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TITLE: Understanding the Mechanisms of Platelet Alloimmunization and Its Prevention

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14. ABSTRACT

The purpose of our experiments is to identify methods of preventing alloimmunization to donor platelets in a dog platelet transfusion model. We have established DLA Class II typing to select antigen incompatible donor-recipient pairs for our transfusion experiments. In addition, we are evaluating potential allostimulatory WBC that must be removed to prevent platelet alloimmunization or WBCs that must remain to induce tolerance to donor platelet transfusions. Flow cytometry techniques using antisera that detect various classes of WBCs are being used to identify cells that are removed *versus* those that remain using different leukoreduction filters. The results of these white cell characterization studies will be correlated with the results of donor platelet transfusion experiments where different filters have been used to leukoreduce donor platelets prior to transfusion. Our current platelet transfusion experiments have evaluated γ-irradiation alone or combined with leukoreduction to prevent alloimmunization to donor platelets. These experiments have demonstrated that none of these methods prevent platelet alloimmunization. Our next experiments will combine UV-irradiation along with filtration leukoreduction to determine if this combination can prevent alloimmune platelet refractoriness.

15. SUBJECT TERMS
Platelets, Platelet Transfusion, Leukoreduction, γ-Irradiation, UV-Irradiation, Alloimmunization, Tolerance

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INTRODUCTION:

We have now completed the second year of our three-year grant proposal. Our original grant proposal reported our prior studies that had shown that platelets that had been filter-leukoreduced (F-LR), when followed by a soft centrifugation of the filtered platelets to remove additional leukocytes (C-LR) with injection of the supernatant platelets, was highly effective in preventing platelet alloimmunization. However, this procedure was very dependent on the filter used for the F-LR process. Using a PLS-5A filter (Fenwal Corporation), 10/10 recipients (100%) accepted 8 weekly transfusions of donor platelets without becoming refractory to their donor's platelets. Refractoriness is defined as two sequential radiolabeled donor transfusions with less than 5% of the donor platelets circulating in the recipient at 24 hours post-transfusion. If the platelets were only filtered with the PLS-5A filter but not also C-LR, only 4/6 recipients (66%) accepted their donor's platelets.

In contrast, when a PL1B pediatric filter (Pall Corporation) was used, only 6/12 recipients (50%) accepted a full 8 weeks of donor F-LR/C-LR platelet transfusions without becoming platelet refractory. However, if the PL1B F-LR/C-LR platelets were also γ-irradiated, 7/7 recipients accepted all 8 weeks of their donor's platelet transfusions. As the PL1B F-LR/C-LR platelet transfusions had such a poor acceptance rate, studies transfusing only PL1B filtered donor platelets were not done.

One of the major practical problems with these F-LR/C-LR preparations was how to control the C-LR process within the context of a busy platelet transfusion service. However, if a F-LR process combined with γ-irradiation without C-LR was effective, then this procedure could be easily performed and quality controlled within a blood center. Therefore, the primary focus of our studies over the last 1½ years (6 months were spent getting study approvals, technical staff trained, and the initial dogs DLA-typed and in place) has been to evaluate adding γ-irradiation to donor platelets that were F-LR using either the PLS-5A or PL1B filters. In addition, we have continued our studies on the wbc's that remain after F-LR or F-LR/C-LR to characterize allostimulatory wbc's, we have refined our DLA typing techniques to ensure that only DLA mis-matched donor/recipient pairs are utilized in our studies, and, finally, we have further enhanced our antibody detection techniques.

BODY:

Methods.

Experimental Design of the Platelet Transfusion Experiments.

The experimental design of our studies is as follows:

1) Select DLA-mismatched crossmatch negative random donor/recipient pairs.
2) Platelets are obtained weekly from a single donor.
3) Donor dog's platelets are modified/unmodified (standard) as per protocol.
4) Donor dog's platelets are radiochromium labeled prior to recipient transfusion.
5) Serial blood samples are drawn from the recipient to determine recovery and survival of the donor dog's platelets.
6) Primary Endpoint: Refractoriness is defined as <5% of the donor dog's platelets still circulating in the recipient at 24 hours post-transfusion after two sequential transfusions.

