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CONTINUOUS MEASUREMENT OF THE CHANGE IN ERYTHROCYTE VOLUME AFTER ADDITION OF SAPONIN
(Saponin Test)

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Abstract

One μl of whole blood is added to 50 ml aliquots of 0.9 % and 0.5% saline solutions prewarmed at 30°C and mixed to get homogeneous suspensions of erythrocytes. Into each of these suspensions two hundred μl of 1% aqueous solution of saponin is put 40 seconds after addition of blood to the saline solutions. The changes in mean erythrocyte volume (MCV) are able to observe continuously in an automatic blood cell counter equipped with a recording assembly. The results of the observations are recorded in graphic curves. The curves analyzed for the expansion ratio (E), the time (in sec.) required for attaining the maximum MCV (T max), and the time (in sec.) necessary for erythrocytes to reach the critical point of complete rupture due to erosive effect exerted by saponin (T end). This observation or test is called Saponin test.

Saponin test of erythrocytes was carried out with heparinized blood samples collected from normal subjects. The results obtained showed that initial mean corpuscular volume (IMCV) was 85-91 fl, maximum value of mean corpuscular volume (MMCV) 108-118 fl, E 128-137%, T max 28-33 sec., T end (in 0.9% saline) 38-44 sec. and T end (in 0.5% saline) 30-57 sec. The test was applied to the examination of physicochemical properties of erythrocytes of the patients with hereditary spherocytosis (HS), β-thalassemia (β-Thal), iron deficiency anemia (IDA), diabetes mellitus (DM) and hepatobiliary disorders. The test results were examined in comparison with the lipid compositions of relevant erythrocyte membranes which were analyzed simultaneously.

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β-Thal gave a pathognomonic pattern of saponin test. (Decrease in IMCV and MMCV, increase in E, and prolongation of T max and T end). IDA was discriminated from β-thalassemia by normal or slightly shortened T max and T end. HS was characterized by remarkable decrease in E. The erythrocytes of hepatobiliary disorders in which increase in free cholesterol of the cellular membrane was commonly evident were very sensitive to saponin test. Their T max and T end were shortened. The abnormality in saponin test was not significantly remarkable in DM.

The saponin test is thought to be a new test recommended for the differential diagnosis of β-thalassemia from iron deficiency anemia.

INTRODUCTION

When Saponin is added to an erythrocyte suspension in an isotonic or hypotonic saline solution, erythrocytes gradually swell and reach their maximum volume after about 30 seconds. Then, their volume begins to increase and approximately 40 seconds following the addition of Saponin they rupture and present ghost images[1].

Recently, in our laboratory through continuous measurement of the volume of erythrocytes by means of an automatic blood cell counter, dynamic changes in the erythrocytes induced by saponin have become observable, and the curves indicating the changes in the mean erythrocyte volume of various diseases have been reported elsewhere[2].

Saponin acts on cholesterol, one of the components of the erythrocyte membrane, to form circular micelles[3] that scale off from the membrane and leave circular flaws through which the contents of the erythrocyte seep out and result in ghost cell.

This new method of continuous observation of erythrocyte volume after addition of saponin was called Saponin test. In our experience it proved to be potentially useful for the differential diagnosis of some hematological diseases.

MATERIALS AND METHODS

Tests were carried out on heparinized venous blood specimens collected from normal adults and patients with iron deficiency anemia (IDA, 16 cases), β-thalassemia (β-Thal, 5 cases), hereditary spherocytosis (HS, 5 cases), diabetes mellitus (DM, 27 cases) and disorders of the hepatobiliary tract (6 cases).

An automatic blood cell counter, Model CC-108, which enables us to measure continuously the change of blood cell volume at 30°C was employed for the test. It was equipped with a thermostat and a set of recording machines (Toa Medical Electronics, Kobe) (Fig. 1).

