Nuclei from Normal and Leukemic Mouse Spleen

II. The Nucleic Acid Content of Normal and Leukemic Nuclei

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In an earlier study of the distribution of pentosenucleic acid (PNA) among the various fractions derived from normal and leukemic spleen homogenates by differential centrifugation, it was found that the PNA concentration was greatly increased in a crude, unwashed "nuclear fraction" of leukemic spleen (11). It therefore seemed desirable to determine the nucleic acid content of uncontaminated nuclei. Since in leukemia the PNA is also elevated in the cytoplasm (11), a method for the separation of nuclei in a neutral medium in which contamination with cytoplasmic nucleoprotein should be minimal was developed. The isolation and analysis of nuclei from normal spleen have been reported in the first paper of this series (15). In this paper data on nuclei from spontaneous and transplanted leukemia are presented and compared.

The nuclei prepared from the spleens of mice with transplanted leukemia contain over 4 times as much PNA (per nucleus) as those from normal spleen; their desoxypentosenucleic acid (DNA) and nitrogen contents are also greatly increased. In spontaneous leukemia, on the other hand, the nuclear PNA is but slightly increased, and the DNA and nitrogen contents are normal.

MATERIALS AND METHODS

Mice of the Akm strain (2) were used. The normal mice and those used in the experiments on transplanted leukemia were 2–3 months old. The line of transplanted lymphatic leukemia, 9421, was the same as that used in previous studies (11, 12). Leukemic spleen, minced in saline and injected intraperitoneally, produced advanced leukemia in about 10 days. The spleen weights then averaged about 400 mg., an increase of about 200 per cent over the normal values. The mice with spontaneous leukemia were about 10 months old. Their spleens were very large, weighing from 400 to 1,800 mg.

The nuclei were isolated by a procedure similar to that used on normal spleen (15), except that an increased amount of calcium chloride was added to the 0.88 M sucrose medium. With normal spleen, a concentration of 0.0018 M calcium chloride is sufficient to prevent stickiness and clumping of the nuclei, without causing any aggregation of the cytoplasm. In both spontaneous and transplanted leukemia, however, it was found necessary to increase the amount of calcium chloride to 0.0023 M to obtain the same effect. Staining technics, nuclear counts, and nucleic acid and nitrogen analyses were carried out by the same technics that were used for normal spleen (15).

Control experiments on the effect of calcium chloride were made as follows: One gm. of normal spleen was homogenized for 2½ minutes in a Potter-Elvehjem homogenizer with 9 cc. of 0.88 M sucrose (11). Four cc. of homogenate was then measured into a clean ground glass homogenizer. Two cc. of sucrose was added, and homogenization was continued for ½ minute. The homogenate was then transferred to a 15-cc. centrifuge tube. A second 4-cc. sample of the first homogenate was treated in the same way, except that 2 cc. of 0.88 M sucrose containing 0.0064 M calcium chloride was added, to give a final calcium chloride concentration of 0.0018 M. Both tubes were centrifuged at 1,000 g for 15 minutes. The volumes of the nuclear and cytoplasmic fractions were recorded and the cytoplasm suspension removed for nucleic acid analysis.

A similar experiment was carried out on transplanted leukemia spleen. Here the calcium chloride concentration of the 2 cc. of added sucrose containing 0.0064 M calcium chloride was added, to give a final calcium chloride concentration of 0.0018 M. Both tubes were centrifuged at 1,000 g for 15 minutes. The volumes of the nuclear and cytoplasmic fractions were recorded and the cytoplasm suspension removed for nucleic acid analysis.

RESULTS AND DISCUSSION

The isolated nuclei appeared round, unclumped and undistorted, and resembled morphologically those seen in stained sections of leukemic mouse spleen. The large nucleoli showed up clearly.
The results of the nitrogen, DNA, and PNA analyses are shown in Chart 1. All three are significantly increased in transplanted leukemia, while in spontaneous leukemia only a slight increase in PNA is found.

