Allogeneic adsorptions: a comparison of the traditional method with a modified PEG adsorption method

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The purpose of this study is to demonstrate the benefits of enhancing adsorptions with PEG. Allogeneic adsorptions were performed on 20 patient samples containing warm reactive autoantibodies with two volumes of adsorbing RBCs; results using unenhanced adsorptions were compared with those using PEG-enhanced adsorptions and with using untreated adsorbing RBCs and ficin-treated adsorbing RBCs. Two volumes of adsorbing RBCs, one volume of serum, and one volume of PEG were used. The number of adsorptions, average time saved, and presence or absence of underlying alloantibodies were compared for the two methods and types of adsorbing RBCs. Modified PEG-enhanced adsorptions resulted in a 69 percent decrease in adsorbing time. PEG adsorptions removed all autoantibodies and detected 18 of 19 underlying alloantibodies. The unenhanced method did not remove autoantibodies in two samples and identified only 15 of 19 underlying alloantibodies. As expected, reductions in the number of adsorptions and adsorbing time were observed. The modified-PEG adsorption is an improved method that may replace the current unenhanced method. ***Immunohematology* 2010;26:104–108.**

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Patients with warm-reacting autoantibodies who demonstrate immune hemolysis are generally symptomatic with critically low hemoglobin levels, requiring urgent transfusion to treat their clinical symptoms. The evaluation of these patients' samples requires adsorption procedures, which are labor-intensive and time-consuming. PEG is added to the adsorption mixture to decrease the number of adsorptions performed, shorten the length of the adsorbing procedure, and facilitate complete removal of the autoantibody.[1] PEG is a water-soluble polymer that enhances antibody interaction by excluding water molecules during antigen and antibody interactions, allowing for a closer proximity of antibodies to antigens.[2] PEG can be an irritant in routine testing because it enhances reactivity of autoantibodies. However, in the adsorption procedure, it is helpful by because it enhances the adsorption of autoantibody onto the adsorbing RBCs.[3] The purpose of this study is to investigate whether double-volume allogeneic adsorptions performed with either untreated or enzyme-treated (ficin) allogeneic adsorbing RBCs using PEG as an enhancement are more efficient and more effective in removing autoantibody, while leaving alloantibody in the absorbed serum, than adsorptions performed without PEG.

Previous PEG adsorption studies used a testing ratio of one volume of serum, one volume of adsorbing RBCs, and one volume of PEG. The 1:1:1 (serum to RBCs to PEG) ratio has led to questionable underlying alloantibody recovery and the inability to completely remove the autoantibody in some cases. Although the PEG adsorption method requires less time than the established reference method, time saved must be optimized while maintaining the integrity of the test and the ability to identify clinically significant alloantibodies.

Prior studies performed using untreated, ZZAP, or ficin-treated adsorbing RBCs with PEG enhancement show promising results. Leger and Garratty[4] studied 39 samples in which all alloantibodies after PEG adsorption exhibited reactivity greater than or equal to that observed in the ZZAP method. The alloantibodies recovered in their study included antibodies from all of the major blood groups. Barron and Brown[3] had mixed results with respect to alloantibody recovery. In their study, three alloantibodies were identified in the enzyme methods that were not identified in the PEG method; however, one antibody was found in the PEG method that was not found in the enzyme method. The authors also found that five of the antibodies reacted more strongly after PEG adsorptions, although it seems very weak alloantibodies may have been lost with the PEG method. Judd and Dake[5] found 7 of 12 samples had decreased alloantibody reactivity, notably attributed to immunoglobulin precipitation observed when using PEG. The authors of both of these studies commented that their observations were inconsistent with other studies.

Alloantibody recovery can only be measured if the autoantibody is completely removed. Liew and Duncan[1] reported complete autoantibody removal in all six samples tested. In their study of 16 samples, Cheng et al.[6] reported 3 samples in which the adsorptions did not remove all autoantibody with the unenhanced method, whereas all autoantibodies were removed with the PEG-enhanced method. Both methods in the Cheng study used untreated adsorbing RBCs.[6] Judd and Dake[5] reported four samples with the PEG method and five with the ZZAP method in which the autoantibody was not completely removed. The present study has been designed considering the success and failures of other studies.

One advantage of the PEG-enhanced adsorption is the time saved in performing the enhanced adsorption

procedure compared with the unenhanced adsorption procedure. Patients exhibiting autoimmune hemolytic anemia can have severely low hematocrits and exhibit life-threatening symptoms as a result of anemia. Decreasing the time required to perform the adsorption procedure will expedite the treatment of these patients. Leger and Garratty[4] saved 16.75 hours in their study of 39 patients. Barron and Brown[3] found that the PEG method averaged 30 minutes to perform, whereas the unenhanced method averaged 161.5 minutes. Cheng et al.[6] reported a 40 percent decrease in the number of adsorptions performed and an 85 percent decrease in the time needed to perform the adsorptions in a study of 16 samples using untreated adsorbing RBCs with PEG enhancement. Using double-volume adsorptions may save even more time.

