Presentation by Dr. Robert Keller – 1998 Conference on the Laboratory Science of HIV

ABSTRACT

It has been previously demonstrated that HIV infection is associated with reduced levels of intracellular GSH. This reduction favors vial replication and disease progression. Longitudinal assays of intracellular GSH levels in peripheral blood mononuclear cells [PBMC] can reveal trends that may be reversed through the administration of supplements capable of restoration of GSH levels.

Heparinized peripheral blood [PB] specimens were immediately placed in small portable ice baths and held in this state until PBMC Ficoll-Hypaque enrichment is performed. As intracellular GSH concentrations are volume dependent, the Bayer Technicon H.3RTX automated hematology system was used to both quantitate and characterize the composition of the PBMC populations with respect to cell volume and type. PBMC samples were standardized at 5 x10 to the 6^{th} exponent cells/ml and treated with meta-phosphoric acid to form a cryoprecipitate. At this point, specimens may be held indefinitely at -85 degrees C, which permits batch analysis. Duplicate specimen aliquots were assayed in a 96 well microtiter plate using glutathione oxidoreductase and NADPH. Color development is monitored ever two [2] minutes for a total six [6] minutes using the Bio-Tek EL808 Ultra microplate reader. Patient specimens were compared to a working calibrator curve. Low, normal and elevate patient cryoprecipitates were pooled to form controls. Results are reported in nm x 10 to the 6^{th} exponent cells/L [reference range: 1000 – 1500 nmol x 10 to the 6^{th} exponent cells].
Lineage and subset specific GSH levels were obtained in the same fashion by inserting a magnetic negative selection step after the initial PBMC FH isolation and prior to cryoprecipiation.

RESULTS:

This assay measures total glutathione [thiols] and includes all forms of glutathione [thiols]; it is the sum of reduced [GSH] and oxidized GSSG glutathione and mixed disulphides [GSSR and ProSSG]. Non-protein-bound glutathione is the sum of GSH, GSSG and GSSR.

Before releasing this assay to the IBT Clinical Division we documented the importance of the conditions of specimen holding prior to the preparation of the cryoprecipitate. We also noted that in PB specimens that were not held in an ice bath prior to FH enrichment, the values tended to rise rather than fall as compared to freshly collected, immediately enriched parallel samples and those held in the ice bath. This may be explained in part y the increased frequency of neutrophilic granulocytes which may occur in older PB specimens subjected to FH enrichment. Are must be taken to return these ice-bath held specimens to room temperature [20 – 22 degrees C] prior to the FH enrichment step due to the effects of temperature on the specific gravity of the FH solution.

The Technicon H.3 RTX [Bayer, Tarrytown, NY] automated hematology system was used to characterize the cells of the PBMC population. Small and medium lymphocytes were contained within the rectangular are to the lower left with larger reactive lymphocytes in the are a above commonly referred to as the LUC region [large, peroxidase negative cells]. Monocytes, being weakly peroxidase positive generated signals in the triangular are to the right of the LUCs. During the separation process it is common for some monocytes to activate and enlarge their volume generating signals which fall in the upper neutrophil map Figure 1 illustrates a typical differential performed on a FH enrichment PBMC population. To best fit the algorithm generated cluster boundaries the gains on the peroxidase X and Y axis were increased from those used in the performance of a routine CBC/DIFF.

In developing the reference range for this assay we initially collected fasting PB specimens, however, with the passage of time using our collection and holding methods we have determined that PB specimens may also be collected in a non-fasting state without significant change as long as they are subjected to the appropriate holding conditions. The current reference range was generate by non-fasting adult reference donors [N = 33].

We have employed this assay in a longitudinal manner on patients [N=317] with a variety of impaired immune function conditions primarily HIV associated but including hepatitis C and chronic immune dysfunction syndrome patients over a period of 9 months at the time of this writing. The results have reflected for the most part the GSH deficiencies described in the literature in HIV-infected individuals not receiving supplemental therapy for intracellular GSH. In individuals diagnosed in early periods of infection, the GSH values in some cases were well above our reference range.

