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Methotrexate reduces vaccine-specific immunoglobulin levels but not numbers of circulating antibody-producing B cells in rheumatoid arthritis after vaccination with a conjugate pneumococcal vaccine

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ABSTRACT

Background: Treatment with methotrexate (MTX) in patients with rheumatoid arthritis (RA) leads to decreased total immunoglobulin (Ig) levels and impairs vaccine-specific IgG antibody levels following pneumococcal vaccination. The mechanisms by which MTX exerts these effects in RA are unknown. We aimed to evaluate whether MTX reduces vaccine-specific serum Ig levels and their functionality in RA patients following vaccination with pneumococcal conjugate vaccine, and if numbers of antigen-specific circulating plasmablasts are affected.

Methods: Ten patients with RA on MTX and 10 RA patients without disease modifying anti-rheumatic drug (DMARD) were immunized with a dose 13-valent pneumococcal conjugate vaccine (Prevenar13). Circulating plasmablasts producing total IgG and IgA as well as specific IgG and IgA against two pneumococcal capsular serotypes (6B and 23F) were enumerated using ELISPOT 6 days after vaccination. IgG levels against both these serotypes were determined with ELISA before and 4–6 weeks after vaccination. Positive antibody response was defined as $\geqslant$2-fold increase of pre-vaccination antibody levels. The functionality of vaccine specific antibodies to serotype 23F was evaluated by measuring their ability to opsonize bacteria using opsonophagocytic assay (OPA) in 4 randomly chosen RA patients on MTX and 4 RA patients without DMARD.

Results: After vaccination, RA patients on MTX showed significant increase in pre- to postvaccination antibody levels for 6B ($p < 0.05$), while patients without DMARD had significant increases for both 6B and 23F ($p < 0.05$ and $p < 0.01$, respectively). Only 10% of RA on MTX and 40% of RA patients without DMARD showed positive post-vaccination antibody responses for both serotypes. Increased opsonizing ability after vaccination was detected in 1 of 4 RA patients on MTX and 3 of 4 patients on RA without DMARD. However, numbers of circulating total and vaccine-specific IgG- or IgA-producing plasmablasts did not differ between RA patients with or without MTX.

Conclusions: MTX treatment in RA leads to reduced vaccine-specific antibody responses and their functionality compared to untreated RA following pneumococcal vaccination using polysaccharide-protein conjugate vaccine. However, since there was no reduction in numbers of circulating total or vaccine-specific antibody-producing plasmablasts after vaccination this effect is probably not due to reduced activation of B cells in lymphoid tissue.

Clinical trial registration: NCT02240888.

1. Background

Methotrexate (MTX) is a folic acid antagonist used for the treatment of various malignancies. Low dose MTX ($\leqslant$25 mg/week) seems to have more anti-inflammatory than anti-proliferative properties and is widely used for treatment of several chronic inflammatory diseases including rheumatic diseases, psoriasis or inflammatory bowel disease [1]. The effectiveness of MTX in rheumatoid arthritis (RA) has been demonstrated in

Abbreviations: RA, rheumatoid arthritis; MTX, methotrexate; DMARD, disease modifying -rheumatic drug; Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; ELSPOT, enzyme-linked immunospot assay; OPA, opsonophagocytic assay.

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numerous studies and MTX is currently considered as an anchor disease modifying anti-rheumatic drug (DMARD) in RA [2]. However, the mechanisms by which MTX performs its disease-modifying effects are not entirely elucidated and several hypotheses have been presented of which one is suppression of B cell function [1–5].

B cells play an important role in the pathogenesis of RA. A variety of autoantibodies including antibody against citrullinated peptides (ACPA) can be found years before clinical onset of disease. The presence of ACPA and rheumatoid factor (RF) in patients with established RA are recognized markers of more severe disease. The importance of B cells in RA has re-gained attention since B cell depletion therapy has been proven efficacious in RA. The effects of MTX on the B cell compartment in patients with juvenile chronic arthritis has been studied and revealed significantly lower serum immunoglobulin (Ig) levels and proportions of transitional B cells in these patients compared to patients treated with TNF inhibitors or to patients not receiving DMARDs [6]. In RA we previously reported decreased total Ig levels in patients treated with MTX compared to TNF inhibitors [7]. Also, MTX treatment in systemic lupus erythematosus (SLE) patients was associated with decreased levels of total IgG, IgA and IgM compared to SLE patients without MTX treatment [8]. We and others have also observed lower vaccine-specific IgG levels following pneumococcal and influenza vaccination in arthritis patients treated with MTX compared to those on TNF inhibitors, to those without DMARD and to healthy controls [9–13].

