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Bacterial Interference with L-Forms[1]

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During the course of studying the effect of normal nasal flora on the growth of L-forms, a clear zone of inhibition was observed around colonies of many coagulase-negative staphylococci. Subsequent investigation demonstrated that *Staphylococcus aureus* and some *S. albus* strains produce a substance which is capable of markedly inhibiting the growth of stable staphylococcal and streptococcal L-forms. This interfering substance is separable from the staphylococcal organism and is diffusible through 1.5% agar, but not through a dialysis membrane. It is heat-stable.

Interference with the growth of one organism by a second organism or its products has been well documented. Many reports have demonstrated interference in vivo or in vitro among bacteria and viruses (1, 5, 6, 8–10). During the course of studying the effect of normal nasal flora on the growth of known L-forms, it was observed that there was a zone of inhibition of L-forms around colonies of some coagulase-negative staphylococci. This initiated studies of these and other bacterial organisms with respect to interference with the growth of L-forms.

MATERIALS AND METHODS

Stable *Staphylococcus aureus* L-forms, strains 212 and ATCC 6538 p, were obtained from Benjamin Kagan, Cedars-Sinai Hospital, Los Angeles, Calif. Other staphylococcal L-forms were induced in the laboratory by use of penicillin or lysostaphin. The parent strains of these L-forms were *S. aureus* strain 502A, *S. aureus* phage type 80/81, and *S. aureus* phage type 42D. Stable streptococcal L-forms, strains GL8-L, 64X392-L, and 60X298, were obtained from Roger Cole of the National Institute of Allergy and Infectious Diseases.

The media used for stock passage of L-forms was Brain Heart Infusion (BHI, BBL) with 5% NaCl and 20% unheated horse serum. The solid medium used was BHI Agar with 5% NaCl and 20% horse serum. BHI with 5% NaCl and without serum was used in all inhibition studies in which liquid medium was used.

Stock cultures of vegetative bacteria were maintained on Trypticase Soy Agar slants (BBL) at 4 C.

RESULTS

Effect of normal nasal flora on growth of L-forms on solid media. Cultures acquired from the nares of three laboratory assistants were spread on a lawn of staphylococcal L-forms 212 and ATCC 6538 p. A 2- to 3-mm zone of L-form inhibition was noted at 24 hr of incubation around colonies of coagulase-negative staphylococci from one of the volunteers, but no inhibition was noted around other vegetative organisms present. This inhibitory *S. albus* was designated strain D and saved for future study.

The inhibitory activity of a variety of bacteria was tested in the following experiment. Lawns of broth cultures of ATCC 6538 p and 42D L-forms and of an L-form from *S. aureus* phage type 54/53/47/42E/76 were allowed to dry on L agar plates. Loopsful of test bacterial strains were touched to the surface of the plates. The test strains were coagulase-positive staphylococci of phage types 54/53/47/42E/7/6 and 80/81, group A beta-hemolytic streptococci, alpha-hemolytic streptococci, gamma-hemolytic streptococci, neisseriae, strains of *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas*, diphtheroids, and several coagulase-negative staphylococcal strains. Observation at 24 hr of incubation showed a 1- to 2-mm zone of inhibition around *S. aureus* strains and a 2- to 3-mm zone of inhibition around certain *S. albus* strains, but none around any of the other organisms tested.

Influence of bacteria grown simultaneously with L-forms in broth. Tubes containing 10^2, 10^3, or 10^4 colony-forming units (CFU) of *S. albus* strain D per ml in BHI were inoculated with 10^2, 10^3, or 10^4 CFU/ml of staphylococcal L-forms 212 and ATCC 6538 p. L-form growth was determined by plate counts at 24 and 48 hr (Table 1). In all tubes containing *S. albus*, a marked decrease in the number of L-forms was noted compared with the L-forms growing in control tubes of broth alone. This effect was most frequently noted when the

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TABLE 1. *Interference with L-forms by Staphylococcus albus*

S. albus inoculum	L-form inoculum[a]					
	10^2		10^3		10^4	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
10^2	3×10^2	2×10^2	3×10^3	2×10^3	1×10^4	1×10^5
10^3	6×10^1	1×10^2	2×10^3	5×10^2	1×10^3	4×10^2
10^4	1×10^2	1×10^2	4×10^2	2×10^2	5×10^3	1×10^4
Control	1×10^4	1×10^6	2×10^4	1×10^6	1×10^4	2×10^6

[a] L-form strain 212. Statistically similar results obtained with L-form strain ATCC N538 p.

inoculum of coagulase-negative staphylococci was equal to or exceeded the number of the L-form inoculum.

