PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B, Vol. 70 (2016), No. 4 (703), pp. 175–181.
DOI: 10.1515/prolas-2016-0023

PERIPHERAL BLOOD MONONUCLEAR CELLS' PROLIFERATIVE RESPONSE TO HUMAN PARVOVIRUS B19 ANTIGENS IN PATIENTS WITH RHEUMATOID ARTHRITIS

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This study aimed to determine peripheral blood mononuclear cells' (PBMC) proliferative response to parvovirus B19 (B19) antigens in rheumatoid arthritis (RA) patients and possible changes in proliferative response due to chemotherapy. Serum and blood samples of 52 RA patients and 25 sex and age matched healthy individuals were examined for the presence of anti-B19 IgG and IgM class antibodies and virus specific DNA sequence by the recomLine B19 test and nested polymerase chain reaction, respectively. The PBMC proliferative activity was estimated on the 3rd and 6th day of PBMC cultivation in the presence of virus and B19 VP1/VP2 peptide, using thymidine incorporation assay. On the 3rd day, PBMC response to B19 antigens was detected in 74.1% RA patients with active, in 44.8% — with remote and in 40% — with latent stage of persistent B19 infection, while in the control group the response was observed only in two individuals with active viral infection. On the 6th day, the response was found in 50% RA patients with active, 68.9% — with remote and in 80% — with latent stage of latent persistent infection as well as in 41.1% remotely infected control individuals. The highest PBMC mean stimulation indices were detected in the RA patients and control persons with active infection as well as in RA patients with latent stage of persistent viral infection. On the 3rd and 6th day, strong proliferative response was significantly more frequently observed in RA patients not receiving methotrexate treatment, compared to the patients receiving methotrexate treatment in different combinations with other drugs. RA patients had more frequent and faster response to B19 antigens than apparently healthy persons.

Key words: *autoimmune disease, B19 infection, cellular response to VP1/VP2 B19 peptide.*

INTRODUCTION

Parvovirus B19 (B19) is a non-enveloped virus with a linear single-stranded genomic DNA. In spite of its small size, B19 is associated with a wide spectrum of diseases in humans, including autoimmune diseases (Broliden *et al*., 2006; Servant-Delmas *et al*., 2010; Rogo *et al*., 2014; Page *et al*., 2015). The B19 genome encodes three major proteins: two capsid proteins VP1 (84 kDA) and VP2 (58 kDA), the non-structural protein NS1 (77 kDA), and two small polypeptides (7.5 and 11 kDA). The capsid proteins VP1 and VP2 are almost identical and differ only in an additional 227 amino acids at the amino terminus of VP1 — the so-called VP1-unique region (VP1u) (Heegard and Brown, 2002; Rogo *et al*., 2014). The VP1u region is exposed to the surface of viral capsid and contains an immunodominant site with several linear epitopes that underline its importance for humoral immune response (Rosenfeld *et al*., 1992; Saikawa *et al*., 1993). The virus-specific humoral response is well documented, while data on cellular immune response are limited. In 1996, Poblotcky *et al.* determined cellular immune response against recombinant VP1, VP2, VP1u and NS1 proteins in remotely infected donors and showed that seropositive individuals respond to at least one of the capsid proteins and demonstrated that CD4+ T lymphocytes are the major population of reactive cells (von Polotzki *et al*., 1996). The important role of cellular immunity in controlling of B19 replication was further supported by Franssila *et al*. (2001), who reported stimulation of proliferation of CD4+ T cells by B19 VP1/2 capsids in recently

