BACTERIAL INTERFERENCE IN EXPERIMENTAL BURNS*

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The phenomenon of bacterial interference has attracted renewed interest because of its evident significance in the epidemiology of human disease due to *Staphylococcus aureus*. Interference has been studied clinically as a factor in staphylococcal colonization of hospital and outpatient populations and has also been employed successfully in the control of outbreaks of staphylococcal infection (1–3). Yet the phenomenon is poorly understood and laboratory examination of its occurrence has been limited to the chick embryo model, where interference by coagulase-negative staphylococci with lethal *Staph. aureus* infection has been demonstrated (4, 5). The present studies of interference between strains of *Staph. aureus* in experimental burns in rabbits were undertaken to develop an animal model which might be useful in exploring the mechanism(s) underlying this phenomenon and to obtain observations of some of the qualitative, quantitative, and temporal factors in bacterial interference.

Materials and Methods

Experimental Burn Lesions.—Adult New Zealand rabbits (2.5–3 kg) were anesthetized with sodium pentobarbital (60 mg intravenously) and by ether inhalation and were burned on one or both shaved flanks by an apparatus modeled after that of Körlof (6), consisting of a copper cylinder (7.5 cm diameter and 10 cm height) which contained an electric coil in connection with a voltage regulator (Adjust-A-Volt, Staco, Inc., Standard Electrical Products Div., Dayton, Ohio). The cylinder was filled with distilled water, heated to a temperature of 90°C, and was applied to the anesthetized animal for 30 sec.

Staphylococcal Strains.—Only coagulase-positive staphylococci were employed. Strain 502A, provided by Dr. Henry Shinefield, is lysed by several group III phages, most consistently in our experience by type 7, and is sensitive to most antibiotics including penicillin, (<0.1 unit/ml) but relatively resistant to tetracycline. Strain Q461 was recovered from an adult female with osteomyelitis, is phage type 80/81, and is highly resistant to most antibiotics including penicillin (>100 units/ml) and streptomycin (>100 γ/ml). Strain Q481

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was isolated from the furuncle of a hospital laboratory worker, is type 29/52, and is penicillin-sensitive (<0.1 unit/ml). Strain Q451 was recovered from an adult male with chronic spinal osteomyelitis, is type 3C/55/71, and is penicillin-sensitive (<0.1 unit/ml). Strain K417 was recovered concomitantly with Group A streptococci from the throat of a child with pharyngitis, is type 3A/3B/3C, and is penicillin-sensitive (<0.1 unit/ml).

Cultures.—Staphylococci for animal inoculation were prepared from stocks of frozen, exponential phase broth cultures (Trypticase soy broth, Baltimore Biological Laboratory, Baltimore, Md.); 0.5 ml of the thawed stock culture was inoculated into 50 ml of Todd-Hewitt broth (Difco Laboratories Inc., Detroit, Mich.), incubated at 37°C for 18 hr, harvested, washed in 0.85% sterile saline, and resuspended in saline. The concentration was adjusted to approximately 10^9 colony-forming units (C.F.U.) per ml, determined by standardized optical density readings in a Coleman Jr. spectrophotometer (Coleman Instruments, Inc., Maywood, Ill.) at 620 μm. Further dilutions were made in saline to the desired concentration for animal inoculation. 1 ml of staphylococcal suspension was dropped on the burn with a syringe and small caliber hypodermic needle and spread with a sterile applicator. This procedure was followed for both initial colonization and for "challenge" of previously colonized lesions.

Each burn lesion was cultured at 48-hr intervals (or more frequently) with a broth-saturated applicator which was applied to both a blood agar plate (6% sheep blood in Tryptose blood agar base, Difco), and a similar plate also containing 0.5 units per ml of penicillin G. Preliminary experiments indicated that the penicillin-containing plates completely inhibited growth of the penicillin-sensitive strains employed but permitted growth of a penicillin-resistant strain, Q461, even in very small inocula (1–10 C.F.U.). Several staphylococcal colonies (usually four per plate) were selected for phage-typing by standard methods (7). Initially, the full set of 22 phages provided by the Communicable Disease Center, Atlanta, Ga., was applied to all isolates; subsequently, selected phages were used and the full set was employed only when an isolate failed to type with these.

Enumeration of Staphylococci in Broth Cultures.—Colony counts were performed by standard methods, inoculating duplicate and triplicate pour plates of Trypticase soy agar (Baltimore Biological Laboratory) from 10-fold dilutions in saline of broth cultures. Plates were incubated at 37°C and were counted when the colonies had attained sufficient size, usually after 48 hr. For enumeration of the respective counts of strain 502A or strain K417 and strain Q461 in mixed culture, a second series of pour plates was used, containing Trypticase soy agar and 0.5 unit/ml of penicillin G. Thus the count in antibiotic-free plates represented total staphylococci, the count in penicillin plates represented penicillin-resistant staphylococci (strain Q461) and the difference represented penicillin-sensitive staphylococci (strain 502A or K417). In preliminary experiments, varying known quantities of strains 502A and Q461 were mixed together and counted by this technique; the results indicated this to be a reliable procedure.

Enumeration of Staphylococci in Lesions.—For a given count, three separate biopsies of the burn lesion were obtained with sterile, 6 mm Keyes skin punches (V. Mueller Surgical Products, Inc. Chicago, Ill.). Each tissue plug was weighed in a sterile test tube on a microbalance (Mettler Instrument Corp., Princeton, N.J.) suspended in 2 ml of saline and homogenized with a Potter-Elvehjem tissue grinder (A.S. Aloe Co., St. Louis, Mo.), all under aseptic conditions. The homogenate was checked for purity on blood agar, and colony counts were performed on the homogenate. When more than 10% of the colonies on a blood plate were not staphylococci, counts from that particular preparation were excluded from the experimental analysis.