While the yields of nuclei were low (10–20 per cent) (15), the isolation procedure was the same for normal and leukemic spleen, except for the CaCl₂ concentration. It is therefore probable that about the same proportion of cells was lost during filtration, either because the cells were larger or because they were imbedded in fibrous connective tissue, and that the same proportion of smaller nuclei was lost in the supernatants during the sedimentation and washing steps. The close agreement in nitrogen values between normal and spontaneous leukemic nuclei supports this explanation.

The use of calcium chloride raises the question of whether cytoplasmic PNA has been precipitated by the calcium, since this might lead to contamination of the nuclei. Schneider (16) has found that calcium concentrations similar to those used here precipitate significant amounts of cytoplasmic PNA from liver homogenized in distilled water. Although no agglutination of cytoplasm or nucleoprotein precipitate such as that found in the liver homogenates (16) was ever observed, it seemed advisable to check the effect of calcium chloride by chemical analysis. The results of these control experiments (Table 1) are difficult to interpret.

While the concentration of PNA in the cytoplasm is definitely lower when calcium has been added, the total volume of cytoplasm is significantly increased. As a result, the total amount of PNA left in the cytoplasm is the same for normal spleen, with or without 0.0018 M calcium chloride, and 14 per cent lower for leukemic spleen when 0.0023 M calcium chloride is used.

Chart 1.—The nitrogen, DNA, and PNA contents of nuclei isolated from mouse spleen, in mg. X 10⁻⁴ per nucleus. N = normal spleen; S = spontaneous leukemia spleen; and T = transplanted leukemia spleen.
A second suggestion as to whether the nuclei are contaminated with cytoplasmic PNA may be gleaned from a comparison of the isolated nuclei with the whole “nuclear fraction” obtained by one centrifugation in sucrose alone (11). The PNA: nitrogen ratio (0.08) for the isolated normal nuclei is similar to the ratio found on the whole “nuclear fraction,” 0.07; while for leukemic spleen the ratio for the isolated nuclei is 0.20, definitely lower than the ratio of 0.26 found for the whole “nuclear fraction.” These ratios are also somewhat lower than those found for normal and leukemic spleen nuclei isolated with the aid of citric acid (1).

The reasons for the effect of calcium in preventing stickiness of nuclei have not been investigated in detail. In the experiments on whole spleen reported above, the nuclear fraction from 0.4 gm. of spleen occupied a volume of 0.3 cc. in sucrose with calcium, and of 1.0–1.2 cc. in sucrose alone, after centrifuging 15 minutes at 1,000 g. Only a little more packing is obtained after 30 minutes at 20,000 g. Once they have been packed by centrifuging, even at low speed, the nuclei clump badly and cannot be resuspended. Similar observations have been made by others (1). The calcium prevents this, perhaps by decreasing the solubility of the nucleohistone. Phase contrast photomicrographs of nuclei isolated with the use of calcium show condensed chromatin (15), while in nuclei suspended in 0.88 M sucrose alone the chromatin appears to be evenly dispersed (14). Why a higher concentration of calcium is required for leukemic spleen has not been determined; but it should be noted that when citric acid is used to prevent stickiness of spleen nuclei the leukemic nuclei require more acid than the normal ones (1).

It may be seen (Chart 1) that in transplanted leukemia the DNA per nucleus was markedly increased (to 1.45 times the normal value), while in spontaneous leukemia no such change occurred. It would be of great interest to check these findings on isolated nuclei by determining the number of nuclei per gram of whole spleen and calculating the DNA per nucleus from the DNA content of the whole tissue, as was done by Price and Laird (13) for liver. So far, however, it has not been found possible to obtain reliable nuclear counts on whole spleen. When the organ is homogenized before the removal of the connective tissue fibers, microscopic examination of the homogenate shows clusters of nuclei trapped in networks of fibers, and reliable nuclear counts have not yet been obtained (15). Without values for DNA per nucleus on whole spleen, there is no check on the loss of DNA during the isolation procedure. That any loss of DNA has taken place during the isolation seems unlikely, however, since the value obtained for normal nuclei, $6.5 \times 10^{-8}$ mg. per nucleus, is the same as the values found for mouse and rat spleen nuclei isolated in citric acid (15). Nuclear counts on whole spleen would also serve as a measure of the number of cells per unit weight of tissue (13). Since the total DNA is slightly lower in transplanted leukemia (14 mg. per gram) than in normal spleen (15 mg. per gram) (11), the elevated DNA per nucleus found in transplanted leukemia would indicate that the number of cells per gram of spleen may be considerably reduced.

