The Effects Of Azathioprine On Differential Leukocyte Counts In Mice

Patrick Roche Jr.
Carroll College

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THE EFFECTS OF AZATHIOPRINE ON
DIFFERENTIAL LEUKOCYTE COUNTS IN MICE

Submitted in Partial Fulfillment of the Requirements for
Graduation with Honors in Combined Sciences: Biology and Chemistry
at Carroll College, Helena, Montana

Patrick A. Roche, Jr.

March 24, 1981
This thesis for honors recognition has been approved for the Department of Combined Sciences: Biology and Chemistry by:

Joseph Harrington
Professor of Biology
Advisor

Dr. Arthur E. Westwell
Professor of Chemistry
Reader

Rev. O. L. Hightower
Professor of Modern Languages
Reader

March 24, 1981
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ABSTRACT

Twenty-four, ten to fifteen-week-old C3H.SW female mice were used to determine the effects of azathioprine on differential leukocyte counts. Three groups consisting of six mice each were given various dosages of azathioprine for a period of twelve days. Six control animals were administered saline for the same period. The first experimental group received the recommended dosage of azathioprine (0.04 mg/10 g body weight per day); the second group received one and one-half times the normal dosage; and the third group received two times the normal dosage. Blood smears were taken from each of the animals for three days prior to the injections and every day during the injection period. These blood films were then stained with Wright's stain and differential counts of these cells were carried out.

Experimental evidence suggests that azathioprine, when injected intravenously to mice, results in a fall in lymphocyte percentages of total leukocyte populations and a corresponding rise in neutrophil percentages. With regard to monocyte, eosinophil and basophil percentages, changes due to the injection of azathioprine were negligible.
Azathioprine (AZP) is an immunosuppressive agent which has been found to cause a "modest fall in blood lymphocyte populations" when used as a therapeutic agent in man (1). This fall in lymphocyte count seems to be a rather slow process, thus it is generally believed that a continuous inhibition of lymphopoiesis or differentiation occurs throughout AZP treatment periods. In nearly all reported instances of AZP treatment, this fall in blood lymphocytes has been noted, but the degree to which this fall in lymphocyte populations occurs has not been examined in great detail.

Since the discovery that 6-mercaptopurine in appropriate dosages can suppress primary antibody responses and induce specific tolerance (Schwartz and Dameshek, 1959), AZP and other thiopurines have been widely used as immunosuppressants. These agents have been applied in the treatment of many diseases whose pathogenesis may be mediated by immunological mechanisms. In a few of these cases, the thiopurines have been found to have an "established, if limited, usefulness in therapy" (1).

AZP has been particularly useful in preventing the rejection of kidney transplants and in the treatment of immune diseases (4). Chemically, AZP is 6-(1-methyl-4-nitro-imidazol-5-yl)thiopurine (Figure 1), and as an antimetabolite, it interferes with the normal metabolic pathway involving nucleic acids, leading to an inhibition of RNA and DNA synthesis (5).

While not completely understood at this time, AZP is believed to act specifically by blocking the conversion of inosinic acid to adenylosuccinate. It is then eventually incorporated into 6-methylthiopurine ribonucleotide.
which is an important inhibitor of protein synthesis. This incorporation of AZP into "nonsense messenger" RNA results in the synthesis of defective proteins and antibodies which are similar in structure to the normal proteins and antibodies, but are unable to perform their specific functions. As a result, when the immune response of the organism is initiated, these defective materials are produced in place of the actual proteins and antibodies and a failure of the immune response occurs.

The superiority of AZP as an immunosuppressant is, at this time, not completely understood although it has been shown to suppress both cellular and humoral immunological reactions (7), inhibit the rosette formation of lymphocytes with sheep red blood cells (8) and inhibit the inflammatory reaction (5).

Depression of leukocyte counts, and in particular, lymphocyte counts has been directly associated to the effects of AZP on the bone marrow. Depending on the dosages of AZP, results in this area show a range of features from "mild depression of the myeloid series to frank aplasia" (11). Evidence also suggests that these effects are not only dependent on the dosage of AZP, but also upon the time of exposure to the drug.

