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COMPARISON OF VALIDITY OF MICROLYMPHOCYTOTOXICITY AND FLOWCYTOMETRY METHODS WITH PCR FOR HLA-B27 ANTIGEN TYPING

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ABSTRACT

The Human Major Histocompatibility Complex (MHC) plays a crucial role in transplantation, transfusion, paternity test and assessment of susceptibility to some diseases associated with HLA-B27. Three of the most fashionable methods for determination of HLA antigens in clinical and research laboratories are microlymphocytotoxicity (MLCT), flowcytometry and polymerase chain reaction (PCR).

The purpose of this study was to compare the sensitivity, specificity, and positive and negative predictive values of MLCT and flowcytometry methods with PCR as a gold standard method in determination of HLA-B27 antigen.

In the present study, all three above-mentioned techniques have been used for 36 patients suffering from ankylosing spondylitis and 31 healthy volunteers. Specific antisera and monoclonal antibodies against HLA-B27, and allele specific PCR have been used in MLCT, flowcytometry and PCR methods respectively.

The results show that sensitivity, specificity, positive and negative predictive values of MLCT method as compared with PCR technique were 83.3%, 100%, 100% and 88.1% respectively. Moreover, sensitivity, specificity, positive and negative predictive values of flowcytometry compared to the PCR technique were 100%, 94.6%, 93.8% and 100% respectively.

Based on the results, the flowcytometry method in determination of HLA-B27 is more valid than MLCT in this regard, particularly in research programs. The similarity between the results of our study and those studies done in Europe suggests the probability of resemblance between HLA-B27 subtypes in Europe and in Iran.

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Keywords: Ankylosing spondylitis, Flowcytometry, HLA-B27, Microlymphocytotoxicity, PCR.

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INTRODUCTION

The major histocompatibility complex (MHC) class I

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molecules are heterodimers, composed of a heavy chain (α chain) and an invariant chain called β2- microglobulin. In human, the three distinct MHC loci are designated HLA-A, -B and -C which their products are found on most nucleated cells and thrombocytes. The encoding genes, predominantly located on chromosome 6, show a high degree of allelic polymorphism, with more than 100 class I alleles described to date. The function of these molecules is to present self- peptides or antigens derived from intracellular microorganisms to cytotoxic CD8+ T cells.[11]

Several HLA allotypes appear to be linked to various diseases. The most frequently requested typing of a disease-correlated HLA allele is that of HLA-B27. This allele, present in 3-6% of the Iranian normal population,[14] is strongly associated with seronegative spondyloarthropathies, especially ankylosing spondylitis (AS). A total of 90-95% of patients with AS have the HLA-B27 antigen; because of this solid association, HLA-B27 has become an important classification tool in rheumatology.

Screening for HLA-B27 is conventionally based on serological methods either MLCT or flowcytometry analysis which detect the antigen at the cellular surface. Therefore, these techniques are sensitive to down regulation or conformational changes of the antigens. Another major drawback of serological identification is the cross-reactivity of antibodies with different HLA class I antigens, owing to the extensive homology within the class.[9]

Recently, DNA typing of HLA-B27 by polymerase chain reaction (PCR) is increasingly accessible. Since this technique only relies on the detection of the HLA- B27-specific DNA sequence and directly determines the allelic DNA and is not influenced by conformational antigenic changes, it is an ideal test for HLA- B27.

The aim of this study was to compare the validity of MLCT and flowcytometry methods with PCR as a gold standard method in typing for HLA-B27 antigen. This was the first time that these three techniques had been studied together (previous studies had compared two of these three methods). Since typing of HLA-B27 is a routine and important test in rheumatology, it is crucial to define the sensitivity and specificity of each possible method.

MATERIAL AND METHODS

36 patients suffering from ankylosing spondylitis (AS) comprising the total number of patients who had been visited in Dr. Shariati hospital, ward of rheumatology, during 6 months were selected as a population with high probability of having the HLA-B27 antigen. 31 healthy volunteers had been chosen as a population with high probability of lacking the HLA - B27 antigen. These two groups were matched in all respects except in being affected by AS or not.

