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Enhancing Pre-Transplant Flow Cytometry with Pronase: Applications and Insights.

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Abstract:

Pre-transplant Flow cytometry cross-matching (FCXM) is a widely used technique for evaluating organ transplant compatibility. It is crucial for determining the risk of graft rejection and tracking immune responses. Flow cytometry is optimized to identify donor-specific antibodies (DSA) directed toward the human leukocyte antigens (HLA) present in the donor' s cells. Many of these antibodies can mount an immune response to the transplanted organ causing early allograft rejection. However, one of the main disadvantages of FCXM is the limited accessibility of human leukocyte antigen (HLA) epitopes on donor cell surfaces, which makes it difficult to identify DSAs with low affinity. This can lead to a saturation of all available binding sites when they encounter a mismatched donor, especially among sensitized patients who previously had varying degrees of exposure to foreign HLA antigens from transplants, blood transfusions, or pregnancies. Recent research has demonstrated that pronase, a proteolytic enzyme can dramatically boost FCXM sensitivity by cleaving extracellular proteins on the donor cell surface and revealing buried HLA epitopes. This enhancement enables the detection of DSAs that would otherwise be missed by traditional approaches, resulting in a more accurate assessment of transplant compatibility and better prediction of AMR risk. This review looks into the processes by which pronase improves FCXM sensitivity, its therapeutic applications in organ donation, and its potential benefits in treating sensitized patients. It also covers the problems, limitations, and future directions of pronase-enhanced FCXM, providing insight into its role in transplant immunology and personalized medicine.

Key words: Kidney transplantation, rejection, donor specific antibodies, flow cytometry, cross match, pronase.

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Email: haniarizwan5@gmail.com
DOI: 10.53778/pjkd91289
Received Feb 25, 2025 accepted March 28, 2025 PJKD 2024;9(1):27-32

Introduction:

Solid organ transplantation saves the lives of thousands of patients suffering from end-stage organ failure. Nevertheless, one of the principal problems in clinical transplantation is antibody-mediated rejection (AMR), which occurs when the recipient's immune system produces antibodies against the donor's human leukocyte antigens (HLA), thus long-term results are of prime importance.[1] This initiates a cascade of immune responses that culminate in graft rejection, dysfunction, and ultimately, failure.

Early identification of donor-specific antibody (DSA) is essential for determining the likelihood of antibody-mediated rejection and influences pre-transplant choices including desensitization procedures and donor selection.[2]

Flow cytometry cross-matching (FCXM) assay is one of the best semi-qualitative methods that can identify HLA antibodies attaching to T-cells and B-cells simultaneously. The procedure begins with isolating lymphocytes (either T or B cells) from the donor' s blood.[3]
Possible donor-specific antibodies (DSA) in the recipient's serum are incubated with the donor cells. In the presence of DSAs, they attach themselves to the surface of the donor cells. After incubation, fluorescently conjugated anti-human IgG antibodies are added, binding only to the DSAs of the lymphocytes. They are then analyzed using a flow cytometer.[4]

The flow cytometer works by passing cells through a laser beam, causing them to produce light of specific wavelengths. Detectors collect the emitted light and measure its fluorescence intensity. The emitted light is captured by detectors, which measure the intensity of its fluorescence. Cells attached to DSA will produce a stronger fluorescence signal compared to non-attached cells. That way, it can detect when there are DSAs. A positive indicates suspect abstraction and is associated with significant fluorescence, suggesting AMR may be occurring. FCXM is used to predict such transplant rejection and direct clinical decision-making.[5]

While FCXM is an effective tool for detecting specific antibodies, factors such as restricted access to HLA epitopes on donor cells may compromise sensitivity. This may produce False-negative results, especially if DSAs target low-expression or buried epitopes on the donor cell surface. As a result, transplant facilities might miss crucial antibodies that might cause early allograft rejection.[5] To overcome these obstacles, scientists and medical professionals have turned to Pronase, a proteolytic enzyme that can increase the sensitivity of FCXM.[6]