Materials and Methods

Twenty patient serum or plasma samples containing warm-reactive autoantibodies were provided by a local reference laboratory. With each patient, the same type of sample (serum or plasma) was consistent for all work on each given patient. Henceforth, these samples are all referred to as serum samples. Patient samples were preferentially selected for the study to include as many underlying alloantibody specificities as possible. Eight samples with no underlying alloantibodies were included in the study as the negative control group. Because some patients have PEG-dependent autoantibodies, a LISS detection method was initially used in an effort to identify and exclude the patients with PEG-dependent autoantibodies from the study. If the patient had a current or past RBC phenotype determined, a report accompanied the sample to aid only in the selection of the adsorbing RBCs and alloantibody identification. All identifying information was removed from the samples except for the patient's historic phenotype.

The strength of the autoantibody reactivity was determined by performing an antibody detection test with LISS (NHANCE, Immucor, Norcross, GA) enhancement.[7] Reactivity strength (as with all testing performed) was graded using the 0 to 4+ scale, as described in the current edition of the AABB *Technical Manual*.[7]

The PEG, ficin, and *Glycine soja* reagents were prepared using standard methods by the participating Immunohematology Reference Laboratory (Blood Systems Laboratories, Tempe, AZ). PEG (Sigma, St. Louis, MO) and ficin (Sigma, St. Louis, MO) were prepared from powder, and G. soja (Fearn, Mequon, WI) was prepared from soja seeds. Adsorbing RBCs were prepared as untreated (washed) and ficin-treated.[7]

Unenhanced Adsorption

Two volumes of adsorbing RBCs (untreated or ficin-treated) combined with one volume of patient serum were incubated for 30 minutes at 37°C (inverted to mix 2–3 times throughout the incubation). At the end of 30 minutes, each adsorption tube was centrifuged for 10 minutes at 3500 rpm, and the serum was harvested. After the adsorption procedures, the absorbed sera were tested using LISS antiglobulin technique against phenotypically similar RBCs or the adsorbing RBCs to determine when autoantibody was completely removed, after which alloantibody detection and identification were performed.

Modified PEG-Enhanced Adsorption

Two volumes of adsorbing RBCs (untreated or ficin-treated) combined with one volume of patient serum and one volume of PEG were incubated for 10 minutes at 37°C (inverted once during the incubation to mix the contents of the tube). The adsorption tube was then centrifuged for 10 minutes at 3500 rpm, and the serum-PEG mixture was harvested. Four drops of the harvested serum-PEG mixture were then tested with one drop of phenotypically similar RBCs or with the adsorbing RBC, incubated at 37°C for 10 minutes, washed four times with saline, and then analyzed with anti-IgG reagent. Negative reactions were verified with IgG-coated RBCs. Four drops of the serum-PEG mixture were used to account for the dilution caused by adding PEG to the adsorption mixture.

Following the reference laboratory protocol, a maximum of four double-volume adsorptions were allowed to completely remove the autoantibody. If the autoantibody was not completely removed with four double-volume adsorptions, then alloantibody identification was not performed. When more than four double-volume adsorptions are performed, a dilutional effect may result (residual saline in the adsorbing RBCs that cannot be completely removed, which increases with each subsequent adsorption), and antibody detection results could be invalid.[7] The serum-PEG mixtures were analyzed the day of preparation because of immunoglobulin precipitation that has been observed on storage.[8] Interpretation of allogeneic adsorptions are limited as the procedure will remove antibodies to high-frequency antigens present on the adsorbing RBCs.

Time saved by performing fewer and shorter adsorptions was averaged for all samples tested with each method. Time required for each adsorption was determined by adding the adsorption time (30 minutes no enhancement and 10 minutes modified PEG-enhanced) to the time required to harvest the adsorbed serum (10 minutes).

Results

All of the modified PEG-enhanced adsorptions completely removed the autoantibodies, allowing for alloantibody detection and identification. In comparison, autoantibody was not completely removed after four double-volume, unenhanced adsorptions in two samples. One untreated, unenhanced adsorption did not completely remove the autoantibody, whereas the ficin-treated cells without enhancement did completely remove the autoantibody in this sample.

