Methyl-Green-Pyronin as a Differential Nucleic Acid Stain for Peripheral Blood Smears

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IT HAS been known for many years that desoxyribosenucleic acid (DNA), largely confined to the nucleus in mammalian cells, can be stained preferentially by methyl green and by leukobasic fuchsin, as in the Feulgen stain. The exact function of DNA is unknown but it may be connected with the reproduction of the gene elements during cell division.[1] Its quantity remains constant once the cell ceases to divide.

Ribosenucleic acid (RNA) is predominantly found in the cytoplasm but is also present in nucleoli. What role this nucleic acid plays in cell metabolism is not definitely known, but is thought to be important in protein synthesis.[1] In general, as the cell matures the mechanism for formation of protein becomes progressively less active paralleling a continuous decrease in cytoplasmic RNA.[2] The most immature cells contain the greatest amount of RNA. This fact applies to most cells of the body, not excluding those of the erythroid and myeloid series. Thus, it is well established that the myeloblast, for example,[1, 3] has a high concentration of RNA in the cytoplasm and a small amount in its nucleoli. With increasing maturity the cells of the myeloid series lose their RNA so that the mature segmented granulocyte is thought to contain no cytoplasmic RNA.[3] However, the plasma cell is considered to have a large amount of RNA which it retains with development.[4] The mature lymphocyte, also, contains some RNA which becomes marked upon antigenic stimulation.[5] The entire subject of nucleic acid metabolism and cellular localization by histochemical and other means has been extensively reviewed in several excellent papers recently[3, 6-9] and hence will not be discussed here.

Whereas the localization of DNA has been a relatively simple problem, histochemical identification of RNA has been quite difficult and possible of accomplishment only with the elaborate and complicated ultraviolet method of Caspersson.[10] Ribonuclease has been employed for the enzymatic degradation of RNA using a nonspecific stain such as eosin methylene blue.[7] However, in 1951 Taft reported[10] that pyronin Y was a specific stain for RNA but that other pyronins, such as pyronin B were of no value in this regard. This work apparently has been substantiated[11-12] so that now the combination of methyl-green and pyronin, originally introduced by Pappenheim, is again being employed as a differential nucleic acid stain. Although it has been applied widely in studies of tissue cells,

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to our knowledge it has not been used in any intensive study of cells as they appear in peripheral blood smears.

It is logical to assume that although the majority of the segmented granulocytes appear to be of the same age morphologically they are actually in different stages of maturity. Accordingly, in clinical conditions accompanied by a leukocytosis, the percentage of these relatively "immature" well segmented cells should be increased. Furthermore, in some systemic diseases, nucleic acid metabolism is disturbed and this might be reflected in the appearance of RNA in peripheral blood cells.

Method and Materials

Simultaneous peripheral white blood counts and differential counts using Wright's stain and methyl-green-pyronin Y were obtained in patients with a variety of clinical disorders. In many instances patients were studied repeatedly as their clinical conditions warranted. Laboratory technicians and other employees served as the control group. A peripheral blood count of 10,000 white blood cells per cu. mm. was arbitrarily chosen as the upper limit of normal.

The stain employed in this study is an adaptation of suggestions made by Kurnick[11] and Taft[6] with certain modifications. First, 0.5 Gm. methyl green (National Aniline, N. Y.) is dissolved in 100 ml. M/10 acetate buffer at pH 4.4 and extracted repeatedly with chloroform until clear. Then 0.2 Gm. pyronin Y (Matheson Coleman & Bell, East Rutherford, N. J.) is added. Blood smears are prepared by the coverslip method, and after drying are fixed in Carnoy's solution[14] for 10 minutes. With this fixative, mature red blood cells hemolyze and are not seen on the stained slide. When methyl alcohol, the usual fixative for blood smears is used, cytoplasmic staining with pyronin Y is uniformly extreme and intense in all cells, is not affected by ribonuclease, and hence is of no value in this study.

Ribonuclease (Armour) was employed as a control on the pyroninophilia in selected smears. A solution of 1 mg. per ml. distilled water was prepared and brought to 60 C. In the test it was kept at 37 C. for 1–2 hours while the slides were immersed prior to staining. Distilled water alone was without effect.

