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Frequency and causes of refractoriness in multiply transfused patients

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Abstract The use of leukocyte-depleted blood components has become the standard therapy for multiply transfused patients during the past few years, as a measure to reduce the frequency of alloimmunization and refractoriness. We assessed frequency and causes of refractoriness, defined as a repeated 24-h post-transfusion platelet count below 20000/μl, in 145 consecutive patients who received three or more single-donor platelet concentrates during a 1-year period. Flow-cytometric detection of anti-platelet antibodies and a glycoprotein-specific ELISA were applied for the diagnosis of alloimmunization. Forty patients (27.6%) had at least one episode of refractoriness. In 25 of these 40 patients (62.5%), nonimmune factors (fever, sepsis, coagulopathy, splenomegaly) alone were the cause. In 15 refractory patients alloantibodies were detected. In seven patients (17.5%), alloimmunization alone caused an inadequate transfusion response, while in eight refractory patients (20.0%) alloimmunization and fever or sepsis were present. HLA antibodies were detected in 17 patients (11.7%); three patients (2%) had platelet-specific antibodies in addition to HLA antibodies; in two patients panreactive platelet antibodies were detectable. All 17 patients had a history of previous transfusions or pregnancy. We did not observe primary immunization in patients transfused exclusively with filtered (leukodepleted) blood products. Our data suggest that alloimmunization in patients with a negative risk history can be prevented by the exclusive use of leukodepleted blood components.

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Introduction

Refractoriness to platelet transfusions is a severe complication in patients with aplastic thrombocytopenia, which can result in life-threatening bleeding. Refractoriness is usually defined as a repeated insufficient platelet increment after consecutive platelet transfusions. Two major causes of a decreased platelet increment can be distinguished, i.e., immune and nonimmune factors. Alloimmunization occurs most frequently against the HLA, and rarely against the HPA system. Several nonimmune factors have been identified, including splenomegaly, fever, sepsis, and disseminated intravascular coagulation [5, 13]. In addition, the quality of platelet concentrates (PCs) has an influence on the platelet increment.

Alloimmunization against HLA antigens is usually induced by contaminating leukocytes in PCs or red cell concentrates (RCCs). Several clinical trials have shown that leukodepletion by filtration of blood components is effective in preventing primary alloimmunization, as recently reviewed by Heddle and Blajchman [7]. However, in most of these randomized trials, patients with nonimmune factors known to cause refractoriness as well as patients with a "positive risk history" (i.e., previous transfusions and pregnancies) have been excluded, in order to better estimate of the effect of leukodepletion in the prevention of refractoriness. Additionally, neither the frequency of alloimmunization nor the frequency of refractoriness gives information on the clinical course and outcome. Thus, the clinical relevance of these studies is limited [7, 20].

The aim of our study was to determine the frequency and causes of refractoriness and the frequency of alloimmunization in a nonselected, hemato-oncological

patient population transfused exclusively with leukodepleted blood components during a 1-year period.

Methods

Patients and treatment regimen

All patients with hemato-oncological disease possibly requiring multiple transfusions were screened for HLA antibodies prior to the first transfusion and were typed for HLA class I with the microlymphocytotoxicity test (LCT) [22]. These patients received leukodepleted blood components exclusively. Random single-donor platelet concentrates from HLA-/HPA-typed donors [12] were initially used. In cases of refractoriness, patients' sera were tested for HLA and platelet-specific antibodies, using the LCT and flow cytometry as screening tests and the MAIPA as confirmation assay. Additionally, the patient's history was thoroughly checked for the following factors: fever or sepsis, splenomegaly, disseminated intravascular coagulation, history of prior pregnancies or transfusions. When antibodies were detectable, the patients received HLA-/HPA-compatible, or best-match platelets, which were negative in platelet cross-matching. The clinical outcome of patients with HLA/HPA antibodies was followed up till May 1996.

In all, 274 patients received 2389 single-donor platelet concentrates at the University of Göttingen during 1995. Causes and frequency of refractoriness were determined in 55 female and 90 male patients who received three or more single-donor platelet concentrates. None of the remaining 129 patients was refractory to random single-donor platelet concentrates.

