Use of 8-Methoxypsoralen and Long-Wavelength Ultraviolet Radiation for Decontamination of Platelet Concentrates

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Transmission of viral diseases through blood products remains an unsolved problem in transfusion medicine. We have developed a psoralen photochemical system for decontamination of platelet concentrates in which platelets are treated with long wavelength ultraviolet radiation (UVA, 320-400 nm) in the presence of 8-methoxypsoralen (8-MOP). Bacteria, RNA viruses, and DNA viruses ranging in genome size from $1.2 \times 10^5$ to $10^{10}$ daltons, encompassing the size range of human pathogens, were inoculated into platelet concentrates and subjected to treatment. This system inactivated 25 to 30 logs/h of bacteria Escherichia coli or Staphylococcus aureus, 6 logs/h of bacteriophage fd, 0.9 log/h of bacteriophage R17 and 1.1 logs/h of feline leukemia virus (FeLV) in platelet concentrates maintained in standard storage bags. Platelet integrity and in vitro function before, immediately following photochemical treatment, and during prolonged storage after treatment, were evaluated by measuring: (1) extracellular pH; (2) platelet yields; (3) extracellular lactate dehydrogenase (LDH) levels; (4) platelet morphology; (5) platelet aggregation responsiveness; (6) thromboxane $\beta_2$ (TXB-2) production; (7) dense body secretion; and (8) alpha granule secretion. These assays demonstrated that this photochemical inactivation system inactivated bacteria and viruses in platelet concentrates with minimal adverse effects on the in vitro function of platelets in comparison to untreated control concentrates maintained under current, standard blood bank conditions.

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Photochemical treatment of platelet concentrates inoculated with microorganisms for measurement of killing efficiency. The contents from three 50-mL bags of platelet concentrates (Alameda-Contra Costa County Blood Bank, Oakland, CA), each containing 3.5 to 8.5 x 10^10 platelets of the same blood type, were pooled. All platelet concentrates were subjected to routine blood bank testing procedures, and were released for experimental use 24 hours after donation. A stock of Escherichia coli, Staphylococcus aureus, R17, fd (all from ATCC, Rockville, MD) or FeLV (a gift from Dr P. Fischinger) was added to the pooled platelet concentrates to the indicated final titers. After inoculation with microorganisms, the pooled platelet concentrate was evenly divided into three portions and transferred into three PL732 bags (Fenwal Laboratories, Deerfield, IL). 8-MOP (Aldrich, Milwaukee, WI), dissolved in dimethyl sulfoxide (DMSO), was added to two bags to a final concentration of 1 mg/mL. The bags were then flushed with air, through a sterile filter system, at 2 psi for one hour followed by storage at room temperature on a tumbling platelet agitator (Model PA8, Helmer Labs, St Paul, MN). Platelet samples were taken for functional analysis before irradiation, immediately after irradiation, and at 24-hour intervals from the beginning of the treatment until 96 hours after initiation of treatment, 120 hours after donation. The control concentrate for these experiments consisted of a standard concentrate without the addition of 8-MOP, DMSO, or UVA exposure. Because the photodecontamination technique required the addition of a small amount of DMSO (0.3%) for solubilization of 8-MOP, no comparison was performed with platelet concentrates containing DMSO alone. We compared the function of PCD-treated platelets with standard concentrates. Untreated standard concentrates were handled and stored the same as the treated units except they were not exposed to UVA.

