TECHNICAL NOTE

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Identification of Fetal Bloodstains by Enzyme-Linked Immunosorbent Assay for Human α-Fetoprotein

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ABSTRACT: A rapid and highly sensitive enzyme-linked immunosorbent assay (ELISA) for determination of human α-fetoprotein (AFP) using commercially available reagents was devised and applied to identification of fetal bloodstains. When experimentally prepared bloodstains, 1 by 2 mm in area, were submitted to analysis, only fetal bloodstains showed positive reactions in the present ELISA. The reactions did not change significantly when these bloodstains were stored at room temperature for one week. The present ELISA seems to be suitable for forensic science practice.

KEYWORDS: pathology and biology, blood, immunoassay, proteins, α-fetoprotein, fetal bloodstains, ELISA

Identification of fetal bloodstains is important in certain cases of infanticide, abortion, and concealment of delivery. For this purpose, immunoelectrophoretic detection of fetal antigens, hemoglobin F [*1-2*], or α-fetoprotein (AFP) [*3-4*] has been used. Recently, highly sensitive methods for identification of fetal bloodstains using the radioimmunoassay kit for AFP were reported [*5-8*]. This enables the detection of AFP from fetal bloodstains as small as 0.1 mm^2 [*6*] or those stored for a long period [*5, 7, 8*]. However, radioimmunoassay requires expensive counting equipment and radioactive probes constituting a major health hazard. Recently, the solid phase enzyme-linked immunosorbent assay (ELISA) has begun to be used in the field of legal medicine [*9-12*]. The technique sacrifices none of the sensitivity and still can be used for the same applications as radioimmunoassay.

In the present study, a new and rapid ELISA method for detection of AFP using commercially available reagents was devised and applied to identification of fetal bloodstains.

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Materials and Methods

Materials

Fetal blood samples were obtained from newborns at the time of delivery from the umbilical cord. Retroplacental blood samples were collected from pregnant women in parturition, and venous blood samples from healthy adults of both sexes. Bloodstains were made on a piece of filter paper (Whatman, No. 2, Maidstone, England), allowed to dry at room temperature, and examined one day and one week after preparation.

Reagents

Goat anti-human AFP, rabbit anti-human AFP, alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (IgG), and standard human AFP were purchased from Medical and Biological Laboratories Ltd. (Nagoya, Japan), Nordic Immunological Laboratories (Tilburg, Netherlands), Kirkegarred & Perry Laboratories Inc. (Gaitersberg, U.S.A.), and The Green Cross Corporation Ltd. (Osaka, Japan). IgG in goat anti-human AFP was partially purified by a standard ammonium sulfate precipitation method [*13*] and the protein concentration was adjusted to 4 mg/mL in 0.01*M* phosphate buffered saline (PBS), pH 7.6. This preparation was stored in 0.1-mL aliquots at −70°C. Other antibodies were tenfold diluted with PBS containing 1% bovine serum albumin and were stored in 0.1-mL aliquots at −70°C. Standard AFP was also stored in 0.1-mL aliquots at −70°C. Thawed solutions of these reagents can be stored at 4°C for at least one month in the presence of 0.1% sodium azide.

Principle of the Present ELISA Method

A sandwich method of enzyme immunoassay for determination of human AFP reported by Maiolini and Masseyeff [*14*] was modified in the present study. The present method differs in two major respects from the original technique: a polystyrene ELISA plate was selected as solid phase material instead of CNBr-activated microcrystalline cellulose, and the antigen was identified by the combined use of rabbit anti-human AFP and alkaline phosphatase-labeled anti-rabbit IgG instead of the single use of the glucose-oxidase labeled anti-human AFP prepared by itself. Thus, a simple and highly sensitive ELISA was devised using commercially available reagents.

The principle of the present method is illustrated in Fig. 1. The wells of a microtiter plate are coated with IgG of goat anti-human AFP, and the remnant surfaces of the wells are blocked with bovine serum albumin (Step 1). Specimens, rabbit anti-human AFP, and enzyme-labeled second antibody are added to the wells in that order (Steps 2 through 4). After each step, the wells are washed with PBS containing 0.05% Tween 20 (PBS-Tween). Finally, the activities of the bound enzyme are measured spectrophotometrically.

