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# Enhanced Content of IgG in Burkitt's Lymphoma Cells after Treatment with the Topoisomerase II Inhibitor, Lucanthone

**Robert Bases[1]*, Rukmani Lekhraj[2], Xudong Tang[3], Jinghang Zhang[3], Zhi Duan[4], Jennifer T Aguilan[5] and Edward Nieves[6]**

[1]Departments of Radiology and Radiation Oncology, Albert Einstein College of Medicine, Montefiore Medical Center, NY 10467, USA
[2]Departments of Neurosurgery, Montefiore Medical Center, Bronx, NY 10467, USA
[3]Departments of Microbiology and Immunology, Albert Einstein College of Medicine, NY 10461, USA
[4]Departments of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
[5]Departments of Pathology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
[6]Departments of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461, USA

## Abstract

Burkitt's lymphoma cells (CRL-1647) which had survived treatment with lucanthone contained 3.6 fold more IgG than untreated cells, although most of the cellular immunoglobulins were still IgM. DNA activation induced cytidine deaminase (AID) was increased 5 fold in these surviving cells, consistent with active Class Switch Recombination (CSR).

Progeny of the small fraction of cells which had survived 20 h exposure to 8 μM lucanthone before rescue were cloned. $1.5 \times 10^8$ cloned cells contained ~1 μg of cytidine DNA deaminase, as determined from affinity column isolation of the enzyme, assayed by digestion of a 30 nt $^{32}P$ labeled specific DNA substrate. Before lucanthone treatment, little AID could be detected. After the second treatment, a six fold increase in AID was found. In confirmation, Western blot determinations of AID from lysates of lucanthone treated cells showed 5 fold increased AID content. These results suggest that lucanthone led to increased IgG content of surviving cells, consistent with their increased AID activity. The surviving cells were also more resistant to the standard lucanthone treatment, as determined in clonogenic assays.

IgG could not be detected in the cell membranes of CRL cells before or after lucanthone by immunostaining and flow cytometry, but both cell types secreted 80 kDa and 25 kDa immunorelated protein.

Lucanthone, formerly used to safely to treat hundreds of thousands of schistosomiasis patients, might be considered as a means to promote IgG synthesis in macroglobulinemia patients.

**Keywords:** Cytidine deaminases; Class Switch Recombination (CSR); Lucanthone; Clonogenic assay; Activation Induced Deoxycytidine deaminase (AID)

## Introduction

The role of DNA Activation Induced Deoxycytidine deaminase (AID) in class switch recombination (CSR), i.e., switching from synthesis of IgM to IgG inlymphocytes, has been well established [1,2]. Furthermore, AID deficiency has been implicated in the hyper-IgM syndrome [3]. The proto oncogene, c-myc, which regulates antibody secretion, in significantly involved in CSR [4].

We considered ways to enhance CSR in Burkitt lymphoma cells, as a model to learn how to enhance CSR in clinical situations. AID deaminates DNA cytidines on single stranded DNA [5] leaving DNA uridines that are then removed by ubiquitous cellular uracil DNA glycosylases. Abasic sites and double strand breaks result, which may be essential for CSR. We sought to mimic the deamination step by treating Burkitt lymphoma cells with lucanthone, an inhibitor of topoisomerase II [6], whose ability to create Abasic sites and double strand breaks in DNA of HeLa cells had been demonstrated [7,8]. As a caveat, we note that DNA deamination activity of AID might not alone represent its physiological function [9,10].

Burkitt lymphoma cells CRL-1647 synthesize IgM but little IgG. Here we describe experiments to learn if IgG synthesis was stably enhanced many generations after the lucanthone treatment of Burkitt lymphoma cells.

Continuous exposure to concentrations of 8 μM lucanthone beyond 2-4 h progressively sterilized HeLa cells. This lethal effect must be taken into account in the results to be described here i.e., could the increase in IgG synthesis result from selection of a special cell population?

Lucanthone's effects on HeLa cells were readily reversible [11]. Furthermore, it has an unblemished clinical safety record [12]. It was given to hundreds of thousands of patients who were treated for schistosomiasis in the past. Currently, lucanthone is used clinically as an adjuvant to radiation therapy [13]. We encountered no hematologic toxicity or other toxicity when care was taken to avoid interference from concurrent medications. Lucanthone was well tolerated when administered to patients daily for several weeks, maintaining serum concentrations of 8 μM lucanthone. The *in vitro* studies described here employed 8 μM lucanthone. Lucanthone is promptly eliminated by the liver and kidneys in experimental animals. HeLa cells suffered no loss of clonogenic survival for 2 h in 8 μM lucanthone when growth medium was then changed. But a thousand fold loss of survival was found if lucanthone removal was delayed 20 h [11]. Serum lucanthone

***Corresponding author:** Robert Bases, Professor of Radiology and Radiation Oncology, Albert Einstein College of Medicine, Montefiore Medical Center, 111 East 210th Street, Moses Research Building, 301, Bronx, NY 10467, USA, Tel: 914-380-2757; Fax: 914-632-3056; E-mail: rbases@verizon.net

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concentrations of 8 μM are readily achieved, and are well tolerated. The 8 μM lucanthone in cell culture increased the frequency of DNA abasic sites and double strand DNA breaks [7,8]. A study of lucanthone concentration dependence would be of interest but is beyond the scope of this initial report. The Burkitt lymphoma cell line was chosen because it was a B cell line which contained IgM but little or no IgG, so that it was a good target for study.

## Materials and Methods

### Cells

CRL-1647 Burkitt human lymphoma cells (0L) were purchased from American Type Culture Collection (ATCC), Manassas, VA 20108. They were grown in suspension at 37°C in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum in 8% $CO_2$ in a humidified atmosphere. The cell culture doubling time was 24 h. Media and sera were from ATCC. After two serial treatments with lucanthone, the lymphoma cells are designated: 2L.

