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Absorption Test Using Latex Particles as the Indicator System for the Species Identification of Bloodstains and Muscles

In the practice of forensic medicine, various methods [*1-11*] have been applied to determine whether a bloodstain is of human or animal origin. In 1901, Uhlenhuth [*1*], Wassermann and Schütze [*2*], Stern [*3*], and Nuttall and Dinkelspiel [*4*] found the specific precipitation of antiserum and corresponding serum proteins and recommended forensic science use of this phenomenon for the species identification of bloods. The test by means of the interfacial or ring precipitin technique [*12*] has been widely employed. This method is simple but has poor reproducibility [*13*], and specific antisera are not easily prepared [*14*].

Wiener et al [*5*] in 1949 reported the anti-globulin inhibition test, in which anti-human globulin was neutralized by sera or bloodstain extracts and the absorbed antiserum was examined to determine its capacity to agglutinate indicator erythrocytes sensitized with incomplete antibody. Since the neutralization effect was reported to be antigen-specific, the practical applications of this test method have been studied by other workers [*15-19*].

Cayzer and Whitehead [*11*] and Whitehead et al [*20*] recently reported a latex agglutination test in which latex particles were coated with anti-human or anti-animal serum immunoglobulin and tested against bloodstain extracts. Their method, though rapid, occasionally produced cross-reactions.

The purpose of this study is to offer a specific, sensitive, and relatively simple test method for the species identification of biological materials. The method has proved to be an effective tool for the species identification of bloodstains and muscles.

Materials and Methods

Bloodstains

Fresh human and animal blood was applied to cotton strings or sheets of gauze that were then air-dried for at least 1 h at room temperature. The quantity of the dried blood was calculated based on the weight difference of the stained and unstained fabrics. As the fixation procedure, the stained fabrics were dipped into acetone or methanol for 5 min or heated in an oven for 5 min at 100°C.

Muscles

Human and animal muscles were preserved at −20°C and were washed with deionized water and weighed before being used. Most primate samples, including chimpanzee, were the gifts of Prof. Michio Okajima, Tokyo Medical and Dental College.

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Human IgG

The human IgG solution was prepared according to the method of Kendall [*21*]. Stock solution containing 2.80 mg/ml of human IgG was stored at −70°C. Immunoelectrophoretic analysis of this solution showed no detectable proteins other than IgG.

Antisera

Antisera to human IgG were prepared by injecting the pads of rabbits with an emulsion containing human IgG and Freund's complete adjuvant [*22*]. Some rabbits received one or two more injections. Twenty-one to 28 days after the final injection the rabbits were killed and the antisera collected. Sodium azide was added to the antisera to a final concentration of 0.1% and the mixture was stored at −70°C until use. Other batches of rabbit antisera to human IgG, to bovine serum, or to chicken serum, and goat antisera to rabbit serum, were obtained from Behringwerke AG (Marburg-Lahn, West Germany), Miles Laboratories (Ill.), or Hyland Laboratories (Calif.).

Coating of Latex Particles with Human and Animal Serum Proteins

The method used to prepare the latex particles was developed in cooperation with Takeda Chemical Industries (Osaka). This type of latex particles (SDL-43) showed high stability at both acidic and alkaline pH [*23*]. The latex particles were coated by the process shown in Table 1.

Titration

Antisera to be titrated were serially diluted with glycine buffer (7.505 g of glycine, 5.846 g of sodium chloride, and 1 g of sodium azide dissolved in 2000 ml of deionized water, adjusted to pH 8.2 by adding 1*N* sodium hydroxide). A thick glass slide with ten wells, into each of which was placed a drop of one of the dilutions and an equal amount of indicator latex particles, was rotated for 10 min at 120 rpm on a slide test rotator (Kayagaki Works, Tokyo). The slide was then placed on a black table to read the agglutination. Results were recorded by the patterns illustrated in Fig. 1, and the titer was indicated by the maximum dilution causing weak agglutination.

