

Testing for weak D

Two recent papers (Wagner *et al.*, 2005 [1], Gassner *et al.*, 2005 [2]) have rekindled the discussion on the immunogenicity of weak D and DEL and therefore on the minimum number of D sites on red cells required for inducing a primary or secondary immune response. The above papers strongly suggest that the frequency of donors, typed D-negative in routine testing and whose red cells are capable of inducing an anti-D immune response, is greater than suspected at present. Thus, the question remains whether donors typed D-negative in routine testing should be further typed for weak D and DEL, particularly when their red cells are to be used for girls or women of childbearing age. To obtain relevant information on this issue, the following questions were sent to specialists in the field. We obtained 17 contributions to this Forum.

Question 1: Have you seen anti-D immunizations after the transfusion of red cells from donors typed D-negative in routine testing? If so, which technique was used for D typing and has the responsible donor(s) been found to be weak D or DEL, and by which technique? If you encountered such donors, has the number of D sites on their red cells been determined?

Question 2: Which technique do you currently use for typing for RhD?

Question 3: Have you used *RHD* genotyping to detect weak D and DEL and, if so, how many D-negative donors were found to be weak D? Again, has the number of D sites on the red cells of such donors been determined?

Question 4: With regard to detecting weak D:

(a) Is it obligatory in your country/centre to retype donors found to be D-negative in routine testing, with the indirect antiglobulin test (IAT)?

(b) Because DEL and probably other very weak Ds are not detected even in the IAT, would you recommend making *RHD* genotyping mandatory? If so, of all D-negative donors, only of C- and/or E-positive D-negative donors, and only of donors whose red cells are to be transfused to girls or women of the childbearing age?

(c) A related problem is D-positive/D-negative chimeras. In many (probably most) cases, the D-positive red cell population is too small to cause problems in routine testing, but even a few millilitres of normal D-positive red cells would be enough to induce a primary immune response. Do you think that it is worthwhile to test for D chimerism and if so, which technique would you recommend, and should this technique be

recommended for all donors or only for donors whose red cells will be transfused to girls and women of childbearing age?

The answers to the questions contain a wealth of interesting information and discussion. It is impossible to include all this in an editorial. It is therefore essential to read the individual answers to appreciate the value of this Forum.

Only a few cases, i.e. a total of seven definitely proven cases of anti-D immunization in D-negative recipients induced by red cells from donors typed D-negative in routine testing, have been recorded in the 17 countries/centres. However, some of the contributors believe that there may have been unreported cases. The number of D sites on the red cells of the donor, determined in some of these cases, was very low (10–70 sites). For comments, see below.

For the first RhD typing of donors (generally two) mostly monoclonal anti-Ds are used, usually in an automated system (Olympus PK72000). More importantly, in virtually all countries/centres, the red cells of first-time donors found to be D-negative by the first typing are then tested in the indirect antiglobulin test (IAT) to detect weak D.

The percentage of donors, who, at serological typing are found to be D-positive in the IAT varies from 0.02 in Spain to 4.1% in Denmark. This percentage depends of course on the frequency of weak D (and partial D) in the population as well as on the sensitivity of the test(s) used for the first serological typing.

Typing for RhD at the DNA level has only been performed in a few centres, so far. Schönitzer (Innsbrück) *RHD*-typed 738 D-negative, but C- or E-positive samples. Eleven of them were found to be weak D or DEL. Frey and Mendez (Switzerland; this article) tested 54 donors with ambiguous results on RhD typing and found 52 to be weak D, the number of D sites on the red cells varying from 840–3592 sites. Lomas-Francis and Westhoff (USA; this article) found that the majority of discrepant 'D-negative donors' had either the Crawford or the R_0^{Har} phenotype.

On the whole, making *RHD* genotyping mandatory is not recommended at present, the main reason being that too little is known about the immunogenicity of red cells, only typed D positive by genotyping (see below). Some contributors feel that, should *RHD* genotyping be recommended, it should only be for C- or E-positive donors and/or only for donations for girls and women of childbearing age. In Poland and the Netherlands, a project has been set up to determine the frequency of donors serologically typed D-negative, C- or E-positive but found to be weak D by *RHD* typing.

D-positive/D-negative chimerism is not considered a real problem. The sensitivity of the currently used single donor *RHD* genotyping assay, especially of a quantitative PCR-based assay, would be sufficient to detect *RHD* alleles in D-positive/D-negative chimeras (de Haas *et al.*, the Netherlands). Otherwise, flow cytometry could be used but general application is not recommended unless new data about the significance of chimerism becomes known.

Comments and conclusions: There seems to be no doubt that the frequency of weak D or DEL donors, typed D-negative, even in the IAT, is greater than previously expected, as shown by Gassner *et al.* [2] and Schönitzer (Austria). Garratty (USA) calculated that in southern California alone each year the red cells from at least 120 weak D or DEL donors, typed D-negative serologically, are transfused to D-negative recipients. Yet, no cases of unexpected anti-D immunization have been recorded. Furthermore, as mentioned above, only seven cases of unexpected anti-D immunization have been seen in the vast population represented by the countries participating in this Forum. It seems therefore reasonable to conclude that red cells only found to be D-positive by *RHD* genotyping, i.e. very weak D or DEL cells with a very small number of D sites, only very rarely induce a primary anti-D immune response in D-negative recipients. It is perhaps safe to conclude that, in the rare cases that such a primary immune response occurs, it is due to hyper-responsiveness of the recipient and that such recipients are very rare. Because of these data and considerations, we could agree with the opinion of most contributors to this Forum that routine *RHD* genotyping should not be recommended, unless new data would contradict the results reported in this Forum.

However, several of the contributors are in favour of only transfusing D-, C- and E-negative red cells to D-negative recipients, certainly to girls and women of childbearing age. In fact, other red cell alloantigens (e.g. Kell, Fy^a, etc.), which are not taken into account when selecting donors for transfusion, may be much more important with regard to alloimmunization than very weak D and DEL.

Finally, in several countries/centres, genotyping for red cell alloantigens is being set up with the ultimate goal of it being used to replace serological typing. The problem of recognizing very weak D and DEL will then automatically be solved, as will the problem of D-positive/D-negative chimerism.

