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Rapid and sensitive identification of human blood by an ELISA-ABC method using a biotinylated antibody against human HbA_0

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Summary. A direct enzyme-linked immunosorbent assay (ELISA) using an avidin-biotin complex (ABC) system for the identification of human blood is described. In this ELISA-ABC method, in which biotin-labeled goat IgG antibody against human HbA_0 was used, it was possible clearly to distinguish human blood from the blood of other species, including that of Japanese monkeys. It took about 3 h to obtain the results. Human Hb concentrations ranging from 22 ng to 169 µg produced a positive reaction, and the minimum detection limit in terms of the highest possible dilution of human blood was 1:640,000.

Key words: Human blood identification, ELISA-ABC method – Enzyme-linked immunosorbent assay (ELISA) – Avidin-biotin complex (ABC) system

Zusammenfassung. Beschreibung einer direkten ELISA-Methode unter Verwendung des Avidin-Biotin-Complexes zur Identifizierung winziger Mengen menschlichen Blutes. In dieser ELISA-ABC-Methode wurde ein Biotin-gebundener IgG-Antikörper (Ziege) gegen Human-HbA_0 verwandt. Mit dieser Methode war menschliches Blut von dem Blut japanischer Affen und anderer Tiere sehr leicht unterscheidbar. Positive Reaktionen wurden nach einer Untersuchungszeit von etwa 3 Std. gewonnen. Minimale Hämoglobin-Präparationen zwischen 22 ng und 169 µg erwiesen sich als ausreichend, um eindeutige positive Reaktionen zu erzeugen. Der Test reagierte bis zu einer Blutverdünnung von 1:640.000-fach positiv.

Schlüsselwörter: Avidin-Biotin (AB)-Komplex-System – Blutidentifizierung, ELISA-AB-Komplex-Methode

Introduction

Medico-legal practice requires a rapid and sensitive method that allows the identification of human blood from minute samples. Recently, enzyme-linked

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immunosorbent assays (ELISA) have been applied for the species identification of blood or bloodstains, because such methods are both sensitive and easily automated [1–6]. Since, in these ELISA methods, antisera or antibodies against human serum proteins are used as the primary antibody, the results merely indicate whether human serum protein components are present in the sample. Moreover, the troublesome problem of reactivity of the secondary antibody arises when an indirect ELISA method is applied. Fletcher et al. [2] reported that, when wells were coated with bloodstain extracts from mice, rats, or humans all exhibited a positive reaction when subjected to an ELISA method using enzyme-labeled sheep antimouse IgG as the secondary antibody.

In 1983, Madri and Barwick [7] reported that the use of the avidin-biotin peroxidase complex (ABC) technique in ELISA improved the method's sensitivity as compared to the conventional enzyme immunoassay using an enzyme-labeled antibody. In the present study, we prepared a biotinylated antibody against human HbA_0 and attempted to apply the ABC system to an ELISA method for the identification of minute samples of human blood.

Materials and methods

Biotinylated antibody against human HbA_0. A biotin-labeled goat antibody against human HbA_0 was prepared as follows: a crude goat serum against human HbA_0 was isolated in a way similar to that described previously [8]. Specific goat immunoglobulin G (IgG) against human HbA_0 was separated from the absorbed antiserum by DEAE-cellulose column chromatography using 0.01 *M* phosphate buffer (pH 7.35). Only the first fraction was pooled and concentrated to the original volume of the applied antiserum. Biotin labeling of goat antihuman HbA_0 IgG was performed according to the method of Nerurkar et al. [9] with slight modifications. In brief, 60 µl freshly prepared N-Hydroxysuccinimidobiotin (1 mg/ml in dimethyl sulfoxide; Pierce Chemicals, USA) was added to 1 ml goat antihuman HbA_0 IgG solution (1 mg/ml in 0.1 *M* $NaHCO_3$, pH 8.3). After incubation at room temperature for 4 h with occasional gentle stirring, the mixture was extensively dialyzed against phosphate-buffered saline (PBS; pH 7.2) containing 0.1% NaN_3 to remove unconjugated biotin ester and dimethyl sulfoxide. The biotinylated antibody solution was stored at 4°C.

Blood samples. Stroma-free, washed erythrocyte lysates and sera were obtained from human subjects and from 12 species of animals and were stored at −80°C until use. Fresh human whole blood samples from six normal healthy adult donors were separately frozen overnight at −80°C before testing.

