Use of Gel Microcolumn Assay for the Detection of Drug-Induced Positive Direct Antiglobulin Tests

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Drugs can result in broad variety of hematologic abnormalities including positive direct antiglobulin test. In this study, we evaluated gel microcolumn assay for the detection of drug-induced antibodies. Direct antiglobulin test was performed by conventional tube and by gel microcolumn assay in 139 hospitalized patients. Drug in vitro studies were done in 34 patients with positive direct antiglobulin test by tube test and gel microcolumn assay using serum and eluate. None of them had signs of hemolytic anemia. A total of 1,000 blood samples from donors were used as control group. Gel microcolumn assay was more sensitive than in tube test for direct

antiglobulin test ($P < 0.01$). Positive direct antiglobulin test was more frequent in patients than in donors ($P < 0.01$). Drug in vitro studies were positive with at least one drug in 76.5% of patients with positive direct antiglobulin test by immune complex and/or adsorption mechanisms. We found a high incidence of positive drug in vitro tests in positive direct antiglobulin test patients. Gel microcolumn assay showed appropriate results for drug in vitro studies. The combination of tube and gel microcolumn assay can improve detection of drug-induced positive direct antiglobulin tests. *J. Clin. Lab. Anal.* 19:219–227, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Drugs sometimes can cause a broad variety of hematological abnormalities, including positive direct antiglobulin tests and immune hemolytic anemia (1). Many drugs have already been described as causing positive direct antiglobulin test (2–4). Drug-induced antibody formation either against drug itself or against intrinsic red cell antigens it is not uncommon (5). Approximately 30% of fatal blood dyscrasias are secondary to drug administration but, only occasionally patients will develop immune hemolytic anemia (6). Although rare, drug-induced immune hemolytic anemia can be severe, sometimes accompanied by vascular collapse, renal failure, and a fatal outcome (7–9). Recently, a cohort study demonstrated an association between blood dyscrasias and antibiotics, especially cephalosporins (10). The majority of drug-induced immune hemolytic anemia are case reports, and cephalosporins (second- and third-generation) are by far the most common cause of drug-induced immune hemolytic anemia (11–14). When a positive direct

antiglobulin test is suspected to be drug-induced, adequate in vitro tests must be done and the physician must be informed of this hypothesis because the cause is not always obvious, especially if the drug was given prophylactically for surgery (15).

Direct antiglobulin test can be performed by tube, gel microcolumn assay, column agglutination technology, enzyme-linked antiglobulin test and other techniques (16,17). Gel microcolumn assay is a modified serological technique that has been used for ABO and Rh typing, direct antiglobulin test, detecting antibodies, red cell phenotyping and other applications (18,19). This technique has been found to be sensitive and easy to

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perform and may allow reliable detection of antigen-antibody reactions (20,21). It can also be used with monospecific reagents such as IgA, IgM, IgG, and C3d providing a direct antiglobulin test profile that can be useful in many serological investigations (16).

Salama et al. (22) compared the conventional tube test to gel microcolumn assay for drug antibody detection, but they only used drug antibodies that were initially detected by conventional tube test for comparison, therefore there was no possibility of finding gel only antibodies.

Difficulties in investigating drug antibodies arise because many drugs or metabolites may elicit positive results on a direct antiglobulin test by one or more mechanisms (23); however, only some drugs can cause hemolytic anemia (24).

The purpose of this study was to compare drug-induced direct antiglobulin tests and drug in vitro studies performed with tube agglutination and gel microcolumn assays.

MATERIALS AND METHODS

Study Design

This investigation, a prospective study, consisted of three parts. In part 1, peripheral blood samples were tested by gel microcolumn assay and conventional tube test technique for direct antiglobulin test simultaneously. The EDTA samples used in both techniques were tested within 12 hr of specimen collection. All samples were drawn while the patients were receiving the drug(s). Patients' samples obtained from all patients admitted at Hematology Unit at Clinicas Hospital, University of São Paulo School of Medicine and Brigadeiro Hospital were studied prospectively. All of these patients were onco-hematological patients. Blood samples from 1,000 consecutive donors at our blood donor center were used as control group. The blood donors included in this denied taking any drug or herbal drugs in the last thirty days prior the donation. This study was approved by the Ethical Committee of Clinicas Hospital, Brigadeiro Hospital and, Fundação Pro-Sangue. Written informed consent was obtained from all patients and donors enrolled. The patient and donor demographics are given in Table 1.