References

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Question 1: We have published two cases of anti-D immunization caused by transfusion of red cells initially typed D-negative but which were indeed extremely weak D variants with less than 30 D sites per red cell [1,2]. We have seen no further cases so far. However, we observed alloanti-D in several young individuals with a history of blood transfusion. According to the Austrian transfusion guidelines, D-positive red cells should not be administered to D-negative patients, and must not be given to girls and women of childbearing age. Thus, alloanti-D in young individuals may well have resulted from weak D or DEL red cell transfusions, together with other causes like unnoticed D-positive pregnancy, neglect of transfusion guidelines or potential needle sharing in intravenous drug addicts. In our experience, some unexplained anti-D immunizations were responsible for severe hemolytic disease of the fetus and newborn.

Question 2: At our institution, automated RhD typing is performed on Olympus PK7100 equipment by using polyclonal and blended monoclonal anti-D reagents as well as a combination of monoclonal anti-C, anti-D and anti-E. Recipients' samples negative with anti-D but positive with blended anti-C/anti-D/anti-E are further tested in gel matrix with polyclonal anti-D, -C, -E, -c and -e, whereas such donor samples are additionally tested with blended monoclonal anti-D in the indirect antiglobulin test in gel matrix. *Addendum:* Roughly 2–3% of donor samples typed D-negative but C- or E-positive by the Olympus PK7100 are D-positive by gel matrix IAT.

Question 3: We have not used *RHD* genotyping to discover extremely weak D variants on a routine basis. Weak D variants are identified by serology only, and are further specified by molecular analyses.

Question 4:

(a) In Austria, it is obligatory to retype apparently D-negative but C- and/or E-positive donors with anti-D in the indirect antiglobulin test.

(b) *RHD* genotyping of donors typed D-negative by serology would lead to recognition of extremely weak D variants including DEL and could therefore prevent some cases of anti-D alloimmunization. However, too few data are available

on the effective immunogenicity of extremely weak D variants. Due to reduced D antigen dose, at times combined with marked D epitope loss, the immunogenicity of extremely weak D variants may well be in the range of other blood group antigens (e.g., Fy^a, Jk^a and others) primarily disregarded in donor typing. Moreover, our data indicate that extremely weak D variants likely to be missed by routine serology are rare in Central Europe. Therefore, we currently cannot recommend molecular *RHD* typing of all donor samples nonreactive with anti-D reagents. Within the near future, it can be expected that the RH genetics will be of increasing complexity due to migration and ethnic mixture. The introduction of large-scale automated microarray technology [3] will have to meet the requirements of the changing population genetics, and promises to allow also for simple and cost-effective recognition of even the weakest D variants.

(c) D-positive/D-negative chimeras and spontaneous mosaics are most reliably recognized serologically using gel centrifugation technique. Blood centers routinely employing this comparatively expensive technique for donor typing will be at an advantage for the detection of mixed-field agglutination with anti-D reagent. Protecting D-negative girls and women of childbearing age from exposure to even small amounts of D-positive red cells is mandatory to prevent anti-D induction. Nevertheless, the frequency of RhD chimerism/mosaicism in donor populations needs to be determined to estimate the relevance of this phenomenon.

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Question 1: We have seen one anti-D immunization after the transfusion of red cells from donors typed D-negative in routine testing. This is the immunization described by Gassner

et al. 2005, mentioned in the introductory remarks of the questionnaire.

Question 2: Rh D typing is performed in duplicate in Austria. In our centre the first test is done in gel cards (DiaMed, Cressier, Switzerland) containing human antibodies, the second test is performed with liquid reagents from monoclonal sources.

Question 3: In our centre in Innsbruck we tested 738 samples serologically RhD-negative but positive for C or E. The results were the following: 1 weak D type 26, 2 weak D type 5, 1 RHD category VI type I, 5 DEL (M295I), 1 DEL (IVS3+1G > A), 1 DEL (1252T(Tins) 1253), 29 D negative RHD-CE(2-9)-D, and 1 D negative RHD-CE(4-7)-D hybrid – all among 738 phenotypes (dd, C- or E-positive).

Question 4:

(a) The Austrian guidelines for blood group serology and transfusion medicine (issued by the Austrian Association of Blood Group Serology and Transfusion Medicine) require the IAT for RhD negative samples if the antigens C and/or E are present.

(b) As we do not care about the possible exposure to other antigens (C, Cw, c, E, etc.) that could induce antibodies, at the moment, we would not recommend *RHD* genotyping

(c) D chimerism seems to be a very rare condition and its frequency is not well documented. Using our routine technique, the gel test, a second cell population becomes visible if its concentration exceeds 3% (clearly visible at 5%). The chance that an individual has two cell lineages with a concentration of a second cell population of less than 3% should be very low. The fact that RhD-positive individuals can lose their positivity over the course of several months or years, merits more consideration and interest. This observation was made in our institution in several donors and recipients respectively. The condition is currently under investigation. These individuals show temporarily two cell populations with anti-D and finally they lose their positivity completely. The condition was observed in individuals with malignancy of the haematopoietic system. From this point of view, it makes sense to look for D+/D– chimeras.

I want to emphasize that with the exception of the answer to question (4a) all answers reflect my personal view on D weak testing.

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R. Fontão-Wendel & S. Wendel

Question 1: No, we haven't seen any anti-D immunization after transfusion of red blood cells (RBC) from donors typed D-negative in our service (total of 1835 RHD-negative recipients transfused with 11 285 RHD-negative red blood cells).

Recently, there was a case report describing an anti-D alloimmunization by a weak D type 1 donor in a Brazilian patient [1]. In this report, the first donation of the donor involved was considered RHD-negative but 4 months later, when the donor returned for another donation, the donor was typed as RHD-positive (weak D). The first donation was transfused into a 76-year-old woman who has no history of previous pregnancy or blood transfusions; and because of this transfusion, she developed anti-D plus anti-C. The donor was genotyped as weak D type 1, with a low antigen density (~357 D antigen per cell), but could also be detected serologically by some polyclonal anti-Ds. According to the authors, this report elucidates the importance of controlling properly the strategy and the quality of reagents used in a routine RHD typing.

Question 2: We use microplate technique for typing blood donors. The protocol for microplate technique is the following: 1% bromelin-treated RBC suspension is incubated with the antibody using U-shaped bottoms microplate assay. After 37 °C incubation, the microplate is centrifuged and placed in a 70-degree angle to the bench-top support device. The definition of results is based on the presence (positive) or absence (negative) of a button in the middle of microplate wells.