In studies involving the use of AZP in inflammatory bowel disease, A. C. Campbell, et al. have shown that treatments with AZP over long periods do result in a fall in lymphocyte populations with neutrophil populations also being affected. The difference between lymphocyte and neutrophil populations was found to be insignificant. And, "at no time during the trial period did a significant difference occur in anything but lymphocyte counts. This fall in lymphocyte populations was gradual and the difference between these two groups only became significant after relatively long periods of time" (1).
All of the effects of AZP seem to result from its conversion to 6-mercaptopurine and other metabolites in vivo. Although the exact mechanism by which AZP induces lymphocytic (and in some cases erythrocytic) changes is uncertain, it is probably related to the known effects of 6-mercaptopurine and its nucleotide forms on the biosynthesis of nucleic acids (10).

It is believed that AZP quickly leaves circulation and is split extensively to mercaptopurine which is then subject to catabolic destruction to form a variety of oxidized and methylated derivatives, among which, 6-thiouric acid seems to predominate. This is formed by the oxidation of mercaptopurine by xanthine oxidase. The proportions of the different metabolites vary from individual to individual depending upon the relative amounts of the catabolic enzymes available.

Although AZP is mainly eliminated from the system by metabolic destruction, small amounts of unchanged AZP along with mercaptopurine are eliminated by the kidneys. Therefore, the biological effectiveness as well as the toxicity of AZP may be increased as much as twofold in the anuric patient.

Blood levels of AZP and the mercaptopurine that is derived from it are extremely low (less than 1 μg/ml) at therapeutic doses. This has been determined by studies involving radioactive material. Thus, the chief toxic effects of AZP that have been determined to date include hematologic manifested by leukopenia, anemia, thrombocytopenia and bleeding.
FIGURE 1

Structure of Azathioprine, 6-(1-methyl-4-nitro-imidazol-5-yl)thiopurine

\[
\begin{align*}
\text{CH}_3 & \quad \text{N} \\
\text{N} & \quad \text{NO}_2 \\
\text{S} & \quad \text{N} \quad \text{H}
\end{align*}
\]
MATERIALS AND METHODS

Mice

Twenty-four C3H.SW female mice obtained from the McLaughlin Research Center, Great Falls, Montana, were used for analysis in an attempt to determine the effects of azathioprine on differential leukocyte counts. All mice were approximately ten to fifteen weeks old at the outset of the experiment. The mice were separated into four groups (one control and three experimental) with each group consisting of six randomly selected mice. The animals were then separated into groups of three and caged in wire mesh and plastic cubicles (12" x 9" x 6"). A diet of Purina Rat Chow (1.01% calcium, 0.75% phosphorus) and tap water was administered to the animals throughout the experiment.

Drug Treatment

Azathioprine is a purine analog which is a derivative of mercapto-purine. Chemically, AZP is 6-(1-methyl-4-nitro-imidazol-5-yl)thiopurine. As the sodium salt, AZP is sufficiently soluble in water to make a 1.0 percent solution which remains stable for approximately two weeks when stored at 15 to 30 degrees Centigrade. After this time it undergoes increasing hydrolysis to form mercaptopurine. From the sodium salt (Sigma Chemical Corp., St. Louis, Missouri), 1.0 percent solutions were made and stored in an incubator at 16 degrees Centigrade.

Each experimental group was injected intravenously into the tail veins for a period of twelve consecutive days. The first group received dosages
of 0.04 mg/10 g body weight each day; the second group 0.06 mg/10 g body weight each day and the third group 0.08 mg/10 g body weight each day. The control group received injections of 0.01 mg/10 g body weight of a 1.0 percent saline solution daily for the length of the experiment.