Evaluation of platelet concentrate quality and function. Extracellular pH of the platelet concentrates was measured using a standard pH meter (Model PHM82, Radiometer America, Cleveland). The platelet counts in the platelet concentrates were measured using an Elzone Particle Data counter (Particle Data, Inc, Elmhurst, IL). The morphology scores of the platelet concentrates were determined as described by Kunicki et al.15 LDH was determined spectrophotometrically using a modification of the Wacker method.34 The concentration of LDH released into the supernatant plasma was calculated from the ratio of LDH activity in 1 mL of platelet concentrate supernatant to the total LDH activity in 1 mL of Triton X-100 lysed platelet concentrate.35 Platelet aggregation was performed using a dual channel aggregrometer (Payton Associates, Inc, Buffalo) as described previously.40 A23187, a calcium ionophore (Sigma, St Louis), at 1.4 mg/mL was used to induce platelet aggregation. At the completion of platelet aggregation stimulated by A23187, the supernatant fraction was recovered by centrifugation at 2,000 g for 15 minutes at 20°C. The amount of thromboxane B2 (TXB-2) produced in response to A23187 was measured by radioimmunoassay (New England Nuclear, Wilmington, DE).

Two assays were used to measure platelet activation: dense body secretion measured by the fluorescent mepacrine dense body staining assay, and alpha granule secretion detected by the GMP-140 expression assay. Platelet dense bodies were labeled with mepacrine (50 µmol/L), a dense body-specific probe.41 Platelet dense body content was evaluated by measurement of the fluorescence distribution of mepacrine stained platelets by fluorescence activated flow cytometry with a Becton-Dickinson FACs Analyzer (Becton-Dickinson, Inc, Mountain View, CA) as previously described.42 Platelet activation also results in alpha granule secretion, which can be detected by measurement of GMP-140 expression. GMP-140 is a...
unique alpha granule membrane glycoprotein that is expressed only on the surface of activated platelets. The expression of GMP-140 was measured using the murine monoclonal antibody S-12, which has been prepared against GMP-140 and is specific for that protein. Platelets were separated from plasma using a simple discontinuous arabinogalactan gradient, followed by the addition of 0.05 μg of S-12 per 5 x 10⁶ platelets. For enhancement of sensitivity in the detection system, an F(ab')₂-biotin conjugated fragment of goat anti-mouse immunoglobulin (1.7 μg/5 x 10⁶ platelets, TAGO, Burlingame, CA) was used as the second antibody. The detection system consisted of avidin-conjugated FITC (2.5 μg/5 x 10⁶ platelets, Becton-Dickinson, Inc). Analysis was performed on 10⁶ platelets as previously described for the measurement of platelet-associated immunoglobulin.

RESULTS

Inactivation of R17, FeLV, fd, E coli, and S aureus in platelet concentrates. The photochemical inactivation kinetics of microorganisms inoculated into platelet concentrates were determined over the six hours of photoinactivation treatment for the experimental sample and the two controls that were not irradiated (Fig 1). R17 was inactivated at an initial rate of 0.9 log/h and FeLV was inactivated at an initial rate of 1.1 logs/h in platelet concentrates (Fig 1D and E). Fd was inactivated at an initial rate of 6 logs/h in platelet concentrates (Fig 1C). E coli and S aureus were each inactivated at an initial rate of at least 25 to 30 logs/h in the platelet decontamination system (Fig 1A and B). The rates of inactivation were approximately proportional to the genomic size of the respective microorganisms.

Inactivation of fd, R17, E coli or FeLV was not observed in the platelet concentrates treated with UVA only or 8-MOP only. A loss of up to 2 logs of S aureus was observed after four hours in both UVA only and 8-MOP only control samples. However, there was obvious platelet clumping in
the samples containing *S. aureus*, which presumably was the reason for the loss of *S. aureus* titer with time.

**Effects of PCD treatment on platelet yields, integrity, and extracellular pH.** No significant differences in platelet morphology scores and in platelet yields were observed between the control and treated platelet concentrates immediately after six hours of treatment (Table I). Since LDH is released from the cytoplasm into the extracellular phase when platelets are severely injured, we measured the supernatant plasma LDH levels before and after UVA treatment of the platelet concentrates as an index of cellular injury. In experiments II, III, and IV (Table I), the LDH activity was measured 24 hours after initiation of treatment rather than immediately after treatment. In both cases, there was no significant difference between the treated and untreated platelet concentrates. After six hours of UVA irradiation, the pH of treated concentrates varied from an increase of 0.06 pH units to a decrease of 0.26 pH units. In all cases the pH was maintained in the range between 7.1 and 7.7 pH units. No significant difference between the pH of treated and untreated platelet concentrates could be demonstrated immediately following PCD treatment.