### Cell lysates

Cells were sedimented from phosphate buffered saline without $Ca^{++}$ or $Mg^{++}$, resuspended in lysis buffer [14] with 10 μM Aprotinin and sonicated with 20 one-second strokes, leaving 1-2% unbroken cells. Lysates of $10^7$ to $10^8$ cells that were clarified by centrifugation at 15,500 g for 12 min contained approximately 1 μg/μl of protein [15].

### Western blots

For most experiments, 7 cm mini gels, purchased from BioRad Laboratories, Los Angeles, CA were used. Buffer without SDS or methanol, containing 25 mM Tris, pH 8.3 and 192 mM glycine were used for gel electrophoresis and Western blot transfer.

Detection of IgM and IgG in cell lysates was made in Western blots, using goat anti-human IgG pre-coupled to horseradish peroxidase (SC 2453) (Santa Cruz Biotechnology, Inc., Santa Cruz California, 95060).

This antibody reacts with IgM and more strongly with IgG. An advantage is that both IgG and IgM can be determined in the same cell lysate aliquot in the same gel lane. Quantitation is made by reference to immunodensities of reference standards in the gel. To characterize the relative abundance of IgG and IgM in an aliquot, relative immunodensities were determined: IgG/(IgG+IgM). For untreated Burkitt lymphoma cells in this report the ratio was 0.20 ± 0.05 SE, from 4 separate experiments. After transfer of immunoglobulins to nitrocellulose membranes and blocking with 5% milk proteins, membranes were stained with antibody, rinsed, and immunofluorescence was induced with ECL reagent RPN2209 [GE Health Care, Amersham, Buckinghamshire.

[HP7 9NA, U.K] After exposure to x-ray film, gel band IgG and IgM were scored by densitometry. Complete transfer of proteins from gels to nitrocellulose membranes was verified by loading one lane of each gel with a mix of multicolored purified proteins encompassing the sizes of IgM and IgG (Kaleidoscope-BioRad). After transfer, bands were found in the membranes, none in the gels.

Human IgG, 11.2 mg/ml was bought from Pierce Biotechnology Rockford Illinois, product #31154. Human IgM (myeloma), 4.5 mg/ml, product 31146 was from (Pierce).

Actin was determined in cell lysates by electrophoresis in tris glycine mini gels and transfer to nitrocellulose membranes. The actin bands were stained first with mouse anti actin (Cal Biochem mAb, JLA 20 CAT #CP01) at 1:5000 dilution for 2 h followed by goat anti-mouse IgM pre-coupled to horse radish peroxidase (JA 1200) at 1:2000 dilution for 2 h. Immunofluorescence then was induced by the ECL reagent and bands detected by exposure to X-ray film were scored by densitometry. Cell lysates had been prepared without mercaptoethanol, to prevent interference with IgM. Because of this, actin aggregates species were encountered in the gel lanes. Their sizes were from ~$10^5$ MW to ~$10^6$ MW. Therefore, the densities of all the actin reactive species in each lane were determined together.

### Nucleic acid gel electrophoresis assay for AID

A single stranded substrate 30 nt DNA, TTT TTT TTT TTT TTA GCA TTT TTT TTT TTT, was purchased from Life Technologies, InVitrogen Carlsbad, CA 920081. 5' 32P end labeling was accomplished with T4 polynucleotide kinase from InVitrogen according to the manufacturer's instructions, using 5 pmol of substrate and 25 μCi of [γ 32P] ATP, 3000 Ci/mmol, Perkin Elmer, Shelton, CT 06484. After labeling, the reaction was stopped by addition of EDTA to 5mM and incubation at 65°C for 5 min. The 30 nt long labeled product was purified by centrifugation through a Micro Bio spin 6 column, BioRad, to remove smaller species [5].

Enzyme activity was measured by cleavage at the single cytidine, which released 32P labeled 16 nt product, accompanied by undigested 30 nt substrate. Usually, 10 μL reaction mixtures containing 0.5 pmol of 32P labeled substrate in 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 1 mM DTT were incubated with 5 μL of sample at 37°C for 15 min. Cell lysates were pretreated by digestion with 10 μg/ml of pancreatic RNase for 30 min at 37°C.

Uracil DNA glycosylase, 1 μL (1 unit/μL), InVitrogen, was added, followed by 15 min of incubation at 37°C to remove uracil products of AID digestion. The resulting abasic sites were cleaved by adding 1 μL of 2 N NaOH to the reaction mixture and heating for 5 min at 95°C. Then 10 μL of formamide were added.

After these additions, 10-15 μL of TBE sample buffer (BioRad, 161-0768), were added and the samples were placed in the 30 μL sample wells of 15% acrylamide TBE urea gels (BioRad 161 _ 1117). After electrophoresis for 60-75 min, the gels were exposed to x ray film for 60 min or more. The radioautograms were scanned by quantitative densitometry. Radiographic densities of faster migrating 16 nt DNA enzyme lysis products were quantitated and expressed as fractions of the sum of residual 30 nt substrate and 16 nt product densities.

### Oligodeoxycytidylic affinity column purification of AID

1 ml HiTrap affinity columns (GE Health Care, Biosciences, Uppsala, Sweden SE 75184) were first prepared by binding oligodexoxy cytidine (American Biosciences, Piscataway, NJ 08855) according to the manufacturer's directions. We expected that few of the primary amino groups of the polydeoxycytidylic would be eliminated by the binding step at 4°C; 600 μg of oligo dC were bound to the column. Cell lysates from $1.5 \times 10^8$ cells were introduced for binding at 4°C and after 125 min, the column was washed at 4°C to eliminate unbound AID and other proteins. The column was kept in Phosphate Buffered Saline (PBS) at 4°C to prevent cytidine deaminase digestion of the column bound cytidylic amino groups.