Diffusibility of the inhibitor. A lawn of *S. albus* strain D was placed on the surface of an L-form agar plate and allowed to incubate for 4 hr. A 0.45-μm cellulose acetate filter (Millipore Corp., Bedford, Mass.) was placed on the plate, and its surface was inoculated with 0.1 ml containing 10^4 CFU of L-forms. The L-forms used were staphylococcal strains 212 and ATCC 6538 p and streptococcal strains GL8-L, 60X298-L, and 64X392-L. At 72 hr of incubation, there was no growth of L-forms.

A second experiment was performed in which L-form agar was covered with a lawn of each of the above L-forms. A 0.45-μm filter was placed on the surface of the lawn and spotted with loopsful of an 18-hr culture of strain D *S. albus*. At 72 hr, there was marked inhibition of all L-form growth immediately under the area of the filter where the coagulase-negative staphylococci were growing. Control plates of L-forms on the filters alone showed heavy growth.

In a third experiment, loopsful of an 18-hr culture of strain D were spotted on L-form agar and allowed to dry. These were overlaid with L agar 2 to 3 mm in depth and inoculated with lawns of L-forms. After 48 hr of incubation, L-form growth was completely inhibited directly above the strain D growth.

Inhibition of L-forms by bacterial filtrates. BHI cultures (24 hr) of *S. albus* strain D, a noninhibitory *S. albus*, and *S. aureus* phage type 80/81 were centrifuged for 30 min at 3,000 rev/min. The supernatant fluid was filtered through 0.45-μm Swinney filters. Each filtrate was divided into four 1-ml portions. To each tube, one L-form strain containing approximately 10^3 CFU in 0.1 ml was added. The L-forms tested were staphylococcal L-forms 212 and ATCC 6538 p and streptococcal L-forms GL8 and 64X392. All tubes were incubated at 37 C for 72 hr. There was heavy L-form growth in the broth and the filtrate of the noninhibitory *S. albus*, but complete inhibition of L-form growth in the *S. aureus* and *S. albus* strain D filtrates (Table 2).

An experiment was conducted to determine whether the parent bacterial strain of a tested L-form could also be inhibited by filtrate of the D strain of *S. albus* and a noninhibitory *S. albus* strain. Samples of the above filtrates and BHI broth were inoculated with 10^3 CFU of *S. aureus* phage type 54/53/47/42E/76 and 10^3 CFU of an L-form established from the latter organism. At 24 hr, in all tubes inoculated with the *S. aureus* there were 5×10^7 organisms. In the D strain filtrate, there was no L-form growth. In the broth and noninhibitory filtrate, 10^6 L-forms were present.

Effect of heat-killed staphylococci on L-form inhibition. Overnight cultures of *S. aureus* 80/81 and *S. albus* strain D were placed in a boiling-water bath for 30 min, centrifuged, and separated from their supernatant fluids. The sediment was diluted in broth to an approximate concentration of 10^4 cells/ml. Half was washed three times with sterile saline and rediluted to approximately 10^4 cells/ml. To broth cultures containing 10^3 CFU/ml of L-forms 212 and ATCC 6538 p, 10^4 washed or unwashed heat-killed organisms were added.

Controls were 10^4 CFU of living coagulase-

TABLE 2. *Interference with L-form growth[a] by staphylococcal filtrates*

Filtrate	L-form growth		
	24 hr	48 hr	72 hr
S. albus strain D	0	0	0
S. albus noninhibitory	8×10^6	7×10^6	2×10^6
S. aureus 80/81	0	0	0
BHI control	1×10^5	2×10^5	3×10^6

[a] Inoculum, 10^3 L-forms of strain 212.

	10⁴	
	24 hr	48 hr
	× 10⁴	1 × 10⁵
	× 10³	4 × 10²
	× 10³	1 × 10⁴
	× 10⁴	2 × 10⁶

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	L-form growth	
	48 hr	72 hr
	0	0
)⁶	7 × 10⁶	2 × 10⁶
	0	0
)⁵	2 × 10⁵	3 × 10⁶

s of strain 212.

positive and coagulase-negative organisms per ml in broth containing 10³ CFU of L-forms per ml. At 24 hr, there were 10⁴ CFU of L-forms per ml in the tubes with unwashed heat-killed cells, 10⁴ CFU of L forms per ml in the tubes with washed killed cells, and no growth in the controls. At 48 hr, there were 10⁶ CFU of L-forms per ml in unwashed heat-killed cells, the same in washed killed cells, and no growth in the controls (Table 3).