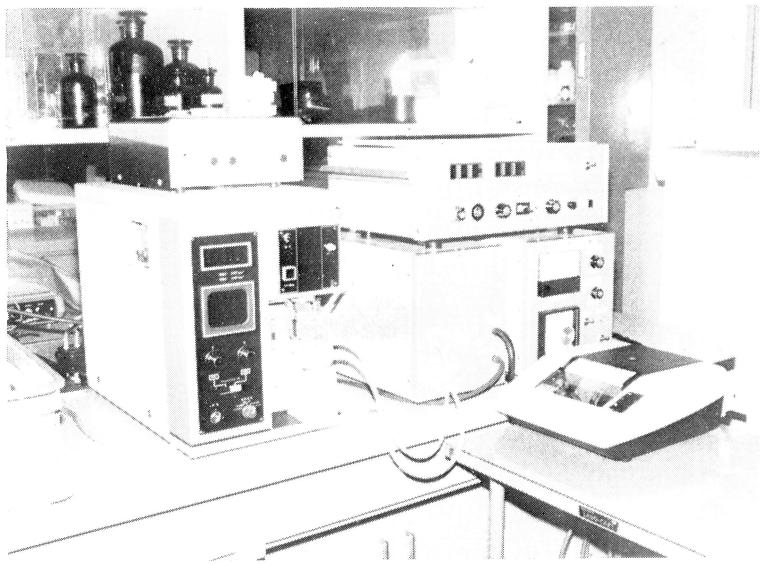

Fig. 1. Assembly of equipment

One μl of whole blood was mixed with 50 ml of 0.9 % saline solution (isotonic solution) prewarmed (to 30°C) in a breaker. The same treatment was also carried out using 0.5 % saline solution (hypotonic solution). Two hundred μl of 1 % saponin solution were added to them 40 seconds after the whole blood had been mixed and the changes in the mean cell (erythrocyte) volume (MCV) were recorded every second by means of the automatic blood cell counter described above.

The expansion ratio of the erythrocytes (E), T max (sec.) i.e. the time required to attain the maximum volume, and T end (sec.), i.e. the time necessary for reaching the point of erythrocyte collapse were reckoned from the curves depicting the changes in MCV (Fig. 2).

Analysis of the lipids of the erythrocytic membrane was carried out by the Iatroscan method[4] and FC (free cholesterol), PE (phosphatidyl serine), PC (phosphatidyl choline) and SM (sphingomyelin) were separated and determined quantitatively.

RESULTS

Fig. 2 illustrates the time course of the change in the mean cell volume (MCV) after addition of saponin to the eryhrocyte suspension.

The MCV prior to addition of saponin is called initial MCV (IMCV) and the length of time (sec.) required to attain the maximum MCV following the

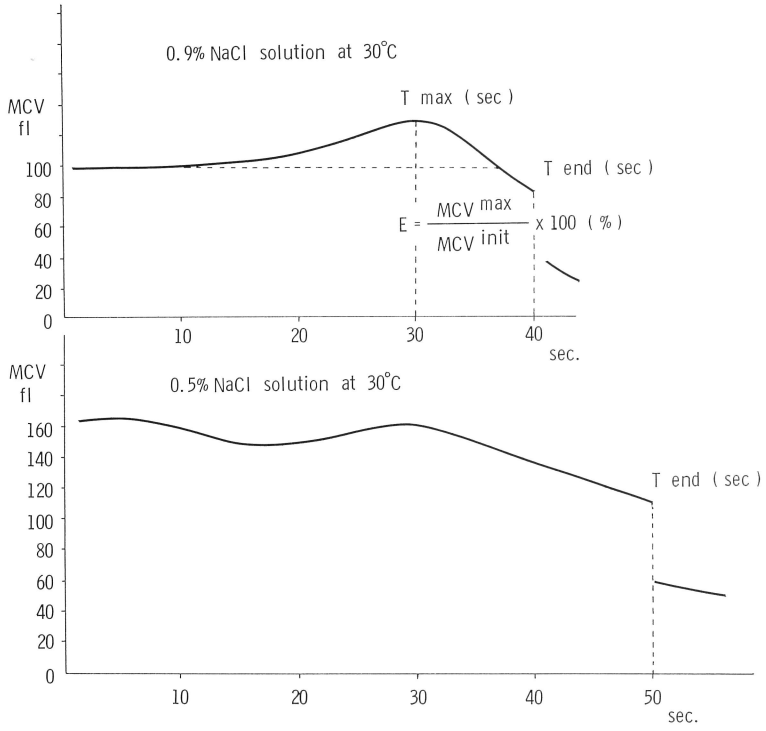

Fig. 2. Profile of erthrocyte volume changes after addition of saponin solution

addition of Saponin is called T max. Maximum MCV (MMCV) stands for the value of MCV at this point.

