Acta Medica Okayama

Volume 60, *Issue* 2 2006 *Article* 9

APRIL 2006

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Abstract

Deficiency of glucose-6-phosphate dehydrogenase (G6PD) causes acute hemolytic anemia triggered by oxidative drugs such as primaquine. It is therefore essential in malaria-endemic areas for malaria patients to be confirmed for their G6PD activity before taking primaquine. The WST-8 method, a newly established screening method for G6PD deficiency, has been demonstrated to be suitable for field conditions, particularly for on-site malaria surveys. Here we report a laboratory evaluation by this method of the reactivity of blood-spotted filters. A time-course experiment was conducted to evaluate the reactivity of blood samples spotted onto 4 types of filter paper, Whatman 31ET Chr (ET), 3MM Chr (3MM), P81, and Advantec No. 2 (AD2). The rank of the relative reaction intensity was ET $>$ 3MM = AD2 $>$ P81. Blood-spotted filters stored at 4 degrees centigrade gradually decreased G6PD reactivity with the passage of storage time, whereas those stored at room temperature rapidly reduced their reactivity. Unexpectedly, saponin supplementation reduced the reactivity of blood-spotted filters. In conclusion, 1) ET is the most suitable filter for the WST-8 method ; 2) blood-spotted filters stored in cold condition can be assayed within 14 days, or those stored at room temperature should be tested within 3 days ; and 3) reaction mixtures should not contain saponin.

KEYWORDS: glucose-6-phosphate dehydrogenase, G6PD deficiency, blood-spotted filter, WST-8 method, malaria

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*PMID: 16680190 [PubMed - indexed for MEDLINE]

Acta Med. Okayama, 2006
Vol. 60, No. 2, pp. 127-134

Acta Medica Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

Reactivity of Blood Samples Spotted onto Filter Papers in the WST-8 Method for Screening of G6PD Deficiency

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Deficiency of glucose-6-phosphate dehydrogenase (G6PD) causes acute hemolytic anemia triggered by oxidative drugs such as primaquine. It is therefore essential in malaria-endemic areas for malaria patients to be confirmed for their G6PD activity before taking primaquine. The WST-8 method, a newly established screening method for G6PD deficiency, has been demonstrated to be suitable for field conditions, particularly for on-site malaria surveys. Here we report a laboratory evaluation by this method of the reactivity of blood-spotted filters. A time-course experiment was conducted to evaluate the reactivity of blood samples spotted onto 4 types of filter paper, Whatman 31ET Chr (ET), 3MM Chr (3MM), P81, and Advantec No. 2 (AD2). The rank of the relative reaction intensity was ET > 3MM = AD2 > P81. Blood-spotted filters stored at 4℃ gradually decreased G6PD reactivity with the passage of storage time, whereas those stored at room temperature rapidly reduced their reactivity. Unexpectedly, saponin supplementation reduced the reactivity of blood-spotted filters. In conclusion, 1) ET is the most suitable filter for the WST-8 method; 2) blood-spotted filters stored in cold condition can be assayed within 14 days, or those stored at room temperature should be tested within 3 days; and 3) reaction mixtures should not contain saponin.

Key words: glucose-6-phosphate dehydrogenase, G6PD deficiency, blood-spotted filter, WST-8 method, malaria

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common hereditary disorders prevalent in malaria endemic areas, probably because G6PD-deficient erythrocytes are resistant to malaria [1, 2]. G6PD deficiency may cause various types of hemolytic anemia, most typically an acute hemolytic attack after taking certain oxidative drugs such as primaquine [3, 4].

Primaquine has been widely used for the radical treatment of *Plasmodium vivax* malaria. In addition, its gametocytocidal action is effective to cut the transmission of *P. falciparum* gametocytes in endemic areas [5, 6]. Primaquine-induced hemolytic crisis is a serious problem in chemotherapeutic malaria control activities. Thus, primaquine should not be administered to malaria patients before confirming their G6PD activity [7].

We have visited malaria endemic areas in Asian countries to introduce rapid methods of malaria diagnosis [8] and G6PD testing [9]. Using these methods, patients are notified of the results of a blood examination within 60 min and are able to receive antimalarial medicine, including primaquine [10].

