Value of Interferon-γ Enzyme-Linked Immunospot Assay in Identifying Sensitizing Drugs in Patients with Anti-tuberculosis Drug Hypersensitivity Reactions (Postprint)

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Abstract

Background Anti-tuberculosis drug hypersensitivity reactions are common in clinical practice. Drug provocation test is currently the only effective method to identify the sensitizing drug; however, it can induce recurrent allergic reactions or even anaphylactic shock, posing life-threatening risks in severe cases. Additionally, some patients may abandon treatment, leading to drug resistance and dissemination of Mycobacterium tuberculosis. Given that the currently standard anti-tuberculosis regimen requires combination therapy with four drugs, how to timely and accurately identify the sensitizing drug that induces hypersensitivity reactions is an urgent clinical problem. Objective To evaluate the clinical value of IFN-γ enzyme-linked immunospot assay in rapidly identifying the sensitizing drug responsible for inducing hypersensitivity reactions in such patients. Methods Fifty patients with newly treated drug-sensitive pulmonary tuberculosis who developed drug hypersensitivity reactions during treatment and were hospitalized in our hospital from January 2021 to December 2022 were enrolled. Peripheral blood mononuclear cells were collected from patients during the acute phase of hypersensitivity. Drug-specific IFN-γ-releasing cells were detected using enzyme-linked immunospot assay. Drug provocation test was employed as the gold standard for determining which drug induced the hypersensitivity reaction. The clinical value of enzyme-linked immunospot assay in identifying hypersensitivity reactions induced by the anti-tuberculosis quadruple regimen (isoniazid, ethambutol, rifampicin, pyrazinamide) was analyzed. Results The sensitivity of enzyme-linked immunospot assay in identifying hypersensitivity reactions induced by isoniazid, ethambutol, rifampicin, and pyrazinamide was 69.2%, 61.5%, 75.0%, and 66.7%, respectively; the specificity was 97.3%, 100.0%, 100.0%, and 100.0%. Conclusion IFN-γ enzyme-linked immunospot assay can serve as an effective in vitro diagnostic method for sensitizing drug identification

during the acute phase of anti-tuberculosis drug hypersensitivity.

Full Text

The Value of IFN-γ Enzyme-Linked Immunospot Assay in Identifying Causative Agents in Patients with Hypersensitivity Reactions to Antitubercular Drugs

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Abstract

Background: Hypersensitivity reactions to antitubercular agents are common in clinical practice. The drug provocation test (DPT) is currently the only effective method for identifying causative agents, but it may trigger recurrent allergic reactions or even anaphylactic shock, posing life-threatening risks. Some patients consequently abandon treatment, leading to drug resistance and dissemination of *Mycobacterium tuberculosis*. Since conventional anti-tuberculosis regimens require combination therapy with four drugs, the urgent clinical challenge is to identify the specific drug inducing hypersensitivity reactions in a timely and precise manner, particularly in critically ill patients.

Objective: To evaluate the clinical value of the IFN-γ enzyme-linked immunospot (ELISpot) assay for rapidly identifying causative agents in patients experiencing hypersensitivity reactions to antitubercular drugs.

Methods: We enrolled 50 patients with primary pulmonary tuberculosis who developed hypersensitivity reactions during anti-tuberculosis treatment at Jiangxi Chest Hospital between January 2021 and December 2022. Peripheral blood mononuclear cells (PBMCs) were collected from patients during the acute phase of allergic reactions. Drug-specific IFN-γ-releasing cells were detected using the ELISpot assay, with DPT serving as the gold standard for determining the causative drug. We analyzed the clinical value of ELISpot for identifying hypersensitivity reactions induced by the four-drug anti-tuberculosis regimen (isoniazid, ethambutol, rifampicin, and pyrazinamide).

Results: The sensitivity of ELISpot for identifying hypersensitivity reactions due to isoniazid, ethambutol, rifampicin, and pyrazinamide was 69.2%, 61.5%, 75.0%, and 66.7%, respectively, with specificities of 97.3%, 100.0%, 100.0%, and 100.0%.

Conclusion: The IFN-γ ELISpot assay can serve as an effective in vitro diagnostic tool for identifying causative agents in patients with antitubercular drug hypersensitivity during the acute phase of allergic reactions.

Keywords: Tuberculosis; Antitubercular agents; Hypersensitivity; Enzyme-linked immunospot assay; IFN-γ

Introduction

Hypersensitivity reactions represent a significant adverse effect that tuberculosis clinicians frequently encounter during patient treatment. When tuberculosis patients develop hypersensitivity reactions during combination therapy with four drugs, timely and accurate identification of the causative agent presents a considerable challenge, particularly in critically ill patients. Although several in vitro diagnostic methods—including skin patch testing (SPT), basophil activation testing (BAT), and drug lymphocyte stimulation testing (DLST)—have been employed for diagnosing drug hypersensitivity and identifying causative agents, their low sensitivity often necessitates the use of drug provocation testing (DPT), which exposes patients to the risk of recurrent allergic reactions.

