A Simple Spot Screening Test for Fast Detection of Red Cell NADH-Diaphorase Deficiency

By JEAN-CLAUDE KAPLAN, ANNE-MARIE NICOLAS, ALENA HANZLICKOVA-LEROUX AND ERNEST BEUTLER

A rapid spot screening test for NADH-diaphorase deficiency is described. Nitrited blood is added to a reaction mixture freshly prepared from stable stock reagents. Spots are made at intervals on filter paper and examined for defluorescence as NADH is oxidized. All of six patients with NADH diaphorase deficiency studied have been correctly identified using this technique, but it is not suitable for the detection of heterozygotes.

CONGENITAL METHEMOGLOBINEMIA due to NADH-diaphorase deficiency ("NADH-dependent methemoglobin reductase deficiency") is a relatively rare clinical disorder characterized by lifelong cyanosis. The diagnosis of this disorder may be established by several methods: the exploration of the methemoglobin reduction capacity in intact nitrite red cells; the quantitative determination of the enzyme activity itself in hemolysates. The latter estimation can be performed by measuring the rate of reduction of dichlorophenol-indophenol (DCIP) or of the methemoglobin-ferrocyanide complex in the presence of reduced NAD (NADH). The methemoglobin reduction test and the method of Scott are tedious and time-consuming because they require repeated washings of the nitrite-treated red cells. The method of Hegesh, although simpler and more specific, requires as a substrate a relatively fresh preparation of enzyme-free hemoglobin. These drawbacks have prompted us to devise an easy method for rapid diagnosis of NADH-diaphorase deficiency. It is based on the ultraviolet spot test principle already described for fast detection of various other red cell enzyme deficiencies.

MATERIALS AND METHODS

Principle of the Test

Whole blood is added to a mixture containing a hemolyzing agent, NADH and DCIP. In the presence of NADH-diaphorase, the dye is reduced by NADH. During the reaction NADH, which fluoresces when illuminated by long wavelength ultraviolet light, is trans-
Fig. 1.—UV spot-test for NADH-diaphorase: normal blood. 1. Reaction mixture without blood (nitrite added). 2. Nitrited normal blood plus reaction mixture without DCIP. 3. Normal blood without nitrite plus reaction mixture without DCIP. 4. Normal nitrited blood plus complete reaction mixture.

formed into NAD, which is not fluorescent under such conditions. Pretreatment of the blood sample with sodium nitrite oxidizes the hemoglobin, thus preventing its direct reducing effect upon DCIP.

Test Procedure

Five μl. of a freshly prepared 0.18 M (1.24%) sodium nitrite aqueous solution are added to 0.1 ml. of heparinized or ACD whole blood. After being thoroughly mixed, the sample is allowed to stand at room temperature for 30 minutes. Twenty μl. of the nitrited whole blood are added to a test tube containing 0.04 ml. of 1 per cent saponin and 0.2 ml. of a reaction mixture containing 0.70 mM NADH, 0.19 mM DCIP and 0.27 mM sodium EDTA in a 0.06 M Tris-HCl buffer, pH 7.6. This mixture is freshly prepared from stable stock solutions by adding 1.0 ml. of 0.06 M Tris-HCl buffer containing 0.27 mM sodium EDTA and 10 μl. of 19 mM DCIP (6.25 mg./ml.) to a preweighed vial of 0.5 mg. dry NADH (Sigma). The test tube containing the blood with the reaction mixture is incubated at 37°C. Every 10 minutes, a drop is spotted on Whatman No. 1 filter paper. Dry spots are examined under a long wave ultraviolet lamp.

RESULTS

Normal blood defluoresces in less than 30 minutes (Fig. 1). When DCIP is omitted, or in the absence of blood, no defluorescence is observed. If hemolyzed blood is heated at 56°C for 2 hours before being added to the reaction mixture, there is no defluorescence. The omission of nitrite results in a delay of the defluorescence time. Plasma alone does not produce any defluorescence of the reaction mixture. A suspension of washed nitrited red cells in saline gives the same result as unwashed nitrited whole blood. If red cells are suspended in various amounts of plasma or saline, the increased fluorescence time is proportional to the dilution factor. The same propor-
Fig. 2.—UV spot test for NADH diaphorase: deficient and normal blood. (For experimental conditions, see text). 1. Reaction mixture alone. 2 and 4. Reaction mixture plus normal blood. 3 and 5. Reaction mixture plus deficient blood.

Functionality is observed with diluted hemolysates. The hematocrit value influences the defluorescence time because the combined reduction of red cell number and hemoglobin-quenching effect are additive, both contributing to prolong the defluorescence time. We recommend readjusting the PCV to normal whenever it is lower than 35 per cent. This can be done either by removing the excess of plasma or by suspending the packed cells in an appropriate amount of saline.

The usefulness of the ultraviolet spot test was verified by applying it to six homozygotes with congenital methemoglobinemia. Their red cell NADH-diaphorase activity was less than 10 per cent of normal. In these subjects, the defluorescence time was found to be considerably increased (Fig. 2). On the other hand, blood samples from two heterozygous subjects did not give clearly abnormal results. Therefore we do not recommend this procedure for detection of the carrier state.

The technique has been applied to 70 blood samples, without any false positive results. It is noteworthy that in the few young infants tested, the defluorescence time was not significantly increased in spite of their lowered red cell NADH-diaphorase as compared to normal blood. The blood of two subjects with high reticulocyte counts, 11 per cent and 24 per cent, respectively, gave normal results.

**Discussion**

Since the whole procedure can be carried out within an hour, using only readily available equipment and reagents, we consider the fluorescent spot test a simple and fast method for diagnosis of NADH-diaphorase deficiency.
The stability of the reagents for the test is of particular importance, since the average laboratory is only rarely called upon to make the diagnosis of NADH-diaphorase deficiency. Only the nitrite solution must be freshly prepared, and this can be accomplished in a few minutes. All of the other solutions are stable for many months in the frozen state and can merely be added to a preweighed vial of NADH when the occasion for performing an examination for NADH-diaphorase deficiency presents itself.

Since it can quickly provide an all or none answer on capillary blood samples, it should be valuable for screening methemoglobinemic, or even simply cyanotic infants, as well as adults with methemoglobinemia.

REFERENCES

A Simple Spot Screening Test for Fast Detection of Red Cell NADH-Diaphorase Deficiency

JEAN-CLAUDE KAPLAN, ANNE-MARIE NICOLAS, ALENA HANZLICKOVA-LEROUX and ERNEST BEUTLER