Staining schedule: The staining procedure, carried out in coverslip staining dishes, is as follows: (1) Rinse smears in 95 per cent alcohol for a few seconds. The time for all the rinsing procedures must be determined by trial and error. (2) Rinse in distilled water for a few seconds and drain carefully. (3) Stain in methyl-green-pyronin Y for 30 minutes. (4) Rinse in distilled water for a few seconds. (5) Rinse in normal butyl alcohol for a few seconds. (6) Drain and mount.

Scoring method: A scoring technic recently suggested[15, 16] was found suitable for comparing smears. Pyroninophilia in each of the first one hundred cells of the myeloid series was graded 0 to 3+ with that in the cytoplasm of the mature lymphocyte invariably being sufficiently intense to warrant a 3+ and thus serve as a control for the staining on the particular slide. The sum of the values obtained by multiplying the number of cells by their rating was recorded as the "score."

Results

A total of 660 peripheral blood smears were obtained on different occasions on 411 patients with 67 diseases. Normal individuals showed remarkably consistent cytoplasmic pyroninophilia when studied repeatedly. Scores in 40 controls ranged from 17 to 161 with a mean of 88. Three smears were above 150 and 50 per cent fell below 87. Examination of only segmented mature granulocytes in five normal bone marrows resulted in an average score of 145 with a range of 127 to 163. The conditions studied and the results are listed in table 1.

TABLE 1.—*Average Leukocyte Cytoplasmic RNA Scores in Various Conditions*

Diagnosis	No. of pts.	Average W.B.C.	Per cent of leuckocytes rated				Total aver. score
			0	1+	2+	3+	
Cushing's syndrome	1	5925	64	29	5	2	45
Paroxysmal nocturnal hemoglobinuria	2	4650	67	22	9	2	46
Iron deficiency anemia	1	9450	49	36	10	5	71
Gouty arthritis (chronic)	4	9557	49	35	11	5	72
Hyperthyroidism	3	7958	40	45	11	4	79
Pernicious anemia (in remission)	4	7568	40	43	14	3	80
Normal	40	7575	30	54	14	2	88
Multiple sclerosis	5	15600	39	39	17	5	88
Myocardial infarction	2	14850	20	69	9	2	93
Acquired hemolytic anemia	2	14175	29	51	14	6	97
Multiple myeloma	6	11416	22	54	19	5	107
Polycythemia vera	6	15333	17	54	23	6	118
Malignancies	14	18605	23	44	22	11	119
Rheumatoid spondylitis	4	7450	15	56	23	6	120
Rheumatoid arthritis	19	10772	13	56	22	9	127
Refractory anemia	2	2962	24	36	29	11	127
Sickel cell anemia	2	18712	20	46	20	14	129
Erythroblastosis fetalis	1	18570	14	48	29	9	133
Myelofibrosis	3	14800	14	45	30	11	138
Hemochromatosis	1	5850	6	60	22	12	140
Lupus erythematosus	2	7950	11	51	25	13	141
Hodgkin's disease	4	10550	9	50	30	11	143
Infections (bacterial)	44	13923	17	36	29	18	147
Portal cirrhosis	4	15862	7	47	34	12	151
Chronic myelogenous leukemia	6	96545	14	38	27	21	154
Rheumatic heart disease (active)	1	11000	4	44	35	17	165
Pernicious anemia (in relapse)	3	4841	7	34	32	27	179
Idiopathic thrombocytopenic purpura	4	12958	5	35	31	29	183
Hypothyroidism	1	5200	3	29	45	23	188
Chronic lymphatic leukemia	2	189500	3	27	42	28	195
Acute leukemia stem cell (treated)	3	6050	6	23	32	39	205

With our technic, cells seen in the peripheral blood and marrow may be characterized as follows:

1) *Segmented neutrophil.* There is usually very little pyronin-staining material to be seen in the cytoplasm. When present, the intensity usually is sufficient to warrant only a 0 to 1+. Pyroninophilia appears as a moderately coarse pink granulation with no regular pattern of distribution. The nucleus is prominent,

staining green to blue-green but containing some purple areas. Toxic granulations and Döhle bodies fail to take either stain.

2) *Lymphocyte.* The nucleus is clumped and stains blue-green with a small amount of pink material being visible. The cytoplasm is quite distinctive showing a rim of solidly staining red. The mononuclear cell in infectious mononucleosis has a cytoplasm that is magenta in color, both the nucleus and cytoplasm appearing vacuolated. It often does not have the thin rim of the normal lymphocyte and in general simulates the appearance of a very early, immature form.