**Blood Analysis**

Blood samples were taken from the experimental animals for three days prior to the initiation of the injection procedure and each day during the procedure. Samples were obtained by removing the tip of the mouse's tail. A drop of blood was then placed on a microscope slide and a smear was made. These blood smears were stained by the Wright's stain technique as follows: Air-dried blood films were placed with the smear surface upwards on a staining rack in a level, horizontal position. The entire surface of the slide was covered with Wright's stain (a solution of polychrome methylene blue and eosin in methyl alcohol) and allowed to stand for approximately three minutes. Buffer solution (pH 6.86) from a dropping bottle was added to the slides so that a layer piles up over the stain and no liquid spills off the edge of the slide. Gently blowing across the fluid mixed the stain and buffer. The slide was then allowed to stand for five minutes. The slide was rinsed thoroughly by flushing with distilled water; the reverse of the slide was carefully wiped with a soft, absorbant tissue and allowed to air dry by standing on one end. Differential counts were then carried out for each slide by identifying and counting one hundred consecutive leukocytes on a differential counter. This was done through the use of a microscope with a mechanical stage. The slide was moved from left to right, then down one vertical field, then again from right to left. This is done without counting the same area twice.
The number of each type of leukocyte is expressed as a percent of the total number of white cells. Atypical leukocytes, hypersegmented neutrophils (more than five lobes), plasmacytes and other abnormal and immature leukocytes were also noted.
RESULTS AND OBSERVATIONS

Lymphocyte Percentages

The mean percentage of lymphocytes compared to the overall leukocyte population in the control group was considerably higher than the lymphocyte percentages of the three experimental groups (Table 1). This difference of lymphocyte percentages ranged from 33 percent fewer lymphocytes in the 0.04 mg/10 g group to a high of 48 percent in the 0.08 mg/10 g group. The control group experienced a fairly constant lymphocyte percentage level throughout the experimental period, while all three experimental groups experienced a gradual drop in lymphocyte percentages over the period (Figure 2).

Neutrophil Percentages

The mean percentage of neutrophils in the control group was somewhat less than the percentages found in the three experimental groups (Table 2). The difference between experimental and control group neutrophil percentages ranged from a low of 32 percent in the 0.06 mg/10 g group to a high of 46 percent in the 0.08 mg/10 g group. The neutrophil percentages of the experimental groups experienced a gradual rise through the trial period, seeming to correspond directly to the fall in the lymphocyte percentages throughout the period. The neutrophil percentages of the control group remained more or less constant throughout the trial period (Figure 3).
Basophil, Eosinophil and Monocyte Percentages

The overall percentages of the basophils, eosinophils and monocytes remained relatively constant throughout the experimental period in all of the experimental groups as well as in the control group. These cell percentages did not differ more than one or two percent at any given time during the trial period (Figures 4, 5, 6). As a result, the effect of AZP on these cell percentages was determined to be insignificant.
TABLE 1

Standard Deviation and Mean for Lymphocyte Percentages of Total Leukocyte Populations

<table>
<thead>
<tr>
<th></th>
<th>Control 0.01 mg/10 g saline</th>
<th>Exp. #1 0.04 mg/10 g AZP</th>
<th>Exp. #2 0.06 mg/10 g AZP</th>
<th>Exp. #3 0.08 mg/10 g AZP</th>
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</thead>
<tbody>
<tr>
<td>Standard Deviation</td>
<td>8.0</td>
<td>98.4</td>
<td>137.1</td>
<td>158.1</td>
</tr>
<tr>
<td>Mean</td>
<td>41.8</td>
<td>27.8</td>
<td>25.9</td>
<td>21.6</td>
</tr>
</tbody>
</table>

TABLE 2

Standard Deviation and Mean for Neutrophil Percentages of Total Leukocyte Populations

<table>
<thead>
<tr>
<th></th>
<th>Control 0.01 mg/10 g saline</th>
<th>Exp. #1 0.04 mg/10 g AZP</th>
<th>Exp. #2 0.06 mg/10 g AZP</th>
<th>Exp. #3 0.08 mg/10 g AZP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Deviation</td>
<td>12.7</td>
<td>203.2</td>
<td>209.0</td>
<td>289.7</td>
</tr>
<tr>
<td>Mean</td>
<td>50.0</td>
<td>68.1</td>
<td>66.1</td>
<td>73.0</td>
</tr>
</tbody>
</table>
FIGURE 2

Lymphocyte Percentages
of Total Leukocyte Populations

0.04 mg/10 g body weight
0.06 mg/10 g body weight
0.08 mg/10 g body weight
control
FIGURE 3