Absorption Test Using Coated Latex Particles

A piece of stained fabric (0.5 to 5 mg of dried blood) or of muscle fragments (10 to 100 mg) was dipped into 0.2 ml of an antiserum reagent (agglutinating titer = 1:8) in a tube and then allowed to stand for 2 h at room temperature. The absorbed antiserum was titrated to determine the decrease in titer.

TABLE 1—*The latex particles coating methods.*

1 volume of 1% latex particles in glycine buffer
+
1 volume of 0.3 mg/ml human IgG in glycine buffer
Leave for 30 min at room temperature
+
1/30 volume of 30% bovine serum albumin
Leave for two days at 4°C
Centrifuge for 10 min at 5000 rpm
Discard supernatant
Resuspend precipitated latex particles into two volumes of glycine buffer containing 0.1% bovine serum albumin

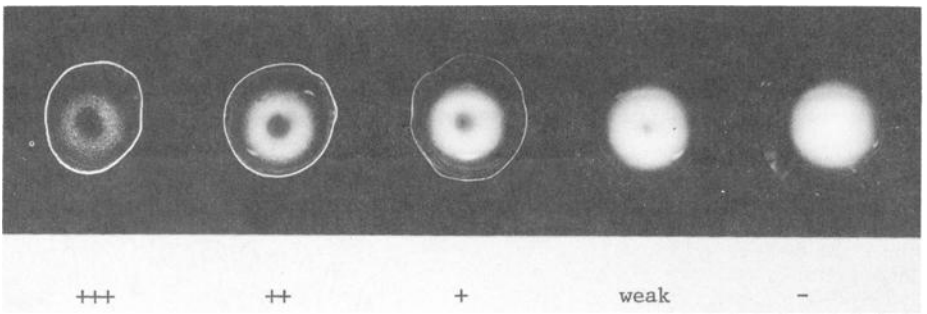

FIG. 1—*Degree of latex particle agglutination.*

Methods for Preliminary Studies

In the latex particle agglutination test, an antiserum to human IgG was titrated by different latex particle reagents that had been coated by the method shown in Table 1 except that a 1:100 dilution of sera was used instead of human IgG. In the inhibition test, one drop of an antiserum for human IgG (agglutinating titer = 1:8) was mixed with equal quantities of serially diluted serum samples of human or animal sera on a well slide. After 2 h at room temperature, one drop of the human IgG–latex particles suspension was added to the absorbed mixture. Results were read by the method described above, and the maximum dilution causing complete inhibition was determined to be the inhibition titer.

Results

Preliminary Studies

Two methods for the identification of human serum were compared. One was the agglutination of latex particles coated with human or animal sera, and the other was the inhibition of antibody by serially diluted human or animal sera. Table 2 compares the results obtained by these two methods using the same lot number of rabbit antiserum to human IgG. Cross-reaction occurred with the latex particle agglutination tests using horse and rat sera, whereas the cross-absorption phenomenon was not found in the inhibition of antibody tests using horse, rat, chicken, or rabbit sera. Repeated experiments using four other rabbit anti-human IgG sera showed similar results.

Species Identification of Bloodstains by Absorption Test

The anti-human IgG was titrated after it had been absorbed by the bloodstains to measure the reduction in the anti-human IgG agglutinating capacity. Table 3 shows

TABLE 2—*Comparison of two methods: agglutination of serum-coated latex particles and inhibition of latex particle agglutination.*

Methods	Agglutination or Inhibition Titers by Sera of				
	Human	Horse	Rat	Chicken	Rabbit
Latex particle agglutination	2560	160	40	below 10	below 10
Inhibition of latex particle agglutination	6400	below 10	below 10	below 10	below 10

TABLE 3—*Decrease of anti-human IgG agglutinating activity after absorption with human and animal bloodstains (1 to 20 months after preparation).*