For RHD typing, we use two different kinds of anti-D: one monoclonal (IgG + IgM, clones TH-28 and MS-26) and one commercial blend of polyclonal anti-D (human source). Both are tested simultaneously with control sera from the same manufacturer. Additionally, we also test with anti-CD (polyclonal antibody) and anti-CDE (monoclonal antibody, clones: MS-24, MS-26, MS-201; MS-80). Negative reactions by microplate are confirmed by conventional tube test (immediate spin, followed by a 37 °C incubation and IAT test). For RHD typing in patients, we use the same reagents (anti-Ds), tested only by tube (as described above).

Question 3: No. We have no data related to weak D donors detected by molecular methods whose serological results were defined as RHD-negative. However, we have detected D category patients (confirmed by genotyping techniques), initially typed as RHD-positive, who produced anti-D because of immunization by transfusion of RHD-positive donors. We consider in our centre that the occurrence of D category recipients developing anti-D is more problematic than weak D donors sensitizing RHD-negative recipients. So far, we found five patients under D category (2 D^{III}, 1 D^{VII}, and 2 yet under identification) in a total of 12 665 RHD+ recipients transfused with 79 901 red cells units (1: 2 533 recipients).

Question 4:

(a) Yes, as required by the Brazilian guidelines (www.anvisa.gov.br/sangue/legis/index.htm) [2].

(b) Although we think that the safest blood transfusion must be provided for all patients, some considerations should be raised:

- Millions of red blood cells units are transfused annually all around the world to RHD-negative patients, and so far,

this is the first case reported in the literature of an anti-D immunization by the rare phenotype DEL. Thus, the possibility of having another case looks remote.

- The implementation of new technologies with higher costs for transfusion medicine should be evaluated based on clinical evidences of effectiveness for the population under study. In countries with limited financial resources such as Brazil where the annual health expenditure per capita is around US\$700 Purchasing Power Parity (PPP; world health report) [3], the decision of genotyping all donors routinely for RHD would be highly unlikely.
- (c) The same answer applied to item *Question 4b* (above).

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Question 1: In Québec, Héma-Québec collects 250 000 units per year. No case with anti-D immunization after transfusion of D-negative red cells has been clearly documented.

Question 2: Rh(D) typing is carried out with the Olympus PK 7200 on 2% red cell suspension in 0.2% bromelin, using two different lots of potent polyclonal anti-D. When both direct D determinations are negative, the IAT (tube agglutination) is carried out with another lot of polyclonal anti-D and poly-specific anti-human globulin.

Question 3: No routine RHD genotyping is performed to detect weak D and DEL on D-negative donors.

Question 4:

(a) It is required that Rh(D) typing is carried on Olympus PK 7200 on all repeat donors. IAT (tube agglutination) is carried out only on the first donation for Rh(D)-negative donors. All Rh(D) typing results of each donor must be concordant with the preceding Rh(D) typing results.

(b) Genotyping of RHD is complex because of the high polymorphism of the gene. New mutations are identified frequently. The assay design to detect all polymorphism would be an ongoing project. We could limit the test performed to the most prevalent ones. As mentioned by Gassner *et al.*, [1] it would be appropriate to study our population genetic data in order to devise a molecular typing strategy for RHD typing.

(c) No case of RH(D) donor chimerism with anti-D immunization after transfusion has been documented in Quebec. Cases of chimerism are rare, and systematic screening on all donors for D-positive/D-negative chimeras cannot be justified at this time. RHD genotyping can efficiently detect D-positive samples and an individual diagnostic analysis of transfusion events can be performed on those rare cases.

Reference

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Question 1: We have recorded one case of development of anti-RhD in a 61-year-old female RhD-negative patient presumably caused by the transfusion of buffy coat-depleted red blood cell (RBC) suspensions typed as RhD-negative.

All the involved donors (11) had previously been tested negative for RhD in IAT.

After the immunization event, all involved donors were retyped in IAT. Fortunately, one of the donors was also tested in an antibody consumption test normally used for screening for fetomaternal hemorrhage, and this single donor was found positive. The consumption of antibodies would have corresponded to a bleeding of approximately 1 ml of packed RhD-positive RBCs with a phenotype estimated to have 10 000–20 000 D sites per RBC. If the blood volume is estimated to 2000 ml packed RBCs, the reaction corresponds to an average number of D sites of 1/2000 of the normal, i.e. 5–10 D sites per RBC. Supplementary examination of the donor in flow cytometry yielded a uniform weak reaction with several distinct anti-RhD antibodies. Genotyping for *RHD* showed the presence of a normal, intact *RHD*.

Question 2: All donors tested negative for RhD are subsequently retested in IAT using gel card technique with at least one antibody that detects the DVI variant.

Question 3: We have not used genotyping on a systematic basis for testing of serologically RhD-negative donors.

Question 4:

(a) In Denmark, it is obligatory to retype with IAT all donors found RhD-negative in routine agglutination technique.

(b) With our present appreciation of the problem, it does not seem justified to use genotyping for all D-negative donors. We are considering labelling C- or E-positive, D-negative donors as RhD-positive.

(c) All donor samples yielding mixed field reaction in routine serological testing are re-typed in flow cytometry with anti-D reagents where chimerism down to 4 RhD-positive RBCs per 10 000 RhD-negative RBCs would be readily detected. The antibody consumption test normally used for screening for fetomaternal haemorrhage would be very well suited for routine detection of RhD-positive/RhD-negative chimeras. Alternatively, *RHD* genotyping could be used. Based on our present appreciation of the problem we would not introduce a separate set of blood donors for girls and women of childbearing age.

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V. Kretschmer & R. Karger

In order to ensure a reasonable interpretation of our answers, we would like to describe the way Rh(D) testing is performed in Germany because our experiences and conclusions certainly relate to the particular procedures used.

Until 1996, German guidelines prescribed all D-negative donors to be tested for C and E, and to label D-negative, C- or E-positive donors as Rh-positive even when anti-D IAT-negative. Therefore, until 1996, D-negative recipients only received 'truly' D-negative, i.e. also DEL-negative, blood. In 1996, the guidelines were changed. Rh(D)-negatives still have to be confirmed by additional testing in the IAT, also directed against D^{VI}, e.g. by using blended reagents, but D-negative, C- or E-positive donors are now designated as Rh-negative. Red cell concentrates of those donors may be generally used for D-negative recipients, except in the case of chronic transfusion or transfusion of women of childbearing age, where all major Rhesus antigens should be considered, although this is not mandatory. Thus, the problem of immunizing patients after transfusion of DEL-positive units could only occur after 1996.