To release cytidine deaminases from the column, elutions were made with 0.5 ml of 0.2N NaCl in 0.04M Glycine at pH 3.0 and then 0.5 ml of 0.5N NaCl in 0.1 M Glycine at pH 3.0, followed by PBS. 5.0 μL aliquots of each 500 μL column fraction were assayed for cytidine deaminases.

Purified AID was purchased from Enzymax, Lexington, KY 40503.

### In-gel trypsin digestion

Excised gel bands were reduced with 10 mM TCEP (tris-carboxy-ethylphosphine, Sigma, USA), alkylated with 55 mM iodoacetamide (Sigma, USA) and digested with trypsin (Trypsin Gold, Promega, USA) in 25 mM ammonium bicarbonate pH=8 (Sigma, USA), 0.01% ProteaseMax (Promega, USA) at 50°C for 1 h.

### Mass spectrometry analysis

LC-ESI-MS/MS (liquid chromatography electrospray ionization mass spectrometry) analysis of the peptide digests was done by C18-Reversed Phase (RP) chromatography using an Ultimate 3000 RSLCnano System (ThermoScientific, USA) equipped with an Acclaim PepMap RSLC C18 column (2 µm, 100 Å, 75 µm × 15 cm, Thermo Scientific, USA). The UPLC was connected to a TriVersa NanoMate nanoelectrospray source (Advion, USA) and a linear ion trap LTQ-XL (ThermoScientific, USA) mass spectrometer with ESI source operated in the positive ionization mode. The MGF files generated from the raw LC-ESI-MS/MS data were searched by Mascot (version 2.5, Matrix Science, USA) against Swissprot AC database version 2016-05 (551,193 protein sequences) with the following search parameters: trypsin; two missed cleavages; peptide charges of +2 and +3; peptide tolerance of 2.5 Da; MS/MS tolerance of 0.8 Da; Carbamidomethylation (Cys) for fixed modification; deamidation (Asn and Gln) and oxidation (Met) for variable modifications. A decoy database search was also performed to measure false discovery rate. The Mascot search results were validated by Scaffold version 4.1.1 (Proteome Software Inc., USA).

## Results

### Lucanthone treated cells contain more IgG

Goat antihuman IgG reacts with both IgG and IgM. IgG was much more reactive, as noted in the legend for Figure 1A. For ease of comparison we scored both their immunoreactivities. Estimates were also made of protein content in gel bands, by reference to standard IgM and IgG gel band protein contents. Figure 1A and Figure 1B clearly show that lymphoma cells which survived lucanthone 1L contained 3.6 times more IgG (by relative immunofluorescence) than untreated cells (0L). Furthermore, when estimated from protein content without lucanthone treatment, IgM was 398 times more abundant; after lucanthone treatment, IgM was 53 times more abundant indicating increase in relative IgG abundance.

Figure 1B shows results of determinations made in another experiment with a CRL-1647 cell lysates. Enhancement of IgG was again found in the cells from this culture, which also had grown from cells which survived treatment with 8 µM lucanthone. From these 2 experiments we found that lucanthone survivors of one lucanthone exposure contained 3.6 fold enrichment of IgG, determined from Western blots of Figure 1A and 1B; $p \leq 0.01$. Survivors of a second lucanthone exposure exhibited a small increase in IgG/(IgG+IgM).

In an experiment (not shown) the ratio for 1 × lucanthone survivors was 0.75 ± 0.0067 SE; for the 2 × lucanthone survivors it was 0.85 ± 0.010 SE, the difference was significant, $p<0.001$. In 5 separate experiments with survivors of one lucanthone treatment, the IgG/IgG+IgM was 0.57 ± 0.09SE, significantly different from results with 4 separate experiments with untreated cells (0.20+-0.05SE) at $p>-0.01$.

Determination of enhanced content of IgG in cell lysates in individual lanes did not depend upon comparisons between individual lanes.

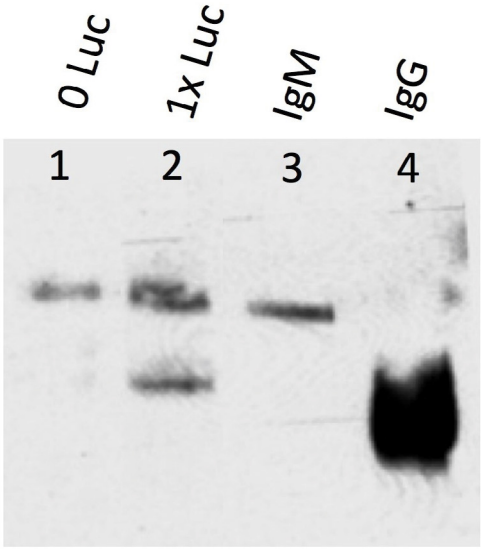



**Figure 1A:** Treatment of the Burkitt lymphoma cells with 8 µM lucanthone for 18 hours, followed by recovery in normal growth medium for 22 days; the relative abundance of cellular IgG was increased in cell lysates. Lane 1: no lucanthone; lane 2: 8 µM lucanthone; lane 3: IgM 4.5 ng; lane 4: IgG. 1.1 ng. Relative immunoreactivity of IgG/(IgM+IgG) increased from 0.075 to 0.32. Input to lane 1-lysate from 1.2 × $10^5$ cells. Input to lane 2 lysate from 3 × $10^5$ cells. Determination of immune protein content in lysates was made by comparing their IgG and IgM gel band densities with their standards. Without lucanthone treatment, 107 cells contained 0.42 ng IgG and 167 ng IgM. After lucanthone, 107 surviving progeny contained 4.6 ng IgG and 242 ng IgM.

