Immunosuppression by Cyclophosphamide in NZB X NZW Mice With Lupus Nephritis

By T. P. Casey

Severe lupus nephritis accompanied by positive antinuclear factor tests in the peripheral blood occurs in NZB X NZW F1 hybrid mice. It has been shown that the renal lesions can be profoundly modified by betamethasone phosphate. Equally impressive benefit was achieved with cyclophosphamide in what was probably the first statistically significant demonstration of benefit from antimitotic drugs in spontaneously occurring autoimmune disorders. The immunosuppressant effect of the drug was demonstrated indirectly by diminished antibody response to exogenous antigens.

It was considered important to see if the beneficial effects of cyclophosphamide on lupus nephritis in NZB X NZW hybrids could be confirmed; also whether such benefit was accompanied by evidence of decreased autoantibody production. Recent reports have shown a carcinogenic effect in C57BL mice from 6-mercaptopurine (6-MP) and in NZB/BL and NZB X NZW mice from azathioprine. It was considered of value to report that no such effect was noted with cyclophosphamide.

Materials and Methods

The characteristics of the NZB X NZW mice, methods of estimation of haematocrit and of total leucocyte counts, of Coombs’ tests, LE cell tests and latex nucleoprotein tests (Hyland) and of blood urea estimation have been previously described. Treated mice were given 2 mg. of cyclophosphamide in 0.2 ml. of normal saline, intraperitoneally once a week. Control mice were injected with 0.2 ml. of normal saline.

Mice With Established Autoimmune Disease

Twenty female mice aged 249 ± 3 days were matched into pairs on the basis of age, weight and whether their blood ureas were normal or not. The mice in one group were treated for 174 days when surviving mice were electively killed. Blood tests were carried out 35, 56, 120 and 150 days after therapy began as well as before death, if possible. The urine was tested each week for albumin using Albustix (Ames).

Mice Before Establishment of Autoimmune Disease

Twenty female mice aged 82±2.8 days were matched into pairs on the basis of age, weight. The mice in one group were treated for 294 days when surviving mice were electively killed. Blood tests were carried out approximately 100, 150, 170, 220 and 270
IMMUNOSUPPRESSION

IMMUNOSUPPRESSION

days after therapy began as well as before death, if possible. The urine was tested each month for albumin.

RESULTS

Mice With Established Autoimmune Disease

The 10 control mice died. In the treated group 8 of the 10 mice were still alive when they were electively killed to terminate the experiment. The mean age at death of the treated mice was 404 ± 15.8 days compared with 335 ± 17.2 days for the controls (P < 0.01). This underestimates the beneficial effect on survival because most of the treated mice were well when electively killed. At the commencement of the experiment the 2 groups were similar with mean body weights of 30.4 ± 1 Gm. The mean body weight of the treated mice at the end of the experiment was 34.2 ± 3.1 Gm. As renal failure developed the controls lost weight and had a mean weight of 23.7 ± 1.2 Gm. at postmortem (P < 0.01).

Renal Data

Blood Urea. Two of the 10 mice in the treated group had elevated blood urea levels before treatment. One of these reverted to normal from a level of 120 mg./100 ml. and 6 months later was 64 mg./100 ml. The other dropped from 475 mg./100 ml. to 216 mg./100 ml. but the mouse died after 5 weeks of therapy. During therapy only one previously normal mouse became uraemic. This contrasts with the untreated group where elevation was seen in 2 initially but later in 9 of the 10 controls (P = 0.0078).

Albuminuria. All of the control mice had developed 3+ or 4+ levels of albuminuria. 5 treated mice with initial levels of 2+ or less remained at this level. In only 1 mouse was there clear evidence of diminution in degree of already established albuminuria during therapy.

Kidneys. In treated mice the kidneys weighed 369 ± 19 mg., compared with the heavier kidneys in the controls which weighed 522 ± 38.2 mg. (P < 0.01). Histologically the latter all showed severe lupus glomerulonephritis. Severe lesions were present in 2 of the treated mice. In the other 8 there were mild to moderately severe lesions comprising enlargement and tufting of glomeruli, glomerular basement membrane thickening and wire looping, hyaline change particularly in the axial regions, interstitial damage and minor degrees of cast formation. There was almost complete absence of the fibrinoid necrosis, hypercellularity and focal necrosis typical of the active stages of the disease. Lymphoid tissue was markedly reduced in the kidneys of treated mice.

