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ORIGINAL ARTICLE

Development of an immunochromatographic strip test for rapid detection of a potent neutralizing anti-interferon gamma antibody in adult-onset immunodeficiency

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Abstract

Background: Adult-onset immunodeficiency (AOID) is associated with the presence of anti-interferon gamma autoantibodies (anti-IFN-γ autoAbs), which neutralizes IFN-γ signaling and leads to susceptibility to intracellular opportunistic infections. However, measuring neutralizing autoAbs is not practical for general clinical laboratories.

Objective: This study aims to develop a competitive immunochromatographic (IC) strip test for detecting autoAbs that recognize the same epitope as a potent neutralizing antibody.

Methods: The competitive IC strip test detecting autoAbs that recognize the same epitope as mouse anti-IFN-γ monoclonal antibody, clone B27 (B27 mAb) was fabricated and used to determine B27 epitope recognizing autoAb (B27 AAb) in AOID plasma. The competitive ELISA was used as a comparative method.

Results: The efficacy of the IC strip test was compared with competitive ELISA and found a percent positive agreement of 91.30%, a percent negative agreement of 79.31%, and a percent overall agreement of 84.62%.

Conclusion: The results from the competitive IC strip test were consistent with those from competitive ELISA, indicating that the generated IC strip could detect B27 AAb in AOID plasma.

Key words: Adult-onset immunodeficiency, Autoantibody, Neutralizing antibody, Anti-IFN-γ autoantibodies, Competitive IC strip test

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Introduction

Adult-onset immunodeficiency (AOID) is a disease in previously healthy adults that is characterized by phenocopies of primary immunodeficiencies (PIDs).[1] AOID patients have an increased susceptibility to opportunistic infections due to the presence of neutralizing anti-IFN-γ autoAbs.[2] Nontuberculous mycobacteria (NTM) are the most common intracellular pathogens found in AOID patients, with an incidence as high as 85%.[2,3] Other common opportunistic infections in AOID patients include varicella-zoster virus (VZV), *Talaromyces marneffei*, *Salmonella* spp., and *Cryptococcus* spp. The most common sites of infections are the lymph nodes, lungs, bones, skin, and bloodstream.[3,4] The treatment of AOID aims to control symptoms and prevent infections, and immunomodulatory therapy is the primary treatment option.

However, treatment response varies, and some patients may not respond to therapy. Monitoring anti-IFN-γ autoAb levels and neutralizing antibodies during treatment may be helpful in predicting treatment response and modifying therapy accordingly. Several methods are available for measuring anti-IFN-γ autoAbs, such as enzyme-linked immunosorbent assays (ELISAs) and flow cytometry detecting IFN-γ-mediated STAT1 phosphorylation.[5] However, these methods require time, expensive equipment, and well-trained technicians.

Currently, the epitopes recognized by neutralizing anti-IFN-γ autoAbs have been examined. Epitope mapping of patient autoAbs has identified amino acids 121-131 of IFN-γ that is linear epitope located in the C-terminal region.[6] The amino acid sequence of this antigenic determinant is highly homologous to a sequence in the Noc2 protein of *Aspergillus* spp., which is cross-reactive with patient autoAbs.[6] Moreover, epitope mapping of neutralizing monoclonal antibodies (mAbs) using human-bovine chimeric proteins have been uncovered two conformational epitopes including regions A and E.[7] By phage display random peptide library, amino acids 27-40 have been designated as epitope of neutralizing anti-IFN-γ mAb, clone B27 (B27 mAb).[8] Recent report indicated that B27 mAb demonstrates remarkable neutralizing activity, implying that the epitope of B27 mAb is crucial for cell activation.[9,10] Using the synthetic octapeptide homologs of IFN-γ, some mAbs react with the epitope was localized to residues 84-94.[11] It is suggesting that there are several clones of neutralizing antibodies which recognize the different epitopes of IFN-γ. The pathogenic autoAbs are classified into three groups based on their binding sites including autoAbs that block the binding to IFN-γ receptor 1 (IFN-γR1), autoAbs that inhibit IFN-γR1-IFN-γR2 heterodimerization, and autoAbs that mediate antibody-dependent cellular cytotoxicity.[12]

