Role of Enzyme-Treated Cells in RBC Antibody Screening Using the Gel Test: A Study of Anti-RH1, -RH2, and -RH3 Antibodies

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The role of enzyme-treated cells (ETCs) in red blood cell (RBC) antibody screening has been the subject of controversy, and its place in the clinical routine remains to be determined. In this work, plasma samples containing anti-RH1 (anti-D; N = 10), anti-RH2 (anti-C; N = 10), or anti-RH3 (anti-E; N = 10) antibodies were studied. The samples were diluted in nonbuffered or buffered normal saline, as well as in a pool of AB plasma samples. Titers and scores were

determined by means of the gel test, using the indirect antiglobulin test (IAT) as well as ETCs, with R_0r , $r'r$, or $r''r$ test cells. Our results showed that compared to the IAT, ETCs allowed a clearer detection of anti-RH2 and anti-RH3, but not of anti-RH1 antibodies. Based on our study, it is not clear whether the ETC phase of the gel test should be maintained for RBC antibody screening. *J. Clin. Lab. Anal.* 21:61–66, 2007. © 2007 Wiley-Liss, Inc.

Key words: antibody identification; enzyme; gel test; immunohematology; laboratory medicine; papain; RH system

INTRODUCTION

The history of red cell compatibility tests was reviewed by Beck and Tilzer (1) about 10 years ago. Cross-matching has been progressively replaced by the type-and-screen procedure, which has been adopted by most large hospitals around the world. In the late 1980s, antibody screening using an indirect antiglobulin test (IAT) as well as enzyme-treated cells (ETCs) was obligatory in most countries. The role of proteolytic enzymes, such as bromelain, papain, and ficin, in detecting red blood cell (RBC) antibodies has been discussed over the years and has become a controversial (and sometimes emotional) subject. The place of ETCs was questioned in the British guidelines for pretransfusion compatibility procedures published in 1996 (2). In 1987, Contreras et al. (3) reported the lack of clinical consequences of naturally occurring anti-RH antibodies. Six years later, a cornerstone paper on enzyme-related antibodies was published by Issitt et al. (4), who demonstrated the lack of clinical significance of “enzyme-only” antibodies. In 1999, Clark et al. (5) presented evidence that routine antenatal enzyme antibody screening had no clinical value for predicting hemolytic disease in newborns. These three publications completely challenged routine practice and changed the opinions of investigators. Subsequently, enzyme-based tests were progressively abandoned by many labora-

tories, notably in the United Kingdom (6). In the last published version of the guidelines for compatibility procedures prepared by the Working Party of the British Committee for Standards in Haematology Blood Transfusion Task Force, the use of ETCs in antibody screening was neither expressly recommended nor definitively condemned (7). In contrast, the authors strongly recommended their use for antibody identification. Similar recommendations were recently published in the French literature (8). In Switzerland, until 2002 the use of ETCs for antibody screening was mandatory. Currently, tests should detect anti-RH1 concentrations of <10 ng/mL, which can readily be achieved with most modern commercial IAT technologies.

The observation of IAT- and enzyme-only anti-RH3 antibodies (Fig. 1), and the lack of clear data regarding the role of ETCs in RBC antibody screening prompted us to compare the two phases of the gel test by evaluating samples that contained anti-RH1, -RH2, or -RH3 antibodies, which accounted for about 40% of all anti-RBC antibodies identified in our laboratory.

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Fig. 1. Patterns of agglutination in three different anti-RH3 samples with the gel test (A) enzyme-only, (B) IAT-only, and (C) usual pattern, with agglutination in both phases. Positive reactions were observed only with R²R² and r''r test cells.

MATERIALS AND METHODS

Routine Antibody Screening and Identification

We performed routine antibody screening and identification using the gel test developed by Lapierre et al. (9). All reagents were obtained from DiaMed (Cressier, Switzerland), and samples were tested according to the manufacturer's recommendations. Antibody screening and identification were performed with the IAT as well as with two-stage papain-treated RBCs.