Observations by other workers on the DNA content of nuclei of neoplastic cells are quite variable. In a single experiment on leukemic mouse spleen, normal values were found (1). Increased amounts of DNA were observed by Stowell in leukemic nuclei (17), although Davidson, Leslie, and White (5), studying unspecified types of human leukemia, failed to observe any change in DNA per nucleus. In tumors of the liver the DNA per nucleus is not increased (3, 9, 13); but elevations to 2 and 3 times the normal values have been observed in mouse ascites tumors (7, 8), and a marked elevation is reported for GRCH 15 tumor in the fowl (4).

It is doubtful whether the increase in DNA in transplanted leukemia can be ascribed to a primary neoplastic change; its most probable cause is an increased mitotic rate. An even more striking elevation has been observed in regenerating rat liver by Price and Laird (13), who found that 24 hours after partial hepatectomy the DNA per nucleus was increased to 1.8 times the normal value; up to this time mitoses were not observed. During the second and third days mitoses reached a maximum frequency, and the average DNA per nucleus fell sharply.

An explanation of these findings on a cellular basis is not easily obtained by chemical analysis of mixed populations of nuclei. Cytochemical methods, in which individual nuclei are studied, give
more detailed information. In the pronephros, a tissue of high mitotic rate, of a newly hatched larva of *Ambystoma opacum*, Swift (18) found the normal amount of DNA (Class I) in telophase nuclei; twice this amount (Class II) in prophase; while in interphase the values were distributed over a broad curve between Class I and Class II, with most measurements falling at the lower end. He concludes that “through much of the interphase the nuclei must keep the Class I amount of DNA, since the majority of the measured resting cells have this value. Then, while still appearing microscopically as a resting nucleus, DNA is built up, and, when the doubled amount is reached, prophase is initiated.” Similar results were obtained on embryonic mouse liver.

It therefore seems that, in the absence of extensive polyplody, the average DNA per nucleus is correlated with the mitotic rate of the tissue. Regenerating liver (DNA 1.8 x normal) triples in weight in about 3 days; in transplanted leukemia (DNA 1.45 x normal) the spleen triples in weight in about 10 days; while in spontaneous leukemia and in liver tumors, where growth is slow, no increase in DNA is seen.

The increase in the PNA content of the nuclei in the transplanted leukemia to over 4 times the normal value is even more striking. PNA has been found in chromosomes by Mirsky and Ris (10) and by Kaufmann, McDonald, and Gay (6). Isolated residual chromosomes prepared from leukemic mouse spleen by the procedure of Mirsky and Ris contained about twice as much PNA as those from normal spleen (12).

A simple calculation, however, shows that the PNA isolated with the chromosomes can account for only a fraction of that found in the whole nucleus. In the whole chromosomes from normal spleen, the ratio of PNA to DNA was about 0.015.

The chromosomes isolated from one nucleus (presumably containing 6.5 x 10^-9 mg. of DNA) would therefore have contained 6.5 x 0.015 = 0.10 x 10^-9 mg. of PNA. Since 0.29 x 10^-9 mg. per nucleus was found, the excess nuclear PNA not in the chromosomes was 0.19 x 10^-9 mg. per nucleus. By the same reasoning, in transplanted leukemia 9.4 x 0.08 = 0.38 x 10^-9 mg. of the nuclear PNA would have been found in the isolated chromosomes; the nuclear PNA not in the chromosomes would be 1.25 - 0.28 = 0.95 x 10^-9 mg. per nucleus, a fivefold increase over the normal value.