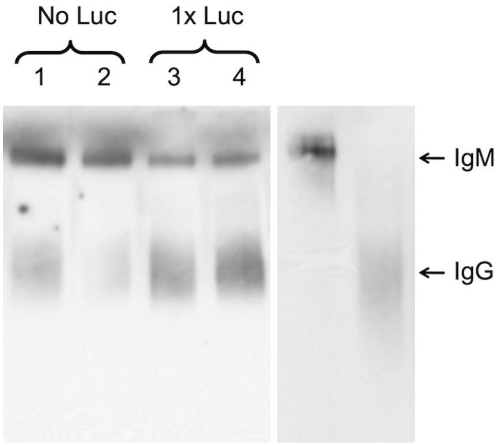



**Figure 1B:** Treatment of Burkitt lymphoma cells with 8 µM lucanthone for 24 h followed by recovery for 24 days in normal growth medium. The relative abundance of cellular IgG was increased in cell lysates. Lanes 1 and 2: No lucanthone. Input to lanes: Lysates from 2 × $10^5$ cells. Relative immunoreactivity: IgG/(IgG+IgM) 0.18 and 0.10. Lanes 3 and 4: Lucanthone, 8 µM for 24 h, followed by recovery. Input to lanes: Lysates from 2 × $10^5$ cells. Relative immunoreactivity IgG/(IgG+IgM) 0.46 and 0.50. Lanes 1-4: 2 min ECL exposure. IgM and IgG standards were from 5 s ECL exposures of the gel.

The actin content of lysates of Figure 1B as well as lysate from cells which had survived two lucanthone treatments (2L cells) was compared in Figure 1C. Lucanthone survivor's actin content was two to three

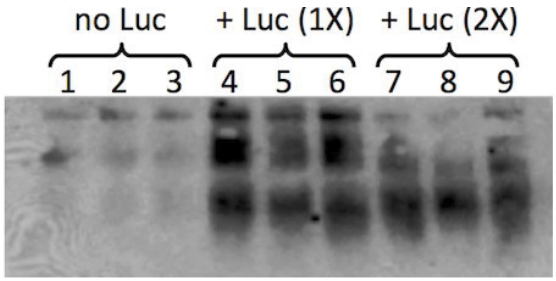



**Figure 1C:** The aggregate actin related densities from cell lysates were scored. Cell lysates had not been treated with heat and dithiothreitol before electrophoresis, resulting in several actin positive bands over a range of sizes. The range of sizes from $2 \times 10^5$ to $8.3 \times 10^4$. Actin content of the cells in the study of Figure 1B was compared by immunofluorescence, as described in the Methods section. Lysates from $2 \times 10^5$ cells of each of the three groups were compared. Lanes 1, 2, 3–No lucanthone; relative immunodensities: 955, 859, 1117, mean: 977. Lanes 4, 5, 6–Lucanthone survivors; relative immunodensities: 2776, 2442, 2777, mean: 2665. Lanes 7, 8, 9–2 times lucanthone treatments; relative immunodensities: 2326, 2001, 2487, mean: 2272.

folds greater than obtained with untreated cells, over a wide range of molecular sizes. Determination of their post lucanthone enhanced IgG was not related to actin content (Figure 1C); both IgG and IgM in each lane were normalized by the same factor.

ELISA assays for the four IgG isotypes in 0L and 2L cells' lysates show enrichment of the four isotypes in 2L cells, confirming in 4 separate assays, the determinations made by Western blots previously (Figure 2).

We eliminated the possibility that IgG isotypes had been absorbed to 2L cells from the fetal bovine serum in growth medium. Testing the serum by ELISA assays failed to detect such antigenicity (not shown). Because all four IgG isotypes were involved, RT PCR studies would probably not be helpful.

## Aid is enhanced in lucanthone survivors

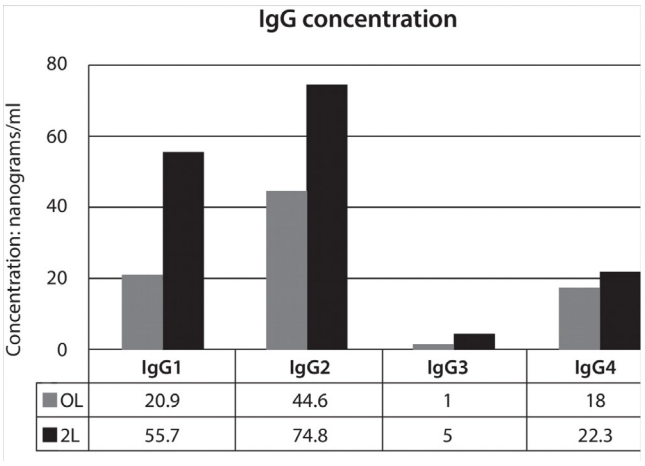
Single cell survivors of 8 μM lucanthone were cloned by terminal dilution in a 96 well plate. One clone, A-7, was grown and tested for IgM and IgG by relative fluorescence. As expected, IgG cell content was enhanced, as measured by immunofluorescence, to 0.49 and 0.36 in the uncloned and cloned cells (not shown).

To learn if cytidine DNA deaminase was present in A-7 cells, cell lysates were purified by binding to polydeoxycytidine containing column and eluting, as described in Materials and Methods. Three major fractions contained enzyme activity (Figure 3).

Previously, two major fractions were found when bone fide AID was analyzed on a similar column [16]. AID activity of these 2L cells was increased (Figure 4). AID from 3/8 as many cells assayed in the experiment of Figure 3 yielded 2.5 fold more AID in the experiment of Figure 4, comparing the areas under the elution curves of Figures 3 and 4. Therefore, an overall six fold increase in AID activity was achieved. Untreated lymphoma cells contained little detectable AID activity (Figure 4).

