BACTERIAL INTERFERENCE IN HUMAN AND CANINE RESIDENT CUTANEOUS MICROFLORA

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TABLE OF CONTENTS

INTRODUCTION

It is now well established that the resident, indigenous bacterial populations inhabiting various parts of the human body possess the capability of inhibiting the growth of contaminating pathogens and other transient microorganisms. This phenomenon is called "bacterial interference". It is apparently a very important protective mechanism for maintaining normal healthy body habitats and it has been implicated in preventing infection of the skin, throat, respiratory tract, and bowels. The bacterial interference phenomenon has been studied *in vitro* and *in vivo* and the mechanism of interference appears to be due to either the elaboration of some, as yet, unknown extracellular product, or the alteration of the environment to make it uninhabitable for the pathogens.

Many of the studies that have been conducted concern the indigenous microflora of the throat and nasal passages. Very little work has been done on interference using the cutaneous microflora of man and animals. This habitat would seem to be an equally important area of bacterial interference especially in regard to the skin infection known as impetigo which is caused by *Staphylococcus* and *Streptococcus*. For example, it is possible that the disease impetigo commences as a result of failure by the resident cutaneous microflora to successfully inhibit these pathogenic organisms. Bacterial interference is therefore implicated very strongly as a defense mechanism against impetigo.

It should be pointed out that the skin of man and animals is a complex environment with many different nutritional niches. The two main nitrogen sources are urea and the several amino acids found in keratin. The typical resident nonpathogenic strains utilize urea as a nitrogen source whereas typical pathogenic strains utilize amino acids as a nitrogen source. Therefore, competition for nitrogen is not implicated as a mechanism for interference. In fact, since vitamins, carbohydrates, and lipids are all abundant on the skin it seems unlikely that competition for nutrients (in this particular habitat) is the primary mechanism for interference, and therefore, another interfering mechanism must exist. The production of an extracellular antibiotic substance by the interfering (resident) strains would seem a logical alternative.

Since the bacterial populations on the various skin surfaces of the body remain constant for years, it is also possible that a cooperative interference phenomenon may have evolved to prevent any new species from invading these already inhabited environments. For example, two different strains of bacteria could pool their biochemical machinery in producing antibiotic; one species might respond to an invading microorganism by producing an extracellular product which in turn would stimulate another resident bacterium to produce another antibiotic substance. The first strain, which could be called the detector strain, would have to possess the biochemical equipment enabling it to detect the presence of a "newcomer". Such equipment might include the ability to respond to atypical microbial byproducts (produced by transient organisms or pathogenic organisms). This response (of the detector strain) in the form of an extracellular protein would stimulate a second strain to synthesize an antibiotic to inhibit the growth of the pathogen.

The present work represents an attempt to study the bacterial interference of skin microorganisms with the idea of learning:

a. The types of resident cutaneous bacteria which are capable of producing interference.

b. If the interference results from an extracellular product.

c. The magnitude of the interference potential of the skin microflora in relation to interference potential recognized in microflora for other body habitats.

d. The ability of different resident cutaneous bacterial types to cooperatively produce bacterial interference.

For the purpose of this study, the term <u>test strain</u> indicates a nonpathogenic species of bacteria which is included in the resident, indigenous population of an animal. These test strains will be assayed for their ability to inhibit the growth of various pathogenic strains of bacteria. These pathogenic strains are designated as <u>target strains</u>.

P L A N O F P R O C E D U R E

a. Organisms will be obtained from various areas of the skin surface of man and animals. These will be identified and assayed for interference against various target strains (known to cause skin infection). It is the aim of this part of the research to discover which test bacteria are capable of interfering with which target strain according to the assay described below.

b. Once we have detected interference, we will attempt to deduce just exactly what it is we have measured. Have we merely observed a nutritional phenomenon or have we observed the elaboration of an extracellular product produced by the test strain which interferes with the target strain?

c. After interference has been detected, the relative magnitude of the interference potential of various members of the skin microflora will be measured. The magnitude of interference will be determined by the size of a zone of inhibition in a target lawn of bacteria. Also, the degree of inhibition within a zone will be taken into account, i.e. is the zone of inhibition an area of absolutely no bacterial growth or merely slowed down bacterial growth?

d. Cooperative interference will be measured to see if in fact two test strains are capable of greater interference than one test strain alone. This means that a combined culture of two or more test strains will produce more antibiotic than either organism grown individually. Since the fact that there is more than one organism growing in a culture does not alter the final cell population, any increase in antibiotic production (over and above that of the test strain which is most inhibitory) will be interpreted as cooperative antibiotic production (interference). Implicit in this statement is the fact that for cooperative interference to be achieved, an increased amount of interference must be seen from a combined culture of test strains which is equal in cell number to a culture of only one test strain. To test the combined cell cultures for the predominance of one bacterial species over another, gram straining was done and it was found that all test strains grew equally well in the presence of each other.