In Part 2 of this study, extra EDTA blood samples of all direct antiglobulin test positive results were collected for retesting, for elution studies and for immunoglobulin and complement fraction detection test. Clot blood samples were collected for unexpected antibody screening testing by gel microcolumn assay and conventional tube test of all patients and donors in which a positive direct antiglobulin test

TABLE 1. Patient and donor demographic data

	Donors (n = 1,000)		<i>P</i>	Patients (n = 139)		<i>P</i>
	Positive DAT	Negative DAT		Positive DAT	Negative DAT	
SEX						
Male	3	710		18	59	
Female	4	283		23	39	
TOTAL	7	993	0.10	41	98	0.05
Age (years)						
Mean	35.4	30		50.5	44.3	
Median	37	27	0.13	50	43	0.21

DAT, direct antiglobulin test.

was found. Antibody identification was performed by gel microcolumn assay and conventional tube test whenever an unexpected antibody screening was positive.

Part 3 of this study compared the results of antibodies detection to drugs by gel microcolumn assay and conventional tube test. The use of red cell panel enzyme-treated and not treated, and time of incubation was also evaluated.

Patients with positive direct antiglobulin test due to autoimmune hemolytic anemia, hypergammaglobulinemia and cold agglutinins were excluded from this study prior to sampling whenever these information were available from the patient's history or from medical records. The study group was composed only by patients with positive direct antiglobulin test of unknown etiology and in use of drugs. None of them had signs of hemolysis. All 139 patients were in use of drugs at the moment the blood sample had been collected. Although no patient presented any clinical or laboratory sign of hemolysis at the time this investigation was conducted, whenever we have detected a positive DAT due to drugs it was informed to the patient's physician and discontinued whenever possible.

METHODS

Direct Antiglobulin Tests in Patients and Donors

The direct antiglobulin test were performed in conventional tube test using polyespecific antiglobulin reagent (monoclonal rabbit anti-human IgG and anti-C3d; DiaMed Latino America, Lagoa Santa, Brazil) and anti-IgG (monoclonal rabbit anti-human IgG, DiaMed, Latino America, Lagoa Santa, Brazil) and 6% bovine albumin as a control (25). Control red blood cells coated with IgG (Controcell, Biotest S/A, Itapeirica Daserra,

Brazil) were tested with each batch of patient samples. The direct antiglobulin tests by gel microcolumn assay were performed with LISS-Coombs cards (monoclonal rabbit anti-human IgG and anti-C3d; DiaMed Latino America, Logoa Santa, Brazil) as described elsewhere (26). Monospecific anti-IgG, -IgA, -IgM, -C3c, and -C3d ID-cards (DC-Screening I; DiaMed, Cressier Sur-Murat, Switzerland) were used in all positive direct antiglobulin tests results samples to detect immunoglobulin and/or complement fraction involved. Positive reactions were graded from 1+ to 4+ according to manufacturer's instruction (27).

Elution Studies

Elution studies were performed by using commercial acid elution kits (28) in all positive direct antiglobulin tests patients and donors samples (28,29).

Unexpected antibody screening and identification

Unexpected antibody screening and identification were performed using serum and eluate by conventional tube test and gel microcolumn assay in all positive direct antiglobulin test patients' and donors samples with the purpose to investigate one cause of positive DATs. For conventional tube test antibody screening and identification were performed using polyethylene glycol (PEG) in indirect antiglobulin test as described elsewhere (25) red blood cells reagents for antibody screening and identification were obtained from Gamma Biologicals, Inc. (Duet Plus[®], Panel Two[®], Houston, TX). Anti-IgG antiglobulin reagent was purchased from DiaMed (DiaMed Latino America, Logoa Santa, Brazil). All negative antiglobulin tests were verified with IgG-coated red blood cells (DiaMed Latino America, Logoa Santa, Brazil).

