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# AZATHIOPRINE (IMURAN) ADMINISTRATION AND THE DEVELOPMENT OF MALIGNANT LYMPHOMAS IN NZB MICE

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## SUMMARY

Azathioprine in doses that made NZB mice more anaemic did not cause reversal of their positive Coombs tests. When given to young NZB mice it did not delay development of the Coombs positive state.

Azathioprine had a carcinogenic effect. Six out of eight NZB mice treated from the age of 2 months developed malignant lymphomas particularly involving the thymus.

Caution is suggested in the use of immunosuppressant agents in non-malignant conditions, especially in the young, until the risk of carcinogenesis has been evaluated.

## INTRODUCTION

It has been shown (Casey, 1968) that the administration of 6-mercaptopurine (6-MP) does not benefit NZB mice which develop autoimmune haemolytic anaemia (AIHA) (Helyer & Howie, 1963; Holmes & Burnet, 1963). Its popularity as an immunosuppressant drug has led to widespread usage of azathioprine, an analogue of 6-MP. However, the evidence that antimitotic drugs are advantageous in AIHA is scanty, and it was, therefore, considered of interest to study also the effects of administration of azathioprine on NZB mice.

It is the purpose of this paper to report that in a controlled therapeutic trial administration of azathioprine to NZB mice did not benefit their AIHA. In addition, it was found that there was a high incidence of malignant lymphomas particularly affecting the thymus of young mice given intensive and then moderate long-term therapy with azathioprine.

## MATERIALS AND METHODS

The NZB inbred mouse strain and the haematological techniques used to study them have been documented (Helyer & Howie, 1963; Casey & Howie, 1965). Coombs tests were scored numerically and their significance statistically evaluated.

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*Morbid anatomical techniques*

Helly's fluid was used as fixative. Sections were cut at 3 $\mu$ and all were stained by haematoxylin–eosin and Unna–Pappenheim stains. In addition, the kidney sections were stained with periodic acid Schiff–Alcian Blue as well as the Martius Scarlet Blue stain for fibrinoid (Lendrum *et al.*, 1962).

*Azathioprine*

One-hundred milligrams of azathioprine were dissolved in 2·4 ml of N/10 sodium hydroxide by shaking and then standing the vial in warm water for 5–10 min. Following this 7·6 ml of normal saline was then added, giving a final concentration of 1 mg azathioprine in 0·1 ml. Each treated mouse was given a dose of 0·3 ml into the thigh muscles. For a 30-g mouse this corresponds to a dosage of 100 mg/kg/injection. Control animals were given the solvent.

*Azathioprine administration for 6 weeks to NZB mice with established autoimmune haemolytic anaemia*

Twenty-four NZB male mice aged 11 months were matched into two similar groups of twelve on the basis of strength of Coombs tests. The group to be treated were given one injection of azathioprine three times a week for 4 weeks and then twice in the 5th week and once in the 6th week. Blood samples were obtained from all mice before treatment, and from surviving mice after 4, 5 and 6 weeks of therapy and, if practicable, before death. Haematocrit, Coombs tests, total leucocyte counts and blood urea estimations were performed on each sample.

Mice found dead were subjected to post mortem examination. Tissues were examined histologically only in mice killed when very ill, and not in those found dead.

*Azathioprine administration for 6 months to NZB mice, commencing before the onset of autoimmune haemolytic anaemia*

Sixteen NZB male mice aged 2 months were matched into two similar groups on the basis of body weight and haematocrit readings. The eight mice to be treated were given one injection of azathioprine three times a week for 4 weeks, two injections in the 5th week and then one injection a week for a total treatment period of 6 months.

As well as a pre-treatment specimen blood was obtained from surviving mice after 4, 5, 6, 8, 12, 22 and 26 weeks of azathioprine treatment. They were tested for haematocrit, Coombs tests and blood urea. Urine was tested each month for albumin using 'Albustix' (Ames). Surviving animals were then selectively killed.

Post mortem examination was carried out on all animals, except two control mice which died with an infective diarrhoea and whose bodies were found in a poor state of preservation. Histological examinations were carried out unless precluded by autolytic changes.