Bloodstains		Dilutions of Absorbed Anti-Human IgG					
Species	Quantity, mg	1	2	4	8	16	Buffer
Control (unstained fabrics)	...	+++	++	++	+	−	−
Human (RF negative)	1.12	−	−	−	−	−	−
Human (RF positive)	1.12	−	−	−	−	−	−
Monkey (*Macaca irus*)	1.20	++	+	weak	−	−	−
Monkey (*Macaca mulatta*)	1.12	++	+	weak	−	−	−
Dog	1.24	+++	++	+	weak	−	−
Cat	1.14	+++	++	+	weak	−	−
Goat	1.20	+++	++	++	weak	−	−
Rabbit	1.12	+++	++	++	weak	−	−
Rat	1.20	+++	++	++	weak	−	−
Mouse	1.40	+++	++	+	weak	−	−
Guinea pig	1.35	+++	++	++	weak	−	−
Chicken	1.36	+++	++	++	weak	−	−

the results obtained with human and animal bloodstains. The absorption by human bloodstains completely removed the anti-human IgG agglutinating capacity, but absorption by animal bloodstains did not. No nonspecific agglutination was observed throughout these tests, and the rheumatoid factor (RF) in a human bloodstain did not agglutinate the suspension of human IgG-latex particles. Small quantities (such as 0.05 mg) of freshly dried human blood completely absorbed the anti-human IgG of titer 1:8 (Table 4). After 8 and 40 months, the quantity of bloodstain required for complete absorption of the anti-human IgG increased to 0.33 and 0.55 mg, respectively.

After storage for one year at 4°C, the indicator suspension of human IgG-latex particles neither caused nonspecific agglutination nor showed decreased sensitivity toward the anti-human IgG.

The absorption test gave positive results on unfixed bloodstains. Approximately equal amounts of bloodstains were required for unfixed stains and for those fixed by acetone treatment or heating; after methanol fixation, however, at least six times as much was necessary to obtain positive results.

The absorption test required a very small quantity (0.3 to 1 μl) of anti-human IgG because some potent anti-human IgG rabbit immune sera could be diluted 160 to 640 times to provide the solution of 1:8 titer.

To ascertain the utility of the absorption technique, anti-rabbit serum protein reagent prepared in goats was absorbed by bloodstains of various species, and then the antiserum was titrated by using latex particles coated with rabbit serum proteins. As shown in Table 5, a rabbit bloodstain completely absorbed the agglutinating capacity, whereas the other bloodstains did not. Similar experiments using anti-chicken serum protein reagent prepared in rabbits and latex particles coated with chicken serum proteins proved that only a chicken bloodstain completely absorbed the agglutinating capacity of the antiserum.

Species Identification of Muscles by Absorption Test

Table 6 shows the degree of reduction of anti-human IgG reagent (titer = 1:8, 0.2 ml) absorbed by about 50 mg of muscle fragments from human and various animal sources. Complete loss of the antibody activity was brought about only by human muscles, and the tests with monkey muscles showed partial inhibition. The chimpanzee muscles did not completely absorb the anti-human IgG even though at least 200 mg was used.

TABLE 4—*Absorption test using human bloodstains in different amounts and periods of preservation.*

Quantity, mg	Periods of Preservation	Dilutions of Absorbed Anti-Human IgG					
		1	2	4	8	16	Buffer
Control (unstained gauze)	...	+++	++	++	+	−	−
0.05	1 day	−	−	−	−	−	−
0.55	1 day	−	−	−	−	−	−
0.11	8 months	weak	−	−	−	−	−
0.33	8 months	−	−	−	−	−	−
0.11	20 months	weak	−	−	−	−	−
0.33	20 months	weak	−	−	−	−	−
0.55	20 months	−	−	−	−	−	−
0.11	40 months	weak	−	−	−	−	−
0.33	40 months	weak	−	−	−	−	−
0.55	40 months	−	−	−	−	−	−

TABLE 5—*Decrease in agglutinating activity of anti-rabbit serum protein reagent after absorption by human and animal bloodstains.*

Bloodstains		Dilutions of Absorbed Anti-Rabbit Serum					
Species	Quantity, mg	1	2	4	8	16	Buffer
Control (unstained fabrics)	...	+++	++	++	+	−	−
Human	1.12	+++	++	+	weak	−	−
Monkey (*Macaca irus*)	1.20	+++	++	+	weak	−	−
Dog	1.24	+++	++	+	weak	−	−
Cat	1.14	+++	++	+	weak	−	−
Goat	1.20	+++	++	+	weak	−	−
Rabbit	1.12	−	−	−	−	−	−
Rat	1.20	+++	++	++	weak	−	−
Mouse	1.40	+++	++	+	weak	−	−
Guinea pig	1.35	+++	++	+	weak	−	−
Chicken	1.36	+++	++	+	weak	−	−