and remotely infected persons (Franssila *et al*., 2001). In 2004, the same team showed that B19 VP1 contains an important B-cell epitope and that VP2 is the major target for B19 specific T helper cells during years after natural infection (Franssila and Hedman, 2004). Lindner *et al.* investigated T cell responses directed against VP1u region in peripheral blood lymphocytes of donors using stimulation of peripheral blood nuclear cells (PBMC) with autologous B cells producing VP1u. All persons with recent, acute or persistent B19 infection had significant numbers of interferon-gamma secreting lymphocytes while in PBMC of donors with past B19 infection and seronegative individuals, interferon-gamma secreting lymphocytes were not detected (Lindner *et al*., 2005). T-cell response in relation to peptide level was determined in healthy seropositive individuals (past infection) and persistently infected persons (with B19 DNA detected in bone marrow during a follow-up period from 2 to 8 years) using 210 overlapping peptides representing entire NS1 and VP1 proteins. In this study, remotely infected individuals had discordant distribution of cellular immune response compared to persistently infected persons. Healthy seropositive individuals had significantly more frequent response to NS1 than to VP1 peptides, while in persistently infected persons more frequent response to VP1 than to NS1 was observed (Isa *et al*., 2006). Kasprowicz *et al*. (2006) reported on a study of anti B19 CD4+ T-cell immune responses among remotely infected and acutely infected persons with acute arthropathy using a set of 70 overlapping 20-mer peptides covering VP1/VP2 proteins. This study showed that both acutely and remotely infected persons have broad CD4+ responses, however, the vast majority of acute and remotely infected persons respond to epitope LASEESAFYVLEHSSFQLLG (Kasprowicz *et al*., 2006).

In the present study we investigated the frequency and quickness of B19 specific PBMC proliferative response to the virus and VP1/VP2 peptide LASEESAFYVLEHSSFQLLG in rheumatoid arthritis patients and apparently healthy individuals, and tested the possible effect of methotrexate therapy on B19 specific proliferative response.

MATERIALS AND METHODS

Subjects. 52 RA patients and 25 sex and age matched healthy individuals (control group) were enrolled in this study. 45 of 52 RA patients and 24 of 25 in control group individuals were females. The mean age of RA patients was 56.06 ± 1.54 (range 20–82) years and of the control group individuals — 55.48 ± 2.62 (range 26–82) years. Specimens from RA patients and control group individuals were collected at the same time. RA patients were recruited from the Rīga East University Hospital Clinic "Linezers" and Clinic "Gaiļezers". All patients had fulfilled the 1987 or 2010 American College of Rheumatology classification criteria for RA (Arnett *et al*., 1988; Aletaha *et al*., 2010). For RA diagnosis setting plain radiography was used (van der Heijde, 1996). Magnetic resonance imaging was used to determine early changes and erosions in wrists (Moller *et al*., 2008). Staging was based on Venables and Wheeless recommendations (Wheeless, 2012; Venables and Maini, 2013). The study was approved by the Ethics Committee of the Rīga Stradiņš University and all participants gave their informed consent prior to the examination.

Detection of anti-B19 IgM and IgG class antibodies. Virus specific IgG and IgM class antibodies were detected using the recomLine Parvovirus B19 IgG and IgM test (Microgen, Germany) according to manufacturer's recommendations.

Detection of B19 DNA sequences. The presence of B19 genomic sequences was detected by nested polymerase chain reaction (nPCR). DNA was purified from the peripheral blood leukocytes (PBL) and cell-free blood plasma by proteinase K digestion overnight and standard phenol-chloroform extraction technique. To assure quality of the PBL DNA and to exclude contamination of plasma DNA by cellular DNA, PCR was performed with primers that recognized the 200-bp fragment of the beta-globin gene. B19 NS1 nPCR was performed as described previously (Barah *et al*., 2001).