Tantular and Kawamoto have recently developed a

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simple and rapid G6PD-deficiency diagnosis method [11] based on a new formazan substrate, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8) [12], and a hydrogen carrier, 1-methoxy-phenazine methosulfate (1-methoxy PMS), which is more light-resistant than PMS [13]. With this WST-8 method, a diagnosis of G6PD deficiency can be achieved both qualitatively by observing color development and quantitatively by reading absorbance values on ELISA microplate readers.

Apart from malaria control activities, the WST-8 method will also be quite useful for studying the geographic distribution of G6PD enzyme polymorphism and possibly for newborn screening for G6PD deficiency. For such purposes, and when on-site diagnosis is not essential, it would be more convenient and desirable to collect blood samples as blood-spotted filters, bring them back to the laboratory, and examine them later. Processing many samples at the same time will make it easier precisely evaluate individual G6PD activity, especially for the detection of heterozygous females. Although it has been mentioned that blood-spotted filters can be used as blood samples in the WST-8 method [11], the details of this method have not been reported.

The purpose of the present study was to evaluate the reactivity of blood-spotted filters in the WST-8 method by measuring their reaction intensity and retentivity during the storage period. We also examined the effects of saponin on the G6PD reactivity of blood-spotted filters.

Materials and Methods

Chemicals. WST-8 and 1-methoxy PMS were purchased from Dojindo Laboratories (Kumamoto, Japan). Both chemicals are included in a commercial kit (CCK-8) for a cell-counting system at concentrations of 5 mM WST-8 and 0.2 mM 1-methoxy PMS in 0.15 M NaCl [14]. Glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Oriental Yeast Co. (Tokyo, Japan). Saponin extracted from Quillaja bark was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Reaction mixtures. The reaction mixtures included were: (1) 50 mM Tris-HCl buffer pH 7.2, which contained 5 mM $MgCl_2$ and 0.1% saponin; (2) the substrate mixtures of 2.5 mM G6P, 0.2 mM NADP in H_2O; and (3) the WST-8/1-methoxy PMS mixture [11]. These reaction mixtures are mixed in a 1.5 ml microtube as in Table 1 immediately before the G6PD assay.

Sample preparation. For this study, venous blood obtained from a Japanese donor (MA) with normal hemoglobin concentrations (14.5 g/dl) and G6PD activity (8.0 IU/g Hb) was used throughout the study. Ten milliliters of venous blood was taken into a heparinized syringe and immediately divided into 2 conical tubes. The blood in one tube was incubated at 56℃ in a water bath for 30 min in order to inactivate the G6PD activity, while the other was kept at room temperature (25–28℃) for 30 min. Both normal- and heat-inactivated blood were spotted onto four kinds of filter paper, *i.e.* 31ET Chr (ET), 3MM Chr (3MM), and P81 from Whatman International Ltd. (Maidstone, UK), and No. 2 filter (AD2) from Advantec Toyo Ltd. (Tokyo, Japan)(Table 2).

Two sheets were prepared for each filter paper, onto which 10 μl of blood was applied using a micropipette, resulting in a circular blood spot. One hundred blood spots from normal blood were made onto one sheet, and another 100 spots from the heat-inactivated blood were made onto the other sheet. The remaining blood (normal and heat-inactivated) was used as whole blood samples. The filter sheets were then dried for 60 min at room temperature. Circular pieces 6 mm in diameter cut from the dried blood spots were put into 1.5 ml microtubes containing the reaction mixtures (Table 1). After collecting the fresh samples, the blood-spotted sheets were kept in black containers and stored either at 4℃ in a refrigerator or at ambient conditions (room temperature 25–28℃; relative humidity 30–40%).

At various time points, fresh blood (2 ml) was taken and divided, heat-inactivated, spotted onto filters, dried, and cut out as described above. These fresh samples and old samples cut from the stored blood-spotted sheets were used for the G6PD assays.

