Immunology of Celiac Disease

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Abstract

There is no treatment for Celiac Disease, other than to follow a gluten-free diet lifestyle. Yet, this does not work for all patients. In our study, we were trying to find out why this happens. We need to observe the behavior of pro-inflammatory cytokines: IL-1β, IL-6, IL-8, IL-12 p70, INF-γ, and TNF-α to determine the concentration levels in healthy patients, active CD (celiac disease) patients, CD patients on a gluten free diet, and refractory disease patients.

Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 proteins. The six bead populations are mixed together to form the BD™ CBA. The capture beads, PE-conjugated detection antibodies, and recombinant standards or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD™ CBA Analysis Software.

We did not obtain the expected results, (increased serum levels of inflammatory cytokines in active and refractory CD patients). Our results show increase in the level of IL8 and INF-γ in active celiac disease patients *and* gluten-free died patients. The current data is still being analyzed on an individual patient-by-patient basis to determine if there is a correlation with the biopsy grade for each patient.

Introduction

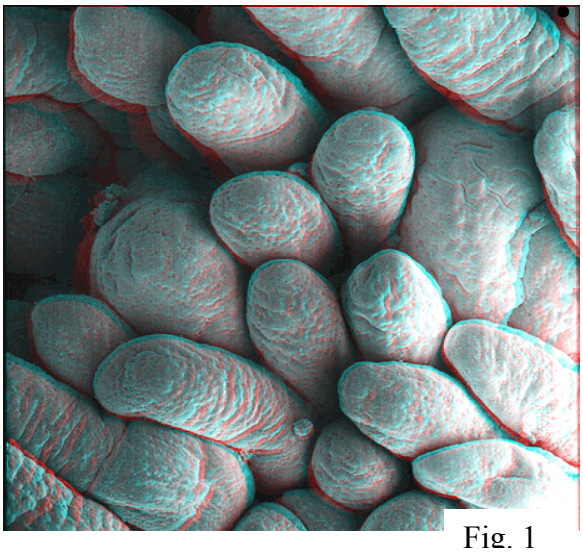


Fig. 1

Affecting one out of 250 people in the United States, Celiac Disease is often misdiagnosed, or underdiagnosed. Celiac Disease is an autoimmune disorder damaging the villi (singular: villus) in the small intestine interfering with the absorption of nutrition from the food one consumes. (leading to malnutrition). (Abul K Abbas, MBBS: Andrew H Litchman, MD, PHD. Cellular and Molecular Immunology. Pgs- 1- 104.)

Villi appear as finger-like structure (fig. 1) protruding from the wall of the small intestine. This particular physical structure increases the surface area of the small intestine. The villi of a person with this genetic disorder is eroded away (fig.3)

There is no treatment for this disorder; the patients are advised to follow a gluten-free diet for life. Yet, this lifestyle doesn't always work, and the patient continues having problems. The villi of a person on a gluten-free diet should grow back in two to three months (up to two years for an elderly). Of course, this is not true for refractory disease patients.

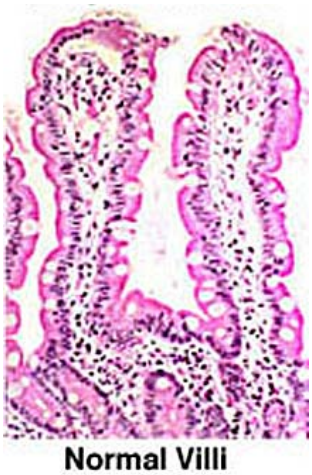


Fig.2

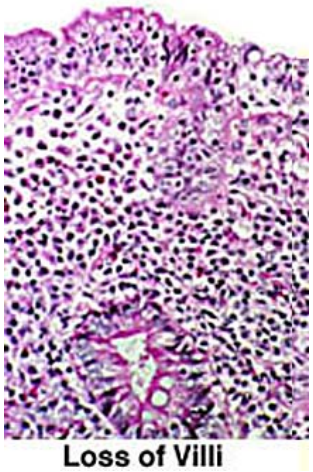


Fig. 3

Our major goal in our study was to figure out why this happens. Yet, to do so, we first had to observe the behavior of a few inflammatory cytokines, (IL-1β, IL-6, IL-8, IL-12 p70, IFN-γ, and TNF-α) in: healthy patients, active CD (celiac disease) patients, CD patients on a gluten-free diet, and refractory disease patients.