Another series of experiments using anti-animal serum proteins and human or animal muscles was also performed. When rabbit antiserum to bovine serum proteins was absorbed by muscles of human or animal origin, the activity of the antibody reagent was completely absorbed only by bovine muscles (Table 7). Similar experiments using anti-rabbit serum proteins showed complete absorption of antibody activity by rabbit muscles, whereas the titer of the antiserum was scarcely decreased after absorption by muscles from other species, including man.

Discussion

The preliminary experiments of this study indicated that the direct reaction of antisera to human serum proteins and to latex particles coated with serum proteins caused cross-reactions. The latex particle agglutination-inhibition reaction, despite the use of such cross-reactive antisera, showed highly specific results. It has been demonstrated that antibody activity was completely neutralized by absorption by corresponding serum proteins, but the absorption of antibodies by noncorresponding serum proteins was in-

TABLE 6—*Decrease of anti-human IgG agglutinating activity after absorption by human and animal muscles.*

Muscles	Dilutions of Absorbed Anti-Human IgG					
	1	2	4	8	16	Buffer
Human	−	−	−	−	−	−
Chimpanzee	+	weak	−	−	−	−
Crab-eating macaque (*Macaca irus*)	++	+	−	−	−	−
Savannah monkey (*Cercopithecus aethiops*)	++	+	−	−	−	−
Patas monkey (*Erythrocebus patas*)	++	+	−	−	−	−
White-throated capuchin monkey (*Cebus capucinus*)	++	+	−	−	−	−
Spider monkey	++	+	−	−	−	−
Tupaia (*Tupaia glis*)	++	+	−	−	−	−
Cattle	+++	++	+	−	−	−
Cat	+++	++	+	−	−	−
Goat	+++	++	+	weak	−	−
Rabbit	+++	++	+	weak	−	−
Mouse	+++	++	+	−	−	−
Chicken	+++	++	+	weak	−	−
Bluefin tuna	+++	++	+	+	−	−
Control (unabsorbed anti-human IgG)	+++	++	++	+	−	−

TABLE 7—*Decrease in agglutinating activity of anti-bovine serum protein reagent after absorption by human and animal muscles.*

Muscles	Dilutions of Absorbed Anti-Bovine Serum					
	1	2	4	8	16	Buffer
Human	+++	++	+	−	−	−
Monkey (*Macaca irus*)	+++	++	+	−	−	−
Cattle	−	−	−	−	−	−
Cat	+++	++	+	−	−	−
Goat	+++	++	+	−	−	−
Rabbit	+++	++	+	−	−	−
Mouse	+++	++	+	−	−	−
Chicken	+++	++	+	−	−	−
Bluefin tuna	+++	++	++	+	−	−
Control (unabsorbed anti-bovine serum)	+++	++	++	+	−	−

complete. Indicator latex particles were agglutinated if some of the antibodies remained unabsorbed; accordingly, noncorresponding serum samples showed no inhibitory effect in the agglutination-inhibition test.

For practical applications, a simplified technique, the absorption test, was designed; it also caused specific absorption. Human bloodstains and muscles could be differentiated from the samples of some monkeys, including chimpanzee.

Besides excellent specificity, the absorption test has other advantages such as sensitivity, small consumption of antisera, and simplicity. The simplicity was brought about partly by the long-lasting stability of indicator latex particles.

Fixation of bloodstains by organic solvent has some practical advantages. Test samples contaminated with oily substances could be cleaned by washing with acetone. This treatment did not alter the bloodstains' antigenicity for the absorption test.

Summary

This paper reported the species identification of bloodstains and muscles by means of an absorption test in which antisera were absorbed by bloodstains or muscles and then the absorbed antibody capacity was titrated by using latex particles coated with corresponding serum proteins. This test method was proved to be simple, specific, and sensitive.

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