Western blot analyses in Figure 5 showed that AID in 2L cells was 5.4 fold more abundant than in 0L cells, as expected from previous results shown in Figures 3 and 4.

Western blot determination of AID from 0L and 2L cells (Figure 5) showed that from $2.4 \times 10^6$ 0L cells 0.025 ± 0.011 micrograms were found; from $2.4 \times 10^6$ 2L cells 0.13 ± 0.025 μg were found, indicating a 5.4 fold increase in AID in the lucanthone treated cells. A confirming experiment (Figure 6) showed that normalizing for actin content in 0L and 2L lysates indicated 7.7 fold increases in AID.




| | IgG1 | IgG2 | IgG3 | IgG4 |
|---|---|---|---|---|
| OL | 20.9 | 44.6 | 1 | 18 |
| 2L | 55.7 | 74.8 | 5 | 22.3 |

**Figure 2:** Legend for ELISA assay of 0L/2L cells. Lysates were prepared by sonication of $1.5 \times 10^8$ 0L cells and $2.0 \times 10^8$ 2L cells; supernatants were harvested after centrifugation at 12,365 g for 16 min. Equal concentrations of the four isotypes of IgG were compared by ELISA assays, as shown in Figure 2, using reagents from Life Technologies ref 991000.

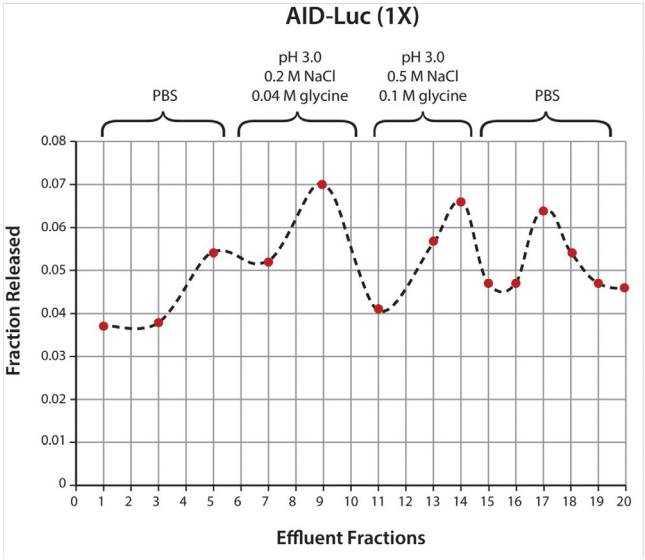



**Figure 3:** Binding and release of cellular deoxycytidine deaminase from an affinity column containing ~600 μg of bound 100 nt long polydeoxy cytidine oligomers. $1.5 \times 10^8$ CRL-1647 ($A^{-7}$) cells cloned after 18 h treatment with 8 μM lucanthone were sonicated and then sedimented at 12,365 g for 16 min. Supernatants were treated with 10 μg/ml of DNAase free pancreatic ribonuclease and then bound to the column for 125 min at 0°C. Serial elutions were made with phosphate buffered saline; 0.2 N NaCl in 0.04 M glycine pH 3.0; 0.5 N NaCl in 0.1 M glycine, pH 3.0 and finally with phosphate buffered saline. 0.5 ml fractions were collected. Enzyme activity in the fractions was determined by measuring release of 16 nt $^{32}P$ labeled DNA fragments from $^{32}P$ labeled 30 nt DNA substrate. Release is expressed as the fraction of 30 nt DNA released as 16 nt DNA product, determined from autoradiographs of each gel lane (•-•).

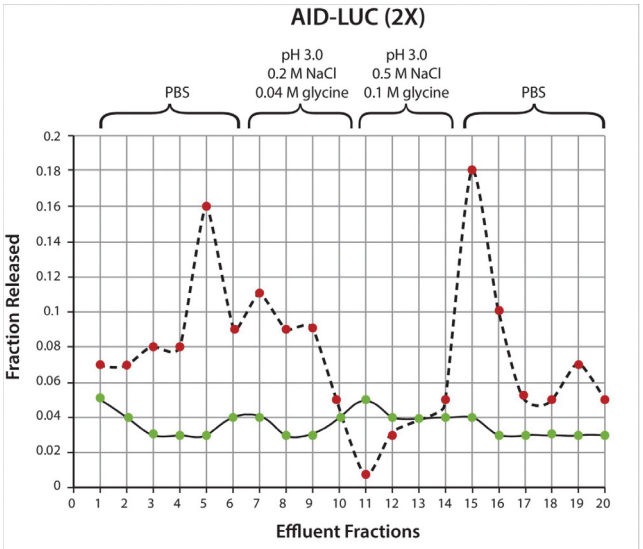
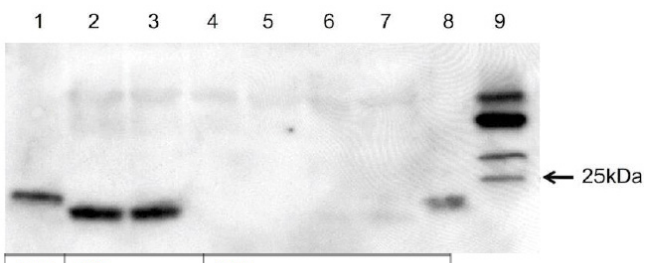



**Figure 4:** AID from 4 × $10^7$ cells which had survived the second serial treatment with 8 µM lucanthone was determined by elution from the polydeoxycytidine column, as described for Figure 3 (●-●). Little or no AID activity was recovered in a lysate from 3.4 × $10^7$ untreated CRL-1647 lymphoma cells (●-●).