For gel microcolumn assay antibody screening and identification red blood cells reagents were obtained from DiaMed Latin America, Logoa Santa, Brazil (Diacell[®] and Diapanel[®]). Antibody detection and identification in gel microcolumn assay were performed as described elsewhere using LISS/Coombs Cards (DiaMed Latin America, Logoa Santa, Brazil) (21).

Drug In Vitro Tests

Drugs in vitro tests were performed using eluate and patient's serum for the detection of antibodies to drugs and for ex vivo demonstration of drug/anti-drug complexes by conventional tube test (25,30) and gel microcolumn assay. Drugs that are given intravenously were obtained from the pharmacies in the powder form in order to prepare desired concentration for drug in vitro studies. When drugs were formulated in tablets or capsules, incubation at 37°C was necessary to their

complete dissolution. Control red blood cells were harvested from a pool of five voluntary blood donors group O, with negative direct antiglobulin tests results and compatible with the patient's serum. Bromelin treated red blood cells were prepared by two-stage enzyme technique, using Bromelin (Microcolumn Assay; Sigma Chemical Co., St. Louis, MO). Red blood cell suspensions for conventional tube test were prepared in saline solution at 5% and for gel microcolumn assay, in Diluent 2 (DiaMed Latino America, Brazil) at 0.8%. ID-NaCl/Enzyme Cards and LISS/COOMBS Cards (DiaMed Latino America, Logoa Santa, Brazil) were used for gel microcolumn assay with bromelin-treated and untreated red blood cells respectively. Fresh normal sera with less than 6 hr of collection were used as source of complement.

Detection of Drug-Induced Antibodies by Adsorption Mechanism (AD), Modified from Brecher (25)

Drug-treated red blood cells were prepared by incubating 10 volumes of drug (penicillin 60-mg-per-mL in phosphate buffered saline (PBS) pH 7.3; cephalosporins 40-mg-per-mL in PBS pH 6.0; ranitidine and erythromycin 30-mg-per-mL in PBS pH 7.3; dipyrone 12.5-mg-per-mL in PBS pH 7.3) and added to 1 volume of washed, packed O red blood cells for 1 hr at 37°C, except for penicillin and erythromycin that were incubated for 1 hr at room temperature. After the incubation, the tubes were washed 4x in saline solution. For conventional tube test, 50 µL of a 5% drug-coated red blood cells suspension in saline was added to 100 µL of serum or eluate. In parallel, 50 µL of a 5% untreated red blood cells suspension from the same donor was added to 100 µL of serum or eluate as negative control. The tubes were incubated for 1 hr at 37°C, centrifuged and examined macroscopically for agglutination and or hemolysis. The results were tested by the indirect antiglobulin test (IAT) using commercial anti-IgG human globulin serum. After centrifugation the results were graded and recorded. For gel-test, 50 µL of a 0.8% drug-treated red blood cells in Diluent 2 (DiaMed Latino America, Logoa Santa, Brazil) and 25 µL of serum or eluate were dispensed in LISS/COOMBS Cards (DiaMed Latino America, Logoa Santa, Brazil). Cards were incubated at 37°C for 1 hr followed by centrifugation for 10 min. Untreated red blood cells suspension at 0.8% in Diluent 2 from same donor was tested in parallel as control-negative. The results were considered positive whenever reactivity (agglutination and/or hemolysis) was observed of serum and/or eluate with drug-treated red blood cells but not with untreated red blood cells.

Testing by the Immune Complex Method (IC), Modified from Brecher (25)