**Platelet aggregation and thromboxane *β*-2 production after PCD treatment.** Platelet aggregation was measured 18 hours after completion of irradiation, 42 hours after treatment. Treated and untreated platelet concentrates aggregated in response to A23187 (14 μg/mL). Before treatment, the platelet concentrates aggregated to 56% to 76% of the maximal response with A23187; in contrast to freshly drawn control platelets, obtained from platelet rich plasma, which aggregated to 80% to 82% of maximum under similar conditions. Although the maximal aggregation response decreased after six hours of treatment and 18 hours of storage in all cases, the posttreatment samples maintained aggregation equivalent or superior to the untreated controls. Comparison of the results from five replicate experiments did not demonstrate any significant difference (Table I). At completion of aggregation, the supernatant fraction was assayed to measure TXB-2 production (Table I). Average TXB-2 production in PCD treated concentrates was 1,240 pg/mL ± 70(SD) and 1,475 pg/mL ± 192(SD) in control concentrates. In each experiment, control and treated concentrates contained equal numbers of platelets in the aggregation cuvette. Although TXB-2 production in three of four experiments was less in PCD-treated platelet concentrates, this difference was not significant (*P* = .09).

**Platelet activation after PCD treatment.** Loss of dense bodies (DB) during photochemical inactivation treatment was used as an index of platelet activation. The distribution of dense body associated fluorescence was measured as the

| Table 1. Platelet Integrity and In Vitro Function Evaluated Immediately Following Pseudal Photochemical Inactivation |
|---|---|---|---|---|---|---|---|---|
| **Expt** | **Time of Assay (h)** | **Extracellular pH** | **Morphology Scores** | **Platelet Counts (x 10^9/L)** | **% Extracellular LDH** | **% GMP-140 Activation** | **TXB-2 Production (pg/mL)** | **DB Mean Ratios** |
| | | **Cont** | **Treated** | **Cont** | **Treated** | **Cont** | **Treated** | **Cont** | **Treated** | **Cont** | **Treated** | **Cont** | **Treated** |
| I | 0 | 7.56 | 7.56 | 276 | 276 | 0.744 | 0.744 | 6 | 7.65 | 7.30 | 258 | 251 | 0.653 | 0.650 |
| | 6 | 7.48 | 7.48 | 264 | 261 | 1.07 | 1.07 | 12 | 7.38 | 7.29 | 254 | 256 | 0.938 | |
| | 24 | | | | | | | |
| II | 0 | 7.61 | 7.61 | 263 | 263 | 1.08 | 1.08 | 14 | 7.37 | 7.36 | 249 | 242 | 1.20 | 1.06 |
| | 24 | | | | | | | |
| III | 0 | 7.47 | 7.47 | 255 | 255 | 0.835 | 0.835 | 7 | 7.63 | 7.53 | 245 | 239 | 0.612 | 0.723 |
| | 24 | | | | | | | |
| IV | 0 | 7.43 | 7.43 | 264 | 264 | 0.875 | 0.875 | 8 | 7.33 | 7.45 | 266 | 237 | 0.982 | 1.02 |
| | 24 | | | | | | | |
| V | 0 | 7.29 | 7.29 | 271 | 271 | 1.68 | 1.68 | 4 | 7.17 | 7.20 | 254 | 237 | 1.61 | 1.59 |
| | 24 | | | | | | | |
| VI | 0 | 7.30 | 7.30 | 271 | 271 | 1.27 | 1.27 | 6 | 7.27 | 7.26 | 264 | 254 | 1.36 | 1.35 |
| | 24 | | | | | | | |