In the figures representing selected patients you will note that the typical frequency of small/medium lymphocytes is between 15-20%. Because size [volume] is a key factor in tGSH determination, knowledge of the cell volume profile of each sample is invaluable in interpretating change.

In Figure 2, a HIV+ individual, note the low PBMC tGSH values prior to the institution of the nutritional supplement indicated by the arrow. The progressive increase in intracellular tGSH reflects the effects of this therapy in this patient. Figure 3 also reflects the profile of a HIV+ patient. Again, nutritional therapy was initiated in June of this year. Figure 4 reflects the changes in a patient who initiated in June of this year. Figure 4 reflects the changes in a patient who initiated the nutritional therapy 3/98 taking twice the recommended dose. Upon feeling better he discontinued the supplement. Figure 5 illustrates a transient increase followed by decrease during a severe infection.

METHODS and MATERIALS:

Heparinized peripheral blood [PB] specimens were immediately placed in small portable ice baths and held in this state until PBMC Ficoll-Hypaque enrichment is performed. In most HIV+ individuals 10 to 12 ml of blood is adequate for this assay.

Prior to the Ficoll-Hypaque enrichment the B-D green top vacutainers were removed from the ice bath, placed on a rocker and brought to ambient room temperature. FH of 1.077 SG was employed using the underlay technique and a 30 min. centrifugation step at 400 xG at 20 degrees C in a temperature controlled centrifuge. PBMCs collected at the interface were washed 1X with cold [4 degrees C] DPBS [calcium /magnesium free] suspended in 2.0 ml of cold [4 degrees C] DPBS and vortexed. A 0.5 ml aliquot was removed for quantitation and morphologic identification using the Bayer TehniconH.3RTX automated hematology system. Specimens containing greater than 5% neutrophils were subjected to the FH step a second time which in all cases to date resolved any granulocyte contamination.

The cell volumes were established through the use of Size Calibration Standards [Flow Cytometry Standards Coportation, San Juan, PR] by plotting the relative channel number [linear or log] of the peak maximum against the appropriate size value of the microbed standards and assigning each channel of the Y [volume] axis on the H.3RTX a fLvalue.

PBMC samples were standardized at 5 x10 to the 6 power cells/ml, 200 ul removed and treated with metaphosphoric acid [MPA] to form a cryoprecipitate. Cryoprecipitates were then held at -85 degrees C for batch assay. In those instances wherein extreme lymphocytopenia occurred the PBMCs collected were diluted in a lesser total volume to equal a final concentration of 5 x10 to the 6^{th} power cells/ml. The sensitivity of the assay is such that the cell concentration can be lowered to 2.5 x10 to the 6^{th} power cells if necessary.

Immediately prior to analysis the sample MPA extracts were diluted 1:20 with assay butter [100 mmol/L Na 2 PO4 and 5 mmol EDTA adjusted to pH 7.5 with 1 molar NaOH. 50 ul of the MPA extract from each patient is assayed in duplicate in a 96 well microtiter palte [flat-bottomed]. Calibrators and controls are also run in duplicate. 50 ul of 1.26 mmol DTNB [5,5'-dithiobis-(2-nitro-benzioc acid) is added to each well followed by 50ul of glutathione oxidoreductase solution and the plate is allowed to stand for a minimum of 5 min. at 22 degrees C. The reaction is initiated by the addition of 50ul of NADPH solution to each well. Timing is critical. Within the next 30-120 seconds after addition of NADPH the plate is transferred to the reader [Bio-Tek EL808].

The plate is read at 2 minutes intervals for a total of 6 min. at 410 nm. The samples are compared to the calibrator curve, the duplicate results averaged and reported. CVs on duplicate assays should be in the range of 1-5.2% [mean 3.9%] for the assay. Specimens with CVs greater than 10% are repeated.