Determination of PBMC proliferative activity. PBMC were isolated from heparinized blood by Ficoll-Hypaque centrifugation (400 × g, 30 min) and were washed twice in PBS. The proliferative activity of PBMC was estimated on the 3rd and 6th days by the incorporation of ^{3}H-thymidine into the cells cultivated in the presence of B19 (final concentration — 10^{6} viral genomes/ml) or VP1/VP2 B19 peptide LASEESAFYVLEHSSFQLLG (Caslo Laboratory ApS, Denmark) at final concentration 10 μg/ml (27). Peripheral blood serum of a highly positive individual was used as the B19 containing material with the viral load calculated by real-time PCR. PBMC were isolated from heparinised blood by Ficoll-Hypaque density gradient centrifugation, washed in RPMI-1640 medium (GIBCO), centrifuged and re-suspended in RPMI-1640 with 10% foetal bovine serum supplemented with penicillin (10^{5} U/L) and streptomycin (100 mg/L) (GIBCO). PBMC were seeded into 9–12 wells of two 24-well plates at concentration 1 × 10^{6} cells per well. For each specimen 3–4 wells were used. On the 3rd or 6th day of cultivation, 2 μCi/well of ^{3}H-thymidine (25 Ci/mmol, Amersham, England) were added and the cells were incubated for 4 hours. Then the cells were collected and transferred to Millipore filters (diameter of pores 1.5 μm). Filters were washed twice with PBS and three times with 5 ml of 5% Trichloroacetic acid to precipitate DNA. The DNA was fixed by 1 ml of 96% ethanol and air dried at 37 °C. The incorporation of ^{3}H-thymidine was measured using a Packard liquid scintillation counter. The PBMC response was considered positive if incorporation of ^{3}H-thymidine in virus or peptide stimulated cells increased by at least twice in comparison with the negative control (media). The data were expressed as counts per minute (Δcpm): mean cpm (test antigen), mean cpm (media) and as stimulation indices (SI). SI = mean cpm (test antigen)/mean cpm (media). The PBMC stimu-

lated by phytohaemagglutinin M (GIBCO) (2 μg/ml) served as a positive control.

Statistical analysis. Data were analysed using GraphPad Prism 6.0 software. The frequency and quickness of PBMC responses were compared between the patients with and without active viral infection as well as between the patients and a control group using the Fisher's exact test. Significance of differences on the 3rd and 6th day in PBMC responses between RA patients who received methotrexate and without methotrexate treatment and controls was tested using the Kruskal-Wallis test (as nonparametric ANOVA) and Dunn's multiple comparisons as post-hoc test. The mean values are expressed as median with interquartile region (IQR) as variability.

RESULTS

The radiological examination of RA patients showed that 4 of 52 RA patients had the I radiological stage of RA , 13 patients had the II stage, and 35 patients had erosive arthritis with III stage (33 patients) and IV (2 patients) radiological stages. The majority of the patients had prolonged duration of the disease. In the RA patients with I, II, III, and IV radiological stages, the mean disease duration was 3.0 ± 2.0, 4.2 ± 0.96 10.5 ± 1.5 and 34.5 ± 24.5 years, respectively. Disease duration up to one year was observed in 12 of 52 patients. In three of them, bone erosions were revealed within 3–11 months after appearance of the disease clinical symptoms. The majority of patients had received disease modifying anti-rheumatic drugs (DMARDs) like hydroxychloroquine, sulfasalazine, methotrexate and leflunomide. Sixteen of 52 (30.8%) RA patients had received only DMARDs; but DMARDs in combination with glucocorticoids and/or non-steroid anti-inflammatory drugs (NSAIDs) was received by 23 (44.2%) patients. DMARD taken separately or in different combination with glucocorticoid or/and NSAIDs was received by 39 patients. Twenty-five patients received methotrexate in different combinations, 14 patients — DMARDs without methotrexate, 13 patients had received glucocorticoids or/and NSAIDs (Table 1).