## RESULTS

*Effect of azathioprine on NZB mice with established AIHA*

*Mortality.* The twelve treated mice died. Eight were found dead and four were killed because they had declined in general condition, lost weight, stood in a hunched position

TABLE 1. Summary of mortality, body weights, haematocrits and leucocyte counts, in NZB male mice aged 320±3 days treated with azathioprine for up to 6 weeks, compared with control mice of a similar age

| Time | No. of mice | | Mean body weight (g) | | | Mean haematocrit (%) | | | Mean WBC ($mm^3$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treated | Control | Treated | Control | *P* | Treated | Control | *P* | Treated | Control | *P* |
| Pre-treatment | 12 | 12 | 34·7±0·6 | 36·3±0·6 | | 41·4±1·1 | 43·1±0·7 | | 9167±474 | 9333±838 | |
| 4 weeks | 7 | 12 | 34·8±1·0 | 35·2±0·6 | | 18·1±2·1 | 36·8±1·8 | 0·001 | 3757±558 | 7542±538 | 0·001 |
| 5 weeks | 5 | 12 | 36·5±0·7 | 36·3±0·5 | | 12·0±1·4 | 37·5±1·7 | 0·001 | n.t. | n.t. | |
| 6 weeks | 1 | 12 | 32·5 | 37·0±0·8 | | 7·0 | 37·1±1·8 | | n.t. | n.t. | |
| Post mortem* | 12 | 12 | 29·2±0·8 | 39·0±0·8 | 0·001 | 8·5±0·5 ($n$ = 4) | 37·3±1·2 | 0·001 | 738±221 ($n$ = 4) | 14208±1189 | 0·001 |

± Standard error of the mean for all means given.
*P* Stated if significant.
n.t. Not tested.
*n* Indicated if less than number stated at left of Table.
* Control mice not killed until mean age 419±3 days whereas treated mice died at mean age 348±5 days.

with rapid respirations and appeared to be moribund. The first mouse to die did so in the 1st week of therapy and only one mouse survived to the end of the 6 weeks. The time of death and the decreasing population of treated mice are indicated in Table 1. All of the controls survived. They were killed for another purpose some time later.

*Haematocrit.* In all treated mice there was a marked fall in haematocrit. This fall was striking when the mean haematocrits of the surviving treated mice are compared with those of the untreated mice seen in Table 1. The average haematocrit of the four animals killed when moribund was 8%.

*Coombs test.* No statistically significant change was detected in the strength of Coombs tests in the treated animals.

*Leucocyte counts.* Peripheral blood leucocyte counts tended to fall during therapy (Table 1). Leucopenia had developed in the four terminally killed animals, as judged by cardiac blood leucocyte counts showing a mean of $738 \pm 221/mm^3$.

*Blood urea estimations.* These showed no significant change during therapy except that in one treated mouse the blood urea rose to 256 mg/100 ml, terminally. With this exception mortality in the treated group could not be attributed to renal failure.

*Post mortem examination.* Allowing for the difference in body weight shown in Table 1, there were no significant differences in thymus, liver or kidney weight. No macroscopic thymic tumours were noted in any of the twelve treated or twelve control mice. For spleens of treated animals the mean weight and standard error of the mean were $71 \pm 8$ mg ($n = 8$), compared with $306 \pm 74$ ($n = 12$) in the controls ($P < 0{\cdot}01$).

Histologically in the treated mice studied there was moderate lymphoid depletion of lymph nodes, thymus and lungs. Haemopoietic and lymphoid tissue was markedly reduced in the spleen. There was patchy hepatocellular necrosis.

*Effect of azathioprine administration begun before onset of AIHA in NZB mice*

*Mortality.* Five of the eight treated animals died during the experiment. The first died in the 3rd week during the initial intensive therapy. It had blood in the stomach and probably died as a result of the therapy. The other four mice that died had large malignant thymic tumours. Four of the control mice died or were killed during the experiment for various reasons. Two of these control mice had severe infective diarrhoea in the 6th week of the experiment and were not examined post mortem. A third control mouse died with pyelonephritic lesions, especially in one kidney, accompanied by scarring and peritoneal adhesions with intestinal obstruction. The blood urea in this animal was 186 mg/100 ml at death. The fourth control mouse was killed while apparently well when its treated partner was found to have a tumour of the thymus.

*Haematocrit.* Haematocrit changes are shown in Table 2. There was a significant fall in the treated animals during the first 6 weeks when intensive therapy was being administered. Subsequently the haematocrit readings were more similar to those of the controls. They showed no advantage over the control animals.