R E V I E W O F T H E L I T E R A T U R E

It is now well documented that bacterial interference is strongly implicated in protecting an animal from invasive organisms. Cutaneous bacterial interference, therefore, is probably very active in preventing skin infection, a common example of which is impetigo. This is typically an endemic disease characterized by lesions on the hands, wrists, ankles, and back. It is caused by two different invasive pathogenic microoganisms, group A Beta hemolytic Streptococcus pyogenes and Staphylococcus aureus. The streptococci are the organisms typically responsible for the primary infection. The fist lesions usually appear within eight to twelve days after the organisms can be detected on the skin. In a recent screening of patients who developed lesions, 76% were found to harbor group A Streptococcus (11).

After a primary skin infection has resulted from the pathogenic Streptococcus, pathogenic strains of Staphylococcus aureus begin to establish a secondary infection. The most common kind of lesion found therefore, is one in which both a pathogenic Streptococcus and a pathogenic Staphylococcus can be isolated.

The streptococci are transmitted typically from external endemic sources. After the organisms have established a cutaneous infection, they can, and often do, spread to the throat, the kidneys (nephritis) and the heart (rheumatic fever) causing lesions in all these areas(8). The staphylococci, on the contrary, originate in the nasal passages and then spread to the skin where they may cause new skin lesions or infect lesions that already exist (10).

It is obvious that inhabitation of the skin by a pathogenic strain of streptococci or staphylococci could result in a case of impetigo. But the question is why are not people continually plagued with this disease throughout their lives? What allows these pathogens to cause infection only at certain times of the year, i.e. the summer months (11), and what biological properties do these pathogens have to allow them to successfully compete for survival on the human skin with the resident microbial population already present?

A partial answer to these questions appears to be bacterial interference. The ability of the resident cutaneous microflora to inhibit the growth of pathogens is the primary defense mechanism which can prevent infection from starting. This has been well substantiated in the literature. For example, in order to establish an experimental model of impetigo, pathogenic bacteria must be injected beyond the zone of bacterial interference, i.e. intradermally. This has been shown by administering an intradermal injection of pathogenic streptococci or staphylococci to Syrian hamsters (6). The injections resulted in experimental lesions which remained essentially a pure culture of the injected pathogen (4). Experimental lesions established in this manner were very slow to heal naturally and many of the antibiotics and germicides commonly used in treating skin infection proved ineffective in promoting the lesion to heal. These included topical scrubbing with PHisohex (which made already existing lesions worse and resulted in satellite lesions) and topical application of gentamicin or bacitracin (which were ineffectual). Injection of penicillin proved effective in promoting healing of already existing lesions. Prophylactic administration of benzathrine penicillin made experimental lesions much harder to establish by injection of streptococcus or staphylococcus (7).

If, however, an experimental situation is set up where the normal bacterial population of a body site is left intact, and then that area is exposed to a pathogen, the existence of bacterial interference becomes clear, i.e. infection is prevented. In fact, there have been cases reported in which the only way to prevent staphylococcal lesions from reoccurring, after they were cured with antibiotic therapy, was to inoculate the patients with a nonpathogenic strain *Staphylococcus aureus* 502A. This nonpathogenic bacteria (which integrated itself into the patient's resident cutaneous microflora) successfully inhibited the pathogenic strain of *Staphylococcus aureus* from reestablishing lesions in 73% of the patients who had been suffering from chronic reoccurrences of lesions after they had been cured with antibiotic therapy (2).

A similar technique has been used in curtailing nursery epidemics of staphylococcal disease in newborn infants. By inoculating the nasal mucosa of infants with a nonpathogenic strain of *Staphylococcus aureus* they are rendered much more resistant to staphylococcal skin infection. The protective mechanism implicated was bacterial interference (21).

Bacterial interference has also been studied in vitro. Pathogenic groups of streptococci were found to be inhibited by membrane filtered supernatant fluids from cultures of staphylococcal skin isolates. The bacterial substance which was purified also inhibited pneumococci and corynebacteria. The substance was found to be a protein and was detected in the broth culture after 24 to 48 hrs of incubation (5).