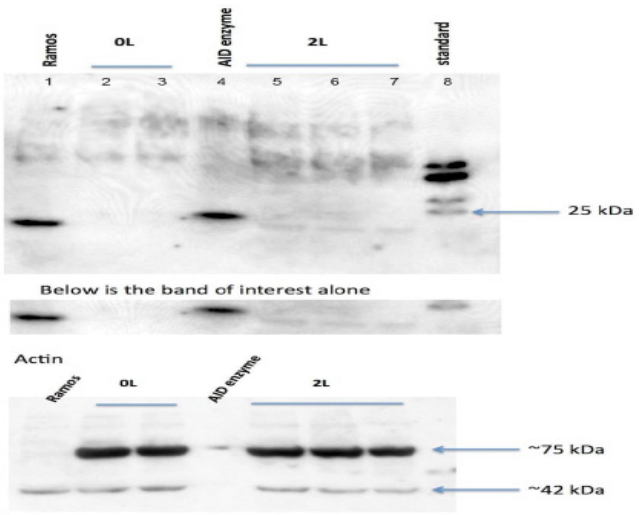

| Lane | AID | Cells | |
|---|---|---|---|
| 1 | 0.5µg | ENZYMAX STANDARDS | |
| 2 | 0.72µg | 3x$10^6$ RAMOS | |
| 3 | 0.66µg | 3x$10^6$ RAMOS(p) | |
| 4 | 0.037µg | 2.4x$10^6$ 0L | }mean 0.025µg |
| 5 | 0.014µg | 2.4x$10^6$ 0L | |
| 6 | 0.11µg | 2.4x$10^6$ 2L | }mean 0.140µg |
| 7 | 0.16µg | 2.4x$10^6$ 2L | |
| 8 | 0.50µg | ENZYMAX | |
| 9 | ENZYMAX MW MARKERS | 50 kDa, 40 kDa, 30 kDa, 25 kDa | |

AID enrichment 2L / 0L = 5.6

**Figure 5:** AID from 0L cells and 2L cells were determined from immunodensities in Western blots using rat mAB #49595 (Cell Signaling).

Actin content of 0L and 2L cells was much greater than in Ramos cells. Ramos cells' AID content was four fold greater than the AID content of 2L cells, but elevated IgG was not found in Ramos cell survivors of lucanthone treatment and little or none.

IgG was found in untreated Ramos cells (not shown). Despite the AID activity in Ramos cells, IgG enhancement was not observed. So far, we have not been able to induce (or select) for cells with enhanced IgG content in Ramos 6.4 human Burkitt lymphoma cells or in a transplantable mouse lymphoma MOPC 460d (a kind gift of Dr. Michael Potter, National Cancer Institute, USA).




| Lane | AID | Cells | |
|---|---|---|---|
| 1 | 0.2µg | 3x$10^6$ RAMOS | |
| 2 | 0.003µg | 2.4x$10^6$ 0L | }mean 0.007µg |
| 3 | 0.011µg | 2.4x$10^6$ 0L | |
| 4 | 0.2µg | ENZYMAX STANDARD | |
| 5 | 0.049µg | 2.4x$10^6$ 2L | |
| 6 | 0.042µg | 2.4x$10^6$ 2L | }mean 0.038µg |
| 7 | 0.024µg | 2.4x$10^6$ 2L | |
| 8 | ENZYMAX MW MARKERS | 50 kDa, 40 kDa, 30 kDa, 25 kDa | |

AID enrichment 2L/0L=5.4, corrected to 7.7; 0L cells 7 ng, 2L cells 38 ng; From 3 × $10^6$ Ramos cells 200 ng were obtained; Antibodies were stripped from the nitrocellulose membrane, and it was restrained with mouse anti actin, followed by horseradish peroxidase labeled goat anti-mouse antibodies; AID density/actin density at 42 kDa, 20/628, 75/717 for 0L cells; AID density/actin density at 42 kDa 346/645, 295/515, 170/395 for 2L cells; 0L Normalized AID is 0.066; 2L Normalized AID is 0.51. Therefore, 0.51/0.066=7.7 fold increase in AID in 2L cells; For 8 × $10^6$ Ramos cells, 1349 AID Units found

**Figure 6:** AID from 2.4 × $10^6$ 2L cells and 2.4 × $10^6$ 0L cells were determined, comparing the immunodensities in Western Blots using rat mAb #49595 from Cell Signaling Technology and bone fide AID from Enzymax.

## Resistance of lucanthone survivors to retreatment, determined by clonogenic assays in polyornithine coated plastic dishes

Normally CRL-1647 lymphoma cells grow in suspension and do not attach to plastic surfaces. To evaluate their clonogenic fraction, plastic dishes were first coated with polyornithine [17]. Macroscopic colonies were counted in single-cell assays after 12 days of incubation. Parallel studies of growth in uncoated 96 well plastic plates, after terminal dilution confirmed the clonogenic fractions were the same. The clonogenic fraction of 0L cells was 0.097 ± 0.03 SE. After recovery, the clonogenic fraction of log phase survivors of one or two lucanthone challenges [20 h in 8 µM lucanthone], were 0.18 and 0.11, respectively.

By contrast, when these cell cultures were challenged by 8 µM lucanthone for 20 h and immediately tested, the clonogenic fraction of naive cells was $1.5 \times 10^{-4} \pm 0.7 \times 10^{-4}$, but for the survivors of the one or two serial exposures to lucanthone the clonogenic fractions were $1.3 \times 10^{-3}$ and $2.9 \times 10^{-3}$. Lucanthone survivors were therefore tenfold more resistant to the standard challenge. They also exhibited enhanced AID content (Figures 5 and 6). These findings are compatible with selection of lucanthone resistant cells with enhanced IgG content and AID content.

