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A simple and reliable method to blood type monkeys using serum samples

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Summary

Monkeys are frequently used in experimental transplantation research because of their physical traits and availability. As ABO incompatibility may result in humoral injury, it is important to identify the ABO blood typing of monkeys before transplantation. However, monkeys lack expression of ABH antigens on red blood cells, which makes accurate determination of the blood type difficult. The gel agglutination assay has been widely used as a routine blood grouping test clinically for more than 10 years. In this study, we evaluated the efficacy and the interference factors of using the gel system (including the direct gel system and the reverse gel system) for ABO typing in rhesus monkeys ($n = 38$) and cynomolgus monkeys ($n = 26$). Immunohistochemistry assay was used to obtain the accurate blood type data of monkeys. The results revealed that the direct gel system was ineffective in blood typing of monkeys, whereas the reverse gel system assay, which is based on preabsorbed serum, provided reproducible results that were confirmed by histologic analysis. We conclude that the reverse gel system assay with use of preabsorbed serum is a simple and reliable method for ABO typing of monkeys.

Introduction

In man, the oligosaccharide antigens under the control of the ABO genetic system and their precursor, the H structure, are present on red blood cells (RBCs), vascular endothelium, and exocrine secretions [1,2]. These structures serve as important blood group antigens that are involved in incompatible blood transfusions and organ transplantations.

Like humans, non human primates (including anthropoid apes, New- and Old-World monkeys) also express the ABH specificities of the ABO histo-blood group system on vascular endothelium, epithelial cells, and exocrine secretions. However, these ABH antigens are absent or only weakly expressed on the erythrocytes of Old and New World monkeys [3]. Macaques, a typical representative of Old World monkeys, are frequently used in experimental transplantation research because of their physical traits and availability. As the sera of macaques regularly contain natural anti-A and anti-B antibodies directed against antigen(s) absent from the animal's body secretions [3], the A and/or B antigens expressed on vascular endothelial cells can be a target for the host reaction of immune rejection, and may form a transplantation barrier [3–5].

The ABH carbohydrate barrier must be avoided or clearly identified during experimental transplantation; hence, knowledge of the ABO blood type of these monkeys is essential. Non expression of A or B antigen on monkey RBCs makes accurate determination of blood type difficult. Using the fact that monkeys secrete ABH substances, the saliva hemagglutination inhibition assay has been previously widely used, but it is complex, expensive and time consuming, and can only be performed in

relatively few laboratories [6,7]. Another reliable method to ABO type monkeys is by immunohistochemical staining of tissues, but it is limited because of the trauma associated with organ biopsy.

The detection of serum anti-A and anti-B antibodies would represent an easy and convenient way to blood type monkeys, but some traditional attempts failed because of the frequent occurrence of confusing results, which might be caused by some interference factors. In this study, by using different serum samples, a method of the gel system, which has been widely applied in clinics, was evaluated systemically for ABO grouping of rhesus and cynomolgus monkeys.

Materials and methods

Animals

Outbred male rhesus monkeys (Macaca mulatto) ($n = 38$) and cynomolgus monkeys (Macaca fascicularis) ($n = 26$), ranging in age between 2 and 5 years, were obtained from Kunming Institute of Zoology of Chinese Academy of Sciences, Kunming, China and Guangzhou Landao Biotechnology Corporation & South China Primates Research Center, Guanzhou, China. Monkeys were housed in the primate facility at the Experimental Animal Center of Tongji Medical College according to the University's Research Animal Resources guideline. All animals were prepared for other kidney transplant research.

Sample preparations

Venous blood was collected from monkeys into ethylene diamine tetra-acetic acid (EDTA)-containing tubes for preparing plasma and into tubes without anticoagulants for serum before any immunoprogram. One milliliter of sera, 1 ml of plasma and 1 ml of fresh RBCs were collected from each monkey.

Normal renal tissues were obtained from monkeys that were used as recipients in life-supporting kidney transplantation experiments (both native kidneys were removed during the surgery). The tissue samples were fixed in 10% buffered formalin. Tissue and section processing followed general recommendations.