A total of 19 alloantibodies were identified after adsorption (Table 1). Fifteen alloantibodies (79%) were detected in the unenhanced method, and 18 alloantibodies (95%) were detected with the modified PEG-enhanced method. Three of the alloantibodies demonstrated stronger reactivity after modified PEG-enhanced adsorptions. The antibodies identified with the modified PEG-enhanced adsorptions and not with the unenhanced adsorptions all belong to the Rh blood group system (two each of anti-C and anti-E; Table 2). The antibody identified by unenhanced adsorption but not with the modified PEG-enhanced method was anti-e. An anti-C was identified with modified PEG-enhanced adsorptions, with ficin-treated adsorbing RBCs only. Not all samples included in the study contained alloantibodies to differentiate true alloantibody activity from method-sensitive autoantibody activity that can confuse the final interpretation.

Using PEG as an enhancement medium conserved time in the adsorption procedure. Unenhanced adsorptions require 40 minutes, whereas modified PEG-enhanced adsorptions require only 20 minutes. Unenhanced adsorptions averaged 85 minutes, whereas modified PEG-enhanced adsorptions averaged 27 minutes. The enhanced method saved an average of 57 minutes per sample when comparing the total time to perform both methods. The unenhanced method required a total of 54 hours, whereas the modified PEG-enhanced method required 17 hours, saving 37 hours with enhancement. Enhanced adsorptions with untreated adsorbing RBCs alone saved 19.3 hours in addition to the time saved by omitting ficin treatment of the adsorbing RBCs.

For all patient samples tested, a 2 × 2 analysis of variance was calculated. The fixed factors were type of adsorbing RBCs (ficin-treated vs. untreated), and adsorption method (modified PEG-enhanced vs. unenhanced). The two dependent variables were number of adsorptions and time required to remove autoantibodies, and these were analyzed separately (Table 3).

Table 1. Alloantibodies identified

Specimen number*	Underlying antibody identified anti-	Unenhanced adsorption		Modified PEG-enhanced adsorption	
		# of adsorptions	Reaction strength	# of adsorptions	Reaction strength
2	e C	2	2+ Not detected	1	3+ 1+†
4	E S	2	1–2+ 3+	1	1–2+ 3+
6	E Jk^b S	2	3+ 1+ W+	1	3+ 2+ W+
7	E	2	3+	1	3+
9	K	2	2+	1	2+
10	E S K	2	1+ W+ 2–3+	2	1+ W+ 3+
13	E	2	Not detected	1	1+
14	K e	3	2+ 1+	4	2+ Not detected
15	C	1	1+	2	2+
16	K	2	1+	2	1+
18	C	2	Not detected	1	1+
19	E	3	Not detected	1	W+

*Eight samples with no underlying alloantibodies identified.
†Identified with serum adsorbed with ficin-treated adsorbing RBCs. For all other antibodies, results were equivalent for ficin-treated and untreated adsorbing RBCs.

Table 2. Alloantibodies not detected with one of the methods

Specimen number	Underlying antibody identified anti-	Unenhanced adsorption method		Modified PEG-enhanced adsorption method	
		Strength of reactivity	#Ads	Strength of reactivity	#Ads
2	e C*	2+ Not detected	2	3+ 1+	1
13	E	Not detected	2	1+	1
14	K e	2+ 1+	3	2+ Not detected	4
18	C	Not detected	2	1+	1
19	E	Not detected	2–4	W+	1

*Detected with modified PEG-enhanced adsorptions performed with ficin-treated adsorbing RBCs only. In all other specimens, the unenhanced method required two adsorptions and the PEG-enhanced method required only one adsorption, and the antibodies reacted stronger than those antibodies missed in these specimens.

Table 3. Mean number of adsorptions and time to perform adsorptions by experimental condition

Conditions	Number of adsorptions*	Mean time per adsorption (min)*	Estimated total time for 20 samples (hours)
Modified PEG-enhanced method			
Ficin-treated (n =19)	1.33 (0.21)	27.27 (4.96)	
Untreated (n = 20)	1.34 (0.22)	26.67 (4.74)	
Average PEG enhanced	1.35 (0.15)	26.97 (3.43)	8.99
Unenhanced method			
Ficin-treated (n =19)	2.12 (0.26)	85.00 (5.81)	
Untreated (n = 20)	2.11 (0.24)	84.44 (5.47)	
Average unenhanced method	2.12 (0.18)	84.72 (3.99)	28.24

*Standard deviation in parentheses.

