The Effect of A-Methopterin on Sensitive and Resistant Leukemic Cells in Vitro*

J. KIELER† AND E. KIELER†

(Chemotherapy Service, Memorial Center for Cancer and Allied Diseases, the Division of Experimental Chemotherapy, Sloan-Kettering Institute, and the Sloan-Kettering Division of Cornell University Medical College, New York, N.Y.)

The effect of folic acid antagonists on leukemia in man and in mice has been frequently reported. The eventual failure to inhibit the growth of the malignant cells with these compounds has been interpreted as a manifestation of resistance. In mice this course of the disease has been shown to be due to a selection of spontaneously resistant mutants (12). This selective effect of folic acid antagonists has been utilized in the development of resistant strains of mouse leukemia (3, 13). No morphological differences have been demonstrated between sensitive and resistant cells, and the biochemical differences, which in all probability exist, are mostly unknown. In the literature dealing with this problem consideration has been given to the possibility of (a) a decreased permeability of the resistant cells to the drug, (b) an increased detoxification of the antagonist, and (c) metabolic differences between sensitive and resistant cells.

In an attempt to elucidate these problems the effect of A-methopterin (4-amino-N³ -pteroylglutamic acid) on sensitive and resistant leukemic cells in vitro was examined.

EXPERIMENTAL

The experiments were carried out with the highly malignant leukemia, line I, and its subline I/A. This “blast” leukemia, originally described as lymphoecytic (14), is carried in the original strain of mice, C58, but is transplantable to other strains. It generally kills the host in less than 1 week. However, considerable prolongation of the survival time or complete cures can be obtained by treating the transplanted animals with folic acid antagonists. Resistance toward various antimetabolites has been developed in this strain of leukemia, as in others, by Dr. Burchenal and his group. Subline I/A has been carried for several generations in C58 mice, and shows complete resistance to A-methopterin.

Spleen tissue from C58/M mice inoculated with leukemia line I or subline I/A was cultivated according to the following technic. Embryonic mouse heart fibroblasts were grown on coverslips attached to the flattened wall of roller tubes. When large growth areas had developed, the explants were separated from the zones of proliferating cells and removed. In the resulting central defects small fragments of spleen tissue were fixed by means of a plasma clot. After the replacement of the coverslips in the roller tubes, 1 ml. of fluid medium was added to each tube. The medium was composed of Gey’s solution, horse serum, human placent serum, and chick embryonic extract in the proportion of 4:3:1:2. Small amounts of penicillin and streptomycin were added.

In the course of 24-48 hours the empty space between the spleen explant and the inner border line of the fibroblast capsule is seen to be filled with proliferating leukemic cells. These will eventually infiltrate the fibroblast capsule, and those which penetrate the capsule undergo rapid disintegration. In the leukemic growth zone between explant and capsule the dominating cell type is a fairly large, round cell with basophilic cytoplasm and large, round nucleus rich in chromatin and nucleoli. These cells correspond very well to the leukemic cells seen in smear preparations from the same spleen. They are the first cells to appear in the inner growth zone, and in well growing cultures are much more numerous than the second type of cells to be described. The latter are very large, elongated cells with big oval nuclei with two or three nucleoli. These cells are basophilic too, but do not stain so deeply as the leukemic cells...
cells. They resemble large fibroblasts, but are not found in the capsule before this is infiltrated by spleen cells. During mitosis they are clearly distinguishable from the mitotic leukemic cells because of their size. This type of cell is presumably derived from the reticulum of the spleen, and they will be referred to as reticulum cells.

When the cultures grow old, the fibroblasts of the capsule invade the leukemic growth zone, just as the cells of the latter to an increasing extent infiltrate the fibroblast tissue. It then becomes more and more difficult to differentiate between the three cell types during mitosis. However, the clear distinction between fibroblast capsule and leukemic growth zone can be restored by transplantation of the spleen tissue to a new capsule. We thus have the advantage of the fibroblast support, which has been shown to be essential for the growth of leukemic cells in vitro (1), and at the same time a pure zone of spleen cells in which the leukemic cells can easily be recognized even during mitosis. The main difficulty is to choose the right size of the leukemic explant. If this is too small, the proliferating cells deteriorate before they fill the intracapsular space and obtain the necessary support from the fibroblasts. If, on the other hand, the explant is too large, the inner growth zone is so crowded with cells that satisfactory preparations for microscopic examination cannot be obtained.

Long-term experiments can be carried out by this technic. In a preliminary experiment even after 3 months of cultivation no decrease could be detected in the ability of these cultures to cause leukemia, when injected into mice intraperitoneally. In such long-term experiments the fluid medium was renewed 2-3 times weekly, and once weekly the spleen tissue was transferred to a new culture with a new fibroblast capsule. The growth of the cultures generally improved after one to two passages, when all the erythrocytes had disappeared.

