On the Growth Activity of Virus-induced Leukemia Cells*†

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Leukemia is characterized by a neoplastic and malignant growth of cells deriving from the hematopoietic organ. The present investigation is an attempt to evaluate the growth activity of leukemia cells in quantitative terms for comparison with the growth of the homologous, normal hematopoietic cells.

Virus-induced erythroleukemia in chicken provided the experimental material. Two different systems of measuring were chosen for assessing the growth activity.

First, growth as manifested by cell division was recorded by direct counting of mitotic figures in cytological preparations. Astaldi and Mauri's (2) stathmokinetic test—i.e., the accumulation of mitotic figures at various times after colchicine treatment—was also applied.

Second, the DNase activity in correlation with the cellular nucleic-acid content was determined. In earlier investigations we found this enzyme activity to parallel the variations in proliferative activity during regeneration of bone marrow cells (8). These findings are in agreement with other results on the DNase pattern in nonmalignant growth (3, 6). On the other hand, a lack of parallelism between growth activity and the DNase activity has been found in several human and chemically induced experimental tumors (6, 7).

One object of the present investigation was, therefore, to establish the DNase pattern during virus-induced tumor growth, a problem hitherto not attacked.

MATERIALS AND METHODS

Young chickens of a White Leghorn strain (EDO) and 2-4 weeks old were used. Before and during the experiment they had free access to a standardized chicken food (Fors). Erythroleukemia was induced by intravenous inoculation of a virus suspension obtained from a leukemic spleen-tissue extract as described earlier (10). The given dose insured 100 per cent "take" with an average latency period of about 10 days (12). Untreated animals of the same age and size served as normal controls.

For the mitotic frequency counts, animals were given injections intraperitoneally on the 9th-10th day after the virus inoculation of 2-4 mg. of N-deacetyl-N-methyl-colchicine (Colcemid, Ciba) and sacrificed after 0-10 hours. This dose (10 mg/kg body weight), given in a concentration of 1 mg/ml, corresponded to the 50 per cent lethality dose (LD50) as shown by a separate experiment. Air-dried smears, stained with May-Grünwald Giemsa azure-eosin were prepared from the bone-marrow of both femurs of each chicken. Paraffin sections, stained with hematoxylin and eosin, were also made for the histological evaluation of the bone-marrow involvement (12).

For the biochemical analyses chickens weighing approximately 500 gm. were used. The animals were sacrificed by exsanguination, and bone marrow from the femurs was taken out as soon as possible and immediately frozen on dry ice. A small sample of each marrow was put aside for a cytological and histological check. After being thawed, the material was homogenized in distilled water for 4 minutes with a Bühler homogenizer at 40,000 r.p.m. The tissue concentration was about 10 per cent. Precautions were taken to keep the temperature of the homogenate close to 0°C.

The DNase activities were determined as described earlier (3). As a substrate, thymus DNA, prepared according to Hammarsten (11), was used. The activities were measured as the increase in acid-soluble products from DNA, produced under standard conditions. The DNase activities are expressed as μg. DNAP liberated per hour per mg. tissue DNAP or per mg. dry weight.

The nucleic acids were determined as described earlier (4, 5).
RESULTS

All virus-induced leukemias in the present experiments exhibited the typical course observed in our earlier experience (1, 10, 12).

CYTOLOGICAL FINDINGS

Chart 1 and Table 1 show the values for the accumulation of mitotic figures in normal erythroblasts and leukemia cells 0-10 hours after the Colcemid treatment. A successive rise of the mitotic ("stathmokinetic") index occurs during this period in all three developmental stages of the normal erythroblasts. The basophilic and polychromatic erythroblasts give higher figures than do the proerythroblasts. A maximal arrest of mitosis occurs after about 8 hours in accordance with earlier studies on the duration of the colchicine effect (9). A similar pattern is obtained for the leukemia cells, but with two significant deviations. First, there is a lag in their reaction to Colcemid. Not until 4 hours after the treatment does an increase in arrested leukemic cell mitosis occur. Second, the maximal values at 8 hours are all above the values for normal erythroblasts. The standard errors (Table 1) were established in normal erythroblasts and erythroleukemic cells and found to be from ±0.3 to ±2.1. The rapid accumulation of arrested mitoses in erythroleukemic cells in the period 4 and 8 hours after the treatment indicates a more active proliferation of the leukemia cells. The difference between the average mitotic frequency of normal erythroblasts and leukemia cells after 8 hours was calculated and found to be 19.6 per cent. The probability of this being due to chance is <0.001, and the difference is therefore highly significant.