Procedures

Unless otherwise mentioned, all the steps below were performed at room temperature. The wells of a polystyrene microtiter plate (129B, Dynatech Ltd., Sussex, England) were coated with 100 μL of 50-fold diluted solutions of IgG of goat anti-human AFP in 50m*M* sodium carbonate buffer (pH 9.6). After incubation for 10 min, the IgG was pipetted out. Subsequently, 150 μL of blocking buffer (PBS containing 1% bovine serum albumin) was added to each well and allowed to react for 10 min. The buffer was then pipetted out with two washes of PBS-Tween, and 100 μL of PBS-Tween containing either standard AFP or a piece of bloodstain (1 by 2 mm in area) was added to each well. After incubation for 60 min, the specimens were pipetted out with three washes of PBS-Tween, and 100 μL of 50-fold diluted rabbit anti-

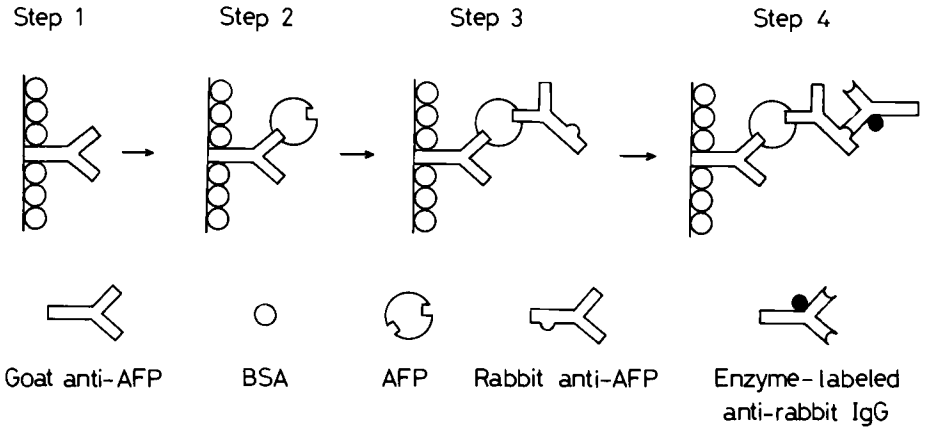

FIG. 1—*Principle of the present ELISA method.*

human AFP in blocking buffer was added to each well followed by incubation for 10 min. The antiserum was pipetted out with three washes of PBS-Tween, and 100 μL of a 500-fold diluted solution of enzyme-labeled anti-rabbit IgG in blocking buffer was added to each well. After incubation for 30 min, the reagent was pipetted out with three washes of PBS-Tween. Finally, a reaction medium (100 μL; 1*M* diethanolamine containing 1m*M* magnesium chloride, pH 9.3) and a substrate solution (10 μL: 100m*M* of *p*-nitrophenyl phosphate in 0.001*N* hydrochloric acid) were added to the wells and incubated for 2 h at 37°C. At the end of incubation, 50 μL of the mixture was added to 600 μL of a stop solution (0.35*N* sodium hydroxide) and the absorbance at 405 nm was measured with a Hitachi 557 spectrophotometer. Measurements were made in duplicate.

Results

Preliminary experiments revealed that the coating of the plate with IgG of goat anti-human AFP was completed within 10 min. The binding of AFP to the antibody-coated plate was completed by 10 min for AFP standards and by 60 min for fetal bloodstains. When the second antibody, rabbit anti-human AFP, was reacted, the ELISA reactions observed after 10 min of incubation reached around 70% of those observed after 30 min of incubation. When the third antibody, the enzyme-labeled anti-rabbit IgG, was reacted, the ELISA reactions observed after 30 min of incubation reached around 60% of those observed after 1 h of incubation. The extention of the incubation period of the second or the third antibody was, however, associated with a significant elevation of the control reactions observed without addition of AFP. Thus, relatively short incubation periods were selected in the present ELISA system. These short incubation periods adopted did not cause a significant decrease in the sensitivity and the specificity of the method.

A typical standard curve for human AFP in the range of 0 to 150 ng/mL is shown in Fig. 2. The sensitivity of the assay was around 3 ng/mL. When 1 by 2 mm of bloodstains was analyzed in the present assay system, AFP was not found in pregnant women stains or adult stains but found in fetal stains, and the reactions of fetal bloodstains showed only a slight decrease during one week of storage at room temperature (Table 1). In the present ELISA system, the absorbance at 405 nm below 0.02 were regarded as negative reactions. The range of the negative reactions should probably be determined in each ELISA system. As shown in Table 2, ELISA reactions of the standard AFP were not influenced significantly by the presence of the blood extract. Usually, the presence of AFP in the fetal bloodstains could easily be observed with the