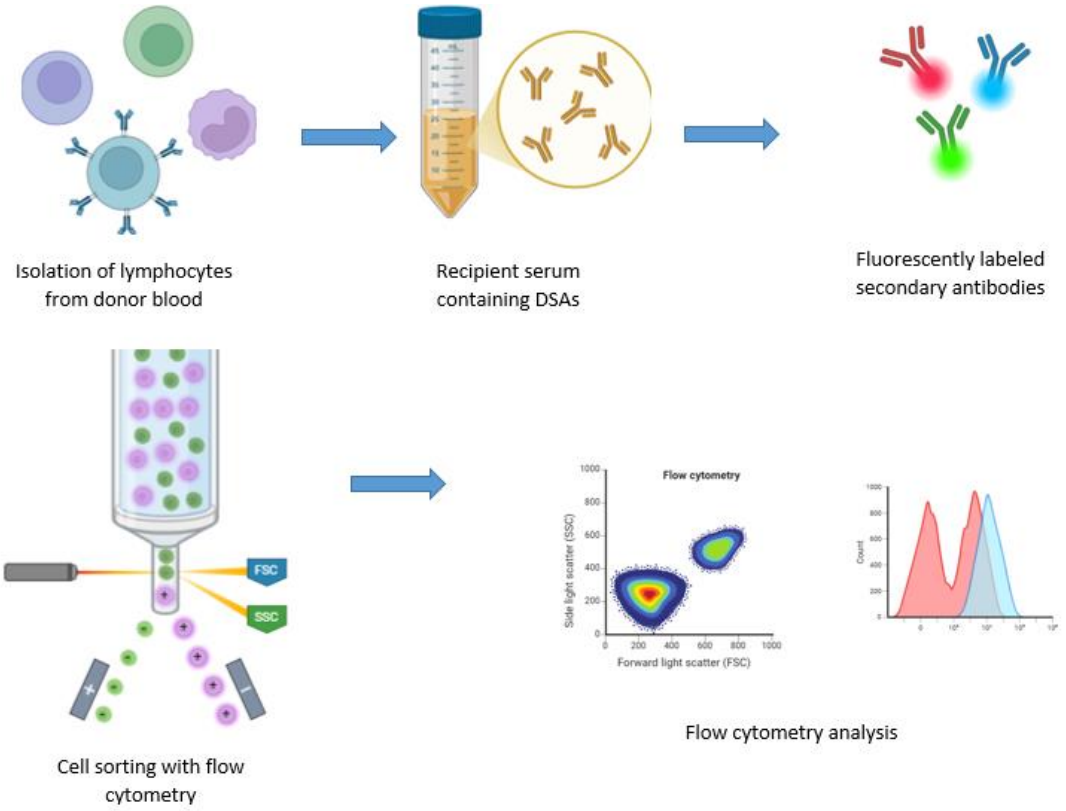
Principles of Flow Cytometry Cross-matching:

Flow cytometry is widely used in the field of transplantation for both pre-transplant cross-matching and continuous monitoring of immune reconstitution. It has a significant role in the immunological recovery of the patient following transplantation.[7] Flow cytometry is a versatile tool used in histocompatibility laboratories as it can detect and track anti-donor antibodies with great sensitivity, and can simultaneously recognize several cell types and antigens. In a clinical setting, this ability plays a vital role in identifying patients who may be at increased risk of early transplant rejection. By recognizing these individuals, healthcare providers can initiate timely interventions, ultimately leading to improved patient outcomes.[8]

The process involves mixing donor lymphocytes, typically drawn from peripheral blood with the recipient's serum. Using a flow cytometer, the objective is to evaluate the way the recipient's antibodies attach to the donor's cells. Using fluorescently labeled secondary antibodies, flow cytometry makes it possible to identify antibodies that adhere to the donor's cell surface. The fluorescence intensity reveals

important details about the recipient's antibody response and its potency. A positive cross-match is indicated by the flow cytometer measuring the degree of binding between the recipient's antibodies and the donor's cells if the recipient has antibodies that target the donor's HLA, Figure 1.[9]

Fig1: The figure shows the process of flow cytometry cross-matching. 1) Isolation of lymphocytes (T and B cells) from donor blood. 2) Incubation of donor lymphocytes with recipient serum containing DSAs. 3) Addition of fluorescently labeled antibodies that will bind with the DSAs attached to donor lymphocytes. 4) Flow cytometry analysis of fluorescence intensity indicating the presence of DSAs.



