detected by quantitative precipitin technics. 7. An attempt to demonstrate univalency of the type-specific mouse-protective antibody failed.

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Development of Resistance to 4-Amino-N^{10}-Methyl Pteroylglutamic Acid (Amethopterin) by *Leuconostoc citrovorum*.* (19676)

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In children with acute leukemia, whose disease initially responds well to treatment with 4-amino-N^{10}-methyl pteroylglutamic acid (Amethopterin), there develops in the leukemic process, after 6 to 18 months, refractoriness to therapy(1-4). The elucidation of the mechanisms through which this resistance is established would be of extreme importance to the practical chemotherapy of this disease, and hence have been studied in several different systems. The induction of resistance to amethopterin in 2 strains of mouse leukemia has been demonstrated(5,6). The development has also been reported of an amethopterin-fast strain of *Streptococcus faecalis* which, in the absence of amethopterin, maintained its quantitative requirement of pteroylglutamic acid (PGA), but was capable of establishing maximum growth in media containing amethopterin, 4-amino PGA (aminopterin), and 4-amino pteroyl aspartic acid (amino-an-fol) in the absence of PGA(7,8). Studies with this resistant *S. faecalis*(9) have given some insight into the mechanism of the resistance, as well as the role of PGA and citrovorum factor (CF) in the process of cell metabolism.

Since CF has been shown to be a more direct competitor of aminopterin and amethopterin in *S. faecalis*(10), mouse leukemia (11), and human leukemia(12), it was thought again that a biological system specifically requiring CF might be of value for further study of amethopterin resistance.

Method. *Leuconostoc citrovorum* (ATCC No. 8081) was grown for 18 hours on the CF assay medium of Sauberlich and Baumann (13) in the presence of 1 mγ/ml of leucovorin (synthetic CF Lederle),‡ the culture was centrifuged and washed once with saline and inoculated into the same medium with amounts of amethopterin ranging from 0-500 mγ/ml and incubated at 37°C. In 24 hours there was half maximum growth at 50 mγ/ml

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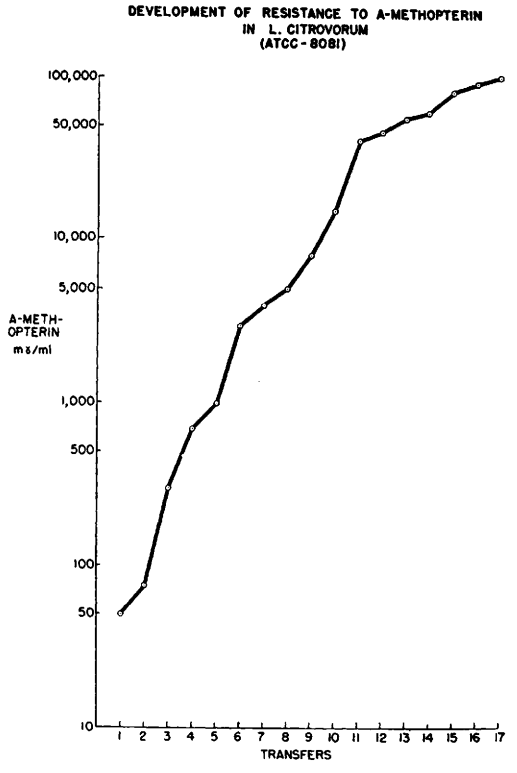

FIG. 1.

with maximum growth at the 0, 5, and 25 mγ/ml levels. After 7 days of incubation there was still no growth in the tubes containing 100 mγ/ml. After 6 days, tubes containing 25, 50, and 75 mγ/ml of amethopterin and 1 mγ/ml of leucovorin were inoculated from the 50 mγ tube and good growth was observed in 24 hours. This procedure was followed using one drop of the culture containing the highest concentration of amethopterin to inoculate 5 ml of fresh medium as soon as possible after growth appeared. Thus, through 17 transfers covering a period of 51 days a culture with a 2000-fold increase in resistance to amethopterin was selected. Comparative studies with the parent strain have been carried out both in the presence and absence of leucovorin.