DISCUSSION:

Glutatione is important in the defense against oxidative stress and plays a key role in the maintenance of cellular thiol redox status. Because it is conjugated to many xenotiocs and is essential for the optimal functioning of numerous enzymes is is critical for cellular viability in general and lymphocyte function in particular. Several groups have reported

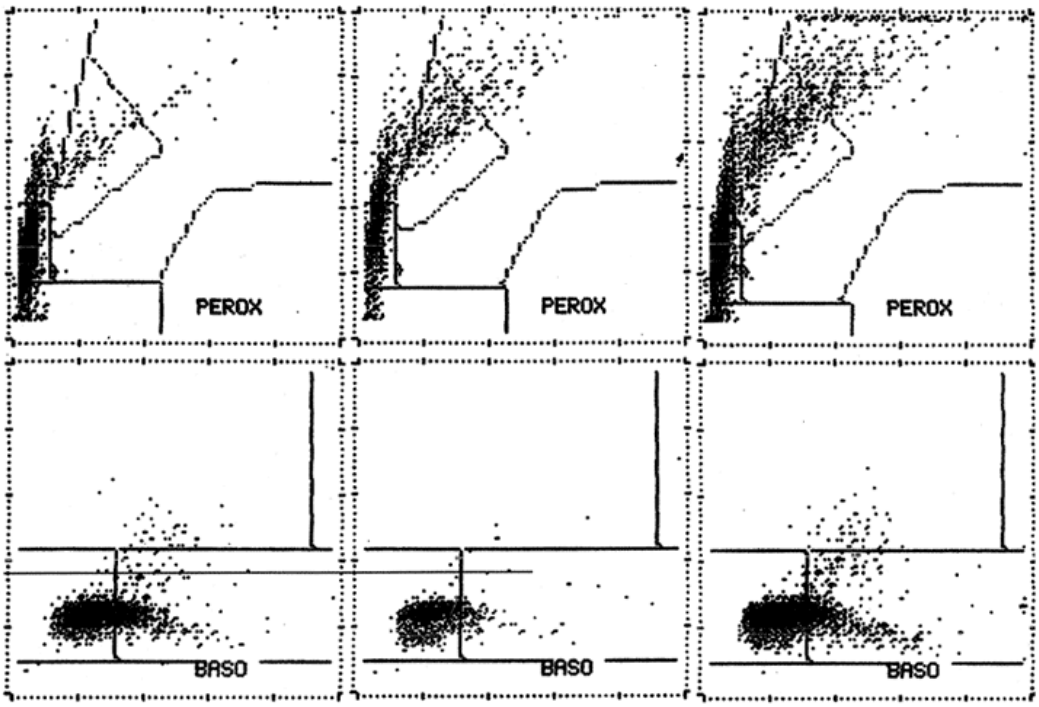
decreased glutathione levels in PBMCs and imunocompetent CD4+ and CD8+ cells of HIV-infected individuals.

This microtiter plate based assay permits the batching of specimens for optimal laboratory efficiency. The actualassay is rapid the labor intensive portion being the FH enrichment and the preparation of the precipitate itself.

The longitudinal studies have documented the efficacy of the nutriotional supplement [ALL VitImmune Inc.] which was designed to promote GI absorption and increase intracellular glutathione in liver and lymphoid cells in those individuals with low GSH values.

This assay appears sensitive to modest change and therefore capable of providing valuable information in a rapid fashion to the clinician.

Figure 1 H.3RTX Differential Cytograms on PBMCs obtained by FH enrichment



The values obtained for the total WBC are then used in the adjustment of the cell concentration to 5 x 106 cells/ml prior to the cryo-precipitation. Tthe frequency of the major cell types is shown below from the left to the right samples. Lymphocyte totals are shown in red.

	1	2	3
Lymphs	93.7	58.7	75.0
LUCs	3.4	22.4	17.2
Monocytes	1.7	10.3	3.5
Other	0.7	8.3	3.8