Reagents and buffers. As ABC reagents, we used the Vectastain ABC kit PK-4000 (avidin-biotin-peroxidase complex) and the ABC-AP kit AK-5000 (avidin-biotin alkaline-phosphatase complex) from Vector Laboratories (Burlingame, Calif). The horseradish peroxidase (PO) substrate consisted of 0.4 mg/ml o-phenylendiamine (Nakarai Chemicals, Japan) in 50 m*M* citrate/phosphate buffer (pH 5.3) containing 0.015% H_2O_2 [10]. The alkaline phosphatase (AP) substrate comprised 4 mg/ml p-nitrophenyl phosphate (Research Organics, USA) in 1 *M* diethanolamine buffer (pH 9.8) containing 0.01% $MgCl_2 \cdot 6H_2O$ and 0.02% NaN_3 [11]. The stopping solutions used were 1 *M* H_2SO_4 for the PO substrate, and 1 *M* NaOH for the AP substrate. As the dilution and coating buffer, we used 0.012 *M* PBS, pH 7.2. The blocking buffer was PBS containing 0.5% casein (Difco Laboratories, USA), while the washing buffer was PBS containing 0.05% Tween 20 (PBS-T). As the dilution buffer for the biotinylated antibody and ABC reagent, we applied PBS-T containing 0.02% bovine albumin (Nakarai Chemicals).

Standard ELISA-ABC procedure. The wells of flat-bottomed microplates (Nunc-Immuno Plate; Nunc, Denmark) were coated with 100 µl diluted antigens (blood samples) for 15 min at room temperature. After discarding the coating antigen solution using a thin pipette, 200 µl blocking solution was added to each well for 15 min at room temperature. The plates were washed three times with PBS-T using Immuno Washer NK-100 (Inter Med, Japan) before 100 µl biotinylated antihuman HbA_0 solution (diluted 1:100) was added to each well and incubated for 15 min at 37°C and then for 15 min at room temperature.

After washing the plate ten times as already described, one drop of ABC reagent (in the ABC-PO system) or ABC-AP reagent (in the ABC-AP system), which had been prepared according to the instructions contained in the Vectastain ABC kit, was added to each well and incubated for 15 min at room temperature. The plates were again washed ten times and were then developed in the dark using 100 µl PO substrate solution for 15 min or the AP substrate solution for 30 min. In the case of the ABC-PO system, the reaction was terminated by the addition of 100 µl 1 *M* H_2SO_4, and the optical density (OD) was measured at 492 nm using an EIA Reader (Titertek Multiskan MCC; Flow Laboratories, Finland); when AP substrate was used, 100 µl 1 *M* NaOH was added to stop the reaction, and the absorbance was measured at 405 nm.

A reagent 'blank' value for PBS (0.038 ~ 0.044 in the ABC-PO method, 0.075 ~ 0.085 in the ABC-AP method) was automatically subtracted from all sample readings. The minimum OD value accepted as a positive result was 0.5 in the ABC-PO system, and 0.2 in the ABC-AP system.

Results

Hemolysates (36 g Hb per 100 ml) obtained from human subjects and from 12 animal species were serially diluted with PBS to concentrations ranging from 1:500 to 1:2,048,000 and were examined using ELISA-ABC methods. The highest OD values of the human and animal hemolysates are listed in Table 1,

Table 1. Species specificity of the ELISA-ABC method using a biotinylated antibody against human HbA_0

Hemolysate source	Highest OD value obtained by ELISA	
	ABC-PO	ABC-AP
Human	1.741	1.086
Japanese monkey	0.222	0.085
Dog	0.110	0.052
Cat	0.119	0.057
Rat	0.149	0.081
Rabbit	0.180	0.081
Cow	0.080	0.026
Horse	0.116	0.053
Pig	0.098	0.031
Goat	0.044	0.009
Chicken	0.180	0.073
Goose	0.200	0.080
Dove	0.065	0.054

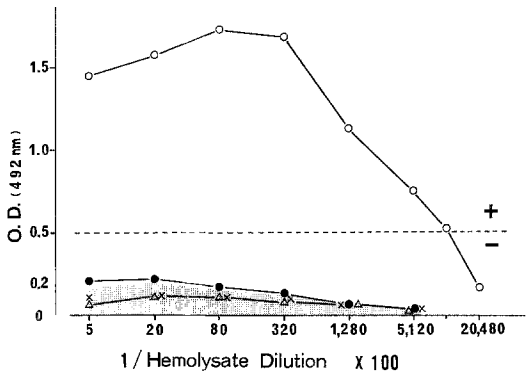

Fig. 1. Findings using the ELISA-ABC-PO method for variously diluted hemolysates (original concentration, 36 g Hb per 100 ml) obtained from humans (○), Japanese monkey (●), dog (△), cat (×), and other animals (▨), i.e., rat, rabbit, cow, horse, pig, goat, chicken, goose, and dove