Drops of the washed and unwashed heat-killed cells were placed on lawns of L-forms. After 72 hr of incubation, no interference with L-form growth was seen. In the same experiment, live staphylococci inhibited L-form growth.

Effect of heated staphylococcal supernatant fluid on L-form inhibition. Overnight cultures of the 80/81 and D strains were centrifuged for 30 min at 3,000 rev/min. The supernatant fluids were decanted and tested for inhibitory activity after being heated in boiling water for 30 min to kill the remaining bacteria. A supernatant fluid of a noninhibiting *S. albus* was prepared in the same manner and used as a control. The heated supernatant fluids were inoculated with 2 × 10⁴ CFU of staphylococcal L-forms 212 and ATCC 6538 p per ml. Both the D strain and 80/81 supernatant fluids showed complete inhibition of L-form growth at 24 hr. In the noninhibitory *S. albus* supernatant fluid, 5 × 10⁵ CFU/ml were present and 8 × 10⁴ CFU of L-forms per ml were counted in a broth control (Table 4).

Reversal of inhibition by addition of fresh BHI. Supernatant fluids prepared as described above were diluted with broth in the following ratios: 10:0, 10:1, 4:1, 3:2, 1:1, 2:3, 1:4, and 1:10. To the coagulase-negative strain D supernatant dilutions, 2 × 10³ CFU of L-form ATCC 6538 p were added. At 24 hr, all tubes containing supernatant-broth ratios greater than 3:2 had no viable L-forms. When 2 × 10⁴ CFU were inoculated into each of the *S. aureus* supernatant dilutions, at 24 hr no growth was found in tubes containing supernatant-broth ratios greater than 2:3 (Tables 5 and 6).

TABLE 3. *Effect of heat-killed staphylococcal organisms on L-form growth*

Organism[a]	L-form growth[b]	
	24 hr	48 hr
S. albus		
Heat-killed washed	2 × 10⁴	5 × 10⁶
Heat-killed unwashed	2 × 10⁴	6 × 10⁵
Control: live *S. albus*	0	0
S. aureus		
Heat-killed washed	1 × 10⁴	2 × 10⁶
Heat-killed unwashed	6 × 10⁴	1 × 10⁶
Control: live *S. aureus*	0	0

[a] Inoculum of *S. albus* and *S. aureus*, 8 × 10⁴ CFU. Statistically similar results were obtained when L-form strain 212 was tested.

[b] Inoculum of L-form strain ATCC 6538p, 2 × 10³ CFU.

TABLE 4. *Effect of heating staphylococcal supernatant fluid to 100 C on growth of L-forms*

Prepn	L-form[a] growth	
	24 hr	48 hr
S. aureus 80/81 supernatant fluid	0	0
S. albus "D" supernatant fluid	0	0
Noninhibitory *S. albus* supernatant fluid	5 × 10⁶	4 × 10⁶
BHI	8 × 10⁵	6 × 10⁶

[a] L-form strain 212, inoculum 2 × 10⁴ CFU.

Role of *p*H in the inhibition of L-forms. A supernatant fluid of *S. albus* strain D was prepared as in previous experiments, and the *p*H was found to be 6.32. The *p*H of the media used for the preparation of the supernatant fluid had been determined to be 7.15. A 7.5% solution of sodium bicarbonate was added to the supernatant fluid to adjust the *p*H to 7.15. As a control, an equal volume of sterile saline was added to a second tube of supernatant fluid. To each tube and to a broth control, 10⁵ CFU of L-form 212 per ml were added. Supernatant fluid with sodium bicarbonate and supernatant fluid with saline showed no growth of L-forms at 72 hr, whereas the broth control yielded 10⁶ CFU of L-forms per ml.