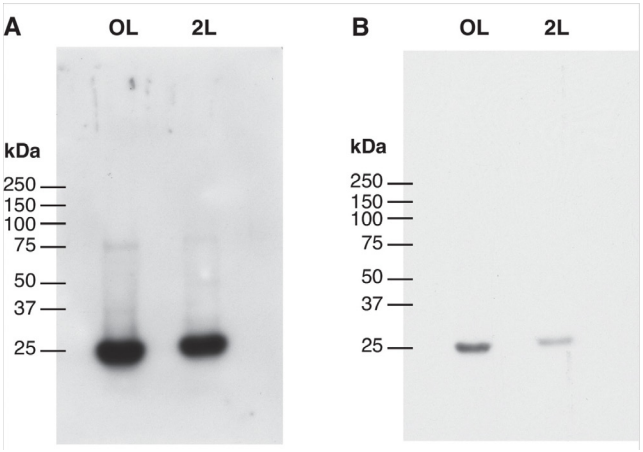



**Figure 7: (A)** 0L cells and 2L cells in the log phase growth were re-suspended in medium RPMI 1640 without serum at 2.4 × $10^6$/ml. After incubation at 37°C in 5% $CO_2$ for 6 h, 200 ml of cell free supernatant were concentrated 2,000-fold by membrane filtration (10 kDa MWCO). Two aliquots of 15 µl each of the concentrated 0L (293 µg) and 2L (298 µg) were subjected to 1D-SDS-PAGE. One replicate gel was used for Western blot analysis while the second replicate gel was stained with Coomassie and used for mass spectrometry analysis. Western blot analysis showed 80 and 25 kDa protein bands were immunoreactive with goat anti-human IgG/IgM antibody conjugated to horseradish peroxidase. Protein identification of the 80 and 25 kDa gel bands was performed by in-gel trypsin digestion and peptide digests were analyzed by LC-ESI- MS/MS. Western blot 80 kDa Ig from the 2L cells were scanned; 9.0 density units were determined after 30 minutes exposure. With 0L cells 17.3 density units were found. So far, absolute amounts of Ig gel proteins cannot be determined because structures and specific immunoreactivity of the 80 and 25 kDa proteins have not yet been determined. Nevertheless, results here are in agreement with previous results from mass spectrometry noted for the Ig mu chain C region. **(B)** A 30 second exposure of the Western blot of Figure 8A was made to reduce the intensity of the 25 kDa immuno proteins. For the 25 kDa proteins from 2L cells 25.2 density units were found; for 0L cells 70.9 density units were found. Therefore, the 25 kDa immunoproteins from 0L cells were nearly three times as abundant secreted immunoproteins. Nevertheless, 2L Ig kappa chains secreted were 1.4 times more abundant than 0L kappa chains, previously noted.

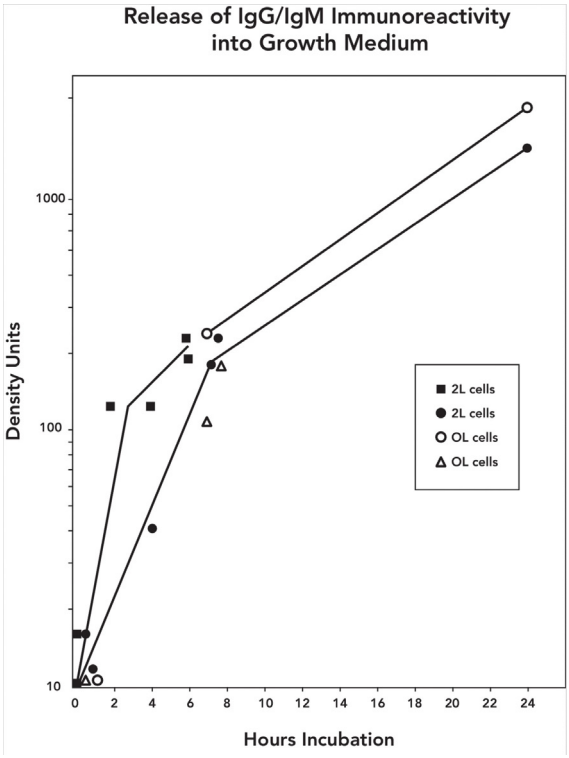



**Figure 8:** Time course of release of IgG/IgM immunoreactivity into growth medium of 0L and 2L cell cultures. Western blot assays for IgG/IgM immunoreactivity were made on cell free supernatants from 0L and 2L lymphoma cell cultured at 2.4 × $10^6$ cells/ml in RPMI 1640 with 10% fetal bovine serum. Cell supernatants were sedimented at $10^3$ g for 2 min to eliminate cells before assays. Samples were heated at 100°C for 5 min in dithiothreitol, cooled and placed on mini gels for electrophoresis without SDS. 15 µL of cell free supernatants were loaded on each gel lane. Comparison of the ~80 kDa densities from 0L and 2L cells with authentic IgG standards in the gel. One ml of the 2L cells released 240 ng of IgG/Ig immunoreactive proteins, assuming that the 80 kDa species possess characteristics similar to those of IgG, as yet unknown.

Despite much effort, we failed to detect IgG on or in the cell membranes of 0L cells and 2L cells by immuno-flow cytometry. Nevertheless, we found that both cell types continuously released IgG and IgM immunorelated 80 and 25 kDa species into their cell culture fluids (Figures 7 and 8). Secreted Ig related proteins were characterized by LC-ESI MS/MS.