Immunohistochemistry

In order to accurately determine the blood type of monkeys, A/B antigen was detected by immunohistochemical staining of formalin-fixed and paraffin-embedded kidney sections. Briefly, after deparaffinization and rehydration, slides were incubated in 3% hydrogen peroxide for 10 min, PBS containing 10% of goat serum for 30 min and with a primary monoclonal antibody diluent (Changchun Institute of Biological Products, Jilin, CHN) specific for anti-A or -B antigen (overnight incubation at 4 °C). After washing, sections were stained with a secondary biotinylated anti-mouse antibody (Vector, Burlington, Ontario, CA, USA) (room temperature; 30 min), followed by streptavidin-peroxidase (DakoCytomation, Glostrup, Denmark) (room temperature; 30 min). Subsequently the slides were incubated in DAB chromogen for 5 min at room temperature. Then the sections were counterstained with hematoxylin, and coverslipped.

Gel test method

To evaluate the efficacy and the interference factors of the gel system for determining ABO typing of monkeys, both the direct test and the reverse test were performed. For the reverse gel system, results from the plasma, non-preabsorbed sera, and preabsorbed serum samples were compared and evaluated. The gel agglutination assay was performed using commercially available six-well gel cards (DiaMed-ID Micro Typing System, Cressier FR, Switzerland), where anti-A, anti-B and anti-D were predispensed in three of the chambers for direct ABO typing (Fig. 1a), and no reagents were predispensed in the last three of the chambers for reverse ABO typing (Fig. 1b). Gel testing was performed according to the manufacturer's instructions. RBCs from monkey specimens were suspended at a concentration of 5% in diluent (DiaMed Diluent-2). Human A_1, B and O reagent RBCs (Shanghai Blood/Biomedical Co., Ltd, Shanghai, CHN) were centrifuged and resuspended in diluent to a level of approximately 0.8% suspension.

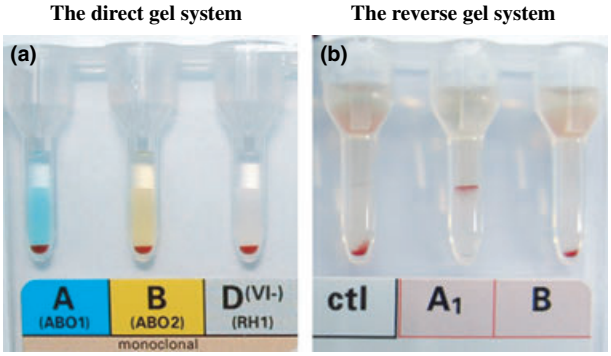

Figure 1 The gel card used for ABO typing. (a) The direct gel system. The first three columns are used for the direct ABO typing (anti-A, anti-B and anti-D are predispensed). (b) The reverse gel system. The last three columns are used for the reverse ABO typing without predispensed reagents in the chambers. The A1 microtube in this card showed a typical positive result, the others showed typical negative results.

For direct ABO typing, 10 μl of monkey specimen RBC suspension were pipetted into the reaction chamber of the A, B or D microtube respectively (Fig. 1a). For reverse ABO typing, a volume of 50 μl of reverse A_1 or B reagent RBC suspension and 50 μl of EDTA-stabilized plasma was pipetted into the appropriate reaction chambers. As a control, reverse O reagent RBC suspension and EDTA-stabilized plasma was pipetted into the 'ctl' column in the same proportion (Fig. 1b). After 15 min of incubation at 37 °C, the samples were centrifuged for 10 min using the preset cycle of the centrifuges supplied by DiaMed. Following centrifugation, the cards were examined for agglutination and/or hemolysis. Agglutinated RBCs became trapped in or above the gel and unagglutinated RBCs moved through the microtube and formed a pellet at the bottom (Fig. 1). Positive reactions were graded from 1+ to 4+ according to the manufacturer's instructions. But as a control, positive reactions were not expected in the 'ctl' column, for the reverse O reagent RBC did not express A or B antigen.

Further tests were performed on specimens with false positive results, which were determined when positive reactions occurred in the 'ctl' microtube. In these tests, modifications were made to the reverse gel test. Clear serum without any depositions was used instead of plasma. Additionally, to eliminate non specific binding from anti-human heteroagglutinins, serum samples were preabsorbed on human type O RBCs [8]. To do this, 200 μl concentrated O RBCs were resuspended with 200 μl of serum to be typed, and incubated at 37 °C for 30 min. After incubation, the RBC/serum samples were centrifuged at 900 *g* for 5 min at 4 °C. The absorbed serum was aliquotted into a new tube, avoiding any transfer of RBCs.