PBMC of RA patients responded to B19 antigens (virus and/or VP1/VP2 peptide) more frequently and faster, in comparison to the control group. On the 3rd day of cultivation, in the presence of B19 antigens (virus or VP1/VP2 peptide), PBMC proliferation was observed in 48.0% (25/52) of RA patients and only in 8.0% (2/25) of healthy individuals (p = 0.00068). On the 6th day of cultivation, PBMC of 63.5% (33/52) of RA patients and 32.0% (8/25) of healthy individuals responded to B19 antigens (p = 0.01436). Analysis of PBMC response to B19 antigens depending on the presence of B19 infection markers showed that the lymphocytes of all RA patients (4/4) and all control group persons (6/6) without B19 markers did not respond to B19 antigens. The PBMC of both control group persons with active B19 infection (B19 DNA in plasma and/or IgM antibodies) and 74.1% (10/14) RA patients with the active virus infection reacted to B19 antigens (virus and/or VP1/VP2 peptide) by proliferation on the 3rd day. Among RA patients, PBMC proliferative response to B19 antigens on the 3rd day was detected also in 44.8% (13/29) remotely infected patients who had IgG class antibodies only, while in the control group, no response was found in remotely infected persons on the 3rd day (p = 0.00071). On the 6th day, PBMC proliferative response to B19 antigens among remotely infected persons was detected in 68.9% (20/29) RA patients and 41.1% (6/17) control group persons, respectively (p = 0.0347). In RA patients who had IgG class antibodies and the B19 genomic sequence in peripheral blood leukocytes (latent stage of persistent infection), PBMC proliferation in response to B19 antigens (virus and/or VP1/VP2 peptide) was observed in 40.0% (2/5) and 80.0% (4/5) individuals on the 3rd and 6th days, respectively. In the control group, the latent stage of persistent virus infection was not detected. PBMC proliferative response to B19 VP1/VP2 peptide among RA patients with active infection was detected in 64.2% (9/14) and 57.1% (8/14) individuals on the 3rd and 6th days, respectively. In the control group, active B19 infection PBMC proliferation in response to this peptide was observed in both of the individuals, but on the 6th day only. In remotely infected RA patients, the proliferative response to B19 VP1/VP2 peptide was detected in 27.5% (8/29) and 24.1% (7/29) patients on the 3rd and 6th days, respectively, while in the remotely infected control group, PBMC proliferation in response to the B19 peptide was found in 35.2% (6/17) cases on the 6th day only. PBMC proliferative response to the B19 peptide in RA patients who had IgG class antibodies and B19 genomic sequence in peripheral blood leukocytes was detected in 40.0% (2/5) and 80.0% (4/5) cases on the 3rd and 6th days, respectively. Thus, the proliferative response to the VP1/VP2 peptide was observed in RA patients as well as in control group persons, but in RA patients this response was significantly faster than in the control, as on the 3rd day it was detected in RA patients only (p = 0.009; 0/19 vs 10/34). The PBMC mean stimulation indices of RA patients and control persons are shown in Table 2. The highest PBMC mean stimulation indices were observed in RA patients and control persons with markers of active infection as well as at with markers of latent stage of persistent infection.

To evaluate the possible effect of methotrexate on B19 specific cellular immune response to the B19 antigens, PBMC proliferative response (mean count per minute) of RA pa-

Table 1

GROUPS OF RA PATIENTS DEPENDING ON THE RECEIVED TREATMENT

Used therapy	MTX alone	DMARDs with MTX with/without GC or NSAIDs	DMARDs without MTX with/without GC or NSAIDs	GC with/without NSAIDs	NSAIDs alone
Number of RA patients	7/52 (13.5%)	18/52 (34.5%)	14/52 (27.0%)	6/52 (11.5%)	7/52 (13.5%)

MTX, methotrexate; DMARDs, disease modifying anti-rheumatic drugs; GC, glucocorticoids; NSAIDs, non-steroid anti-inflammatory drugs

Table 2

MEAN STIMULATION INDICES OF PBMC BY THE B19 ANTIGENS IN RA PATIENTS AND CONTROLS

Patients with markers of active infection (n = 14)		Remotely infected patients		Patients with latent stage of persistent infection		Patients without markers of virus infection (n = 4)	
3 days peptide/ virus	6 days peptide/ virus	IgG only (n = 29)		IgG with B19 DNA in PBL (n = 5)		3 days peptide/ virus	6 days peptide/ virus
		3 days peptide /virus	6 days peptide/ virus	3 days peptide/ virus	6 days peptide/ virus		
2.00 ± 0.18	2.17 ± 0.16	1.55 ± 0.12	1.63 ± 0.09	1.87 ± 0.55	2.63 ± 0.63	0.99 ± 0.10	1.04 ± 0.05
1.76 ± 0.15	2.24 ± 0.14	1.49 ± 0.12	1.88 ± 0.12	1.30 ± 0.22	2.67 ± 0.64	1.13 ± 0.03	1.07 ± 0.07
Control persons with markers of active infection (n = 2)		Remotely infected control persons (IgG only) (n = 17)				Control persons without markers of virus infection (n = 6)	
3 days peptide/ virus	6 days peptide/ virus	3 days peptide/ virus		6 days peptide/ virus		3 days peptide/ virus	6 days peptide/ virus
1.78 ± 0.03	2.21 ± 0.12	1.06 ± 0.04		1.56 ± 0.20		1.11 ± 0.10	0.96 ± 0.04
2.01 ± 0.01	2.62 ± 0.10	0.99 ± 0.03		1.46 ± 0.16		1.04 ± 0.08	0.99 ± 0.06