Drugs were diluted in a 1-mg-per-mL solution in PBS pH 7.3. Four sets of test mixtures were prepared, two for bromelin-treated red blood cells suspension (one for reading after 2 hrs incubation at 37°C, and other after 12 hrs incubation), and two for non-bromelin treated red blood cells suspension (one for reading after 2 hrs incubation at 37°C, and other after 12 hrs incubation). Each set consisted of five test mixtures: tube 1 - patient's serum plus drug; tube 2-patient's serum plus normal serum plus drug; tube 3-patient's serum plus normal serum plus PBS pH 7.3; tube 4-normal serum plus drug; tube 5: normal serum plus PBS pH 7.3. For all sets in conventional tube test we used 100 µL of patient's serum, 100 µL of drug, 100 µL of normal serum and 50 µL of red blood cells suspension at 5%. After incubation and examination for agglutination and hemolysis, red blood cells were washed in PBS and tested by the IAT using polyespecific AGS reagents. For gel microcolumn assay the sets of test mixtures were prepared using 50 µL of red blood cells suspension at 0.8% and 50 µL of patient's serum, normal serum or drug. After incubation at 37°C for 2 hr all cards were centrifuged and examined for agglutination or hemolysis. Reactivity observed in any of tests containing patient's serum to which the drug was added, and absence of reactivity in the corresponding control tests indicated a drug/anti-drug interaction. Whenever negative results were observed after incubation at 37°C for 2 hr, incubation time was extended to 12–18 hr in order to detect possible weak antibody-antigen reactions. In eluate, the same procedure was performed by conventional tube test and gel microcolumn assay using non-bromelin treated red blood cells and reading after 2 hr incubation at 37°C.

Ex Vivo Demonstration of Drug/Anti-Drug Complexes

Ex vivo demonstration of drug/anti-drug complexes was performed in one patient in use of diclofenac, because the preceding methods results were negative. Clotted blood samples and urine specimens were collected from a volunteer (one of us, CRS), immediately before and 1 hr after taking diclofenac (25,31,32). The urine specimens, neutralized at pH 7.0, were used as the source of drug metabolite. Two sets of test mixtures were prepared, one for bromelin-treated red blood cells suspension, and one for non-bromelin-treated red blood cells suspension for conventional tube test and for gel microcolumn assay. Each set consisted of six test mixtures: tube 1: test serum plus volunteer serum or urine; tube 2: test serum plus volunteer serum or urine plus; tube 3: normal serum plus volunteer serum or

urine; tube 4: test serum plus PBS pH 7.3; tube 5: serum test plus normal serum; tube 6: complement plus PBS pH 7.3. For all sets in conventional tube test, 100 µL of patient's serum, 100 µL of diclofenac and 50 µL of red blood cells suspension at 5% were used. After incubation for 2 hrs at 37°C, all tubes were centrifuged and examined for agglutination and/or hemolysis. The red blood cells were washed four times in 0.9% saline, followed by an antiglobulin test performed using 100 µL of anti-IgG reagent. For gel microcolumn assay, 25 µL of each test mixture and control was dispensed to the top of the microtubes (LISS/COOMBS Cards) and added 50 µL of bromelin treated red blood cells in each microtube for one set and 50 µL of non-bromelin treated red blood cells to each microtube for another set. After incubation at 37°C for 2 hrs and centrifugation, the cards were examined for agglutination and/or hemolysis. Reactivity in any of the tubes and/or cards containing test serum and volunteer serum or volunteer urine, and absence of reactivity in all control tubes indicated an antibody against a metabolite of diclofenac.

STATISTICS

All statistical calculations were performed using software (SAS Institute, Inc., Cary, NC). Results were expressed as mean $\pm$ SD. Data were analyzed by chi-square test 2-by-2 with Yate's correction, t-Student test and Fisher exact test, where applicable. A *P* value less than 0.05 was considered significant (33).

RESULTS

Patient and donor demographic and data are presented on Table 1.

One hundred and thirty-nine patients were enrolled in this study. The study group was made of 62 females and 77 males. Females patient's proportion with positive direct antiglobulin test was higher than males proportion (*P* = 0.05). Patient's age varied from 14 to 85 years old in patients and from 18 to 60 years old in donors. We found no statistical significant difference between direct antiglobulin test positive and negative results in patients according to age (*P* = 0.21).

In our study, direct antiglobulin test was found positive in 41 samples (29.5%) and remained positive when retested. Of these, only 9 (6.5%) showed positive direct antiglobulin test by conventional tube test, *P* < 0.01. All 41 DAT positive blood samples in patients were positive by gel microcolumn assay.

IgG was detected in all direct antiglobulin test positive samples in this study. Complement fraction (C3d) was associated to IgG in only 2 (5%) patients.

Unexpected antibody screening was positive in 3 (7.3%) positive direct antiglobulin test patients.

Anti-Fy^a, anti-C^w, anti-K1 were identified by gel microcolumn assay and conventional tube test. These patients presented negative elution test and were included in this study. Autocontrols were performed for the samples with positive antibody screens, positive DATs and negative elutions.

