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EVALUATION OF DRIED STORAGE OF PLATELETS FOR TRANSFUSION:
PHYSIOLOGIC INTEGRITY AND HEMOSTATIC FUNCTIONALITY.

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Attachment: Report from subcontract principal investigator, Marjorie S. Read, Ph.D.,
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Administrative Activity:

On August 20, 1993, a meeting (site visit) was held in Dr. Read’s laboratory at Chapel Hill involving representatives of Armour Pharmaceutical Co., Research and Development Division, Scott Murphy, M.D., acting as independent advisor to Armour, and Alan Rudolph, Ph.D., of the Naval Research Laboratory (ONR). The meeting consisted of a review of all project findings to date (1989 - present) to evaluate the prospects of commercialization of dried human platelets for transfusion. Data was presented to show the typical yields, retention of platelet integrity and function, and possible in vivo application. The discussion that proceeded was generally very favorable and supported a progression toward GMP/GLP large-scale production of pharmaceutical grade human platelet preparations as a next step. We view this outcome as a validation of our efforts.

Scientific Progress:

In anticipation of the process adaptations that may be necessary for large-scale, GMP production of lyophilized platelets, we have examined our current research procedure step-by-step for its effect on platelet reactivity and integrity. Three attempts were made to perform the stabilization, washing, and final resuspensions completely in the closed sterile system of a Fenwal blood collection triple-pack bag set. Clumping of platelets after the stabilization step prevented adequate yields unless there were multiple entries into the platelet bag with washing buffer. Therefore, further development along these lines will be discussed with technicians at Armour Pharmaceutical to define a reasonable approach to use of a closed system. As an alternative, we also went to the local hospital’s gas sterilization unit (ethylene oxide at 60 - 80°C) in order to sterilize platelet preparations produced in our "open" research process (capped centrifuge tubes as opposed to sterile blood bag packs). The gassed preparations lost hypotonic shock response, had no aggregation reactivity, and reconstituted at a 20 - 30% lower yield. This option seems infeasible.

Previous results have shown that the light fixation used in our stabilization process prior to freeze-drying does not completely abrogate responsiveness of the platelets upon rehydration. However, we have now examined platelet responsiveness after various stabilization permutations prior to freezing and drying. Even after only 2 minutes exposure of fresh washed platelets to 1.8% paraformaldehyde, or 5 minutes exposure to 0.002 - 0.02% permanganate, the platelets lost all aggregation response to ADP or collagen and lost half to 2/3 of the hypotonic shock response. The washed platelets prior to stabilization already showed reduced response relative to fresh citrated platelet-rich plasma. Yet, the rehydrated platelets appear to be more responsive that the above. We conclude that to some extent the effects of the fixation step are reversible after the lyophilization and rehydration process.

Other details of processing under evaluation currently include physical parameters of lyophilization. Temperature probes were placed inside vials with platelets during the standard drying cycle in our Virtus 600 Unitop to monitor equilibrium with the lower stage condenser. Even after 120 hours run time, the
temperatures between sample and condenser never equalized. Analysis of residual water was not available, but the yield upon rehydration was less than optimal at 120 hours drying time versus the usual empirical endpoint of 60 - 80 hours (powder in vial appears cracked and fluffy). More work needs to be done to optimize lyophilization conditions, and it would help this effort to have a determination of water content in the dried state. We are pursuing this with Armour.

One of the techniques we propose to use to evaluate activation of rehydrated platelets is detection of calcium flux with cytoplasmic probes like aequorin. After much effort with several published methods, we have now successfully demonstrated loading of rehydrated para-platelets with sufficient quantities of aequorin in DMSO to produce a measurable signal. The actual activation experiments are still pending.

For long-term storage experiments, two permanganate platelet preparations (0.005% and 0.02%) were made recently and put away dried in small aliquots at room temperature (R.T.), 4°C, or -70°C. The 0.005% perm-platelets were prepared from lipemic plasma and showed a poor yield (39%) and compromised reactivity at first workup, therefore these vials were discarded. The twelve month workup of para-platelets was completed in this reporting period. The data strongly suggest that storage of dried platelets at room temperature is inadequate to prevent significant changes over 12 months. Besides the loss of binding of antibodies to GPIb and GPIV at R.T. documented at 9 months workup in our last report, we now found deterioration of hypotonic shock response and ristocetin-induced aggregation: the R.T.-platelets had half as much shock-response as platelets stored at 4°C or -70°C, and had no response remaining to ristocetin as opposed to a normal response in the other platelets. Although the data do not indicate a significant difference in results of storage at 4°C versus -70°C, storage at R.T. should be ruled out as an acceptable means of preservation of dried platelets.
Report Period June 1, 1993 - September 30, 1993
University of North Carolina at Chapel Hill

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Principal Investigator: Marjorie S. Read, Ph.D.
Co-PI: Robert Reddick, MD
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Studies Conducted during the reporting period.