Question 1: No, we have never seen anti-D after transfusion of seemingly D-negative blood, although we always trace back the origin of the immunization by careful investigation of the pregnancy and transfusion history of our patients with anti-D. In particular, former emergency transfusions with D-positive blood can easily be identified in our electronic database, which reaches back to 1996. Such transfusions are by far the most common transfusion-related cause of anti-D immunization, because of temporary shortages of D-negative blood. To our knowledge, anti-D after transfusion of seemingly D-negative blood has never been reported in Germany so far.

Question 2: In Germany, direct agglutination with the gel technique using two different monoclonal anti-D reagents is usually employed. The anti-D reagents are required not to detect D^{VI}, at least when recipients are typed. For blood donors, it is mandatory to add an IAT if they are negative with anti-D reagents in the direct agglutination tests. We also include and recommend an auto-control in the IAT in order to prevent false-positive typing in case of red cell-coating with IgG.

Question 3: No.

Question 4:

(a) Yes (see also answer 2).

(b) We do not believe that it is necessary or advisable to perform *RHD* genotyping in D-negative blood donors for several reasons. (1) There are plenty of blood groups, against which a recipient may be immunized, that are more immunogenic than DEL. In the mentioned case report of Wagner *et al.* [1], in only one out of four transfusion episodes of DEL-positive blood development of anti-D was found. We believe it is unlikely that the anti-D was a primary immune response in this case because the anti-D could be detected as soon as 8 days after transfusion of the DEL-positive unit. Such a rapid response is very unlikely to be a primary response, in particular when the low antigenic stimulus is taken into account. In the literature, primary immunization has not been reported before 11 days after exposure to a considerably higher immunogenic stimulus [2]. (2) If we correctly interpreted the somewhat cryptic economic analysis in the article of Gassner *et al.* [3], the prevention of one anti-D immunization would cost about US\$18 500 through *RHD* genotyping. However, prevention of immunization does not necessarily involve prevention of an unfavourable clinical event. In contrast to several other antibodies, anti-D can easily and safely be detected by current screening techniques and, consequently, is only very rarely involved in delayed haemolytic transfusion reactions. Thus, anti-D is virtually only a problem in HDN. The impact and costs of *RHD* genotyping for preventing HDN have to be shown. The costs of *RHD* genotyping for prevention of an instant of HDN due to a missed DEL phenotype in a blood donor are supposed to be several times higher than in the analysis provided by Gassner *et al.* In our opinion, it would be a far more economic approach to preclude the administration of blood of D-negative, C- or E-positive donors to D-negative

patients, at least to women of childbearing age. This would hardly reduce the supply of D-negative units but would solve the discussed problem altogether.

(c) We do not believe Rh(D) chimerism to be a clinically relevant problem either. The same reasoning as outlined in answer 4b applies.

We would like to conclude our contribution with a general remark. Transfusion medicine specialists as members of the medical community ought to assume responsibility for how our limited health care resources are spent in order to provide really effective and beneficial health care for our patients (good economic practice or GEP). In our opinion, this general perspective should always be born in mind when new diagnostic or therapeutic measures are proposed.

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F. Morelati, N. Revelli & M. A. Villa

Question 1: No anti-D alloimmunization was detected at our institution in Rh(D)-negative patients transfused only with red cell units from donors typed as Rh(D)-negative in agglutination routine testing. Our laboratory does not perform the evaluation of the RhD antigen density.

Question 2:

New patients and blood donors are tested on two occasions for Rh type and phenotype respectively. The tests are done by two technicians, using two walk-away instruments (AUTOVUE Ortho-Clinical Diagnostic System, Raritan, NJ; and GALILEO, Immucor, Norcross, GA). The former and the latter devices are based on microcolumn and liquid phase technologies, respectively. As suggested by the European guidelines [1], each sample is tested in duplicate with blood grouping anti-D reagents that should not detect the D^{VI} variant in a direct agglutination method. Rh(D)-negative blood donors, babies born to mothers

considered for Rh prophylaxis, pregnant women considered for Rh prophylaxis, cord blood and stem cell donors are tested for weak D (D^u test), while this test is not mandatory for Rh(D)-negative transfusion candidates.

During night shifts, we perform a slide manual method in duplicate or a slide manual method in combination with the microcolumn method using different sources of blended anti-D. As reported by some investigators [2], our current combination of anti-D reagents reacts strongly (i.e. without antiglobulin test) with most D variants and weak Ds. Moreover, microcolumn technology gives the strongest reactions with most D weak forms. If we detect any discrepancy between the two anti-D reagents, we perform further investigations: (1) D^u test; (2) tests using a set of monoclonal commercial anti-D reagents able to detect the most frequent Rh(D) variants (Diagast, Lille cedex, France; Immucor Gamma; Diamed AG, Cressier s/Morat, Switzerland); and (3) molecular testing. Based on the combination of the results observed with the tests listed in points 1, 2 and 3 above, we determine the Rh(D) status of patients and blood donors and consider patients who need to be transfused: pregnant women, newborns and blood donors separately. Weak D in blood donors is classified as D-positive so that their units are not used for D-negative recipients. Similarly, a newborn with a weak D variant is considered Rh(D)-positive with regard to Rh prophylaxis of the mother. Conversely, a pregnant woman or woman of child-bearing age with a D variant is regarded as Rh(D)-negative.

Question 3:

The RHD genotype is performed only as a second level investigation using two commercial kits (CDE-SSP and Weak D SSP, Inno-Train, Kronberg, Germany or RH-type and partial D-type, BAgene, Lich, Germany) able to detect partial D-types including DEL antigen. As reported before, we do not perform the evaluation of the number of D sites on these samples.

