Effect of Methisazone and Other Drugs on Mouse Hemopoietic Colony Formation In Vitro

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Methisazone (N-methylisatin β-thiosemicarbazone) was shown to inhibit the development of hemopoietic colonies in vitro when incorporated in the culture medium. The activity of methisazone was compared with that of several antimetabolite and antibiotic drugs. Although methisazone reduced the number of colonies, it did not affect the size of those colonies which did develop, and evidence is presented which shows that it acted only at the early stages of colony development. Some implications of these findings are briefly discussed.

It has been shown that the antiviral drug methisazone (N-methylisatin β-thiosemicarbazone) can have an immunosuppressive effect and a suppressive effect upon the response of hemopoietic colony-forming cells (granulocyte-macrophage progenitor cells) to adjuvant stimulation in vivo. The purpose of this paper is to describe the effects of methisazone on hemopoietic colony formation in vitro and to compare these effects with those of some other drugs, in particular with the thymidine analogue 5-iodo-2-deoxyuridine.

MATERIALS AND METHODS

Drugs. Methisazone was provided by D. J. Bauer, Wellcome Research Laboratories, Beckenham, England. For most experiments, the powder was dissolved in dimethylformamide (23 mg in 0.5 ml) added to 900 ml of distilled water. It was redissolved by autoclaving at 10 psi for 10 min, and nine parts were mixed with one part 10 times concentrated Eagle's medium (BHK Eagle's, Burroughs Wellcome, England) to give a concentration of 100 μM in single-strength medium. In some later experiments where a higher concentration was required, the powder was dissolved in 0.1 N sodium hydroxide and diluted to appropriate concentration in tissue culture medium.

Rifampin powder was provided by Lepetit Pharmaceuticals Ltd., Maidenhead, England. 5-Iodo-2-deoxyuridine (IUDR) was obtained from Schwarz BioResearch, Inc.; Chloramphenicol succinate from Park, Davis & Co., and daunomycin from May and Baker Ltd. These drugs were dissolved in sterile normal saline, and further dilutions were made in Eagle's medium.

Escherichia coli endotoxin O111:b4 (Difco) was dissolved in sterile distilled water and further diluted in Eagle's medium.

Bone marrow culture and addition of drugs. Marrow plugs from a single femur of two normal C57BL mice (2 to 3 months old) were flushed into collecting medium (BHK Eagle's supplemented with 10% fetal calf serum and 10% Trypticase soy broth). The colony culture system was essentially that described by Metcalf and Foster (10). In brief, modified Eagle's medium containing 0.3% agar (Difco) was held at 37°C, and bone marrow cells were added to give a concentration of 5 × 10^4 cells per ml. Colony-stimulating factor, which is essential for growth of mouse colonies, was provided by mouse embryo-conditioned medium (4) and was incorporated in the agar medium. Cultures were performed in 35-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.).

In most experiments, 0.1 ml of drug solution was placed in the culture dish, and 1-ml samples of the cell suspension in agar medium were added and mixed before gelling. Cultures were incubated at 37°C in sealed humidified boxes containing 10% CO_2 in air. Colonies were counted by use of an Olympus dissecting microscope, usually after 7 days. In some experiments, cells either were preincubated with drug in collecting medium before agar culture or the drug was added to cultures at intervals after their initiation. These experiments are specifically referred to in Results.

Colony size. Colony size was measured by counting the number of cells per colony at 40 times magnification of the culture. This gives quite accurate and reproducible results provided the size of individual colonies does not exceed 50 to 60 cells (8). All estimates of colony size were based upon the mean number of cells per colony from 30 to 50 sequential colonies in three or four identical cultures.

Cytological examination of colony cells. Colonies were removed onto a glass slide with a fine Pasteur pipette. A glass cover slip was placed over the colonies which were then stained by running 0.6% orcein in 60% acetic acid between slide and cover slip.

RESULTS

Effect on colony number. Figure 1 shows the dose-response effect of methisazone on the
number of colonies in 7-day cultures stimulated by a concentration of conditioned medium which gave maximal stimulation of colonies in control cultures. The points represent the mean colony number in drug-containing cultures, expressed as a percentage of the colony number in control cultures from duplicate plates for each drug concentration in each of five separate experiments. Twofold increases in methisazone concentration caused a gradual decrease in colony number, with 50% colony inhibition occurring at a concentration of 13 μM (3 μg/ml). The solvent, dimethylformamide, when tested alone did not have any effect on colony growth at the concentrations present in the relevant methisazone solutions. The activity of methisazone was then compared with that of several other drugs. Table 1 gives the 50% colony-inhibiting concentrations and shows that with the exception of daunomycin, which was highly active, the activity of methisazone was comparable to that of the other drugs.