The objective of the present study was to investigate whether the effect of MTX on vaccine-specific serum immunoglobulin levels could be due to reduced activation of B cells by analyzing total and vaccine-specific circulating plasmablasts in RA patients. To this end, RA patients with or without MTX treatment were immunized with one dose of a 13-valent pneumococcal conjugate vaccine. Six days after vaccination we enumerated circulating plasmablasts producing total and vaccine-specific IgG and IgA, as well as vaccine-specific serum antibody levels before and 4–6 weeks after vaccination. As vaccine antigens in the immunoassays we chose two different pneumococcal capsular polysaccharides (6B and 23F) included in the vaccine since these two antigens are known to be associated with invasive infections [14].

2. Methods

2.1. Study patients

Twenty patients with RA fulfilling the ACR 1987 classification criteria for RA [15], who were regularly followed up at the Rheumatology department in Lund, Skåne University Hospital Sweden, were prospectively included in the study between December 2012 and September 2013. Ten randomly chosen RA patients treated with MTX in a stabile dose without any other DMARD and 10 RA patients not taking any DMARD were included into the study. In order to avoid the possible immunological effect of smoking or prednisolone, only non-smokers and patients not treated with systemic steroids were recruited. In addition, only patients who had not received pneumococcal vaccination within five years prior the study entry were eligible. However, none of patients had received pneumococcal vaccination previously. All participants were immunized with a single dose (0.5 ml) of 13-valent pneumococcal conjugate vaccine (Prevenar13) administrated intramuscularly. Blood samples were taken immediately before vaccination, after 6 days and 4–6 weeks following vaccination.

2.2. Isolation of lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway). Interface PBMCs were collected, washed three times with phosphate buffer (PBS) (HyClone™, Logan, USA) and resuspended in complete medium consisting of Iscove's medium (Sigma- Aldrich, St. Louis, USA) supplemented with 5% fetal bovine serum (FBS; HyClone™), 50 μg/ml gentamicin (Sigma-Aldrich) and 1 mM L-glutamine (Sigma-Aldrich). Cell suspensions were kept on ice prior to being assayed for numbers of antibody-secreting cells (ASCs).

2.3. Enumeration of antibody-secreting cells by ELISPOT

Frequencies of total and serotype-specific ASCs using unstimulated freshly isolated PBMCs were determined by enzyme-linked immunospot assay (ELISPOT). Wells of mixed cellulose ester membrane 96 well MultiScreen HA plates (Millipore, Billerica, MA) were coated with 50 and 200 μg/ml of pneumococcal polysaccharide type 23F (Danish designation 23F) or type 6B (Danish designation 6B) (ATCC Manassas, VA, USA). For detection of total IgA and IgG, wells were coated with 5 μg/ml of affinity purified goat antibodies to the $F(ab')_2$ fragment of human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Control wells were coated with 10 μg/ml of bovine serum albumin (BSA; Sigma-Aldrich). The plates were coated overnight at 4 °C and then washed four times with PBS, blocked with complete medium (Iscoves medium containing 5% FBS) and incubated at 37 °C in 5% CO_2 for 30 min. The blocking was removed and different dilutions of cells in complete medium were added to the plates and incubated for 4 h at 37 °C in 5% CO_2. Plates were washed three times with PBS and four times with PBS with 0.05% Tween 20 and incubated with peroxidase-conjugated affinity purified goat anti human IgA or IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:1000 in PBS -Tween with BSA at 4 °C overnight. Plates were then washed four times with PBS-Tween and three times with PBS and exposed to 0.3 mg/ml of 3-amino-9-ethylcarbazole (Sigma) and 0.015% H_2O_2 in 0.1 M sodium acetate, pH 5.0 for 15 min. The plates were rinsed with tap water and dried in the dark. The spots were enumerated under magnification (x40) in a blinded fashion from at least two dilutions [16].

2.4. Detection of vaccine-specific antibody levels in serum

Levels of serotype-specific pneumococcal IgG to 23F and 6B were measured using the WHO standard ELISA for quantitation of human IgG antibodies specific for *S. pneumoniae* capsular polysaccharides (Pn PS ELISA), as previously described [17]. Briefly, ELISA plates were coated with 1 μg Pn PS 6B or 23F. In order to diminish nonspecific binding to capsular polysaccharides, dilutions of human sera were absorbed with pneumococcal PS and then added to the ELISA plates. Goat anti-human IgG antibodies, conjugated with alkaline phosphatase, followed by addition of the substrate, nitrophenyl phosphate, were used for the detection of serotype-specific antibodies (anti-6B and anti-23F IgG). The optical density, proportional to the amount of anti-6B and anti-23F IgG present in the serum, was measured with an ELISA plate reader at 405 nm. The assay was calibrated with an international reference serum that was kindly provided by Dr. C. Frasch, Bethesda, MD, USA [18]. The lower limit of detection was 0.01 mg/l. A vaccine antibody response was defined as a $\geqslant$2-fold increase in pre-vaccination specific antibody level.