Inhibition of Nucleic Acid and Protein Synthesis in Mouse Spleen Cells In Vitro by Azathioprine

GRACE Y. CHAN AND ROBERT L. STONE

Biological Research Division, Eli Lilly Research Laboratories, Indianapolis, Indiana 46206

Received for publication 20 August 1970

The effect of azathioprine on macromolecular biosynthesis was studied in mouse spleen cells cultured in vitro. The rate of incorporation of $^3$H-thymidine, $^3$H-uridine, and $^3$C-leucine into acid-insoluble material was used to measure deoxyribonucleic acid, ribonucleic acid, and protein synthesis. Results indicate that azathioprine inhibited nucleic acid and protein synthesis at levels which did not decrease cell viability.

Azathioprine [6-([1'-methyl-4'-nitro-5'-imidazolyl]-thiopurine] like its parent compound 6-mercaptopurine is an immunosuppressive agent and has been found to be effective against a variety of transplantable rodent tumors and leukemias (1). In the suppression of the formation of antibody in mice to sheep red blood cells, azathioprine has been found to be superior to 6-mercaptopurine as determined by inhibition of hemagglutinating titers (6).

Azathioprine is an antimetabolite and interferes with purine metabolism. The metabolism of azathioprine has been investigated in mice and in man (2, 3). However, in vitro studies of its effect on the various cells involved in the immune response are lacking.

The present communication describes the inhibitory effect of azathioprine on deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis by mouse spleen cells in vitro.

MATERIALS AND METHODS

Preparation of spleen cell suspensions. Methods for the preparation of spleen cell suspensions were essentially as described by Mishell and Dutton (5). Spleens were removed aseptically from Swiss white mice weighing between 15 and 20 g. They were washed once with Eagle's Minimum Essential Medium (MEM). Glassware precoated with silicon was used throughout the experiments. The cells were teased out from the spleens in MEM with the aid of sterile forceps. Cell suspensions were centrifuged at 200 × g for 10 min, the supernatant was discarded, and the cell pellet was resuspended in MEM supplemented with 10% inactivated fetal calf serum. The number of cells was determined by counting in a hemacytometer after elimination of erythrocytes with 0.1 M HCl. The cell suspensions were gassed with 5% CO₂ and incubated in a water bath at 37 C.

Solubilization of azathioprine. Azathioprine (Imuran, Burroughs Wellcome & Co., New York, N.Y.) was solubilized in dimethylsulfoxide (DMSO). Solutions of DMSO containing different amounts of azathioprine were added to cell suspensions (15 X 10⁶ cells per ml of culture) to a final concentration of 2% DMSO.

Cytotoxicity tests. Levels of azathioprine solubilized in DMSO were added to mixed samples of cell suspensions as described above and incubated at 37 C. At intervals, samples of cells were removed to determine their cell count per milliliter of culture and viability. Cell counts were performed in a hemacytometer with appropriate dilutions in phosphate-buffered saline (PBS, pH 7.4)-0.1 M HCl, and the viability of the cells was determined by a dye-exclusion procedure with trypan blue (0.4%). Controls included cell suspensions to which either PBS alone or DMSO (2% final concentration) was added.

Incorporation of precursors. The precursor, $^3$H-thymidine, $^3$H-uridine, or $^3$C-leucine (all at 0.2 μCi/ml), was added to the cell suspensions. Cells were next incubated with drug solutions containing radioactive precursor at 37 C. At different time intervals up to 2 hr, a fixed volume of cells from duplicate tubes was removed and immediately chilled in ice to stop further incorporation. The cells were then centrifuged at 200 X g for 10 min and were washed once with Hanks balanced salt solution. They were resuspended in distilled water to achieve cell disruption. Cold trichloroacetic acid was added to a final concentration of 10%. The solution was mixed well, and the precipitate was collected by centrifugation at 450 X g for 10 min. It was rewashed in cold 5% trichloroacetic acid and solubilized in 0.5 ml of NCS (Nuclear-Chicago Corp., Des Plaines, Ill.). The amount of radioactivity was then counted in a toluene-based scintillant, and the results were extrapolated to disintegrations per minute.

RESULTS

Results summarized in Table 1 show that at 2- or 0.2-mg concentrations azathioprine did
TABLE 1. Determination of viability of mouse spleen cells treated with azathioprine incubated at 37°C

| Treatment of cells | Expt. no. | Per cent of viable cells | Total no. of cells per 0.5 ml |
|-------------------|-----------|--------------------------|----------------------------|
|                   | 0.5 hr | 1 hr | 2 hr | 0.5 hr | 1 hr | 2 hr |
| 2 mg of azathioprine in 2% DMSO<sup>b</sup> per ml of culture | I | 91 | ND<sup>a</sup> | 73 | ND | ND | ND |
|                   | II | 80 | 77 | 79 | 7.3 x 10<sup>4</sup> | 8.1 x 10<sup>4</sup> | 7.0 x 10<sup>6</sup> |
| 0.2 mg of azathioprine in 2% DMSO per ml of culture | I | 92.5 | ND | 93 | ND | ND | ND |
|                   | II | 79 | 77 | 81 | 8.4 x 10<sup>4</sup> | 8.4 x 10<sup>6</sup> | 6.6 x 10<sup>6</sup> |
| 2% DMSO control | I | 91 | — | 93 | 8.5 x 10<sup>5</sup> | ND | 9.5 x 10<sup>6</sup> |
|                   | II | 83 | 86 | 72 | 7.2 x 10<sup>6</sup> | 9.8 x 10<sup>6</sup> | 7.4 x 10<sup>6</sup> |
| PBS<sup>c</sup> control | I | 96 | ND | 88 | 9.2 x 10<sup>4</sup> | ND | 8.4 x 10<sup>6</sup> |
|                   | II | 85 | 79 | 84 | 7.4 x 10<sup>4</sup> | 8.8 x 10<sup>6</sup> | 7.4 x 10<sup>6</sup> |