Effect on colony size. An interesting aspect of colony inhibition by methisazone was that, although it reduced the number of colonies per culture, it did not reduce the size of those colonies which did develop. Table 2 shows the mean colony size for control and methisazone-containing cultures with several drug concentrations and at different stages of colony development. Great variation in colony size is characteristic of this culture system, which made it difficult to establish significant effects. However, the results show that mean colony cell counts were consistently about the same for control and methisazone-containing cultures, whereas results with other drugs all suggested a decrease in mean colony size. This indicated that methisazone had a more selective mode of action on colony development than the other drugs. To clarify this observation, several other aspects of colony inhibition by methisazone were investigated, and a comparison was made with the effects of IUDR.

Relationship between degree of colony stimulation and drug inhibition. When marrow cells are cultured with different concentrations of colony-stimulating factor, there is a linear relationship between the amount of stimulating factor and the number of colonies obtained (3, 4). With levels of stimulation below maximum, the number of colonies, as a percentage of that obtained with optimal stimulation, is independent of the number of marrow cells per culture (McNeill and Fleming, unpublished data), and it seems likely that the relationship between colony number and degree of stimulation represents a heterogeneity of colony-forming cells in their responsiveness to colony-stimulating factor. The observation that methisazone decreased colony number but not size could be explained if the drug had a selective effect on a subpopulation of colony-forming cells, e.g., on those most responsive to stimulation. This was investigated by measuring the degree of colony inhibition resulting from a constant amount of drug when the amount of colony-stimulating factor was varied. Groups of cultures were set up containing 2.5 μg of methisazone per ml, 1 μg of IUDR per ml (concentrations which were known to give about 50% inhibition with maximal stimulation of cultures), or no drug, and with embryo-conditioned medium in twofold dilution steps. The results of such an experiment are given in Table 3 and show that a greater proportion of colonies were inhibited with the lower levels of colony stimulation. However, Table 3 also shows that IUDR had the same effect, and therefore this selectivity cannot explain the lack of reduction in colony size with methisazone.

Addition of methisazone at different times. Table 4 shows that, when marrow cells were incubated in collecting medium which contained concentrations of each drug known to result in marked inhibition if included in the agar medium, there

![Graph](http://aac.asm.org/Downloadedfromhttp://aac.asm.org/ on November 1, 2017 by guest)

**FIG. 1.** Dose-response effect of methisazone in colony inhibition.

**TABLE 1.** Drug concentrations in medium resulting in 50% inhibition of colony number

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc</th>
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<tbody>
<tr>
<td>Methisazone</td>
<td>3 μg/ml</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>12 μg/ml</td>
</tr>
<tr>
<td>5-Iodo-2-deoxyuridine</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>6 ng/ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>200 μg/ml</td>
</tr>
<tr>
<td>Rifampin</td>
<td>10 μg/ml</td>
</tr>
</tbody>
</table>
was no reduction in the colony-forming potential of the cells. Neither drug had, therefore, any short-term toxic effect on colony-forming cells.

Figure 2 shows the result of an experiment in which methisazone or IUDR was added to cultures during the early stages of the 7-day incubation period. The drugs were placed on top of the cultures in 0.1-ml volumes to give a final concentration of 8 μg of methisazone per ml or 4 μg of IUDR per ml after diffusion into the culture medium. These results show that there was a difference in the effect of the two drugs. Methisazone caused only slight colony inhibition when added 24 or 48 hr after the start of the culture period. Even after 12 hr, there was a slight loss of effect, but there was no significant difference between the inhibition obtained by drug addition at 0, 3, or 6 hr in this or similar experiments. IUDR, on the other hand, gave the same degree of inhibition irrespective of when it was added. The greater colony number obtained after addition of IUDR at 48 hr compared with 12 hr does not indicate an escape from inhibition after addition of the drug, because this represented the number of recognizable colonies in the cultures on the second day of incubation before the drug was added. The IUDR then arrested further colony growth.