Hypothesis

The goal of our experiment was to find the cytokine concentration in the serum of: the healthy patients (the controls), active CD patients, CD patients on a gluten-free diet, and refractory disease patients. We hypothesized that we would see an increased serum level of inflammatory cytokines in patients with active and refractory celiac disease; and normal levels of cytokines in gluten-free diet patients

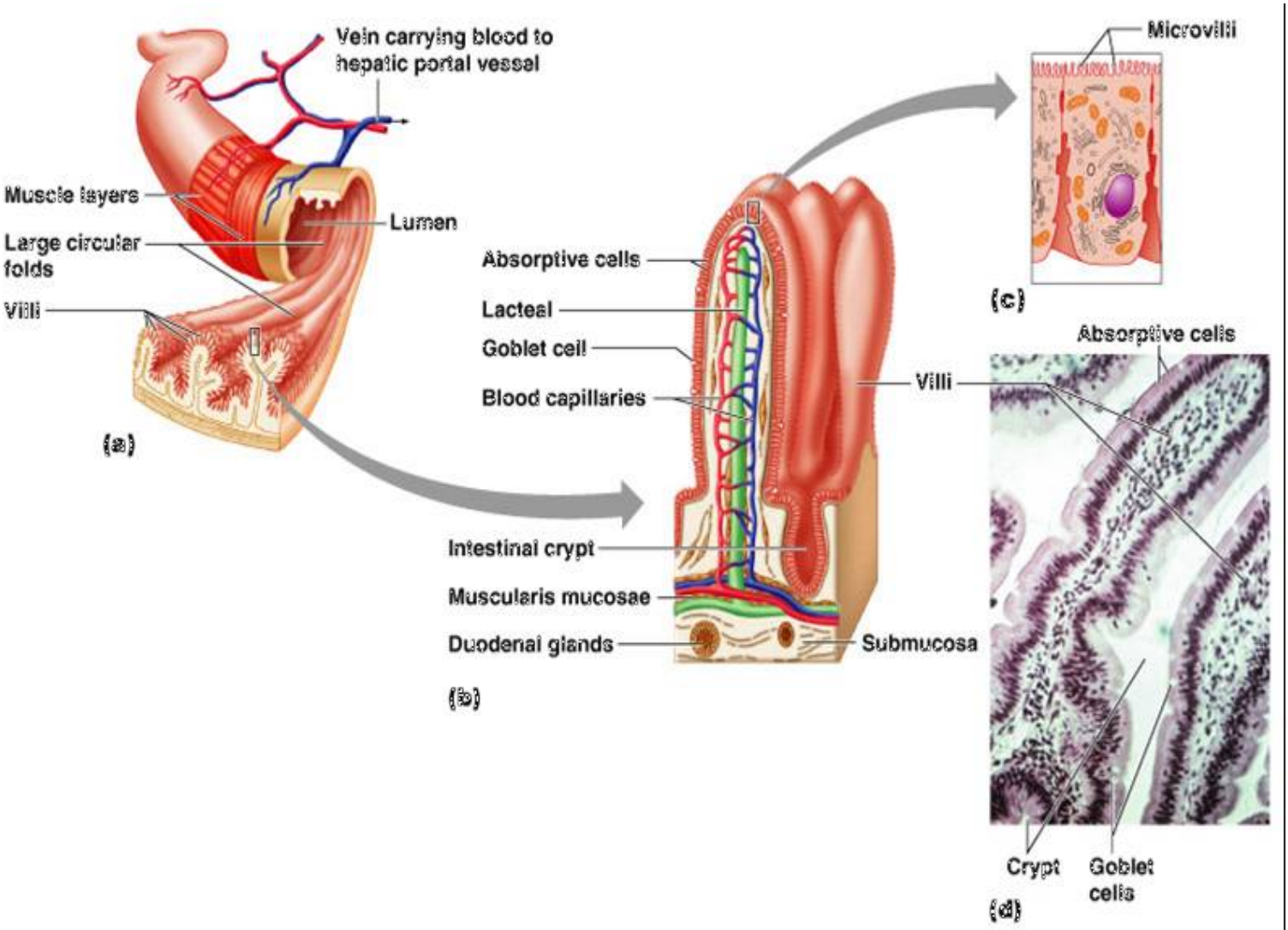

Vein carrying blood to hepatic portal vessel
Muscle layers
Lumen
Large circular folds
Villi
(a)
Absorptive cells
Lacteal
Goblet cell
Blood capillaries
Intestinal crypt
Muscularis mucosae
Duodenal glands
Villi
Submucosa
(b)
Microvilli
(c)
Absorptive cells
Crypt
Goblet cells
(d)

Fig.4

Materials and Methods

After a long preparation period and procedure, our main machinery that would give us our data was the flow cytometer.