Tests for Production of Bacterial Inhibitors by Staphylococci in Cultures.—The staphylococcal strains under study were examined for inhibition of other staphylococci on solid media by methods described by Parker and Simmons (8). Their modification of the Fredericq technique detects "deferred antagonism" by a strain grown on agar from which the growth is scraped

away and the plates sterilized with chloroform; other strains are cross-streaked to detect inhibitors released into the agar by the first strain. In addition, Barrow's test for "simultaneous inhibition" (9) was employed because of its simplicity and sensitivity; the "passive" strain was streaked onto Trypticase soy agar or blood agar from an overnight broth culture and after drying, the streak was stabbed with a wire loaded with the "active" strains grown on agar. Supernates of overnight broth cultures (100 ml amounts) were also examined for antistaphylococcal activity after centrifugation, filtration through a membrane filter with average pore diameter of 0.45 μ (Millipore Filter Corp., Bedford, Mass.), and dialysis in cellulose bags (Union Carbide Corp., Visking Div., Chicago, Ill.) at 4°C against two 500 ml changes of fresh broth. For these purposes, a completely dialyzable medium was employed (10). The possibility that an inhibitor of low molecular weight was produced but removed by dialysis was examined by dialyzing 100 ml of culture filtrate against a large volume (500 ml × 2) of fresh medium diluted 1:10. The latter was then concentrated to approximately 100 ml by pervaporation in cellulose bags, the pH readjusted to 7.8, and the concentrate examined for its capacity to support staphylococcal growth.

Tests for Production of Bacterial Inhibitors In Vivo.—Homogenates of burn lesions colonized with strain 502A were streaked on Trypticase soy agar and cross-streaked with Q461 staphylococci; penicillin (10 μ/ml) and streptomycin (20 γ/ml) were incorporated into these plates to prevent overgrowth of contaminants. Similar homogenates were introduced into wells of antibiotic plates seeded with a lawn of Q461 from a 4-hr broth culture.

To test the possibility of an inhibitor for Q461 produced in vivo but active only in the presence of multiplying 502A organisms, agar plates without antibiotics were poured in successive layers; the deeper layer was poured with approximately 10^9 C.F.U./ml of strain 502A and the upper layer was seeded with a lawn of strain Q461. Wells were cut through both layers and filled with tissue homogenate from animals colonized with strain 502A.

Passive Transfer Experiments.—For collection of serum for passive transfer, burned and infected animals were sacrificed with 300 mg of intravenous pentobarbital and immediately exsanguinated through the heart. Tissue homogenate was prepared from sacrificed animals by immediate excision of the infected burn through the subcutaneous plane; small pieces of tissue were put through a hand-operated tissue press with pores of approximately 1.5 mm diameter and the product was then homogenized with the Potter-Elvehjem apparatus. The homogenate was centrifuged for 15 min at 1500 rpm to remove chunks of tissue visible in the gross. Sterilization of the resultant suspension was performed by filtration (sintered glass) or by incubation for 2 hr at 37°C with 50 μnits/ml of penicillin G and 50 γ/ml of streptomycin and was confirmed by subsequent inoculation on blood agar plates.

RESULTS

Gross and Histological Appearance of Burn Lesions.—The circular burned areas which averaged 7.5 cm in diameter exhibited immediate, well-demarcated blanching, followed by eschar formation and occasionally by sloughing at 7–10 days. These were examined at 24 hr by full excision or punch biopsy and sections were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin, periodic acid–Schiff, and Gram stains. Normal rabbit skin was similarly processed for comparison. In burned but uninfected lesions, the epithelium was intact but the keratin layer was condensed and dark-staining. In all layers of the epidermis the nuclei were pyknotic and there was loss of cytoplasmic detail and margins. The superficial collagen of the dermis and, to a lesser extent, the deep collagen were light-staining and the bundles were

fragmented and indistinct. These were interpreted as changes of early necrosis. Infected lesions were not markedly different, with variable (often absent) subepithelial infiltration of polymorphonuclear leukocytes in moderate numbers. Microabscesses were rarely seen. No appreciable difference was observed between lesions infected with strains 502A and Q461. In some specimens, scattered Gram-positive cocci were seen in the epithelial and subepithelial layers; they were often absent and were never seen in clusters.

Consistency of Colonization of Burn Lesions by Staphylococci.—The ability of the two principal strains studied (502A and Q461) to colonize fresh burn lesions in various inocula is summarized in Table I. Colonization (persistence as the predominant organism for at least 7 days) was infrequent with numbers less

TABLE I

Frequency of Colonization of Burned Rabbits Achieved with Various Inocula of Strains 502A and Q461

Strain	Inoculum size	No. of rabbits inoculated	No. of rabbits colonized
	C.F.U.		
502A	$<10^4$	17	0
	10^4	6	1
	10^5	6	6
	10^6	10	8
Q461	$<10^4$	3	0
	10^4	6	3
	10^5	6	5
	10^6	10	10

than 10^5 C.F.U. and occurred with consistency at 10^5 C.F.U. and greater. There was no apparent difference in the inherent capacity of either strain to colonize lesions. Additional experiments indicated that 10^6 C.F.U. of either strain colonized most lesions when inoculated at 24 and 48 hr. In subsequent experiments, unless otherwise noted, an inoculum of 10^6 C.F.U. was selected for both initial colonization and for "challenge."

Interference by Strain 502A with Artificial Colonization by Strain Q461.—When freshly burned rabbits were colonized with strain 502A and the lesions were inoculated 24 or 48 hr later with strain Q461 (10^6 C.F.U.), the latter organisms, although present transiently in small numbers in some animals, were ultimately eliminated from most lesions. Fig. 1 summarizes a representative experiment of this kind. All nine animals satisfy the definition of interference as the term is used in this paper: the failure of the challenge organism to appear consistently, even in small numbers, in serial cultures of the lesions (on selective and unselective media). The ability of strain 502A to prevent infection in this man-

ner by strain Q461 was examined in 20 rabbits and in 18 of these interference occurred (Table II, line 1).

