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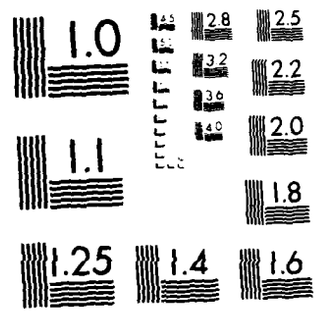

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Enzymatic Conversion of Red Cells for Transfusion

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Enzymatic Studies

Alpha-Galactosidase

In order to perform the inital pre-clinical and clinical studies referred to in the Renewal, large quantities of alpha-galactosidase will be needed. To provide for this we have developed our own procedure for the isolation and purification of coffee bean alpha-galactosidase using both classical and affinity chromatographic methods. On the order of 5,000 units of crude enzyme can be extracted from 1 kg of green beans. Initial studies indicate that at least 70% of the crude material is recoverable in purified form essentially devoid of contaminating exoglycosidase and protease activities. The purified enzyme exhibits the same kinetics for the removal of B antigenicity from the surface of the red cell as the enzyme we have used previously which we purified from a starting commercial preparation. Type B cells converted to type O by this enzyme do not exhibit any membrane stickiness, are not polyagglutinable and are not lysed during treatment in numbers greater than observed with our previous enzyme preparation (1% or less). Thus these results indicate that both our purified enzyme preparation obtained de novo from coffee beans and the one we purified from a commercial preparation act upon B cells in the same way to produce the same converted type O erythrocytes.

Alpha N-acetylgalactosaminidase

We have been searching in both the animal and microbial kingdoms for the appropriate alpha N-acetylgalactosaminidase (A-zyme) needed to convert group A cells to group O. With regard to the former, we are currently working with an enzyme present in the limpet Patella vulgata. This enzyme has been reported to destroy A blood group activity present in blood group substances when used at pH 4. However, it becomes progressively inactive with increasing pH as a result,

it is thought, of the enzyme's dissociating into subunits. We have isolated the enzyme and have shown that it can remove A activity from preparations of red cell membranes at pH 4.

It can also inactivate A blood group activity from blood group substance at pH 5.7 although it appears to retain only about 10% of the activity it has at pH 4. We do not as yet know whether this enzyme will remove A activity from red cell membranes at pH 5.7. We have prepared a derivative of dextran which allows us to bind the intact enzyme to dextran at pH 4. Under these conditions, the molecule should be unable to dissociate into subunits and thus will presumably retain activity at the higher pH needed to remove antigenic activity from red cells while maintaining their viability. Using this approach we have been able to couple some of the enzyme to dextran, however, thus far no reduction in the loss of enyzyme activity with increasing pH has been obtained. Further binding and crosslinking studies are projected.

Our starting point in our search for A-zymes in the microbial kingdom has been the isolation and cultivation of soil microorganisms. We are screening bacteria of known pedigree as well as bacteria selected randomly from samples of soil. We have found many strains which can hydrolyze synthetic substrate specific for A-zyme activity. Enzymes isolated from a few of these also appear to destroy A blood group activity associated with blood group substances. We are now in the process of determining whether these putative A-zymes are active against red cell membrane group A antigenicity. At the same time, we continue to screen other types of microorganisms for A-zyme activity.

In Vivo Survival Properties of Enzymatically Converted Type B Human Erythrocytes

Table 1 summarizes results obtained from various in vitro studies used as indicators for possible changes in the membrane integrity of enzyme treated cells. As shown in the first column, less than 1% of the cells lyse as a result

of treatment, most of the lysis being due to the end over end mixing procedure we employ. Furthermore, treated cells do not exhibit any membrane stickiness since they resuspend in the same manner as untreated cells following centrifugation, and are not agglutinated by their own serum or the sera of A or O individuals.

When fragility studies are performed with such cells the results tabulated in the next column are obtained. A comparison of the % sodium chloride producing 50% hemolysis of treated cells, with cells taken from plasma reveals that those treated with free enzyme, buffer and enzyme dextran conjugate are not significantly more susceptible to osmotic shock. The fragility curves themselves as reported last year also indicate that no select portion of the cell population has been rendered more fragile. We have searched for changes in membrane structure which could result from enzyme treatment, possibly due to perturbation of the cell membrane surface as a result of removal of terminally alpha-linked galactose residues. These would come not only from B antigens but also from P_1 antigen, if present, and trihexosyl ceramide which is a constituent of all erythrocyte cell surfaces. We used as markers acetylcholinesterase which is bound to the outer membrane surface and cholesterol which is involved among other things, in the maintenance of proper membrane fluidity. As can be seen from the results in the third and fourth columns, the levels of both are essentially unchanged following enzyme conversion.