In order to investigate whether or not cultivation in vitro would influence the sensitivity of the leukemic cells to A-methopterin (C58 × BALB)F1, mice were given inoculations of sensitive and resistant cells which had been grown in vitro for one to four passages. Two cultures were injected intraperitoneally into each of two mice. When leukemia developed, the spleens from these mice were homogenized and suspended in saline, and dilutions containing ten million cells per ml. were prepared. A larger number of animals were then inoculated intraperitoneally with 0.1 ml. of the diluted suspension. One half of the mice was treated with A-methopterin, 3 mg/kg body weight every other day, while the other half was kept as untreated controls. After a maximum of ten injections treatment was discontinued. The results of the experiment as presented in Table 1 are average values for twenty mice. It is seen that cultivation per se did not significantly influence the sensitivity of the cells.

The effect of A-methopterin on the leukemic cells in vitro was studied by exposing cultures to various concentrations of the drug for 4 hours, fixing them in methyl alcohol, and staining them with May-Grunwald-Giemsa stains. The number of mitoses per one thousand nondividing leukemic cells (mitotic coefficient) was determined for each culture. The average values for four to six cultures are presented in Table 2.

Inhibition of the mitotic activity was seen in
the sensitive cultures at a concentration of 0.01 mg/ml, while in the resistant cultures no inhibition occurred at concentrations below 1 mg/ml. No significant change in the distribution of the mitotic phases was observed. Apart from differences in the mitotic activity no differences were observed between the two strains. At concentrations below 1 mg/ml, most of the cells looked normal. Signs of cytoplasmic degeneration were not conspicuous, and pyknosis and other nuclear abnormalities were rarely seen. Abnormal chromosomes did not occur. At 1 and 10 mg/ml, a considerable shrinking of all cells in both strains was the only detectable sign of intoxication. Apart from a few mature leucocytes, which disappeared from the cultures in the course of a day or two, signs of cell differentiation were not seen in either treated or untreated cultures.

These results seem to indicate that A-methopterin is a specific inhibitor of mitosis, while a direct, damaging effect on the cell is only caused by excessively high doses. In order to substantiate this impression the effect of A-methopterin on the cells of the intestinal crypts in mice, treated with aminopterin, and he concluded that an inactivation of the antagonist had taken place. A spontaneous recovery was observed when the compound was withdrawn, although some arrested metaphases were still seen 17 hours later. The effects described by Burchenal were observed after 15 minutes to 4 hours of treatment. However, cultures exposed to the inhibitor for 4 hours or more resumed their activity. Consequently, this author believed that an inactivation of the antagonist had taken place.

Since the development of resistance might be explained by an increased detoxification of A-methopterin, the concentration of this drug in the medium was measured after 1, 2, 4, and 6 days of incubation, using the technic described by Burchenal et al. (4). Samples of 0.5 ml of the fluid medium were withdrawn from each roller tube containing four explants and diluted with equal amounts of distilled water. After boiling, 0.09 ml of each sample was delivered to filter paper discs placed on agar plates inoculated with S. faecalis (ATCC No. 8043). The inhibition of bacterial growth was determined after 24 hours of incubation. Each experimental group contained two roller tubes, and duplicate determinations were carried out in all cases. The A-methopterin concentration was computed by comparison with a standard curve based on controls run simultaneously as described by Burchenal et al. (4). The results are presented in Table 3. The initial concentrations of A-methopterin in these experiments were 50 mg and 25 mg/ml. No significant decrease in these concentrations was found in any of sixteen sensitive and sixteen resistant cultures even after 6 days of incubation.

### DISCUSSION

The present experiments show that A-methopterin acts principally as a mitotic inhibitor, while the direct, damaging effect on the cells is only of moderate intensity. Mitotic inhibition by folic acid antagonists has previously been described in other types of cells in vivo as well as in vitro. Gunz (6) observed that aminopterin produces marked mitotic inhibition of human leucemic cells in vitro. Hughes (7) found that dividing chick fibroblasts were arrested at metaphase by A-methopterin and aminopterin. This author also noticed that no spindle was formed in the treated cells. Using a similar material Jacobson (8) found a decrease of the ratio of anaphases after treatment with aminopterin, and he concluded that this drug interferes with the processes which allow the chromosomes to split. In Hughes' experiments the cultures were exposed to the antagonist for less than 40 minutes. A spontaneous recovery was observed when the compound was withdrawn, although some arrested metaphases were still seen 17 hours later. The effects described by Jacobson were observed after 15 minutes to 2 hours of treatment. However, cultures exposed to the inhibitor for 24 hours or more resumed their mitotic activity. Consequently, this author believed that an inactivation of the antagonist had taken place.

A decreased proportion of anaphases in vivo was observed by Jacobson (8) in the bone marrow of man and mice treated with folic acid antagonists. Dustin (5) described a "radiomimetic" effect on the cells of the intestinal crypts in mice,
and Thiersch (18) observed chromosomal segregation in erythroblasts from human and dog bone marrow.

While the present results confirm the observations of a depressing effect on the mitotic activity, they fail to demonstrate a selective effect on any particular phase. This may be due to the longer exposure to the drug. Thus, the present investigations indicate that in addition to the immediate arresting effect of folic acid antagonists on cells in metaphase, these drugs also have a prolonged inhibiting effect on mitosis. The absence of any phase specificity in long-term experiments suggests an interference with metabolic reactions related to the initial stimulus to division. In this respect leukemic cells may be more sensitive to folic acid antagonists than other cells, since Jacobson (8) found that a variety of cells resumed their mitotic activity when exposed for 24 hours or more.