Effect of addition of amino acids and vitamins on inhibition of L-forms by staphylococcal supernatant fluids. Various amino acids and vitamins were added, one at a time, to portions of heated 80/81 and strain D supernatant fluids. The amino acids used, and the amount of each added (in milligrams per 100 ml), were as follows: L-arginine HCl, 2.10; L-cystine HCl, 1.20; L-histidine HCl, 1.05; L-isoleucine, 2.62; L-leucine, 2.62; L-methionine, 0.75; L-lysine HCl, 3.65; L-phenylalanine, 1.65; L-threonine, 2.38; L-tyrosine, 1.81; L-tryptophan, 0.40; and valine, 2.34. The vitamins added, each in an amount of 100 g/100 ml, were nicotinamide, calcium pantothenate, pyridoxyl HCl, thiamine HCl, riboflavine, and nicotinic acid. Approximately 10⁴ CFU of L-forms were added to each tube, with broth and heated supernatant fluids used as controls. Plate counts at 24 and 48 hr showed

TABLE 5. *Influence of the addition of fresh BHI to heated S. albus supernatant fluid on L-form[a] growth*

Ratio of supernatant fluid to BHI	L-form growth	
	24 hr	48 hr
10:0	0	0
10:1	0	0
4:1	0	0
3:2	10	10^4
1:1	10^3	10^5
2:3	10^2	10^5
1:4	10^5	10^5
1:16	10^4	10^6

[a] Inoculum: 2×10^3 CFU of L-form ATCC 6538 p.

TABLE 6. *Influence of the addition of fresh BHI to heated S. aureus supernatant fluid on L-form[a] growth*

Ratio of supernatant fluid to BHI	L-form growth	
	24 hr	48 hr
10:0	0	0
10:1	0	0
4:1	0	0
3:2	0	0
1:1	0	10^2
2:3	10^2	10^3
1:4	10^3	10^3
1:10	10^4	10^6
0:10	10^5	10^6

[a] Inoculum: 2×10^4 CFU of L-form ATCC 6538 p.

no reversal of inhibition by any of the amino acids or vitamins tested.

Inability of inhibitor to be dialyzed. Flasks were prepared containing 100 ml of BHI in which a sac of dialysis tubing containing 10 ml of sterile BHI was freely suspended. Amounts of 1 ml of 6-hr cultures of *S. albus* strain D, *S. aureus* 80/81, and a noninhibitory *S. albus* strain were inoculated separately into three of these flasks and incubated at 37 C for 18 hr. The liquid from the flasks and from the dialysis tubing sacs was then filtered through 0.45-μm filters. Amounts of 1.8 ml of each filtrate and of BHI were then inoculated with 0.2 ml of a 5×10^4 CFU/ml dilution of L-form 212. Plate counts were made at 24, 48, and 72 hr of incubation. No growth occurred at 24, 48, or 72 hr in the flask filtrates of the D or 80/81 strains. There was no inhibition of growth compared with the broth control from the noninhibitory *S. albus* filtrate or in the filtrates of the material inside the dialysis tubing from all three flasks (Table 7). The dialysis tubing, obtained from Arthur H. Thomas Co., Philadelphia, Pa., is known to restrict a molecular weight of 12,000 or greater.

DISCUSSION

The paucity of isolation of L-forms in vivo is in marked contrast to the relative ease of their in vitro propagation (4, 7). The in vitro interference with both streptococcal and staphylococcal L-form growth by many staphylococci may be a factor in the failure of L-forms to propagate or be detected in vivo.

Most strains of *S. albus* and all strains of *S. aureus* tested exhibited this ability to interfere with the growth of L-forms. The interfering substance has the following characteristics: (i) it is separable from the staphylococcal organism, (ii) it is diffusible through agar, (iii) it is heat stable, and (iv) it is not diffusible through a dialysis membrane. Adjustment of *p*H to neutrality did not affect the activity of the inhibitor. Addition of large proportions of fresh BHI partially reversed the interfering effect.

To our knowledge, this is the initial report of bacterial interference with L-forms. Other studies have demonstrated inhibition of bacteria by products of *S. aureus* and *S. albus* (2, 3, 7–10), but these mechanisms and properties differ from those observed in the present investigation.

TABLE 7. *L-form growth in broth dialyzed against staphylococcal strains*

Prepn	Organism	L-form 212 growth[a]		
		24 hr	48 hr	72 hr
Staphylococcal filtrate	*S. albus* strain D	0	0	0
	S. aureus 80/81	0	0	0
	Noninhibitory *S. albus*	8×10^6	7×10^6	2×10^6
Dialysate	*S. albus* strain D	1×10^7	1×10^7	5×10^5
	S. aureus 80/81	1×10^6	9×10^6	4×10^5
	Noninhibitory *S. albus*	2×10^5	7×10^5	1×10^6
BHI control		1×10^5	2×10^5	3×10^6

[a] Inoculum of L-forms: 10^4 CFU.

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