If the resident cutaneous microflora is instrumental in inhibiting the growth of pathogenic bacteria, then it would seem that antibiotic production is generally greater in nonpathogenic bacterial strains than in pathogenic strains. A study has been done using coagulase positive (pathogenic) staphylococci and coagulase negative (nonpathogenic) staphylococci. It was found that many isolates of both kinds of staphylococci inhibited the growth of the pathogenic indicator strain Staphylococcus aureus Oxford 209P. The coagulase negative bacteria were, however, found to be more active in antibiotic production than were the coagulase positive bacteria (15). It seems therefore, that antibiotic production (interference capabilities) is more commonly a part of the biochemical equipment of resident nonpathogenic organisms (which are coagulase negative) than transient pathogenic organisms.

Since the resident cutaneous microflora is primarily invaded by gram positive bacteria it would seem adaptively significant for the resident species to produce antibiotics which are particularily active against gram positive bacteria. This phenomenon of skin isolates interfering with gram positive rather than gram negative bacteria has been found using *Staphylococcus aureus* strains of human origin. These particular strains were all isolated from skin infections and were all pathogenic. It was found, interestingly enough, that these pathogenic bacteria inhibited many other pathogenic groups including other strains of *Staphylococcus aureus* and *Streptococcus*. These inhibitory strains were also active against some coagulase negative staphylococci and corynebacteria. Consequently, it seems that pathogenic bacteria are in fact capable of inhibiting resident nonpathogenic bacteria. This study is quite interesting because it indicates that there is probably an "antibiotic war" constantly being waged on the skin. The resident bacteria produce antibiotics against invasive pathogens and the invaders actually produce antibiotics against the resident bacteria in their struggle to establish a permanent residence. Thus, efficient antibiotic production is probably one decisive factor in determining which strains of bacteria successfully compete on the skin for an ecological niche (1, 18).

Nutrition is also an important factor in determining which strain will survive in a competitive situation. It has been reported that coagulase negative staphylococci will utilize specific amino acids in the allantoic fluid of chick embryos and thereby prevent the growth of a virulent coagulase positive strain. The particular amino acid used by the coagulase negative strain in this case was threonine. By adding this to the medium, the growth of the coagulase positive bacteria could be restored (19).

Bacterial interference has also been shown to exist in other areas besides the skin. The throat is one area that has been studied extensively. It has been shown that the normal flora of children who are susceptible to throat infection is much less inhibitory to *Streptococcus pyogenes* than the normal throat flora of children who are not susceptible to infection. The resident bacteria in this case were viridans streptococci and the inhibition of growth was caused by the induction of an acidic medium and depletion of essential substrates by the viridans streptococcus (3, 20). This was proved conclusively by showing that inhibition could be abolished by restoring the basal nutrients of the medium (glucose and salts) or by buffering the medium.

There is also evidence that the interference mechanism maintains the status quo of the resident microbial populations. Many organisms in the normal pharynx studied *in vitro* are capable of preventing the growth of other bacterial species with which they normally coexist *in vivo* or test organisms to which they are exposed experimentally. Wiping out the predominant bacterial species (e.g. alpha hemolytic streptococci) with massive antibiotic therapy (such as used after major surgery) can completely disrupt the microbial status quo of a body area. Such a condition can, and often does, result in an infection. The throat for example becomes overrun with bacteria not routinely found there. When antibiotic therapy is withdrawn, the original predominant species may appear, and the normal flora is reestablished (22).

Bacterial interference also plays an important role in maintaining the bacterial status quo of the bowels. By irradicating the intestinal microflora with antibiotics, experimental animals can be rendered much more susceptible to bowel infection and infection in general. When the normal intestinal microflora is irradicated or severely disrupted, an animal has virtually no defense mechanisms against enteric pathogens like *Vibrio cholerae* and the bowel infections which often result are usually fatal. The mechanism hypothesized to be responsible for bacterial inhibition in the bowels is the depletion of carbon sources by the resident microflora which prevents pathogens from establishing infection. When the resident microflora is irradicated, an ideal environment is opened up for any pathogen that it finds suitable, e.g. *Shigella flexneri* or *Proteus vulgaris* (9, 12, 13, 14).

This work will contribute insight into the ability of the human and canine cutaneous microflora to inhibit pathogenic organisms. It will represent an attempt to show that the members of the resident indigenous microflora of these two animals cooperatively produce bacterial interference.

