Development of Resistance to 2,4-Diamino-5(3'4'-Dichlorphenyl)-6-Methylpyrimidine (SK5265) by *Streptococcus faecalis*.* (20171)

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The discovery by Hitchings *et al.*(1) of the inhibitory effect of a series of 2,4-diamino pyrimidines on *L. casei* led to their screening for anti-tumor effects in experimental animals. Clarke *et al.*(2) first noted the inhibitory action of these compounds on Sarcoma 180 in mice, and since that time they have also been shown to have an effect on various strains of transmitted mouse leukemia(3). Certain of these compounds have potent antimalarial activity(4) and cause many of the symptoms of antifolic toxicity in experimental animals(3, 5). The administration of 2,4-diamino-5-(3'4'-dichlorphenyl)-6-methylpyrimidine (SK 5265) to both dogs(6) and patients(7) causes a megaloblastosis in the bone marrow. With these compounds remissions are achieved in a certain percentage of children with acute leukemia(7). Since acute leukemia eventually develops resistance to all forms of therapy now known it was felt worthwhile to study the development of resistance to this new agent. In order to provide another system for the study of resistance to SK 5265, attempts were made to develop an SK 5265-fast strain of *Streptococcus faecalis* (ATCC 8043), an organism ordinarily requiring folic acid and very sensitive to the inhibitory action of SK 5265(8).

Method. Streptococcus faecalis (ATCC 8043) was grown for 18 hours at 37°C on Difco Folic Acid Assay broth in the presence of 1 mγ ml of pteroylglutamic acid (PGA). When inoculated into similar broth containing the same amount of PGA and concentrations of SK 5265 varying from 3-300 mγ/ml, there was not half maximum growth in any of the tubes, but there was slight growth just visible after 48 hours in tubes containing 3, 10, 30 and 100 mγ/ml. Subcultures from the tube with the highest concentration of the drug were made into another set of tubes containing varying concentrations of the compound by a technic somewhat similar to those used to develop resistance to amethopterin in *S. faecalis*(9) and *Leuconostoc citrovorum* (10).

Results. A culture from the tube containing 100 mγ/ml of SK 5265 was inoculated into tubes containing 30, 100 and 300 mγ/ml, but even after 96 hours no growth occurred in concentrations above 30 mγ/ml. On the third transplant generation, however, growth occurred at 100 mγ/ml and on the fourth at 300 mγ/ml. As can be seen from Fig. 1, resistance to SK 5265 appeared very gradually and in a step-wise fashion. On the fourth transfer some growth occurred at 300 mγ/ml but from there to the 12th transfer no further increase in resistance was achieved. By the 16th transfer some growth occurred at 3 γ/ml. After a total of 54 transfers, growth occurred in 20-24 hours in liquid medium containing 1 mγ/ml of PGA and 100 γ/ml of SK 5265, and the organism is now being maintained on such a medium. In more quantitative experiments half maximum growth (HMG) occurred in the presence of 45 γ/ml of SK 5265 whereas with the parent organism HMG had occurred at 0.2 mγ/ml. No morphologic differences between the sensitive and resistant variants could be detected in Gram stained preparations. The resistant organism, *S. faecalis*/D, however, had lost its requirement for added PGA and grew on Difco Folic Acid Assay Medium without supplementary PGA in the presence or absence of SK 5265. This phenomenon has been confirmed in a second experiment in which resistance to SK 5265 was again induced in the parent strain of *S.*

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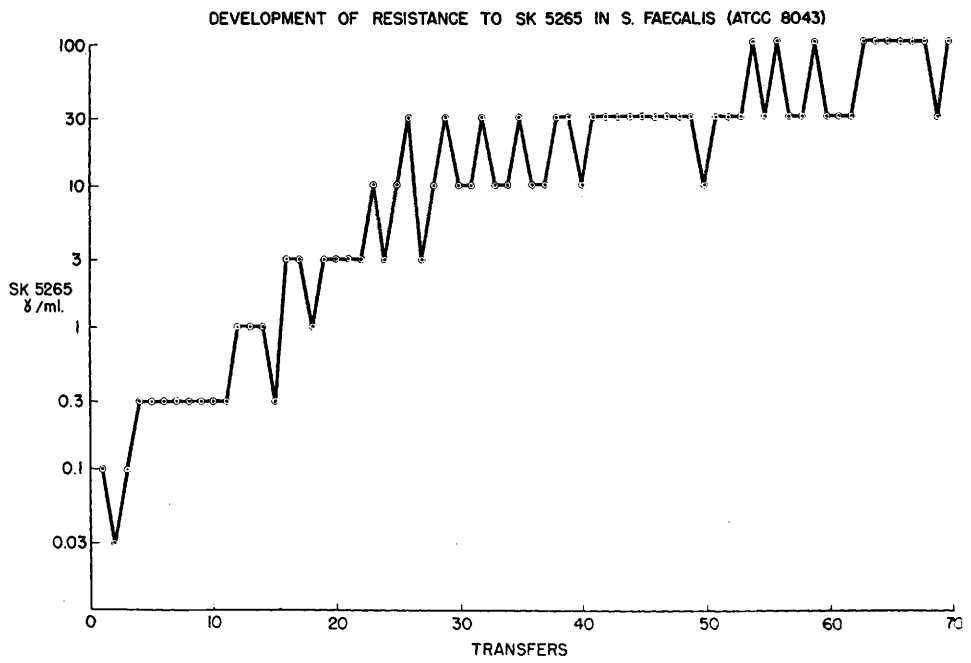