## Quantitation of the ~80 kda and ~25 kda bands from 0L and 2L supernatants

0L and 2L cells in the log phase growth were re-suspended in medium RPMI 1640 without serum at 2.4 × $10^6$/ml. After incubation at 37°C in 5% $CO_2$ for 6 h, 200 ml of cell free supernatant were concentrated 2,000-fold by membrane filtration (10 kDa MWCO). The concentrated supernatants 0L (298 µg) and 2L (293 µg) were also loaded on a gel and analyzed by Western blot and probed with SC 2453 antibody against immunoglobulin chains. The intensities of the bands from the immuno-reactive Ig mu heavy chain (~ 80 kDa) and kappa light chains (~25 kDa) detected by Western blot (Figures 7A and 7B) were quantified using ImageJ to estimate the ratios of these two immuno-related proteins from the OL and 2L supernatants. Quantitation of the ~80 kDa Ig mu chain was based on a 30 min exposure, 0L was 2 × higher compared to 2L. For the ~25 kDa kappa light chains, since the bands at 30 min exposure appeared to be overexposed, a more reliable quantitation was obtained based on the 30 s exposure. 0L is 3 × higher compared to 2L for the ~25 kDa kappa light chains.

## Protein identification by LC-ESI-MS/MS

Based on 99% protein and 95% peptide probabilities and a minimum of 2 unique peptides, a total 122 proteins were identified from both the 80 and 25 kDa bands. Three immunorelated proteins were identified for both 0L and 2L cells. The Ig mu chain C region was identified from the 80 kDa gel band while both the Ig kappa chain C region and Ig kappa chain V-II region were identified for the 25 kDa gel band.

Label free quantitation based on normalized emPAI values (exponentially modified Protein Abundance Index) was used to estimate the ratios of immuno-related proteins identified for 0L and 2L cells. The Ig mu chain C region from 0L cells was 0.69 while Ig mu chain C region from 2L cells was 0.31. Hence, 0L was 2 × higher compared to 2L cells for the Ig mu chain C region. The normalized emPAI values for both Ig kappa chain C and V-II for the 0L cells was 0.84 while Ig kappa chain C and V-II for the 2L cells was 1.16. Hence, the Ig kappa chains for 2L cells were 1.4 × higher than 0L cells.

## Discussion and Conclusion

Here we describe four novel results on the effects of lucanthone on

human Burkitt lymphoma cells. Surviving cultures after two consecutive 20 h treatments with 8 μM lucanthone (2L cells) exhibited enhanced cell content of IgG relative to IgM content. The 2L survivors contained enhanced levels of AID. They were much more resistant to challenge by further lucanthone treatments, as determined by clonogenic assays. Mass spectrometry determinations on secreted Ig suggest reduced IgM related proteins and increased IgG related proteins, consistent with these results.

These results show that CRL-1647 Burkitt lymphoma cells which survived the exposure to 8 μM lucanthone contained relatively more IgG than unexposed cells. Moreover, this was a stable feature, expressed in progeny of a single cell which had been cultured over many generations.

Was the enhancement because of selection and proliferation of a pre-existing minor population or induction of a new character in the survivors?

Lucanthone survivors expressed deoxycytidine deaminase (Figures 5 and 6). This enzyme is an essential feature of CSR; its presence in the lucanthone survivors is consistent with their enhanced elaboration of IgG. The relatively small amount of IgG contained in CRL-1647 cells prior to lucanthone exposure (0L cells) suggests small amounts of AID activity. If AID provided survival value to some cells, then this population would be expanded, possibly accounting for the results described here.

Studies on the elaboration of IgG messenger RNA and on the schedule of DNA rearrangements in lucanthone treated cells are of interest but are beyond the scope of this initial report and would involve 4 different m RNA's. Recent studies have implicated hn RNP's as cofactors for AID [18]. Their role, if any, in lucanthone's effects need further consideration. Finally, the roles of selection or induction of IgG enhancement warrants further study, perhaps by fluctuation tests.

Overall, results here demonstrated that lucanthone treatments of Burkitt's lymphoma cells (0L) produced progeny cells (2L) which contained 5 fold more IgG and AID than the parent cell line. IgM was still the predominant Ig species. So far, we have not found enhanced release of IgG related 80 kDa species from 2L cells (Figure 8). Both 0L cells and 2L cells continuously release this material over many hours.

We cannot yet account for the large differences in the content of 80 kDa Ig's in 2000 fold concentrated supernatants from 0L and 2L cell cultures (Figure 7), in contrast to little or no difference in 80 kDa Ig's from freshly harvested un-concentrated 0L and 2L cell supernatants (Figure 8). The isolation from gels and the concentration process, which were needed for mass spectrometry analyses, might to be responsible for differences in amounts of 80 kDa and 25 kDa Ig's after preparation for mass spectrometry. We have found a small increase in IgG related 26 kDa Ig from the 2L cells, but this must be confirmed and is not yet certain. Secretion of Ig has been noted in CHO cells and human leukemic cells [19-21].

The full identity of the secreted Ig species remains to be established. Do they attach specifically to antigens? What might be the effect of these secreted immunospecies on the immune response of Burkitt lymphoma patients if their lymphoma cells also secrete them?

Overall, two mechanisms which might account for the increased IgG in these Burkitt's lymphoma cells after treatment with lucanthone. Increased cell content of AID, by a mechanism presently unknown, might have stimulated class switching. But interference with orientation specific joining [22,23] by inhibition of topoisomerase II [6] should also be considered. Perhaps one orientation was favored by inhibition of DNA strand orientation [22,23].

Recently Kanu, et al. found increased APOBEC 3 cytidine deaminases (related to AID) in cancer cells after treatment with several chemotherapeutic agents [24], in general agreement with results of increased AID cytidine deaminase described in this report.

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### Author Contributions

Conception and design: R. Bases, Rukmani Lekhraj; IgG and IgM and AID assays: R. Bases; Isotype determinations: Xudong Tang; Flow cytometry measurements of cell surface Ig: Jinghang Zhang. AID measurements: Zhi Duan; Mass Spectrometry: Jennifer Aguilan, Edward Nieves

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