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Aerobic exercise training as a potential source of natural antibodies protective against human immunodeficiency virus-1

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Despite the effectiveness of HAART in controlling HIV-1 replication, the emergence of drug-resistant viruses in infected patients and the severe side effects caused by the currently used drug regimens and the lack of an effective vaccine necessitate the continued search for new therapeutic strategies for prevention and therapy of HIV disease. Previously we reported that natural autoantibodies, recognizing peptide FTDNAKTI (peptide NTM1) derived from the C2 domain of HIV-1 gp120, contribute to the control of HIV disease. Here we demonstrated that sera from well-trained athletic (HIV-negative) subjects showed high reactivity with peptide NTM1. This result confirms that aerobic exercise training stimulates production of natural autoantibodies, which recognize peptide NTM1. Bioinformatics analysis indicates that these natural autoantibodies could slow down disease progression by blocking the superantigenic site on HIV-1 gp120. The results suggest that aerobic exercise training may be a promising non-toxic and inexpensive adjunctive anti-HIV therapy.

Since the first case of HIV/AIDS was identified (Gottlieb et al., 1981), AIDS has emerged as the largest and the most devastating public health pandemic of our time, affecting approximately 70 million people worldwide, with >25 million dead (Berkley, 2003). There is consensus that the development of an effective and safe preventive HIV vaccine represents the best strategy to control the AIDS epidemic, and consequently, this endeavor remains a top public health priority. Unfortunately, despite the deployment of enormous scientific and financial resources worldwide toward a preventive vaccine strategy, no vaccine candidate is on the immediate horizon (Veljkovic et al., 2008a). There also are strong indications that the AIDS vaccines currently tested in humans are not only ineffective, but potentially harmful (Veljkovic et al., 2008a). In addition, current medical therapy of HIV disease is extremely toxic (multiple side effects and drug interactions), expensive, and has the potential to induce drug-resistant HIV strains. Clearly, other less toxic, inexpensive, non-drug modalities must be pursued in order to slow down the spread of HIV infection and to decrease the burden of HIV infection and treatment.

In the development of new strategies for prevention and therapy of HIV disease, many scientists are focused on the rare group of HIV-infected patients called long-term non-progressors (LTNP) who are able to maintain high $CD4^+$ T-cell counts for 10 or more years in the absence of antiretroviral therapy. Although several factors have been associated with this non-progression (for a complete review see Saez-Cirion et al., 2007), there is no consensus on the most relevant mechanisms underlying this phenomenon. Despite the rarity of LTNP (only $\sim 5\%$ of HIV-infected persons), analysis of such persons offers a unique opportunity to improve our understanding of the pathogenesis of HIV infection and mechanisms that control disease progression.

Investigating the difference in the spectrum of antibodies directed against the envelope glycoprotein gp120 of HIV-1 between LTNP and HIV-infected individuals who developed AIDS within 5 years of the onset of infection, Neurath et al. (1990) found that antibodies recognizing the C-terminus of the second conserved domain (C2) of gp120 are significantly more prevalent in asymptomatic carriers than in AIDS patients. Accordingly, it was proposed that the absence or disappearance of detectable antibodies reacting with the C-terminus of C2 of HIV-1 gp120 may represent a possible factor contributing to the development of AIDS (Neurath et al., 1990). It

has been also showed that antibodies that recognize the C2-derived peptides [RSANFTDNAKTIIVQLNQ SVEIN (peptide NTM) and FTDNAKTI (peptide NTM1)] are more prevalent in LTNP than in progressors (Djordjevic et al., 2007), as well as in asymptomatic than in symptomatic HIV patients under the antiretroviral therapy (Veljkovic et al., 2004). The therapeutic potential of antibodies reactive with peptide NTM is additionally demonstrated in a unique clinical experiment which showed that passive immunization of an AIDS patient with human HIV-negative plasma enriched with NTM-reactive antibodies restored the immune network and slowed disease progression (Veljkovic et al., 2001).

Despite the presence of the strongest T-cell epitope of gp120, which is active *in vitro* (Mathiesen et al., 1989), and an exposed B-cell epitope, the C-terminus of the C2 region encompassing peptide NTM is not immunogenic in humans (Mathiesen et al., 1989; Sastry & Arlinghaus, 1991). This gives rise to an important question about the origin of antibodies recognizing this domain of gp120. Based on the structural and informational similarity between peptide NTM and vasoactive intestinal peptide (VIP) (Veljkovic et al., 1992), and immunological cross-reactivity between these two peptides (Metlas et al., 1991; Veljkovic et al., 2003), it has been proposed that antibodies that are reactive with the C-terminus of C2 of gp120 represent natural anti-VIP antibodies.