As is shown in Table II, we have also looked for changes in the metabolic capabilities of converted cells. Both ATP and 2,3 DPG levels of enzymatically treated cells are at least 90% of that of untreated cells at 1-1 1/2 hrs. incubation; the time used for our <u>in vivo</u> studies. Methemoglobin formation under our treatment conditions as is shown in the third column, is minimal and, as is seen in the last column, enzyme treatment does not lower the converted

cells partial oxygen pressure value nor, as not included here, change the shape of its oxygen dissociation curve. The results presented in Tables I and II demonstrate that enzymatic conversion does not impair cell deformability nor normal oxygen binding and exchange.

Based on these membrane and metabolic results, and the successful in vivo studies we reported last year, when enzymatically converted gibbon erythrocytes were found to have normal survival rates after being returned to the original donor animals, we felt we had provided the basis for performing in vivo survival studies with converted human erythrocytes. For this initial study, 3 volunteers, group B, O and A individuals in good health, were used.

They were selected on the basis of preliminary studies that established Rh compatibility (Rh+), the lack of high titer antibodies to minor blood group red cell antigens and the absence of detectable antibody activity toward the alpha-galactosidase preparation to be used for removal of group B antigenicity. The next step was to treat a small amount of cells obtained from our group B volunteer with alpha-galactosidase. These converted cells were tested against the group B donor's own fresh serum as well as similar sera from the group O and A individuals using the customary pre-transfusion serological procedures at room temperature and 37°. We could not detect any agglutination or hemolysis indicating that in vitro compatability had been achieved and that these volunteers could be used to determine the in vivo survival characteristics of enzymatically treated cells. Approximately 30 ml of blood were collected using citrate phosphate-dextrose as anticoagulant (sterile procedures and solutions were used throughout) centrifuged and plasma and buffy coat carefully removed. The packed cells were resuspended in normal saline (0.15 M NaCl) centrifuged, and the topmost layer of erythrocytes aspirated to remove any remaining white cells and platelets. This was repeated once with 4.2 ml of cells which were

then resuspended in 10 ml of normal saline and labeled at 95% efficiency by adding 200 μCuries of isotope and incubating at room temperature for 15'. Cells were washed 3 times by centrifugation and resuspension in normal saline and then 3 times at 5'intervals in phosphate-citrate saline (PCS) pH 5.7 to adjust their pH to that of the buffer. Packed cells (3.2 ml) were then mixed with 2000 units of alpha-galactosidase in PCS to a final volume of 11.2 ml and 350 μl were removed to a pilot tube to provide samples for monitoring the removal of B antigenicity. Both reaction mixtures were incubated at 26°, aliquots removed at various times from the pilot tube, washed and tested for agglutinability using a 5% suspension of cells and 2 drops of commercial human anti-B antisera or group A cell absorbed A, B antisera. The mixture was allowed to stand at room temperature for 10 minutes, was centrifuged for 1' at 3200 RPM and then examined for agglutination microscopically. At 60 minutes the cells were no longer agglutinable, however, since the limit of detection of the agglutination reaction is believed to be a approximately 2000 antigenic sites per cell, the incubation was continued for 30' in order to insure that all susceptible group B antigens were converted to H activity. We then tested an aliquot of cells from the primary reaction mixture and obtained the same results. The enzyme solution was removed following centrifugation and the packed cells were washed four times with 45 ml of phosphate buffered saline (PBS) to remove any remaining traces of the enzyme preparation and allowed to remain 15' in the last wash to insure equilibration to the pH of this buffer, pH 7.3. Following centrifugation, the cells were resuspended in 12.5 ml of PBS to a final volume of 15.5 ml. Three aliquots of 5 ml each containing about 1 ml of converted cells were taken up in preweighed syringes and the weight of cells to be administered was measured. Transfusion was performed quantitatively be several back and forth rinsings of each syringe with the recipient's blood. An aliquot of the remaining 0.5 ml of

cells was used as a standard to determine the total amount of radioactivity administered (Footnote, Table III).