G6PD assay. Table 1 shows the reaction components for the G6PD assay in the present study. Two sets of reaction tubes were prepared: one for color photographs and the other for quantitative

Table 1 Components of the reaction tubes

	Tubes for blood-spotted filter			Tubes for whole blood			
	Normal (μl)	Heat-inactivated (μl)	No substrate (μl)	Normal (μl)	Heat-inactivated (μl)	No substrate (μl)	No blood (μl)
Tris-HCl buffer with or without 0.1% saponin*	400	400	400	400	400	400	400
Substrate mixtures of G6P and NADP	400	400	–	400	400	–	400
Distilled water	–	–	400	–	–	400	5
WST-8/1-methoxy PMS mixture	30	30	30	30	30	30	30
Whole blood	–	–	–	5	–	5	–
Whole blood, heat-inactivated	–	–	–	–	5	–	–
Blood-spotted filter	1 piece	–	1 piece	–	–	–	–
Blood-spotted filter, heat-inactivated	–	1 piece	–	–	–	–	–

* Saponin was included in the reaction mixture for evaluation of the reactivity of the blood samples spotted onto four kinds of filter. To assess the effect of saponin on the G6PD reaction, both saponin-containing and -excluded buffers were used.

measurement of color development. The G6PD assay was started by adding a piece of blood-spotted filter or 5 μl of whole blood to the reaction tube, then mixed vigorously for 5 sec using a vortex mixer. One set of the reaction tubes was arranged in a row, and color photographs were taken at 20 min, 40 min, 60 min, 90 min, and 120 min after the start of the reaction. For quantitative measurement, 100 μl of the reaction mixture was pipetted from each tube into wells of a 96-well microtiter plate, and absorbance was measured at 450 nm with a reference at 595 nm. The absorbance of each sample was corrected by subtracting the absorbance of the corresponding blank (normal blood or spotted blood in reaction mixtures without the substrates). Immediately after the measurement, the mixture in the microtiter wells was transferred back to the original tubes, keeping the chromogenic reaction in progress. The absorbance measurements were carried out at 20 min, 40 min, 60 min, 90 min, and 120 min after the beginning of the reaction. The G6PD assay was carried out at room temperature (26–28°C) throughout the present study.

Effects of saponin on the reactivity of blood-spotted filter. Blood-spotted filter samples were prepared and stored at 4°C as described above. On the 14th day, the G6PD assay was carried out. The reactivity of each blood-spotted filter or whole blood sample was compared between 2 reaction mixtures, one with saponin and the other without.

Results

Reactivity of blood-spotted filters: fresh samples. Fresh samples (spotted blood and whole blood) were tested for their G6PD reactivity during the time scale of 20–120 min. The reaction tubes with normal blood samples in the complete reaction mixture showed orange color development as over time (Fig. 1), whereas those with heat-inactivated sample or those with normal blood but lacking substrates in the reaction mixture did not change their color (Fig. 2).

Color change developed slower in reaction tubes with blood-spotted filters than in those with whole blood in the early phase (20–60 min; Fig. 1). Among the blood-spotted filters tested, ET showed the highest color development, followed by 3MM and AD2 with very similar reactivity, and with P81 showing the lowest level of development. At 60 min after the start of the reaction, the tubes with whole blood reached a plateau in their color development, and thereafter the reaction showed saturation, with very similar color intensity of the tubes with blood-spotted filters except P81, which did not reach saturation during the observation period.

Reactivity of blood-spotted filters: changes in reactivity during storage. After the time-course experiment with fresh samples (designated as day 0), we decided to use the absorbance at 40 min for a comparison of reactivity among the stored blood-spotted filters. Fig. 2 shows the orange color development at 40 min after starting the reaction by