The results were the same for the first samples and the subsequent samples.

Direct Antiglobulin Test Results in Blood Donors

Positive direct antiglobulin test was found in seven blood donors (0.7%). All of them were positive by gel microcolumn assay and only one of these was also positive by conventional tube test (0.1%), ($P = 0.01$). No positive direct antiglobulin test was solely found by conventional tube test. Donor's age mean was 35.4 years (median = 37) for positive direct antiglobulin test and 30 (median = 27) for negative results, with no statistical significant difference ($P = 0.13$). All direct antiglobulin test positive results in donors were due to IgG and eluate was non-reactive.

When direct antiglobulin test positive results in patients and blood donors were compared, a statistical significant difference was found, ($P < 0.01$).

Drug In Vitro Tests Results in Patients

Drug in vitro tests were performed in 34 of 41 direct antiglobulin test positive results (mean = 9.1 tests/patient) using eluate and serum (Table 2). It was not possible to further investigate seven patients (4 deaths, 2 early discharges and 1 undetermined auto-IgG, indistinguishable from autoimmune hemolytic anemia caused by warm antibodies). Drug in vitro tests were positive in 26 (76.5%) of 34 patients, with at least one drug, in at least one of tests performed. Drug in vitro studies were found positive with more than one drug for the same patient in 13 (38.2%). Of these, in four cases (11.7%) occurred between chemically related drugs

(penicillin's and cephalosporins). We performed 65 laboratory tests to demonstrate immune complex formation involving drugs and 42 for detection of drug-induced antibodies by adsorption mechanism in eluate (Tables 3 and 4). Eighty-six drug studies in vitro were performed by adsorption and 118 by immune complex mechanisms using serum (Tables 5 and 6). Ex vivo demonstration of drug/anti-drug complexes was performed in one patient who was using sodium diclofenac and other drugs by conventional tube test and gel microcolumn assay in serum and urine as ex vivo source of diclofenac, giving both positive results.

Comparison Between Tube and Gel Test

We did not find statistical significant difference between conventional tube test and gel microcolumn assay for drug in vitro studies using eluate ($P = 0.31$) and serum ($P = 0.15$) by adsorption mechanism (Tables 4 and 6), and using serum by immune complex mechanism ($P = 0.06$) (Table 5).

Conventional tube test method showed to be more sensitive than gel test ($P = 0.03$) for drug in vitro studies by immune complex mechanism using eluate (Table 3).

We used enzyme treated and non-enzyme treated RBCs for drug in vitro studies by immune complex mechanism, both incubated for 2 hr and overnight. In 118 drug in vitro studies by conventional tube test we found 14 (11.9%) positive results using enzyme treated red blood cells, and 11 (9.3%) with non-treated red blood cells, ($P = 0.69$). Of drug in vitro studies by gel microcolumn assay, only one (0.8%) was positive with enzyme-treated red blood cells and 15 (12.7%) showed positive results using non-treated red blood cells, ($P < 0.01$).

Finally, incubation time was found to be relevant in investigating drug-induced direct antiglobulin test. Most of drug in vitro studies by immune complex mechanism showed positive results after overnight incubation for both methods ($P < 0.01$) (Fig. 1).

TABLE 2. Comparison of tube and gel test for complete drug *in vitro* studies

Sample	Mechanism	Number of tests	Positive results					
			Tube only		Gel only		Tube+Gel	
			<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Eluate	IC	65	4	6.1	0	0.0	5	7.7
	AD	42	3	7.1	1	2.4	0	0.0
	Total	107	7	6.5	1	0.9	5	4.7
Serum	IC	118	17	14.4	8	6.8	8	6.8
	AD	86	0	0.0	2	2.3	1	1.2
	Total	204	17	8.3	10	4.9	9	4.4

IC, circulating immune complex mechanism; AD, adsorption mechanism; *n*, number of positive studies results.