BIOCHEMICAL FINDINGS

Nucleic acids.—Table 2 is a summary of the results obtained as compared with some of the results on normal and regenerating chicken bone marrow from an earlier study (8). As may be seen, the PNAP content/mg dry weight was rather low in the normal, resting bone marrow, the average being 2.09. It increased during regeneration induced by phenylhydrazine, and a further increase was found in erythroleukemia, the average being 5.22. The DNAP-content/mg dry weight did not show any consistent changes in these varying states.

The PNAP/DNAP quotients are rather low in normal, resting bone marrow, and the increase noticed during regeneration after phenylhydrazine is statistically significant (8). These ratios show a considerable and consistent increase in erythroleukemia as compared with the values found during regeneration.

DNase activities.—The DNase activities are presented with reference to the tissue DNAP as well as per mg dry weight (Table 2). The mode of calculation does not influence the results in this material, and the patterns observed in the different states were identical. The lowest values were observed in the normal group, a considerable increase being found in the regeneration group and in erythroleukemia. It should perhaps be noticed that the highest values in the erythroleukemic group were higher than the maximal values observed in the regeneration group.

PNAP/DNAP quotients in relation to DNase activities.—During normal growth there is a consistent parallelism between PNAP/DNAP quotients and DNase activities, i.e., high quotients are invariably accompanied by high enzymic activities, and a decrease in the ratios is associated with a decrease in DNase activities (8, 6, 8).
In erythroleukemia this was not the case. In this material there was an inverse, atypical pattern. The highest PNAP/DNAP quotients were found in tissues with a DNase activity within the normal, relatively low range.

**DISCUSSION**

"Leukemia is a morbid condition... characterized by widespread proliferation of the leukocytes and their precursors in the tissues of the body" (14). The leukemic cell counts in the periph-

**TABLE 1**

**AVERAGE MITOTIC INDICES OF ERYTHROLEUKEMIA CELLS AND NORMAL ERYTHROBLASTS**

<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>0 (per cent)</th>
<th>2 (per cent)</th>
<th>4 (per cent)</th>
<th>6 (per cent)</th>
<th>8 (per cent)</th>
<th>10 (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal erythroblasts from control animals, Exp. series no. 1</td>
<td>2.2±0.4</td>
<td>12.1±1.1</td>
<td>20.8±1.3</td>
<td>47.5±1.6</td>
<td>53.9±1.6</td>
<td>44.4±1.5</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 2</td>
<td>2.7±0.5</td>
<td>10.8±1.0</td>
<td>22.1±1.3</td>
<td>46.8±1.6</td>
<td>51.2±1.6</td>
<td>37.4±1.6</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 3</td>
<td>2.6±0.3</td>
<td>13.4±1.1</td>
<td>20.7±1.3</td>
<td>38.0±1.5</td>
<td>52.4±1.6</td>
<td>54.0±1.6</td>
</tr>
<tr>
<td>Leukemia cells, Exp. series no. 1</td>
<td>2.9±0.5</td>
<td>3.9±0.6</td>
<td>5.6±0.5</td>
<td>46.2±1.6</td>
<td>72.8±1.5</td>
<td>8.9±1.0</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 2</td>
<td>3.3±0.6</td>
<td>2.0±0.4</td>
<td>5.8±0.7</td>
<td>40.2±1.6</td>
<td>72.8±1.5</td>
<td>53.0±1.6</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 3</td>
<td>2.5±0.5</td>
<td>3.2±0.6</td>
<td>5.2±0.6</td>
<td>63.3±2.1</td>
<td>71.3±1.8</td>
<td></td>
</tr>
<tr>
<td>Normal erythroblasts remaining in the leukemic bone marrows.</td>
<td>2.5±0.5</td>
<td>12.1±1.1</td>
<td>21.2±1.3</td>
<td>44.1±1.5</td>
<td>52.5±1.8</td>
<td>51.9±1.8</td>
</tr>
</tbody>
</table>