Interference by Strain 502A with Natural Cross-Infection.—To determine the frequency of spontaneous cross-infection between animals by strain Q461, pairs of freshly burned rabbits—one member of each pair inoculated with strain Q461 and the other with sterile saline—were placed in single cages and followed by serial cultures. Cross-infection of the saline-inoculated animal by strain Q461 occurred in 8 of 11 pairs (Fig. 2, left panel). All three of the saline-inocu-

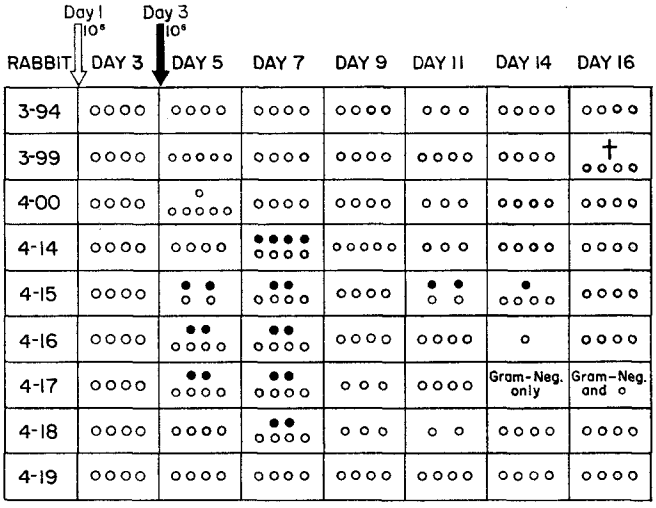

FIG. 1. Bacterial interference in an experiment involving nine rabbits. The open arrow indicates inoculation of 10^8 C.F.U. of strain 502A in all animals; the solid arrow indicates inoculation of an equal quantity of strain Q461. Each open circle indicates a colony of 502A isolated from a lesion and each solid circle indicates a colony of Q461.

TABLE II

Interference Produced by Various Staphylococcal Strains

Colonizing strain*	Challenge strain	Frequency of interference‡
502A	Q461	18/20
Q461 (80/81)	502A	13/19
Q481 (29/52)	Q461	11/12
Q451 (3C/55/71)	Q461	5/11
K417 (3A/3B/3C)	Q461	14/18

* Numbers in parenthesis indicate phage types.

‡ The denominator indicates the number of animals successfully infected by an "interfering" strain and subsequently inoculated with a "challenge" strain. The numerator indicates the number of animals in which interference occurred as defined in the text.

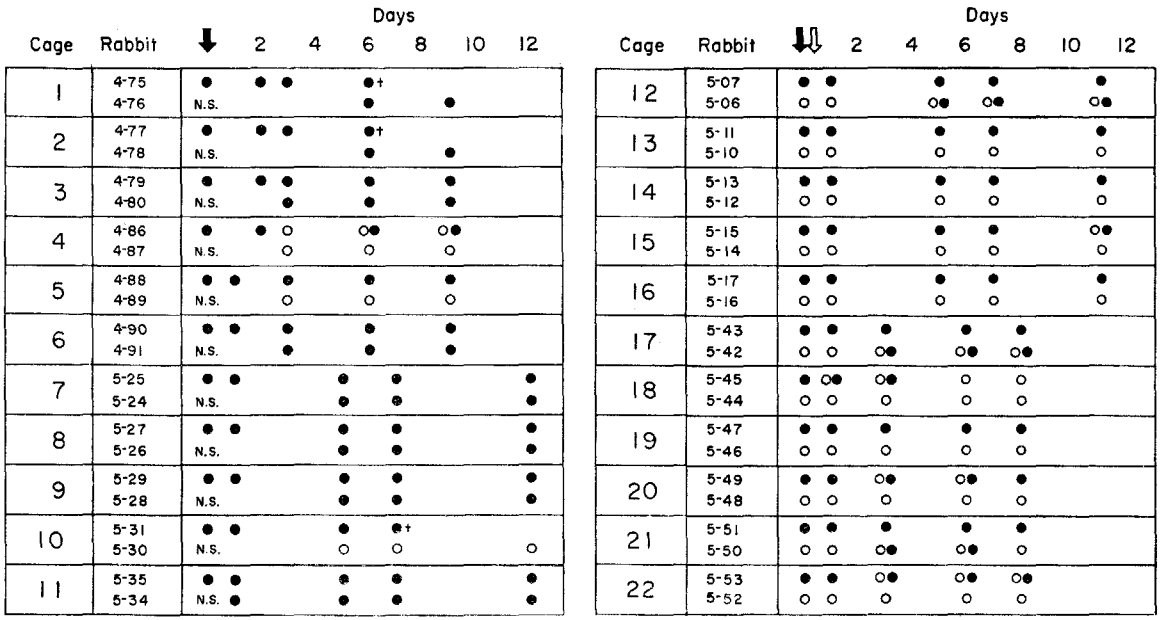

FIG. 2. Cross-infection between pairs of rabbits in single cages. On the left one animal received strain Q461 (solid arrow) and the other was inoculated with sterile saline (N.S.). On the right one animal received strain Q461 (solid arrow) and the other 502A (open arrow). Multiple colonies were identified from all cultures; solid circles indicate presence of Q461 and open circles presence of 502A.

lated animals which failed to acquire strain Q461 (cages 4, 5, 10) proved to be colonized by a staphylococcus with the phage-type and antibiotic sensitivity of strain 502A, presumably the result of cross-infection from other animals housed

