ASHI U

Title: (d) Pronase

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Date prepared/revised: January 14, 2013
Section 2: Objectives

- Understand the basics of Fc receptors.
- Understand the clinical rationale for using pronase-digested lymphocytes in flow cytometric crossmatches.
- Understand the general testing process of pronase digestion.
- Understand the pros and cons of pronase digestion.
- Understand the ASHI Standards that are applicable to pronase digestion.
**Section 3: Introduction**

Pronase is a proteolytic enzyme that removes Fc receptors and other Fc receptor-like surface molecules on cells of the immune system. Fc receptors are cell surface molecules that bind to the Fc region of immunoglobulins, facilitating phagocytosis or antibody-dependent cell-mediated-cytotoxicity of the immunoglobulin-coated pathogen or cell. ‘Pronase E’ is the name given to a group of proteolytic enzymes produced by *Streptomyces griseus*. There are at least 10 proteases in this mixture: five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases, and one zinc carboxypeptidase.

Fc receptors are present on numerous cells of the immune system and are antibody class specific and isotype selective. Fc receptors are most commonly found monocytes, neutrophils and activated B cells, but can also be found on platelets, eosinophils and some immunoregulatory T cell subsets. There are numerous groups of Fc receptors, based on the class of immunoglobulin they bind and the distribution on cells of the immune system. For example, there are three groups of IgG receptors: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), with multiple subtypes of each group, all sharing a common immunoglobulin domain structure that is susceptible to pronase digestion.

Surface molecules such as Fc receptors present on B lymphocytes can contribute to non-specific auto reactivity in a flow cytometric crossmatch. Antibodies present in the patient serum can bind to Fc receptors on the donor B cells solely based on antibody class or isotype, causing higher background fluorescence than on T cells. Higher background fluorescence on the B cell flow crossmatch can reduce the sensitivity of the assay and make interpretation difficult for the laboratory and clinicians.

The utilization of pronase-digested cells can be advantageous as pronase cleaves the Fc receptor, reducing the opportunity for nonspecific binding of antibody to the Fc receptor. The use of pronase-digested lymphocytes can decrease the background in the assay and increase the sensitivity and specificity of the B cell flow cytometric crossmatch.

Additionally, pronase has been used for crossmatches when the patient is being treated with Rituximab, a humanized anti-CD20 monoclonal antibody. Due to the structural similarity between Fc receptors and CD20, pronase cleaves the CD20 from the surface of B lymphocytes, eliminating the target of Rituximab. As a consequence CD19, and not CD20, must be used to delineate B cells in a population of pronase-digested cells.
It is important to note that not all laboratories and clinicians agree on the advantages of using pronase-digested cells in the flow cytometric crossmatch. Many laboratories have found that pronase-digested cells can be advantageous for increased sensitivity of the B cell crossmatch, but not for the T cell flow crossmatch since Fc receptors are only found on a small subset of T cells. In the T cell crossmatch, background fluorescence can actually increase when using pronase-digested cells. Laboratories may feel that pronase digestion renders the lymphocytes too fragile for crossmatching. The use of frozen lymphocytes in a crossmatch may not be possible, as these cell preparations may be too fragile to withstand the stress of pronase digestion. It may also be challenging for laboratories to obtain enough cells for crossmatching following digestion. Additionally, some clinicians may feel that the pronase crossmatch is too sensitive for their clinical decision making process. Such limitations are important to note and discuss with clinicians if pronase-digested cells are to be used in the flow cytometric crossmatch.

The same ASHI standards apply for the flow cytometric crossmatch regardless of whether digested or undigested cells are used. It is important to note that each laboratory must validate the pronase concentration and digestion time. Laboratories must also independently validate the positive/negative cutoff that will be used for crossmatches using pronase-digested cells. Due to changes in background fluorescence, it may be necessary to use a different cutoff with digested and undigested cells.
Section 4: Methodologies

Pronase digestion is performed on a lymphocyte preparation that has been purified for flow cytometry. Validation of pronase concentration and digestion time is required prior to clinical use. It is important that the pronase concentration and digestion time used is high enough to yield appropriate removal of Fc receptors, but not too high as to damage the cells or cleave bystander molecules such as CD19. Each new lot or batch of pronase must pass quality control before use.

