ELSEVIER

Journal of Clinical Virology xxx (2004) xxx–xxx

JOURNAL OF CLINICAL VIROLOGY

www.elsevier.com/locate/jcv

Antibodies reactive with C-terminus of the second conserved region of HIV-1gp120 as possible prognostic marker and therapeutic agent for HIV disease

Nevena Veljkovic[a,*], Donald Branch[b], Radmila Metlas[c], Jelena Prljic[a], Roberto Manfredi[d], William W. Stringer[e], Veljko Veljkovic[a]

[a] *Laboratory for Multidisciplinary Research, Institute of Nuclear Sciences, P.O.Box 522, 11001 Belgrade, Serbia and Montenegro*
[b] *Canadian Blood Services and University of Toronto, 67 College Street, Toronto, Ont., Canada M5G 2M1*
[c] *Diapharm, Quay House, South Explanade, St. Peterport, Guernsey, Channel Island GY1 4EJ, UK*
[d] *Division of Infectious Diseases, Department of Clinical and Experimental Medicine, University of Bologna, Bologna, Italy*
[e] *Department of Medicine, UCLA School of Medicine, Harbor-UCLA Medical Center, 1000 West Carson Street, Box 459, Torrance, CA 90509, USA*

Abstract

It has been reported that antibodies reactive with peptide RSANFTDNAKTIIVQLNQSVEIN (peptide NTM) derived from the C-terminus of the second conserved domain of HIV-1 envelope glycoprotein gp120 could represent an important factor in control of the HIV disease. In order to check this notion we (i) tested reactivity with peptide NTM serum samples collected from 310 consecutive HIV-1 infected patients with a $CD4^+$ lymphocyte count ranging from 10 to 800/μL and (ii) performed the longitudinal study that included 107 sera samples collected from 29 HIV patients. Results of these studies demonstrated correlation between presence of anti-NTM antibodies in sera of HIV patients and disease progression measured by the $CD4^+$ cell count. Based on these findings we proposed the anti-NTM antibodies as useful prognostic marker for HIV disease.

Keywords: Antibodies; Serum samples; HIV patients

1. Introduction

The most effective way to control the HIV/AIDS pandemic would be the development of a safe and effective HIV vaccine. Unfortunately, despite the deployment of enormous scientific and financial resources worldwide in the past 15 years, no vaccine candidate is on the immediate horizon (Veljković et al., 2001a; Kohler et al., 2002). There also are strong indications that AIDS vaccines currently tested in humans are not only ineffective but also harmful (Veljković et al., 1997, 2003a, 2003b). In addition, current medical therapy of HIV disease is extremely toxic (multiple side effects and drug interactions), expensive, and constantly risks development of drug-resistant HIV strains. Clearly, other less toxic, inexpensive, non-drug modalities must be pursued in order to slow the spread of HIV infection and to decrease the burden of HIV infection and treatment.

It has been demonstrated that antibodies with specific affinity (or cross reactivity) to the HIV-1 envelope protein (gp120 surface antigen, residues 280-302, RSANFTDNAKTIIVQLNQSVEIN, designated peptide NTM), which represent, in fact, naturally occurring autoantibodies (designated as anti-VIP/NTM antibodies) with a specificity to vasoactive intestinal peptide (VIP) (Veljković et al., 1992, 1993), may be directly involved in control of HIV disease progression (Neurath et al., 1990; Veljković et al., 2001b; Branch et al., 2002).

In order to investigate whether a correlation between HIV-1 disease progression and the anti-VIP/NTM antibodies levels could be found, we have tested reactivity of sera collected from 310 consecutive HIV-1 infected patients with peptide

* Corresponding author.
E-mail address: druid@beotel.yu (N. Veljkovic).