Since isolated chromosomes contain nucleoli (10), the true PNA content of the chromosomes themselves is probably less than the values found on chemical analysis; the extra-chromosomal PNA associated with the isolated nuclei may therefore be even greater than these calculations indicate. Also, how much PNA may have been lost during the isolation of the chromosomes cannot be determined. It does appear, however, that the leukemic nuclei contain large amounts of PNA not associated with the chromosomes. This finding is in agreement with results obtained by cytochemical methods; Thorell (19) has shown by ultraviolet absorption that the large and numerous nucleoli characteristic of malignant lymphocytes are rich in PNA. Whether any of this excess PNA, like the DNA, is ascribable to the malignant state, and not just to the rapid growth of the tissue, cannot be determined at the present time. Thorell (19), in a careful study of the PNA content of the developing myelocyte, found that in the primitive myeloblast the PNA content of both nucleoli and cytoplasm was elevated. This is strikingly parallel to the present findings in transplanted leukemia. In spontaneous leukemia, where growth and cell division are relatively slow, the PNA is only slightly increased either in the nuclei or in the cytoplasm.

A rough calculation of the nitrogen distribution in normal and transplanted leukemic nuclei is shown in Table 2. The amount of histone has been assumed to equal the amount of DNA. Although this is considerably less histone than the amount found in the chromosomes from calf thymus (10), it is in better accord with the DNA : N ratios found for mouse spleen chromosomes. The residual chro-

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**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Transplanted Leukemia</th>
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<tbody>
<tr>
<td><strong>DNA</strong></td>
<td>Total N cont.</td>
<td>Total N cont.</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>9.4</td>
</tr>
<tr>
<td><strong>Histone</strong></td>
<td>6.5</td>
<td>1.17†</td>
</tr>
<tr>
<td><strong>PNA</strong></td>
<td>0.29</td>
<td>1.23</td>
</tr>
<tr>
<td>Residual chromosome</td>
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<td>0.35</td>
</tr>
<tr>
<td>protein</td>
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<td></td>
</tr>
<tr>
<td>Sum of nitrogen</td>
<td>2.59</td>
<td>3.76</td>
</tr>
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<td>fractions</td>
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<tr>
<td>Total nitrogen</td>
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<tr>
<td>Nitrogen not accounted</td>
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<td>2.54</td>
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<td>for:</td>
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<tr>
<td>Isolated chromosomes</td>
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<tr>
<td>DNA: N ratio</td>
<td>2.4</td>
<td>2.71</td>
</tr>
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<td>Total nitrogen in</td>
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<td>3.62</td>
</tr>
<tr>
<td>chromosomes</td>
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</tr>
<tr>
<td>Residual chromosome</td>
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</tr>
<tr>
<td>nitrogen</td>
<td>0.53</td>
<td>0.43</td>
</tr>
<tr>
<td>by difference</td>
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</tr>
<tr>
<td>16 per cent of total</td>
<td>0.47</td>
<td>0.38</td>
</tr>
<tr>
<td>* 14 per cent of NA.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>† 16 per cent of histone.</td>
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</tr>
</tbody>
</table>

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mosome nitrogen has been calculated from the DNA:N ratios for whole chromosomes and the percentage of nitrogen recovered in residual chromosomes (12).\(^3\) Approximate as these values are, they indicate the presence of more nonchromosome nitrogen in the leukemic nucleus (2.41 \(\times\) 10\(^{-9}\) mg.) than in the normal nucleus (1.00 \(\times\) 10\(^{-9}\) mg.). This, also, is in agreement with morphological observation of the frequent large nucleoli present in leukemic lymphocytes.

**SUMMARY**

Nuclei have been isolated from the spleens of mice bearing spontaneous or transplanted leukemia.

In spontaneous leukemia, which develops slowly, the PNA per nucleus is increased to 1.6 times the normal amount, and there is no change in DNA or nitrogen.

In transplanted leukemia, which develops rapidly, the PNA, DNA, and nitrogen per nucleus are increased to 4.2, 1.45, and 1.69 times their respective amounts in normal spleen.

The differences in nucleic acid content found in the nuclei of the transplanted leukemia are similar to those reported for rapidly growing tissues, while the nuclei from the spontaneous leukemia more closely resemble the normal nuclei. Thus, the changes seem to be characteristic of rapid growth rather than of any primary neoplastic process.

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