Microlymphocytotoxicity test

For detection of HLA-B27 antigen, 3 different antisera against HLA-B27 were used in this study (Biotest AG, Germany). Peripheral blood lymphocytes were collected based on density gradient using Ficol 1077 and were inoculated into the wells of a Terasaki plate by Hamilton syringe. Antiserum, which previously had been inoculated into those wells, will react with lymphocytes which have HLA-B27 antigen on their surface, and make them susceptible to become killed by rabbit complement. The eosin stain penetrates into the mortal cells and makes them distinguishable from unstained live lymphocytes under the inverted microscope.[6]

Flowcytometry analysis

Peripheral blood mononuclear cells were stained with monoclonal antibodies recognizing epitopes of HLA-B27 (Anti-HLA-B27 fluorescein isothiocyanate (FITC)/CD3 phycoerythrin (PE) monoclonal antibody. Becton Dickinson, California, USA). Lymphocytes were gated according to size and granularity, and analyzed separately. Samples with a median fluorescence 1 (FL1) channel result greater than or equal to the decision marker should be considered HLA-B27 positive. Samples with a median channel result lower than the decision marker should be considered HLA-B27 negative. FL1 is a signal from anti-HLA-B27 FITC and the decision marker is encoded in the suffix of the reagent lot number listed on the vial label of every kit.[6]

HLA-B27- specific PCR

PCR was carried out using standard methods. Briefly, DNA was directly prepared from peripheral blood (with EDTA) with salting out method. The reaction mixture was composed of 1 μL reaction buffer, 16.9 μL distilled water, 1 μL DNA (50 ng) and 0.1 μL Taq polymerase (5 unit/μL). DNA was amplified using oligonucleotide primers specific for exon 3 of the HLA-B27 gene (Biologische Analyzen system GmbH. [BAG, DNA based typing kits], Germany). Results were analyzed by gel electrophoresis.[6]

Statistical analysis

Estimation of sensitivity, specificity, positive and negative predictive values (PPV & NPV) of techniques were calculated as follows:

Sensitivity = TP/(FN + TP)
Specificity = TN/(FP + TN)
PPV = TP/(FP + TP)
NPV = TN/(FN + TN)

While, TP is true positive; TN is true negative; FP is false positive and FN is false negative.[6]

RESULTS

All three above-mentioned techniques were used for 67 individuals under the study (patients and healthy volunteers). According to the results of PCR as a gold standard method, 30 individuals were HLA-B27 positive and 37 persons were

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HLA-B27 negative. To assess the sensitivity, specificity, positive and negative predictive values of MLCT and flowcytometry methods, the results of these techniques were compared with those of the PCR method.

The results of comparison between MLCT and PCR are as follows: in MLCT technique, 25 persons were found to be HLA-B27 positive and 42 persons HLA -B27 negative. Interestingly, comparing results revealed 5 false- negative cases in this part of the study. Therefore, the sensitivity of this technique is 83.3%. On the other hand, there were no false-positive results using the MLCT method. Thus, the specificity of this method is 100%. Furthermore, the positive and negative predictive values of MLCT were 100% and 88.1% respectively.

In comparison between flowcytometry and PCR, no false-negative result was found. Therefore, the sensitivity of flow cytometry is 100%. On the other hand, 2 out of 37 persons who were typed by PCR as HLA-B27 negatives, were HLA-B27 positives with flowcytometry technique. The specificity, positive and negative predictive values of flowcytometry were calculated as 94.6%, 93.8% and 100% respectively.

DISCUSSION

Although it seems that the results could be anticipated easily, we were supposed to have differences in real figures. Furthermore, because of genetic variations in different populations, we needed to have a study specifically in the Iranian population.