Flow cytometry is an analysis tool that allows for the discrimination of different particles on the bases of size and color. Multiplexing is the simulation assay of many analytes in a single sample. This help us accomplish our project even faster. The BD™ CBA cytometric Bead Array (CBA) employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple saluble analytes.

Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample.

The BD™ CBA Human Inflammation Kit was used to quantitatively measure Interleukin-8 (IL-8), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein levels in a single sample.

In the kit we received, the following reagents were provided:

- Bead Reagents (all need to be stored at 4°C)

Human Inflammation Capture Beads (A1 – A6): The specific capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest to dimmest.

Cytometer Setup Beads

- Antibody and Standard Reagents (all need to be stored at 4°C)

Human Inflammation PE Detection Reagent: A 75-test vial of PE-conjugated anti-human IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 antibodies formulated for use at 50 µl/test.

Human Inflammation Standards: Two vials containing lyophilized recombinant human proteins.

PE Positive Control Detector: A 10-test vial of PE-conjugated antibody control formulated for use at 50 µl/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

FITC Positive Control Detector: A 10-test vial of FITC-conjugated antibody control formulated for use at 50 µl/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

- Buffer Reagents (all need to be stored at 4°C)

Wash Buffer: Two 130 ml bottles of phosphate buffered saline (PBS) solution (1×), containing protein and detergent, used for wash steps and to resuspend the washed beads for analysis.

Assay Diluent: A single, 30 ml bottle of a buffered protein solution (1×) used to reconstitute and dilute the Human Inflammation Standards and to dilute test samples.

Serum Enhancement Buffer: A 10 ml bottle of a buffered protein solution (1×) used to dilute mixed Capture Beads when testing serum or plasma samples.

Aside from the reagents supplied by the BD™ CBA Human Inflammation Kit, a few had to be purchased separately:

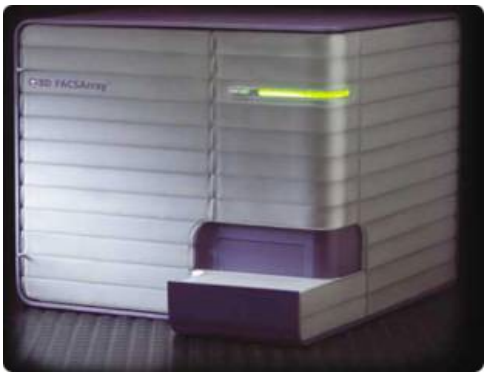
• A flow cytometer equipped with a 488 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan™ or BD FACSCalibur™ instruments) and BD CellQuest™ Software.

• 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008).

• BD CBA Software, (Cat. No. 550065).

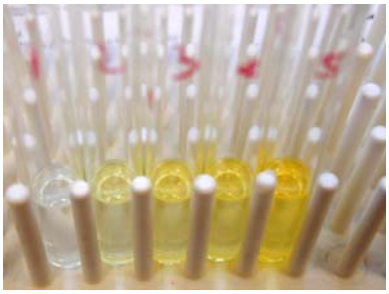
• BD CaliBRITE™ 3 Beads, (Cat. No. 340486).

Machines used:



Flow Cytometer

Centrifuge

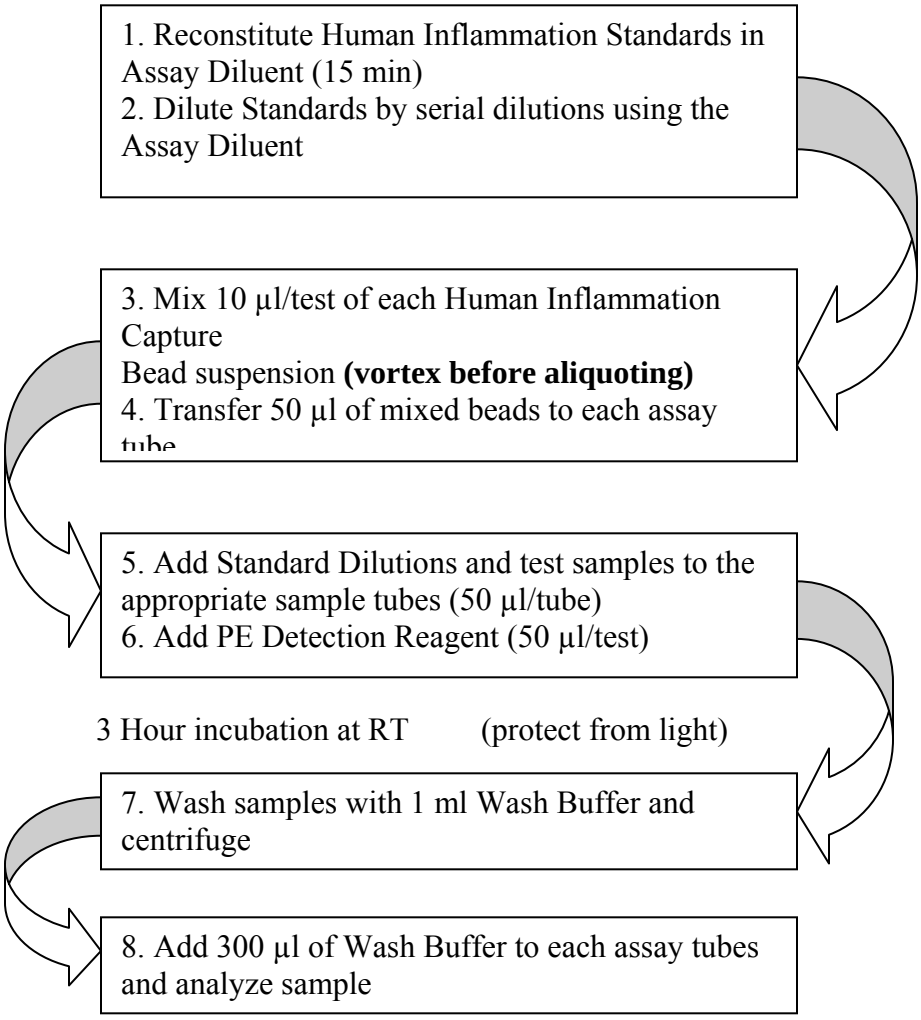


12x75 mn tubes

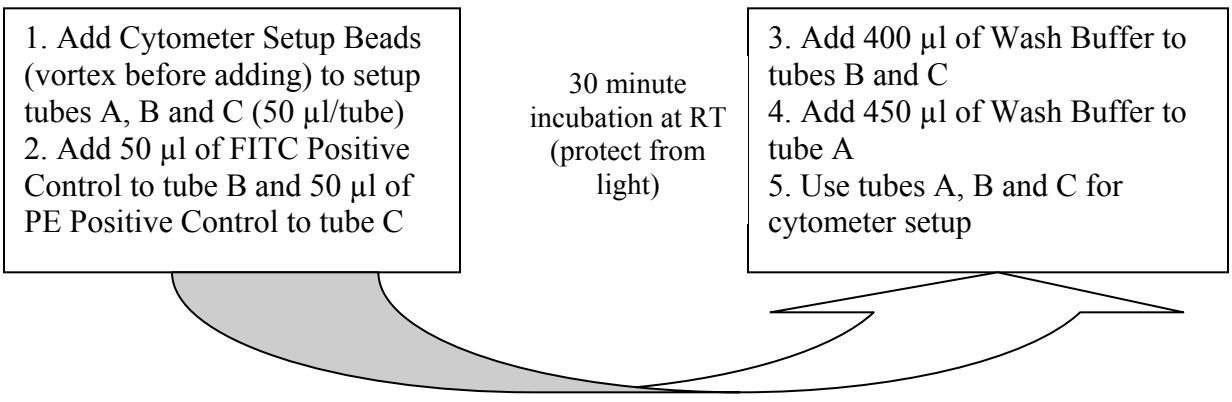
Vortex

Overview of Procedure:

BD™ CBA Human Inflammation Kit Assay Procedures
Culture Supernatant Assay Procedure



Cytometer Setup Bead Procedure



Basic overview of protocol:

1. Addition of the capture beads that contain each cytokine to both the standards and the serum samples

2. To this mixture, the antibodies to each cytokine conjugated to a fluorescent dye called PE is added; three hour incubation