*Coombs test.* Coombs test results are summarized in Table 2. The treated mice showed no significant difference from the controls. It can be seen that there was no delay in onset of positive Coombs tests in the treated group.

*Blood urea.* With the exception of the one control mouse already mentioned, blood urea estimations were all normal.

TABLE 2. Summary of mortality, body weight, haematocrit and Coombs tests in NZB male mice aged 67±5 days treated with azathioprine for up to 6 months compared with control mice of a similar age

| Time | No. | | Mean body weight (g) | | Mean haematocrit (%) | | | Coombs tests | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Treated | | | Control | | |
| | Treated | Control | Treated | Control | Treated | Control | *P* | − | ±† | ⩾2† | − | ±† | ⩾2† |
| Pre-treatment | 8 | 8 | 28·3±0·6 | 28·6±0·5 | 47·8±1·1 | 46·0±0·8 | | 8 | 0 | 0 | 8 | 0 | 0 |
| 4 weeks | 7 | 8 | 30·4±0·7 | 30·8±0·4 | 29·1±3·3 | 42·5±0·7 | 0·01 | 2 | 2 | 3 | 3 | 3 | 2 |
| 5 weeks | 7 | 7 | 31·4±0·5 | 31·5±0·1 | 24·0±4·0 | 42·1±1·8 | 0·001 | 1 | 1 | 5 | 1 | 4 | 2 |
| 6 weeks | 7 | 6 | 33·6±0·7 | 33·5±0·9 | 29·6±4·0 | 43·0±2·4 | 0·02 | 1 | 2 | 4 | 0 | 4 | 1* |
| 8 weeks | 7 | 6 | 34·5±0·6 | 33·6±1·4 | 41·4±0·8 | 41·7±3·2 | | 4 | 2 | 1 | 2 | 3 | 1 |
| 12 weeks | 7 | 5 | 36·9±0·7 | 38·6±0·1 | 38·6±1·3 | 43·0±1·2 | 0·05 | 0 | 3 | 4 | 3 | 1 | 1 |
| 22 weeks | 4 | 4 | 41·4±0·7 | 43·1±2·7 | 36·7±1·1 | 40·0±1·5 | | 0 | 4 | 0 | 0 | 0 | 4 |
| Post mortem | 8† | 6‡ | 37·9±2·5 | 41·5±3·3 | 39·5±2·5 | 37·0±1·5 | | 0 | 0 | 4 | 0 | 0 | 6 |

* One test omitted.
† Four treated mice killed, four found dead.
‡ Six control mice killed, two found dead.

*Albuminuria.* Albuminuria developed in a similar pattern in both groups. Albuminuria to levels between 2+ and 3+ was present in all mice surviving the 6 months' therapy.

*Kidneys.* The glomerulonephritis seen in NZB mice was present in treated and untreated groups, never being more than mild to moderate in severity. Pyelonephritic scars were seen in half of each group. These were more striking in the treated group. They were old and often multiple.

*Lymphoid tissue: Thymomas and thymus.* Six of the eight treated mice had malignant lymphomas particularly affecting the thymus. Included in these six are four that died during the experiment and two killed to terminate the experiment after 6 months. No malignant lymphomas were seen in the six control animals studied. The difference in incidence in the two groups is significant ($P = 0{\cdot}009$). The mean thymus weight was $245 \pm 109$ mg in the treated group with tumours compared with $33 \pm 4$ mg in the untreated group. All of the tumours were locally infiltrating. Usually there was a large mediastinal mass replacing the thymus, filling the upper part of the chest and constricting the heart. The first animal to die with a large thymic tumour did so at the age of 165 days, some 97 days after commencing azathioprine therapy. Other deaths were after 138, 140 and 179 days. The further two tumours seen were in mice still apparently well when killed to terminate the experiment 188 days after commencing azathioprine. At this time the mice were 256-days old. Histologically the tumours were lymphoblastic in type and presented a 'starry-sky' appearance.

*Other lymphoid tissues.* The mean weight of the spleen was $441 \pm 163$ mg ($n = 5$) in the treated mice with tumours compared with $183 \pm 26$ mg ($n = 6$) in the controls. In at least four of the six mice with affected thymuses tumour was seen in spleen, lungs, liver, kidney and lymph nodes in varying combinations.