The expansion ratio of erythrocytes volume is determined according to the following formula :

$$E = MMCV/IMCV \times 100\ \%.$$

T end denotes the length of time (sec.) elapsing until erythrocyte collapse by the action of saponin is reached and, therefore, MCV can no longer be measured.

There are two sorts of values for T end obtained in saponin test, i.e. T end (0.9) and T end (0.5). The figures in parentheses indicate the concentration of the saline solutions in which the erythrocytes are suspended. The measurement of the change in both of these MCV's were carried out at 30°C. The values (mean and range) of IMCV, MMCV, E, T max, T end (0.9), and T end (0.5) obtained in normal adult subjects are shown in Table 1.

The results of saponin tests carried out on representative diseases are given

TABLE 1. Normal values in saponin test

	IMCV (fl)	MMCV (fl)	E (%)	T max (sec)	T end(0.9) (sec)	T end(0.5) (sec)
Mean	88	114	132	31	41	42
Range	85—91	108—118	128—137	28—33	38—44	30—57

in Fig. 3.

In HS (hereditary spherocytosis), E was decreased and T max and T end were shortened. The characteristic finding was diminution of T end (0.5).

The analysis of the lipid composition of the erythrocyte membrane disclosed that the composition ratio of the outer lipid layer to the inner lipid layer, i.e. (PC + SM)/(PE + PS) was 1.32, This value was larger than that obtained in normal subjects (1.09), and the PE of the inner lipid layer was reduced, whereas FC was increased (Table 2).

TABLE 2 Lipid composition (mg/g Hb) of red cell membrane

	Total Lipids	FC	Outer surface		Inner surface		PC+SM / PE+PS
			PC	SM	PE	PS	
Normal Subject (mg/g Hb)	14.95±2.59 (100)	4.63±0.65 (31.0)	2.91±0.40 (19.5)	2.51±0.74 (16.8)	3.01±0.44 (20.1)	1.89±0.36 (12.6)	1.09
IDA (mg/g Hb)	17.22±2.30 (100)	5.72±0.74 (33.4)	3.31±0.53 (19.5)	3.00±0.51 (17.5)	3.07±0.63 (17.6)	2.11±0.42 (12.0)	1.26
Hs (mg/g Hb)	12.56±1.67 (100)	4.54±0.42 (37.2)	2.40±0.48 (18.8)	2.20±0.33 (17.4)	2.06±0.60 (16.0)	1.36±0.34 (10.6)	1.32
Acute hepatitis (mg/g Hb)	13.05±1.74 (100)	5.01±0.60 (39.4)	2.84±0.70 (20.6)	1.87±0.40 (14.3)	1.70±0.30 (13.0)	1.63±0.40 (12.7)	1.42
Chronic hepatitis (mg/g Hb)	12.96±1.68 (100)	4.48±0.60 (34.6)	2.75±0.60 (21.2)	1.97±0.45 (15.2)	2.20±0.30 (17.0)	1.56±0.38 (12.0)	1.26
Cirrhosis of the liver (mg/g Hb)	14.51±2.20 (100)	5.33±1.00 (36.7)	3.12±0.50 (21.5)	2.20±0.26 (15.2)	2.09±0.46 (14.4)	1.77±0.57 (12.2)	1.38
Obstructive jaundice (mg/g Hb)	17.4±1.00 (100)	6.73±1.00 (38.7)	5.30±0.30 (30.5)	1.91±0.35 (11.0)	2.18±0.53 (12.5)	1.28±0.13 (7.3)	2.13
β-Thalassemia (mg/g Hb)	19.14 (100)	6.18 (32.3)	3.22 (16.8)	3.23 (16.9)	3.71 (19.4)	2.80 (14.6)	0.99
α-Thalassemia (mg/g Hb)	25.79 (100)	8.55 (33.2)	4.84 (18.8)	4.19 (16.2)	4.78 (18.5)	3.43 (13.3)	1.06

The figures in parentheses represent percentages of lipid compostions of the erythrocyte membrane.