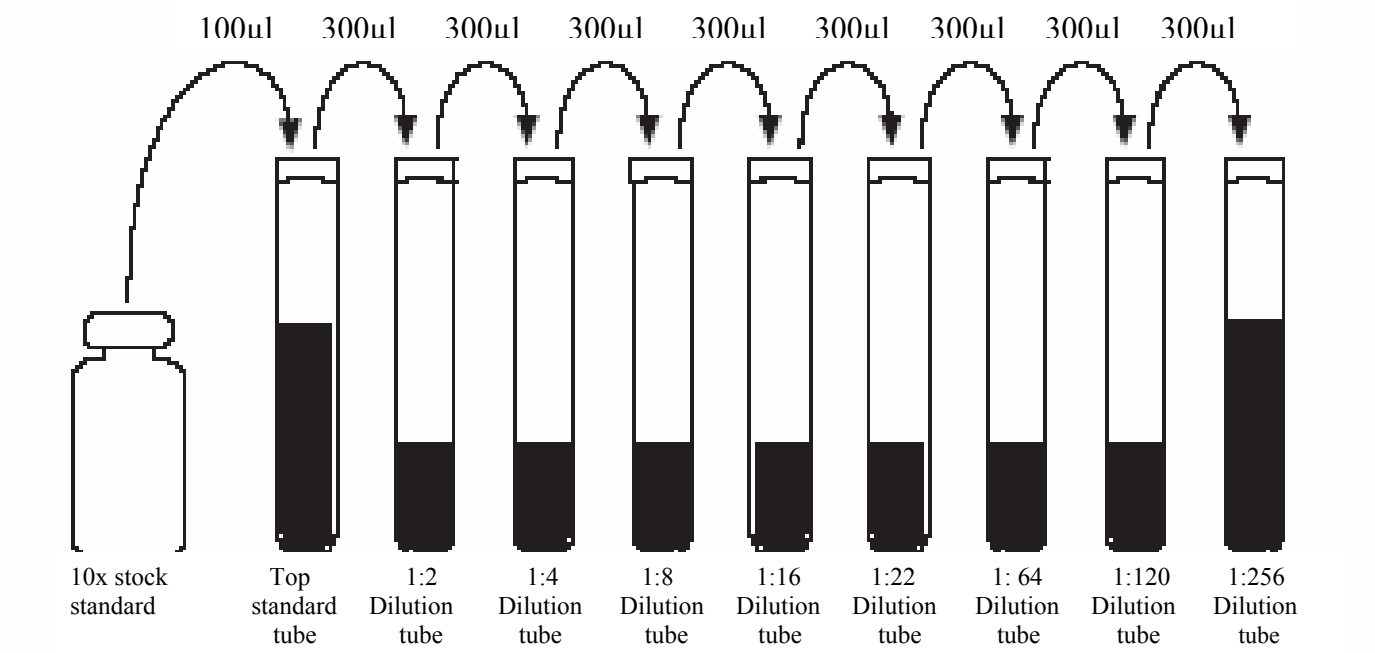
3. After incubation, the samples and standards are washed once and analyzed on a flow cytometer

The Procedure in Details

Preparation of Human Inflammation Standards

The Human Inflammation Standards are lyophilized and should be reconstituted and serially diluted before mixing with the Capture Beads and the PE Detection Reagent.

1. Reconstitute 1 vial of lyophilized Human Inflammation Standards with 0.2 ml of Assay Diluent to prepare a 10× bulk standard. Allow the reconstituted standard to equilibrate *for at least 15 minutes* before making dilutions. Agitate vial to mix thoroughly. *Do not vortex.*
2. Label 12 × 75 mm tubes and arrange them in the following order: Top Standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
3. Add 900 µl of Assay Diluent to the Top Standard tube. 4. Add 300 µl of Assay Diluent to each of the remaining tubes.
4. Add 300 µl of Assay Diluent to each of the remaining tubes.
5. Transfer 100 µl of 10× bulk standard to the Top Standard tube and mix thoroughly.
6. Perform a serial dilution by transferring 300 µl from the Top Standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 300 µl from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mix thoroughly (see ***the figure below.***) The Assay Diluent serves as the negative control



Preparation of Human Inflammation Standard Dilutions:
the approximate concentration (pg/ml) of recombinant protein in each dilution tube is shown in Table 1.

Table 1. Human Inflammation Standard concentrations after dilution

Protein (pg/ml)	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Human IL-8	5000	2500	1250	625	312.5	156	80	40	20
Human IL-1β	5000	2500	1250	625	312.5	156	80	40	20
Human IL-6	5000	2500	1250	625	312.5	156	80	40	20
Human IL-10	5000	2500	1250	625	312.5	156	80	40	20
Human TNF	5000	2500	1250	625	312.5	156	80	40	20
Human IL-12p70	5000	2500	1250	625	312.5	156	80	40	20

Preparation of Mixed Human Inflammation Capture Beads

The Capture Beads are bottled individually, and it is necessary to pool the bead reagents (A1 – A6) immediately before mixing them together with the PE Detection Reagent, standards, or samples. It is recommended that this procedure be used for preparing the mixed Human Inflammation Capture Beads for experiments in which cell culture supernatant samples will be analyzed. For experiments testing serum or plasma samples, refer to ***Preparation of Mixed Human Inflammation Capture Beads for Serum and Plasma Sample Analysis***, below.