Blood Samples

EDTA plasma samples containing low-titer antibodies detectable in IAT with anti-RH1 (N = 10), -RH2 (N = 10), or -RH3 (N = 10) were prospectively collected and stored at -40°C . After the samples were thawed, doubling dilutions were performed using nonbuffered 0.15 M NaCl, buffered ($\text{pH } 7.3 \pm 0.2$) normal saline (Immusol; Medion Diagnostics, Düringen, Switzerland), or a pool of blood group AB plasma samples without alloantibodies.

Titers, Scores, and Strengths of the Reaction of the Last Positive Dilution in IAT

R₀r, r'r, and r''r untreated cells and two-stage papain-treated RBCs were used to determine the titers and scores of the 30 antibodies selected for the study. Doubling dilutions of plasma samples (1/1, 1/2, 1/4, 1/8, 1/32, etc.) were assigned an absolute number (1, 2, 3, 4, 5, etc.), and scores were determined according to the sum of the strengths of the reactions (Fig. 2) observed at all plasma dilutions that showed a positive result.

Finally, the strengths of the reaction of the last positive dilution in the IAT were compared with those of the corresponding dilution in ETCs. All of these tests were performed with the same lots of reagents.

Statistical Analysis

A one-way repeated-measures analysis of variance (ANOVA), paired *t*-test, and Wilcoxon signed-rank test were used as appropriate.

RESULTS

Effect of the Milieu on Titers, Scores, and Strengths of the Reaction of the Last Positive Dilution in the IAT

None of the pairwise multiple-comparison procedures (Dunn's method) showed a significant difference due to the milieu on the titers determined by the IAT. By contrast, the titers determined in ETCs were slightly higher when performed with samples diluted in AB plasma ($P < 0.05$) compared to those determined using nonbuffered or buffered saline. No significant differences were observed between titers determined in nonbuffered and buffered NaCl. The same statistical observations were made when the scores were evaluated (Fig. 3).

Titers, Scores, and Strengths of Agglutination at the Last Positive Dilution in the IAT

In the following section, the means of the values obtained for each sample in nonbuffered saline, buffered

Fig. 2. Strengths of agglutination for antibody scoring in the gel test.

Fig. 3. Effect of the milieu on the scores of 30 different anti-RH antibodies determined with IAT (A) or ETC (B). Statistical analysis (all pairwise multiple-comparison procedures) revealed that the scores measured in AB plasma were significantly higher ($P < 0.05$) than those obtained in 0.15 M NaCl or buffered normal saline (Immosol).

saline, and the AB plasma pool are used for data comparison.

For all antibodies ($N = 30$) tested, significant differences were observed between the means of the titers (paired t -test: $P < 0.001$; Fig. 4A) as well as between the mean of the scores (paired t -test: $P < 0.001$; Fig. 4B) when IAT was compared with ETC. A significant difference was also observed when the means of the strengths of agglutinations at the last positive dilution in IAT were compared with those observed at the same dilution in ETC (paired t -test: $P < 0.001$; Fig. 4C). Of note, in three of 30 cases the strengths of the reaction observed in ETC were equal to or lower than those observed in IAT.

Anti-RH-1, Anti-RH-2, and Anti-RH-3

When the analyses were made according to the specificity of the antibodies, different results were obtained, as shown in Figs. 5–7. For anti-RH1, the mean of the titers evaluated in IAT did not differ from that obtained in ETC (Wilcoxon signed-rank test; $P = 0.519$, Fig. 5A). Five samples were characterized by titers in ETC that were lower than or equal to those obtained in IAT. In contrast, the mean of the scores was statistically significantly higher in ETC (Wilcoxon signed-rank test; $P = 0.027$, Fig. 5B). Only one sample was characterized by a lower score in ITC (Figs. 5A and 8). The strengths of the agglutination at the last positive

Fig. 4. Titers (A), scores (B), and strengths of agglutination at the last positive dilution in IAT (C) of 30 different anti-RH antibodies. Each individual value corresponds to the mean of values obtained in 0.15 M NaCl, buffered normal saline, and AB plasma determined with IAT or ETC. For all three parameters, statistically significant differences were observed (paired *t*-tests).