Expt: Roman numerals indicate individual experiments. Time of assay: 0 = the beginning of photochemical treatment; 6 = 6 hours, the completion of photochemical treatment; 24 = 24 hours after the beginning of photochemical treatment. % Extracellular LDH = the amount of LDH released is expressed as a percentage of total releasable platelet LDH. Aggregation was induced by 14 μg/mL A23187. The light transmission of platelet concentrates was initially set at 0%. The light transmission of platelet poor plasma was set at 100%. The extent of platelet aggregation was quantified by the increase in % transmittance. The level of TXB-2 production was quantified by a radioimmunoassay. DB = dense body content expressed as the normalized mean ratio. Normalized mean ratio = mean channel ratio for fluorescence: volume, normalized with respect to that obtained with fresh platelets. % GMP-140 activation = percentage of platelet population expressing the alpha granule activation glycoprotein GMP-140 as detected by S-12 monoclonal antibody. Cont = control untreated platelet concentrates. Treated = platelet concentrates treated by 8-MOP/UVA for 6 hours.
mean channel of the ratio of fluorescence intensity to volume distribution. The ratio of fluorescence to volume was used instead of fluorescence alone in order to compensate for changes in platelet autofluorescence due to alterations in platelet volume, which occurred independent of dense body staining or secretion. In the absence of DB secretion, increases in platelet volume, due to platelet stimulation results in small increases in platelet fluorescence. The fluorescence to volume ratios for experimental samples were compared to those for platelets isolated from freshly drawn whole blood obtained at the time of the assay. The latter were used as the closest approximation to native platelets. Values for experimental samples were divided by those for the fresh platelets to obtain a normalized mean channel ratio in comparison to native platelets. Because the assays for DB content must be performed immediately, different fresh platelet-rich plasma controls were used at each time point and the results between experiments were compared using a paired t-test. At the initiation of treatment there was no significant difference between control and treated platelet concentrates when the bags were sampled immediately after addition of 8-MOP but before UVA irradiation. The average mean ratio for control concentrates was 0.895 ± 0.229(SD) and for treated concentrates 0.895 ± 0.229. After PCD treatment and storage for 18 hours (24 hours after initiation of treatment), the average mean ratio for control platelets was 0.960 ± 0.339(SD) and for treated concentrates 0.943 ± 0.341(SD). This difference was not statistically significant.

The level of GMP-140 expression before and following photochemical inactivation treatment was used as an additional index of platelet activation. The degree of platelet activation was expressed as a percentage of platelet population expressing GMP-140 detected by S-12 monoclonal antibody. Freshly drawn platelets from normal subjects (N=14) demonstrated an average of 3.7% ± 1.0(SD) activated platelets. After exposure to bovine thrombin (0.005 IU/10⁶ platelets), more than 75% of platelets were activated as indicated by GMP-140 expression. This assay appears to be extremely sensitive for detection of activated platelets, although others have also observed that not all platelets express GMP-140 after activation with potent agonists. Platelet concentrates (N=6) before treatment, 24 hours after donation, exhibited an average of 33.6% ± 12.6(SD) activated platelets. Control and treated platelet concentrates were sampled 18 hours after completion of treatment and 24 hours after initiation of treatment. The average proportion of activated platelets in the control concentrates increased to 44.6% ± 12.8(SD), while the treated concentrates demonstrated an increase to 54.3% ± 10.6(SD). This difference achieved a low level of statistical significance (P <.02) by paired t-test analysis.