Figure 2

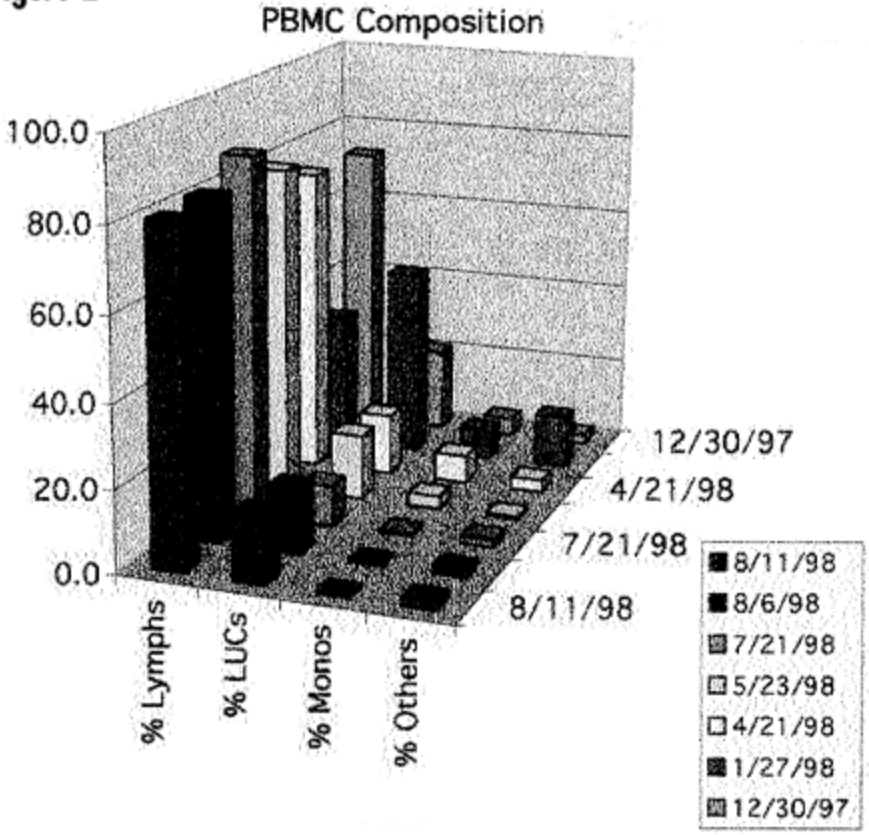

PBMC Composition
100.0
80.0
60.0
40.0
20.0
0.0
% Lymphs
% LUCs
% Monos
% Others
12/30/97
4/21/98
7/21/98
8/11/98
8/11/98
8/6/98
7/21/98
5/23/98
4/21/98
1/27/98
12/30/97

Figure 2

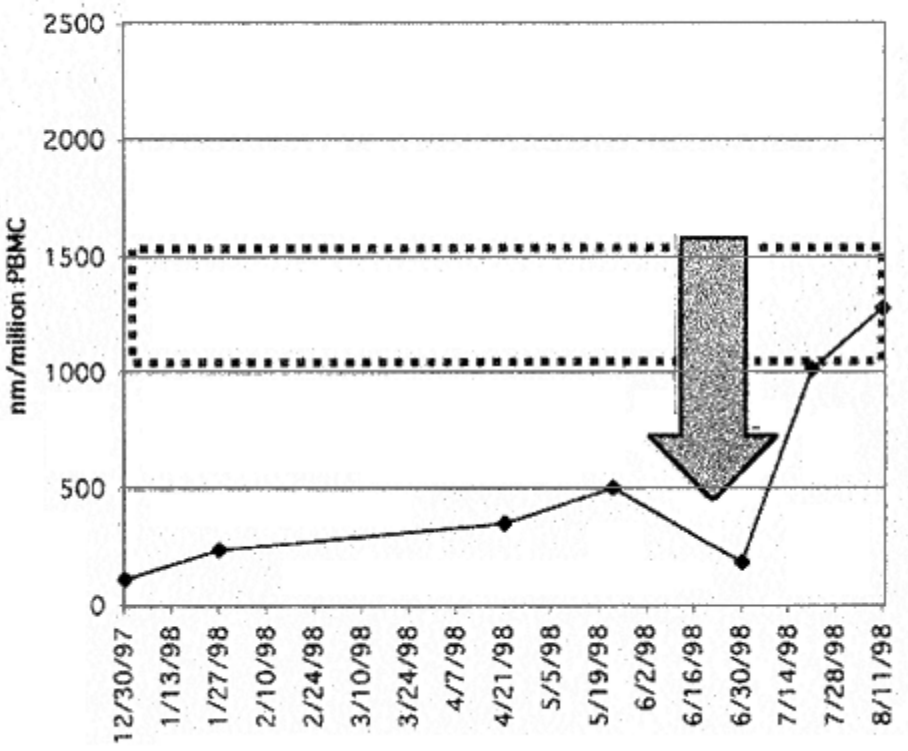
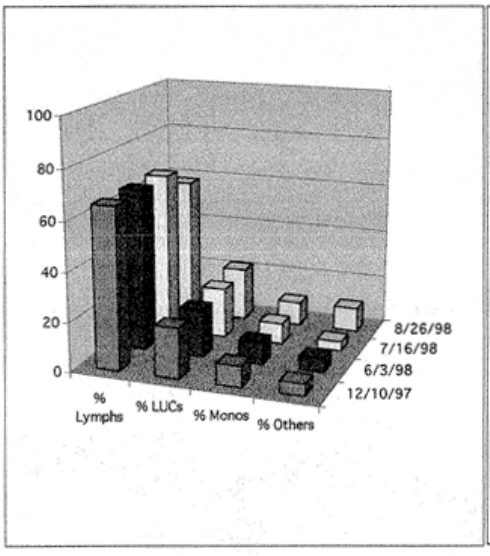