Our previous study reported that mAbs recognizing distinctive antigenic determinants inhibit the biological activity of IFN-γ to different degrees.[10] We identified the epitope recognized by a population of anti-IFN-γ autoAbs in patient sera related to an efficient B27 mAb. Remarkably, patient sera carrying B27 AAb showed blockade effects of IFN-γ-mediated signaling. Monitoring this population of neutralizing antibodies may provide more information for therapeutic decisions. In this study, we propose a lateral flow immunoassay for detecting the population of neutralizing antibodies in clinical samples. The IC strip test is based on competitive binding of autoAb to neutralizing mAb, B27 mAb. This simple and convenient method will provide an assessed method for a certain neutralizing anti-IFN-γ autoAb detection in AOID patients. Detection of epitope-specified neutralizing autoAbs might be useful for developing personalized therapy. By providing a practical and reliable method for detecting B27 AAb, this study aims to contribute to the development of more effective and personalized treatments for AOID patients.

Materials and methods

Samples

The 52 AOID patient plasma used in this study were obtained from Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. All patients were positive for anti-IFN-γ autoAbs by indirect ELISA as described below. The research protocol was approved by Siriraj Institutional Review Board, SIRB Protocol No. 490/2562 (EC2). Ethics approval and informed consent from participants were obtained in accordance with the guidelines of the Helsinki declaration.

Detection of anti-IFN-γ autoAbs by indirect ELISA

To examine anti-IFN-γ autoAbs in patient plasma, indirect ELISA was performed. The recombinant IFN-γ used in this study was prepared as describe elsewhere.[10] The wells of microtiter plate were coated with 50 μL of 2.5 μg/mL recombinant IFN-γ in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The coated wells were washed three times with 0.05% Tween 20 in phosphate-buffered saline (PBS) and blocked with 200 μL of blocking buffer (2% skimmed milk in PBS) at room temperature. After 1 h, the plates were washed and 50 μL of patient plasma at dilution 1:5000 in blocking buffer was added and incubated for 1 h. The plates were then washed and added rabbit-anti-human IgG conjugated HRP (KPL, Gaithersburg, MD, USA) at dilution 1:5000. After 1 h incubation, 50 μL of TMB substrate was added and incubated for 15 min. The reaction was stopped by adding 50 μL of 1N HCl and optical density (OD) was measured at 450 nm.

Determination of B27 epitope recognizing autoAb by competitive ELISA

In order to detect B27 neutralizing antibody in AOID samples, competitive binding of anti-IFN-γ autoAb to B27 mAb was performed by competitive ELISA. The wells of microtiter plate were coated with 50 μL of 2.5 μg/mL recombinant IFN-γ in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times with 0.05% Tween 20 in PBS and coated wells were blocked with 200 μL of blocking buffer (2% skimmed milk in PBS) for 1 h at room temperature. Each patient plasma was diluted 1:250 and combined with equal volume of 0.1 μg/mL B27 mAb (ImmunoTools, Friesoythe, Germany). After removing the blocking buffer, the mixture of antibodies was added to the coated wells and incubated for 1 h. The wells were subsequently washed for five times before adding 50 μL of goat anti-mouse immunoglobulin conjugated HRP (KPL, Gaithersburg, MD, USA) at dilution 1:3000 and incubated for 1 h. After washing, 50 μL of TMB substrate were added and the enzymatic reaction was stopped with 1N HCl. The OD was measured at 450 nm. The relative level of B27 AAb was calculated by % binding of B27 mAb without plasma - % binding of B27 mAb with plasma and represented in percent inhibition (% inhibition).