Question 4:

- (a) In Italy it is obligatory to retype Rh(D)-negative donors with indirect antiglobulin test (Du test) but there are no national requirements for anti-D activity in regard to Rh variants such as DVI. The test for weak D (Du test) is performed in our facility on all Rh(D)-negative blood donors at the first donation, using a validated method based on the use of two anti-D monoclonal blended reagents, a commercial Rh control reagent as negative control and microcolumn technology.
- (b) We do not recommend making RHD genotyping mandatory. Nonetheless, very weak D with a very small number of Rh(D) sites could not be detected, as reported by Grassner *et al.* [3] and Mota *et al.* [4].
- (c) Although anti-D immunization due to chimerism represents a potential serious risk, we never detected immunization due to chimeras. Blood cell chimerism rarely occurs in humans. The phenomenon is very difficult to detect in agglutination methods and it may be inaccurately interpreted. To solve it, the development of fast and economic methods, based on flow cytometry or molecular typing, may be a

promising future perspective. Before such tests become routine for large-scale use in blood donors, a cost-benefit evaluation is necessary. In the meantime the use of chimera-typed blood donors may be appropriate for Rh(D)-negative donors, e.g. for the transfusion of girls and women of childbearing age.

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Question 1: We detected one case of anti-D immunization in a polytransfused patient analyzing the data of 2052 blood units given to 216 subjects. The patients studied had the ccdee phenotype and were transfused with RhD-negative red cells and the median follow-up was 126 days (31-844). The donor blood group was determined with the microplate agglutination method and for weak D with the IAT. At a following red cell typing, the donor was confirmed ccdee and weak D-negative by the IAT. The blood unit was given to us from another transfusion centre and no further tests were performed.

The RhD-negative blood units that carry the -C- or -E- antigens are characterized by a low immunogenic potential when transfused to a recipient with the ccdee phenotype [1]. However, in our centre, the ccdee phenotype is mainly transfused with ccdee units, taking particular care for women of childbearing age. Analysing the data of 5480 blood units given to ccdee patients, we detected 62 blood units with the phenotype Ccdee or ccdEe or CCdee. This policy is able to reduce the probability of exposure to the DEL antigen that is

known to occur in the Ccee, CcEe, or CCee phenotypes [2]. Furthermore, it solves the problem also for the most frequent weak Ds, except for the weak D type 4, which frequency is 1.30 % of all the weak Ds and for the rare type 11 [3]. The weak D type 4 carries a sufficient number of D sites to be detected by serology; the weak D type 11 instead carries a very low number of D antigens.

Question 2: We perform RHD typing using the microplate agglutination method in a fully automated system, employing the monoclonal RUM-1 and the blended antibody MS26/TH28 [4]. The weak D test is performed using the solid phase red cell adherence methodology, employing the blended MS26/TH28 antibody.

Question 3: We did not use *RHD* genotyping to detect weak D and DEL.

Question 4:

(a) In Italy it is mandatory to retype donors found to be D-negative using the IAT or methods with the same sensitivity.

(b) The policy used in our centre for RhD-negative transfusions can be considered disadvantageous when applied to other blood banks with a different organization (see answer to *Question no. 1*). Two recent reports detailed cases of anti-D immunization generated by red cells with a very low number of RhD molecules in the RBC surface. Furthermore, the use of anti-D monoclonals with low efficiency and difficulties for weak D detection can render a correct RhD typing unlikely. A suitable solution may be RHD genotyping, with an adequate evaluation of costs. In a previous report, the author suggested the RhD genotyping in all donors using pools to reduce the costs [4]. The RhD genotyping of all donors would render easy the management of blood supplies, ensuring safety in all the RhD negative transfusion including women of childbearing age.

(c) Red cell chimerism is not detected in routine blood typing when the minor cell population is lower than 5–30%. The source of red cell chimerism in humans was detected in twins [5]. This kind of information could be employed to select blood donors to submit to *RHD* genotyping and then define the possible presence of RhD chimerism.

A univocal solution for points (b) and (c) of the question may be *RHD* genotyping in all donors, in pools as suggested in a previous report [6]. The *RHD* genotyping could render easy and effective the characterization of RhD, with a unique solution capable of detecting both weak Ds and chimerism.

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Question 1: In the Netherlands, 'unexplained' anti-D immunizations are incidentally recorded, but a systemic review of the reported cases is lacking and underreporting may be likely. Before labelling an observed anti-D in a transfused patient as 'red cell transfusion-induced', it should be excluded that: (i) anti-G has been developed upon transfusion of C-positive D-negative red cell units, (ii) platelet transfusions from D-positive donors have triggered the anti-D immunization, and (iii) anti-D is passively acquired upon transfusion of a plasma product. If red cell-induced anti-D immunization is likely, the donors are called into repeat serological analysis with the indirect antiglobulin test (IAT) and, since recently, to perform *RHD* genotyping. The number of D sites on the red cells is not determined.

Question 2: For D typing, new donors are tested with an Olympus PK 7200 with two different anti-D reagents. When both reagents give negative results, an indirect antiglobulin test is performed to detect weak RhD expression. This is repeated twice (first and second donation). Donations from regular donors are tested with only one anti-D reagent with the Olympus PK 7200.

Question 3: Indeed the two recent papers of Wagner *et al.* and Gassner *et al.* re-opened the discussion on the immunogenicity of weak D or DEL phenotypes. The frequency of donors with a weak D or DEL phenotype may be considerable [1,2]. In the Netherlands, this year, at all four divisions of Sanquin Blood Supply, we will start a study to evaluate the risk of anti-D immunization by serologically typed D-negative donations. Moreover, we aim to assess the cost-efficiency of the contribution of *RHD* genotyping in improving the safety of blood transfusion, with special emphasis on anti-D-immunization prevention in women under the age of 45. A cohort of 10 000 serologically D-negative donors, with a positive selection for C- or E-positive,

D-negative donors, will be *RHD* genotyped. Subsequently, serological analysis of the red cells from *RHD* allele-positive serologically D-negative donors will be extensively performed. Finally, look-back studies and case-finding studies have been planned to evaluate the risk of anti-D immunization.

Question 4:

(a) In the Netherlands, it is not presently obligatory to retype a D-negative donor with the indirect antiglobulin test. However, all regional blood banks are performing the indirect antiglobulin test the first two donations, when a new donor is found to be D-negative.

(b) If the outcome of the planned Dutch study (*Question 3*) points to a relevant risk for women under 45 to become anti-D immunized upon red blood cell transfusion, this may be a reason to start molecular *RHD* typing in the routine screening. However, *RHD* genotyping of (subsets or pools of) serologically D-negative donors will be costly. *RHD* genotyping is part of the assays currently developed in blood group genotyping microarrays, which may be the future blood group antigen typing platforms [3,4]. These microarrays are developed to be informative for a broad range of clinically relevant blood group systems, hence, informative for typing the whole donor cohort [5]. Thus, all alternatives: single *RHD* donor typing, *RHD* genotyping of pooled samples from serologically D negative donors and *RHD* typing as part of microarray-based typing, will be taken into account in a cost-efficiency study.