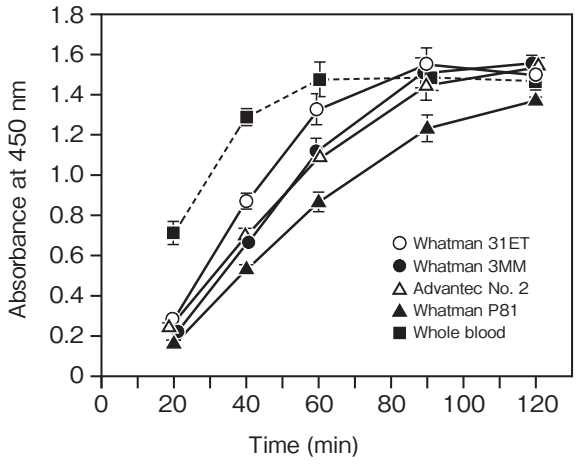

Fig. 1 Time-course of color development in reaction tubes with blood-spotted filters or whole blood. Each value represents the mean ± SD (n = 5).

different blood-spotted filters stored at 4°C or room temperature for 1, 14, and 42 days.

In order to analyze the reactivity among blood-spotted filters, the relative reactivity of each reaction tube was calculated by 2 different methods. The relative reaction intensity (RRI) was defined as the absorbance of the reaction tube with blood-spotted filter expressed as a percentage of that of the corresponding whole blood sample. RRI of each filter at day 0 was 68% (ET), 52% (3MM), 54% (AD2), and 42% (P81). The relative reaction retentivity (RRR) was defined as the absorbance of the reaction tube with blood-spotted filter expressed as a percentage of that of the corresponding fresh blood-spotted filter sample. RRR at day 0 was therefore 100% in all 4 filters.

Based on the RRI of blood-spotted filters, each filter reduced its reactivity with the passage of storage time (Fig. 3). Samples stored at 4°C gradually reduced their reactivity, while the rank order of RRI was the same as that found in the fresh sample experiment, *i.e.* ET > 3MM = AD2 > P81, within the observation period (Fig. 3A). On day 14, the RRI of each filter was 63% (ET), 44% (3MM), 48 % (AD2), and 28% (P81). The color changes of the 4 filters were still distinguishable (Fig. 2). On day 42, although the RRI of each filter was further reduced to 50% (ET), 36% (3MM), 37% (AD2), and 17% (P81), the color changes of the former 3 filters were still distinguishable, but that of P81 was weak (Fig. 2). On the other hand, samples stored at room temperature rapidly lost their reactivity (Fig. 3B). On day 3, the RRI of each filter was 62% (ET),