Biotinylation of recombinant IFN-γ

Recombinant IFN-γ was labelled with biotin using EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Thermo Scientific, Rockford, IL, USA) as manufacturer's protocol. Briefly, recombinant IFN-γ was dissolved in PBS and incubated with the appropriate volume of Sulfo-NHS-LC-Biotin solution on ice for 2 h. Excess biotin was removed by Zeba™ Spin Desalting Columns. Biotin incorporation was detected by ELISA as described elsewhere.[13]

Colloidal gold preparation and conjugation

Colloidal gold (CG) nanoparticles were prepared as described previously.[13] The 6HIS-maltose binding protein-monomeric streptavidin (6HIS-MBP-mSA) was conjugated to colloidal gold using the optimized protocol.[13] Colloidal gold conjugation was determined by UV-Vis spectrophotometer. The 6HIS-MBP-mSA-CG were combined with biotinylated IFN-γ to form the complex of 6HIS-MBP-mSA-CG conjugates and biotinylated IFN-γ (mSA-CGC/biotinylated IFN-γ) upon the biotin-streptavidin interaction.

Fabrication of a competitive IC strip test for detecting B27 epitope recognizing autoAb

The IC strip test operates on the principle of competitive assays, wherein the binding competition between patient anti-IFN-γ autoAbs and B27 mAb indicates the presence of B27 AAb in plasma. A schematic diagram illustrating the competitive IC strip test (created with BioRender.com) is presented in **Figure 1**. In the presence of B27 AAb, only the control line was observed, whereas the absence of B27 AAb resulted in two bands at the test line and control line. To manufacture a competitive IC strip, the mSA-CGC/biotinylated IFN-γ complexes and mouse IgG-CG conjugates (mouse IgG-CGC) was applied to a glass fiber (conjugate pad). B27 mAb and goat anti-mouse immunoglobulin (KPL, Gaithersburg, MD, USA), both at a concentration of 1 mg/mL, were precisely jetted onto a laminated nitrocellulose membrane at the test line and control line zones, respectively. Subsequently, the conjugate pad and nitrocellulose membrane were incubated at 37°C for 4 h and desiccated at room temperature in desiccators. The assembly of immobilized nitrocellulose membrane, absorbent pad, conjugate pad, and sample pad was performed, and the resultant assembly was cut into separate strips using a strip cutter. Each individual strip was then carefully placed in an aluminum package along with a small desiccant pack.