TABLE III

Failure of Interference to Occur between Lesions on Opposite Flanks

Rabbit	Flank	Inoculum Day 1	Culture Day 2	Culture Day 3	Inoculum Day 3	Culture Days 4–5	Culture Day 7	Culture Day 9
3–58	Right	502A (10^6)*	502A (4)*	502A (3)	—	502A (4)	502A (4)	502A (4)
	Left	—	0	0	Q461 (10^6)	Q461 (8)	Q461 (8)	Q461 (8)
3–93	Right	502A (10^6)	502A (2)	502A (4)	—	502A (4)	502A (4)	502A (4)
	Left	—	—	—	Q461 (10^6)	Q461 (8)	Q461 (8)	Q461 (8)
7–86	Right	502A (10^6)	4+‡	4+	—	502A (3)	4+	502A (2)
	Left	—	0	0	Q461 (10^6)	Q461 (3)	4+	Q461 (2)
7–87	Right	502A (10^6)	4+	4+	—	—	4+	502A (2)
	Left	—	1+	0	Q461 (10^6)	Q461 (3)	4+	Q461 (2)
7–90	Right	502A (10^6)	4+	4+	—	502A (3)	4+	502A (2)
	Left	—	1+	1+	Q461 (10^6)	Q461 (3)	4+	Q461 (2)
7–91	Right	502A $(10)^6$	4+	4+	—	502A (3)	4+	502A (2)
	Left	—	1+	0	Q461 (10^6)	Q461 (3)	4+	Q461 (2)
7–92	Right	502A (10^6)	4+	4+	—	502A (3)	4+	502A (1)
	Left	—	0	0	Q461 (10^6)	502A (2)	4+	502A (1)
						Q461 (1)		Q461 (1)
7–93	Right	502A (10^6)	4+	4+	—	502A (3)	4+	502A (2)
	Left		0	0	Q461 (10^6)	502A (1)	4+	Q461 (2)
						Q461 (2)		

* Numbers in parentheses refer to inoculum size and to number of colonies identified by phage-typing or antibiotic sensitivity.

‡ Grading (0 to 4+) refers to presence of *Staph. aureus*, not identified by typing or sensitivity.

in nearby cages. The influence of 502A colonization on cross-infection with strain Q461 was examined in paired rabbits in which one member of each pair was colonized with strain 502A and the other with strain Q461 (Fig. 2, right panel). In contrast to the high rate of infection of saline-inoculated animals, cross-infection with strain Q461 occurred in only 3 of 11 pairs (cages 12, 17, 21).

Strain Specificity of Interference.—The experiments summarized in Table II were designed to determine whether strain 502A demonstrated a unique or

superior capacity over other strains to block staphylococcal superinfection. Lesions were first infected with strain Q461 and subsequently inoculated with strain 502A in the standard quantity of 10^6 C.F.U. As shown in the table, colonization was prevented in 13 of 19 animals. When unrelated strains of *Staph. aureus* (Q481, Q451, and K417) were used to colonize burn lesions, subsequent superinfection by strain Q461 (10^6 C.F.U.) failed to occur in a significant number of animals (Table II).

Examination for Interference in a Second, Uninfected Lesion.—When rabbits burned on both flanks were colonized on only one flank with strain 502A, there was no interference with colonization by Q461 when the latter was inoculated 48 hr later into the opposite flank (Table III). There was minor cross-infection of the uninoculated flank in two animals (7–92 and 7–93) but this evidently did not attain a sufficient degree of colonization to prevent infection by strain Q461.

TABLE IV

Frequency of Bacterial Interference with Varying Intervals between Inoculation of "Interfering" and That of "Challenge" Strain

Interval between 502A and Q461 inoculation	Frequency of interference*
hr	
Simultaneous	2/8
3	1/10
6	2/12
9	5/5

* See explanatory note for Table II.

Critical Time Requirement for Interference.—To determine the minimal time interval between the inoculation of strain 502A and the challenge with strain Q461 required for interference, the experiments summarized in Table IV were conducted. Both strains usually persisted when inoculated simultaneously (6 of 8 animals). Strain 502A did not interfere consistently with strain Q461 when inoculated 3 and 6 hr before challenge. However, with an interval of 9 hr, interference occurred in 5 of 5 animals and, as shown in earlier experiments (Fig. 1, Table II), interference was consistent at longer intervals (24–48 hr). Although interference could be demonstrated after an interval of 5 days, the maximum interval permitting interference was not determined.

Examination of In Vivo Rates of Growth.—In vivo growth curves were examined for two strains. In five experiments with strain 502A and two with strain Q461, the findings were essentially as shown in Fig. 3. Both strains 502A (Fig. 3 *a*) and Q461 (Fig. 3 *b*) demonstrated rapid multiplication in the initial 24 hr after inoculation. Generally, samples obtained in the first 2–3 hr showed counts of 10^1–10^2 C.F.U./mg tissue. Counts at 3–4 hr varied between 10^1–10^4

C.F.U./mg tissue and, at 24 hr, reached a level of 10^5–10^7 C.F.U./mg. Similar colony counts were obtained for the duration of the experiments (5–10 days).

Rates of in vivo growth of staphylococci were also determined under the conditions of bacterial interference, i.e., the sequential inoculation of strains 502A and Q461. In three experiments, when strain Q461 in the usual dose of 10^6 C.F.U. was inoculated into a lesion already colonized by 502A to a level of 10^5–10^7 C.F.U./mg tissue, strain Q461 was not detected subsequently in pour plates containing a selective concentration of penicillin, indicating failure of