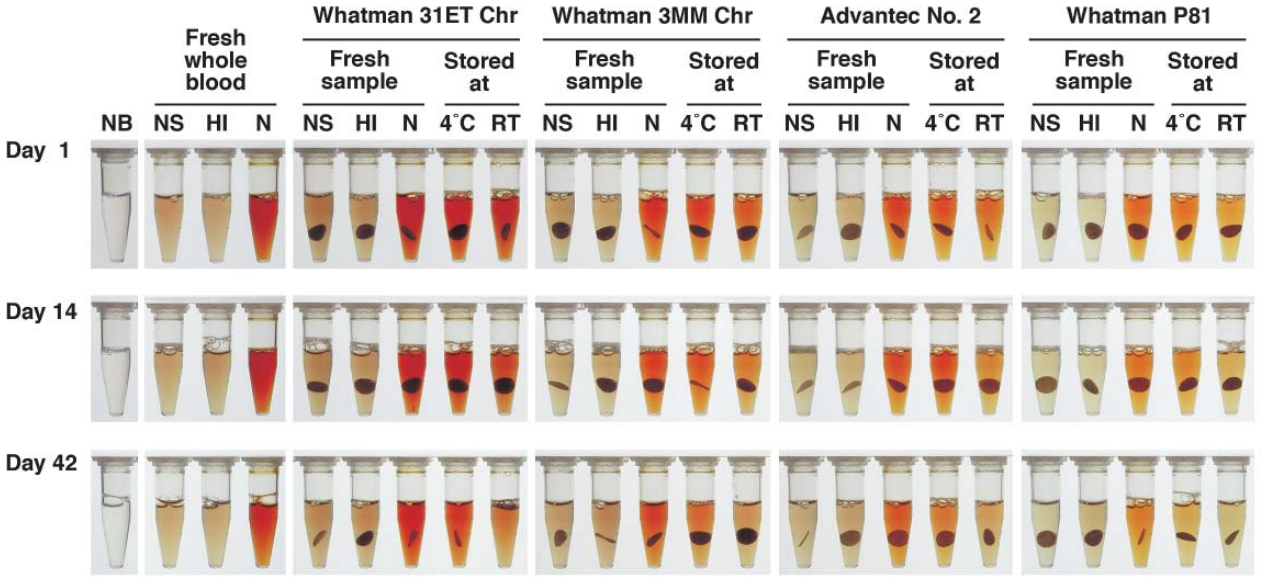

Fig. 2 Reaction intensity and retentivity of stored blood-spotted filters. Blood-spotted filters were prepared on day 0 and stored at 4°C or room temperature (25–28°C). At various time points, the filters were subjected to G6PD assay, and color photographs were taken at 40 min after the beginning of the reaction. Pictures taken on day 1, day 14, and day 42 are presented. At each time point of the G6PD assay, blood was taken from the same donor (MA) for the preparation of fresh samples. NB, no blood control; NS, no substrate control; HI, heat-inactivated blood; N, normal blood (for details, see Table 1).

41% (3MM), 44% (AD2), and 27% (P81), which was comparable to that of the corresponding filter stored at 4℃ for 14 days. On day 14, the RRI of each filter was considerably reduced to 32% (ET), 19% (3MM), 23% (AD2), and 12% (P81), and the color change of the latter was obviously diminished

Table 2 Specifications of the filters used in the study

Filter	Material	Thickness (mm)	Weight (g/m^2)	Absorption speed	Applications
Whatman					
31ET Chr	α-cotton cellulose	0.50	190	225 mm/30 min	Chromatography, electrophoresis
3MM Chr	α -cotton cellulose	0.34	185	130 mm/30 min	Blotting, chromatography, electrophoresis
P81	cellulose phosphate (cation exchanger)	0.23	85	125 mm/30 min	Ion exchange chromatography
Advantec					
No.2	α -cotton cellulose	0.26	125	80 mm/10 min	Qualitative filtration

These information are obtained from the catalogues of Whatman International Ltd. and Advance Toyo Ltd.