tients receiving methotrexate in different combinations and RA patients without methotrexate treatment was compared on the 3rd and on 6th days (Fig. 1). The results showed that in both RA patients groups (with and without methotrexate treatment) the mean count on 6th day was significantly higher than that on 3rd day ($p < 0.024$ and $p = 0.006$). Significantly higher mean count was observed also on the 3rd as well as on the 6th day in RA patients who did not receive methotrexate, compared to the count on the 3rd and 6th days in the control group (Fig. 1). There were no significant differences in the mean count per min between RA patients with and without methotrexate treatment as well as between RA patients who received methotrexate and the control group. Taking into account the high individual variabilities among RA patients with and without methotrexate treatment, a qualitative analysis was performed, in which RA patients and control group individuals were divided into two subgroups depending on the PBMC mean count. The mean count detected without addition of B19 antigens (negative control) was taken as 100%. One subgroup in each group included patients and control persons who had mean count similar to the negative control (the medium), while the second subgroup included patients and control persons with mean PBMC count higher than in the negative control. In this analysis, significant difference in PBMC proliferative response between RA patients with and without methotrexate therapy on the 3rd day and on 6th day were found (Fig. 2). On the 3rd day, the number of the patients with high mean count in the group of RA patients receiving methotrexate was significantly lower than in the group of RA patients without methotrexate treatment ($p < 0.025$), but significantly higher than in the control group ($p < 0.0374$). The number of the patients with high mean count in the group of RA patients not receiving methotrexate was also significantly higher in comparison with the control group ($p < 0.0001$). On the 6th day the number of persons with high mean count in the group of RA patients receiving methotrexate was also significantly lower than in the group of patients without methotrexate treatment ($p < 0.012$), but similar to the number observed in the control group. Significantly higher number of patients with high mean count revealed in the RA patients without methotrexate treatment compare to the control group on the 3rd day was also observed on the 6th day ($p < 0.0001$). Thus, the highest number of patients with strong PBMC proliferative response to B19 antigens was detected in RA patients who did not receive methotrexate treatment.

DISCUSSION

Association of B19 infection with RA has been demonstrated in several studies, while B19 specific cellular immune response in these patients was unknown (Takahashi *et al*., 1998; Murai *et al*., 1999; Stahl *et al*., 2000; Kozireva *et al*., 2008; Kakurina *et al*., 2015). Although B19 specific cellular immune responses have been investigated very poorly, the existing studies indicate the important role of these responses in the control of acute and persistent B19 infection (Fransilla *et al*., 2001; Tolfvenstam *et al*., 2001; Isa *et al*., 2005; Norbeck *et al*., 2005; Isa *et al*., 2006; Streitz *et al*., 2008). Recently, using a set of peptides to B19 VP1/VP2 proteins, Kasprowicz *et al.* (2006) showed that the majority of both acutely (10/12) and remotely (6/7) infected individuals responded to one of the examined VP1/VP2 peptides (Kasprowicz *et al*., 2006). Taking into account this observation, we used this peptide in our study to determine the frequency and quickness of PBMC proliferative responses among RA patients and healthy persons with active infection and remotely infected RA pa-