Despite being a useful method for identifying donor-specific antibodies (DSAs), flow cytometry has some drawbacks that may provide false-negative results. One major obstacle is the presence of HLA antigens on donor cell surfaces is necessary for the detection of HLA antibodies. Antibody binding may be impeded by elements such as the presence of particular immunoglobulins or the structural arrangement of HLA molecules. This is particularly true when the cell surface epitopes are not readily accessible or exposed, which hinders the ability of antibodies to bind efficiently.[10] The inherent heterogeneity in the composition of lymphocyte membranes, which can vary among donors, presents another problem. This variation may affect the expression of HLA molecules on the cell surface, potentially leading to inconsistent cross-match test findings. When it comes to identifying antibodies that target conformational epitopes, which might not be easily accessible for detection, these variables can restrict the sensitivity and accuracy of conventional flow cytometry.[11] The detection of low levels of donor-specific antibodies (DSAs), which can exist in extremely minute numbers in a recipient's

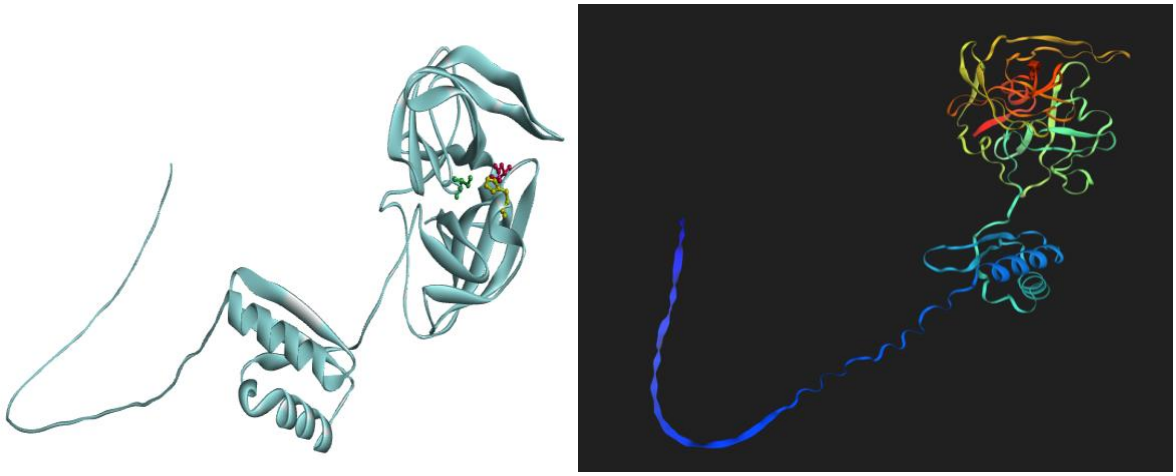
serum, is one of the main problems in transplant immunology. These antibodies have the potential to greatly influence graft rejection, yet they are frequently hard to detect because of their low concentration or because specific HLA molecule epitopes may be obscured.[12]

The role of Pronase in enhancing low cytometry sensitivity:

Pronase, a combination of proteolytic enzymes, is frequently employed in biological research because of its capacity to break down proteins, Figure 2. As a broad-spectrum protease, it can degrade a variety of proteins, including cell surface antigens and membrane proteins. Pronase is a helpful tool in a variety of immunological tests, particularly where antigen masking or low antigen expression on cell surfaces may limit detection. It is used in flow cytometry to improve the sensitivity and quality of cell surface staining.[13]

