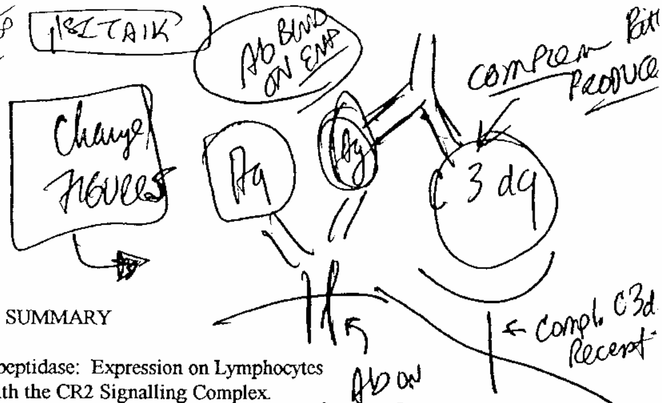

Cell Signalling
Oral Presentation I: March 10,1997
Professor: Bradley Stith

SUMMARY

Gamma-Glutamyl Transpeptidase: Expression on Lymphocytes and Association with the CR2 Signalling Complex.

Specific Aim #1 of a Master's Thesis
Expression of Gamma-Glutamyl Transpeptidase on Human PBL

Overview:

The first presentation will review lymphocyte activation and discuss the importance of gamma-glutamyl transpeptidase and glutathione in the cell. Background, methods, results, and conclusions will be discussed.

Background:

The purpose of this project was to use an antibody to an enzyme, which our lab developed, to study the relationship between the activation of immune cells and the function of that enzyme. This project evolved from the study of mechanisms by which a Complement receptor found on B cells is involved in the activation of those cells. Our lab has developed an antibody during this search which recognizes a well documented cell surface enzyme (GGT). The enzyme's action is involved with the chemical cycle of a tripeptide which can be chemically protective once it is assembled within the cell. The enzyme-tripeptide pathway and the events which occur during B cell activation have not been linked previously. Such a link is important because many diseases, especially those involving autoimmunity, present secondary complications that result from inappropriate stimulation of immune cells.

The ectoenzyme, gamma-Glutamyl Transpeptidase (GGT), was discovered approximately twenty years ago for its role in facilitation of intracellular synthesis of glutathione (GSH), a powerful antioxidant. Glutathione cannot enter the cell directly and must be cleaved into its components by GGT. The mechanisms by which the components enter the cell have not been defined. Once they are intracellular, they are resynthesized into glutathione which is consequently used in a variety of protective pathways.

Our lab sought to develop an antibody which inhibits an *in vitro* model of B cell activation. The result was an antibody to GGT. Previously, GGT has not been associated with this model nor with the molecules involved in this model This suggests that GGT may have a role in lymphocyte activation.

The following experiments look at GGT expression on subsets of Human peripheral blood lymphocytes (PBL). The subsets examined were unactivated/naive T helper cells, unactivated/naive T cytotoxic cells, activated/memory T helper cells, activated/memory T cytotoxic cells, B cells, and resting B cells. The cell surface marker for unactivated/naive T cells and activated/memory T cells is CD45RA and CD45RO, respectively. CD45 is membrane spanning molecule which has protein tyrosine phosphatase activity (PTP). Although multiple isoform gene transcripts can be found in T cells, the RA isoforms are primarily expressed on unactivated/naive cells and RO on activated/memory cells. The surface marker for Helper cells is CD4, a molecule which recognizes the MHC II of other immune cells. Cytotoxic cells are identified by CD8 which is a molecule that recognizes the MHC I on all nucleated somatic cells. Both CD4 and CD8 interact with the MHC complex to enhance the association of the

respective cells. A signal transducing molecule, CD19, is a cell surface marker unique to B cells. It has effects on phospholipase C (PLC) levels, calcium levels, and protein tyrosine kinases within the cell.

Methods:

1) Isolation of Leukocytes: Blood was donated from 5 normal human donors (3 male, 2 female). The heparinized blood was diluted 2X with phosphate buffered saline (PBS) and layered over ficoll-hystopaque to remove erythrocytes and serum. The cells were centrifuged for 25 minutes at 1500 RPM without braking. The leukocyte layer (immediately above the ficoll-hystopaque) was removed and washed 2 times with PBS at 2000 RPM for 5 minutes (4°C). The cells were counted and resuspended to 5×10^6 cells /ml in FACS buffer (PBS/1% Bovine Serum Albumin (BSA)/.01% Sodium Azide). Each sample of 100µl contained 5×10^5 cells.