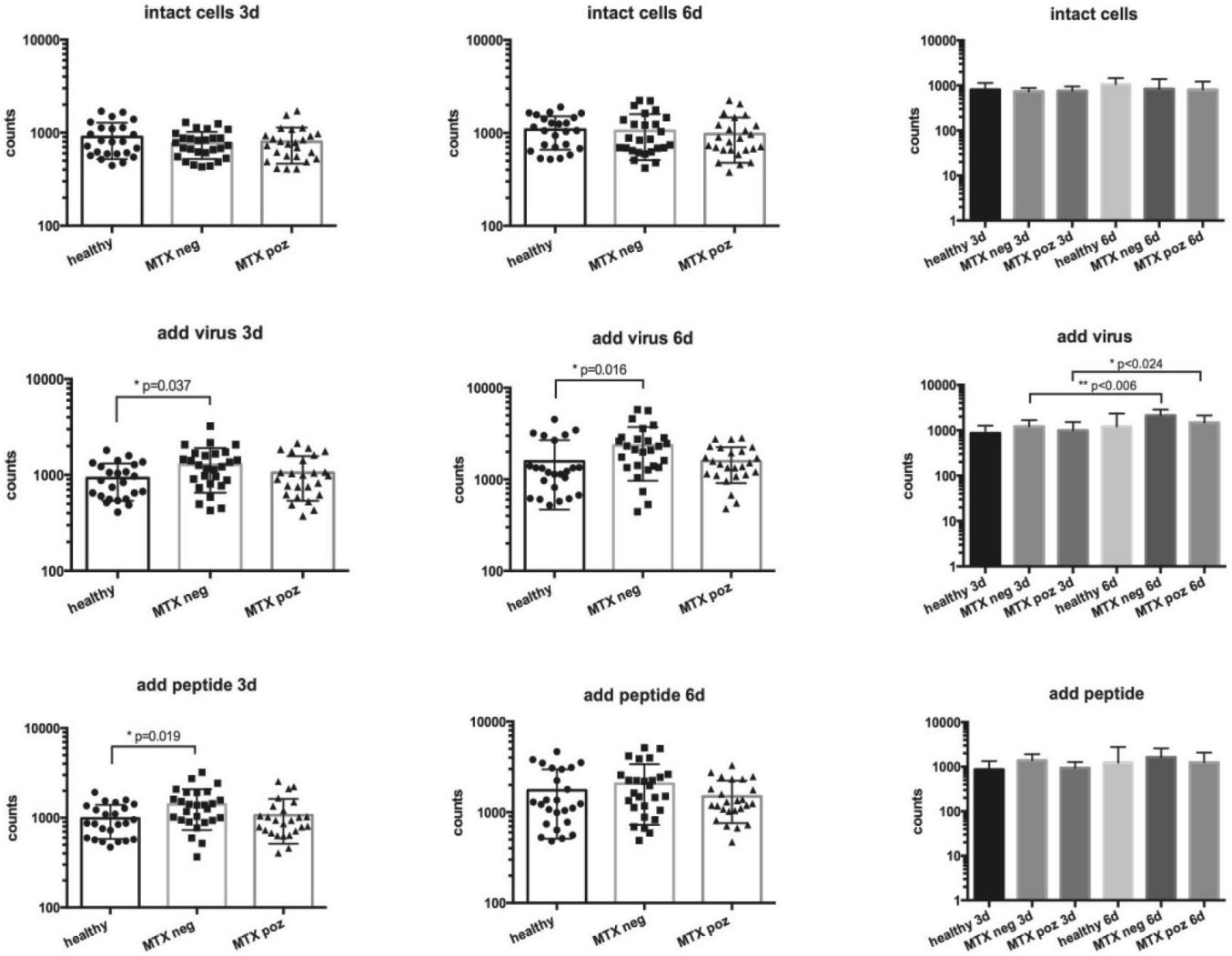

Fig. 1. PBMC proliferative response to B19 antigens in RA patients with and without methotrexate treatment and control persons

tients and healthy persons (control group). In addition, we estimated dependence of B19 specific PBMC proliferative response on the applied therapy, as this aspect had not been previously examined.