In this study, the validity of MLCT and flowcytometry was assessed which showed similarities with other studies in the field. As an example, Kirveskari et al. in 1997, re-tested 20 patients who were typed originally by MLCT, with PCR. According to the MLCT method, 10 patients were HLA-B27 positive and 10 were HLA-B27 negative. Using PCR, all 10 who typed HLA-B27 positive in the beginning were also positive by PCR, while 2 out of 10 patients originally tested as HLA-B27 negatives, were positive with PCR.[11] In other words, sensitivity and specificity of MLCT according to the above-mentioned report was 83.3% and 100% respectively. As it is seen, these results are very similar to those of our study.

Albrecht and Muller in 1987 have reported that 10% of the typing done by flowcytometry must be repeated because of indeterminate results.[2] A study by Reynolds et al. in 1994 gave a sensitivity of 100% and specificity of 85% for flowcytometry.[10] Two studies by Halstaert et al. and Janssen et al. in 1997 indicated a sensitivity of 100% and 97.6% with a specificity of 97.4% and 95.9% respectively[10,12] Reynolds et al. in 1996, using flowcytometry found that some HLA-B27 positives fall into an intermediate zone and require additional testing.[12]

Our results are in conformity with previous studies by others, mostly in Western Europe. Similar to Albrecht and Muller's findings, two of our patients who typed HLA-B27 negative by MLCT and PCR methods, had an intermediate pattern for flowcytometry with a slight shift to positive zone (Figs. 1 & 2) which were considered as positive in flowcytometric results. Since more than 50% of their events were

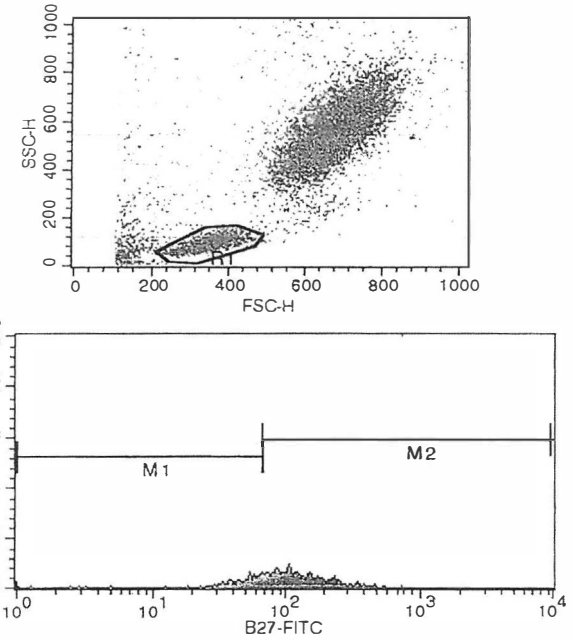

Fig. 1. A patient with intermediate flowcytometric pattern.

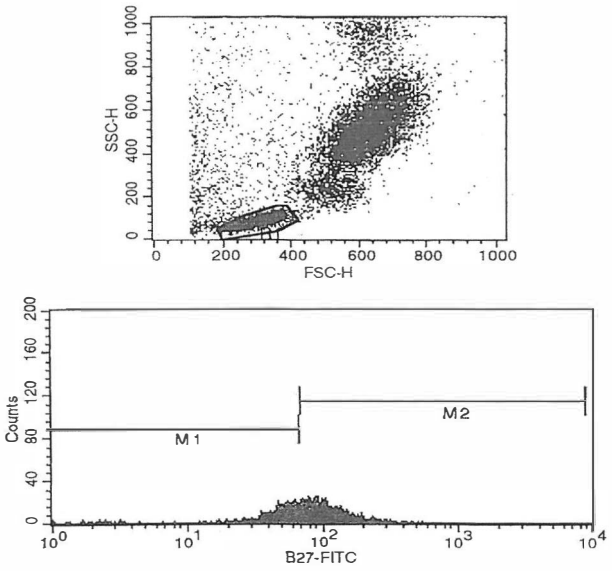

Fig. 2. Another patient with an intermediate flowcytometric pattern.