TABLE 3. Results of drug *in vitro* studies in eluate by immune complex mechanism

Drugs	Number of tests	Positive results			Total
		Tube only	Gel only	Tube+ Gel	
Dipyron	12	1	0	0	1
Ranitidine	12	0	0	0	0
Ceftazidime	11	3	0	0	3
Amphotericin B	5	0	0	5	5
Acetaminophen	6	0	0	0	0
Ceftriaxone	4	0	0	0	0
Sulfonamides	4	0	0	0	0
Furosemide	3	0	0	0	0
Cefpirome	2	0	0	0	0
Meropenem	1	0	0	0	0
Diclofenac	1	0	0	0	0
Penicillin G	1	0	0	0	0
Ampicillin	1	0	0	0	0
Naproxen	1	0	0	0	0
Aspirin	1	0	0	0	0
Total	65	4	0	5	9

$P=0.03$.

TABLE 4. Results of drug *in vitro* studies in eluate by adsorption mechanism

Drugs	Number of tests	Positive results			Total
		Tube only	Gel only	Tube+ Gel	
Dipyron	13	0	0	0	0
Ceftazidime	12	1	1	0	2
Ranitidine	7	1	0	0	1
Ceftriaxone	4	0	0	0	0
Cefpirome	3	0	0	0	0
Meropenem	1	0	0	0	0
Penicillin G	1	1	0	0	1
Ampicillin	1	0	0	0	0
TOTAL	42	3	1	0	4

$P=0.31$.

DISCUSSION

The incidence of positive direct antiglobulin test varies from 3.5% to 8% in hospitalized patients using conventional tube test (34). Our results by conventional tube test (6.5%) are similar to those observed by Freedman (35).

It has been reported that the incidence of positive direct antiglobulin test in healthy individuals increases with aging (36). This phenomenon has not been entirely clarified until the moment. In our series, there was no statistical significant difference in age for patients and blood donors with direct antiglobulin test positive

TABLE 5. Results of drug *in vitro* studies in serum by circulating immune complex mechanism

Drug	Number of tests	Positive results			Total
		Tube only	Gel only	Tube+ Gel	
Dipyron	25	1	2	1	4
Ranitidine	23	4	2	1	7
Ceftazidime	15	0	2	0	2
Amphotericin B	9	3	0	3	6
Paracetamol	5	1	0	0	1
Ceftriaxone	8	1	1	0	2
Sulfametoxazol	4	0	0	0	0
Furosemide	12	3	1	0	4
Cefpirome	3	1	0	0	1
Meropenem	1	0	0	0	0
Diclofenac	2	0	0	0	0
Penicillin	1	1	0	0	1
Ampicillin	4	0	0	2	2
Cefotaxima	1	0	0	0	0
Naproxen	2	1	0	1	2
Cefalothine	2	1	0	0	1
Alopurinol	1	0	0	0	0
Total	118	17	8	8	33

$P=0.06$.

TABLE 6. Results of drug *in vitro* studies in serum by adsorption mechanism

Drug	Number of tests	Positive results			Total
		Tube only	Gel only	Tube+ Gel	
Dipyron	28	0	0	0	0
Ranitidine	19	0	1	0	1
Ceftazidime	10	0	0	0	0
Ceftriaxone	3	0	0	0	0
Cefpirome	1	0	0	0	0
Meropenem	1	0	0	0	0
Penicillin	4	0	0	0	0
Ampicillin	1	0	0	0	0
Cefotaxime	2	0	0	0	0
Erythromycin	2	0	1	1	2
Cefalothin	15	0	0	0	0
TOTAL	86	0	2	1	3

$P=0.15$.

results (mean = 50.5 years and mean = 35.4 years) and those with negative direct antiglobulin tests (mean = 44.3 years and mean = 30 years), respectively. Consequently, age had no influence in our data.

According to sex, drug induced direct antiglobulin test positive results in this study were more frequent in females. There is no information in the literature relating the frequency of drug-induced positive direct antiglobulin test and sex, but drug-induced immune

Fig. 1. Summary of number of samples tested and positive results according to study design. DAT, direct antiglobulin test; IC, immunecomplex mechanism; AD, adsorption mechanism; Enz, enzyme-treated red blood cells; NonEnz, non-enzyme treated red blood cells; 2 h, 2-hr incubation; 12 h, 12-hr incubation.

hemolytic anemia is more frequently in females. It is not clear, if the higher incidence in females is due to genetic predisposition or different immune response or if females do take more drugs than male (37).