The majority of RA patients [64.2% (9/14)] and 100% (2/2) of control group persons with active infection as well as 41.4% (12/29) and 35.2% (6/17) of remotely infected RA patients and control persons, respectively, responded to the applied peptide. Our data on response to the peptide in persons with active B19 infection and remotely infected individuals are consistent with the results of Kasrowicz *et al.* (2006). However, the quickness of proliferative response between remotely infected RA patients and control group persons differed. The subset of remotely infected RA patients responded to this peptide on the 3rd day, while none of the remotely infected control group individuals had responded. Therefore, the PBMC of remotely infected RA patients are able to respond to VP1/VP2 peptide quickly than the PBMC of healthy individuals. Significant differences in proliferative response between RA patients receiving and not receiving methotrexate treatment were observed. Strong PBMC proliferative response to B19 antigens on the 3rd and 6th day was more frequently observed in RA patients without methotrexate treatment than in patients receiving methotrexate in different combinations with other drugs. Methotrexate treatment probably has effect on B19 specific PBMC proliferative response. However, on the 3rd day the number of persons with strong proliferative response in the group of RA patients receiving methotrexate significantly differed from the number in the control group, while on the 6th day this difference was not observed, which does not allow to make a strong conclusion on the diminishing effect of methotrexate treatment on B19 specific cellular response.

This is the first study identifying the frequency and quickness of PBMC proliferative response to B19 antigens including VP1/VP2 peptide in RA patients with active and remote B19 infection as well as dependence of this response on the applied therapy. We found that RA patients with B19 infection markers responded to the virus antigens faster than healthy persons. Among RA patients receiving methotrexate, the number of individuals with strong response to B19 antigens was significantly lower than among RA patients without methotrexate treatment.

