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Abstract

Our experience with Brachet test on twenty-four leukemic patients has shown a high degree of reliability of the test for differentiating acute leukemias. A standard method has been described which is simple enough to be carried out routinely. The test, however, is not without pitfalls and need be interpreted with some caution. From the fact that urine hydrolysis can be closely simulated by the enzymatic action of pure DNase solution, it is suggested that the urine factor responsible for the nuclear lysis is DNase excreted in the human urine. The possible mechanism and implication of the test have been discussed in relation to the results obtained.

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BRACHET-TEST (URINE HYDROLYSIS TEST) AS AN AID TO DIFFERENTIATION OF LEUKEMIC CELLS

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Better selection of antileukemic agents, which is only possible on the exact knowledge of the type of leukemia, is of considerable importance for a prognostic evaluation of a given leukemic patient. Nevertheless, it is not necessarily easy to determine the type of leukemia, particularly of the acute type.

It appears, therefore, any technic which will aid in differentiating immature leukemic cells deserves attention. Being stimulated by the reports of THOMA[1] and GARDNER *et al.*,[2,3] we have investigated the validity of Brachet-test in differentiating leukemic cells. The test is based on the fact that exposure of blood films to human urine results in the differential extraction of the nuclear chromatin.

It is the purpose of this paper to report our experience with the Brachet-test on twenty-four leukemic patients who were admitted to our Medical Service. Factors influencing the test were also studied.

MATERIALS AND METHODS

A slight modification of the methods described by THOMA[1] and GARDNER *et al*[2]. was emplyed. Five peripheral blood or bone-marrow smears were prepared on a leukemic patient. They were fixed in methanol for three minutes and air-dried. Approximately 100 ml. of freshly voided urine from a normal or leukemic individual was collected in a beaker, and its pH was adjusted to 6.4 with either 0.1 *N* NaOH or 0.1 *N* HCl. And the urine was heated to 60 °C in a water bath. Four of the 5 blood films were immersed in the urine for 2, 4, 6 and 8 minutes, respectively, before they were taken out and rinsed thoroughly in running tap water. After being air-dried, the blood films were stained with May-Giemsa or methyl green-pyronin stain. The one remaining slide was stained as control without urine hydrolysis.

Apart from urine, pure enzyme solutions were made up with crystallized DNase and RNase ($\times 1$) obtained from Worthington Biochemicals Corp., U. S. A. They were used in a concentration of 200 μg. per ml. of double-distilled water. The RNase solution was handled as with urine, but because of the limited

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amount of the expensive DNase, the method was modified as follows. The DNase solution stored in the refrigerator was pre-heated to 37 °C prior to each use. A few drops of the solution were put on the methanol-fixed blood films in the humidified incubator at 37 °C. The duration of the enzyme action was empirically set to from one to two hours. Following this treatment, the slides were stained with May-Giemsa stain as before.

RESULTS

Stained blood films were examined microscopically to see the degree of urine hydrolysis. Regardless of the source of the specimens, normal or leukemic, it was shown that the nuclear chromation of neutrophilic cells was lysed, while the nuclei of lymphocytes, monocytes or eosinophils were unaffected. Among the four slides exposed to urine for different lengths of time, one slide was selected for microscopic observation, in which distinction between lysed neutrophils and unaffected lymphocytes or eosinophils was most apparent. Mature segmented neutrophils and their precursors as well showed lysis of the cell nuclei. This characteristic behavior of the neutrophilic series provided the basis for differentiating myelogenous leukemia from other types of leukemia (Figs. 1, 2 and 3).

Brachet test was performed on twenty-four leukemic patients who were consecutively admitted to our Medical Service since June 1961. Correlation between the clinical diagnosis and degree of urine hydrolysis is shown in Table I. They comprised 6 cases of acute lymphocytic leukemia, 8 cases of acute

Table I. Urine Hydrolysis of Leukemic Cells in Various Types of Leukemia

	Blasts			More mature forms			No. cases
Degree of hydrolysis / Clin. diag.	+	+～−	−	+	+～−	−	
Acute lymph. leuk.			6			6	6
Acute myelo. leuk.		8		7	1*		8
Chronic myelo. leuk.		6		6			6
Monocytic leuk.		1**	3	1**		3	4
							Total 24

* Brachet negative neutrophilic cells possibly showing treatment effect.
** From the standpoint of Brachet-test, this case was regarded as acute myelogenous leukemia.

