3×10^2	2×10^4	2×10^8
Infected with strain K	G	3×10^2	1×10^4	3×10^7

normal allantoic fluid is shown in Table V. The values listed represent the means of three duplicate sets of experiments. Growth of both challenge strains in allantoic fluid previously infected with strain P did not differ materially from that in uninfected fluid. Inhibition of both challenge strains was noted, however, in allantoic fluid from eggs previously injected with interfering strain K, and they achieved numbers 1 to 2 logarithms less than those observed in allantoic fluid from control eggs.

Bacterial growth in vivo

Eggs were injected with interfering strains, challenged 2 days later, and sacrificed at various intervals for bacterial counts. Bacterial numbers in allantoic and amniotic fluids and in minced embryo were determined. Lots of 12 control eggs injected with saline and then with the challenge strain 2 days later and 12 eggs injected with the interfering strain and then with the challenge strain were sacrificed 24, 48, 72, 124, and 144 hours after injection of the challenge strain.

In vivo growth curves of challenge strain G in the presence of interfering strain P are shown in Figure 2. Each point represents average bacterial counts from a minimum of 12 eggs. Bacterial multiplication occurred promptly in allantoic fluid, but lagged behind that observed in control eggs for the first 48 hours after challenge. In control eggs, bacterial growth achieved a concentration of 10^9 bacteria per ml of allantoic fluid within 24 hours and persisted at this level. Eggs injected with challenge strain G alone all succumbed to infection within 96 hours, and no counts could be determined for a period beyond 72 hours after challenge. Growth of the challenge strain in allantoic fluid of eggs previously injected with interfering strain P was 10^6 bacteria per ml at 24 hours, 10^8 at 48 hours, and 10^9 at 72 hours. A similar pattern of delayed growth before achievement of maximal bacterial numbers also occurred in amniotic fluid. Despite the initial inhibition of bacterial growth of the challenge strain, inhibition was only temporary, and the numbers of bacteria present in allantoic and amniotic fluids reached numbers similar to those present in controls within 72 hours. In contrast, the numbers of the challenge strain in the embryos of eggs previously injected with interfering strain P never attained the magnitude observed in control eggs. The challenge strain achieved levels of from 10^4 to 10^5 per g of control embryo before death, whereas counts did not exceed 10^3 per g of embryo even 6 days after challenge in eggs previously infected with the interfering strain.

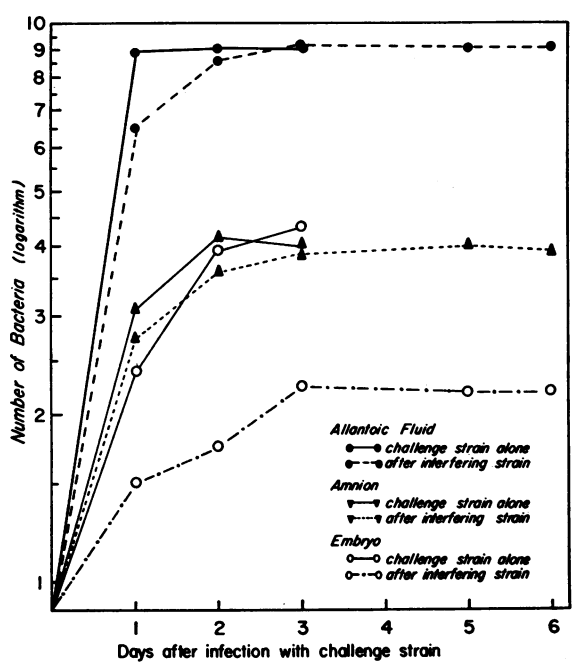

FIG. 2. GROWTH CURVES OF CHALLENGE STRAIN G IN ALLANTOIC AND AMNIOTIC FLUIDS AND MINCED EMBRYOS FROM CONTROL EGGS AND EGGS PREVIOUSLY INJECTED WITH INTERFERING STRAIN P. Each point represents the mean of counts from 12 eggs.

Similar studies were performed utilizing strain K as the interfering strain and OB as the challenge strain. These are illustrated in Figure 3. Results with these two strains differed from those observed with interfering strain P and challenge strain G, in that inhibition of growth of challenge strain OB persisted throughout the entire period of study. Strain OB could not be detected in amniotic fluid or embryonic tissue for periods as long as 6 days after its injection, and its maximal numbers in allantoic fluid failed to exceed 10^7 bacteria per ml. In both sets of studies, the interfering strain attained counts of 10^8 in the allantoic fluid within 24 hours and persisted at this level through the period of investigation.