M E T H O D S

A. Isolation of Test Strains

Test strains of resident cutaneous bacteria were obtained by rubbing a wet sterile swab firmly against the skin and using this swab to inoculate a tube of nutrient broth. After 24 hrs of incubation at 37° C, the broth was diluted by 10^{-8}. One tenth of a ml of the final dilution was spotted on a nutrient agar plate and spread evenly over the surface. The plates were incubated for 24 hrs at 37° C after which time individual colonies were checked to determine the diversity of bacteria associated with that part of the skin. Identification of the various test strains was based on colony morphology, gram staining, and the standard diagnostic biochemical tests indicated below:

a. carbohydrate fermentation

b. coagulase production

c. high or low salt tolerance

d. nitrate reduction

e. catalase production

f. indole production

g. hydrogen sulfide production

B. Procedure for Assaying Bacterial Interference

After the test strains have been identified, they were assayed for their ability to inhibit the growth of various target strains. For the purpose of this study, target strains were isolated from infected skin, i.e. an organism was considered pathogenic only if it has been obtained from a clinical skin infection. Thirteen strains of pathogenic Staphylococci and 11 strains of pathogenic Streptococci were used.

The assay procedure was a modification of the technique used by Crowe et al. (3) in their recent study on inhibition of pathogenic streptococci by the resident microflora. Their procedure was to spread a test strain of bacteria on an agar plate, overlay these cells with another layer of agar, and then spread a target strain of bacteria on the overlayed surface in high enough concentration to form a continuous lawn of bacteria (area of unbroken bacterial growth). In Crowe's study, as well as in this study, the test strain underneath the overlay agar was considered inhibitory to the strain if it caused zones of inhibition (no growth) in the lawn of target strain bacteria growing directly over a colony of the test strain underneath the overlay agar.

The modification of the Crowe technique used in this study was to place only one colony of test strain on each petri dish. This modification was employed as an attempt to use the size of the zone inhibition to quantitate the effectiveness of the interference. It was much easier and more accurate to measure the size of a zone of inhibition if there was only a single zone on one plate, rather than several overlapping zones.

C. Alternate Method for Assaying Bacterial Interference - Spot Technique

Pathogenic target strains were removed from a blood agar culture plate and inoculated into tubes of sterile distilled water. The tubes were then diluted to give a final optical density of 0.1. The test strains were prepared in the same way.

One tenth of a ml of target strain suspension was spread evenly over the surface of a blood agar plate and one hundredth of a ml of the test strain suspension was spotted in the middle of the same plate. The plates were then incubated at 37° C for 12 hrs. With this method, the target strain grew up in a continuous lawn evenly covering the surface of the agar. The test strain grew as a single large round colony in the middle of the plate. A test strain was considered inhibitory if there was a zone of inhibition (no target strain growth) around it. The width of the band of inhibition served as a quantitative index of inhibition, i.e. the wider the band, the greater the inhibition.

To investigate how nutrition might effect interference, the spot technique was carried out under three nutritional situations: on blood agar, on blood agar which was 1% yeast extract, and on blood agar which was 1% yeast extract and 1% urea.

D. Procedure for Assaying Cooperative Bacterial Interference - Filtered Broth Technique

Spent broth medium from a 24 hr broth culture was sterilized with a Swinnex millipore filter and then assayed for its ability to produce interference. This was done by saturating small discs of filter paper with the spent culture medium of various cultures and placing these on a freshly spread lawn of target bacteria. A zone of inhibition around a disc indicated the presence of an extracellular antibiotic product in the broth which was presumed to be the causitive agent of interference.

In this study, the definition of cooperative interference implies that two test strains of bacteria make more antibiotic when they are growing together than when they are growing separately. If this is the case, a filter paper disc soaked in the culture medium of two test strains growing together should produce a wider zone of inhibition than the zones of inhibition produced when the test strain cultures are assayed individually. By culturing various combinations of test strains together and testing the culture broth, it was possible to detect which, if any, test strains cooperate in antibiotic production. Implicit in this assay is the fact that the various test strains in a combined culture will usually grow at the same rate. The relative population of each test strain in a combined culture was checked by gram staining.

RESULTS AND DISCUSSION

A. Isolation of Bacteria

1. Human Isolates

Two principle types of bacteria were isolated from human skin: gram positive cocci in grapelike clusters which were members of the genus *Staphylococcus* and gram positive pleomorphic rods which fall in the general class of corynebacteria. The isolates obtained in this study were:

Isolate 1 was identified as *Staphylococcus epidermidis* and was the predominent species repeatedly found in the human axillae. It was not detected on other parts of the body and was considered to be a permanent resident of the axillae because of its omnipresence in this region.

Isolate 2 was a member of the general class corynebacteria which was found only on the human arm. This bacterium was not detected on any other part of the skin and therefore was considered to only reside on the arm.

