PREVENTION OF THE EMERGENCE OF DRUG-RESISTANT BACTERIA BY POLYAMINES *

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Since the inception of modern chemotherapy one of the chief barriers encountered has been the emergence of drug tolerant bacteria. The discovery and wide use of sulfonamides, various antibiotics and other chemotherapeutic agents, alone or in combination, has not eliminated this potential barrier.

In this paper we report the prevention of the development of drug-resistant populations of bacteria from various drug-sensitive strains of Staphylococcus aureus and Aerobacter aerogenes. The emergence of resistant cells was prevented in growth systems containing the polyamines, spermine, or spermidine and any one of the following antibiotics namely, streptomycin, penicillin, erythromycin, and to a lesser extent, tetracycline and chloramphenicol. Only the results with streptomycin and penicillin will be described here.

Materials and Methods

Organisms. (a) Staphylococcus aureus (3A), and a derived strain resistant to 100 µg of streptomycin/ml., (b) H.Staph.aureus, strain #1357 sensitive to penicillin, and H.Staph.aureus, strain #1360 resistant to 5 unit/ml of penicillin (obtained from Dr. E. Steers of the University of Pennsylvania Hospital), and (c) Aerobacter aerogenes, and a derived strain, resistant to 100 µg of streptomycin/ml., were used.

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Growth Medium. Staph.aureus (3A) was cultured in a synthetic medium containing 16 amino acids, vitamins and salts (1). The two H. Staph.aureus strains were grown in extract broth containing 0.5% glucose, and A.aerogenes in a minimal salts-glucose medium (2).

General Procedure. An inoculum containing from 1 to 2 x 10^8 cells was added to 5 ml. of the appropriate medium in a 14 x 120 mm test tube. For the staphylococcal strains the inoculated cells were taken from the second daily transplant on extract agar from the stock culture. These cells were suspended in M/15 phosphate buffer at pH 7.7. Cells of A.aerogenes taken from the stock culture were grown twice in salts-glucose medium and washed in pH 7.1 phosphate buffer before use.

All cultures were incubated at 37°. Growth was followed by measuring the increase in turbidity using a Klett-Summerson photoelectric colorimeter with filter #56. The instrument was calibrated against viable plate count and microscopic count for each strain.

Sterile solutions of the antibiotics in distilled water were used. Spermine or spermine phosphate solutions were sterilized by filtration through Millipore disc filters.

For colony counts, pour plates prepared from the synthetic amino acid medium supplemented with 1.5% agar, were incubated at 37°C.

Results

The behavior of various strains of S.aureus (3A) in media containing a) spermine, b) streptomycin, and c) spermine plus streptomycin, is given in Table 1. Spermine alone appears to have little or no delaying action on the growth of these three strains. In streptomycin the resistant strain grows readily and even the sensitive strains produce resistant cultures within 46 hours. The growth of fully resistant cells is not affected by the combined presence of spermine and streptomycin, just one subculture in streptomycin being sufficient to render the cells resistant to this combination. On the other hand cultures of the sensitive strain are prevented from developing into a resistant population when

Table 1.

Prevention of the Emergence of Drug-Resistant Cells from the Drug-Sensitive Population of Staphylococcus aureus (3A) In the Combined Presence of Spermine and Streptomycin, and Their Failure to Affect the Growth of the Streptomycin-Resistant Strain.

Growth System	Drug-Sensitive Normal Strain						Drug-Sensitive strain after 6 subcultures in Spermine 100µg/ml				Drug-Sensitive strain* after one subculture in Streptomycin 100µg/ml			
	Growth Turbidity Readings at Hours of:													
Additions	0	5	28	36	46	>100	0	18	42	>100	0	8	27	47
None	10	20	260				18	315			10	86	305	
Spermine (100 µg/ml)	13	18	188				20	246			18	18	81	224
Streptomycin (100 µg/ml)	13	16	22	37	200		18	27	276		15	48	260	
Spermine + Streptomycin(each 100µg/ml)	15	16	21	17	18	14	20	27	28	24	10	14	38	245

* Similarly, the combined presence of spermine and streptomycin had no effect on the growth of the normal strain after 4 subcultures in streptomycin followed by 6 subcultures in spermine.

spermine is present. Even spermine grown cells are inhibited by spermine and streptomycin together.

Experiments with A.aerogenes yielded results similar to those with S.aureus (3A).

Should spermine exert a bactericidal action, then the viable count of a sensitive inoculum may be sufficiently reduced to deplete the population of resistant mutants. Viable colony counts, however, showed that S.aureus growing in 10 μg spermine/ml alone are not killed but multiply 26 fold in a 9 hour growth period. When growing in 100 μg spermine/ml there was first a 9 hour lag with no increase or reduction in viable count, followed by a 30 fold increase during the second 9 hours. These results show that spermine itself does not exercise a bactericidal action.