FIG. 1.

faecalis. After 13 passages in increasing concentrations of SK 5265 the organism was resistant to 3 γ/ml SK 5265 and would also grow on a Difco Folic Acid Assay Medium with no PGA or SK 5265 added.

When the parent strain of *S. faecalis* (ATCC 8043), an amethopterin resistant strain *S. faecalis*/A, (maintained in a medium containing 100 γ/ml amethopterin and no PGA), and the SK 5265 resistant strain *S. faecalis*/D, were studied for their sensitivity to amethopterin and SK 5265, by the technic of half maximum inhibition (HMI) of growth, the results presented in Table I were obtained. From this it can be seen that in the strain resistant to amethopterin there was an increase in resistance to this drug some 3 million fold, and a cross-resistance to SK 5265, approximately 250 fold. When the organism that had been made resistant to SK 5265 was compared to the parent strain, however, it was noted that there was a 225,000 fold increase in resistance to SK 5265 and a cross-resistance to amethopterin of approximately 10,000 fold.

Discussion. The development of resistance to SK 5265 by *S. faecalis* is analogous to the resistance developed to amethopterin(9). The amethopterin resistant strain (*S. faecalis*/A) maintains the same requirement for CF but a somewhat smaller requirement for PGA (11). This requirement can also be met by many of the compounds that for the parent strain behave as antagonists of PGA(9,11). Although it is impossible to state with certainty at the present time whether this is due to the utilization for growth of these compounds or to a slight contamination of these compounds with PGA or related growth promoting substances, the latter possibility seems more likely. Resting cells of this strain produce 85-250 times as much CF from a given quantity of PGA as do resting cells of the parent sensitive strain of *S. faecalis*(12). The strain of *Leuconostoc citrovorum* made resistant to amethopterin(10), however, maintained the same CF requirement as the sensitive parent strain, and was unable to utilize PGA any more efficiently than the parent strain. Even though a 2000 fold increase in resistance to amethopterin was achieved, the cross-resistance to SK 5265 increased only 10 fold(10). The SK 5265 resistant strain (*S. faecalis*/D), however after 13 generations of exposure to SK 5265 in the presence of PGA developed the ability to grow on Difco

TABLE I. Effect of Amethopterin and SK 5265 on 3 Strains of *S. faecalis*.*

	mγ/ml	
	Amethopterin HMI	SK 5265 HMI
S. faecalis 8043	.1	.2
S. faecalis/A	300000	50
S. faecalis/D	1000	45000

* All media contained PGA at 1 mγ/ml.

Folic Acid Assay medium without either PGA or SK 5265. It also has been observed that *S. faecalis*/D will grow in 18 hours to half maximum growth or more on certain other PGA deficient media in the absence of added PGA. On these same media *S. faecalis* will not grow at all in the 18 hour incubation period. It has been suggested previously in *S. faecalis*(8) and in mice(3) that SK 5265 acts at a higher level than amethopterin in the folic acid citrovorum factor metabolic pathway.

Preliminary experiments indicate that *S. faecalis*/D has become very efficient in the formation of CF from PGA. This alteration along with the greatly decreased PGA requirement indicates that the development of resistance to SK 5265 has followed the same pattern as the amethopterin resistance in *S. faecalis*. This resistance, however, appears to be more outstanding in one part, *i.e.*, the greater decrease in the PGA requirement of this culture. Biochemical studies are underway at the present time to further elucidate these mechanisms.

Summary. 1. By growing *S. faecalis* (ATCC 8043) in a liquid medium containing successively higher concentrations of 2,4-diamino-5 (3′4′-dichlorphenyl)-6-methylpyrimidine (SK 5265), a thousand fold increase in resistance as evidenced by complete inhibition of growth and a more than two hundred thousand fold increase in resistance as demonstrated by the half maximum inhibition technic has been achieved. 2. No morphologic differences between the sensitive *S. faecalis* (8043) and the resistant variant could be detected. 3. The resistant organism has lost its requirement for supplementary PGA in Difco Folic Acid Assay Medium and certain other PGA deficient media.

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