Isolate 3 was a corynebacterium which could only be isolated from the human toes. It was considered to reside only in this area because it could not be isolated from any other skin surface.

Isolate 4 was a corynebacterium, but it was isolated from many skin surfaces which included the back, neck, abdomen, groin, foot, and axilla.

2. Canine Isolates

From the canine skin, three strains of corynebacteria were isolated. These were designated as isolates C_1, C_2 and C_3. They were found on all parts of the skin without any particular strain being limited to any single cutaneous region.

It seems from the above data that the canine skin is a more generalized environment than the human skin, i.e. all of the resident species can inhabit any of the skin surfaces. This situation is quite different from the inhabitation of human skin, in which certain strains of bacteria are found only on certain areas of the skin surface.

This difference is not really unexpected or illogical when one considers the differences between human and canine skin. A major difference between the physiology of man and dog is that dogs are virtually unable to sweat. The hairy skin of a dog lacks ecrine sweat glands and the only skin surfaces from which a dog can sweat are on the footpads (16, 17). This fact, and the fact that the skin of a dog is completely covered with hair (unlike human skin which is not completely covered) could make it a more stable and more uniform environment than human skin. The reasoning behind this statement is that canine skin is protected from desication by the coat of the animal. Also, the skin does not undergo periods of wetness (sweating) and dryness (not sweating). In canine skin there is continued production of oil from the sebaceous glands. This oil provides bacterial populations with a constant amount of nutrition. The human skin, on the other hand, is a very unprotected environment, and probably much less stable than canine skin. It undergoes wetness, dryness, variations in temperature, and it may or may not be exposed to ultraviolet light from the skin. All these factors make human skin a more complex environment than canine skin.

The skin of a dog can in fact be considered as a single uniform environment, i.e. it is not composed of smaller subenvironments like the human skin (e.g. foot, axilla, and groin). The fact that three bacteria were found on all parts of the canine skin implies, however, that its environmental conditions are not perfectly constant. There must be just enough alteration in the environment to allow three different species to exist. It is quite possible that one species predominates over the other two (such data is not available at this time), but the mere fact that all three species can be isolated indicates there must be some continuous alteration in environmental conditions. The reason for this is that if the environmental conditions were absolutely constant, a succession would occur. One bacterial species (the one most suited to the environment) would completely outgrow the others and after enough generations had elapsed, the two less dominated species would become extinct. It is reasonable, therefore, to compare the entire skin of a dog to one individual subenvironment of the human skin, e.g. the axilla from which two species of bacteria can be isolated but where human isolate #1 (<u>Staphyloccus aureus</u>) probably predominates.

The fact that the number of isolates obtained from both man and dog is not that great can also be explained by the relative stability of the skin surface. Such an environment would typically be inhabited by a few species which are particularly adept at surviving in it. Also, considering the antibiotic produced by these organisms, it is very understandable why a wide variety of other species cannot coexist with them.

B. Interference Assays

The Crowe technique (involved overlaying several test colonies with agar and then spreading pathogens on the overlaid agar; our modification in the Crowe technique was to use only one test colony) proved to be unsatisfactory despite our modification and repeated trials. The primary reason for this is that when a colony of the test strain was covered with the overlay agar, the cells invariably diffused throughout the overlay instead of remaining as a single colony. Therefore, the overlay agar became overgrown with the test strain bacteria. Clearly, it was difficult to see interference in such an uncontrolled system where pathogens are mixed in with the test strain in a purely random fashion.

The Spot technique which we developed for this study was used effectively to determine interference. From the results shown in Table 1, it can be seen that the cutaneous microflora of both man and dog seem to be more inhibitory to staphylococci than streptococci (both pathogenic to humans). Human isolates 1 and 2 did not inhibit any of the 24 pathogenic species used. Human isolate 3 was inhibitory to six strains of staphylococci and to two strains of streptococci. Human isolate 4 was inhibitory to eight strains of the staphylococci and one strain of the streptococci.