Thus, two distinct mechanisms of interference appeared to be operative. One involved inhibition of growth of the challenge strain, which was more marked *in vivo* than *in vitro*. The second, exemplified by interfering strain P and challenge strain G, involved mechanisms other than inhibition of the growth of challenge strain G. In this type of interference, bacterial growth in allantoic and amniotic fluids was slightly delayed, but the same numbers of bacteria were achieved as proved lethal to controls. However, inhibition of bacterial growth in the embryo persisted for the entire period of observation. Studies with additional interfering and challenge strains demonstrated that both types of interference described above occurred with these additional strains.

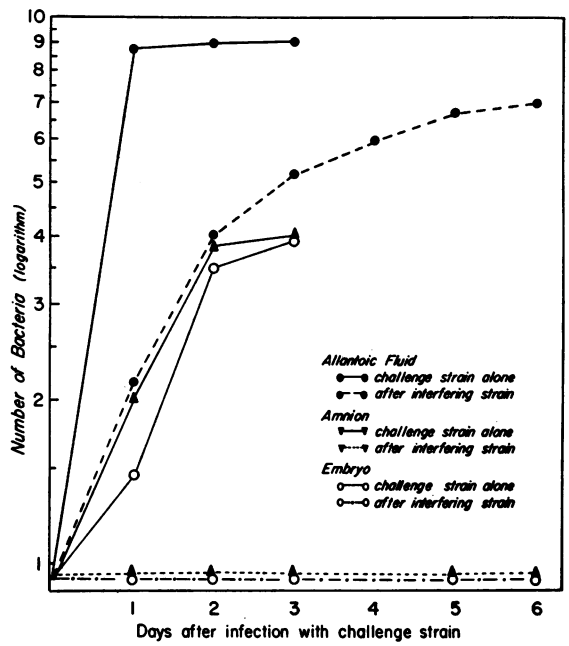

FIG. 3. GROWTH CURVES OF CHALLENGE STRAIN OB IN ALLANTOIC AND AMNIOTIC FLUIDS AND MINCED EMBRYOS FROM CONTROL EGGS AND EGGS PREVIOUSLY INJECTED WITH INTERFERING STRAIN K. Each point represents the mean of counts from 12 eggs.

Transfer of interference

Attempts were made to determine whether the protection afforded by prior infection with an interfering strain was transferable. Allantoic fluid was harvested from lots of 20 eggs at variable intervals after injection of the interfering strain, filtered through 0.45–μ Millipore filters, and injected into control eggs after an equivalent volume of allantoic fluid had been aspirated. No protection against challenge with virulent staphylococci could be detected, even with transfer of quantities of allantoic fluid as great as 5 ml.

Altered virulence of challenge strain

The possibility that attenuated virulence of the challenge strain after injection into eggs previously infected with an avirulent strain resulted from transduction, transformation, or conjugation of the challenge strain was evaluated. Determination of growth curves when challenge strain G was injected 2 days after interfering strain P revealed that after 4 days bacterial counts of the former in allantoic fluid (10^9) exceeded those of the interfering strain (10^8) by at least tenfold. By harvesting allantoic fluid 4 days after injection of the challenge strain, it was possible to dilute out the interfering strain and obtain an inoculum consisting solely of the challenge strain for determination of lethality in additional 10–day–old eggs. Controls consisted of eggs injected with saline and then with the challenge strain, harvested 4 days later, and diluted as above. Comparison of the lethality of allantoic fluid containing strain G from control eggs and eggs that had been injected with both the interfering strain and challenge strain G is shown in Table VI. Twenty-nine of the 30 (97%) ten–day–old embryos injected with 9 cfu of *S. aureus* grown alone in allantoic fluid died. The lethality with 8 cfu of the same strain from allantoic fluid that had previously been infected with interfering strain P was 28 of 30

TABLE VI

Virulence of challenge strain after mixed and single allantoic infection

	Deaths
S. aureus G (single strain in allantoic fluid)	29/30*
S. aureus G (mixed allantoic infection diluted to exclude strain P)	28/30

* Numbers of deaths/number of eggs challenged.

(93%). The failure to demonstrate significant differences in lethality of the challenge strain excluded the possibility that alterations in its virulence as a result of growth in the presence of the interfering strain explained the interference observed.

Bactericidal activity of embryonic blood

Bactericidal activity of whole blood from the embryo was determined in specimens obtained 3 days after allantoic injection of interfering strains P and K or 3 days after saline injection into control eggs. The bactericidal index (BI) was expressed as the bacterial count 3 hours after inoculation of the challenge strain divided by the bacterial count immediately after inoculation. Values in excess of 1.0 indicated bacterial multiplication, whereas values less than 1.0 indicated a bactericidal effect. The BI in blood specimens from control embryos in 38 experiments was 1.06 ± 0.23. Bactericidal activity, BI 0.85 ± 0.19, was significantly greater ($t_{38} = 3.06$; $p < 0.01$) in embryos previously infected allantoically with either interfering strain. Similar enhanced whole blood bactericidal activity was also demonstrable after injection of any of several interfering strains and when additional challenge strains were used as the test organism. All strains tested induced a similar slight but significant increase in the bactericidal activity of embryonic blood against all staphylococci tested as challenge strains.