(c) With the current serological techniques reliable detection of D-positive/D-negative chimeras cannot be achieved. The sensitivity of the currently used single donor *RHD* genotyping assays, especially of quantitative PCR-based assays, is sufficient to detect *RHD* alleles in D-positive/D-negative chimeras. The frequency of such chimeras in the Dutch population is thus far regarded to be very low and may be concluded from the forthcoming *RHD* typing exercise.

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Question 1: We have not seen any anti-D immunizations after the transfusion of typed D-negative donor red cells.

Question 2: Rh(D) typing of all donors is performed using two IgM plus IgG monoclonal blend anti-D reagents; Novoclone (Dominion Biologics Ltd, Dartmouth, Nova Scotia, Canada B3B 1M1) and Gammaclone (Gamma Biologicals, Inc., Houston, TX). In addition, for all first time donors, Anti-C+D+E typing is also performed using the Lorne monoclonal typing reagent (Lorne Laboratories Ltd, Berkshire, RG10 9NJ, UK). In all cases, reactions are read using an automated system with additional visual confirmation of the negative reactions. Where a discrepancy arises and in all cases where the anti-C+D+E test gives a positive reaction the donor sample is sent to the internal Red Cell Reference Laboratory for resolving.

The reference laboratory repeats typing using the Novoclone and Gammaclone reagents and in addition does a third test using an IgM monoclonal anti-D reagent (Diagast Laboratories, Lille, France). The latter reagent may enable the detection of D variant. In all three cases, an IAT tube technique is used. If any one of these reagents gives a positive result, the donor will be categorized as D-positive.

Question 3: We currently do not do Rh(D) genotyping in New Zealand.

Question 4:

(a) Our routine testing incorporates three direct agglutination methods and if all three are negative, an indirect antiglobulin test is not required.

(b) We would require reliable population-based frequency data along with robust specific genotyping assays. The New Zealand population is somewhat heterogeneous and thus such data are crucial in decision-making. At this stage, *RHD* genotyping would not be considered a priority for the service.

(c) The issue of D-positive/D-negative chimeras has not been a part of any of our discussions and any future thoughts on testing for this will be dependent on reliable population-based frequency data. The decision on the appropriate indications for genotyping will be influenced somewhat on our eventual decision to commence *RHD* genotyping. We have to agree with Gassner *et al.* [1] that reliable and detailed

population genetic data will have to be taken into consideration before embarking on *RHD* genotyping.

Reference

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B. G. Solheim

Question 1: No, but until 2003, national reporting was poor.
Question 2: Norwegian regulation requires RhD typing with two different reagents, and retyping of all RhD negative donors with antiglobulin technique. For blood donor typing most Norwegian blood banks now use Diamed DiAClon ABOD gel cards.

Question 3: No

Question 4:

(a) Yes

(b) With C- and/or E-positive D-negative donors, we would recommend that RhD genotyping is considered, but not with all other RhD-negative donors

(c) No, we do not think it would be worthwhile

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B. Zupanska & B. Michalewska

Question 1: Anti-D antibodies were found in six Rh-negative male recipients of Rh-negative blood; however, it has not been proven that they were produced after receiving D weak red cells. We have also observed one RhD-negative woman with no anti-D, although one of numerous Rh-negative transfusions later was found to be D weak; unfortunately, the number of D sites has not been determined.

Question 2: In our country there are 21 regional blood transfusion centres (RBTCs), supervised by our Institute, per-

forming RhD typing in the following way: 8 centres use manual tube technique with two IgM anti-D from different clones (one of them is always RUM 1); 2 centres use the same method but by Olympus PK 80 analyzer; 11 centres use Automatic DiaMed System either with microcolumns or with microplates. Thirteen of 21 centres always re-type RhD-negative donors for weak D by IAT (tube or microcolumn test).

Question 3: As yet, we have not used *RHD* genotyping to detect weak D and DEL. After application of molecular biology for blood group typing, our priority was to develop and introduce into the practice fetal RHD typing from maternal plasma [1]. Now, we plan *RHD* genotyping of C/E+ D-negative donors.

Question 4: The antiglobulin test for weak D detection in donors is not obligatory in Poland since monoclonal antibodies for RhD typing were introduced [2]. We will not recommend mandatory *RHD* genotyping in the near future. However, we plan to genotype C/E+ D-negative donors in our Institute to collect data on *RHD* frequency among serologically D-negative donors in our country. In addition, if we find a weak D or DEL donor we will try to perform look-back in transfused patients since the evaluation of their immunogenicity is very important.

It is very likely that simpler and cheaper procedures of *RHD* genotyping will be available for routine application within few years and they may supplement or even replace serological methods. If this were the case, it would be easier to include all donors for genotyping and not only selected groups due to logistic problems. However, if the costs were too high, it would probably be more practical to genotype only donors whose red cells are to be transfused to girls and women of childbearing age. Fortunately, in recent years most of our RBTCs have implemented genotyping techniques with the introduction of the nucleic acid amplification technique (NAT) for viruses, thus they will be able to undertake new tasks.

We do not have experience with Rh chimeras, so we will not introduce genotyping until more is known. The financial condition of Polish health service is still poor; thus, our priorities have to be chosen very carefully.

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Question 1: No

Question 2: In first time donors we use two monoclonal antibodies of different clones, one detecting DVI red cells, the other not. Donors whose blood gives an unequivocal positive reaction with both anti-D reagents are regarded as D-positive. Donors whose blood is unequivocally negative with both anti-D reagents are regarded as D-negative. These donors are additionally confirmed as D-negative by means of the indirect antiglobulin test (IAT). If the results with both anti-D are discordant or equivocal, the samples are provisionally catalogued as D-positive and are studied comprehensively in the immunohematology laboratory. For repeat donors one anti-D reagent (blended reagent) is employed.

In 2004, a total number of 131 592 blood donations from 109 221 blood donors were processed in our blood transfusion centre. The number of first time donors was 23 107 and 19% of them ($n = 4357$) were D-negative. In the IAT, two of these donors turned out to be D-positive (2 DFR variants in two sisters). From January to July 2005 147 590 blood donations from 123 610 donors were tested. In this period, 25 920 were first time donors and 18% of them were D-negative ($n = 4743$). Again, two donors were found D-positive in the IAT (2 DVI variants) (Table 1).