1386-6532/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2004.09.012

NTM. We also performed the longitudinal study which included 29 HIV patients for analysis of a possible relationship between the presence of anti-VIP/NTM antibodies and time-dependent variation of the $CD4^+$ T-cell count. The results of these studies clearly demonstrated that anti-VIP/NTM antibodies represent an effective prognostic marker for HIV disease progression, as well as for monitoring of effects of antiretroviral therapy. Furthermore, these results strongly support a previous notion that anti-VIP/NTM antibodies can be used in development of immune preparations for therapy of HIV disease. It has also been proposed that physical exercise, which increases the serum level of anti-VIP/NTM antibodies, could be useful as an effective supportive therapy for treatment of HIV disease.

2. Materials and methods

2.1. Human subjects and their sera

For investigation of reactivity with peptide NTM serum samples were collected from 310 consecutive HIV-1 infected patients with a $CD4^+$ T-lymphocyte count ranging from 10 to 800/μL. For controls, we used 600 serum samples collected from healthy HIV-negative blood donors. The longitudinal study was performed on 107 samples collected from 29 symptomatic ($CD4^+ = 65.4 \pm 38.6$) and asymptomatic ($CD4^+ = 707 \pm 236$) HIV patients all receiving antiretroviral therapy. The medium number of samples collected from each patient was ≈4, with the medium time interval between two successively collected samples of 6 weeks (Table 1).

2.2. Peptide

Peptide NTM (RSANFTDNAKTIIVQLNQSVEIN) was synthetized by solid-phase technology by Sigma Chemicals (St. Louis, MO).

2.3. ELISA assay

Polystirene microtiter plates (Greiner, Germany) were incubated overnight at 4 °C with 100 μL of BSA-coupled peptides (1 μg/well) diluted in carbonate buffer, pH 9.6. Plates were washed with phosphate-buffered saline (PBS) -0.05% Tween and non-specific sites were blocked with 200 μL PBS containing 1% BSA for 1 h at room temperature. After further washing, serum specimens were than added to the wells (100 μL/well). Sera were diluted in 0.1% BSA in PBS. Plates were incubated for 1 h at room temperature. After three washing with PBS-0.05% Tween, 100 μL of goat anti-human IgG peroxidase-conjugated antibodies (Sigma), diluted 1:5000 was added and the plates were incubated for 1 h. After five washing, the 2,2′-azino-bis(3-ethylbenzothiasoline-6-sulfonate) (ABTS) substrate was added and the absorbance measured at 405 nm.

Table 1
The longitudinal study for 107 samples collected from 29 HIV patients

Serum	Time (weeks)	CD4 (cells/μL)	O.D. (405 nm)	ΔOD(%)/ΔCD4(%)
S1	0	152	0.685	
	3	225	0.623	−0.18
	6	213	0.693	−2.20
	12	124	0.849	−0.56
S2	0	171	0.823	
	27	283	0.269	−1.03
S3	0	80	0.315	
	6	70	0.438	−3.00
S4	0	171	0.773	
	3	126	0.746	0.11
	18	216	0.675	−0.14
	3	162	0.574	−0.60
S5	0	266	0.753	
	6	238	0.855	−1.18
	3	218	0.756	1.50
	6	229	0.702	−1.40
	12	207	0.294	6.50
S6	0	551	0.099	
	3	452	0.178	−4.40
	6	372	0.414	−7.33
	6	353	0.576	−7.80
	6	421	0.500	−0.68
S7	0	301	0.447	
	3	270	0.445	−0.10
	3	221	0.486	−0.50
	3	320	0.464	−0.11
	3	268	0.324	1.87
	12	260	0.218	11.0
S8	0	754	0.553	
	9	943	0.925	2.68
	15	424	0.897	0.05
S9	0	241	0.316	
	3	272	0.387	1.69
	6	337	0.360	−0.29
	3	307	0.557	−6.00
	3	352	0.441	−1.50
	6	303	0.455	−0.21
S10	0	176	0.880	
	3	206	0.927	0.29
	6	131	0.777	0.44
	9	250	0.519	−0.36
	3	157	0.807	−1.48
S11	0	273	0.891	
	3	360	0.908	0.06
	3	486	0.634	−0.85
	9	462	0.424	−13.4
	6	402	0.761	−6.00
S12	0	367	0.602	
	3	352	0.631	−1.25
	21	252	0.654	−0.14
	3	231	0.725	1.38
S13	0	253	0.405	
	3	283	0.475	1.54
	3	211	0.443	0.24
	3	262	0.384	−0.54
	3	177	0.495	−0.90