1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (eg, 8 unknowns, 9 standard dilutions, and 1 negative control = 18 assay tubes).
2. Vigorously vortex each Capture Bead suspension for a few seconds before mixing.
3. Add a 10 µl aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled "mixed Capture beads" (eg, 10 µl of IL-8 Capture Beads × 18 assay tubes = 180 µl of IL-8 Capture Beads required).
4. Vortex the Bead mixture thoroughly.

The mixed Capture beads are now ready to be transferred to the assay tubes (50 µl of mixed Capture beads/tube) as described in ***BD™ CBA Human Inflammation Kit Assay Procedures***)

Preparation of Mixed Human Inflammation Capture Beads for Serum and Plasma Sample Analysis

It is recommended that the following procedure be followed for preparing the mixed Human Inflammation Capture Beads for experiments in which serum and plasma samples will be analyzed. Use of this procedure will reduce the chances of false-positive results due to the effects of serum or plasma proteins. This procedure may also be used with cell culture supernatant samples.

1. Follow steps 1 – 4 under Preparation of Mixed Human Inflammation Capture Beads, above.
2. Centrifuge mixed Capture Beads at 200 × *g* for 5 minutes.
3. Carefully aspirate and discard the supernatant.
4. Resuspend the mixed Capture Beads pellet in Serum Enhancement Buffer (equal volume to amount removed in step 3) and vortex thoroughly.
5. Incubate the assay tubes for 30 minutes at RT and protect from direct exposure to light.

The mixed Capture beads are now ready to be transferred to the assay tubes (50 µl of mixed Capture beads/tube) as described in ***BD™ CBA Human Inflammation Kit Assay Procedures***, page 15.

Preparation of Test Samples

The standard curve for each protein covers a defined set of concentrations from 20 – 5000 pg/ml. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated standard curve. For

best results, samples that are known or assumed to contain high levels of a given protein should be diluted as described below.

1. Dilute test sample by the desired dilution factor (ie, 1:2, 1:10, or 1:100) using the appropriate volume of Assay Diluent.
2. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing mixed Capture beads and PE Detection Reagent.

BD™ CBA Human Inflammation Kit Assay Procedures

Following the preparation and dilution of the standards and mixing of the capture beads, transfer these reagents and test samples to the appropriate assay tubes for incubation and analysis. The serum/plasma assay procedure should be used for any experiment testing serum or plasma samples. The serum/plasma assay procedure also works for culture supernatant

Culture Supernatant Assay Procedure

1. Add 50 µl of the mixed Capture beads (prepared using the procedure described in ***Preparation of Mixed Human Inflammation Capture Beads***,) to the appropriate assay tubes. Vortex the mixed Capture beads before adding to the assay tubes.
2. Add 50 µl of the Human Inflammation PE Detection Reagent to the assay tubes.
3. Add 50 µl of the Human Inflammation Standard dilutions to the control assay tubes.
4. Add 50 µl of each test sample to the test assay tubes.
5. Incubate the assay tubes for 3 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure

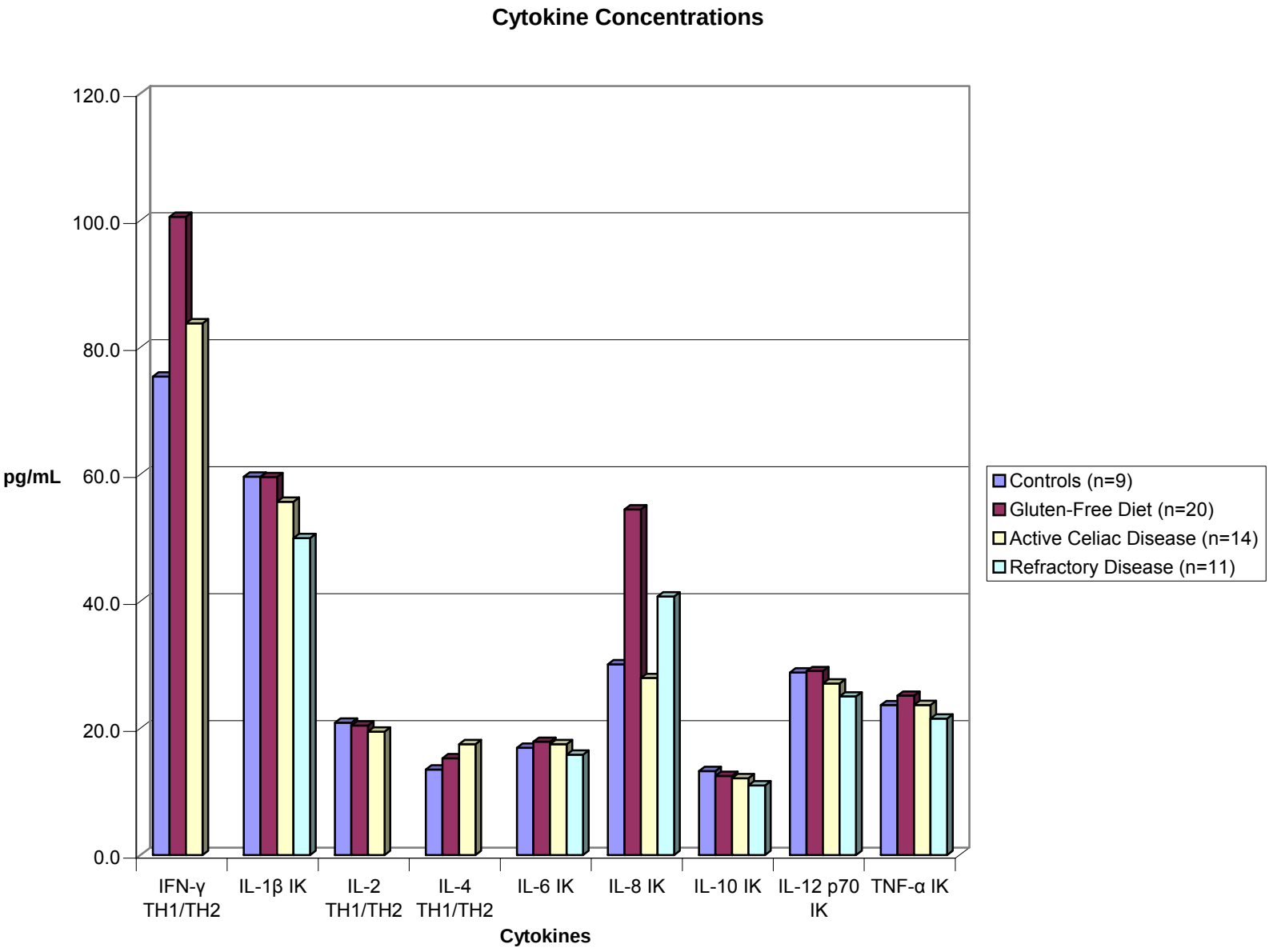
6. Add 1 ml of Wash Buffer to each assay tube and centrifuge at 200 × *g* for 5 minutes.
7. Carefully aspirate and discard the supernatant from each assay tube.
8. Add 300 µl of Wash Buffer to each assay tube to resuspend the bead pellet.
9. Begin analyzing samples on a flow cytometer. Vortex each sample for 3 – 5 seconds immediately before analyzing on the flow cytometer.