Results.

Donor Platelet Transfusion Experiments:

Three different types of transfusion experiments have been completed since initiation of the grant: γ-irradiation of donor platelets; 2) F-LR using the PL1B filter *plus* γ-irradiation; and 3) F-LR using the PLS-5A filter plus γ-irradiation (Table 1). Platelets from a single donor dog are transfused into a single recipient animal for 8 weeks or until the recipient becomes refractory to donor platelets.

TABLE 1

Treatment Of Donor Platelets	Recipients	Acceptance of Donor Platelets (Weeks)	Recipients That Became Platelet Refractory (%)
γ-Irradiation	5	1, 1, 1, 1, 3	100%
PL1B F-LR *plus* γ-irradiation	5	2, 3, 4, 6, 7	100%
PLS-5A F-LR *plus* γ-irradiation*	4	2, 2, 2, 3	100%

***Two more dogs are on study; both have accepted donor transfusions for one week. Antibody assays, in general, correlated with transfusion results.**

We expected that all recipient dogs would become refractory to donor platelets that had been treated only with γ-irradiation. Indeed, all 5 recipients became platelet refractory, and 4/5 accepted only one week of donor platelets before becoming refractory. Although recipients that received donor platelets that had been F-LR as well as being γ-irradiated accepted more donor transfusions, all tested to date have become refractory to their donor's platelets using either filter. Two recipients of PLS-5A F-LR and γ-irradiated platelets are still on study. These results suggest that γ-irradiation cannot substitute for the centrifuge-leukoreduction process as we had originally postulated.

Future Plans.

We see no value in continuing to enroll additional recipients into F-LR *plus* γ-irradiation experiments. Rather, we plan to evaluate F-LR platelets combined with UV-irradiation. In the TRAP Trial,[1] either F-LR or UV-B irradiation were equally effective in preventing platelet alloimmunization in acute myelogenous leukemic patients undergoing induction chemotherapy. However, there was still a residual rate of alloimmunization of 15%-18% in spite of the treatments. Since these treatments have different modes of preventing platelet alloimmunization (i.e., removing the allostimulatory wbc's by F-LR *versus* inactivating the wbc's by UV-B irradiation), possibly combining the two approaches would be better than either one alone. In addition, we are working with the CaridianBCT Corporation who has developed a technique to inactivate bacteria, viruses, and white cells using UV-A irradiation *plus* riboflavin that prevents replication of infectious agents as well as white cells. We will explore both of these types of UV-irradiation procedures when combined with F-LR to determine if platelet alloimmunization can be prevented in our dog model.

[1]The Trial To Reduce Alloimmunization To Platelets Study Group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. N Engl J Med 1997;337:1861-1869.

Dog Antibody Assay Improvements:

In the past year, we have greatly improved our assay techniques for pre-transfusion crossmatch testing to select compatible donor/recipient pairs as well as to detect donor-specific antibodies that may have developed in the recipient following platelet transfusions. Previously, we only screened for IgG antibodies against donor platelets and lymphocytes using flow cytometry techniques. We have enhanced both the sensitivity and specificity of our antibody assays by screening for both IgG and IgM antibodies against platelets, lymphocytes, CD8 cells (cytotoxic T-cells), and B cells. We also normalized the number of white cells captured for analysis so that we can analyze for all the above antibodies in one flow assay. We have further developed the assay so that we only require one fresh EDTA blood sample from both the recipient and donor to run the cells of interest against the recipient's banked frozen serum samples obtained pre-, during, and after their weekly donor transfusions. We believe these more comprehensive antibody assays will allow us to better select donor/recipient pairs for transfusion, and to more clearly understand the mechanisms of antibody response and correlate the antibody findings with the *in vivo* platelet transfusion results.