This point was confirmed by an experiment in
which cultures were allowed to develop for 3 days, the colonies were counted, and the cultures were divided into three groups which had the following additions: (i) 0.1 ml of saline, (ii) 20 μg of methisazone, and (iii) 5 μg of IUDR. Cultures were incubated for a further 4 days and colonies were then counted. The results (Table 5) demonstrate that IUDR arrested colony development at the 3-day stage, whereas methisazone (in a concentration which would cause approximately 90% colony inhibition if added at the beginning of the culture period) caused only a small reduction in the 7-day colony number.

Effect on colony cytology. Colonies are initially composed of cells of the granulocyte series which predominate in cultures examined after 2 to 3 days of incubation, but by the seventh day of incubation most colonies are made up entirely of macrophages (8, 9). Individual colonies can be classified as granulocyte, macrophage, or mixed (8). Since methisazone was most active in the earlier stages of colony development, its action could be mainly on the granulocyte type of colony. Colonies were analyzed in 3-day cultures partially inhibited by either methisazone or IUDR added at the beginning of the culture period. Table 6 shows that there was no change in the relative frequency of the three types of colony with either drug.

**DISCUSSION**

The experiments described have shown that methisazone has an inhibitory effect on the development of granulocytic-macrophage colonies in vitro. In terms of the concentration required to cause 50% inhibition of colonies (3 μg/ml), it compared favorably with chloramphenicol, rifampin, and 6-mercaptopurine; had about the same activity as IUDR; and was much less active than daunomycin. The activity in vitro corresponded to that previously shown in vivo where a daily dose of 46 μg per mouse
TABLE 6. Cytological classification of 3-day colonies in cultures containing methisazone or IUDR

<table>
<thead>
<tr>
<th>Drug</th>
<th>Colony inhibition (%)</th>
<th>Colonies sampled</th>
<th>Colony type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>0</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Methisazone</td>
<td>54</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>IUDR</td>
<td>70</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

resulted in a significant reduction in the spleen colony-forming cell response to the injection of complete Freund’s adjuvant.

Since this drug has been almost exclusively investigated for its effects on virus replication, reports on its antipellicular activity are confined to those cells used in virus experiments. Magee and Bach (7) found a 65% reduction in thymidine uptake by HeLa cells at 50 μg per ml, Munroe and Sabina (11) reported an arrest of MDBK cell division after the third subculture in medium containing 20 μg per ml, and Cho et al. (5) stated that 200 μg was toxic to CV-1 cells. The results of the present experiments show that the hematopoietic colony-forming cells are more sensitive to inhibition by methisazone than these continuous cell lines. It is interesting to note that the dose response effect for methisazone in colony inhibition (Fig. 1) is remarkably similar to that shown for the inhibition of several ribonucleic acid viruses (2).

In contrast to the action of several other drugs, reduction in colony number by methisazone was not accompanied by a reduction in the number of cells per colony (Table 2), and this suggested an action more selective than one involving interference with cell division in a manner applicable to all cells. Selectivity was shown to occur in two ways: (i) those colony-forming cells responding to low levels of colony-stimulating factor were more susceptible to drug inhibition (Table 3), and (ii) methisazone was most active in the earliest stages of colony development (Fig. 2). In these experiments, methisazone was compared with IUDR because IUDR is known to cause arrest of division in a very wide variety of cellular systems and it appeared to reduce colony size also as well as colony number. Like methisazone, IUDR also had a greater inhibiting effect on those colony-forming cells responding to low concentrations of colony-stimulating factor, but, unlike methisazone, it arrested colony development when added to cultures after 2 or 3 days of incubation. The most plausible interpretation of these results is that most colonies are initiated during the first 24 hr of the culture period, and that methisazone has a selective action at this stage. Once the initial steps have been taken, and even though there are no recognizable colonies at that stage, the drug has no effect on subsequent colony development, either in terms of the rate of cell division or morphogenesis.

These results, taken with those reported previously, may have important implications in the search for more satisfactory immunosuppressive or antileukemia agents. They indicate the possibility of using drugs to interfere with specific points in cellular differentiation rather than, as at present, using agents with a rather indiscriminate action on cell division which necessarily involve dangerous side effects. Some short-term clinical experience with oral methisazone has been obtained (1, 6, 12, 13), and the most commonly observed side effect was a variable tendency to cause nausea and vomiting. Although more experimental work is required with methisazone and related compounds before clinical application can be confidently predicted, the present results indicate some interesting possibilities and emphasize the value of the bone marrow culture system in their elucidation.

ACKNOWLEDGMENTS


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LITERATURE CITED