1. **To evaluate the effects of rehydrated platelet transfusions on intravascular clotting.** (Specific Aim 2). We have reported that fresh washed platelets and fixed platelets have similar plasma and platelet antigens on the surface of both types of platelets as measured by specific antibodies. In vitro tests show no enhanced activation of coagulation factors with incubation of plasma and rehydrated platelets. We have also shown that lyophilized platelets generate thrombin at a faster rate than unactivated fresh platelets in the Prothrombinase Complex reaction. Thrombin generation in that assay was dependent on the concentration of platelets. Saturation of the complex occurred with fewer rehydrated platelets than fresh platelets.

   In spite of apparent increased rate of thrombin generation by rehydrated platelets, tests of plasma and serum samples collected from two dogs transfused with rehydrated platelets showed no significant change in factor VIII or FIX levels. The prothrombin times were not different from normal dogs, and there was no detectable change in fibrinogen, no significant change in thrombin clotting times, and no measurable production of fibrinogen degradation products (FDP) as measured by the Thrombo-Welco Test. The partial thromboplastin time test (PTT), a measure of the intrinsic coagulation mechanism was slightly longer than the normal. Factor VIII assays were not conclusive and can not be repeated due to loss of sample.

   These data indicate that there is no resulting pronounced disorder of the hemostatic mechanism in dogs receiving rehydrated platelets under the conditions of our experiments. Since the Thrombo-Welco test was negative, we assume the absence of fibrin monomer and thus no production of circulating thrombin.

   Studies on the lysis of fibrin clots formed in the presence of rehydrated platelets remain to be done.

2. **To evaluate the immune response of dogs to multiple transfusions of dried, rehydrated platelets.** (Specific Aim 3). Studies of the antigenicity of rehydrated platelets are underway. During this reporting period two normal dogs have received multiple transfusions (4 transfusions per dog for a total of 8 transfusions). Each infusate was a mixture of rehydrated platelets prepared from two unrelated dogs. Blood samples were collected from each dog pre- and post-transfusion and tested for RBC, WBC, platelets, diagnostic-multi-chem profile, electrolytes, general chemistries and enzymes. These data are still being tabulated and evaluated. Briefly, WBC count, respiration and temperature varied from transfusion to transfusion. Both dogs showed evidence of allergic response after the fourth infusion that was relieved with benadryl. Neither dog received a fifth transfusion. Our studies to date tend to indicate that in the first dog, bovine serum albumin (BSA) was not completely removed by the washing prior to transfusion. Additional washes were done before treatment of dog 2 and no changes were noted in dog's appearance or behavior until the fourth transfusion. Following the fourth transfusion, the dog demonstrated characteristic allergic response which was treated and relieved with benadryl injections.

   The two animals in this study were pups, 12-14 weeks of age. There were no remarkable differences in the chemistry profile except those seen in
young dogs. Dog 1 received platelets stored in bovine serum albumin and washed twice to remove albumin. 20 minutes post infusion of platelets ($10.8 \times 10^6$), the dog became lethargic and weak but recovered without treatment. We suspected contamination with BSA. The same animal was infused twice more with the same preparation of platelets but with additional washes to remove BSA. No abnormal response was noted. However, on the fourth infusion of rehydrated platelets the dog showed signs of discomfort and was treated and relieved by benadryl. No further infusion were given this dog.

A second pup was treated following the same protocol as above. No reactions were noted until the fourth infusion of rehydrated platelets when a similar allergic reaction as seen with dog 1 was noted. Dog 2 was treated and relieved with benadryl and epinephrine. No further infusions were given.

Post transfusion chemistries were not different from pre-infusion data. The most remarkable and troubling response was a drop in WBC count and a drop in platelet count immediately post infusion. This was noted in both dogs. We do not know the significance of this change. Not enough rehydrated platelets were given to significantly increase the platelet count. Since the rehydrated platelets were not labeled, we can not know if native platelets or rehydrated platelets were sequestered.

Studies are in progress on the recipient dogs’ plasma, serum and platelets for production of antibodies against native and/or rehydrated platelets, BSA, etc. We are looking for any change in the surface related globulin and bound protein of the dogs native platelets. To date we have found a low titer BSA antibody by ELISA. Rehydrated platelets stored in BSA and repeatedly washed still show a BSA band on PAGE. These data indicate that platelets prepared for transfusion into dogs should not be stored in BSA. Data relative to antibody production is still under study, but neither dog has developed thrombocytopenia.

Two additional dogs will be studied using the above protocol but with rehydrated platelets prepared and stored in canine serum albumin.