Table 1

Year	Blood donations	Donors	First-time donors	D-negative	D-positive (IAT)
2004	131 592	109 221	23 107	4357	2 (2 DFR)
2005	147 590	123 610	25 920	4743	2 (2 DVI)

Comments

- The high proportion of D-negative donors is the outcome of intensive and continuous promotion of blood donation among this group of people in our population.
- It seems that weak D is detected by routine typing and donors are typed as D-positive. The IAT seems to be useful in the detection of partial D.
- (Data were provided by Dr J. M. Hernández of our institution).

Question 3: No, we have not. *RHD* genotyping is only used as part of the protocol employed in donors whose results with both anti-D reagents are discordant or equivocal.

Question 4:

(a) Yes, it is.

(b) We think that *RHD* genotyping should not be mandatory for the time being. We need more information concerning the magnitude of the problem in terms of prevalence and in terms of clinical impact of these DEL phenotypes in immunizing

patients. However, it is advisable to perform prospective studies to investigate the exact incidence of DEL in different populations. Moreover, it would be important to study the apparently D-negative donors involved in cases of anti-D immunization by means of *RHD* genotyping. A rational strategy should consist in *RHD* genotyping of the first time D-negative donors to ensure that D-negative blood is provided to women of childbearing age. The second step is to include the remaining donors, giving priority to C- and/or E-positive D-negative donors.

(c) The experience in this field is even more limited but should this problem represent a clinical impact, only donors whose red cells are transfused to women of childbearing age should be tested for the time being by means of *RHD* genotyping.

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Question 1: Our blood service is mainly in charge of screening and selecting suitable blood donors and therefore we possess only incomplete antibody prevalence data of the patient population served. We are not aware of any anti-D immunization of patients who have been transfused with red blood cell concentrates selected by us. However, for years we have considered possible failure in correct typing of weak D/partial D individuals. Therefore, we confirm Rh negativity obtained by routine testing (haemagglutination assay in microtitre plates) applying indirect antiglobulin test (IAT) as well as double-checking of all Rh-negative donors with alternative Rh typing methods such as tube testing and solid phase typing (ID-Gel system, DiaMed, Switzerland). In cases of ambiguous findings in routine and extended tests, corresponding to weak D or partial D phenotype, the blood products will be labelled Rh-positive.

Question 2: RH1 routine typing is performed by haemagglutination assay in microtiter plates using various monoclonal antisera: anti-D (IgM): BS226, BS232, anti-CDE: P3x25513G8/P3x61/P3x234 (Biotest, Dreieich, Switzerland). Negative and ambiguous samples are confirmed by tube testing applying anti-D blend (IgG, IgM), clone TH28 and clone MS26 respectively and conducting IAT to cover category D^{VI} variants. In addition, gel matrix typing system (DiaMed, Switzerland) using polyclonal anti-D is applied.

Question 3: Recently, we investigated 54 donors with ambiguous Rh results by molecular typing [1]. Genotyping for weak D and partial D mutations were exhibited using commercially available PCR-SSC kits (Innotrain, Kronberg, Switzerland) and D epitope density of red blood cells (RBC) was examined by flow cytometry following a modified protocol by Flegel *et al.* [2]. The Table gives a summary of our results:

Genotype as by PCR-SSP	No. of donors (%)	RH1 sites/RBC (median, range)	% RH1 sites relative to R1R1 control (Median)
Weak D	52 (96%)	2201 (220–4267)	9.8%
Type 1	32 (62%)	2375 (1848–3710)	10.4%
Type 2	11 (22%)	1355 (1003–2092)	5.8%
Type 3	3 (6%)	3592 (2454–3960)	16.3%
Type 5	1 (2%)	840	3.5%
Other weak D	3 (6%)	2187 (220–4267)	10.5%
Type 1+2 ^a	1 (2%)	2853	12.0%
Partial D ^{VI}	2 (4%)	3341 (2682–4000)	14.0%

^aOne donor presented a combination of weak D type 1 mutation (T809G) and weak D type 2 mutation (G1154C) that was confirmed by family segregation study.

Regarding prevalence of Rh phenotypes, among 7571 new blood donors, we found 6300 (83.2%) Rh-positive individuals and 1271 (16.8%) Rh-negative donors. 12/1271 (1%) Rh-negative donors showed ambiguous Rh typing results and revealed weak D genotypes.

Question 4:

(a) Yes. For blood donor selection, in Switzerland it is mandatory to confirm Rh negativity by IAT on two independent drawings.

(b) Rh genotyping of Rh negative/ambiguous donors would potentially be a useful approach. However, logistical and economical implications might be demanding. Alternatively, we suggest the standardization of protocols based on flow cytometry to determine RBCs D epitope density. This might provide pertinent information to estimate potential immunization risk. However, it requires establishing the D epitope density threshold that discriminates immunization risk upon transfusion of Rh weak D RBC concentrates. In addition, carriers of weak D genotypes with very low D epitope density may be at risk to generate primary anti-D immune response upon Rh-positive transfusion [3]. Therefore, the RhD density threshold might be critical to direct transfusion protocols in weak D individuals as well as to assign clinically correct Rh labelling of blood donations from weak D donors.

(c) We have no experience in typing of chimeras. However, we think that a flow cytometric approach should cover such situations sufficiently well. Minor RBC populations representing less than 1% are detected unambiguously by flow

cytometry [4]. Once the method is established, product labelling based on D epitope density measurements might diminish immunization events.

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S. A. Kochman & J. S. Epstein

Question 1: The US Food and Drug Administration (FDA) does not currently have a mechanism to track immunization following transfusion and so is unaware of the frequency of such immunizations or the techniques used in testing the donors suspected of causing such immunizations.

Question 2: There are several techniques available to blood collection facilities and transfusion services for typing for RhD; however, FDA does not dictate which methods must be used. For donor testing, the requirements are:

If the test, using Anti-D Blood Grouping Reagent, is positive, the container may be labelled 'Rh-positive.' If the test is negative, the results shall be confirmed by further testing which shall include tests for the 'weak D (formerly Du):' Blood may be labeled 'Rh-negative' if further testing is negative. Units testing positive after additional more specific testing shall be labeled as 'Rh-positive.' Only Anti-Rh Blood Grouping Reagents licensed under, or that otherwise meet the requirements of, this subchapter shall be used, and the technique used shall be that for which the reagent is specifically designed to be effective [1].