## DISCUSSION

NZB mice with established autoimmune haemolytic anaemia tolerated azathioprine badly. With lethal drug dosage they became very anaemic and leucopenic but their Coombs tests showed no lessening in strength. This persistence of the degree of positivity of Coombs tests could be explained by diminished red cell destruction and persistence of autoantibody at a time when red cell production is also diminished by the antimitotic drug. It is unlikely, however, that red cells would survive beyond the usual life span of mouse red blood cells. It is also possible that the half life of antibody is prolonged in the presence of antimitotic drugs.

A similar intensive initial course of azathioprine followed by prolonged administration of smaller doses failed to delay the onset of the Coombs positive state in young NZB mice. This makes it unlikely that the drug would be able to cause real benefit to already affected mice. Eleven-month-old mice receiving the drug for the first time when already affected, tolerated it poorly, as compared with the initially unaffected mice, aged 2 months, in the second experiment. Only one of the latter died in the first 6 weeks on dosage that led to the death of all the older mice within 6 weeks. It has previously been argued with reference to 6-MP (Casey, 1968) that AIHA is the least likely autoimmune disorder to benefit from antimitotic immunosuppressant therapy. One of the main reasons for this contention is that the hyperplastic erythroblastic tissue is seriously depressed by a dosage that

does not achieve suppression of antibody production. The present results point to the same conclusion.

The occurrence of malignant lymphoma in six of the eight mice in the second experiment and in none of the controls is significant. In the NZB strain thymomas and lymphomas occur in up to 25% of mice according to East, de Sousa & Parrott (1965), Mellors (1966) and De Vries & Hijmans (1967), but Helyer & Howie (1963) and Holmes & Burnet (1963), although impressed with the marked lymphoreticular proliferation that occurs regard the incidence of frank neoplasia as being much less. This proliferation is difficult to distinguish from early neoplasia.

It seems likely that the tumours arose in the thymus and spread. The thymus was the only organ always involved, and the histological appearance was of widespread malignant transformation in this organ. It remains possible that tumours were originally multifocal in origin in the lymphoid tissue of the various organs studied.

The drug may alter host factors in such a way that the proliferation which usually gives rise to autoimmune disease is even less under control and gives a frank neoplasia, just as autoimmune diseases in man occasionally go on to neoplastic ones (Dameshek, 1966; Mellors, 1966). However, in the present case the change occurred in relatively young NZB mice before they developed massive lymphoid proliferation. It seems more likely that the carcinogenic effect results from disturbance of host resistance, so that neoplastic cells are not eliminated or allowed to spread more easily. The situation may be closely comparable to the unmasking of, or alteration in the resistance to, a leukaemic or lymphoma-producing virus in mice that have received whole body irradiation (Furth, Okano & Kunii, 1964). Kaplan (1966) indicated that urethane and thio-TEPA may have the same potentiating effect on the leukaemic virus as whole body irradiation. It is of considerable interest that a carcinogenic effect from the antimetabolite 6-MP was shown in the non-related and non-autoimmune C57BL strain by Doell, De Vaux St Cyr & Grabar (1967). The carcinogenic effect of some alkylating agents, well recognized in animals, has not been proven in man (Karnofsky, 1967).

The dosage plan has been given in detail as this may be of importance. Significant species differences occur in the metabolism of purine analogues (Elion, 1967). The great differences in the effect of anti-tumour agents at different dosage levels and in different species have been stressed by Haines, Johnson & Petering (1967). Long-term rather low dosage azathioprine therapy may be comparable to the long-term leukaemogenic low dosage irradiation in mice which Kaplan (1966) discussed. On the other hand, as suggested by Dameshek & Gunz (1964), the relevance of dosage may be in the initial depression of lymphoid tissue that must have followed the first 6 weeks of intensive treatment. Structural alterations may occur in cells which at a later stage transform to neoplastic ones.

In a preliminary communication (Casey, 1967) it was reported that azathioprine treatment is also associated with the development of lymphomas in the NZB × NZW $F_1$ hybrid mice that develop lupus nephritis. Again this effect was seen in young mice and not in old ones. Whether the carcinogenic effect is restricted to mice and/or to this type of drug requires further investigation. In the meantime it is suggested there is need for caution in the use of immunosuppressant therapy in non-malignant conditions, particularly in young patients.

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