Table I. Results of spot technique when canine and human test strains were assayed for their interference of 24 pathogenic target strains.*

	C_1	C_2	C_3	1	2	3	4
1 - SA**	-----	++-+	++--	----	+---	+--+	++++
2 - SA	-----	++++	+---	----	+----	+++-+-++	++++
3 - SA	-----	++++	+++-	----	+----	++-++	++-++
4 - SA	-----	++-+	++++	+---	+----	+++-+	++++
5 - SA	-----	-+++	++++	----	+----	+++-+	++++
6 - SA	-----	-+++	+-+-++++	-----	+---	++--++++	++++
7 - SA	-----	-+++	++++	----	-----	-++++	-+--
8 - Strep***	-----	++-+	+-+-	----	+---	---+	--+-
9 - Strep	+----	++-----	+---	----	-----	----+	+---
10 - SA	-----	-+++	-+++	----	+----	-++-++++	+++----
11 - SA	-----	--+-	+-++	----	+----	--+-+	++-+---
12 - SA	-----	++-+	++-+	----	+----	--+--	-+-+---
13 - Strep	-----	++--+++	+---	---	-----	------	----
14 - Strep	----	+++	+-+	--	---	-+	---
15 - Strep	--	+	-	--	-	-	+
16 - Strep	-----	+-++	+---	---	+---	-+++	++--+++
17 - Strep	-----	++-----	+---	---	+---	--+--	+---
18 - Strep	-----	+++-	+---	---	+----	--+-	+---
19 - SA	-----	++++	++++	---	+---	+++++	+++++
20 - Strep	-----	--	++++---	---	++--	--+++++	---
21 - Strep	--	+	---	--	-	-	-+
22 - SA	-----	+++-	--+	---	+--	+--+	++-
23 - SA	-----	++++	-++	---	+---	++-+	--++---
24 - Strep	-+---	++--	-+--	---	-----	-+--+	++--

* + indicates a positive assay, i.e. test strain inhibited a target strain
− indicates a negative assay, i.e. test strain failed to inhibit the target strain

** SA = Staphylococcus aureus

*** Strep = Group A Beta Hemolytic Streptococcus

It should be pointed out that the width of the zone of inhibition produced by the various test strains was rather constant throughout the study and, as a result, quantitation of the inhibitory effect could not be determined. The zones typically appeared as clear areas (approximately 0.2 - 1 ml in width) of no bacterial growth around the test colonies. A qualitative judgment was thus passed indicating whether or not bacterial interference had occurred.

The greater interference shown against staphylococcal strains appears to be in accord with what is known about human impetigo. In this disease, occurring on both human and canine skin, the streptococci usually initiate the infection and the staphylococci enter as secondary pathogens (11). Possibly bacterial interference is responsible for this sequence of infection because the resident microflora seem to be able to inhibit the pathogenic staphylococci but cannot inhibit the streptococci. Once the streptococci have established infection, bacterial interference breaks down and the way is opened for the staphylococci to infect already existing lesions.

To insure that the inhibtion seen was valid, several control assays were performed. The first assay was a "reverse spot technique", which consisted of exactly the same procedure except that the test strain was spread on the plate and the target strain was spotted on the freshly seeded test lawn. In all cases, the reverse spot technique was negative, i.e. a pathogen could not inhibit a resident strain. On the contrary, the test strain lawn seemed to grow right over the top of the target strain, which indicated its ability to outcompete the target strain.

Another control assay was done to measure the inhibition of the test strains against each other. One test strain was spotted on a freshly spread lawn of another strain. The inhibition of one test strain by another did not occur in any combination.

C. Effects of Nutrition on Interference Using the Spot Technique

The ability of one organism to inhibit the growth of another is not necessarily due to the production of an extracellular antibiotic substance but instead may simply be a competition for available nutrients. This nutritional interference is quite possible under the artifical conditions of a laboratory experiment and it is thus important to distinguish between these two types of interference. Therefore, the spotting assay technique was carried out under three different nutrient situations (blood agar alone, blood which was 1% yeast extract and blood agar which was 1% yeast extract and 1% urea) to test the effects of nutrition on interference. All test strains seemed to retain a constant amount of inhibition in all three nutritional situations. Thus, it did not appear that inhibition of growth was due to nutritional deprivation. None of the test strains which were not inhibitory on standard blood agar become inhibitory with the addition of yeast extract or urea to the blood agar.

From this data, it would seem that an extracellular substance is implicated as the causitive agent of interference and not the other conceivable interpretation that the test colony depletes the nutrients in the agar surrounding it and thereby prevents the target strain from growing. The fact that interference was not influenced by the varying nutritional conditions of the spot technique does not rule out nutritional effects altogether since such a mechanism is well documented in the literature (19). Likewise, the skin is a difficult habitat to duplicate in the laboratory and consequently, the *in vitro* absence of a demonstrable nutritional effect could be misleading since competition for food stuffs could in fact complement or enhance the already demonstrated extracellular antibiotic.

The fact that some test strains were absolutely negative in the spot technique makes the nutritional mechanism for interference appear less likely. If lack of nutrition was producing interference then all test strains should be positive.