Neutrophil Percentages of Total Leukocyte Populations

percent of total leukocytes

number of days

0.04 mg/10 g body weight
0.06 mg/10 g body weight
0.08 mg/10 g body weight
control
FIGURE 4

Basophil Percentages
of Total Leukocyte Populations

number of days

0.04 mg/10 g body weight

0.06 mg/10 g body weight

0.08 mg/10 g body weight

control
FIGURE 5

Eosinophil Percentages of Total Leukocyte Populations

percent of total leukocytes

number of days

0.04 mg/10 g body weight

0.06 mg/10 g body weight

0.08 mg/10 g body weight

control
FIGURE 6
Monocyte Percentages
of Total Leukocyte Populations

number of days

0.04 mg/10 g body weight

0.06 mg/10 g body weight

0.08 mg/10 g body weight

control
DISCUSSION

In this experiment, the effect of Azathioprine on differential leukocyte counts in mice was examined. It should first be pointed out that results from a blood examination of this type do not reflect the specific increases or decreases in individual cell populations. Rather, these results reflect the percentages of individual cell populations with respect to the total leukocyte population of the individual.

The results of this experiment showed a definite decrease in lymphocyte percentages in peripheral blood over the AZP injection period. This decrease in lymphocytes was accompanied by a corresponding rise in neutrophil percentages. The decrease in lymphocytes seems to agree with results of other experiments in which a noticeable decrease in lymphocyte populations was found to be a direct result of AZP treatment (1,2,3). While some suggest that this drop in lymphocyte populations is due to factors other than AZP (folic acid deficiency, chronic liver disease, etc.), it has been shown by Wickramasinghe and others that this fall in lymphocyte count is, in fact, a direct result of AZP treatment (10).

The rise in neutrophil percentages, as pointed out previously, seems to directly correspond to the fall in lymphocyte percentages. Thus, the question arises of whether this rise in neutrophil percentages is a direct result of AZP therapy or simply a consequence of the drop in lymphocyte percentages. Previous studies which included the effects of AZP on neutrophils show that these effects are insignificant or, at most, result only in a slight drop in neutrophil populations (1). Thus, one is led to believe that the rise in
neutrophil percentages is a result of the drop in lymphocyte percentages rather than a result of AZP treatment.

The conclusion that must be made from the results of this experiment is that the intravenous injection of AZP does have a definite effect on the differential leukocyte cell count. These effects are most noticeable in the lymphocyte and neutrophil percentages with a rise in neutrophil percentages and a fall in lymphocyte percentages. From evidence provided by previous investigations (1, 2), it must also be concluded that the fall in lymphocyte percentages is a direct result of AZP treatment while the rise in neutrophil percentages is not a result of AZP treatment, but rather a result of the fall in the lymphocyte percentages. It should further be pointed out that since these examinations were carried out in healthy subjects, the hypothesis of Wickramasinghe et al., that the effects are a direct result of AZP treatment rather than of other factors (10), seems to be supported.

Finally, while the exact route through which AZP carries out its effects on the individual is not yet completely known, the effects in themselves are reason enough for further investigation. The specific clinical implications of these and previous results remains to be seen. Thus, only through further investigation will a more complete understanding of AZP and its effects (beneficial as well as detrimental) come about.

In conclusion, while past studies of the effects of AZP on leukocytes have been concerned primarily with the effects on specific leukocytes, there have apparently been no experiments carried out to examine the effects of the drug on total leukocyte populations. Thus, it was the purpose of this experiment to examine this aspect of AZP by examining the effects of the drug on differential leukocyte counts. In this way, data could be collected and analyzed not just on one leukocyte type, but rather on percentages of
all types of leukocytes in peripheral blood.

The data from this experiment, as pointed out above, does indicate that differential leukocyte counts of peripheral blood are affected by AZP treatment. Whether or not this evidence points to any previously undiscovered abnormal effects of AZP on leukocytes remains to be seen, the purpose of this experiment was merely to examine the possibility that they do, in fact, exist. Having shown that abnormalities in differential leukocyte counts do occur as a result of AZP treatment, it must be left to other investigators to examine the further significance, if any, of these results.