2) Immunofluorescence Staining: The cells were blocked (to reduce non-specific staining) with 50 µl of 10% heat deactivated human serum in PBS for 10 minutes on ice. The primary antibody (either 3A8 or the isotype control UPC-10) was added at a concentration of 1µg/sample and incubated with the cells for 20 minutes on ice. The cells were washed 2 times as above in FACS buffer. Biotinylated sheep F(ab')2 anti-mouse IgG was added as the secondary antibody, incubated, and washed as before. The tertiary staining step included the addition of Avidin-Per-cp to fluorescently label the *biotin-coated secondary antibody + primary antibody* complex, and FITC- (fluoroscein isothiocyanate) or PE- (phyco-erythrin) conjugated antibodies to the following molecules: CD4, CD8, CD45RA, CD45RO, CD19, IgD , GGT, and UPC-10 (isotype control). These additions were incubated and washed as above. After the final wash the cells were fixed in .4 ml of 1% paraformaldehyde.

3) Fluorescence-Activated-Cell-Sorting (FACS): Analysis was conducted at the UCHSC Core Facility on a Coulter *Profiler* Flow Cytometer. The analysis was done on the lymphocyte population and compared in a Per-cp: FITC vs. Phyco-erythrin manner.

Results:

1) There is a population of B cells ($CD19^+$) which stain positive for GGT

2). In both T cytotoxic cells and T helper cells, anti-GGT antibody staining is substantially higher in the memory phenotypes ($CD45RO^+$) than in the naive phenotypes ($CD45RA^+$).

Conclusions:

There are no concrete surface markers to distinguish the activation state of B cells as there are for T cells. This makes the study of this population much more difficult. There is a population of B cells which have expression of GGT and it is possible that these B cells are activated/ memory cells due to the structural association between a signalling complex unique to B cells during activation and GGT (second presentation). To answer that specific question is not within the context of this project. To study the B cell populations in depth, large numbers of cells must be purified further to increase validity of subset evaluation and to decrease potential non-lymphocyte skewing of data.

The expression of GGT in T cells is substantially higher in the memory/activated phenotypes. Although this anti-GGT antibody was developed using a model for B cell activation, T cells are subject to some of the same metabolic pressures during stimulation. So this result reaffirms that a link between the enzyme GGT and lymphocyte activation events exists. Whether the increased expression of GGT is a cause or an effect of activation remains unclear.

Poor Example

DR. STITH'S COMMENTS

NO NAME etc

Initially, good overheads

Not clear speaker

(GNEF NOT GAP)

didn't practice talk

I. History of *ras* genes

A. First oncogenes identified in gene transfer assays

B. N-, K-, and H-*ras* genes

II. *ras* gene products

A. *ras* proteins (first identified in Rat sarcoma virus)

B. Action in the cell

1) Small GTP-binding proteins (guanine nucleotide binding proteins)

2) Example of *ras* protein pathway

C. p21 *ras* proto-oncogene products

1) Homologous to G-proteins; GTP activated, GDP inactivated

2) Damage to DNA activates p53, which activates transcription of p21 gene, p21 protein inhibits Cdks and PCNA

3) Cdks bind cyclins to drive progression through different stages of the cell cycle, PCNA, subunits of DNA polymerase delta, which drives synthesis of DNA ---> two fold effect of p21

D. p21 *ras* oncogenes

1) Mutations usually found on only 2 codons of the *ras* gene causing substitutions for Gly-12 or Gln-61

2) Do not convert to GDP bound form during interaction with GAP

III. 3-D structure of H-*ras* p21 mutants

A. Methods

1) Mutant p21 preparation

2) GppNp exchanged for GDP (GppNp is a slowly hydrolyzing analog to GTP)

3) Crystallization

B. Data Collection

1) X-ray diffraction to electronic area detector

2) Computer controlled vertical oscillations of camera and detector

3) Transferred to larger computer that calculates the crystal structure (taking into account absorption and radiation damage)

C. General comparison of structures

1) Structure of p21 (figure 3 from article)

2) Substitutions (Gly-12-->Arg, Gly-12-->Val, Gln-61-->His, Gln-61-->His, and Asp-38-->Glu)

D. Effects of mutations on p21 activity

1) Important residues to function of p21

2) Regional effects of point mutations

a) D38E

b) G12R

c) G12V

d) Q61H

e) Q61L

E. Conclusions

F. Impacts

Handwritten comments:

- Let's bail out / skip forward
- Time fell for presentation → 50 MIN → Too long intro
- (overgeneralized) Incorrect citation
- methods not finished, no data discussed
- get rid of
- PRACTICE PRONOUNCIATION
- Overhead on method, flowchart needed
- Full of unimpt details
- Need
- LIGATION
- NO DIAGRAM
- at R.T.
- Put in unimportant details, left out impt ones
- where I find the cuts
- "first of all let me tell you about the others!"
- Leu
- Asp acid subst for GLUt.
- error
- NAMES for C NOT EXPLAINED
- end up in "wierd conformations"
- by the ... oh by the way ... go back
- let me see, ... used endonucl. rest (at —— (read), doesnt matter"
- they did this ..., lets see, I had this here, or like reading for 12 (?)