Blood components

Single-donor platelet concentrates were prepared with cell separators from Cobe or Fresenius (Spectra, Cobe, Heimstetten; Fresenius AS100, Fresenius, St. Wendel). A single platelet unit contained $4.86 \pm 1.58 \times 10^{11}$ (mean $\pm$ SD, $n = 75$) platelets. The leukocyte contamination before filtration was $< 5 \times 10^6$ in 90% of platelet concentrates, which is the accepted limit for the prevention of alloimmunization [10]. All platelet concentrates were leukodepleted with filters (LRP 6, Pall GmbH Biomedizin, Dreieich, Germany). After filtration the leukocyte contamination was $< 2.5 \times 10^5$ per unit (mean 2.06×10^4, $n = 75$). Leukodepleted RCCs were prepared by bedside filtration (RC 100 KLE, Pall). All concentrates were CMV-antibody negative and irradiated with 30 Gy prior to transfusion in severely immunocompromised patients. ABO-identical and rhesus-compatible platelet concentrates were selected whenever possible.

Definition of refractoriness

All patients transfused with PCs were registered and their 16- to 24-h post-transfusion platelet count was measured. The transfusion trigger in our hospital is 20000 platelets/μl in nonsurgical patients and 10000 platelets/μl in patients receiving supportive therapy only (e.g. for aplastic anemia). A 16- to 24-h post-transfusion platelet count of less than 20000/μl and 10000/μl, respectively, was considered an inadequate platelet increment. Patients with platelet counts repeatedly below the transfusion trigger after two or more successive platelet transfusions within 48 h were defined as refractory.

Antibody screening

An initial antibody screening with the LCT (5-cell panel) was performed prior to the first platelet transfusion. Positive LCTs were confirmed with flow cytometry [11] and the monoclonal-antibody-specific immobilization of platelet antigens assay (MAIPA) [9]. The clinical history, with special reference to previous transfusions of cellular blood products and pregnancies, was ascertained.

Sera from all refractory patients were analyzed with flow cytometry and LCT. All sera which tested positive in these screening assays were further analyzed with the MAIPA for their platelet glycoprotein specificity. Negative screening tests were not analyzed further, because we had previously demonstrated that the sensitivity of flow cytometry is comparable to that of MAIPA [11]. HLA-antibody specificity was analyzed using the LCT with a commercial panel of 56 cells (Lymphoscreen, Biotest, Dreieich, Germany) and flow-cytometric cross-match results. In all refractory patients the presence of the following nonimmune factors was registered: sepsis or fever, splenomegaly, coagulation disorders, major bleeding.

Flow-cytometric antibody screening

The test principle has recently been described in detail [11]. Briefly, donor cells from five donors were separated with lymphoprep (Nycomed Pharma, Oslo, Norway), washed twice, incubated with the patient's serum, washed twice, and incubated with an FITC-labeled anti-human IgG-antibody (The Binding Site, Birmingham, UK). The median fluorescence of gated platelets and lymphocytes was analyzed by flow cytometry (FACS-Scan and Excalibur, Becton-Dickinson, Heidelberg, Germany). Median-channel fluorescence three times higher than the autologous control of the donor lymhocytes and three times higher than the autologous control of the donor platelets was defined as positive.

MAIPA

Washed platelets of HLA- and HPA-typed donors were incubated with patients' serum for 30 min in U-bottom microplates. After washing, the cells were incubated with the respective monoclonal anti-glycoprotein antibody (anti-GP IIb/IIIa from Immunotech, Hamburg, Germany, or from CLB Amsterdam, Netherlands; anti-GPIb CLB, anti GP Ia/IIa CLB, anti β_2-microglobulin, Immunotech). After 30 min, platelets were lysed in detergent (NP40, Sigma, Munich, Germany, Tween 20, Merck, Darmstadt, Germany), transferred to 1.5-ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuged ($13000 \times g$) at 4 °C. The supernatant was transferred to an anti-mouse IgG coated flat-bottom ELISA plate (Nunc, Denmark) and incubated for 2 h at 4 °C. After washing, a horseradish peroxidase-labeled anti-human IgG (Immunotech, Hamburg, Germany) was used for detection. The substrate reaction was stopped after 15 min with 1 *N* H_2SO_4 and plates were measured at 492 nm on a microplate photometer (ATTC, SLT Labinstruments, Gröding, Austria) [9].

Results

Frequency and causes refractoriness

During 1995, 274 patients were transfused with single-donor platelet concentrates; 145 of these patients received more than two platelet transfusions and were thus able to be analyzed regarding the incidence of refractoriness. Forty of these 145 patients (27.6%) showed at least one episode of refractoriness to platelet transfusions. The causes of refractoriness are shown in Table 1. Nonimmune factors, predominantly fever and sepsis, were responsible for refractoriness in at least 62.5% of patients. Alloimmunization alone was responsible for refractoriness in seven (17.5%) patients.