3) *Basophilic leukocyte.* The nucleus is blue-green and is almost obscured by large dark red granules which are also seen in the cytoplasm.

4) *Eosinophilic leukocyte.* There is little to differentiate this cell from the neutrophil except for what appears to be well defined homogeneous vacuoles in the cytoplasm outlined very faintly in pink. The nucleus assumes the characteristic bi-lobed appearance.

5) *Monocyte.* The cell is covered with innumerable fine pink-red granules, the nucleus being a faint blue-red.

6) *Platelets.* These appear a bright pink-red with irregular indistinct edges.

7) *Myeloblast.* This cell, as well as all the other blasts, is readily identified. The nucleus is almost homogeneous in structure, staining a "muddy" blue-green. The cytoplasm stains diffusely with an intense red, nearly magenta color.

8) *Myelocyte.* This cell is distinctive, the nucleus being almost obscured by very heavy red granulation which appears also in the cytoplasm. The individual granules are larger and more discrete than those seen in the monocyte but smaller than in the basophil.

9) *Metamyelocyte.* The nucleus shows a moderate amount of pyronin-staining material. Cytoplasmic granules are fairly coarse with pyroninophilia usually rating 2+ or 3+.

10) *Band.* The nucleus of the band also contains a good deal of the pyronin stain but cytoplasmic RNA usually is only 1+ or 2+.

11) *Erythroblast.* The nucleus is a distinct blue-green and appears clumped. The cytoplasm is intensely pink and quite homogeneous.

12) *Normoblast.* The nucleus is a bright clear blue-green with little or no pink material. Its clumped structure is similar to that seen with Wright's stain but much more definite. The cytoplasm varies from a lacy appearance to a more homogeneous solid pink-red and is very intense in color. It is quite distinct from the small lymphocyte and easily recognized.

13) *Lymphoblast.* The nucleus is a homogeneous clear blue-green having very little pink-staining material. There is a solid thin rim of magenta-red granularity at the edge of the cytoplasm which distinguishes this cell from the myeloblast.

14) *Monoblast.* The nucleus appears a muddy blue-green with a fine granular faintly pink-staining material covering the entire cell.

15) *Plasma cell.* As with other stains, the nucleus may or may not have a cart-wheel appearance, is eccentrically placed and stains a dull blue-green. The cytoplasm demonstrates a diffuse magenta-pink granularity.

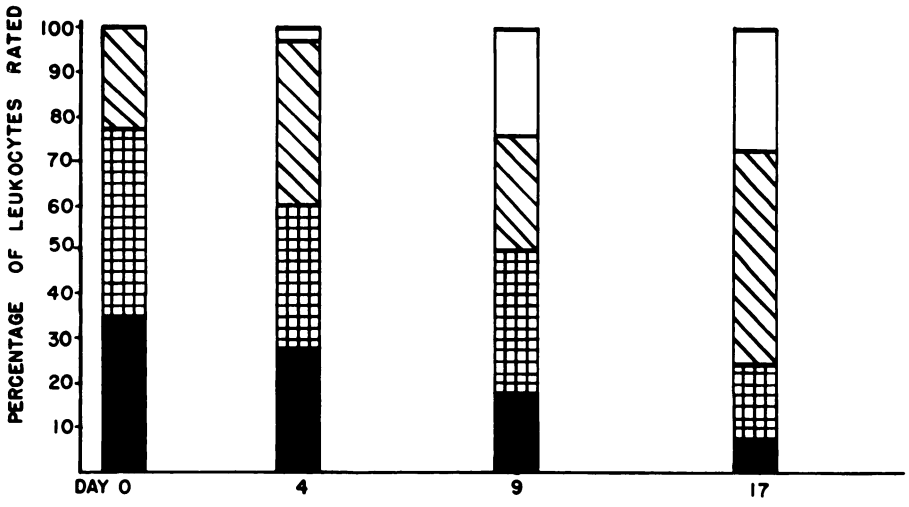

FIG. 1.—Fall in leukocyte cytoplasmic RNA with clinical improvement (penumonia).