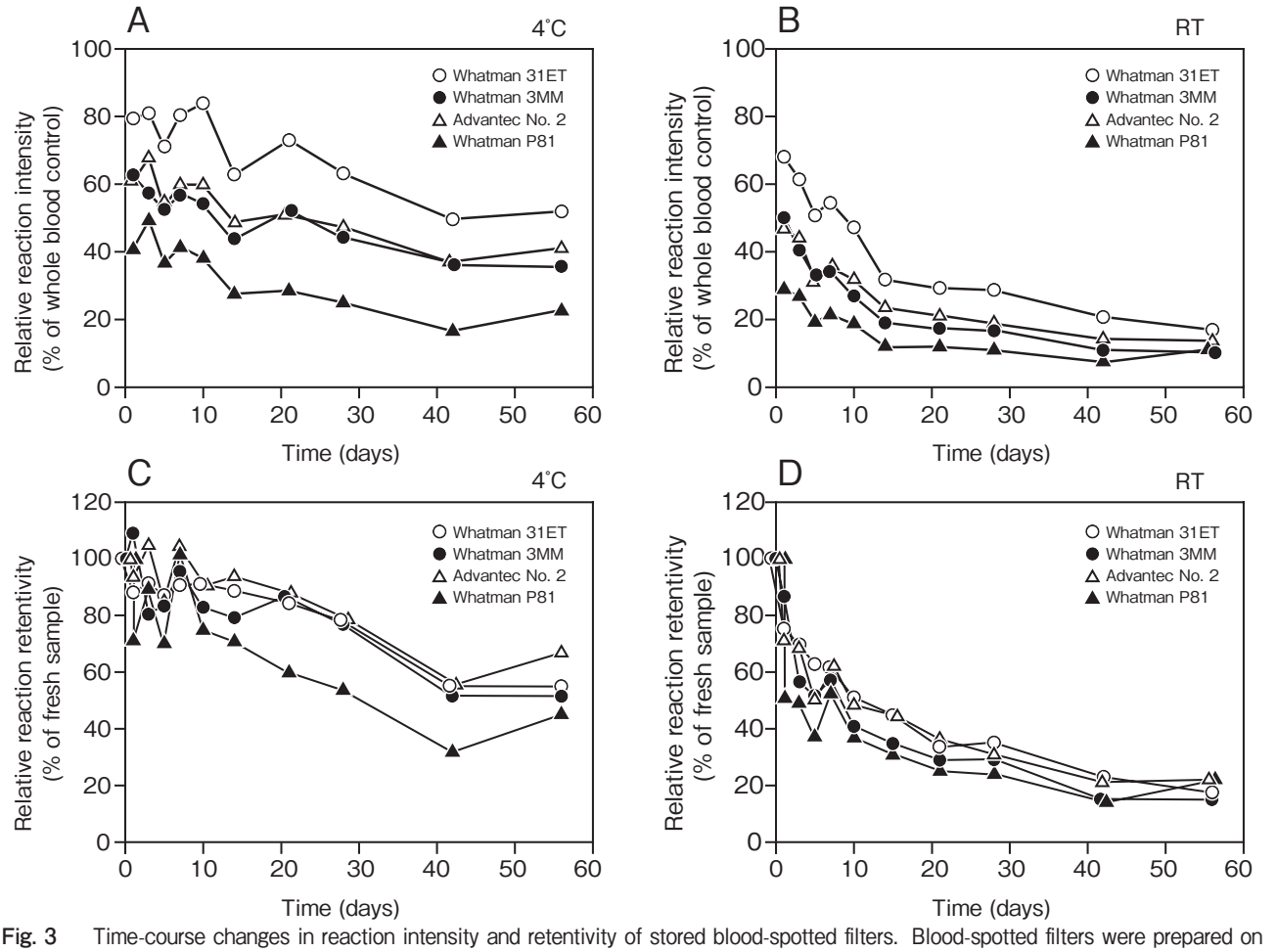

Fig. 3 Time-course changes in reaction intensity and retentivity of stored blood-spotted filters. Blood-spotted filters were prepared on day 0 and stored at 4℃ or room temperature (RT, 25–28℃). At various time points, they were subjected to G6PD assay and absorbance was measured 40 min after the start of the reaction. The relative reaction intensity (**A** and **B**) and relative reaction retentivity (**C** and **D**) were calculated as described in the 'Results' section. The data presented in Fig. 1–3 are from the same data set out of 2 independent experiments with similar results.

(Fig. 2). On day 42, the RRI of each filter was reduced to indistinguishable levels, *i.e.* 21% (ET), 11% (3MM), 14% (AD2), and 8% (P81). Based on the RRR of blood-spotted filters stored at 4℃, the degree of reduction in reactivity among ET, 3MM, and AD2 was very similar (55%, 52%, and 55%, respectively, on day 42), whereas that of P81 (32%) was more apparent than the others (Fig. 3C). As can