Table 1 Causes of refractoriness and frequency of alloimmunization

Causes of refractoriness	*n*	% of refractory patients
Sepsis alone	23	57.5
Sepsis and alloimmunization	8	20.0
Splenomegaly	1	2.5
Coagulation disorders	1	2.5
Alloimmunization alone	7	17.5
Antibody differentiation	***n***	**% of all patients ($n=145$)**
Alloimmunization	17	11.7
Anti-HLA	12	8.3
Anti-HLA and anti-HPA	3	2.1
Alloimmunization (panreactive)	2	1.4

Of 40 patients refractory to platelet concentrates, 28 died within the observation period. Only three of 23 nonimmunized patients with refractoriness due to sepsis/fever survived. All eight patients with alloantibodies and fever/sepsis died. Of seven patients with alloantibodies as the only cause of refractoriness, only one died. This patient suffered from aplastic anemia, had broad multispecific HLA and panreactive platelet antibodies for years, and died of intracranial hemorrhage after 10 years of treatment. The remaining six alloimmunized patients survived until May 1996 and were able to be supported with HLA-/HPA-compatible, cross-matched negative platelet concentrates.

Frequency of alloimmunization

In 15 of 40 patients with transient refractoriness platelet-reactive antibodies were detectable. Additionally, in two nonrefractory patients platelet-reactive antibodies were detected at initial screening. In 11 of these 17 immunized patients, anti-HLA (and platelet-specific) antibodies were detectable prior to the first platelet transfusion. All patients had a history of previous transfusions in other hospitals before transfer to our clinic. It was therefore not possible to further characterize these patients in terms of primary or secondary immunization. Four patients with aplastic anemia had been transfused for several years, when leukodepletion filters were not available, resulting in primary immunization before 1995. The remaining two alloimmunized patients were multiparous women. Anti-HLA antibodies became detectable after the first platelet transfusion. The frequency of alloimmunization was five times higher in women (23.6%) than in men (4.4%; χ^2-test, $p<0.01$). The number of transfused blood compenents, the diagnosis, and the frequency of refractoriness as well as of alloimmunization are shown Table 2.

Characterization of antibodies

Sera from 17 multiply transfused patients were positive for IgG antibodies in flow cytometry and were further characterized. In all cases anti-HLA antibodies were confirmed with MAIPA. Sera from five of these patients were also reactive with platelet-specific antigens in the MAIPA. Sera from two of these five patients reacted with β_2-microglobulin and/or all platelet-specific glycoproteins, and a panreactive antibody was diagnosed. In three patients anti-HPA alloantibodies were confirmed. One patient with myelodysplastic syndrome (MDS) had anti-HPA-1b, anti-HPA-2b, and anti-HPA-5b. A second patient with MDS had anti-HPA-1b and anti-HPA-5b. In the second case these antibodies disappeared and the patient subsequently developed an anti-HPA-2b antibody. The third patient, suffering from AML, had a transient anti-HPA-1b antibody, which disappeared after chemotherapy.

Discussion

The definitions and calculation methods, as well as the threshold values for an adequate response after trans-

Table 2 Frequency of refractoriness and alloimmunization in 145 polytransfused patients (*PC* median of transfused platelet concentrates, *RCC* median of transfused red cell concentrates, *NHL* non-Hodgkin's lymphoma, *AML* acute myelogenous leukemia, *ALL* acute lymphocytic leukemia, *MDS* myelodysplastic syndromes, *AA* aplastic anemia and Fanconi anemia, *CML* chronic myelogenous leukemia, *MM* multiple myeloma)

	n	PC	RCC	Refractory (%)	Immunized (%)
NHL	32	7	10.5	5 (16)	2 (6)
AML	33	25	23	14 (42)	4 (12)
ALL	22	11	12.5	1 (5)	0 (0)
Breast cancer	10	4	6	2 (20)	2 (20)
MDS	8	11.5	11.5	4 (50)	2 (25)
AA	8	38	47.5	4 (50)	4 (50)
CML	6	13.5	12.5	2 (33)	0 (0)
Testicular cancer	6	4.5	10.5	1 (17)	0 (0)
MM	5	6	14	3 (60)	2 (40)
Various	15	5	26	4 (27)	1 (7)
Total	145	9	14	40 (28)	17 (12)