Hemopoietic and Lymphoreticular Effects

Hematocrit. The mean hematocrit results of the 2 groups are compared in Table 1. On no occasion was the mean hematocrit of the treated group less than that of the controls. The hematocrit tended to be higher in the treated mice in the later part of the experiment, as the control mice developed anemia associated with their renal failure.
<table>
<thead>
<tr>
<th>Time</th>
<th>Number</th>
<th>Mean Hematocrit %</th>
<th>Mean WBC/Cu.mm.</th>
<th>Latex Nucleoprotein Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>10 10</td>
<td>43.3±0.9</td>
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<td>31.0</td>
<td>5200±550</td>
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<td>150 days</td>
<td>8 0</td>
<td>37.4±1.7</td>
<td>3500±200</td>
<td>0 0 2</td>
</tr>
<tr>
<td>Postmortem</td>
<td>10 6</td>
<td>32.7±2.3</td>
<td>25.0±3.9</td>
<td>4700±580</td>
</tr>
</tbody>
</table>

T = Treated. C = Control. P stated if significant. Standard Error or Mean given where relevant.
**Total Leucocyte Counts.** After treatment commenced the mean total leucocyte count was always significantly lower in the treated group than in the controls (Table 1).

**Spleen.** The mean weight of the spleen in the treated group was $155 \pm 19.7$ mg, while in the controls it was $195 \pm 63.5$ mg. This difference is not statistically significant. It suggests a reduction in splenic size in the treated group when allowance is made for the fact that the untreated mice were significantly lighter than the treated ones but their spleens were heavier.

**Thymus.** There was no significant difference in weight between the 2 groups. In the treated mice there was less cortical depletion of thymocytes than in the controls. Mitotic figures were frequent in the cortex of treated mice. There was little evidence of the proliferation of medulla.

**Autoantibody Production**

**Latex Nucleoprotein Tests.** The results given in Table 1 show that throughout the treatment period there were significant reductions in the degree of positivity of latex nucleoprotein tests in the treated group compared with the controls.

**LE Cell Tests.** Before treatment, 3 of the 10 mice in the treated group had positive LE cell tests. Subsequently 2 of these mice had negative tests. When pretreatment tests were negative in treated mice, they remained the same. Four of the control mice initially had positive tests and these persisted. During the experiment the remainder of the untreated mice developed positive LE cell tests ($P = 0.016$).

**Coombs’ tests.** Treated and control groups showed no statistically significant results from serial Coombs’ tests. All of the treated and 8 of the controls had weakly positive tests at some stage, only occasional tests being more than weakly positive.

**Mice Before Establishment of Autoimmune Diseases**

The mean age at death of the 10 untreated mice was $283 \pm 14.9$ days. The mean age for the 10 treated mice was $314 \pm 35.8$ days. This includes 2 mice which died 3 and 4 weeks after therapy commenced when aged 96 and 104 days. In one the tissues were too autolytic to examine. In the other there was hepatic necrosis with some regeneration occurring and similar changes were seen in another mouse later. These 2 early deaths may have been side effects of therapy. The remaining 8 treated mice had a mean age of $367 \pm 5$ days which is a highly significant difference from the untreated mice ($P < 0.001$). Because of elective killing to terminate the experiment the survival in the treated group is understated. Four of the 8 treated mice were quite well when killed and 1 had lost weight but no abnormality was found at postmortem. One had a massive tumour of the thymus, 1 had liver necrosis and 1 was found dead with gastrointestinal and pulmonary hemorrhage. The treated mice had a mean weight of $29.7 \pm 3.1$ Gm. at postmortem which was not significantly different from the controls.
Table 2.—Hematoctit, leucocyte counts, latex nucleoprotein tests and number of survivors from 10 NZB X NZW female mice treated with cyclophosphamide from age 82 ± 2.8 days and compared with controls