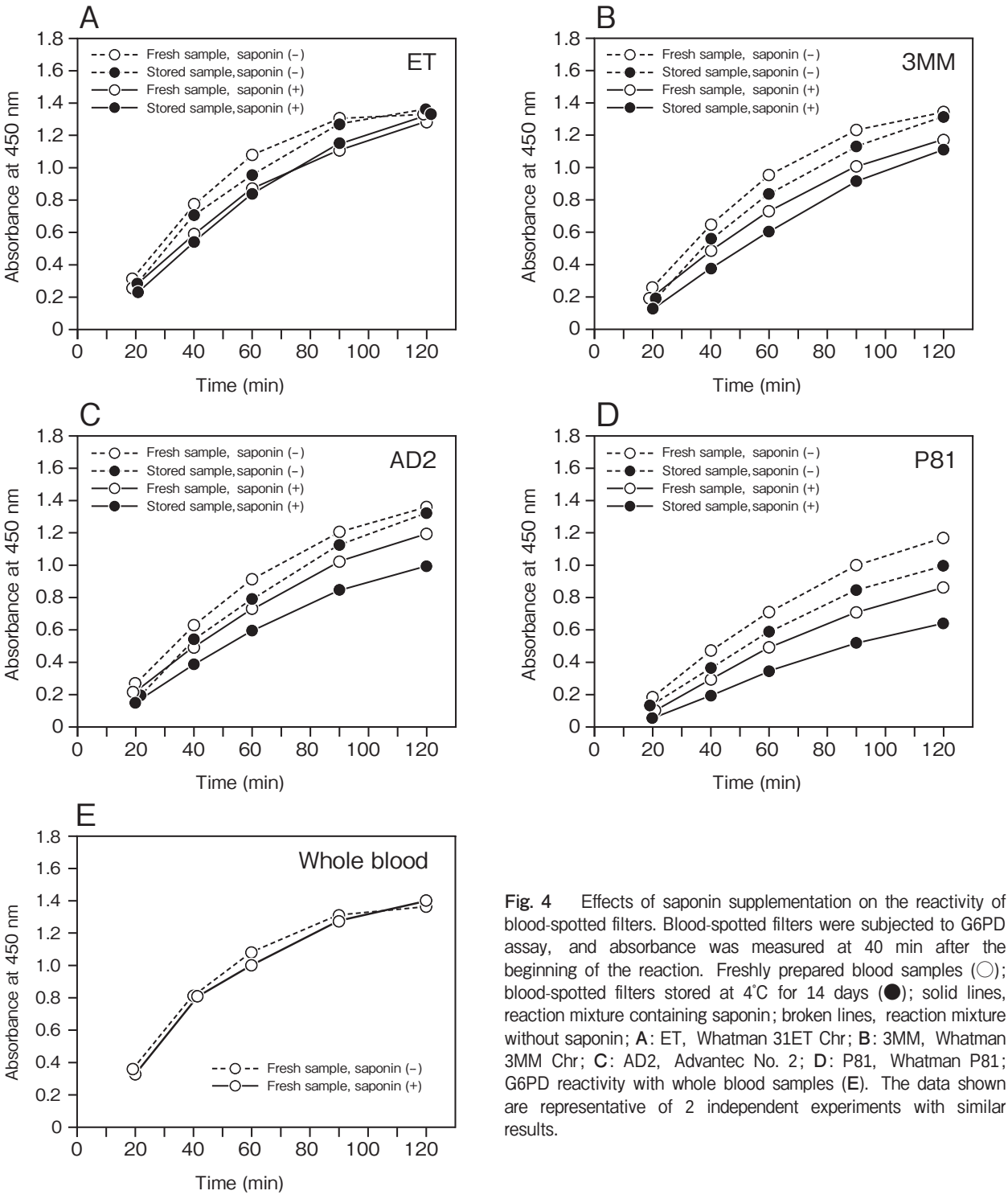

Fig. 4 Effects of saponin supplementation on the reactivity of blood-spotted filters. Blood-spotted filters were subjected to G6PD assay, and absorbance was measured at 40 min after the beginning of the reaction. Freshly prepared blood samples (○); blood-spotted filters stored at 4℃ for 14 days (●); solid lines, reaction mixture containing saponin; broken lines, reaction mixture without saponin; **A**: ET, Whatman 31ET Chr; **B**: 3MM, Whatman 3MM Chr; **C**: AD2, Advantec No. 2; **D**: P81, Whatman P81; G6PD reactivity with whole blood samples (**E**). The data shown are representative of 2 independent experiments with similar results.

be seen in the RRI-based profile, the RRR of each blood-spotted filter stored at room temperature was rapidly reduced (Fig. 3D).

Effects of saponin on the reactivity of blood-spotted filters. With any type of filter tested, reaction tubes with blood-spotted filter but lacking saponin in the reaction mixture showed higher absorbance than those containing saponin (Fig. 4A–E). The inhibitory effects of saponin on the G6PD reactivity were also apparent in a comparison between fresh and stored samples. However, whole blood samples did not show significant difference in reactivity between saponin-lacking and saponin-containing reactions (Fig. 4E).