Direct antiglobulin test by gel microcolumn assay has been described as more sensitive than conventional tube test for newborn and adults samples (16,24,38). However, Tissot et al. (39) found that gel microcolumn assay was less sensitive than conventional tube test, especially for the detection of C3d-coated red blood cells. This apparently contradiction may reflect distinct capacities of various C3d antiserum from different manufacturers to detect C3d fraction. This may explain why gel microcolumn assay was found more sensitive than tube for drug-induced direct antiglobulin test.

In our study, all positive direct antiglobulin tests in patients were due to red blood cells-bound IgG and in only 5% was also positive for C3d, similar results to two other studies (40,41).

The incidence of positive direct antiglobulin test in donors was 0.7% by gel microcolumn assay and 0.1% by conventional tube test in our series. Although these findings are higher than those already published, 1:3,000 to 1:14,000 using conventional tube test (42,43), our sample size was smaller when compared to the number of donors studied by Worledge (65,000 blood donors) (42). Direct antiglobulin test was positive due to IgG in

all donors of our study and in 97% in the reports published by Habibi et al. (43), but contributed to 57% in Gorst et al. (34). Although positive direct antiglobulin test in apparently healthy donors has limited clinical significance, antiphospholipid antibodies and autoimmune hemolytic anemia can rarely cause it (35,44).

There is no reference in the literature on direct antiglobulin test by gel microcolumn assay in blood donors for comparison. Our results show that gel microcolumn assay is a reliable technique for direct antiglobulin test in apparently healthy donors.

We found a high incidence of positive drug studies in positive direct antiglobulin test patients' samples (76.5%). Of these 13 (53.5%) had positive test for more than one drug. Different classes of drugs were responsible for positive results such as antibiotics (especially cephalosporins), diuretics (furosemide), analgesics (dipyrone and acetaminophen) and others. Adequate selection of drugs, underlying diseases and association of techniques may justify our results. Recently, a case report of a massive hemolytic anemia after ceftriaxone in a child with no immune deficiency or hematological disease was published reflecting complex immune response after drug administration (45). In only three patients there was a chemical correlation between them (penicillins and cephalosporins) what in coincident with literature, but there are few reports about the association among non related drugs (46,47). Our finding may be partially explained by secondary immune response alteration caused by underlying disease and/or the sensitization for one drug may facilitate the production of antibodies against others drugs.

Drug-induced direct antiglobulin test can be caused by at least three different mechanisms classified in types I, II, and III. In type I, drugs (penicillins, cephalosporins) become covalently linked in vivo to red blood cells membranes glycoproteins and trigger antibody production. Hemolysis of type II (alfa-methyldopa and procainamide) is characterized by red blood cells-specific autoantibodies that reacts with red blood cells not requiring the presence of drug. A third type of drug-induced hemolytic anemia involves stimulation of antibodies that bind to red blood cells only when the drug is present in soluble form (5,48). Generally, only one mechanism is involved, but patients have been described with the combination of them (49,50).

There is only one work comparing conventional tube test and gel microcolumn assay for cell-drug (haptens) antibody complexes (22). The authors tested serum or plasma samples of patients with immune hemolytic anemia previously identified by conventional tube test. They found similar results for tube and gel test, but did not mention the use of enzyme treated cells or negative controls.

The results presented in this work show that drugs are relatively common cause of direct antiglobulin test. However, in this study, we did not find any clinical or laboratory signs of hemolysis related to drugs. As already reported, in certain situations a positive in vitro drug study does not necessarily indicates that the drug is responsible for the hemolytic picture. Inclusive some drug-induced antibodies (p.e. penicillin) have already been found in apparently healthy individuals, who have used or are using the drug, with no cytopenia, and in these cases a positive direct antiglobulin test has no clinical significance (30). Although drug-induced direct antiglobulin test investigation can be time consuming it is important to identify potential sensitizing medications and to standardize confirmatory testing in order to prevent drug-induced hemolysis (4).

In conclusion, positive drug-induced direct antiglobulin test is not uncommon. Gel microcolumn assay can also be used for drug in vitro studies, although the laboratory investigation for drug-induced direct antiglobulin tests requires association of the tube and gel test methods for best results.

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