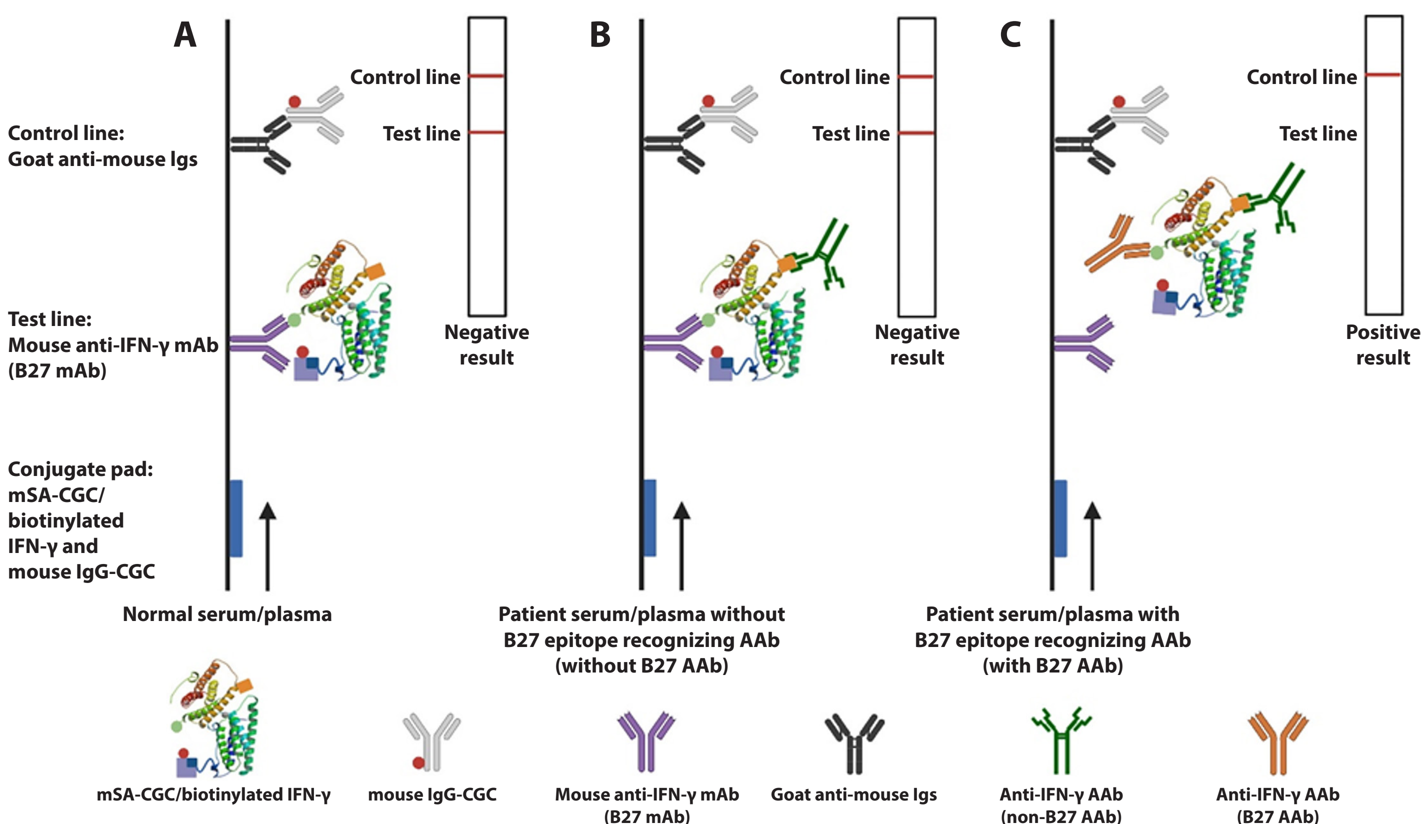

Figure 1. Principle of the competitive IC strip test for detecting B27 epitope recognizing autoAb in AOID plasma.
(A) Schematic diagram represented components of conjugate pad, test line, and control line, and results of serum/plasma without anti-IFN-γ autoAb, (B) illustration of negative result in the absence of B27 epitope recognizing autoAb, and (C) demonstration of positive result in the presence of B27 epitope recognizing autoAb.

Evaluation of competitive IC strip test for detecting B27 epitope recognizing autoAb using B27 mAb

To verify the efficacy of the fabricated IC strip test in detection of B27 AAb, B27 mAb was formerly tested as sample. B27 mAb was diluted in PBS to concentrations 1, 10, and 100 μg/mL. The IC strip test was dipped in 200 μL of different concentration of B27 mAb. The results were read within 10 min. The red-purple band is observed at control line in a positive results.

Evaluation of competitive IC strip test for detecting B27 epitope recognizing autoAb using patient plasma

Patient plasma was diluted at 1:10 in PBS. The competitive IC strip was dipped in 200 μL of diluted plasma for 10 min. The results were monitored by naked eye. The control line must be appeared to indicate the sufficient reaction time and sample volume as well as the quality of IC strip test. In positive results, no band was observed at test line suggesting the presence of B27 AAb in patient plasma. In contrast, there were two bands at test line and control line in the absence of B27 AAb.

Results

Detection of total and neutralizing anti-IFN-γ autoAbs

Total anti-IFN-γ autoAb and B27 AAb, a neutralizing anti-IFN-γ autoAb were examined by indirect ELISA and competitive ELISA, respectively. The optical density at 450 nm and percent inhibition (% inhibition) were compared. The results demonstrated that level of total anti-IFN-γ autoAb correlated with B27 AAb as shown in **Figure 2**.