DISCUSSION

Staining of peripheral blood smears with methyl-green-pyronin, as used in this study, yielded some interesting and important information not apparent with the routine Wright's stain. Assuming that pyroninophilia is a measure of RNA, it was found that there was little of this nucleic acid present in leukocytes in peripheral blood smears of normal people. This confirmed the observations of other investigators, although generally it is thought that RNA is virtually absent from cells more mature than the myelocyte.[1, 3, 7] However, using Carnoy's solution as a fixative and methyl-green-pyronin as described, this has not been our experience. In fact, a small percentage of mature segmented leukocytes normally demonstrate fairly intense pyroninophilia (table 1 and fig. 2). Control studies with ribonuclease demonstrated that this was RNA and not a nonspecific staining reaction of other cytoplasmic materials.

Identification of cells was readily accomplished at all the stages of development. It was unnecessary to resort to the dictum that "a cell is known by the company it keeps." For example, in the cases studied, no difficulty was encountered in differentiating lymphoblasts from myeloblasts.

Results in infections are worthy of comment. The presence of pyroninophilia in the cytoplasm of leukocytes in the peripheral blood showed no correlation with the height of the white blood count or the "shift to the left." Patients with elevated white blood counts of brief duration usually did not demonstrate a rise in their RNA "scores." If treatment were initiated early after the onset of the infection, the "score" invariably did not increase. Occasionally, pyroninophilia did not become marked until leukocytosis had disappeared and the patient had recovered. A similar situation has been reported by Valentine et al.[17] in connection

with leukocyte alkaline phosphatase in the leukocytosis of myocardial infarction. It is logical to assume that only mature cells contribute to the initial leukocytosis of an infection. However, with the continuation of the disease process, myelopoiesis is accelerated. Leukocytes, appearing morphologically quite mature, are then released into the circulation but these cells, if judged by their RNA content, are relatively immature. As a rule, though, the quantity of cytoplasmic RNA decreased as clinical improvement occurred (fig. 1) and the white blood count returned to normal. It is interesting to note that in one case of subacute bacterial endocarditis, the "score" rose immediately following a rise in temperature although the white count did not change appreciably.

That an elevated white blood count alone is in itself not necessary for the increased appearance of RNA in peripheral white blood cells is emphasized by studies in patients with polycythemia rubra vera. Although all had a leukocytosis of some degree, none had a markedly increased "score." In fact, pyroninophilia occurred in some patients with diseases such as pernicious anemia in which leukopenia is a characteristic feature. It is of interest that following a therapeutic response in pernicious anemia, the RNA score falls to normal levels (fig. 3). Davidson et al.,[3] using chemical methods, have reported elevated RNA and total nucleic acid content in the bone marrows of patients with pernicious anemia. With treatment both determinations returned to normal.

The explanation for increased RNA scores in uncomplicated cases of rheumatoid arthritis, gout, idiopathic thrombocytopenic purpura, malignancies, pernicious anemia in relapse, etc., is not apparent at this time. However, it may be

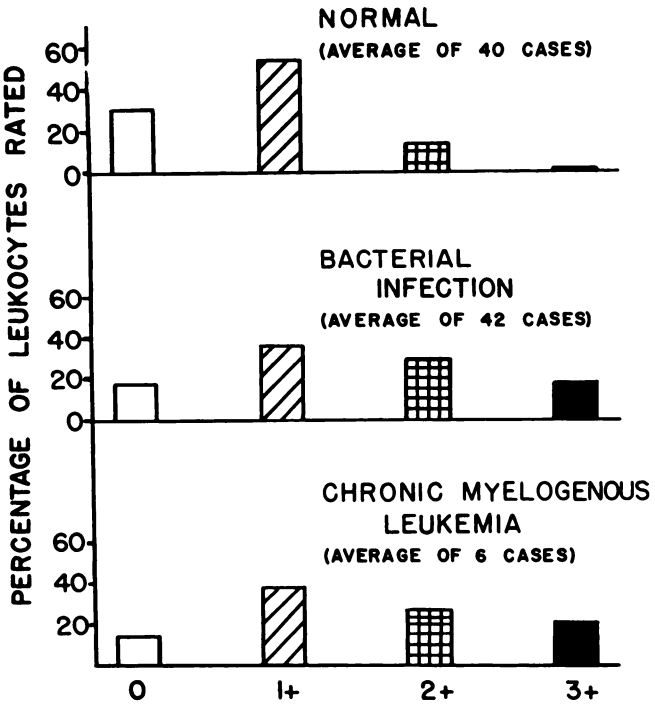

FIG. 2.—Average distribution of cytoplasmic RNA in normal, infection, and leukemia.