Results

ABO typing by immunohistochemistry

Histo-blood group A/B antigens present on the renal interstitium and glomeruli were determined from kidney samples of all monkeys by immunohistochemistry assay, yielding an accurate determination of the ABO phenotype of these animals (Fig. 2). Blood groups B and AB were represented in 74% and 16%, respectively, of the 38 rhesus monkeys that were typed, blood groups A and O were both rare. In the 26 cynomolgus monkeys typed, there was a relatively equal representation of blood types A, B, and AB (23%, 42%, and 35% respectively), blood type O was not found among these monkeys (Table 1).

ABO typing by the direct gel test

With the direct gel test, positive reaction was not found in any of the monkeys typed. All results were exactly the same as shown in Fig. 1a.

ABO typing by the reverse gel test

Using plasma samples, false positive reactions occurred in samples from all monkeys (Table 2), which showed serious false positive reactions in the 'ctl' tubes, and false results in the A_1 or B tubes of the reverse test side as well (Fig. 3).

However, when clear serum samples were used, false positive reactions were significantly attenuated. In 32 of 38 rhesus monkeys, ABO group typing was correctly identified by the reverse gel system as compared with the results from immunohistochemistry assay (Table 2). However, false positive reactions still existed in six rhesus

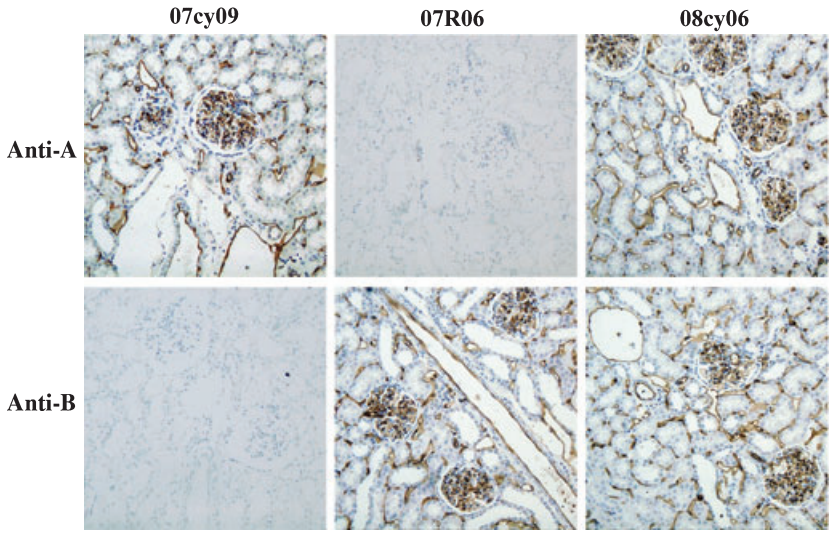

Figure 2 Kidney sections were stained with mouse anti-human A and B monoclonal antibodies, followed by a biotinylated secondary antibody and streptavidin-peroxidase. The chromagen used was DAB (brown) and tissues were counterstained with hematoxylin (blue). Cynomolgus monkey 07cy02, rhesus monkey 07R03 and 07R09 showed positive staining for the A, B and A & B antigen in the interstitium and glomerulus of the kidney, and therefore of blood type A, B and AB respectively (magnification ×200).

Table 1. ABO types of monkeys by immunohistochemical staining.

ABO group type	Rhesus monkeys (n = 38)	Cynomolgus monkeys (n = 26)
A	1 (2.6)	6 (23.1)
B	28 (73.7)	11 (42.3)
AB	6 (15.8)	9 (34.6)
O	3 (7.9)	0 (0)

Values are given as n (%).

monkeys and all 26 cynomolgus monkeys, but the intensity was much weaker than that with plasma samples (Table 2, Fig. 3).

When serum samples were preabsorbed on human type O RBCs, none of the 'ctl' tubes showed false positive reactions, and the results from the A_1 or B tubes were very clear and definitive without any interference (Fig. 3). By this method, the results of ABO blood type determination of all monkeys were consistent with the results obtained from immunohistochemistry assay exactly (Table 2).