Antitubercular drug hypersensitivity is primarily mediated by antigen-specific T cells, with multiple cytokines participating in the immune response. Following drug stimulation, drug-specific T cells trigger calcium influx and increased transcription factor expression, subsequently entering a functional differentiation phase where they produce inflammatory cytokines that induce a cascade of inflammatory reactions. The enzyme-linked immunospot (ELISpot) assay, which detects inflammatory cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2), has recently emerged as an international research focus for identifying causative drugs in hypersensitivity reactions. As a single-cell-level detection method, ELISpot can potentially identify a single drug-specific T cell among thousands of PBMCs. While numerous studies have evaluated ELISpot for β-lactam antibiotic and anticonvulsant drug hypersensitivity, with sensitivity ranging from 13% to 91%, no domestic or international studies have investigated its application for identifying causative agents in antitubercular drug-induced hypersensitivity reactions. This study aims to validate the sensitivity and specificity of ELISpot for identifying causative agents in antitubercular drug hypersensitivity, providing new insights and clinical evidence for in vitro diagnostic methods that can safely, rapidly, and effectively identify causative drugs within a short timeframe, thereby enabling patients to resume regular anti-tuberculosis treatment promptly, reducing the burden on families and society, and decreasing tuberculosis dissemination and drug resistance.

Methods

Study Subjects We selected 50 patients hospitalized in the Infectious Diseases Department of Jiangxi Chest Hospital between January 2021 and December 2022 who were diagnosed with primary drug-sensitive pulmonary tuberculosis and developed hypersensitivity reactions during anti-tuberculosis treatment. Inclusion criteria were: (1) diagnosis of primary drug-sensitive pulmonary tu-

berculosis according to the *Guidelines for the Diagnosis and Treatment of Pulmonary Tuberculosis*; and (2) clinical features and laboratory findings consistent with drug hypersensitivity reaction definitions in the 2nd edition of *Chinese Clinical Dermatology*. Exclusion criteria included: (1) concurrent HIV infection; (2) concurrent chronic kidney disease, diabetes, hematologic disorders, or autoimmune diseases, or patients receiving related treatments.

This study was approved by the Ethics Committee of Jiangxi Chest Hospital (approval number: Gan Xiong Lun Shen Zi (2019) No. 2), and all participants provided informed consent. Study subjects ranged from 16 to 80 years of age, with a mean age of (44.6 ± 19.5) years.

PBMC Isolation from Patients with Antitubercular Drug Hypersensitivity All reagents used in this study were obtained from Tianjin Haoyang Huake Biotechnology Co., Ltd. Collected anticoagulated blood was mixed with an equal volume of RPMI 1640 culture medium. The diluted blood sample was slowly layered over Ficoll lymphocyte separation medium at a 2:1 ratio and centrifuged at 1,000 g for 22 minutes at 18°C. The separated PBMCs were transferred to a 15 mL conical tube, resuspended in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 culture medium, and centrifuged at 600 g for 7 minutes at 18°C for the first wash. After removing the supernatant, cells were gently resuspended in 1 mL RPMI 1640, then brought to 10 mL with RPMI 1640 and centrifuged at 350 g for 7 minutes at 18°C for the second wash. The supernatant was carefully discarded, and cells were resuspended in 500 L AIM-V medium (adjusted according to cell count). A 10 L aliquot of the resuspended cells was mixed with 40 L of 0.4% trypan blue to prepare a 1:5 cell dilution. After mixing, 10 L was loaded into a hemocytometer for viable cell counting, and the cell concentration was adjusted to 2.5×10^6/mL for testing.

ELISpot Assay For each patient, a 96-well plate pre-coated with mouse anti-human IFN-γ monoclonal antibody was divided into four groups of six wells each, arranged sequentially as blank control, isoniazid, rifampicin, pyrazinamide, ethambutol, and positive control wells. The negative control well received 50 L RPMI 1640 culture medium + 50 L IL-2 (50 IU/mL). Drug antigen wells received 50 L of pre-prepared drug solution and 50 L IL-2. The positive control well received 100 L of 10 g/mL phytohemagglutinin (PHA). Each reaction well received 100 L of the cell suspension. Plates were incubated for 24 hours, after which culture supernatant was discarded and each well was washed three times with 200 L sterile phosphate-buffered saline (PBS). Detection antibody was prepared by 200-fold dilution of concentrated labeled antibody reagent in PBS, and 50 L was added to each well. Plates were incubated at 2–8°C for 60 minutes, after which labeled antibody was discarded and plates were washed four times with sterile PBS to remove unbound enzyme-labeled antibody. Fifty microliters of TMB substrate was added to each well and incubated at room temperature for 8 minutes. After repeated washing with distilled water, plates were dried in a 37°C incubator, and the number of dark blue, well-defined spots

in each well was counted and recorded.

Identification of Causative Drugs After collecting baseline patient data, anti-allergic and symptomatic treatments were administered. Once symptoms resolved, DPT was performed under close medical observation. The four suspected drugs were tested sequentially (order determined by clinical situation), starting with small doses and gradually increasing. Each drug trial lasted 3–4 days. If hypersensitivity reactions consistent with the diagnostic criteria occurred, the result was determined as DPT-positive, and the drug was identified as the causative agent.