It is also hard to imagine a bacterium consuming nutrients from places in the agar medium other than the place where it is actually growing. It is true that diffusion of nutrients does take place, but the diffusion should be random in all directions. If some nutrient was depleted in an area surrounding a test colony, there would be an equal probability of more of that same nutrient diffusing into the area from which it had been depleted. It is critical to point out that depletion of nutrients is a process of random diffusion whereby nutrients are depleted when they come into contact with bacterial cells as they diffuse through the agar. Clearly if such a random diffusion process is capable of depleting nutrients from an area then it should also be capable of restoring an equal amount of nutrients to the same area. Thus, the data presented here appears to be the result of an extracellular antibiotic substance rather than a nutritional competition.

D. Cooperative Interference Assays

A combined spot technique (drops of test strains in different combinations spotted on target lawns) was tried as a way of demonstrating cooperative bacterial interference. However, the zones of inhibition produced by this technique were too small to be judged for cooperative interference.

Thus, a membrane filter technique was designed to test antibiotic production of actively growing test colonies consisting of various combinations of test strains. In this assay, a 0.45 u membrane filter was placed on the surface of a blood agar plate and .1 ml drops of an 0.1 OD (450 um) suspension of test strain were placed on the membrane filter paper. The test strain grew on the filter paper as a large colony and any antibiotic produced by the test colony would diffuse down into the agar leaving the bacteria on the filter paper. After 24 hrs of incubation at 37° C, the filter paper and test colony were removed and 0.1 ml of a 0.1 OD suspension was spread over the entire agar surface. If the test strain produced an antibiotic while growing on the filter paper, there should be a zone of inhibition in the target lawn at the spot where the test colony grew. If there is cooperation among the various test strains, then the inhibition produced by the combined growth of two test strains on the membrane filter should be greater than that produced by only one strain.

The results of this assay were very rewarding. When individual test strains were grown on the membrane filter, they produced an extracellular substance which diffused thru the filter and gave a zone of inhibition exactly the size of the test strain growth spot. The edge of the zone of inhibition was very distinct and no growth of the target strain occurred within the zone.

When the various canine test strains were combined and tested for interference, it was found that certain combinations of test strains were more inhibitory than the individual test strains. The criteria used for judging interference were the size of the zone of inhibition and the degree of interference (based on amount of target strain growth) in a zone.

The canine test strains were ranked as they appear in Table II where the number of + signs indicate the relative strength of interference. Test strain C_1 was not inhibitory to any of the pathogens and was ranked as 0. Test strain C_2 was slightly inhibitory and was ranked + (it produced small zones of incomplete inhibition). Test strain C_3 was quite inhibitory (+++) and produced zones of inhibition which were larger (by 2 mm) and more complete than test strain C_2.

Test strain combination C_1-C_2 was more inhibitory than either C_1 or C_2 alone but not as inhibitory as C_3 alone. The zone of inhibition produced by the C_1-C_2 combination of test strains were approximately 1 mm larger than C_2 alone and 1 mm smaller than C_3 alone. The C_2-C_3 (++++) combination was more inhibitory than C_1-C_2 with an increase of 2 mm in the size of the zone of inhibition. The C_1-C_3 combination (+++++) and the C_1-C_2-C_3 (++++++) combination were both more inhibitory than the C_2-C_3 combination. The zone of inhibition produced by C_1-C_3 was approximately 1 mm larger than that produced by C_2-C_3; and C_1-C_2-C_3 produced a zone of inhibition which was approximately 1 mm larger than C_1-C_3. It is important to realize that the increase in the size of the zones of inhibition reported here represents an increase beyond the periphery of the test colony. This was measured by outlining the periphery of each test colony on the bottom of the petri dish. Any increase in the zone of inhibition beyond this outline could then be measured when the membrane filter and the test colony were removed.

Table II - Ranking of Canine Test Strain Combinations*

Test Strains Alone		Combinations of Test Strains	
C_1	0	C_1-C_2	++
C_2	+	C_2-C_3	++++
C_3	+++	C_3-C_1	+++++
		C_1-C_2-C_3	++++++

*The + signs represent the relative strength of inhibition expressed for test strains and combinations of test strains. The more + signs, the greater the interference. O indicates no interference.

Since gram staining revealed that the combined cultures contained approximately equal amounts of each bacteria, it was concluded that cooperative bacterial interference does exist among the canine test strains. A cooperative effect was not seen with the human test strains.