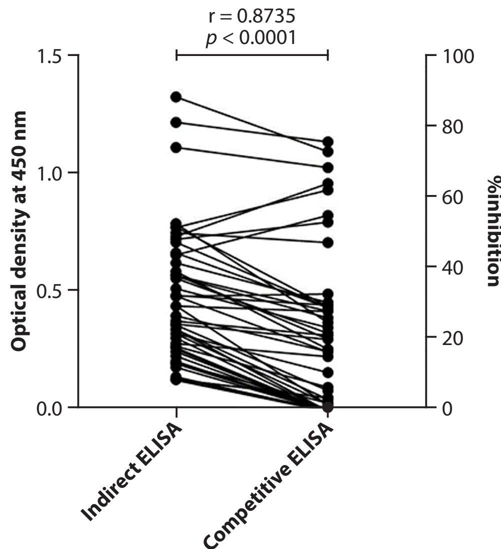

Figure 2. Determination of total and B27 epitope recognizing autoAbs. Total anti-IFN-γ autoAb was measured by indirect ELISA and B27 AAb was detected by competitive ELISA. The correlation of total anti-IFN-γ autoAb and B27 AAb was analyzed by Spearman's correlation coefficients. Statistical significance was calculated using the two-tailed test. The p-value less than 0.05 ($p < 0.05$) is considered statistically significant.

Evaluation of competitive IC strip test for detecting B27 epitope recognizing autoAb using B27 mAb

The competitive IC strip test was tested with B27 mAb in PBS to evaluate whether B27 mAb in solution compete the binding of B27 mAb at test line. The IC strip test was evaluated with various concentrations of B27 mAb as demonstrated in **Figure 3**. The band intensity of test line was decreased in concentration-dependent manner. At 100 μg/mL of B27 mAb, the band at test line was disappeared. There was a faint band at concentration 10 μg/mL of B27 mAb. These data suggested that the generated IC strip test could detect the B27 epitope recognizing antibody. Therefore, this platform was further validated in patient samples.

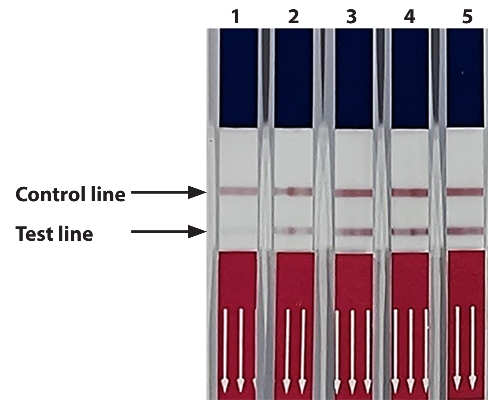

Figure 3. Performance of the competitive IC strip test in detection of mouse anti-IFN-γ mAb, clone B27. The IC strip test was dipped in tube containing 200 μL of B27 mAb which was diluted in PBS for 10 min. Lane 1; B27 mAb 100 μg/mL, lane 2; B27 mAb 10 μg/mL, lane 3; B27 mAb 1 μg/mL, lane 4; PBS, and lane 5; serum from healthy donor.

Evaluation of competitive IC strip test for detecting B27 epitope recognizing autoAb in patient samples

The fabricated IC strip test was validated in 52 AOID plasma compared to competitive ELISA. Example of IC strip test results was shown in **Figure 4**. The results obtained from healthy donor serum and patient sample with 0% inhibition by competitive ELISA were similar to that in PBS. In patient sample with B27 AAb, the band in test line was reduced and absence in patient serum with 55% and 87% inhibition by competitive ELISA, respectively. Control line was observed in all test conditions. These data suggest that the competitive IC strip test could detect the B27 AAb in patient plasma.

Correlation of results obtained by competitive IC strip and competitive ELISA

The competitive ELISA was used as a comparative method in detection of B27 AAb (**Table 1**). At the cut off of percent inhibition ≥ 20 and < 20 by competitive ELISA, the percent positive agreement, percent negative agreement, and percent overall agreement were 91.30%, 79.31%, and 84.62%, respectively.