myelogenous leukemia, 6 cases of chronic myelogenous leukemia, and 4 cases of monocytic leukemia. The clinical diagnosis was established by a simultaneous use of various technics including May-Giemsa staining, phase contrast and fluorescence microscopy, vital staining, and bone marrow tissue culture[5]. Except

one case of monocytic leukemia, the diagnoses by the Brachet-test and by these methods were in full agreement on the other twenty-three leukemic patients. In this instance of monocytic leukemia, urine hydrolysis revealed lysis of the nuclei of some of the blast cells and all of the more mature leukemic cells. From the standpoint of the Brachet-test, therefore, the case was considered to be acute myelogenous leukemia rather than monocytic leukemia.

Attempts to analyze some of the factors influencing urine hydrolysis led to the following observations.

1. Influence of pH.

The pH of urine in the range of 6.0 to 6.8 gave an equally satisfactory differential extraction of the cell nuclei. However, at pH 7.0, rupture of the nuclear membrane and diffusion of the chromatin material into the surrounding area were seen.

2. Inactivation of urine.

Heating urine at 75°C for 15 minutes completely deprived the urine of the lytic action on the nuclear chromatin. Standing urine at a room temperature for 24 hours, likewise, resulted in the almost complete loss of an active agent.

3. Use of sodium chloride solution.

A 0.2 *M* sodium chloride solution was used in place of urine in the otherwise same experimental condition, but no nuclear change was observed. The slide which was kept immersed in the saline solution for one hour, however, showed diffusion of the nuclear material into the adjacent area.

4. Effect of DNase.

The differential extraction of the leukocyte chromatin by means of urine could be closely simulated by pure DNase solution in distilled water, whereas pure RNase sotution in distilled water caused no comparable alteration of the cell nuclei in any leukocyte series.

DISCUSSION

The original investigators[1,6-9] ascribed the differential nuclear lysis of leukocytes to the action of RNase and DNase. They considered that the nuclei of neutrophilic cells are rich in RNA and are susceptible to the action of RNase, while the nuclei of lymphocytes, monocytes or eosinophils contain more DNA than RNA, being susceptible to DNase. Our experiment as well as GARDNER's[2] failed to produce a typical nuclear lysis with pure RNase solution. The reason for this discrepancy is not clear, but it is possible that, the crude RNase which LAVES and THOMA[7] extracted from the cow's pancreas might have contained a small amount of DNase. Moreover, with our present-day knowledge of cytochemistry, it is hard to believe that RNA is a major nucleoprotein of neutrophilic

cells. Methyl green-pyronin staining after urine hydrolysis demonstrated failure of the nuclei of neutrophilic cells to take up a green color of methyl green, indicating loss of DNA from the cell nuclei.

From the finding that sodium chloride solutions in various concentrations also caused removal of the chromatin material, SPRAGUE *et al.*[10,11] regarded urine as a mere salt solution. As stated earlier, we could not induce a clear-cut lysis of the cell nuclei with a 0.2 *M* sodium chloride solution, and we feel, as GARDNER *et al*[2]. thought, that the effect of sodium chloride solutions is a nonspecific dissolution of the nuclear material.

It should be kept in mind that not all of the myeloblasts appearing in acute or chronic myelogenous leukemia show a complete lysis of the nuclear chromatin. Some are quite susceptible to urine hydrolysis but some are entirely retistant to it. In some cases, the degree of nuclear lysis varied considerably among myeloblasts, and the percentage of the susceptible myeloblasts differed from case to case. From the fact that promyelocytes are uniformly susceptible to the action of urine, those myeloblasts similarly susceptible are presumably closer to promyelocytes in their maturation level, having the same DNA composition as promyelocytes. It may be said, therefore, that the degree of urine hydrolysis of myeloblasts in a certain case depends on the level of maturation where the myeloblasts are proliferating. If myeloblasts are closer to stem cells, they are hardly susceptible to urine hydrolysis whereas if they are closer to promyelocytes, they are as susceptible as promyelocytes.