Statistical Analysis Using fourfold table analysis, we calculated the diagnostic value of ELISpot for hypersensitivity reactions induced by isoniazid, rifampicin, pyrazinamide, and ethambutol. Sensitivity was calculated as true positives/(true positives + false negatives) × 100%, and specificity as true negatives/(true negatives + false positives) × 100%.

Results

DPT identified 63 instances of antitubercular drug-induced hypersensitivity reactions. ELISpot detected 11 instances of hypersensitivity reactions to two antitubercular drugs and one instance of reactions to three drugs. The sensitivity of ELISpot for diagnosing hypersensitivity reactions caused by isoniazid, ethambutol, rifampicin, and pyrazinamide was 69.2%, 61.5%, 75.0%, and 66.7%, respectively, with specificities of 97.3%, 100.0%, 100.0%, and 100.0% –.

Table 1 The Diagnostic Value of ELISpot for Isoniazid-Induced Hypersensitivity Reactions (n=50, cases)
Table 2 The Diagnostic Value of ELISpot for Rifampicin-Induced Hypersensitivity Reactions (n=50, cases)
Table 3 The Diagnostic Value of ELISpot for Pyrazinamide-Induced Hypersensitivity Reactions (n=50, cases)
Table 4 The Diagnostic Value of ELISpot for Ethambutol-Induced Hypersensitivity Reactions (n=50, cases)

Discussion

Identifying the causative agent responsible for hypersensitivity reactions during anti-tuberculosis treatment represents a critical challenge for both physicians and patients, as it directly impacts subsequent therapeutic strategies. Antitubercular drug hypersensitivity is considered a delayed-type hypersensitivity reaction that typically occurs hours to days after drug ingestion, mediated by T lymphocytes with involvement of multiple cytokines. T lymphocyte activation begins within minutes of drug stimulation, triggering calcium influx and increased expression of cytokines such as interferon-γ. However, drug-specific T cells are extremely rare within the total PBMC population, with frequencies

of approximately 1 in tens of thousands of PBMCs. Our research group previously used drug lymphocyte stimulation testing (DLST) for in vitro detection of causative agents but observed false-negative results, likely because T cell proliferation in DLST is not always sufficient for detection even when drug-specific T cells are present. In contrast, the IFN-γ ELISpot assay can potentially identify a single drug-specific T cell among thousands of PBMCs.

Similar studies have reported that traditional IFN-γ ELISpot testing for penicillin-induced hypersensitivity is more sensitive than DLST. Our findings demonstrate that ELISpot can detect drug-induced IFN-γ production when T cell responses are robust enough to induce cell division. Since most enrolled cases were sampled during the acute phase of drug hypersensitivity and IL-2 was added during drug-PBMC incubation to stabilize IFN-γ secretion following specific T cell activation, this modified IFN-γ ELISpot method detected drug-specific T cells in over 60% of cases, regardless of the cutaneous reaction type. Notably, ELISpot demonstrated higher sensitivity in patients allergic to multiple tuberculosis drugs: among 50 patients, DPT identified 11 patients allergic to two antitubercular drugs and one patient allergic to three drugs, all of whom were detected by IFN-γ ELISpot.

Regarding concerns that the modified IFN-γ ELISpot method with IL-2 stimulation might produce non-specific IFN-γ and false-positive results, we found that in drug-free control wells, fewer than 5 IFN-γ spots were detected in most cases. These non-specific spots may be associated with persistent immune abnormalities related to drug-induced hypersensitivity syndrome (DIHS) in patients with prolonged rash. However, clinically, some patients allergic to multiple antitubercular drugs showed positive ELISpot results during the acute phase, with positive DPT results after rash resolution. Interestingly, when DPT was repeated 2–3 months later, some previously positive drugs became negative, suggesting that the allergic threshold may be lowered during the acute hypersensitivity phase.

Both domestically and internationally, in vitro diagnostic methods for drug hypersensitivity have become a research focus, with improving sensitivity being the primary objective. This study employed the ELISpot assay and enhanced IFN-γ secretion stability by adding T cell activity modulators, substantially improving detection sensitivity. Overall, our results indicate that the IFN-γ ELISpot assay may serve as a valuable in vitro tool for identifying causative agents in antitubercular drug hypersensitivity cases. This approach will facilitate rapid and safe identification of causative drugs, enable timely adjustment of anti-tuberculosis treatment regimens, increase patient confidence in therapy, and contribute positively to achieving tuberculosis control objectives in China.

Author Contributions: WU Yuqing conceptualized and designed the study and drafted the manuscript; WU Yuqing and LIU Zhou collected and organized data, performed statistical analysis, and prepared figures and tables; LIAO Yongmei revised the manuscript and provided quality control.

Conflict of Interest: The authors declare no conflict of interest.

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Note: Figure translations are in progress. See original paper for figures.