Table 1 (*Continued*)

Serum	Time (weeks)	CD4 (cells/μL)	O.D. (405 nm)	ΔOD(%)/ΔCD4(%)
	6	281	0.376	−0.41
	3	263	0.354	1.00
S14	0	235	0.711	
	9	114	1.053	−0.94
S15	0	73	0.735	
	12	41	0.631	0.32
	6	25	0.694	0.26
	3	13	0.816	−0.38
	3	219	0.369	−0.03
S16	0	446	0.348	
	3	382	0.454	−2.14
	3	481	0.584	1.07
	3	378	0.623	−0.29
	3	510	0.475	−0.68
	3	387	0.630	−1.38
	6	514	0.264	−1.81
	6	328	0.321	−0.61
S17	0	620	0.247	
	12	739	0.054	−4.10
S18	0	62	0.238	
	3	51	0.441	−4.72
	3	58	0.234	−3.35
	3	52	0.562	−14.0
	4	104	0.428	−0.24
S19	0	431	0.532	
	3	423	0.873	−32.0
	3	487	0.359	−2.73
	6	419	0.419	−1.21
	3	344	0.458	−0.50
	12	409	0.437	−0.26
	6	420	0.282	−11.6
S20	0	74	0.454	
	3	50	0.619	−1.12
	3	88	0.406	−0.45
	18	196	0.212	−0.39
S21	0	370	0.153	
	3	345	0.432	−26.0
	3	334	0.630	−15.0
	6	273	0.633	−0.05
	6	435	0.742	0.28
S22	0	674	0.717	
	3	574	0.455	2.40
	3	482	0.445	0.12
	3	579	0.404	−0.45
	6	375	0.772	−2.68
	3	405	0.595	−2.87
	9	486	0.833	2.00
	3	270	0.417	1.13
S23	0	250	0.824	
	3	166	0.823	0.00
	6	290	1.051	0.37
	3	284	0.865	9.00
	9	280	0.615	18.0
S24	0	325	0.620	
	3	453	0.687	0.25
	3	551	0.586	−0.66
	3	390	0.621	−0.17
	6	331	0.542	0.86
	6	235	0.587	−0.27
	3	407	0.721	0.30
	3	454	0.443	−0.11
S25	0	375	0.276	
	3	344	0.204	3.25
	3	305	0.241	−1.63
	6	270	0.301	−0.46
	6	275	0.397	31.0
S26	0	384	0.612	
	15	260	0.682	−0.34
	6	259	0.652	−4.00
	3	152	0.657	−0.02
S27	0	103	0.486	
	18	74	0.545	−0.43
	3	81	0.411	2.67
S28	0	213	0.599	
	9	182	0.820	−0.21
	3	209	0.770	−0.40
	12	146	0.665	−0.50
	3	294	0.862	0.30
S29	0	331	0.688	
	12	324	0.655	−0.05

Col. 1: Time scale, period between two successive sampling; col. 2: $CD4^+$ cells (cells/μL); col. 3: the absorbance of NTM reactive antibodies measured by ELISA (O.D. value at 405 nm); col.4: change of $CD4^+$ cells (%) between two successive sampling.