Blood cells may be separated into two groups depending on the susceptibility of the nuclear chromatin to urine hydrolysis (Table II). However, this distinction is not an absolute one, and with longer urine hydrolysis, even the nuclei of lymphocytes, monocytes or eosinophils show lysis of the chromatin material. It is important, therefore, to be sure that the slide under examination is repre-

Table II. Susceptibility of Blood Cells to Urine Hydrolysis

Susceptible cells	Resistant cells
Myeloblasts	Lymphatic series
Promyelocytes	Monocytic series
Myelocytes	Eosinophilic series
Metamyelocytes	Plasma cells
Band forms	Reticulum cells
Segmented neutrophils	Megakaryocytes
Basophilic series	Platelets
Mature erythroblasts	Immature erythroblasts
Their mitotic forms	Their mitotic forms

sentative of the maximum contrast between lysed neutrophils and resistant lymphocytes.

The mechanism involved in the urine hydrolysis of the nuclear chromation is not entirely clear. It has been shown that the action is influenced by the pH and temperature of the urine. The urine factor, whatever it may be, thermolabile and can be inactivated by heating at 75°C for 15 minutes. Treatment of blood films with pure DNase solution has given a similar result. From these observations, it appears reasonable to assume that an unidentified substance responsible for urine hydrolysis would be DNase excreted in the human urine.

What is the difference, then, in DNA composition between neutrophils and other leukocytes, resulting in the differential nuclear extraction? At the present time, we do not seem to have enough evidence to answer this question.

In the peripheral blood of one patient with acute myelogenous leukemia who was heavily treated with steroid hormones prior to the time Brachet test was done, there were some Brachet negative mature and immature neutrophilic cells. It could not be determined whether the emergence of the Brachet negative neutrophils in this patient was due to the use of steroid hormones or an indication of biochemical alterations of the nucleoprotein by leukemic transformation *per se*. It may be of note that these Brachet negative neutrophils were peroxidase negative. THOMA[1] and LAVES[6] reported X-ray irradiation therapy in leukemia reduced the susceptibility of neutrophilic cells to urine hydrolysis. POLLI *et al*[12]. presented evidence for the presence of cross-linking between DNA molecules induced by Myleran in chronic myelogenous leukemia. NOWELL[13] observed inhibition of human leukocyte mitosis by prednisolone *in vitro*. In view of these reports, it is possible that such antileukemic agents as steroid hormones and Myleran which are most widely used today, act on the DNA level, although their precise mode of action is unknown.

SUMMARY

Our experience with Brachet test on twenty-four leukemic patients has shown a high degree of reliability of the test for differentiating acute leukemias. A standard method has been described which is simple enough to be carried out routinely. The test, however, is not without pitfalls and need be interpreted with some caution.

From the fact that urine hydrolysis can be closely simulated by the enzymatic action of pure DNase solution, it is suggested that the urine factor responsible for the nuclear lysis is DNase excreted in the human urine.

The possible mechanism and implication of the test have been discussed in relation to the results obtained.

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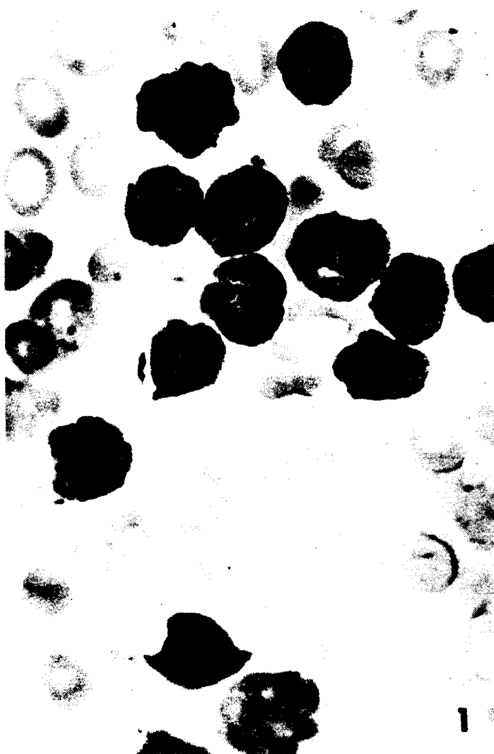

Fig. 1. Bone marrow smear of acute lymphocytic leukemia showing lysis of the nucleus of a band form and unaffected lymphoblasts.

Fig. 2. Bone marrow smear of acute myelogenous leukemia showing complete lysis of the nuclei of the neutrophilic series, a basophil and a erythroblast in contrast to unaffected lymphocyte.

Fig. 3. Bone marrow smear of monocytic leukemia showing the intact nuclei of monocytes, monoblasts, a lymphocyte and a eosinophil, while the nucleus of a segmented neutrophil is completely lysed.