In β-thalassemia, IMCV and MMCV were decreased, whereas E was increased. Furthermore, this disease was characterized by concomitant prolongation

of T max, T end (0.9) and T end (0.5). However, the analysis of the lipid composition of the erythrocyte membrane failed to demonstrate significant difference from the erythrocyte of normal persons.

In IDA, there was slight increase in E, together with shortened T max and T end. As to the values of T max and T end a significant difference was noted between IDA and thalassemia. They were prolonged in β-thalassemia and shortened in IDA (Fig. 3).

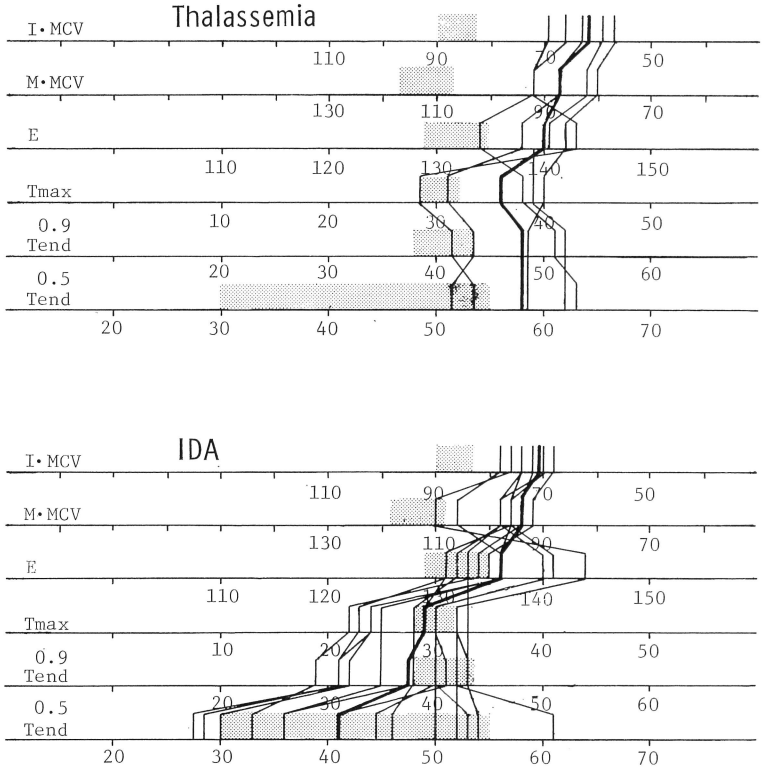

Fig. 3 Diagrammatic representation of the Saponin test in thalassemia and iron deficiency anemia

Increase in the ratio of (PC + SM)/(PE + PS) up to 1.26, elevation of FC and diminution of PE were demonstrable by the lipid analysis of the erythrocyte membrane in IDA however, in β-thalassemia increase of PS in association with normal ratio of (PC+SM)/(PE+PS) was obtained.

The Saponin test in diabetes mellitus was a little different from the pattern seen in normal subjects. It is interesting that T end (0.5) was extraordinarily shortened in some cases.