Allogeneic adsorptions with ficin-treated RBCs versus untreated RBCs by an unenhanced method resulted in no significant differences for number of required adsorptions (F [1, 74] = 0.32; p = 0.57) and time required to remove autoantibodies (F [1, 73] = 0.20; p = 0.66). In contrast, the modified PEG-enhanced adsorption method resulted in significantly fewer adsorptions (mean = 1.31, SD = 0.73) than the unenhanced method (mean = 2.12, SD = 0.44; F [1, 74] = 36.22; p = 0.000), and the modified PEG-enhanced method required less time in minutes on average (mean = 26.97, SD = 3.43) than the unenhanced method (mean = 84.72, SD = 3.99), a significant mean difference (F [1, 73] = 324.78; p = 0.000). The interaction between type of adsorbing RBCs and adsorption method was not statistically significant, and did not support the hypothesis that combining two enhancements (ficin-treated RBCs and PEG) would lead to better results. Ficin treating the adsorbing RBCs only made adsorbing cell selection easier.

The total samples tested included those with underlying alloantibodies identified (n = 12) and those with no alloantibodies detected (n = 8). To study those with identified alloantibodies separately, similar analyses were performed. The same pattern of findings in the subsample was noted. The mean number of adsorptions was significantly lower for the PEG-enhanced method (mean = 1.35, SD = 0.15) compared with the unenhanced method (mean = 2.12, SD = 0.18; F [1,36] = 5.77; p = 0.002), and the average time was less for the PEG-enhanced method (mean = 26.97, SD = 3.43) than with the unenhanced method (mean = 84.72, SD = 3.99; F [1,36] = 120.58; p = 0.000). The comparisons of types of adsorbing RBCs (ficin-treated and untreated) were not significant.

Discussion

For the purpose of this study, all methods used two volumes of RBCs with one volume of serum and one volume of PEG (2:1:1) to encourage complete autoantibody adsorption and to allow subsequent testing of the absorbed serum. This ratio provides more available antigen sites to bind with the autoantibody. Additionally, the use of untreated versus ficin-treated adsorbing RBCs was evaluated with and without PEG-enhanced adsorptions.

The modified PEG-enhanced procedure improved allogeneic adsorption procedures by increasing autoantibody adsorption, decreasing the number of adsorptions performed, and subsequently allowing detection of underlying alloantibodies with stronger post enhanced adsorption alloantibody reactivity. Using two volumes of adsorbing RBCs in combination with the PEG enhancement was comparable only to the Liew and Duncan[1] method by completely removing the autoantibody, allowing antibody detection in all cases.

Previous work regarding enhanced adsorptions is difficult to compare with this study because of the variability of methods and individual technique observed between the studies. The range of variables includes incubation time, untreated RBCs, chemicals used for treatment of adsorbing RBCs (ficin, papain, ZZAP), and the proportions of testing materials (1:1:1 as opposed to 2:1:1 in the current study). In this study, contrary to that of Barron and Brown,[3] weak alloantibodies were recovered with PEG adsorptions. The results of this study agree with previous works by Leger and Garraty[4] and Cheng et al.,[6] but with even increased effectiveness in antibody removal with the utilization of two volumes of adsorbing RBCs. All PEG-adsorbed samples were tested for alloantibodies because the autoantibodies were removed. This is an improvement from the Judd and Dake[5] model.

Antibody detection was possible and the antibody reactivity was stronger in the modified PEG-enhanced method. The four antibodies not detected by the unenhanced method and the one antibody not detected by the modified PEG-enhanced method appear as more weakly reacting antibodies among those detected. Perhaps this is explained by the difference in the number of adsorptions required between the two methods for these samples. The variability between the modified PEG-enhanced and unenhanced adsorptions in regard to antibody detection may be attributable in part to the fact that PEG is often more sensitive in antibody detection than LISS.

Patients with warm autoantibodies have a mean alloimmunization rate of 32 percent.9 Maley et al.[9] showed an average underlying alloantibody rate of 30 to 40 percent, and their population exhibited 12 percent containing multiple alloantibodies. The 20 samples for our study were preferentially selected to include as many alloantibodies as possible in the project timeline. Thus, had the samples all been randomly selected, one would have expected fewer samples to have had underlying alloantibodies. This was not meant to be a true representation of the number of patients exhibiting warm autoantibodies with underlying alloantibodies.

Neither a moderate enhancement nor a synergistic effect was observed when using the two enhancement methods together (ficin-treated RBCs and PEG).

In conclusion, modified PEG-enhanced adsorptions maintained their sensitivity for antibody detection and identification while at the same time reducing the number of adsorptions and the time required to perform the adsorptions, not to mention the added benefit of reagent and labor cost savings. Although the two methods were not statistically different in regard to antibody detection, the clinical difference is potentially quite large in that the modified PEG-enhanced method allowed the detection and identification of four additional antibodies known to be clinically significant. Using the modified PEG-enhanced method to perform allogeneic adsorptions with two volumes of RBCs will expedite the availability of appropriate blood for patients presenting with warm reactive autoantibodies.

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