Properties of PCD-treated platelet concentrates after further storage. Additional experiments were performed to determine the effects of storage on platelet concentrates following photochemical decontamination. Eight different measurements of platelet concentrate quality were measured throughout 96 hours of storage of PCD treated and control concentrates (Fig 2). Platelet concentrates were stored at ambient room temperature (20°C-23°C) on an end-over-end rotating device. The pH of the control concentrates generally increased with storage time, while the pH of the corresponding treated concentrates decreased slightly with time, but remained within an acceptable range. The gradual increase in the pH of the control concentrates with prolonged storage was unexpected, and may have been augmented by the effect of flushing the bags with air after the initial six hour treatment period. Morphology scores, platelet yields, and extracellular LDH concentration of PCD-treated concentrates was comparable to that of standard concentrates throughout the storage period (Fig 2B and C). Similarly, although the aggregation response of PCD-treated concentrates decreased with storage time, it remained equal to the response of standard concentrates. After 48 hours of storage at room temperature, the average amount of TXB-2 produced in response to A23187 stimulation was greater in control platelets than in treated platelets (P <.05), but in one experiment TXB-2 production of treated platelets was greater. PCD-treated platelets exhibited more aggregation after 48 hours of storage than the control platelets based on the GMP-140 platelet activation assay, but this difference was not significant (Fig 2 G). There was no difference in DB content between PCD treated and control concentrates during 48 hours of storage. Both PCD treated and control platelet concentrates deteriorated generally with storage. The rate of deterioration of treated platelet concentrates, however, was not greater than that of the untreated controls.

DISCUSSION

The overall purpose of this study was to develop a method for viral and bacterial decontamination of platelet concentrates. We sought to develop a method capable of inactivating a wide range of viruses and bacteria in platelet concentrates while preserving in vitro platelet function at a level comparable to standard platelet concentrates. We have evaluated the efficacy of a psoralen photochemical inactivation system for the decontamination of platelet concentrates using a group of model microorganisms with genomic sizes that encompass the spectrum of common human pathogens. FeLV is a single-stranded RNA virus with a genome of 3 x 10⁵ daltons. Bacteriophage fd has a genome of 1.6 x 10⁶ daltons of single-stranded DNA. E. coli and S. aureus have double-stranded DNA genomes of 10⁶ daltons. Bacteriophage R17 has a genome of 1.2 x 10⁶ daltons of single-stranded RNA. These studies have shown that UVA irradiation in the presence of 8-MOP can effectively inactivate E. coli, S. aureus, fd, R17, and FeLV in platelet concentrates. The rate of inactivation of these microorganisms was found to be in the following order: E. coli, S. aureus > fd > R17, FeLV. Accordingly, the 8-MOP/UVA process should be effective in inactivating other viral contaminants such as HBV, HIV, or CMV in platelet concentrates, since HBV has a partially single-stranded and partially double-stranded DNA genome of 1.8 x 10⁶ daltons. HIV has a single-stranded RNA genome of 3 x 10⁶ daltons and CMV has a double-stranded DNA genome of 1.6 x 10⁶ daltons.

Our results are in accord with our earlier finding that 8-MOP has a lower binding affinity for RNA than DNA and...
for single-stranded nucleic acid than double-stranded nucleic acid, and 8-MOP has a low binding affinity for small genomes. The present studies also indicate that 8-MOP photochemistry in platelet concentrates is similar to that in buffered solutions inoculated with microorganisms. Photochemical inactivation, using conditions similar to those of the current study, has been demonstrated for enveloped and nonenveloped animal viruses with RNA and DNA genomes.49,50

Most importantly, the inactivation of these model bacte-
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...similar order to that observed for DB nucleotide content in during treatment, which would preclude multi-day storage count, LDH release, aggregation responsiveness, or platelet effects of DMSO alone on platelet function in the present study. Platelets were found to be slightly more activated than platelet concentrates were evident by this assay. Treated platelets with scores of 200 and higher have been reported to be functional in this range. The magnitude of DB loss 72 hours after donation in the present study is of a function of storage time. The magnitude of DB loss 72 hours after donation in the present study is of a... 

The present studies have shown adequate preservation of platelet function, measurement of platelet survival, recovery and hemostatic efficacy of treated platelet concentrates will have to be demonstrated to document preservation of platelet function. In the present investigation, we have only explored a single set of PCD conditions, and it will be important to determine if this decontamination system can be improved by using other psoralen derivatives such as 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) or 4,5'-8-trimethylpsoralen (TMP).

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