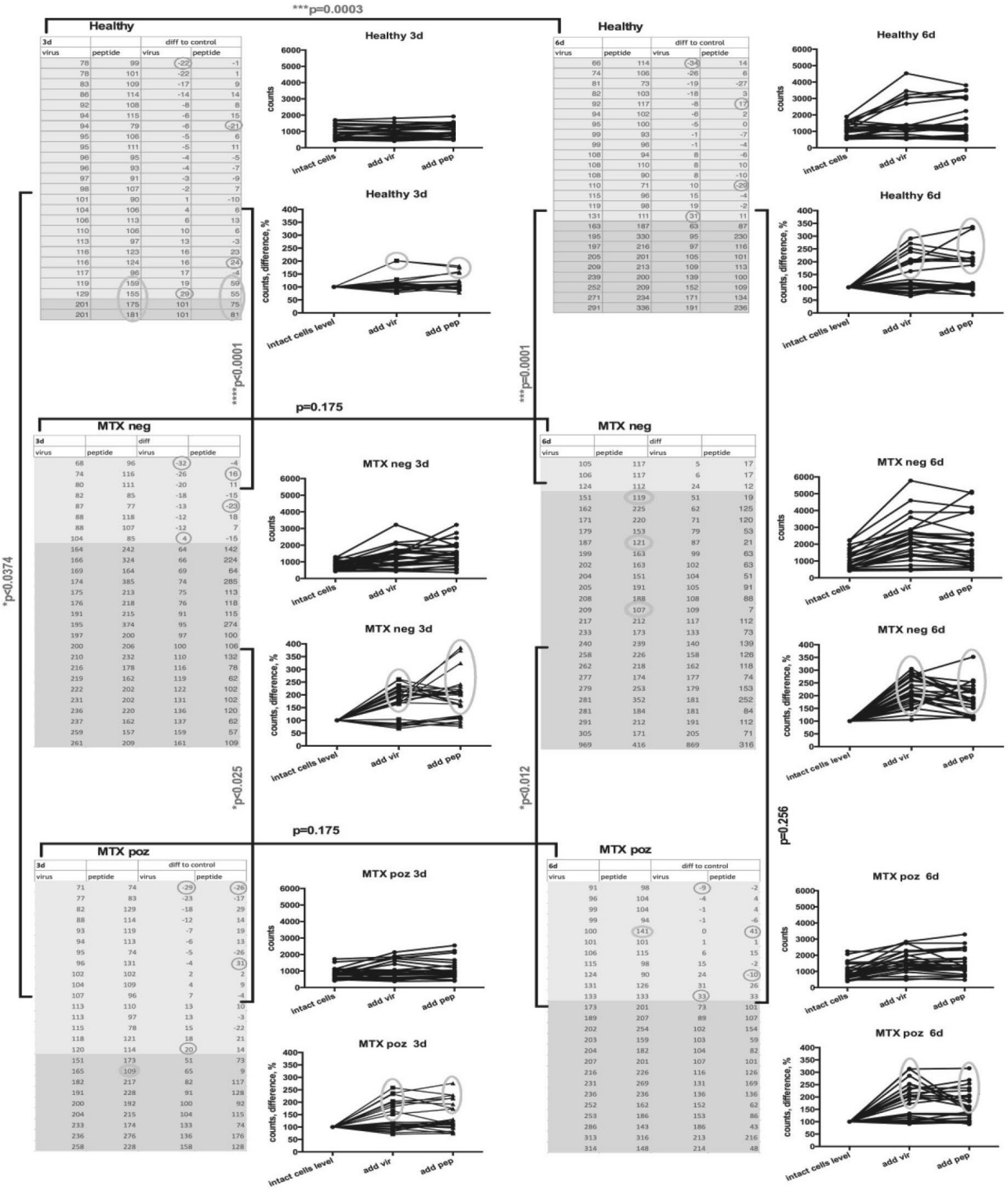

Fig 2. Qualitative analysis of PBMC proliferative response of RA patients to B19 antigens depending on the received treatment

ACKNOWLEDGMENTS

This work was supported in part by the grant No. 09.0112 from Latvian Council of Science and by the National Research Programme in Biomedicine 2014-2017.

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Received 21 December 2015

REIMATOĪDĀ ARTRĪTA SLIMNIEKU PERIFĒRO ASIŅU MONONUKLEĀRO ŠŪNU PROLIFERATIVĀ ATBILDE UZ CILVĒKA PARVOVĪRUSA B19 ANTIGĒNIEM

Šī darba mērķis bija izpētīt reimatoīdā artrīta (RA) perifēro asiņu mononukleāro šūnu atbildi uz parvovīrusa B19 (B19) antigēniem un šīs atbildes iespējamās izmaiņas saistībā ar pielietoto ķīmijterapiju. Lietojot recomLine B19 testu un polimerāzes ķēdes reakciju ar iekšējo praimēšanu, 52 RA slimnieku un 25 dzimumam un vecumam atbilstošu praktiski veselu personu seruma un perifēro asiņu paraugos tika noteikta B19-specifisko IgG un IgM klases antivielu un vīrusspecifisko DNS secību klātbūtne. RA slimnieku un kontroles grupas personu perifēro asiņu mononukleāro šūnu proliferatīvo aktivitāti noteica trešajā un sestajā dienā pēc to kultivācijas vīrusa un B19 VP1/VP2 peptīda klātbūtnē, lietojot timidīna inkorporācijas tehniku. Trešajā dienā perifēro asiņu mononukleāro šūnu proliferatīvā atbilde uz B19 antigēniem tika konstatēta 74,1% RA slimnieku ar aktīvu, 44,8% — ar pārciestu un 40% — ar persistentu B19 infekciju latentā fāzē, turpretī kontroles grupā proliferatīvā atbilde tika noteikta tikai divām personām ar aktīvu vīrusa infekciju. Sestajā kultivācijas dienā proliferatīvā atbilde tika konstatēta 50% RA pacientu ar aktīvu, 68,9% — ar pārciestu un 80% — ar persistentu infekciju latentā fāzē, kā arī 41,1% kontroles grupas personu ar pārciestu infekciju. Augstākie perifēro asiņu mononukleāro šūnu stimulācijas rādītāji tika konstatēti RA pacientiem un kontroles grupas personām ar aktīvu infekciju, kā arī RA slimniekiem ar persistentu infekciju latentā fāzē. Trešajā un sestajā kultivācijas dienā izteikta proliferatīvā atbilde ticami biežāk tika atrasta RA pacientiem, kas nebija saņēmuši metatreksāta terapiju, salīdzinot ar pacientiem, kas metatreksāta terapiju bija saņēmuši dažādās kombinācijās ar citām zālēm. RA pacientiem tika konstatēta biežāka un ātrāka perifēro asiņu mononukleāro šūnu proliferatīvā atbilde uz B19 antigēniem nekā praktiski veselām personām.