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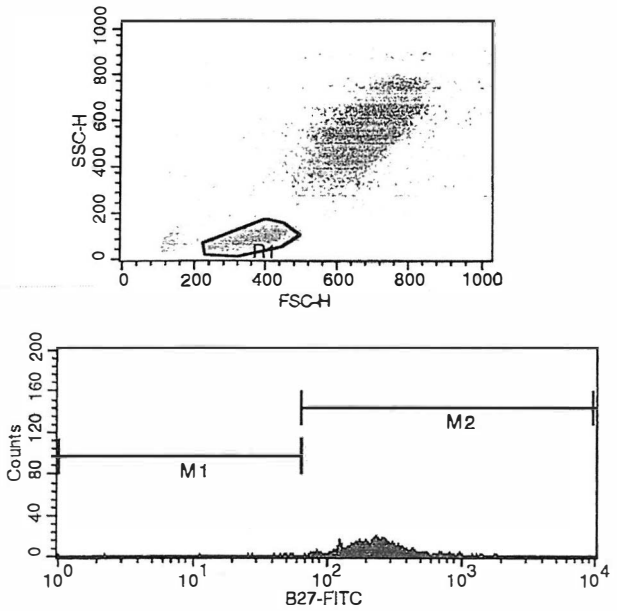

Fig. 3. An HLA-B27 positive case.

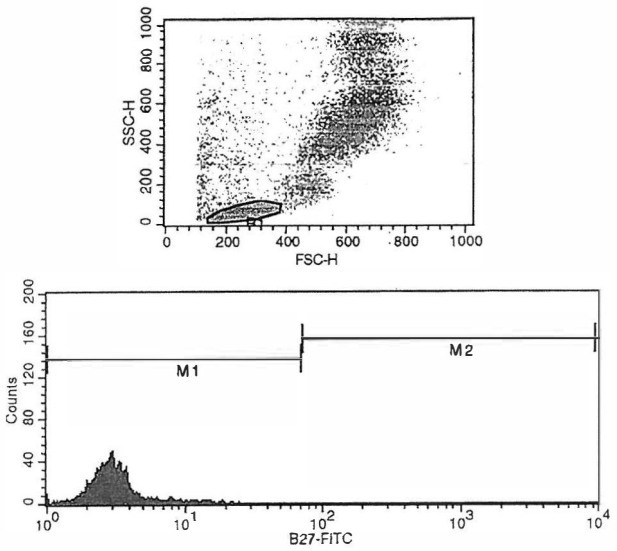

Fig. 4. An HLA-B27 negative case.

in the positive zone, they were considered to be positive. Other cases had a definite pattern of positive or negative results for flowcytometry (Fig. 3 & 4).

Both results of flowcytometry and MLCT techniques (mentioned above) emphasize the probable similarity between HLA-B27 subtypes in Europe and Iran, which needs a separate study for typing the HLA-B27 subtypes with PCR.

The results show that, although the MLCT method, because of more availability of specialist technicians and less cost of materials, is the most routine method in laboratories, only positive results of this test are reliable and false negative results may happen sometimes.[6] Although with regard to clinical findings, the false-negative results using MLCT are less responsible for a wrong diagnosis of ankylosing spondylitis, this technique could not be the first choice in epidemiological studies.[6]

As it was shown in the results, the sensitivity of flowcytometry is equal to PCR as the gold standard method and is more than that of MLCT (100% vs. 83.3%), so the results of flowcytometry in determination of HLA-B27 are more valid than those of MLCT. The specificity of MLCT in this regard, however, is equal to PCR but the difference between MLCT and flowcytometry in this case is little (100% vs. 94.6%). Although in flowcytometry some false positive results may rarely happen, with more experience in handling of the technique and interpreting the different patterns, hopefully the problem of diagnosis of cross-reactivity will be overcome.[6] Furthermore, false-positive results by preincubation of samples with antibodies against cross-reactive HLA antigens for HLA-B27 (particularly HLA-B7) could be prevented.[11]

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