3. Results

Previously, it was reported that antibodies which are reactive with the peptide RSANFTDNAKTIIVQLNQSVEINCTRP (amino acids 280–306 within the C2 second conserved region of the envelope glycoprotein gp120 from the BH-10 isolate of HIV-1) are significantly more prevalent in asymptomatic carriers than in patients who progressed to AIDS, and that this finding could represent an important factor in control of the HIV disease progression (Neurath et al., 1990). It has been proposed that these antibodies represent natural anti-vasoactive intestinal peptide (VIP) antibodies, because the domain of the HIV-1 gp120 encompassing peptide C2 is not immunogenic in humans (Veljković et al., 1992, 1993). Recently we demonstrated that peptide RSANFTDNAKTIIVQLNESVEIN derived from peptide C2 and VIP showed similar reactivity profiles when tested against a battery of sera from 46 AIDS patients (Veljkovic et al., 2003). In order to investigate a possible relationship between anti-NTM antibodies and HIV disease progression, we have now tested 310 serum samples collected from consecutive HIV-1 infected patients with a $CD4^+$ T-lymphocyte count ranging from 10 to 800/μL. As controls, we used 600 sera samples collected from HIV-negative blood donors. In our analysis, we used as cutoff values, the medium absorbencies in an ELISA test calculated for all analyzed HIV-positive and HIV-negative

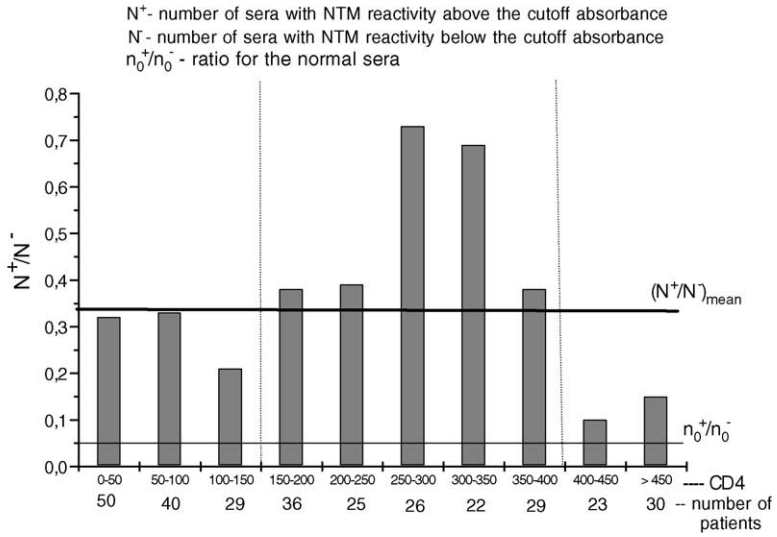

Fig. 1. Reactivity of 310 HIV$^+$ sera with peptide NTM.

samples. These cutoff optical density (OD) values for HIV-positive and HIV-negative sera were 0.6 and 0.2, respectively. In Fig. 1 are presented the distribution of the ratio between the number of HIV-positive sera with reactivity above (N^+) and below (N^-) the cutoff OD value for the entire range of CD4$^+$ T-lymphocyte counts. Results of the same analysis performed for asymptomatic and symptomatic HIV patients are presented in Fig. 2. From results presented in Figs. 1 and 2 can be concluded (i) that sera having the highest reactivity with peptide NTM belong to patients with a CD4$^+$ T-cell count between 150 and 400/μL, and (ii) that sera with reactivity with peptide NTM which is above the cutoff OD value is more frequently found in asymptomatic than in symptomatic HIV patients.

In order to establish a possible relationship between the presence of anti-NTM antibodies and time dependent changes of the CD4$^+$ T-cell count, we performed a longitudinal study. In this study we analyzed 107 samples collected from 29 HIV$^+$ patients with diverse immunological status all receiving antiretroviral drug therapy. The time interval between two samplings varied from 3 to 27 weeks while the average interval was 6 weeks. The results of ELISA tests, time distance between two successive samplings, and CD4$^+$ T-cell counts for each patient are given in Table 1. Results of this analysis are presented in Fig. 3. As can be seen, patients whose sera showed reactivity with peptide NTM above the cutoff OD value, have also, in the analyzed time intervals, an increase of their number of CD4$^+$ T-cells. In contrast, patients whose sera showed reactivity below the cutoff OD value, show a decrease of their CD4$^+$ T-cell count despite receiving antiretroviral therapy.