Blood samples were taken at various times up to 49 days and their radioactivity measured in order to determine the survival of the transfused cells. Using the data obtained from the first blood samples which were taken fifteen minutes following transfusion, we calculated the respective blood volumes of the 3 participants in the study (Table III) and found them to be within the reported average normal values. The results which are essentially a reflection of the amount of radioactivity initally administered as compared with the amount found in the circulation are very similar for all 3 recipients. Although there is always the possibility of a minimal amount of initial loss due to experimental manipulation, the similarity of our values indicates that no selective destruction of enzymatically converted cells by the O and A recipients' immune systems had taken place in the first few minutes following transfusion. The amounts of radioactivity found in samples of whole blood taken at 15, 30 and 60 minutes were essentially unchanged (Table III) and the plasmas of these samples were devoid of ^{51}Cr, demonstrating that rapid destruction of converted cells was not occurring at these time points. These values were averaged and taken to represent 100% survival.

As indicators that enzymatically converted cells were surviving normally in the circulation, we found the following. First, that over 95% of the converted cells are present in the circulation after 24 hours (Table III). Second, that the number of days when 50% (T50) of the converted cells still remain in the circulation ranged from 30-33; times which are in accord with published values for normal survival. Finally that the shapes of the three ^{51}Cr survival curves (Fig. 1) are essentially the same and were not altered during the course of the study, that is, did not "collapse" or show a marked downward shift in slope

which would have indicated an increase in the rate of cell destruction presumably due to an immune response. As further evidence for this, one other set of samples were taken at day 90 and their values fit very nicely to these curves.

These results are the first indication that small quantities of enzymatically converted group B erythrocytes can survive normally in recipients whose immune systems would not tolerate untreated cells. Studies are now planned to determine whether converted cells can be immunogenic when administered in repeated doses or given in larger amounts.

TABLE I

MEMBRANE INTEGRITY OF ∝-GALACTOSIDASE-TREATED HUMAN ERYTHROCYTES

TREATMENT CONDITIONS	MAXIMAL HEMOLYSIS	OSMOTIC FRAGILITY 50% HEMOLYSIS POINT	AChE	CHOLESTEROL
	(%)	(% NaCl)	(%)	(%)
UNTREATED CELLS	-	0.42	100	100
BUFFER	<1.0	0.43	102	98
∝-GALACTOSIDASE	<1.0	0.43	99	102
∝-GALACTOSIDASE-DEXTRAN	<1.0	0.45	99	-

TABLE II

METABOLIC INTEGRITY OF α-GALACTOSIDASE-TREATED HUMAN ERYTHROCYTES

TREATMENT CONDITIONS	ATP (%)	2,3-DPG (%)	METHB (%)	P_{50} (MM HG)
UNTREATED CELLS	100	100	0.6	22.5
BUFFER	97	86	1.3	22.1
α-GALACTOSIDASE	92	91	-	23.7
α-GALACTOSIDASE-DEXTRAN	99	90	-	-

Table III

Blood volume and survival time measurements for ^{51}Cr-Labeled enzymatically converted group B erythrocytes after transfusion to group O, A and B recipients (uncorrected for ^{51}Cr elution).

Recipient's Blood Group	O	A	B
Blood Volume (ml/kg body weight)[1]	59	60	58
30 min survival (%)[2]	99	98	98
60 min survival (%)	98	99	100
24 hr survival (%)[2]	97	97	
T50 (days)[3]	31	30	