Were there only a few bacteria in a sensitive culture comparable in character to those of a resistant culture, these cells should selectively multiply and yield a drug-resistant population in the combined presence of spermine and the antibiotic. That cultures of the sensitive strain do not contain such few cells was readily demonstrated. A mixture of a small number (29) of cells of A.aerogenes resistant to streptomycin and 0.57×10^8 sensitive cells, used as the inoculum in 5 ml of medium, readily yielded a resistant population in the combined presence of spermine and streptomycin. Sensitive cells alone (no resistant cells added) failed to develop in this medium (Table 2).

The penicillin sensitive hospital strain of S.aureus was prevented from emerging as penicillin resistant in the presence of both spermine and 0.1 unit penicillin/ml. While the resistant hospital strain of S.aureus grew readily in a combination of spermine and penicillin it was completely prevented from yielding a population resistant to 50 μg streptomycin/ml in combination with spermine (Table 3).

Experiments to determine the minimal effective doses for S.aureus (3A) showed that 10 μg spermine/ml together with 100 μg streptomycin/ml prevented the emergence of resistant cells. Fifty μg streptomycin with 100 μg spermine/ml produced the same effect on the penicillin resistant hospital strain of S.aureus.

Table 2.

Failure of the Emergence of Streptomycin-Resistant Bacteria from Sensitive Inoculum and the Ready Emergence of Resistant Bacterial Population When Only 28 Resistant Cells were Added to the Inoculum Containing 0.57×10^7 Sensitive Cells/5ml Medium.

Growth System	Drug-Sensitive Normal Strain				Drug-Sensitive Cells + Few Resistant Cells		
	Growth Turbidity Readings at Hours of:						
Additions	0	23	47	73	0	23	47
None	15	138	146	—	16	138	145
Streptomycin 100 µg/ml	15	94	107	—	15	109	150
Spermine 100 µg/ml	15	126	140	—	15	122	147
Streptomycin + Spermine	15	14	14	14	15	122	175

Table 3

Prevention of the Emergence of Drug-Resistant Cells from Penicillin-Sensitive and Penicillin-Resistant Strains of Staphylococcus aureus in the Combined Presence of Spermine and Penicillin or Streptomycin.

Growth System	Staphylococcal Strains	Growth Turbidity Readings at Hours of:					
Additions		0	21	44	68	91	163
None	Sensitive	24	61	161	232		
Spermine (100 µg/ml)	to	24	54	70	90	105	132
Penicillin (0.1 unit/ml)	Penicillin	24	46	89	112	110	123
Penicillin (0.1 unit/ml)+Spermine (100µg/ml)	(#1357)	27	31	31	31	30	33
None	Resistant	21	71	218			
Spermine (100 µg/ml)	to	19	54	144	200		
Penicillin (1 unit/ml)	Penicillin	23	63	265			
Penicillin (1 unit/ml)+Spermine (100 µg/ml)	(#1360)	21	40	137	232		
None	Resistant	25	105	310			
Spermine (100 µg/ml)	to	25	72	162	276		
Streptomycin (50 µg/ml)	Penicillin	24	25	32	93	310	
Streptomycin (50 µg/ml)+ Spermine(100µg/ml)	(#1360)	22	26	28	35	30	31

S.aureus (3A) developed resistance to sulfathiazole and novobiocin, unlike the other antibiotics, in combination with spermine.

Spermidine was as effective as spermine in preventing the development of resistance to streptomycin, but the polyamines putrescine, cadavarine, agmatine and 2-3 diaminopropionic acid were inactive.

In testing the effect of U. V. irradiation on the viability of cultures, it was found that no protection against killing was afforded if the cells were pre-incubated with 100 μg spermine/ml for 40 minutes. In fact, after 5 minutes of irradiation, the viable count of spermine treated cultures was approximately 170 times less than with similar cultures from which spermine had been omitted.

Discussion

The results reported here show that spermine or spermidine in combination with streptomycin, penicillin (or erythromycin) can completely prevent the emergence, from sensitive cells, of populations resistant to the drugs. This effect is not comparable to the usual synergism between two drugs acting together since spermine alone fails to have any bactericidal action on the strains tested and small numbers of resistant cells can survive in medium containing both spermine and streptomycin. Spermine possibly intervenes during development of the resistance mechanism, cells which are already fully resistant, however, are unaffected by the spermine-antibiotic combination.

Following the recent reports that streptomycin exerts its inhibitory action at the ribosome level (3-5) it is interesting to postulate spermine interference at this site. However, since antibiotics of different structures, such as penicillin and erythromycin, act in combination with spermine in a manner similar to streptomycin, final explanation must await future investigations. The results also suggest that the active polyamines may be behaving as antimutagenic agents.

An implication residing in the present observations deserves mentioning. The chemotherapy of cancer, leukemia, and possibly also viral infections and certain other

disease conditions has encountered the phenomenon of drug-tolerance. A reconsideration of the chemotherapy of these conditions from the standpoint of the experience gained here may therefore be required.

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