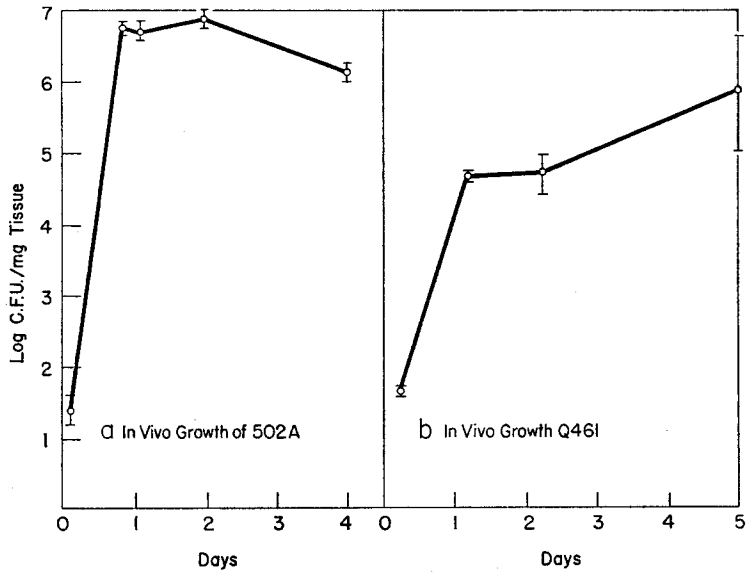

FIG. 3. In vivo growth of strain 502A (*a*) and of strain Q461 (*b*) inoculated at zero time in fresh burn lesions. The points indicate the means and the bars the ranges of counts.

multiplication of the blocked organism. Such an experiment is illustrated in Fig. 4.

Failure of Interference by Heat-Killed Staphylococci.—A saline suspension of 502A staphylococci was killed by heating at 65°C for 30 min and the numerical equivalent of 10^6 C.F.U. was inoculated into each of six fresh burn lesions (Table V). 24 hr later, 10^6 C.F.U. of viable Q461 organisms were inoculated into each lesion with colonization of all six animals, indicating lack of interference following inoculation of killed 502A. Since there was reason to suspect elimination of the killed organisms and since multiplication of the interfering strain appeared to be a prerequisite for interference, larger numbers (equivalent of 10^7–10^{10} C.F.U.) of heat-killed 502A organisms were inoculated simultaneously with 10^6

C.F.U. of living strain Q461. Again, colonization by the latter strain was not prevented (Table V).

Effect of Selective Antibiotic Treatment on Interference.—To determine if interference could be altered by administering an antibiotic specifically antagonistic to the interfering strain, animals colonized with penicillin-sensitive 502A were challenged 24 hr later with the resistant strain Q461; simultaneously, a single

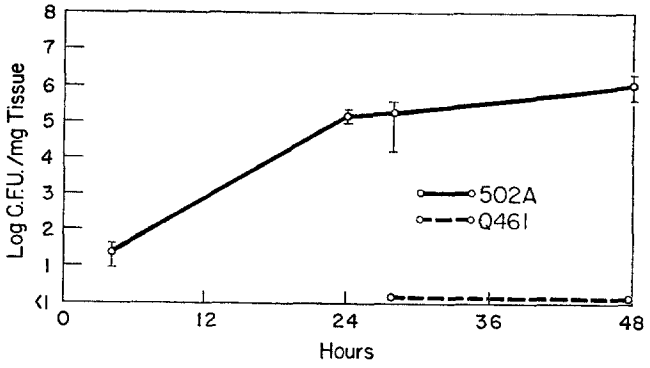

FIG. 4. In vivo growth of strain 502A (solid line) inoculated at zero time and strain Q461 (broken line) inoculated at 24 hr in the same lesion.

TABLE V

Inability of Heat-Killed Staphylococci (502A) to Prevent Colonization (Q461)

Inoculum of killed 502A	Time of Q461 challenge	Frequency of interference*
10^6	24 hr later	0/6
10^7–10^{10}	Simultaneous	0/8
Total		0/14

* See explanatory note for Table II.

dose of procaine penicillin G, 30,000 units, was injected intramuscularly. 14 rabbits were treated in this manner and interference occurred in only 2 of the 14.

Examination for Bacterial Inhibitor Produced In Vitro.—Of the five staphylococcal strains employed in the animal interference experiments, only K417 produced a zone of inhibition when cross-streaked with the other strains or tested against them by the stab method or the chloroform technique. These five strains were also tested against numerous other strains of *Staph. aureus*; only K417 showed antistaphylococcal activity.

The inhibitor produced by strain K417 proved to be active against all staphy-

lococcal strains tested (except those which were also inhibitory), against *Sarcina lutea*,[1] and against strain C51, a nontoxigenic strain of *Corynebacterium diphtheriae* provided by Dr. M. T. Parker. Thus, strain K417 resembles the inhibitor-producing staphylococci of type 71 and related phage patterns which are especially prevalent in impetigo lesions (8).