<table>
<thead>
<tr>
<th>Time</th>
<th>Number</th>
<th>Mean Hematocrit %</th>
<th>T</th>
<th>C</th>
<th>P &lt;</th>
<th>Mean WBC/Cu.mm.</th>
<th>T</th>
<th>C</th>
<th>P &lt;</th>
<th>Latex Nucleoprotein Tests</th>
<th>Treated</th>
<th>Control</th>
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<td>Pretreatment</td>
<td>10</td>
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<td>0.01</td>
<td>3750±330</td>
<td>6300±470</td>
<td>0.001</td>
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<td>0 ± + 2+ 3+ 4+ 5+</td>
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<td>46.3± .7</td>
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<td>4090±510</td>
<td>7800±442</td>
<td>0.001</td>
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<td>6 2 0 0 0 0 0</td>
<td>0 ± + 2+ 3+ 4+ 5+</td>
<td>7 1 1 1 0 0 0</td>
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<td>8000±976</td>
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<td>3</td>
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<td>0 ± + 2+ 3+ 4+ 5+</td>
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<tr>
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<td>40.3± .9</td>
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<td>0 1 3 3 0 0 0</td>
<td>0 ± + 2+ 3+ 4+ 5+</td>
<td>0 0 0 2 5 2 1</td>
</tr>
</tbody>
</table>

T = Treated.  C = Control.  P stated if significant.  Standard Error of Mean given where relevant.
Renal Data

Urea. None of the treated mice developed uraemia in contrast to 8 of the 10 untreated mice (P < 0.001).

Albuminuria. One of the 10 treated mice showed significant albuminuria compared with 9 of the 10 controls (P < 0.001).

Kidneys. The mean weight of the kidneys in the treated mice was 394.7 ± 8.3 mg, which was markedly lighter than the controls with a mean kidney weight of 542.6 ± 30.8 (P < 0.001). Histologically the kidneys in 1 of the treated group showed evidence of severe nephritic lesions. Otherwise the kidneys in 7 of the 10 treated mice could be classed as within normal limits or equivocally abnormal. All of the controls examined had severe active glomerulonephritis with massive lymphoid aggregates in hilum and parenchyma.

Hemopoietic and Lymphopoietic Effects

Hematocrit. There was a significant drop in hematocrit in the early stages of therapy. This disadvantage to the treated group did not persist (Table 2).

Total Leucocyte Counts. During the treatment period the mean total white blood cell counts were lower in the treated group than the controls, the differences being significant until the number of survivors was low (Table 2).

Spleen. In the treated group the mean spleen weight was 127 ± 12.4 mg. In the controls it was 224.8 ± 33.9 mg. (P < 0.02). Histologically in the treated group there was a reduction in the amount of lymphoid tissue with prominent hemopoietic tissue remaining in the red pulp.

Thymus. There was no significant difference in weight of the thymus in the 2 groups. Histologically the untreated mice, which were terminally ill, tended to show more cortical atrophy than the treated mice. One treated mouse was found dead with a massive lymphoma of the thymus.

Autoantibody Production

Latex Nucleoprotein Tests. There was a significant delay in onset of positive latex nucleoprotein tests in the treated mice and a reduction of the degree of positivity of the tests compared with the controls (Table 2).

LE Cell Tests. None of the treated mice developed positive LE cell tests during this experiment. This was significantly different from the controls where 8 out of 10 mice developed positive tests (P < 0.001).

Coombs' tests. No statistically significant differences were noted in the time of onset, incidence or strength of positive Coombs' tests in the 2 groups. All mice had equivocal or weakly positive tests at some stage, with occasional tests more strongly positive.

Discussion

In a study of 450 NZB X NZW F1 female mice8 death from lupus nephritis commenced at about 6 lunar months and about 90 per cent of the animals were dead by the fourteenth month. Development of azotemia was preceded and
paralleled by development of antinuclear factors and albuminuria in a predictable fashion. The arithmetical mean survival time of 280 days was very similar to that of the control mice in the second experiment reported here.

In the first experiment the survival to over 400 days of 8 of the 10 mice treated with cyclophosphamide is very significant compared with the controls and the natural history of the disease. These mice were a selected group initially in that they had already survived to an age of 249 days when treatment was commenced. They would be predicted to have some renal lesions at that time. The further development of these lesions was largely prevented and activity suppressed. Beneficial effects were seen in the lupus nephritis lesions and incidence of uremia. Already existing albuminuria showed little change although survival was increased. Likewise cyclophosphamide therapy had a markedly beneficial effect in the group of young mice treated before the development of renal lesions. The dosage used proved relatively toxic for the younger mice compared with the older.