Two additional experiments were done to further study the phenomenon of cooperative bacterial interference. The first of these was an overlapping drop assay where .05 ml drops of different test strains of bacteria were spotted on a membrane filter (0.45 um). The drops were placed so they just barely overlapped and each drop was a suspension of a different test strain. When the filter papers were removed and target strain spread on the agar surface, any cooperative antibiotic production should be evidenced by an increased zone of inhibition at the overlap of the drops (provided the drops were approximately of equal size). Such an increased zone was not seen in any combinations of test strains. A possible reason for this could be that too few of the various test bacteria were cooperating and any increased antibiotic production could not be seen.

To test if a zone of inhibition produced, using the membrane filter technique, was the result of depletion of essential nutrients in the agar rather than an extracellular antibiotic, the target strain was spread on the original agar surface (i.e. the same agar that nourished the test colony growing on the membrane filter) in 5 ml of overlay agar. By spreading the target strain in an overlay, the target bacteria are provided with a layer of fresh agar and are not subjected to any nutritional limitations. If any antibiotic is present in the original agar surface, it should diffuse up into the overlay of target bacteria and create a zone of inhibition. The results of this assay were positive and zones of inhibition were produced in the overlay surface by positive test strains. Since the experimental conditions of this assay imposed no possible nutritional limitations on the target strain, it seemed clear that interference with these cutaneous isolates was only the result of extracellular antibiotic production.

E. Attempted Isolation of Extracellular Antibiotic Substance

In an attempt to detect an extracellular antibiotic substance techniques were contrived and used:

1. Concentrated filtered broth assay

When the 24 and 48 hr old human and canine test cultures were assayed using the filtered broth technique (see Materials and Methods) no interference to any of the 24 pathogenic target strains was observed. In order to test the possibility that the antibiotic in the culture batch broth was too dilute to produce interference, a technique was devised to concentrate the various solid components of the broth onto filter paper discs. This technique consisted of repeated application of the spent culture medium and subsequent drying. After six applications, the discs were placed on freshly spread lawns of target strains incubated for 24 hrs and checked for interference. None of the test strain discs inhibited any of the target strains, i.e. no bacterial interference was witnessed. The literature indicates that 24 and 48 hr staphylococcal culture broth does contain antibiotic activity (5). In our laboratory, however, when staphylococcal broth cultures (of human origin) were checked for antibiotic activity, they proved negative, i.e. filter paper discs containing the broth did not produce zones of inhibition in the target lawn of bacteria. Since these staphylococcal strains were inhibitory using the membrane filter assay (as were the test strains C_2, C_3, 3 and 4), it was concluded that if an antibiotic was present in the culture broth, it was too dilute or unstable to be measured directly, i.e. saturating filter paper discs with the broth.

2. Plate scraping technique

In still another attempt to demonstrate the presence of an antibiotic in an actively growing test culture, a plate scraping technique was contrived. This technique consisted of adding 2 ml of sterile distilled water to an agar plate which had abundant test strain growth. The colonies of bacteria were then scraped off the agar and stirred into suspension with a glass spreader. The agar surface was agitated with the spreader for a few minutes in an attempt to suspend any bacterial byproducts present on the agar surface. The bacterial suspension on the agar surface was then drawn up into a syringe and sterilized by membrane filtration. The filtrate was absorbed onto filter paper discs and concentrated by the process of repeated application and drying. Approximately 1 ml of filtrate was concentrated on each disc which were then assayed for inhibition by placing them on a freshly spread lawn of target strain bacteria. This technique also indicated the absence of an antibiotic substance, i.e. none of the discs showed interference.

CONCLUSION

This study represented an attempt to learn more about the phenomenon of cooperative bacterial interference. The accomplishments of this work are the following:

1. Bacteria from the various regions of the skin of man and dog were isolated and identified.
2. The following assays were developed to test bacterial interference:
   a. Modified Crowe technique
   b. Spot technique
      1. Nutritional spot technique
      2. Combined spot technique
   c. Membrane filter technique
      1. Overlay membrane filter technique
      2. Double spot membrane filter technique
   d. Concentrated Broth technique
      1. Plate scraping technique

Data has been obtained from the above assays which support the hypothesis that the members of the resident canine cutaneous microflora do cooperate in preventing skin infection. The prevention is implicated, by this study, as being the result of cooperative antibiotic production by the cutaneous microflora.

The fact that cooperation could not be demonstrated using the human microflora is probably a function of the assays used rather than the phenomenon of cooperative bacterial interference. The only real way to measure cooperative bacterial interference is to quantitate the mg of antibiotic produced by combined cultures of various test strains.

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