Further, we examined the connection between variations of reactivity of sera with peptide NTM and variations of CD4$^+$ T-cell count. We hypothesized that the distribution of the variable ΔOD%/ΔCD4% corresponds to the normal distribution, meaning that any observed difference between

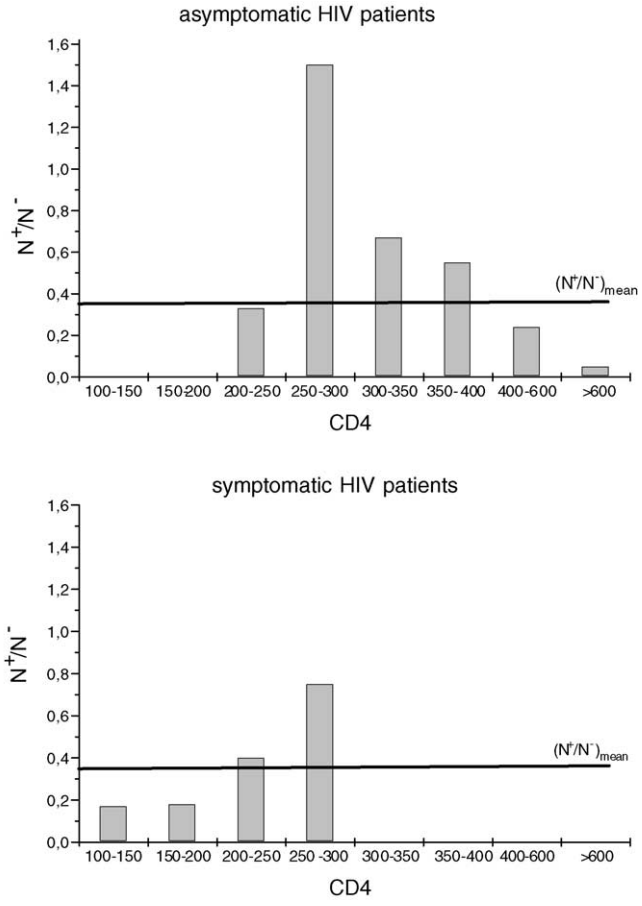

Fig. 2. Reactivity of sera collected from asymptomatic and symptomatic HIV patients with peptide NTM.

mean value of ΔOD%/ΔCD4% and determined value is due to chance. For this purpose, we compared theoretical characteristics of normal distribution to characteristics of distribution of x = ΔOD%/ΔCD4% (Table 2). The range between

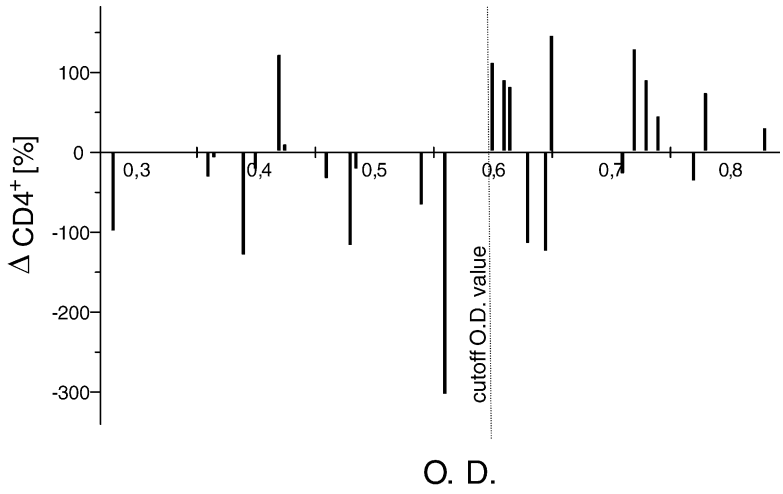

Fig. 3. Distribution of the variable ΔOD%/ΔCD4%.