Results. The gradual development of resistance as shown in Fig. 1 indicates a stepwise resistance pattern very similar to that of *S. faecalis*(7), even though slightly different methods were used in the induction of resistance. The growth rate of this amethopterin-fast organism, designated *L. citrovorum*/A, was approximately half that of the parent strain when high concentrations of amethopterin were used, *i.e.*, the growth obtained in 48 hours with the resistant culture was of the same magnitude as that of the parent strain in 18 hours. Therefore, it was arbitrarily decided to maintain the culture in a medium containing 20 γ/ml of amethopterin and 1 mγ/ml of leucovorin on which maximum growth was obtained in 18-24 hours. However, when small inocula were used, *i.e.*, one drop of a 1-100 dilution of a 20-hour culture, a 48-hour incubation period was found to give more reproducible results.

The requirement for CF (leucovorin) by the *L. citrovorum*/A has not changed from that of the parent sensitive strain, half maximum growth was obtained on 0.15 mγ/ml of leucovorin. Approximately the same amounts of PGA, 10-20 γ/ml, and N^{10}-formyl PGA, 50 γ/ml, were required for half maximum growth of each culture. When tested over a range of from 0.1 mγ to 1 mg per 2 ml of culture medium with prolonged incubation, and in the absence of leucovorin, none of the following compounds could be utilized for growth by *L. citrovorum*/A, amethopterin, aminopterin, amino-an-fol, 9-methyl PGA, 10-methyl PGA, 9,10-dimethyl PGA, or 4-amino-9, 10-dimethyl PGA.

A slight (10-fold) increase in resistance to 2,4-diamino-5(3′4′dichlorphenyl) 6-methyl pyrimidine (SK 5265), was noted (Table I) when both half maximum inhibition and complete inhibition levels were compared. This pyrimidine has been shown to have properties somewhat similar to amethopterin(14-17).

The difference between these 2 strains, therefore, seems to be manifest in the amethopterin resistance and a slight increase in resistance to SK 5265. Morphologically the 2 cultures appear to be identical and the inhibitory action of 5 antibiotics is also similar. Complete inhibition of each strain was obtained with 1.5 γ/ml of penicillin, 5 γ/ml of chloromycetin, 150 γ/ml of streptomycin; with aureomycin and terramycin, 500 γ/ml did not inhibit either strain.

Discussion. The development of resistance to amethopterin by *L. citrovorum* is somewhat

TABLE I. Inhibition of 2 Strains of *Leuconostoc citrovorum* by SK 5265.

CF (mγ/tube)*	mγ/tube of SK 5265	
	H.M.I. × 1000	Complete inhibition × 1000
	L. citrovorum 8081	
1	.5	3
100	5	20
	L. citrovorum/A	
1	5	30
100	50	100

* Total vol/tube was 2 ml.

comparable to that of *S. faecalis*(7). So far, however, it appears to be more related to that of penicillin(18), chloromycetin(19), aureomycin(18), and terramycin(20), since the resistance has not yet affected the physiology of the organism other than its ability to grow in the presence of extremely high concentrations of amethopterin. Unlike streptomycin resistance(21), the development of resistance to amethopterin took place over a long period of time and in a stepwise fashion. It differs from the amethopterin-fast *S. faecalis* in that *L. citrovorum*/A has not yet acquired the ability to utilize amethopterin, aminopterin, or amino-an-fol.

Summary. 1. A two thousand-fold increase in resistance to amethopterin has been induced in *L. citrovorum.* Since growth was slow at this high concentration of amethopterin, the culture was maintained on a medium with 400 times the concentration of amethopterin tolerated by the parent culture. 2. This amethopterin-fast variant has been maintained in the above medium for more than 5 months without any observed physiological or morphological changes.

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