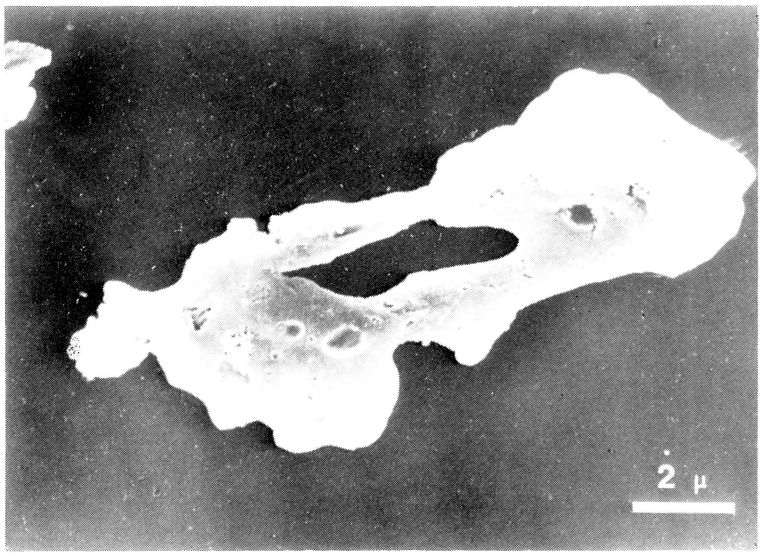

Fig. 4. A erythrcyte ghost eroded by Saponin

The characteristic features of the tests seen in the disorders of the liver and of the biliary tract were the concomitant decrease in E, T max and T end which suggested alteration of the properties of membrane toward increased sensitivity to saponin. The (PC + SM)/(PE + PS) ratio was large due to tremendous diminution of PE, the inner layer lipid component. FC was increased in these disorders (Table 1).

Especially, in biliary obstruction, decrease in PE in association with increase in PC and FC was evident. This finding was thought to be suggestive of a strong affinity of the erythrocyte membrane to saponin.

DISCUSSION

According to the current conception the erythrocyte membrane consists of double layeres of lipids and its inner surface is lined with contractile protein (spectrin)[5-6]. The outer lipid layer is chiefly composed of PC + SM and the inner lipid layer mainly of PE + PS. FC is distributed uniformly in both outer and inner layeres.

Saponin binds with FC of the lipid double layer and forms micelles which are prone to slough off from the membrane and produce flaws of defect after having been shed[3]. In fact our phase-contrast microscopy of erythrocyte ghosts revealed that their membranous surface was studded with flaws which were supposed to have been eroded by saponin (Fig. 4).

Shortened values of T max and T end are therefore thought to be related to the accelerated tendency toward the collapse of erythrocytes due to increased affinity of erythrocyte membrane to saponin.

Increase in FC in the erythrocyte membrane is common in hepatobiliary disorders, hereditary spherocytosis and iron deficiency anemia. It is, accordingly, natural that the erythrocyte membrane is vulnerable to saponin in these diseases and T max and T end are diminished in saponin test. Furthermore, decrease in PE in the inner lipid layer of the erythrocyte membrane may be presumed to be responsible also for the abnormal saponin test in this group of disorders.

In β-thalassemia the erythrocytes show decreased affinity to saponin, and consequently the lengths of times up to the point of erythrocyte collapse, T end (0.9) and T end (0.5), are prolonged. However, in iron deficiency anemia, both of these times are shortened notwithstanding the similarity of erythrocyte morphology (hypochromic microcytosis).

In this way the saponin test successfully detects the delicate difference of the quality of erythrocytes between these two diseases (β-thalassemia and IDA). The lipid analysis disclosed that PE and PS, the constituents of the inner lipid layer of erythrocyte membrane, are somewhat richer in thalassemia in conparison with iron deficiency anemia. In both diseases FC is increased. The lipid composition of the erythrocyte membrane does not seem to be contributory to the elucidation of the causes of different attitude of erythrocytes to saponin test between these diseases.

The erythrocytes of the patient with hepatobiliary disorders tend to be easily ruptured by saponin. There is a remarkable increase in FC, while prominent decrease in PE is noted. The affinity of the erythrocyte membrane for saponin is elevated.

It is interesting that, in hepatobiliary disorders, the erythrocytes show increased osmotic resistance (dynamic observation of the physical properties of the erythrocyte membrane by means of CPC)[7] giving a sharp contrast to their fragility in saponin test or remarkable shortening of T max and T end.

Saponin test is, therefore, recommended to the clinical laboratories as a measure to appraise the alteration of physicochemical properties of erythrocyte membrane which are not demonstrable by the conventional osmotic fragility test.

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