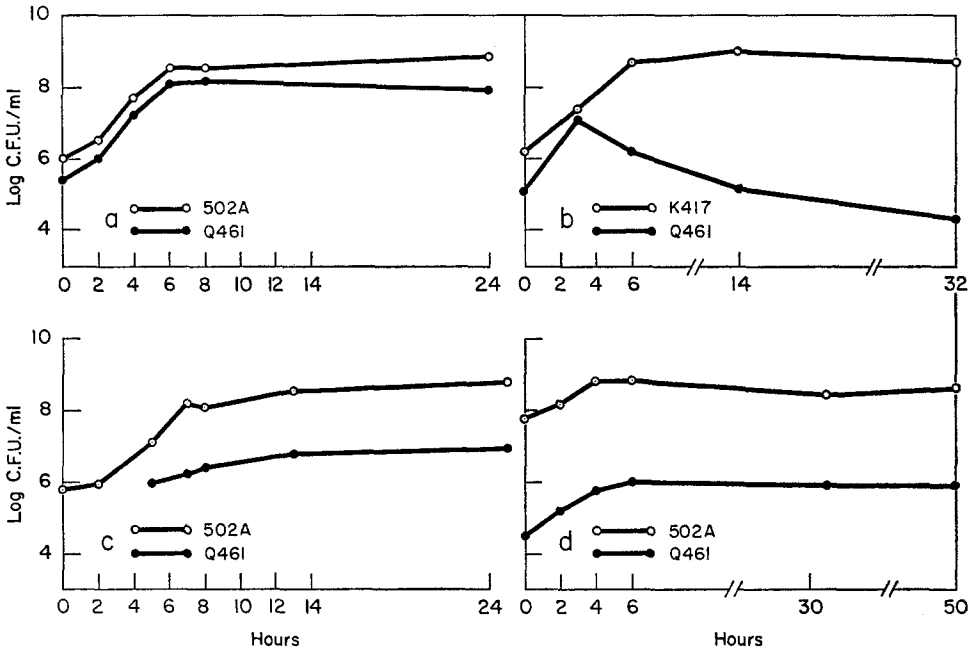

FIG. 5. In vitro growth of staphylococcal strains in mixed broth cultures. Simultaneous inoculation of strains 502A and Q461 (*a*) and of strains K417 and Q461 (*b*). In part (*c*) strain Q461 was inoculated 5 hr after strain 502A and part (*d*) strain 502A was inoculated in a quantity approximately 3½ logs greater than strain Q461.

Strains 502A and Q461 were also examined for mutual inhibition in broth cultures. When both organisms were inoculated in similar quantities into 25 ml of Todd-Hewitt medium, rates of growth were parallel (Fig. 5 *a*). In contrast, when strains K417 and Q461 were inoculated together in broth, there was a phase of parallel and exponential growth followed by a sharp decline in the population of viable Q461 organisms, presumably caused by elaboration of the K417 inhibitor (Fig. 5 *b*).

Growth of strain Q461 in mixed broth cultures occurred even when strain 502A was inoculated 4 hr earlier (Fig. 5 *c*) or simultaneously but in an excess of

[1] Quie, P. G. Unpublished observation.

3 log (Fig. 5*d*). These temporal and numerical relationships were selected to resemble those in vivo when interference occurred.

When Q461 staphylococci were inoculated into the sterile filtrate of an 18 hr broth culture (dialyzable medium) of strain 502A, multiplication did not occur (Fig. 6 *a*). That this was the result of nutritional exhaustion of the medium and/or increased hydrogen ion concentration seemed likely since dialysis (100 ml of filtrate) against fresh medium (500 ml × 2) was found to restore the capacity of the filtrate to support growth of Q461 (Fig. 6 *a*). An alternative possibility of an inhibitor of low molecular weight, removed by the above dialysis, was excluded by the experiment represented in Fig. 6 *b*. Dialysis of 502A culture filtrate against a 1:10 dilution of fresh medium was followed by pervaporation concen-

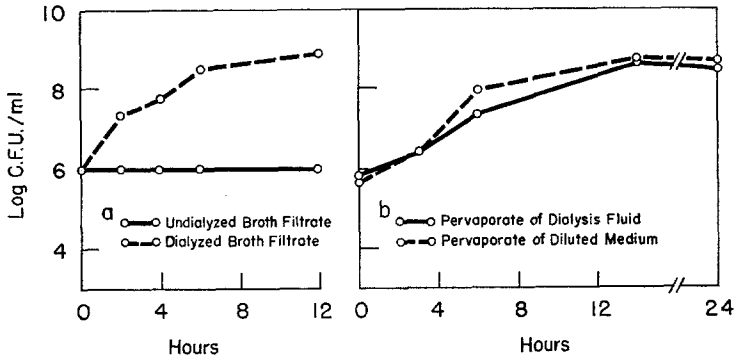

FIG. 6. Growth of strain Q461 in dialyzed and undialyzed broth filtrate of strain 502A (*a*). Q461 (*b*) was grown in the concentrate of fluid used for dialysis of the 502A broth filtrate and in broth which was concentrated in the same manner. (In these experiments, a completely dialyzable medium was used.)

tration of the dialysis fluid to approximate the original concentration. This latter material was found to support the growth of strain Q461. For comparison. Fig. 6 *b* also shows the growth rate of Q461 in diluted and pervaporated medium.

Examination for Bacterial Inhibitor Produced In Vivo.—Strain Q461 was not inhibited when crossed through a streak of tissue homogenate from animals colonized with strain 502A. Neither was there inhibition of a Q461 lawn around wells containing similar homogenate, both with and without a heavy growth of strain 502A in a deeper layer of agar.

Tests for Interference by Passively Transfered, Bacteria-Free Material from Infected Lesion—When serum collected from animals whose lesions were infected with strain 502A was injected in 25 ml amounts directly into the burn lesion (two recipient animals) and intravenously (two recipient animals), there was no interference with colonization when the recipients were challenged 24 hr later with 10^6 C.F.U. of strain Q461. Similarly, when the tissue homogenate of excised

lesions which had been colonized with strain 502A was infiltrated into fresh burn lesions, there was no interference with infection of these lesions 24 hr later by strain Q461. For the latter experiments, the homogenates from two donor animals were combined for injection into one recipient. In two experiments the homogenates were sterilized by filtration through a sintered glass filter and in three experiments sterilization was accomplished by incubation in the presence of penicillin and streptomycin. Only those experiments with sterile homogenates were accepted for analysis; when tissue homogenates were still infected with strain 502A, the presence of viable staphylococci might be expected to interfere with colonization of recipient animals by strain Q461.

In eight of the nine experiments, interference did not occur. Thus with the methods employed, no evidence was obtained that bacterial interference could be passively transferred.

TABLE VI

Effect of Increasing Inocula of Challenge Strain in Overcoming Interference Effect in Rabbits Colonized with 502A

Inoculum of Q461 challenge	Frequency of interference*
10^6	18/20
5×10^6	10/12
10^7	7/13
10^8	1/6

* See explanatory note for Table II.