Table 2
The theoretical characteristics of the normal distribution

Sample mean	x
Sample variance	σ^2
Standard deviation	σ
Coefficient of asymmetry	$\alpha_3 = 0$
Coefficient of flatness	$\alpha_4 = 3$

the minimum and maximum values is divided into 144 intervals. Interval means (x_i) as well as frequencies (f_i) are determined. Five left and right boundary values are excluded from the calculation as inaccurate due to measuring errors. Provisory sample mean is the mean value of the 73rd interval, $m = -0.65$. The difference between the value x_i and the sample mean m is $d_i = x_i - m$. Theoretical characteristics of normal distribution are given in Table 2. The Coefficient of asymmetry and flatness are calculated by the following equations:

$$\alpha_3 = \frac{\Sigma f_i d_i^3}{\sigma^3 \Sigma f_i}$$

$$\alpha_4 = \frac{\Sigma f_i d_i^4}{\sigma^4 \Sigma f_i}$$

The calculated characteristics for the distribution of variable ΔOD%/ΔCD4% are presented in Table 3 and Fig. 4. It is obvious from presented results that the values of theoretical and empirical coefficients are similar. This fact points out existence of a correlation between the change of sera reactivity with peptide NTM and the change of $CD4^+$ cell count.

Table 3
The characteristics of distribution of the variable ΔOD%/ΔCD4%

Sample mean	$x = -0.606$
Sample variance	$\sigma^2 = 5.784$
Standard deviation	$\sigma = 2.045$
Coefficient of asymmetry	$\alpha_3 = 0.095$
Coefficient of flatness	$\alpha_4 = 3.49$

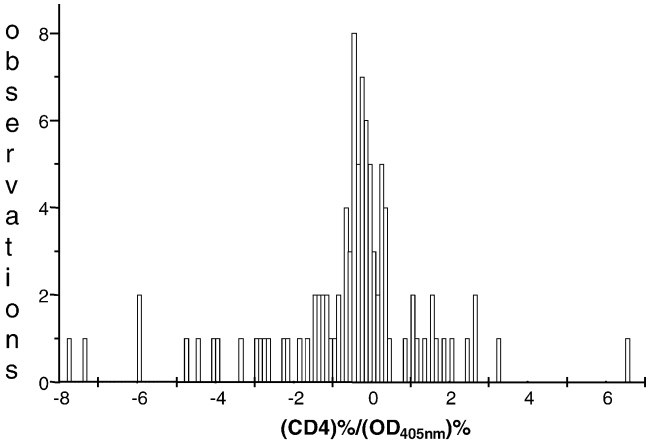

Fig. 4. Distribution of the variable ΔOD%/ΔCD4%.

4. Discussion

There is the consensus opinion that development of an effective preventive HIV vaccine is the unique way to stop the current AIDS pandemic. The main obstacle in this development is the immunologic/antigenic properties of the HIV-1 envelope protein gp120 (Veljkovic et al., 1997), representing the main viral antigenic component. For this reason, gp120-based HIV vaccines are not only inefficient but also dangerous because they could result in vaccines that would make individuals more vulnerable to HIV infection and even accelerate the disease progression by induction of deceptive immune imprinting and destabilization of the immune network (Veljković et al., 2001a; Kohler et al., 2002). Despite these possible consequences, some antibodies naturally generated after HIV infection could play a significant protective role. Such a property was ascribed to anti-NTM antibodies which are reactive with the C-terminus of the second conserved domain of HIV-1 gp120. It has been proposed that absence or disappearance of these antibodies may represent a possible factor contributing to the development of AIDS (Neurath et

al., 1990). It was also reported that passive immunization of an AIDS patient with human HIV-negative plasma enriched with these antibodies restored the immune network and slowed disease progression (Veljković et al., 2001b).