Several studies demonstrated an increase in plasma VIP concentration as reaction to exercise (Galbo et al., 1979; Oektedalen et al., 1983; Oktedalen et al., 1983a, b; Hvidsten et al., 1986; Woie et al., 1986; Opstad, 1987; Paul et al., 1987; Wiik et al., 1988; MacLaren et al., 1995). VIP is a 28-amino-acid peptide with broad biological actions, including strong vasodilator and bronchodilator activities, a neurotransmitter role, and an immunomodulator role (playing an important role in immune homeostasis). Because of these pleiotropic, imunomodulatory, and neuromodulatory activities of VIP, circulating level of this peptide is under tight control. It has been proposed that autoantibodies directed against VIP are potent modifiers of its biological actions and an important regulator of circulating VIP (Paul et al., 1989). Paul and Said (1988) showed that an increased level of anti-VIP antibodies is present in plasma from $\sim$ 30% healthy (HIV-negative) human subjects who habitually performed aerobic muscular exercise (running, cycling, swimming, and/or weight training, three or more workouts per week a year or more before study entry), compared with $\sim$ 2% of healthy, sedentary subjects. These authors suggested that natural anti-VIP antibodies represent an important factor in the regulation of circulating VIP during and after exercise (Paul & Said, 1988). These results indicate that the natural VIP/NTM-reactive antibodies may have been produced in response to increased VIP levels during exercise.

As we have demonstrated in prior publications, natural anti-VIP antibodies which recognize the C2 domain of HIV-1 gp120, encompassing peptides NTM and NTM1, could represent an important host factor in control of the HIV disease progression (Veljkovic et al., 2001, 2004, 2007; Djordjevic et al., 2007). Previously reported results suggested that physical activity could boost production of these natural antibodies with anti-HIV properties (Paul et al., 1987). In order to explore this possibility we investigated reactivity of sera collected from 17 healthy (HIV-negative) well-trained athletes with gp120-derived peptide FTDNAKTI (peptide NTM1).

Materials and methods

Human subjects

Serum samples were collected from 17 water polo players of the elite junior national water polo team of Serbia at the start (Dopsaj et al., 2008), and after 43 days of intensive aerobic exercise training (in the middle of the fifth training microcycle when the aerobic training reaches a maximum; Bompa & Carrera, 2005), just before the 2007 season. All sera were collected a day before the start of the training cycle, and during the first day after the training cycle was completed. During this period, subjects performed 84 trainings in a total duration of 135 h which included 86.01% aerobic training load (32.73% – HR $<$150, low aerobic; 30.48% – HR between 151 and 165, middle and high aerobic; and 22.79% – HR $>$166, submaximal and maximal aerobic load); 9.82% anaerobic training load (5.76% – lactate concentration between 4 and 6 mmol/L, low anaerobic; 4.06% – lactate concentration above 6 mmol/L, submaximal and maximal anaerobic load), and 4.17% – maximal speed training. Four weeks before start of the training cycle, all subjects had active rest, which included low intensity training of 1–2 h a day.

The basic descriptive characteristics of the study population were Age = 18.1 $\pm$ 0.8 years; BH = 190.6 $\pm$ 4.8 cm; BM = 86.4 $\pm$ 9.8 kg; BMI = 23.75 $\pm$ 2.33 kg/m^2; percent body fat = 7.75 $\pm$ 3.06; lean body mass = 80.23 $\pm$ 5.66 kg.

As control we used the sera collected from nine healthy (HIV-negative) individuals doing computer work 6–8 h a day and who do not perform regular physical exercise. The gender and ages of all controls matched to the athletic subjects and had no risk factors that affect the immune system (cigarette smoke, alcohol consumption, use of medications, chronic diseases, etc).

Informed consent and local ethics committee approval has been provided for these human studies.