Effect of Challenging with Large Quantities of Staphylococci Grown In Vitro.—When animals colonized by strain 502A received a challenge of $1\text{–}5 \times 10^6$ C.F.U. of strain Q461, interference with superinfection by the latter organisms was consistent (Table VI). However, with challenge inocula of the order of 10^7 C.F.U., the rate of interference appeared to be less (7 of 13 animals). With challenge doses of 10^8 C.F.U., interference was unusual (Table VI).

Effect of Challenging with In Vivo-Grown Staphylococci and Comparison with Effect of In Vitro-Grown Staphylococci in Similar Inocula.—Preliminary experiments indicated that when Q461 staphylococci were transferred directly (with saline-saturated applicators) from infected animals to lesions of animals colonized with strain 502A, superinfection by strain Q461 was the rule. To determin if this was simply the result of a large inoculum of Q461 organisms, staphylococci from animals infected 24 hr previously were first suspended in saline (by placing an applicator in 2 ml of sterile saline and agitating for 2 min) and enumerated by colony counts; various dilutions of this suspension were then used for inoculation of lesions colonized by strain 502A. Table VII summarizes these experiments in the right column; the results with various inocula of staph-

ylococci grown overnight in broth and diluted in saline are depicted in the center column. These data suggest that in vivo–grown staphylococci were capable of overcoming the interference barrier in significantly smaller quantities than were staphylococci grown in vitro. For example, with inocula of 10^6 C.F.U. ($\pm$ 0.5 log), Q461 organisms grown in vivo fairly regularly superinfected lesions colonized by strain 502A. However, when Q461 was grown in vitro this did not occur with regularity below an inoculum of 10^8 ($\pm$ 0.5 log).

To examine the possibility that this difference was the result of greater clumping of staphylococci collected from lesions than those grown in broth, the numbers of clusters per 100 cocci and the sizes of clusters in the saline suspensions of in vivo and in vitro-grown organisms were quantitated by examining Gram-stained smears of the suspensions. No difference in the tendency to clumping was observed between staphyloccci grown in vivo and in vitro. Thus,

TABLE VII

Frequency of Interference with Challenge Staphylococci Grown In Vitro and In Vivo*

Inoculum size	In vitro-grown	In vivo-grown
10^5 and less	—	17/18
10^6 ($\pm$ ½ log)	23/26	2/11
10^7 ($\pm$ ½ log)	12/19	1/8
10^8 ($\pm$ ½ log)	1/6	0/3

* See explanatory note for Table II.

in vivo–grown Q461 organisms appeared capable of superinfecting lesions colonized by strain 502A in an inoculum significantly smaller (1–2 logs) than broth-grown staphylococci of the same strain.

DISCUSSION

The enhanced ability of the human or animal host, infected with one bacterium, to resist superinfection by related or unrelated microorganisms, is an observation of long standing (11). However, the greatest recent interest in this phenomenon has concerned interference between competing strains of *Staphylococcus aureus*, stimulated primarily by the definitive clinical studies of Shinefield and his associates. These workers demonstrated that the artificial nasal and umbilical colonization of newborn infants (1) and, in later studies, that the nasal colonization of adults (2) by a clinically avirulent *Staph. aureus* (strain 502A) prevented subsequent infection by other epidemic and endemic staphylococcal strains.

The model described in this report appears to offer certain advantages for the study of bacterial interference between strains of *Staph. aureus*. The full

thickness, burn lesion could be consistently colonized by a uniform inoculum of each of several staphylococcal strains examined. Interference with the subsequent establishment of a second strain was a reproducible occurrence, not only under the highly artificial conditions of "direct challenge," but also under the conditions of spontaneous cross-infection between rabbits. In addition, all inoculations took place on the surface of a lesion in an intact animal. For these reasons, the observations described here may have direct relevance to the epidemiology of human staphylococcal disease.

The clinical evidence that staphylococcal strains other than 502A possess the capacity for interference (2) is clearly borne out in the experiments cited here. Indeed, each of the five strains of *Staph. aureus* examined successfully prevented subsequent colonization by a second strain. From this study and from clinical reports, it seems reasonable to predict that any coagulase-positive staphylococcus is capable of interfering with the subsequent colonization by other staphylococcal strains.

The fact that the capacity for interference is clearly not restricted to staphylococci such as strain K417 examined in this study, which are known to produce inhibitors for other staphylococcal strains, constitutes prima facie evidence that the production of such inhibitors is probably not responsible for the interference effect. We found no experimental evidence that strain 502A elaborates antistaphylococcal factors in vitro or in vivo, when examined by several methods on agar and in broth.

Since in vitro interference could not be demonstrated by such a mechanism as the elaboration of inhibitory factors by staphylococci, the active participation of antibacterial defenses of the host animal seemed an attractive possibility. However, from the inconsistent appearance of inflammatory cell infiltrate in the lesion no evidence was obtained that host phagocytic cells play a major role. In addition, several attempts to passively transfer a host factor with the serum and with homogenates of infected lesions were almost uniformly negative. Moreover, the immediate effect of antibiotic therapy in eliminating interference by an antibiotic-sensitive strain even after 24 hr of colonization is not indicative of an important role for host factors in this phenomenon.

When one lesion was colonized by an interfering staphylococcus (strain 502A), interference did not occur when the challenge organism (strain Q461) was inoculated subsequently in a second, uninfected lesion. This finding suggests that whether the basic mechanism of interference involves host or bacterial factors, or both, these factors are operative only in the immediate site of colonization. This observation is also consistent with the inability to transfer interference passively by the local or intravenous injection of a burned, uninfected animal with serum from an animal colonized by strain 502A.

The presence or absence of bacterial multiplication as well as the actual numbers of staphylococci involved appear to play critical roles in bacterial

interference, at least in this model, as indicated by the consistent growth of the interfering and the failure of detectable multiplication by the blocked, challenge strain. In addition, it is likely that the minimal time of colonization by the interfering strain which must lapse before interference occurs is probably related to the achievement of a critical bacterial density by the interfering strain. The importance of multiplication by the interfering strain is also suggested by the effectiveness of prior antibiotic therapy in abolishing interference, both in this study and in clinical experiences (2), and by the lack of interference by heat-killed staphylococci, both in this and in another laboratory model (4). Finally, these studies stress the significance of the actual quantities of staphylococci present in lesions, since the interference barrier could be overcome by larger inocula of the challenge staphylococcus.