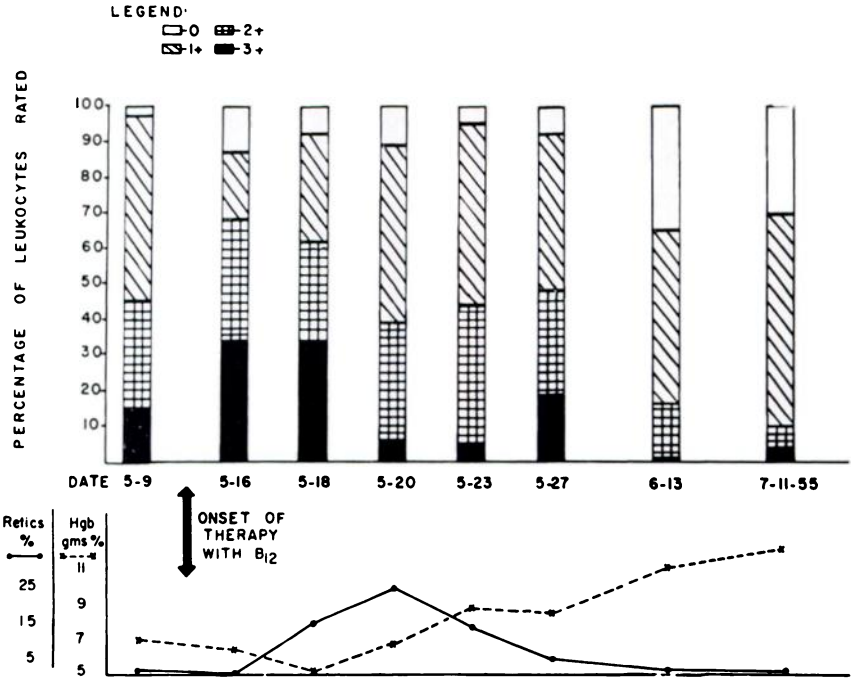

FIG. 3. Changes in leukocyte cytoplasmic RNA with treatment in a case of pernicious anemia.

related to a more rapid release rate of what appear to be morphologically mature well segmented polymorphonuclear leukocytes or to altered RNA metabolism. The fact that the "score" for segmented leukocytes in normal bone marrow is elevated indicates that a shortened time of delivery may be responsible. It is noteworthy that the three patients who were studied with chronic myelocytic leukemia in complete hematologic and clinical remission showed elevated RNA scores.

SUMMARY

A differential nucleic acid stain for peripheral blood and marrow smears using methyl-green and pyronin Y and Carnoy's solution as a fixative is described. Characteristics of the various blood cells are quite distinct so that identification is readily accomplished.

If pyroninophilia is assumed to be a measure of RNA, morphologically identical and mature leukocytes are found to vary in the quantity of cytoplasmic RNA. Since cellular immaturity is associated with increased RNA, this must be an indication of the varying "age" of white blood cells in the peripheral blood. With the use of this stain a much more dynamic picture of white cell physiology is obtained than with other more conventional stains.

The quantity of cytoplasmic RNA in peripheral blood leukocytes, as measured by a scoring technic, was found to be increased as compared to normal in most infections, leukemias, pernicious anemia in relapse, and a variety of other conditions. With treatment, the score fell to normal levels in pernicious anemia and infections.

Summario in Interlingua

Es describite un colorante differential de acido nucleic pro frottis de sanguine peripheric e de medulla, in que es usate verde methylic e pyronina Y e solution de Carnoy como fixativo. Le characteristicas del varie cellulas sanguinee es multo distincte de maniera que lor identification es facile a establir.

Si nos suppone que pyroninophilia es un mesura de acido ribosanucleic (RNA), morphologicamente identic e matur leucocytos monstra variationes in lor quantitate de RNA cytoplasmic. In tanto que immaturitate cellular es associate con augmentos de RNA, isto debe esser un indication del varie "etates" de leucocytos in le sanguine peripheric. Per medio de iste colorante un panorama del physiologia leucocytic es obtenite que es multo plus dynamic que illo obtenite per medio de altere colorantes de character plus conventional.

Le quantitate de RNA cytoplasmic in leucocytes de sanguine peripheric se monstrava augmentate in comparation con le norma in le majoritate del infectiones, in leucemias, anemia perniciose in recidiva, e in un varietate de altere conditiones. Le tractamento reduceva le valores a nivellos normal in anemia perniciose e infectiones.

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