Each value represents one animal. Thus, each experimental series comprises six different chickens. The last set of values (normal erythroblasts in leukemic bone marrows) is pooled from the leukemic series nos. 1-3. One thousand cells of each type were counted in each animal on azure-eosin stained bone-marrow smears. The deviation from the mean values was calculated according to conventional statistical methods. The figures for normal erythroblasts are the calculated mean values for all the three types individually represented in Chart 1. The numerical ratios between these three types were approximately 1:10:10 in the eighteen control animals. In the leukemic animals very few of the earliest normal cell types ("proerythroblasts") were found, but a sufficient number of basophilic and polychromatophic cells, on the other hand, to give representative counts.

**TABLE 2**

**NUCLEIC ACIDS AND DNASE ACTIVITY IN NORMAL, REGENERATING, AND LEUKEMIC CHICKEN BONE MARROW**

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>μg PNAP/mg dry weight</th>
<th>μg DNAP/mg dry weight</th>
<th>PNAP/DNAP</th>
<th>DNase/μg DNAP</th>
<th>DNase/mg dry weight</th>
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</thead>
<tbody>
<tr>
<td>N 1</td>
<td>3.78</td>
<td>6.15</td>
<td>0.62</td>
<td>1.14</td>
<td>7.00</td>
</tr>
<tr>
<td>N 2</td>
<td>1.92</td>
<td>3.13</td>
<td>0.61</td>
<td>1.54</td>
<td>4.76</td>
</tr>
<tr>
<td>N 3</td>
<td>1.77</td>
<td>3.17</td>
<td>0.56</td>
<td>1.58</td>
<td>5.00</td>
</tr>
<tr>
<td>N 4</td>
<td>1.66</td>
<td>2.81</td>
<td>0.54</td>
<td>1.89</td>
<td>3.91</td>
</tr>
<tr>
<td>N 1*</td>
<td>1.76</td>
<td>2.88</td>
<td>0.61</td>
<td>1.55</td>
<td>4.50</td>
</tr>
<tr>
<td>N 2*</td>
<td>1.14</td>
<td>3.20</td>
<td>0.35</td>
<td>0.80</td>
<td>2.40</td>
</tr>
<tr>
<td>N 5*</td>
<td>2.63</td>
<td>2.87</td>
<td>0.92</td>
<td>0.60</td>
<td>2.70</td>
</tr>
<tr>
<td>R 2*</td>
<td>2.49</td>
<td>3.69</td>
<td>0.69</td>
<td>1.95</td>
<td>7.51</td>
</tr>
<tr>
<td>R 3*</td>
<td>3.21</td>
<td>2.76</td>
<td>1.16</td>
<td>4.35</td>
<td>11.11</td>
</tr>
<tr>
<td>R 4*</td>
<td>3.10</td>
<td>3.54</td>
<td>0.88</td>
<td>3.25</td>
<td>12.01</td>
</tr>
<tr>
<td>R 5*</td>
<td>3.71</td>
<td>4.96</td>
<td>0.75</td>
<td>1.75</td>
<td>7.51</td>
</tr>
<tr>
<td>L 1</td>
<td>4.71</td>
<td>3.08</td>
<td>1.53</td>
<td>2.99</td>
<td>9.21</td>
</tr>
<tr>
<td>L 2</td>
<td>4.55</td>
<td>2.68</td>
<td>1.70</td>
<td>4.86</td>
<td>13.02</td>
</tr>
<tr>
<td>L 3</td>
<td>6.30</td>
<td>3.16</td>
<td>1.99</td>
<td>1.76</td>
<td>5.38</td>
</tr>
<tr>
<td>L 4</td>
<td>3.09</td>
<td>2.27</td>
<td>1.36</td>
<td>3.59</td>
<td>8.15</td>
</tr>
<tr>
<td>L 5</td>
<td>5.98</td>
<td>3.11</td>
<td>1.92</td>
<td>4.68</td>
<td>14.59</td>
</tr>
<tr>
<td>L 6</td>
<td>6.08</td>
<td>3.75</td>
<td>1.62</td>
<td>3.50</td>
<td>12.36</td>
</tr>
<tr>
<td>L 7</td>
<td>5.85</td>
<td>3.70</td>
<td>1.58</td>
<td>2.57</td>
<td>9.52</td>
</tr>
</tbody>
</table>