FACS Calibur Characterization of WBC that Remain After F-LR:

During studies of PL1B and PLS-5A filters, we noticed a difference in the amount of CD45 positive particles that passed through these two filters (CD45 is a pan white cell antibody). Much larger numbers of very small particles remained after PL1B filtration than after PLS-5A filtration of the donor dog's platelets. Two

areas of particular differences were the number of CD45 positive particles that were also DM5 (anti canine mature granulocyte) and anti canine CD4 positive.

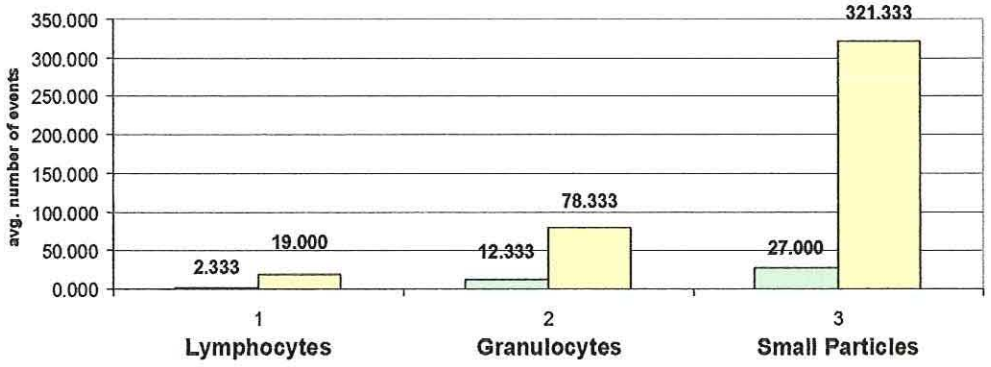
We currently are running DM5 (granulocyte antibody) in an experiment that filters the same volume of the same donor's platelets through the two different filters to directly compare the residual CD45 and DM5 positive particles based on the filter used. Each paired study was performed with blood samples drawn from three different donor dogs (Table 2)

TABLE 2

FACSCalibur Study Summary of All CD45 Positive Filtered Particles
Run on the Same Day on the Same Sample

Study No.	Filter	Fraction	Study Date	Ab	Lymph Gate	Gran Gate	Small Gate
9	PLS-5A	Filtered	08/06/09	DM5	3	14	21
10	PLS-5A	Filtered	09/10/09	DM5	2	14	49
11	PLS-5A	Filtered	09/17/09	DM5	2	9	11
Average					2.333	12.333	27.000
9	PL1B	Filtered	08/06/09	DM5	3	96	441
10	PL1B	Filtered	09/10/09	DM5	18	39	380
11	PL1B	Filtered	09/17/09	DM5	36	100	143
Average					19.000	78.333	321.333

CD45 and DM5 pos. particles through filter PLS-5A vs. PL1B



CD45 positive events are captured in gates set for lymphocytes (1), granulocytes (2), and small particles (3). These small particles may be either membrane fragments or vesicles that type as DM-5 positive. Although these small particles are not cells, they may still be immunogenic and may account for some of the differences found in the transfusion responses of recipient dogs who received donor platelets filtered with PLS-5A *versus* PL1B.

KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated that γ-irradiation alone or when combined with F-LR using either PLS-5A or PL1B filters does not prevent alloimmune platelet refractoriness.
- We have made major advances in identifying the types of residual white cells that escape filtration using either the PLS-5A or PL1B filters.
- We have substantially increased our ability to identify both IgM and IgG antibodies against dog platelets, lymphocytes, CD8 (cytotoxic T cells), and B Cells.

REPORTABLE OUTCOMES:

None.

CONCLUSION:

γ-irradiation, either alone or when combined with filtration leukoreduction, does not prevent alloimmune platelet refractoriness.

REFERENCES:

None.

APPENDICES:

None.