The issue of epitope blockage on cell surfaces brought on by firmly bound or impeded proteins is one of the challenges in flow cytometry. This may lessen sensitivity and make it more difficult to find certain antibodies. The issue of epitope blockage on cell surfaces brought on by firmly bound or impeded proteins is one difficulty in flow cytometry. This may lessen sensitivity and make it more difficult to find certain antibodies. Protein components of the cell membrane, such as glycoproteins and other surface antigens, are broken down by adding pronase to the cell solution prior to staining. Pronase alters or eliminates adjacent glycoproteins and components by acting on the outer surfaces of HLA molecules. By revealing hidden epitopes, this improves antibody binding. Pronase breaks down or modifies the structure of membrane proteins by cleaving peptide links in proteins. Glycosylation and other alterations can be removed by this method, exposing epitopes that would otherwise be difficult to find. Cells are often cleaned after pronase treatment before being labeled using flow cytometry.

Figure 2: Alphafold protein model of pronase. a)The figure highlights the active sites of the residues of pronase, which are critical for its proteolytic activity. b) 3D protein model of pronase, generated using the Swiss-Model.



This increases the fluorescence intensity in the results by improving the binding of fluorescent antibodies.[14] Pronase treatment thereby improves the precision of flow cytometry tests that seek to identify antibodies that preserve HLA molecules while targeting particular conformational epitopes. This is especially crucial in situations when markers are expressed at low levels or when donor-specific antibodies are detected in transplant studies. Pronase can also help reduce problems

in flow cytometry testing, such as background noise or antibody interference, which can result in more accurate and trustworthy data.[15]

Applications of Pronase-enhanced flow ytometry in organ ransplantation:

A primary advantage of pronase application in flow cytometry is its ability to detect the presence of donor-specific antibodies in sensitized patients. Sensitization results from an exposure of the recipient's immune system to foreign HLA antigens which after exposure can induce the development of DSAs. Previous transplant procedures, blood transfusion, or even pregnancy can lead to such a scenario whereby the recipient has been exposed to HLA antigens from another person. These exposures trigger an immune response in the body, whereby antibodies are produced against some antigens. A key problem with this, however, is that sensitized individuals can still have mechanisms in place, even at low levels of DSA, which will trigger AMR to occur. In such cases, any DSA that is present will often be low in strength and unnoticeable over a period of time, making it difficult for FCXM to identify patients who are likely to experience short-term graft rejection. There is little to no chance of preventing transplant failure because standard cross-matching cannot detect low-level DSA as antibodies do not bind tightly enough. Pronase treatment reduces the HLA epitome and thus increases the detection threshold of flow cytometry.[16]

Determining DSA makes it simpler to assess the likelihood of AMR which is the chief cause of primary graft failure especially in heart and kidney grafting patients, thus assisting those carrying out the transplant to make better decisions. For transplant centers, this combination helps avoid unnecessary interventions by increasing the capability to diagnose AMR when pronase is used together with cross-match testing. Such a method can also help tailor-specific donation strategies because it allows examining aspects such as the mechanisms of sensitization, the role of DSA in graft rejection, and others.[17]

Conclusion:

The implementation of pronase enhanced Flow Cytometry Cross-Matching (FCXM) is an important advance in pre-transplant immunological testing. Pronase treatment thereby enhances exposure to HLA epitopes, enabling the detection of low-affinity donor-specific antibodies (DSAs) that are more commonly undetected by methods currently in use. The better sensitivity is particularly helpful for sensitized patients, who are at increased risk of antibody-mediated rejection (AMR). Adding pronase to FCXM is expected to improve the accuracy of transplant compatibility assessments, enabling improved

decisions regarding donor selection, desensitization strategies, and immunosuppressive medications. Nonetheless, obstacles persist in optimizing protease treatment protocols and achieving consistent use of pronase between patient cohorts. Research into additional strategies and optimized protease-based testing will augment the utility of FCXM in transplant immunology. Overall, pronase-enhanced FCXM has the potential to improve transplant outcomes and lower the likelihood of early graft rejection, thereby improving the field of organ transplantation.

Conflict of Interest: None declared

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