A cell count should be performed before and after pronase digestion, as the concentration will decrease after digestion. In cases where cells are very limited, pronase digestion may not be possible. The cells should be used within a few hours of pronase digestion. After an extended incubation, it is possible for live lymphocytes to begin to regenerate the cleaved receptors on the cell surface. Alternatively, pronase digestion may render cells more fragile and lead to a decrease in the percentage of viable cells.

Typically, the channel values will be different for pronase-digested versus non-digested cells. The laboratory must establish a positive negative cutoff channel shift for the pronase crossmatch. This cutoff value may or may not be the same as for the crossmatch with the undigested cells.
Section 5: Click here to link to the web based ASHI U Procedure Manual
Section 6: Frequently Asked Questions

Question: How do we determine the appropriate pronase concentration and digestion time?
Answer: The concentration and digestion time must be high enough to yield appropriate removal of Fc receptors, but not too high as to damage the cells or cleave bystander molecules such as CD19 or HLA molecules. Control studies must be set up looking at a range of pronase concentrations and digestion times in order to determine optimal conditions. Such studies may include evaluation of Fc or Fc-like receptor expression by flow cytometry and crossmatch of the digested cells with previously characterized sera. Additionally, the number of T and B lymphocytes before and after pronase digestion can be compared to ensure minimal cell loss with pronase digestion.

Question: Our clinicians do not use Rituximab. Do we need to run crossmatches with pronase digested lymphocytes?
Answer: The decision on whether or not to use pronase-digested cells depends on the clinicians and your laboratory. Crossmatches with pronase-digested cells can be useful in reducing the background fluorescence on the crossmatch and eliminating non-specific binding to the cells. However, some laboratories also believe that pronase digestion renders the cells too fragile and gives falsely elevated channel shifts.

Question: Can we digest frozen cells with pronase?
Answer: Many laboratories have found that frozen cells are too fragile to withstand pronase digestion. Ultimately it is up to each laboratory to determine if this method will work for them.

Question: Do we need to run our crossmatches with both digested and undigested cells?
Answer: Again, this depends on the clinicians and your laboratory. Some laboratories use pronase crossmatches only, some use both regular and pronase crossmatches, and others use a combination of both (i.e. regular T-cell and pronase B-cell). It is necessary to have an established and validated positive/negative cutoff value for each type of crossmatch being used in your laboratory.
Section 7: Applicable ASHI Standards

ASHI standards do not specifically address pronase digestion. The following standards must be followed for flow crossmatch with or without pronase digestion.

D.5.2.9 Laboratories performing flow cytometry techniques must:

D.5.2.9.1 Establish the optimum serum-to-target ratio.

D.5.2.9.2 Establish the threshold for discriminating positive reactions regardless of the method used for reporting raw data (mean, median, mode channel shifts or quantitative fluorescence measurements). Any significant change in protocol, reagents or instrumentation requires a repeat determination of the positive threshold.

D.5.2.9.3 Define acceptable time periods between processing, labeling and data acquisition. Control samples must be treated in the same manner.

D.5.2.9.4 Use the dilution and/or volume of reagents locally validated prior to use.

D.5.2.9.5 Process antibodies or other reagents from lyophilized powder in order to remove microaggregates prior to use, according to the manufacturer's instructions or locally documented procedures.

D.5.2.9.6 Assess the binding of human immunoglobulin using a fluorochrome-labeled reagent, such as an F(\(ab'\))2 anti-human IgG specific for the Fc region of the heavy chain, or other documented method.

D.5.2.9.7 Use Anti-human immunoglobulin reagents according to manufacturer's protocol or titered to determine the dilution with optimal sensitivity (signal-to-noise ratio). If a multicolor technique is used, the reagent must not demonstrate crossreactivity with the other immunoglobulin reagents used to label the cells.

D.5.2.9.8 Laboratories performing cell-based antibody screening and/or crossmatching by flow cytometry must:

D.5.2.9.8.1 Document that the method used for cell preparation meets or exceeds the laboratory's established criteria for purity and viability; and is sufficient to ensure accurate test results.

D.5.2.9.8.2 Differentiate specific populations (e.g., T cells, B cells and/or monocytes) using monoclonal antibodies that detect the appropriate CD antigen(s), and that are labeled with a fluorochrome different from the one used to detect the binding of the patient's antibody.

D.5.2.9.8.3 For internal labeling, document that the method used to allow fluorochrome-labeled antibodies to penetrate the cell membrane is effective.
Section 8: Literature Cited and Recommended Reading


Protease Type XIV Product Insert. Sigma-Aldrich 8/14/98