The absence of morphological signs of a direct, damaging effect of these compounds on the leukemic interphase cell, except at extremely high concentrations, is in agreement with the results of previous in vivo studies on mouse leukemia (9). Biesele (17) made a similar observation studying the effect of antifolies on embryonic mouse skin and sarcoma MA 387 in vitro. In Gunz' (6) experiments there was no indication of any immediate effects of aminopterin on resting cells, other than an inhibition of their power to divide. Also, Hughes (7) noticed that these compounds exert little or no toxic effect on the culture generally. Woll (19) described cytological degeneration and, possibly, disruption of mitotic figures in Rous chicken sarcoma treated with aminopterin; but apart from this observation the absence of any pronounced toxic effect of folic acid antagonists on the interphase cell seems to be a universal feature.

Another manifestation is the persistent transplantability of the cells even after long exposure to a-methopterin. This is in agreement with the absence of a sterilizing effect in vivo as demonstrated by Burchenal et al. (2). Also, these authors found that the incubation of a saline suspension of leukemic cells with 1 mg a-methopterin/ml for 1 hour would not interfere with their transplantability.

These observations indicate that the difference between a-methopterin-sensitive and -resistant cells may be found in the metabolic processes related to mitosis. This conclusion is likely to be correct, provided that the drug is equally absorbed by the two strains and that no differences exist in the detoxification of the compound.

The absence of any demonstrable difference between the sensitive and resistant cells regarding the toxic effect of a-methopterin during interphase as well as their equal transplantability after long exposure to the antagonist indicate that the absorption is the same. In animal experiments the possibility of a detoxification of the antagonist by the host as well as by the leukemic cells has been considered as an explanation of resistance. A detoxification by the host can be ruled out in the present experiments, and the absence of any decrease in the concentration of a-methopterin in the medium during cultivation seems to indicate that no significant detoxification is carried out by the leukemic cells. However, it should be borne in mind that if the absorption of the drug is very slow, an intracellular detoxification may not influence the concentration in the medium in a measurable way even after 1 week's incubation. Although the latter possibility has not been finally ruled out by the present experiments, the results of these favor the idea of metabolic differences between the two cell types. Pointing in the same direction is the development of sublines of leukemia which are not only resistant, but also partially dependent on antifolics (10, 11, 13). It has been shown that a-methopterin causes a significant increase in formate incorporation into nucleic acids of dependent leukemic cells (15). These observations suggest the presence of a qualitative difference in the metabolism of sensitive and dependent lines. However, in the present and other experiments (16) the difference between sensitive and resistant cells was found to be but a relative one.

At the present we have no satisfactory explanation of these findings. It may, therefore, be useful to consider the possible value of a study of the intracellular distribution of folic acid and its antagonists in sensitive and resistant cells. In view of the evidence that a-methopterin acts during interphase, it may be postulated that resistance is due to a decreased nuclear absorption of the drug, either because the latter is bound in an active form in the cytoplasm or because of changes in the permeability of the nuclear membrane. Thus, in sensitive cells a-methopterin would antagonize cytoplasmic as well as nuclear folic acid, while in the resistant cells inhibition only takes place in the cytoplasm, unless the concentration of the antimetabolite is very high. This would explain the above-mentioned quantitative differences between sensitive and resistant cells. Furthermore, it is conceivable that an accumulation of folic acid in the nucleus would follow the inhibition of the utilization of this substance and its metabolites in the
cytoplasm. If the consequent increase in the activity of nuclear folic acid exceeds the total activity of the untreated cell, then the treated cell would appear dependent.

The speculative nature of this thesis is obvious. It proposes, however, that investigations of the intracellular distribution of folic acid, citrovorum factor, and the various antagonists in sensitive, resistant, and dependent cells may yield valuable information.

**SUMMARY**

A method for the cultivation of leukemic cells in vitro is described. The effect of A-methopterin on sensitive and resistant mouse leukemia in vitro was examined by this method. A significant decrease in the mitotic activity was observed at a concentration of 0.01 mg A-methopterin/ml of fluid medium with the sensitive cultures, whereas a similar inhibition of the resistant cultures was only seen at concentrations of 1 mg/ml or more. Cytoplasmic damage was seen only at very high concentrations (1 mg/ml or more), and in this respect no difference was observed between sensitive and resistant cells. When transferred back to mice, sensitive as well as resistant cultures still caused leukemia, even after 1 week's exposure to A-methopterin at a concentration of 1 mg/ml. These results are discussed. Since no decrease in the concentration of the antagonist could be detected in resistant cultures even after 6 days of incubation, it is concluded that resistance is probably related to nuclear metabolism. The hypothesis is advanced that a decrease of the nuclear absorption of the drug may explain the development of resistance as well as of dependence.

**REFERENCES**

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