Results reported herein show highest reactivity with peptide NTM of sera collected from HIV patients with $CD4^+$ T-cell counts between 150 and 400/μL. It is also shown that anti-NTM antibodies are remarkably more prevalent in asymptomatic than in symptomatic HIV patients. Results of a longitudinal study show correlation between a change of sera reactivity with peptide NTM and a change of $CD4^+$ T-cell count in HIV patients. The results presented also suggest that antiretroviral therapy could be more effective in patients with higher than low levels of anti-NTM antibodies.

In conclusion, this study supports previous suggestions of a role for anti-NTM/VIP antibodies in HIV infection and implicates the use of anti-NTM antibodies as a simple and effective prognostic marker for monitoring of HIV disease progression, as well as for monitoring of effectiveness of the antiretroviral therapy. These results also indicate that the C-terminus of the second conserved region of HIV-1 gp120, and antibodies reactive with this domain, represents a most promising target for future development of therapeutic vaccines and immunopreparations for therapy of HIV disease.

Acknowledgments

This work was supported by the Ministry of Science, Technology and Development of Serbia (Serbia and Montenegro) (contract No. 1993) and Diapharm Ltd.

References

Branch DR, Valenta LJE, Yousefi S, Sakac D, Singla R, Bali M, et al. VPAC1 is a cellular neuroendocrine receptor expressed on T cells that actively facilitates productive HIV-1 infection. AIDS 2002;16:309–19.

Kohler H, Muller S, Veljković V. No hope for an AIDS vaccine soon. AIDS Sci 2002;2:5.

Neurath AR, Strick N, Taylor P. Search for epitope-specific antibody responses to the HIV-1 envelope glycoprotein signifying resistance to disease development. AIDS Res Human Retrovir 1990;6:1183–92.

Veljković V, Metlaš R, Kohler H, Urnovitz HB, Prljić J, Veljković N, et al. AIDS epidemic at the beginning of the third millennium: time for a new AIDS vaccine strategy. Vaccine 2001a;19:1855–62.

Veljković V, Johnson E, Metlaš R. Molecular basis of the inefficacy and possible harmful effects of AIDS vaccine candidates based on HIV-1 envelope glycoprotein gp120. Vaccine 1997;15:437–8.

Veljković V, Muller S, Kohler H. Does VaxGen hide the breakthrough Infections. Lancet 2003a;361:1743–4.

Veljković V, Muller S, Kohler H. AIDSVAX results: an important open question. Vaccine 2003b;21:3528–9.

Veljković V, Metlaš R, Raspopović J, Pongor S. Spectral and sequence similarity between VIP and the second conserved region of HIV envelope glycoprotein gp120: possible consequences on prevention and therapy of AIDS. Biochem Biophys Res Commun 1992;189:705–10.

Veljković V, Metlaš R, Vojvodić D, Čavor LJ, Pejinović N, Dujić A, et al. Natural autoantibodies cross-react with a peptide derived from the second conserved region of HIV-1 envelope glycoprotein gp120. Biochem Biophys Res Commun 1993;196:1019–24.

Veljković V, Metlaš R, Jevtović DJ, Stringer W. The role of passive immunization in hiv-positive patients: a case report. Chest 2001b;120:662–6.

Veljkovic N, Branch DR, Metlas R, Prljic J, Vlahovicek K, Pongor S, et al. Design of peptide mimetics of HIV-1 gp120 for prevention and therapy of HIV disease 2003;62:158–66.

Veljkovic V, Johnson E, Metlas R. Molecular basis of the inefficacy and possible harmful effects of AIDS vaccine candidates based on HIV-1 envelope glycoprotein gp120. Vaccine 1997;15:473–4.