Fig. 5. Titers (A), scores (B), and strengths of agglutination at the last positive dilution in IAT (C) of 10 anti-RH1 samples. Each individual value corresponds to the mean of values obtained in 0.15 M NaCl, buffered normal saline, and AB plasma determined with IAT or ETC. Statistically significant differences were observed only for the scores (Wilcoxon signed-rank test).

Fig. 6. Titers (A), scores (B), and strengths of agglutination at the last positive dilution in IAT (C) of 10 anti-RH2 samples. Each individual value corresponds to the mean of values obtained in 0.15 M NaCl, buffered normal saline, and AB plasma determined with IAT or ETC. For all three parameters, statistically significant differences were observed (paired *t*-tests).

dilution in IAT were not statistically different from those in ETC (Wilcoxon signed-rank test; $P = 0.077$, Fig. 5C). For anti-RH2 and anti-RH3 antibodies (Figs. 7 and 8), statistically significant differences (paired *t*-tests) were observed for the three parameters (titers: $P < 0.001$ and $P = 0.02$, respectively; scores: $P < 0.001$ and $P = 0.001$, respectively; strengths of the last positive

reaction in IAT: $P < 0.001$ and $P = 0.002$, respectively). One out of 10 anti-RH2 samples was characterized by a titer that was identical in ETC and IAT; all of the others showed higher titers with ETC. Two samples that contained anti-RH3 were characterized by titers in ETC that were lower than or equal to those obtained in IAT.

Fig. 7. Titers (A), scores (B), and strengths of agglutination at the last positive dilution in IAT (C) of 10 anti-RH3 samples. Each individual value corresponds to the mean of values obtained in 0.15 M NaCl, buffered normal saline, and AB plasma determined with IAT or ETC. For all three parameters, statistically significant differences were observed (paired *t*-tests).

Fig. 8. Appearance of the agglutinations of doubling dilutions of a sample containing an anti-RH1 characterized by a higher titer and a higher score in IAT compared to the values obtained using ETC.

DISCUSSION

To compare the efficiency of the IAT and ETC phases of the gel test to detect anti-RH1, anti-RH2, and anti-RH3 antibodies, we studied doubling dilutions of samples to evaluate 1) the influence of the milieu, 2) the titers, 3) the scores, and 4) the strengths of agglutination in IAT and ETC at the last positive dilution in IAT. Anti-RH1, anti-RH2, and anti-RH3 antibodies were chosen because there are very frequently observed, and because they are claimed to be better detected in ETC. In comparison with dilution in saline (buffered or not), dilution of samples in AB plasma may have some enhancing effects on the scores of the anti-RH antibodies tested in ETC, but not in IAT. The AB plasma samples were diluted in order to maintain the protein concentration and thus preserve the zeta potential. This influence was not significant in IAT, but clearly influenced the strength of the agglutinations in ETC. The antibody titers were generally but not systematically higher in ETC than in IAT. Indeed, when compared with titers determined in IAT, seven of 30

samples gave values similar to or lower than those obtained in ETC. It is therefore incorrect to claim that enzyme-based tests are more sensitive for detecting anti-RH antibodies. This was true for only 76.7% of the antibodies tested in our series. However, except for one sample, the scores were higher in ETC than in IAT, indicating that most anti-RH1, -RH2, or -RH3 antibodies are enhanced in enzyme-based tests. Finally, when the last positive dilution in IAT was taken into account, the mean of the strengths of agglutination was clearly stronger in ETC than in IAT. In summary, compared to IAT, ETC allowed a clearer but not necessarily better detection of anti-RH2 and anti-RH3, but not anti-RH1 antibodies when studied by the gel test. Based on these results, it is not clear whether the ETC phase of the gel test should be maintained or not.

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