Bacterial interference has been observed in another experimental model, that of embryonated chicken eggs infected with staphylococci (4, 5). Examination of this model has suggested that the interfering coagulase-negative strain inhibited the growth of *Staph. aureus* by the utilization of nicotinamide (12) or of certain amino acids (13). However, in vivo interference has not been prevented by supplying these substances to the eggs.[2] It is possible that depletion of essential growth factors by the interfering strain may be responsible for in vivo interference in experimental burns. The in vitro experiments where strain Q461 was grown in filtrates and dialysates of 502A broth cultures were designed to rule out the production of dialyzable and nondialyzable staphylococcal inhibitors, and exhaustion of nutrients and growth factors was not specifically evaluated.

In the animal model described in this report, staphylococci (strain Q461) which had grown in vivo were found to superinfect a colonized lesion (strain 502A) in significantly smaller inocula than in vitro–grown Q461 organisms. Whether this is a qualitative advantage conferred on the organism by the environment of the host or whether it is result of differences in the growth phase of in vivo–grown and in vitro–grown staphylococci is not clear. However, the consistency of interference in clinical situations and in the present studies of spontaneous cross-infection in animal cages suggests that such natural "challenges" with in vivo–grown staphylococci occur in relatively small inocula and that the quantitative superiority of interfering staphylococci may be of greater significance in the mechanism of this phenomenon.

SUMMARY

A standardized, full thickness, dermal burn in rabbits was used to study interference between strains of *Staph. aureus* inoculated on the wound surface. Several strains appeared equally capable of colonizing lesions and of preventing

[2] Ribble, J. C. Personal communication.

superinfection by other staphylococci inoculated at a later time. In addition, cross-infection between rabbits colonized by different strains (502A and Q461) and placed together in cages was prevented, presumably by the same mechanism. Interference appeared to be a strictly local phenomenon, since it did not occur when an animal was colonized by strain 502A at one burn site and subsequently challenged with strain Q461 at a separate lesion.

For interference to occur, a minimal time interval (9 hr) was required between inoculation of the interfering strain and inoculation of the challenge strain. In vivo growth rates indicated rapid growth in the first 24 hr by the interfering strain but no detectable multiplication by the challenge strain. Heat-killed staphylococci, even in large numbers, were incapable of producing interference. Penicillin treatment of animals colonized by strain 502A (penicillin-sensitive) abolished interference with strain Q461 (penicillin-resistant). These findings indicate that bacterial multiplication by the interfering strain is an essential feature of this phenomenon.

The mechanism of interference between strains of *Staph. aureus* remains obscure. There was no evidence in these studies for direct bacterial antagonism in vitro or in vivo between most of the strains examined; yet, all were capable of producing interference. Attempts to identify antistaphylococcal activity in passively transferred tissue homogenates and serum collected from infected animals were also negative. The ability of large inocula of staphylococci grown in broth to superinfect colonized lesions indicates that the numerical superiority of the interfering strain over the challenge strain is an important aspect of interference. The observation that in vivo–grown organisms may superinfect in significantly smaller quantities is suggestive of a qualitative advantage as well.

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BIBLIOGRAPHY

1. Shinefield, H. R., J. C. Ribble, H. F. Eichenwald, M. Boris, and J. M. Sutherland. 1963. Bacterial interference: its effect on nursery-acquired infection with *Staphylococcus aureus*. V. An analysis and interpretation. *Am. J. Diseases Children.* **105**:683.
2. Boris, M., T. F. Sellers, Jr., H. F. Eichenwald, J. C. Ribble, and H. R. Shinefield. 1964. Bacterial interference: protection of adults against nasal *Staphylococcus aureus* infection after colonization with a heterologous *S. aureus* strain. *Am. J. Diseases Children.* **108**:252.
3. Light, I. J., J. M. Sutherland, and J. E. Schott. 1965. Control of a staphylococcal outbreak in a nursery: use of bacterial interference. *J. Am. Med. Assoc.* **193**:699.
4. Ribble, J. C., and H. R. Shinefield. 1964. Bacterial interference in experimental staphylococcal infections. *J. Pediat.* **65**:1047.
5. McCabe, W. R. 1965. Staphylococcal interference in infections in embryonated eggs. *Nature.* **205**:1023.

6. Körlof, B. 1956. Infection of burns. II. Animal experiments; burns and total body x-irradiation. *Acta Chir. Scand. Suppl.* **209**:117.
7. Blair, J. E., and R. E. O. Williams. 1961. Phage typing of staphylococci. *Bull. World Health Organ.* **24**:771.
8. Parker, M. T., and L. E. Simmons. 1959. The inhibition of *Corynebacterium diphtheriae* and other Gram-positive organisms by *Staphylococcus aureus*. *J. Gen. Microbiol.* **21**:457.
9. Barrow, G. I. 1963. Microbial antagonism by *Staphylococcus aureus*. *J. Gen. Microbiol.* **31**:471.
10. Wannamaker, L. W. 1958. Electrophoretic studies of the extracellular products of Group A streptococci. *J. Exptl. Med.* **107**:783.
11. Sergent, E., and E. Sergent. 1956. History of the concept of "relative immunity" or "premunition" correlated to latent infection. *Indian J. Malariol.* **10**:53.
12. Ribble, J. C. 1965. A mechanism of bacterial interference. *J. Clin. Invest.* **44**:1091.
13. Ribble, J. C. 1966. A mechanism of bacterial interference in allantoic fluid of chick embryos. *J. Pediat.* **69**: 979.