Serum/Plasma Assay Procedure

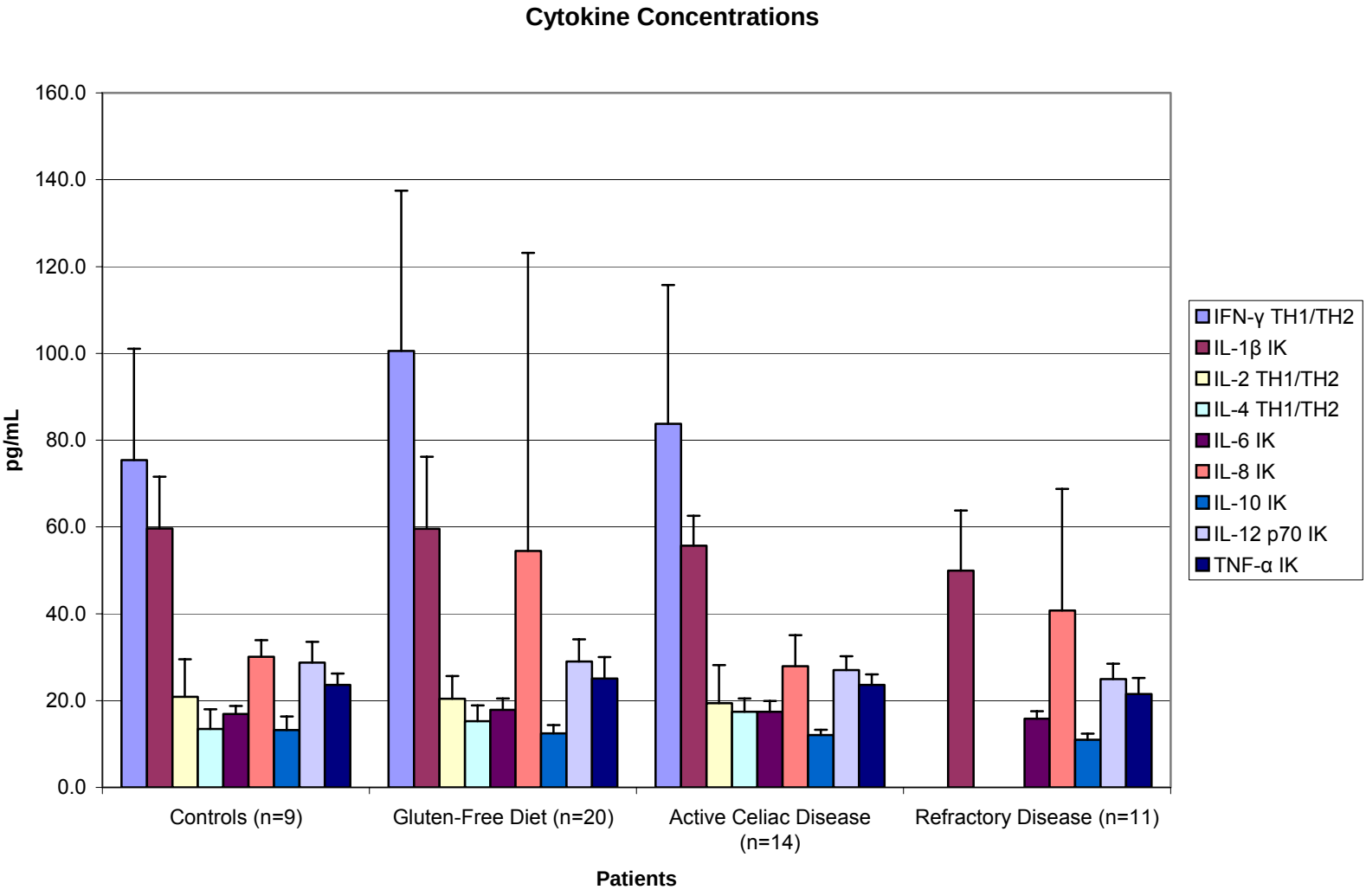
1. Add 50 µl of the mixed Capture beads (prepared using the procedure described in ***Preparation of Mixed Human Inflammation Capture Beads for Serum and Plasma Sample Analysis***,)to the appropriate assay tubes. Vortex the mixed Capture beads before adding to the assay tubes.
2. Add 50 µl of the Human Inflammation Standard dilutions to the control assay tubes.
3. Add 50 µl of each test sample to the test assay tubes.
4. Incubate the assay tubes for 1.5 hours at RT and protect from direct exposure to light.
5. Add 1 ml of Wash Buffer to each assay tube and centrifuge at 200 · *g* for 5 minutes.
6. Carefully aspirate and discard the supernatant, leaving approximately 100 µl of liquid in each assay tube. Aspiration should be done as consistently as possible.
7. Add 50 µl of the Human Inflammation PE Detection Reagent to the assay tubes. Gently agitate assay tubes to resuspend bead pellet.

8. Incubate the assay tubes for 1.5 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure described on pages 17 and 18.
9. Add 1 ml of Wash Buffer to each assay tube and centrifuge at 200 · *g* for 5 minutes.
10. Carefully aspirate and discard the supernatant from each assay tube.
11. Add 300 µl of Wash Buffer to each assay tube to resuspend the bead pellet.
12. Begin analyzing samples on a flow cytometer. Vortex each sample for 3 – 5 seconds immediately before analyzing on the flow cytometer.

Results



Note the concentration of IFN-gamma. The concentration of IFN-gamma on a gluten-free diet patient exceeds the control by a very significant number. This also happens for interlukein-8 (IL-8),



Note: Some of the gluten-free diet patients had very high concentration levels of Il-8 cytokines, where as some had it very low.

Conclusion:

The level of pro-inflammatory cytokines such as *IFN-γ* and *IL-8* seemed to be increased in both patients with *active celiac disease* and celiac disease *patients on a gluten-free diet.*

We are analyzing the data on an individual patient-by-patient basis to determine if there is a correlation with the biopsy grade for each patient.

Reference

- "Intestinal Villi" No date. Online image. Anaglyph stereo images. www.udel.edu/.../ anaglyphpage/anaglyph.html (Fig. 1)

- "Noraml Villi/Loss of Villi." No date. Online image. Celiac Sprue - When Bread Is Poison. 2 April 04. www.gihealth.com/ newsletter/previous/029.html (fig. 2 & 3)

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- BD™ Cytometric Bead Array (CBA) Human Inflammation Kit Instruction Manual.

  DB Biosciences

Acknowledgements

- Dr. Sanil Manavalan
- Dr. Jamal Naiyer
- Sindhu Kunduvaram
- Dr. Sat Bhattacharya
- Harlem Children Society