There are no specific FDA requirements for RhD testing other than the requirement that supplies and reagents shall be used in a manner consistent with instructions provided by the manufacturer [2].

Question 3: The FDA has not performed any *RHD* genotyping nor do they require that it be performed. We are aware that many in Europe and some in USA are performing this testing, albeit as a research test in USA.

Question 4: Regarding the detection of weak D:

(a) Blood collection facilities in USA are required to test D-negative bloods for weak D as described in item 2 above. There are no FDA requirements for transfusion services with regard to RhD testing. AABB standards require transfusion services to confirm the ABO group and D positive/negative type but do not require that the test for weak D be performed on D negative units [3].

(b) FDA does not intend to require *RHD* genotyping at this time or in the near future because of the limited data supporting a decision to make it a requirement and because of limited availability of testing in USA. There are some facilities performing this testing under a research protocol, but no manufacturer has submitted a reagent(s) for premarket review and approval.

(c) FDA would need to see more data on the prevalence of chimeras and the nature of any problems that arise as a result of them before making any recommendations or establishing requirements regarding testing for chimeras. Note also, that as described above, limited availability of the technology and reagents to perform such testing would be taken into consideration.

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G. Garratty

Question 1: No, we have not encountered D alloimmunization proven to be due to serologically tested D-RBCs. Wagner *et al.* [1] reported that 0.6% (50 of 8442) of donors were typed as D-negative by serology, but were RHD by molecular approaches. Gassner *et al.* [2] found that 5.2% of D-negative C+ or E+ donors were RHD. Not all these donors with RHD express the

D antigen on their RBCs and thus have the potential to be DEL immunogenic. In 8442 donors typed as D-negative, Wagner *et al.* only found 20 of the 50 'undetectable D' donors to be DEL or weak D (i.e., D expressed on RBCs), thus only 0.2% (20 of 8442 donors) typed as D- with RHD would seem to have the potential to immunize D-negative recipients. We supply about 400 000 units of RBCs per year (about 60 000 D-negative) to southern California hospitals. Most of these are transfused, so about 60 000/year of units typed by us as D-negative (approximately 1000 would be C+ or E+) are given to D-negative recipients. That means, in southern California, if the statistics are similar to Central Europe (which probably is not so as 30% of our donors are not Caucasian, so more may have weak D/DEL), we are transfusing about 120 (0.2% of 60 000) units which have serologically undetectable D, to D-negative recipients per year, yet we have received very few reports (over the last 10 years) of anti-D, with unknown stimulus (and none that have been shown to be due to a D+ donor being mistyped). I realize that this is not a result of a study/survey but it does not seem a problem in southern California (a very litigious area).

Although recently there have been increasing numbers of reports of recipients of 'weak-D' or 'undetectable D' (e.g., DEL) making anti-D, it should be noted that Gassner *et al.* [1] found that none of the recipients of seven D-negative 'undetectable D' transfusions made anti-D when followed for about 140 days. For many years, we have been transfusing C+, E+, K+ RBCs to Rh+ and Rh- recipients lacking these antigens. I have to wonder if there are more recipients making anti-C, -E or -K than anti-D, stimulated by 'undetectable D' antigen.

Question 2: Donors are typed with a single FDA-licensed anti-D by an automated method (Olympus PK7200), validated to detect weak D.

Question 3: No.

Question 4:

(a) AABB Standard 5.8.2 [3] requires that all D-negative donors are tested using a method designed to detect weak D.

(b) I would not recommend mandating RHD genotyping. It would not be possible for some time in USA as blood centers are not set up (or have staff with pertinent experience) to do such testing. Even if it were possible, I would not make it mandatory but rather a recommendation to perform molecular testing on some, but not all, donors and/or to investigate serological discrepancies. In USA, donors are only tested for ABO and D, if it was so recommended and a cost analysis would be required comparing genotyping of all new D-negative donors versus only testing D-negative C+ and E+ new donors, which would mean typing all new D-negative donors for C and perhaps E (or Rh phenotyping all donors as is done in some other countries). We must keep reminding ourselves that detecting a D gene does not mean it will result in an immunogenic protein on the RBC.

(c) The above arguments for testing serologically 'undetectable D' are similar to those for chimeras.

References

- 1 Wagner FF, Frohmajer A, Flegel WA: RHD positive haplotypes in D negative Europeans. *BMC Genetics* 2001; 2:10
- 2 Gassner C, Doescher A, Drnovsek TD, Rozman P, Eicher NI, Legler TJ, Lukin S, Garritsen H, Kleinrath T, Egger B, Ehling R, Körmöczy GF, Kilga-Nogler S, Schoenitzer D, Petershofen EK: Presence of RHD in serologically D-, C/E+ individuals: a European multicenter study. *Transfusion* 2005; 45:527-538
- 3 American Association of Blood Banks: *Standards for Blood Bank and Transfusion Services*. 23rd edn. Bethesda, MD, American Association of Blood Banks, 2004: 32 (Standard 5-8-2: Determination of Rh type for all collections)

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Question 1:

We are not aware of any, and none have been referred in the last 10 years to either of our reference laboratories that serve large metropolitan areas (Philadelphia and New York). Together, our laboratories represent a total of 725 000 units transfused each year, with 15% being D-negative.

Question 2:

Both centers use the Olympus PK 7200 automated system and at least two different FDA licensed reagents and red cells that are suspended in bromelin.

Question 3:

We use RHD genotyping as a research protocol to aid in the resolution of serological typing discrepancies. In the experience of both our centers, the majority of discrepant 'D-negative' donors are found to have either the Crawford or the R_o^{Har} phenotype.

Question 4:

(a) Yes

(b) This is not possible in USA at present because there is no standard methodology and no FDA licensed procedure for genotyping. Even if it were possible, implementation of new testing requirements/procedures based on rare reports in the literature has always been inappropriate. Additional data would be needed to make a decision regarding the appropriateness of this testing. We strongly support methods to increase awareness and to encourage the follow-up of individuals that present with anti-D and no known stimulus. Avoiding the transfusion of blood from D-negative donors that is C+ and/or E+ to women of childbearing age would have a similar outcome without the additional testing.

(c) No. However, as with our response to 4b, we are in favor of increasing awareness and gathering data.

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