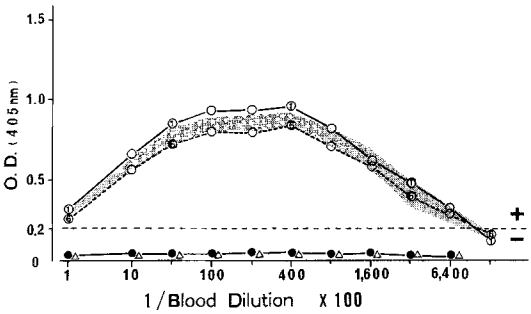

Fig. 2. Findings using the ELISA-ABC-AP method for variously diluted whole blood from six normal adults (donor 1, ① 16.9; donors 2–5, ▨ 16.5 ~ 15.6; donor 6, ⑥ 14.1 g Hb per 100 ml), Japanese monkey (●), and dog (△)

and the values at each dilution point are shown in Fig. 1. These data indicate that the human hemolysates yielded positive reactions at dilutions up to 1:1,024,000, while all of the hemolysates from animals, including the Japanese monkey, failed to react.

Human and animal sera were tested in a similar way, and all gave negative results, with OD values of less than 0.136 and 0.091 for the ELISA-ABC-PO and ELISA-ABC-AP methods, respectively.

Whole blood (14.1 ~ 16.9 g Hb per 100 ml) from six normal adults and from a Japanese monkey and a dog were serially diluted to concentrations ranging from 1:100 to 1:1,280,000 and were tested using the ELISA-ABC-AP method. Only the human blood samples showed specific positive reactions at dilutions ranging from 1:100 to 1:640,000 (Fig. 2). Similar results were obtained using the ELISA-ABC-PO method.

Discussion

The ELISA technique first introduced by Engvall and Perlmann [12] as a method for antigen quantitation has often been applied in the field of forensic medicine. Almost all ELISA methods for the species identification of blood are inhibition ELISA procedures using antisera or antibodies against human serum

proteins as the primary antibody [1–6]. Therefore a positive result may also occur when a sample contains serum protein components, e.g., saliva, semen, sweat, etc. Moreover, the reactivity of the secondary antibody used in indirect ELISA methods [2] presents a serious problem. Positive reactions have been observed in wells coated with bloodstain extracts from mice and rats, because enzyme-labeled sheep antimouse IgG was used as the secondary antibody [2]. On the other hand, a positive result obtained when serum against human HbA is applied is a specific indicator that human blood is present in a sample [13]. In tests to identify human blood, Oshima and Hara [14] applied an inhibition ELISA method using Hb-coated polystyrene beads, in which serum against human Hb served as the primary antibody, while enzyme-labeled goat anti-rabbit IgG was the secondary antibody. They found that human Hb was detectable in samples ranging from 1 to 10 μg and could be distinguished from Japanese monkey blood; however, it took over 2 days to obtain the results.

In the present ELISA-ABC method, a biotinylated IgG antibody against human HbA_0 from goat antiserum was used for the direct determination of the human origin of blood samples. Human blood was clearly distinguishable from animal blood, including that of Japanese moneys, by applying the ELISA-ABC methods using both the ABC-PO and ABC-AP systems. It is of great interest that the concentrated antihuman HbA_0 IgG failed to give a positive reaction with human Hb in a precipitin ring test, despite its high binding activity to human Hb in the ELISA-ABC method. It should also be noted that antisera that can be successfully used in the precipitin ring test are not always suitable for the ELISA-ABC method.

Positive reactions detectable with the naked eye were obtained for human hemolysate (36 g Hb per 100 ml) at dilutions ranging from 1:500 to 1:1,024,000 and with whole blood (14.1 g Hb per 100 ml) at dilutions from 1:100 to 1:640,000 using the ELISA-ABC method. The minimum detection limit of human Hb was 22 ng, so that the detection sensitivity of this method is superior to that of inhibition ELISA procedures [14]. It is especially worthy of note that our direct ELISA-ABC method allows easy identification of human blood even at dilutions that would give negative results in the benzidine test.

For the practice of criminal examination, it is also important to obtain the result of such a test in a short time. The ABC technique allows a short incubation time owing to the extraordinarily high affinity between biotin and avidin [15]. Thus, it takes only 3 h to identify human blood using this ELISA-ABC method.

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