Discussion

The gel agglutination assay was approved by the Food and Drug Administration since 1994 for the detection of A, B, and D and for ABO serum grouping, indirect anti-globulin tests, and direct anti-globulin tests [9]. Gel agglutination technology is based on controlled centrifugation of red cells through dextran acrylamide gel contained within microtubes of the cards. During

Table 2. Outcome of the reverse gel test using different kinds of samples.

	Rhesus monkeys (n = 38)			Cynomolgus monkeys (n = 26)		
Samples	Correct	Incorrect or confusing	Coincidence* (%)	Correct	Incorrect or confusing	Coincidence* (%)
Plasma	0	38	0	0	26	0
Serum	32	6	84.2	0	26	0
Preabsorbed serum	38	0	100	26	0	100

*Compared with the results of immunohistochemical assay.

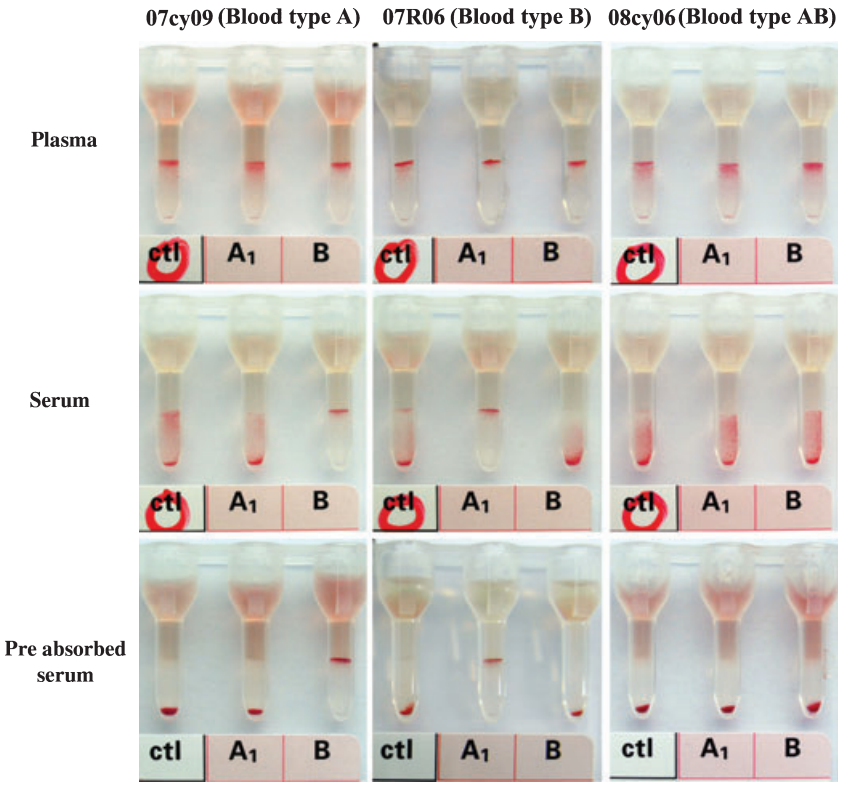

Figure 3 ABO typing by the reverse gel tests with plasma, sera or preabsorbed sera in monkeys with representative ABO groups. With plasma or sera, false positive reactions occurred in the 'ctl' tubes, so the results in the A1 and B tubes were not reliable. With preabsorbed sera, the results were clear: Cynomolgus monkey 08cy09 showed positive for the anti-B antibody in the serum, and therefore of blood type A; Rhesus monkey 07R06 showed positive for the anti-A antibody in the serum, and therefore of blood type B; Cynomolgus monkey 08cy06 showed both negative for the anti-A and anti-B antibody in the serum, and therefore of blood type AB. The determination was consistent with the results of immunohistochemistry assay.

centrifugation, the movement of the agglutinated RBCs is blocked whereas the unagglutinated RBCs pellet at the bottom of the tube. The reactions in the gel test are well defined and stable for hours, which allow a simple and reliable reading. Small sample volumes, standardized performance, technical ease, amenable for ready automation, and increased biosafety; all these factors have made this technology advantageous [10]. Therefore, in many blood centers and transfusion services, the gel assay has being widely used as a routine blood grouping test for human use [11–13]. As non expression of A or B antigen on monkey RBCs makes accurate blood type determination difficult, we evaluated the gel system for ABO typing of rhesus and cynomolgus monkeys in this study.