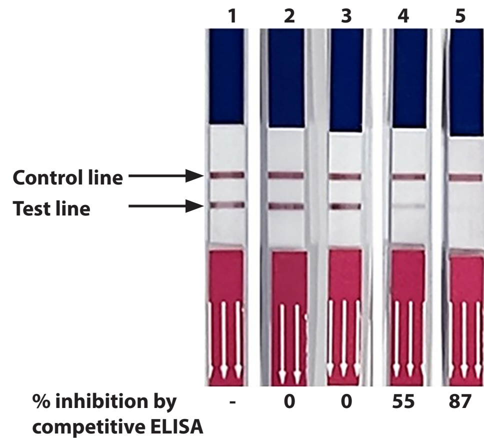

Figure 4. Representative of the competitive IC strip results with different level of B27 epitope recognizing autoAb in AOID samples. A 1:10 dilution of patient serum/plasma in PBS was used. The IC strip test was dipped in 200 μL of diluted serum/plasma for 10 min. Lane 1; result obtained from PBS, lane 2; serum from healthy donor, lane 3; patient sample with 0% inhibition; lane 4; patient sample with 55% inhibition, lane 5; patient sample with 87% inhibition by competitive ELISA.

Table 1. Efficiency of the competitive IC strip test compared with competitive ELISA in patient plasma.

Competitive IC strip test	Competitive ELISA		Total
	% IH ≥ 20	% IH < 20	
Positive	21	6	27
Negative	2	23	25
Total	23	29	52

Discussion

Autoantibodies against IFN-γ can cause immunodeficiency, leading to opportunistic infections such as disseminated nontuberculous mycobacteria (NTM) infections. The severity of the disease is correlated with autoAb levels, with higher titers observed in patients with active opportunistic infections compared to those in remission or stable disease.[14,15] Neutralizing anti-IFN-γ autoAbs block IFN-γ bioactivity by inhibiting its binding to IFN-γR1 or inhibiting IFN- γR1-IFN-γR2 heterodimerization, thus impairing IFN-γ-mediated functions.[12] Investigation of neutralizing anti-IFN-γ autoAbs is essential for evaluating the pathogenic autoAbs. One specific autoAb population, B27 AAb, was identified in AOID patients.[10] B27 AAb exhibits strong neutralizing activity and binds to an epitope on amino acids 27-40 of IFN-γ.[8] In a study of 52 patients, 23 patients (44.23%) exhibited B27 AAb by competitive ELISA. B27 AAb levels correlated with total autoAb levels. However, other autoAb populations might play a role in B27 AAb-negative patients, such as those that recognize amino acids 121-131 of IFN-γ.

The presence of anti-IFN-γ autoAbs has been associated with disease severity and poor outcomes in AOID. Those with high levels of anti-IFN-γ autoAbs had a significantly lower response to treatment and poorer long-term outcomes compared to those with low levels of autoAbs. In addition, the presence of anti-IFN-γ autoAbs was associated with more severe and prolonged disease. These findings suggest that anti-IFN-γ autoAbs may serve as a prognostic marker for AOID. The treatment of AOID aims to control the symptoms and prevent infections. Immunomodulatory therapy is the primary treatment option, and several drugs have been used, including cyclophosphamide, glucocorticoids, immunoglobulins, and rituximab.[3] However, the response to treatment varies, and some patients may not respond to therapy. Therefore, monitoring anti-IFN-γ autoAb levels and activity during treatment may be helpful in predicting treatment response and modifying therapy accordingly.

To detect B27 AAb, a competitive IC strip test was developed and compared to the competitive ELISA. The results showed that the IC strip test strongly agreed with the ELISA for B27 AAb-positive samples but required optimization for B27 AAb-negative samples. The combined use of this test and an IC strip test for total anti-IFN-γ autoAbs could provide more information on autoAbs in AOID patients. Treatment strategies such as IFN-γ administration may not be effective in all patients due to the level and distinct populations of pathogenic anti-IFN-γ autoAbs. An epitope-erased variant of IFN-γ (EE-IFN-γ) that escapes amino acids 121-131 targeting autoAbs has been established and partially restores its function in the presence of patient sera *in vitro*.[6] However, this approach cannot escape other autoAb populations, highlighting the importance of identifying specific autoAb clones for developing targeted treatment strategies. Our previous report demonstrated that IFN-γ mutant modifying amino acids T27, F29, and L30 could escape B27 mAb binding.[8] This IFN-γ variant might be an alternative therapy for B27 AAb-positive patients. The developed competitive IC strip test could serve as a prototype for further developing assays to detect specific neutralizing autoAbs.

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