Discussion

The demonstration that avirulent staphylococci interfere with the lethality of virulent staphylococcal infections in embryonated eggs suggested the use of this model for investigations of bacterial interference. Clinical observations which suggest that bacterial interference contributes to host defense mechanisms have been amply confirmed by demonstrations of the effectiveness of artificial colonization with avirulent bacteria in preventing staphylococcal infections (5, 14–16). The present studies parallel these clinical observations in several respects and provide a convenient tool for experimental studies of interference. Originally, it was thought that interference might be an isolated observation limited to only a few strains of staphylococci, but subsequent experiments indicated a broader significance. The interference induced by prior infection with avirulent staphylococci afforded significant protection, not only against lethal infection with virulent staphylococci, but also against a variety of other bacterial species as well as one virus. In addition, all strains of coagulase–positive and coagulase–negative staphylococci tested that were relatively avirulent for embryonated eggs were capable of inducing interference.

Induction of interference required viable bacteria as the interfering agent. Dead bacteria, even when injected in numbers similar to the maximal bacterial population attained by the interfering strain during *in vivo* growth, failed to afford protection against subsequent infection with challenge strains. The onset of the effects of the interfering strain was quite rapid. They were observed even when the challenge strain was injected minutes after the interfering strain. Achievement of optimal protection required 24 hours, a period sufficient for interfering strains to reach maximal numbers in allantoic fluid (10). These observations suggest that active multiplication of the interfering strain is necessary for the development of interference.

A variety of mechanisms have been shown to cause inhibition of one microorganism by another. These include antibiotic production (17), elaboration of nonantibiotic metabolites (18, 19), bacteriocins (20, 21), exhaustion of essential nutrients (22, 23), and interferon production by infected cells (6–8). None of these mechanisms satisfactorily explains the present observations, however. These experiments indicate that more than one mechanism is operative in the production of interference. Both antibiotic (24–27) and bacteriocin (28, 29) production have been demonstrated with strains of staphylococci. No antibiotic activity against the challenge strains

could be detected in allantoic fluid infected with interfering strains in these studies. We were also unable to demonstrate bacteriocin production with the interfering strains used, although Dr. A. I. Braude [8] found slight bacteriocin activity with interfering strain P against challenge strains OB and G. However, allantoic fluid harvested after infection *in vivo* with strain P supported the growth of these two challenge strains *in vitro* as well as uninfected allantoic fluid. Similarly, the challenge strains grown *in vivo* in allantoic and amniotic fluids in eggs previously infected with strain P attained the same numbers as in control eggs, although achievement of maximal population densities was slightly delayed. In contrast, strain K produced no bacteriocin, but allantoic fluid infected with strain K did inhibit growth of challenge strains *in vitro* and *in vivo*.

Other investigators have reported that nicotinamide depletion resulting from bacterial growth may inhibit multiplication of staphylococci (30, 31). Depletion of nicotinamide or other essential nutrients may explain the growth inhibition of challenge strains induced by strain K observed in the present studies and is presently under investigation. Mechanisms other than exhaustion of growth factors must also be invoked to explain all of the present findings. In addition to the failure to inhibit growth *in vitro* or *in vivo* with interfering strain P, it is unlikely that nutrient exhaustion would provide protection against intravenous challenge. Similarly, it would be unusual for exhaustion of a single growth factor to inhibit the lethality of such a diffuse variety of bacterial species or an obligate intracellular parasite, such as influenza virus. Since interference with lethality occurred despite growth of some challenge strains to numbers previously shown to be lethal (10) and was not attributable to alterations in the virulence of the challenge strain, enhancement of host defense mechanisms appeared likely. Evidence that interference induced by avirulent strains modified host defenses was established by the demonstration of enhanced blood bactericidal activity of the embryo. Increased blood bactericidal activity was observed after infection with all interfering strains, even those inhibiting growth of the challenge strain. Whether the enhanced bactericidal activity reflected alterations in leukocyte activity or opsonizing substances has yet to be determined. In addition, other host defense mechanisms may be affected. Embryonated eggs are generally considered to be relatively devoid of active host defense systems. Only small numbers of phagocytic leukocytes are present before hatching (32), and embryos possess little or no immunoglobulins (33). These and earlier studies have demonstrated that chick embryos are capable of surviving infections with coagulase–negative and some coagulase–positive staphylococci despite multiplication and invasion of these strains in the embryo. The failure of such strains to induce lethal infections indicates that the embryo possesses mechanisms capable of coping with some infectious agents. The present investigations demonstrate that whole blood of embryos is capable of inhibiting staphylococci and that interference further enhances this effect. Additional studies to elucidate further the effects of interference on cellular, humoral, and other specific defense mechanisms of embryonated eggs are in progress.

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