* These values are from Brody and Thorell (8) and have been recalculated per μg. DNAP or per mg. dry weight. Each set of analysis is from a single animal.
† N = normal bone marrow; R = regenerating bone marrow; L = leukemic bone marrow.
eral blood are often high compared with the number of normal homologous cell types, and mitotic figures can also be seen. On the other hand, Astaldi and Mauri 1953 (2) and also Rosin and Goldhaber 1958 (13) observed, on the basis of a direct count of the mitotic frequencies after colchicine inhibition, that acute leukemia "belongs functionally to the group of aplastic bone-marrow diseases" and the "proliferation of leukemic myelocytes is definitely inferior to that of normal myelocytes . . ."

In the present investigation the growth activity of the leukemia cell has been evaluated with two different and independent methods: (a) The cyto
tological counting of the mitotic index and (b) the biochemical determination of nucleic acid levels and the DNase activity. The results show that in most cases of virus-induced chicken erythro-
leukemia the mitotic index, both with and without colchicine inhibition, was in the leukemic cells at least as high as or higher than that in the normal homologous cells from control chickens of the same age. In most leukemia cases the pentose-
nucleic acid values and the DNase activities showed, both per average cell and per unit dry weight, higher values than those found in regener-
ating bone marrow after phenylhydrazine-induced hemolysis. Thus, the virus-induced leukemic cells must be considered to possess a marked and frequently manifested proliferative capacity.

Significant deviations from the pattern observed in normal bone marrow were, however, noticed. No increase in the number of arrested mitoses in erythroleukemic cells was found until 4 hours after the administration of Colcemid. An initial lag of the Colcemid effect was thus observed in the erythroleukemic bone marrow as compared with the controls (Table 1). In the latter material a rise was observed after only 2 hours. As to the cause of this difference, one may exclude any exogenetic factors which might be expected to influence the mitotic process. This statement is based on the observation that the proliferative activity of the remaining normal erythroblasts in the leukemic bone marrows was quite normal (Table 1).

The differences in the response of the different cell types to Colcemid (Chart 1) may explain earlier conclusions based on the mitotic (stathmokinetic) index 4 hours after the administration of colchicine to the effect that there is a general low prolifera-
tive activity of the leukemic cells (13). At this point we also observed a lower mitotic index in the leukemic bone marrow as compared with that of the controls. During the next 4 hours, however, there was an impressive accumulation of mitotic figures in the leukemic cells.

Other causes must also be discussed in connection with discrepancies observed in the mitotic index of leukemic cells. Thus, during the development of erythroleukemia in chicken the predominance of different cell types has been observed at different stages of the disease (1). Type I (2-4 days after the virus-inoculation) is a large, immature, and atypical cell. No hemoglobin can be shown in these cells. Type IV, at the premor-
bund state, is a smaller, intensely basophilic cell appearing in the peripheral blood and containing measurable concentrations of hemoglobin. It is conceivable that the respective proliferative activities of these cell types differ. The results of the mitotic-index counts must be influenced by the predominance of the one or the other cell type at the time of death, which predominance is in its turn influenced by a multiplicity of factors, for instance, the inoculated dose, the general condition of the animal, the degree of infiltration into vital organs such as the lungs, etc.