In the direct gel system, the microtube contains a mixture of gel and specific reagent (anti-A, -B, -D). Such gels are useful for antigen determination. The test RBCs are dispensed on the gel, and centrifuged. Contacting with the specific reagent, the RBCs will be agglutinated and trapped in positive reactions; in negative reactions, they will pellet to the bottom of the tube [10]. The negative results of the direct gel system assay obtained from this study confirmed nonexpression of the A or B antigens on monkey RBCs, which indicates that the direct gel test is not possible to be used in blood typing of rhesus and cynomolgus monkeys.

In the reverse gel system, the gel does not contain any specific reagent, but acts only by its property of trapping agglutinates. The mixture of reagent RBCs and unknown serum are dispensed in the upper part of the microtube above the gel. During the incubation, the A_1 or B reagent RBCs are agglutinated by the anti-A and/or anti-B antibodies contained in the unknown serum, and the O reagent RBCs, as a control, does not agglutinate. After centrifugation under precise conditions, the negative reactions are clearly different from the positive ones [10]. As the sera of macaques regularly contain natural anti-A and anti-B antibodies directed against antigen(s) absent from the animal's bodily secretions, it is possible to use the reverse gel test to blood type monkeys.

Although both serum and plasma samples are allowed to be used for the reverse gel assay in humans according to the manufacturer's instructions, we found severe false positive reactions in the tests using plasma samples. This problem was at least partially solved when serum samples were used. Comparing the difference between plasma and serum, it is more likely that the presence of fibrinogen in plasma may interfere with the results. The fibrin polymers in plasma might inhibit the movement of RBCs or interfere with the assay in some other unknown ways. Although the false positive reaction was significantly attenuated with serum, it still existed, especially in cynomolgus monkeys. This remaining interference might be caused by non specific binding from monkey anti-human heteroagglutinins. In this study, when monkey sera were preabsorbed on human type O RBCs, the reverse gel typing system successfully gave an accurate blood type determination of the monkeys, which were exactly consistent with the results from the immunohistochemistry assay for detecting A/B antigens present on the renal interstitium and glomeruli. Although in a majority of rhesus monkeys, the reverse gel test using untreated serum could also determine ABO group exactly, probably about 15% still showed false results. In order to get reliable and stable results, preabsorbed serum is recommended to be routinely used for blood typing of rhesus monkeys.

There are four grades of positive reactions (1+ to 4+) according to the manufacturer's instructions, but only one grade (4+) of the reactive strength was observed in the present study. This finding indicated that the anti-A and/or anti-B antibodies were regularly present in monkey serum and were strong enough for determination of ABO group type by the reverse gel system assay.

Monkeys have been previously blood typed by detection of ABH antigens in the saliva after administration of pilocarpine [8,14]. This assay requires that one sedate the animal, stimulate salivation, boil the specimen to inactivate enzymes, refrigerate or freeze the sample until tested or shipped to a reference laboratory, finally leading to the performance of a lengthy assay [6,7]. Therefore, some new approaches were developed. Sandra *et al.* [7] described a fluorescent antibody technique that phenotypes the ABH substances on baboon buccal epithelial cells. Busch *et al.* [8] described recently a method of buccal mucosal cell immunohistochemistry to ABO type monkeys. By comparison, the reverse gel assay incorporates standardized pipetting of reagents and specimens and reading of agglutination reactions. The whole process is relatively independent of the skill of the manipulator. The stable reaction endpoints may be reviewed, photographed, or photocopied at a later time. Biosafety is also enhanced in the gel system. The gel technology can be implemented in laboratories of all sizes and expertise levels and lends itself well to incorporation with automated systems [9].

In conclusion, the direct gel system can not be used to blood type monkeys, but the reverse gel system assay with use of preabsorbed serum was validated to be a simple and reliable method for ABO typing of monkeys.

Authorship

SC (Shi) and GC: designed research. SC (Song), QW, JL, YX and HG: performed research. SC (Song) and GC: wrote the paper. TEI, checked the paper.

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