Discussion

In the present study, we compared the reactivity of 4 types of blood-spotted filter samples in the WST-8 method for screening of G6PD deficiency. Among the filters tested, ET was the highest in both reactive intensity and retentivity. It should be noted that due to its high absorption speed (Table 2), it is much easier to make blood-spots onto an ET filter than the other filters. The present results indicate that ET is the most suitable filter for the WST-8 method, especially when rapid judgment is required. 3MM and AD2 were very close in reactivity, probably reflecting the similarity in the material and thickness (Table 2). Although the reaction intensities of these 2 filters were smaller than that of ET, the reaction retentivity was almost the same among the 3 filters (Fig. 3C). Considering that the specifications of 3MM and AD2 are more common and make it easier to obtain equivalent filters than ET, these 2 filters should be the second choice for the WST-8 method when rapid diagnosis is not essential.

In the previous MTT/PMS method by Fujii *et al.*, P81 has been used as a blood carrier [15]. In Fujii's method, as cation-exchange cellulose paper, P81 binds to hemoglobin and G6PD spreads out from the paper at pH 6.5, resulting in a reduced nonspecific reaction of hemoglobin with MTT. However, in our study P81 was the lowest in both reactive intensity and retentivity. It is not likely that P81's unexpectedly low reactivity was due to the higher pH condition in the WST-8 method (pH 7.2) than in Fujii's method (pH 6.5), as the above-mentioned role of P81 is not a factor in the WST-8 method, for which there is no nonspecific reaction between WST-8 and hemoglobin [11]. Although the cause of P81's low reactivity is unknown, it could be said that P81 is not suitable for the WST-8 method under standard conditions.

Under field conditions, because of the limited capacity of cold storage, it is much easier to handle blood-spotted filters than whole blood-containing microtubes. Therefore, when on-site examination is not essential, it is desirable that blood-spotted filters be used to replace whole blood samples. On the other hand, it is very important for field workers to know how long and to what extent blood-spotted filters can retain G6PD reactivity. Based on the results of the present study, we recommend that blood-spotted filters be kept in the cold and assayed within 14 days. In addition, it should also be mentioned that when no cold storage is available, blood-spotted filters that are prepared with appropriate control samples and stored at 25–28℃ can be subjected to the WST-8 method within the limit of 3 days after preparation.

In the early stages of our study, we employed saponin to liberate G6PD from dried blood on the filter by its strong hemolytic effect [16, 17]. However, G6PD reactivity was reduced in the presence of saponin only when blood-spotted filter was used. It has been reported that soybean saponins suppress proliferation of HT-29 human colon cancer cells by reducing protein kinase C activity [18]. Also, Quillaja saponin significantly binds with cholesterol to form micelles [19]. Taken together, the possible explanation for the inhibitory effects of saponin on the G6PD reactivity of filter-spotted blood sample is that some portion of saponin in the reaction mixture may bind with cholesterol in the remaining blood on the filter to form micelles, increasing local concentrations of saponin, resulting in a suppression of the enzyme activity of the filter-bound fraction of G6PD. Considering that saponin supplementation did not improve G6PD reactivity with whole blood samples (Fig. 4E), saponin should not be used in the reaction mixture. It should be noted that the commercial 'G6PD Assay Kit,' which has recently been released from Dojindo Laboratories, does not contain saponin in its reaction mixture.

In conclusion, blood-spotted filters can be used in

the WST-8 method for the screening of G6PD deficiency with the following precautions: 1) ET is the most suitable filter as a blood carrier for the WST-8 method; 2) blood-spotted filters stored under cold conditions can be assayed within 14 days, and those stored at 25–28°C should be tested within 3 days; and 3) the reaction mixture should not contain saponin. Taking into consideration the points mentioned above, the WST-8 method with blood-spotted filters makes it possible to assay hundreds of samples at once for quantitative analysis of their G6PD activity, thus presenting the opportunity for a large-scale survey of G6PD enzyme polymorphism and for the establishment of newborn screening for G6PD deficiency.

Acknowledgements. This study was supported by a Grant-in-Aid for Scientific Research B2 (14406026) from the Japan Society for the Promotion of Science (JSPS), and a Grant for International Health Cooperation Research (16C-1) from the Ministry of Health, Labor, and Welfare of Japan.

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