The DNase activity of the erythroleukemic bone marrow was on an average considerably higher, as compared with that observed in control animals of the same age. The highest values observed in the erythroleukemic group were also well above those found in bone marrow in which regeneration had been induced by phenylhydrazine. There is thus a correlation between the average high mitotic (stathmokinetic) index and DNase activity in the erythroleukemic bone marrow cells, a correlation which also exists in the normal hematopoietic cells (8).

It should be noted, however, that in two animals low DNase activities were observed, these being of the same order of magnitude as the enzymic activities found in bone marrow from normal, nonregenerating animals. As can be seen from Table 2, these relatively low DNase activities were associated with very high PNAP/DNAP quotients.

The chemical data showed average values for the biochemical composition of the existing cell types, although the microscopic control revealed

Figs. 1 and 2.—Erythroleukemic cells in the peripheral blood smear from cases no. L 2 (Fig. 1) and no. L 3 (Fig. 2) in Table 2. Two large, "immature" leukemia cells and a mitotic figure are seen in Fig. 1. Four "mature," small and intensely basophilic cells are observed in Fig. 2. May-Grünwald-Giemsa stain, X 1000.
a vast predominance of a certain atypical and malignant cell type in the leukemic bone marrow. On the basis of earlier investigations (1) in which a continuous increase in the ultraviolet density of the cytoplasm at r A was observable during the development from cell type I to type IV, it could be assumed that the high PNAP/DNAP quotients noted in some of the bone marrow samples (Table 2) reflect a predominance of cell type IV, the hemoglobin-containing, mature erythro-leukemic cell. Examination of bone marrow sections and blood smears taken from the leukemic animals at the time of sacrifice confirms this. Figures 1 and 2 show typical pictures from the different types found in the present material. In Figure 1, showing a smear from an animal with a moderately increased PNAP/DNAP quotient and very high DNase activity, cells belonging to the not fully developed type III can be seen. Mitotic figures are also observed in the peripheral blood. In Figure 2, a smear from an animal with a very high PNAP/DNAP quotient and with a DNase activity at the same level as that found in normal bone marrow, cells of the "mature" type IV dominate the picture. These results strongly suggest a correlation between the degree of development of the virus-induced erythroleukemic cell and the DNase activity, which is in turn correlated with the proliferative activity. These findings on the DNase activity in virus-induced leukemic growth are at variance with those obtained in chemically induced malignant growth in rat liver (6). The latter showed a decrease in DNase activities as well as low PNAP/DNAP quotients. Probably the mechanisms stimulating malignant growth in the chemically induced tumor and the virus-induced erythro-leukemia differ. In the first case a protein-deleting mechanism (6), apparently affecting also proteins concerned with DNase synthesis, is involved. In erythro-leukemia a stimulation of the cellular growth mechanisms takes place in certain phases of pathological cell differentiation. The interaction between the growth stimulation and the pathological cell differentiation results in variations of the strictly defined growth function from case to case and possibly within the same animal in different stages of the disease. The type of leukemia studied in the present investigation cannot, therefore, be described in terms of either high or low growth activity. The pathological cell differentiation induced by the virus must be regarded as the principal change.

SUMMARY

The growth activity of virus-induced chicken erythro-leukemia cells as compared with that of the normal erythroblasts has been studied by measuring the rate of accumulation of mitotic figures after colchicine inhibition and through analyses of the nucleic acids and the DNase activity. The results indicate a higher growth activity, on the whole, in the leukemic cells, even as compared with the stimulated erythropoietic tissue after phenylhydrazine-induced hemolysis. Thus, the highest mitotic frequencies, PNAP/DNAP quotients, and DNase activities were found in the leukemic material. Significant deviation from this general trend was, however, observed in some leukemia cases, i.e., a very high content of pentose nucleic acids combined with low DNase activity and low mitotic rate. This was tentatively interpreted as indicating that the different experimental animals represented different developmental stages of the pathological cell differentiation induced by the leukemia virus.

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