<sup>a</sup> Not done.
<sup>b</sup> Dimethylsulfoxide.
<sup>c</sup> Phosphate-buffered saline (pH 7.4).

not cause significant cell death by the criteria of viability and cell count. By viability tests, 2% DMSO in the culture was not toxic (Table 1); however, in the 2% DMSO control group, there was a consistently lower rate of incorporation (about 10 to 25% lower than in the PBS control group). Since azathioprine was dissolved in 2% DMSO, incorporation rates are compared with those of the 2% DMSO control group and are expressed as percentages of this value. Inhibition of incorporation of <sup>3</sup>H-thymidine (Fig. 1) was noted with 0.2-mg of azathioprine per ml of culture after 30 min of incubation. Here, incorporation was 80% of the DMSO-treated control, decreasing slowly to 76% at 1 hr and to 70% at the end of 2 hr. The effect of the drug at the 2-mg concentration was more prompt and pronounced, reducing the rate to 13% of the DMSO control at 30 min, 8% at 1 hr, and 5% at 2 hr.

The rate of <sup>3</sup>H-uridine incorporation (Fig. 2) showed that 0.2 mg of azathioprine per ml of culture had no pronounced effect even after 2 hr of incubation. Incorporation was 89% of the DMSO control after 30 min of incubation time, 100% at 1 hr, and 92% by 2 hr. At the 2-mg concentration the drug reduced <sup>3</sup>H-uridine incorporation to 41% of the control at 30 min, 34% at 1 hr, and 26% after 2 hr of incubation.

The effect of azathioprine at the 0.2-mg concentration on protein synthesis was somewhat more marked than that on RNA synthesis (Fig. 3). The rate of incorporation of <sup>14</sup>C-leucine as compared to the DMSO control was 98% at 30 min, falling to 89% at 1 hr and 74% at 2 hr. At the 2-mg level, the extent of inhibition was similar to RNA incorporation but less marked than that of DNA incorporation; the amount of trichloroacetic acid-precipitable protein was 47% of the control at 30 min, 27% at 1 hr, and 22% at 2 hr.
inhibits ineffective.

has critical cell to sulfhydryl compounds, antibody administered portedly DMSO. spleen consequently antibody persists, suggesting to antibody antigen injection on of 6-mercaptopurine (4). Mercaptopurine be purinemetabolism, beattained. With antigeninjection of action metabolism. The exact site of the inhibitory action of azathioprine is not resolved by results reported here.

Results reported here indicate that azathioprine inhibits DNA, RNA, and protein synthesis by mouse spleen cells at doses which do not decrease cell viability. At 0.2 mg, azathioprine appears to affect DNA synthesis most, protein to a lesser degree, and RNA least. At a concentration of 2 mg/ml, the effect on DNA synthesis is much more pronounced than on both RNA and protein synthesis. This inhibitory effect becomes apparent as early as 30 min after incubation at 37°C. Several possibilities exist as to the precise mechanism of action of azathioprine in this system. Inhibition of nucleic acid synthesis could be due to the suppression of the synthesis of certain essential enzymes such as DNA polymerase or DNA-dependent RNA polymerase. On the other hand, azathioprine may act at the DNA level, rendering it or the progeny DNA unable to code for RNA (and indirectly) protein synthesis. The latter is hard to visualize in view of the short incubation period (2 hr) used in our experiments. The exact site of the inhibitory action of azathioprine is not resolved by results reported here.

ACKNOWLEDGMENTS

We gratefully acknowledge the help and advice given to us by D. G. Carlson and P. J. Simpson.

LITERATURE CITED

1. Elion, G. B., S. W. Callahan, G. H. Hitchings, and R. W. Rundles. 1961. Comparative antineoplastic and metabolic studies of thiopurines. Proc. Amer. Ass. Cancer Res. 3:222.
2. Elion, G. B., S. W. Callahan, G. H. Hitchings, R. W. Rundles, and J. Lazlo. 1962. Experimental, clinical and metabolic studies of thiopurines. Cancer Chemother. Rep. No. 16, p. 197–202.
3. Elion, G. B., S. W. Callahan, R. W. Rundles, and G. H. Hitchings. 1963. Relationship between metabolic fates and antitumor activities of thiopurines. Cancer Res. 23:1207–1217.
4. Hitchings, G. H., and G. B. Elion. 1969. The role of antimitabolites in immunosuppression and transplantation. Accounts Chem. Res. 2:202–209.
5. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. 126:423–442.
6. Nathan, H. C., S. Bieber, G. B. Elion, and G. H. Hitchings. 1961. Detection of agents which interfere with the immune response. Proc. Soc. Exp. Biol. Med. 107:796–799.