1. Blood volumes were determined as follows:

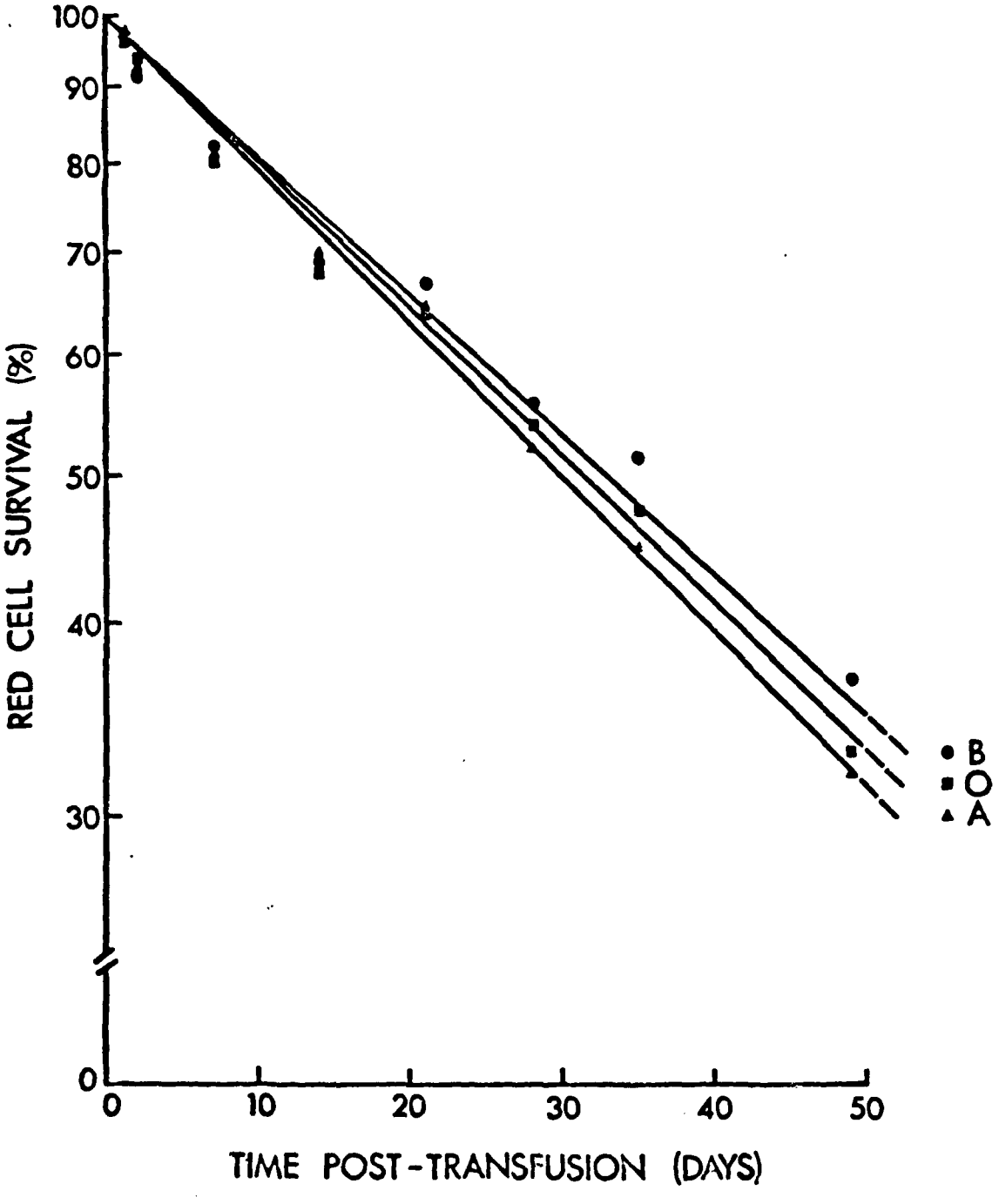
$$\text{Total Radioactivity (CPM) Transfused} = \frac{\text{CPM of Standard X wt of RBC Transfused}}{\text{wt of Standard}}$$

$$\text{Blood Volume (ml/kg)} = \frac{\text{Total CPM Transfused}}{\text{CPM/ml Whole Blood (from 15' sample)/body wt (kg)}}$$

2. The 30' and 60' survivals were calculated using 15' values to represent 100% survival. To obtain the % survivals the 15', 30' and 60' values were then averaged and used to represent 100%.

3. T50's were determined from Fig. I.

Fig. 1. Survival of ^{51}Cr labeled enzymatically converted group B erythrocytes after transfusion to group O, A and B recipients (values uncorrected for ^{51}Cr elution).



Publications List

"Removal of Erythrocyte Surface Antigens by an α-Galactosidase and its Dextran Conjugate," J. Goldstein, J.Y. Kuo, L. Lenny. Joint Meeting of the 18th Cong. International Soc. of Hematology & 16th Cong. International Soc. Blood Transfusion, Montreal, Canada. Abstracts. p. 298, #1634, 1980.

"Nonantigenic Red Blood Cells". J. Goldstein in "Current Concepts of Combat Casualty Resuscitation Symposium". In Press.

"Enzymatic Removal of Blood Group B Antigens from Gibbon Erythrocytes". L.L. Lenny and J. Goldstein. Transfusion, 20, 618, 1980.

"Normal Survival of Enzymatically Converted Group B Erythrocytes in O, A and B Recipients." J. Goldstein, G. Siviglia, R. Hurst, L. Lenny and L. Reich. Transfusion 21, 602-603, 1981.

"Enzymatic Conversion of B Erythrocytes to Group O Yields Normal Survival in O, A and B Recipients." J. Goldstein, G. Siviglia, R. Hurst L. Lenny and L. Reich. Science, 1981. In Press.

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