Figure 3



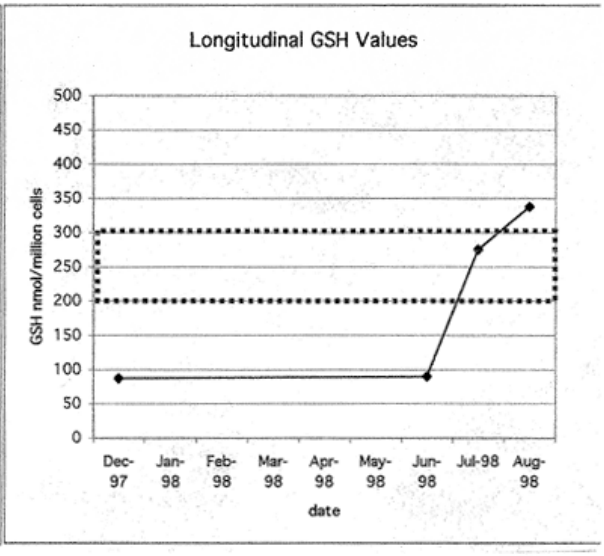


Figure 4

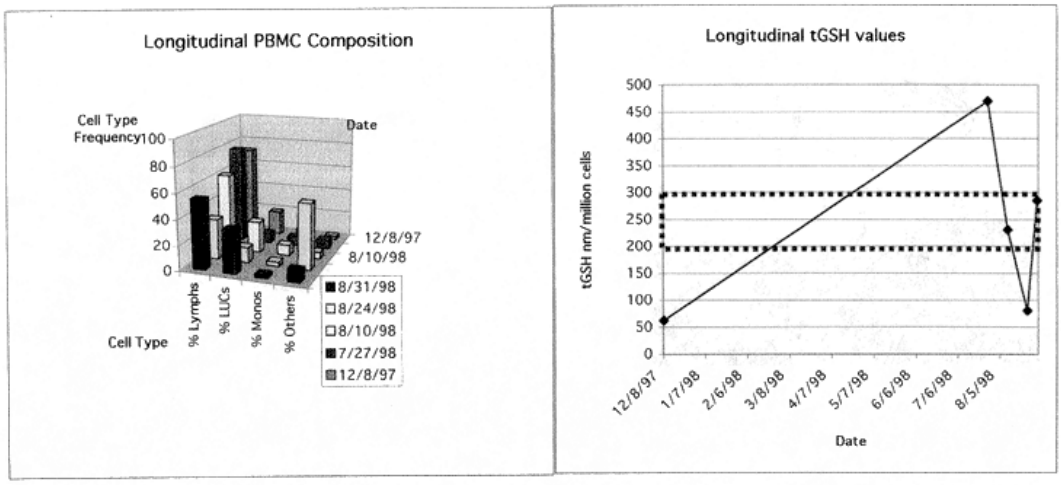


Figure 5