It is considered important to evaluate immunosuppressants at different ages and by different parameters as well as to study different immunosuppressants in a particular situation. Such assessments have been hampered by the previous lack of suitable models of autoimmune disease. The present results with cyclophosphamide confirm and extend those of Russell et al., which were probably the first statistically significant demonstration of the value of alkylating agents in spontaneously occurring autoimmune disorders. It has been possible to show a very similar benefit on longevity and renal lesions from the antimetabolite azathioprine (Imuran) in doses which did not give a clear-cut fall in circulating antibody levels. Such findings, and the earlier demonstration of the marked beneficial effect of the corticosteroid betamethasone, lend substance to the claim that the NZB X NZW hybrid mice should prove useful in settling the controversy as to the degree of reversibility of severe active lupus nephritic lesions in humans.

Significant reductions in frequency and positivity of antinuclear factor tests have been demonstrated here as judged by LE cell tests and latex nucleoprotein tests. This provides the first direct evidence of the immunosuppressant effect of cyclophosphamide in both young and old mice with lupus nephritis. A convincing diminution in lymphoid tissue has been demonstrated. The reason for the beneficial effect on the kidneys is unknown. It is clear from the experiments that strength of antierythrocyte antibody as judged by the Coombs' test is unrelated to the extent of renal disease. Reduction of numbers of immunologically competent cells active against kidney antigens may be important, with reduction of levels of circulating antibody which damage the kidney specifically. Renal damage may be nonspecific and result from local reaction to filtered antigen-antibody complexes. There is no direct evidence linking the decreased levels of antinuclear factors shown or of other autoantibodies with the decreased incidence of renal damage, but this could be the way benefit was achieved. On the other hand immunosuppression by drugs such as cyclophosphamide may be at the level of interference with cellular immune mechanisms or with the inflammatory response.
No carcinogenic effect was found with cyclophosphamide in the dosage used. One malignant lymphoma of the thymus occurred in the group of young mice treated with cyclophosphamide. A high incidence of such a complication has been reported in C57BL mice treated with 6-MP, in the NZB X NZW hybrids and in NZB mice treated with azathioprine (Imuran). It must be stressed that the patterns of dosage were not identical, a more intensive initial dosage with azathioprine being used. Such drugs are being widely used clinically as immunosuppressants in nonmalignant conditions. Further studies of dosage schemes which achieve different degrees of immunosuppression either for short or long terms at different ages, and in different species is urgently required to evaluate the risk of carcinogenesis from antimitotic agents. The NZB X NZW hybrid mice provide a useful model for such studies. In particular they may allow an assessment of whether the carcinogenic effect is related to immunosuppression or separate from it.

**SUMMARY**

Long-term administration of cyclophosphamide to groups of young and old NZB X NZW hybrid mice in controlled therapeutic trials showed:
1. decreased antinuclear autoantibody production as judged by LE cell and latex nucleoprotein tests,
2. marked benefit to lupus nephritis lesions, and
3. no carcinogenic effects.

Attention is drawn to the prospect of using these mice to study the carcinogenic effects of antimitotic drugs with particular reference to the effects of such drugs on immune mechanisms.

**SUMMARIO IN INTERLINGUA**

Le perdurativa administration de cyclophosphamida a gruppos de juvene e vetere muses hybrida NZB X NZW in essayos therapeutic a controlo ha monstrate:
(1) un reducec production de auto-anticorpore antinucleari, a judicar per tests a cellulas LE e a nucleoproteina con latex,
(2) marcate benefici a leisiones de nephritis lupoide, e
(3) nulle effectos carcinogene.

Es signalate le possibilitate de utiliar iste muses pro studiar le effectos carcinogene de pharmacos antimitotic, con referentia particular al effectos de tal pharmacos super le mechanismos immunologic.

**ACKNOWLEDGMENTS**

I would like to thank Associate Professor J. B. Howie and Professor A. Wynn Williams for their helpful comments and Miss J. C. Littlejohn for her competent technical assistance. Mr. C. F. Spears kindly undertook the statistical analysis.

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Immunosuppression by Cyclophosphamide in NZB X NZW Mice With Lupus Nephritis

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