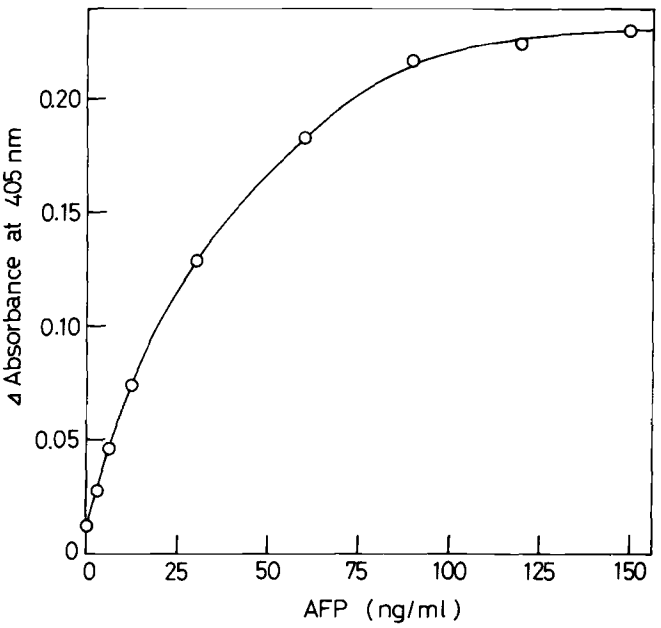

FIG. 2—*Standard curve of AFP.*

TABLE 1—*Results of the present ELISA method applied to detection of AFP in fetal blood stains.*

Source of Bloodstains	Number of Specimens	ΔAbsorbance at 405 nm	
		One-Day Storage	One-Week Storage
Newborns	10	0.204 ± 0.040	0.200 ± 0.019
Pregnant women	6	0.011 ± 0.006	0.009 ± 0.005
Adults	6	0.006 ± 0.003	0.004 ± 0.002
Controls[a]	6	0.008 ± 0.003	...

[a]Pieces of filter paper, 1 by 2 mm in area, were used.

TABLE 2—*Effect of the presence of bloodstain extracts on the ELISA reactions for standard AFP.*

Standard AFP	ΔAbsorbance at 405 nm	
	PBS-Tween[a]	Bloodstain Extract[b]
0	0.007	0.006
12	0.086	0.091
30	0.136	0.147
60	0.183	0.187
90	0.219	0.227

[a]The standards of AFP were prepared in PBS-Tween.

[b]An adult blood stain, 1 by 1 cm in area, was immersed in 5 mL of PBS-Tween followed by incubation for 1 h at room temperature, and the standards of AFP were prepared in the extract.

naked eye since the absorbance at 405 nm of the reaction mixture in the wells reached around 2.0 to 3.5 within 2 h of incubation.

Discussion

The results presented above indicate that the rapid ELISA technique can be used for the identification of fetal bloodstains, and information is obtained within 4 h. Since we aimed to devise a rapid ELISA system for identification of fetal bloodstains applicable to daily practice, relatively short incubation periods were selected. Although complete immunological reactions may not be achieved in some steps, the rapid ELISA is still sensitive enough to detect small amounts of AFP and appears to be suitable for forensic science practice.

The present ELISA method can easily be performed using commercially available reagents without specialized techniques such as preparation of highly purified antibodies or enzyme-antibody conjugates. Partially purified goat anti-human AFP by a standard ammonium precipitation method was sufficient for precoating the ELISA plate. Other reagents can be used without further purification. The sensitivity of the present ELISA is several-fold higher than that of the radioimmunoassay applied to identification of fetal bloodstains by Katsumata et al [*6–8*]. Several ELISA kits for determination of serum AFP levels having comparable sensitivity are now commercially available. Therefore, those kits could be used for the same purpose as the present ELISA method. The main advantage of the present ELISA method is that the same system can be applied to identification of seminal stains [*9*], species identification of blood stains [*10*], detection of methamphetamine [*11*], and ABO grouping of saliva stains [*12*] with minor modifications.

The AFP contents in 1- by 2-mm bloodstains measured by the present ELISA method were 40 to 150 ng. Since this size of the experimentally prepared bloodstains contains around 0.4 μL of blood [*8*], the AFP contents in original cord blood samples were calculated to be 100 to 380 μg/mL which are comparable to the values previously reported [*15*]. It has been reported that the AFP contents in maternal serum are 192.2 $\pm$ 76.3 ng/mL [*15*] and those in normal adult blood are less than 10 ng/mL [*16*]. Therefore AFP contents in 1- by 2-mm bloodstains can be calculated to be around 80 pg for maternal blood and less than 4 pg for normal adult blood which are both far lower than the sensitivity of the present ELISA method (3 ng/mL). Consequently, fetal bloodstains can be differentiated from maternal or adult bloodstains by the present ELISA method provided that bloodstains of 1 by 2 mm in the area of the stained paper are submitted to analysis.

Using radioimmunoassy, it has previously been shown that AFP contents in fetal bloodstains did not decrease significantly during the six months of storage at room temperature [*8*]. In the present study, the AFP contents in fetal bloodstains also showed the least decrease during one week of aging (Table 1).

When AFP is used for the marker of fetal bloodstains, it should be taken into account that some patients having primary hepatoma show high concentrations of AFP in serum [*16*], although that disease is quite rare.

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