fusion of platelet concentrates, vary considerably. Usually, refractoriness is defined as a repeatedly insufficient increase of platelet counts after consecutive transfusions of platelet concentrates. Several calculation methods are used, e.g., platelet increment, corrected count increment, and percentage of platelet recovery. Although these parameters are well suited to the investigation of factors influencing the transfusion response, they have been criticized in a recent review by Heddle and Blajchman [7], because they are not direct measures of the desired clinical effect of platelet transfusions, i.e., the avoidance of bleeding. Thus, new definitions for a successful platelet transfusion have been demanded and put forward [2, 7]. In our evaluation, we used a 24-h post-transfusion platelet count repeatedly below 20000/μl for the definition of refractoriness, which is equivalent to a 1-h CCI smaller than 5.5×10^9 [2]. Although the transfusion trigger of 20000/μl has been questioned, it is the one most commonly used [1]. If a transfusion trigger is defined and it is postulated that platelet counts below this threshold value result in an increased risk of bleeding, it seems rational and of clinical importance to determine whether the transfusion resulted in a platelet count above this threshold value or if further transfusions were needed.

In previous studies, done before the era of leukodepletion, the incidence of refractoriness in multiply transfused patients was reported as high as 70% [4, 6, 8, 13]. A meta-analysis of five studies calculated a common odds ratio of 0.28 for platelet refractoriness in patients receiving leukodepleted cellular blood components compared with patients treated with nonleukodepleted products [7]. We observed refractoriness in 27% of multiply transfused patients, and in the majority of these cases nonimmune factors alone, i.e., fever, sepsis, splenomegaly, or coagulopathy, were the obvious reasons. Novotny et al. [17] observed refractoriness in 34% of 229 patients; nonimmune factors were present in 54%. These data are also supported by a study on the transfusion response in 24 patients, where nonimmune factors were the most frequent causes (67%) for a poor platelet increment. It is apparent from these studies that non-immune factors are now the main causes of refractoriness to platelet transfusions when leukodepleted cellular blood components are used in multiply transfused patients.

We observed no case of primary HLA immunization in our study; this is in accordance with several studies in which immunization rates between 0 and 9% were observed when leukodepleted blood products were used [3, 19, 23]. The great proportion of alloimmunization at presentation in patients with a positive history of transfusions stresses the importance of consistent leukodepletion also in outlying hospitals [20]. The frequency of alloimmunization including secondary immunization was significantly higher in women, confirming the study of Sintnicolaas et al. [21]. They observed no significant reduction of secondary alloimmunization in women with a history of previous pregnancies through transfusion of leukodepleted blood products which were contaminated with less than 5×10^6 WBCs. These authors gave as possible explanations for their observation (a) a contamination with WBC which is possibly still too high, (b) soluble HLA antigens or HLA-bearing microparticles, or (c) HLA antigens on platelets. A further increase of the efficacy and reliability of leukodepletion, and thus a further reduction of alloimmunization, may be possible by using blood components filtered before storage [18].

Antibodies against the HPA system, causing refractoriness, were observed in only 2.1% of our patients, which is in agreement with the observations of Novotny et al. [17]. Thus, the contribution of alloimmunization against platelet-specific antigens to refractoriness was small in our investigation, although sensitive and specific test systems were applied. Using different methods, platelet-specific antibodies in multiply transfused patients have been observed in 2–42% of patients [6, 14, 16, 17]. It should be noted, however, that platelet antibodies which are not directed against the HPA system or platelet glycoproteins IIb/IIIa or Ib/IX are usually transient and usually do not cause refractoriness [6, 14, 16, 17].

Several strategies for the management of refractory patients have been proposed. When antibodies do not have broad panel reactivity, refractory patients can be supported with higher dosages and more frequent administration of random platelets [17]. As an alternative, or when multispecific HLA antibodies are present, HLA-matched platelets can be utilized [15]. We use cross-matched negative platelets from HLA- and HPA-typed donors for alloimmunized patients. With this treatment regimen, only one patient with aplastic anemia and multispecific HLA antibodies died of bleeding complications.

In 1991, C. A. Schiffer stated in an editorial [20]: "Alloimmunization represents the main clinical problem limiting the benefits of platelet transfusions in patients with leukemia and other bone marrow failure states." Meanwhile, immunization is no longer the major cause of refractoriness in these patients and can be prevented and managed in the majority.

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