Bioinformatics analysis

Informational spectrum method (ISM)

The ISM technique is based on a model that assigns to each amino acid a defined parameter describing a physicochemical property involved in the biological activity of the protein and corresponding to electron–ion interaction potential. These values thus determine the electronic properties of amino acids responsible for their intermolecular interactions (Veljkovic et al., 2008b). The obtained numerical sequence, representing the primary structure of a protein, is then subjected to a

discrete Fourier transformation which is defined as follows:

$$X(n) = \Sigma x(m) e^{-j(2/N)nm},\ n = 1, 2, \ldots, N/2 \qquad [1]$$

where $x(m)$ is the mth member of a given numerical series, N the total number of points in this series, and $X(n)$ discrete Fourier transformation coefficients. These coefficients describe the amplitude, phase and frequency of sinusoids, which comprised the original signal. The absolute value of complex discrete Fourier transformation defines the amplitude spectrum and the phase spectrum. The complete information about the original sequence is contained in both spectral functions. However, in the case of protein analysis, relevant information is presented in energy density spectrum (for review see (Veljkovic et al., 2008b), which is defined as follows:

$$S(n) = X(n)X^*(n) = |X(n)|^2,\ n = 1, 2, \ldots, N/2 \qquad [2]$$

Thus, the initial information defined by the sequence of amino acids now is presented in the form of the informational spectrum (IS), representing the series of frequencies and their amplitudes.

The IS frequencies correspond to the distribution of structural motifs with defined physicochemical characteristics responsible for the biological function of a protein. When comparing proteins that share the same biological or biochemical function, the ISM technique allows detection of code/frequency pairs which are specific for their common biological properties, or which correlate with their specific interaction. This common informational characteristic of sequences is determined by cross-spectrum or consensus informational spectrum (CIS), i.e., the Fourier transformation of the correlation function for the spectrum. In this way, any spectral component (frequency) not present in all compared ISs is eliminated. If one calculates a CIS for a group of proteins having different primary structures, and finds strictly defined peak frequencies, it means that the analyzed proteins participate in mutual interaction or have a common biological function.

The ISM was successfully applied in structure–function analysis of different protein sequences, *de novo* design of biologically active peptides, assessment of biological effects of mutations, and identification of new therapeutic targets (for review see Veljkovic et al., 2008b, and references therein).

Peptide conjugates synthesis

The synthesis of the $(NTMs)_4$–SOC_4 conjugate was carried out manually by stepwise solid phase peptide synthesis using the Boc–Gly–OCH_2–Pam resin (1 g, 0.25 mmol/g capacity). Sequential oligopeptide carrier (SOC_4), formed by the repetitive Lys–Aib–Gly moiety, is applied to display analyzed peptides. The synthetic procedure starts with the step-by-step couplings of the protected residues (Boc/Bzl) corresponding to the SOC_4 carrier to the resin. Lysine was introduced as Boc–Lys(Fmoc)–OH. After removal of the Fmoc protective groups from the Lys–$N^{\varepsilon}H_2$ groups by 20% piperidine in dimethylformamide, synthesis of the epitope FTDNAKTI was carried out (Boc/Bzl) by the simultaneous attachment of each residue in four copies.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed with peptide $(NTMs)_4$–SOC_4 by the following procedure: polystyrene microtest plates (Sarstedt, Numbreht, Germany) were incubated overnight at 4 °C with 100 μL of peptides (0.5 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 5% bovine serum albumin (BSA) for 2 h at room temperature. After six washings, serum specimens were added to the wells (100 μL/well). Sera were diluted 1:100 in 5% BSA in PBS. Plates were incubated for 3 h at room temperature. After six washings with PBS–0.05% Tween, 100 μL of goat anti-human IgG alkaline phosphatase-conjugated antibodies (Sigma, St. Louis, USA), diluted 1:2500, were added and the plates were incubated for 30 min at room temperature. After six washings, *p*-nitrophenyl phosphate substrate was added and the absorbance measured at 400 nm after 15 min. Each sample was tested independently twice.

Statistical analysis

The significance of the difference in optical density (OD) values for trained subjects and control was calculated by the non-parametrical Mann–Whitney test, as sample sizes were relatively small. For each comparison, the level of significance P for a directional test and the corresponding critical value of U are given.

Results

Results of ELISA test are presented in Fig. 1. The reactivity of sera with peptide NTM1 significantly increased after training in all tested subjects (OD2/OD1 ratio $\in$ (1.64–4.46); $P = 0.0212$, $U = 85$). It has been reported previously that sera of $\sim$ 5% healthy (HIV-negative) individuals contain increased titer of the VIP/NTM reactive antibodies corresponding to OD value $> (OD_{mean} + 2SD)$ (Veljkovic et al., 2001). In our study, a significantly higher percentage of trained subjects (10 of 17% or 58.8%) had increased titer of NTM1 reactive antibodies before the start of the training period.

The cross-spectrum between gp120(MN) and V_H3 chains of antibodies 18/2, Kim4,6 and Huab2-3, which bind SAg domain on gp120(MN) (Karray et al., 1998) has been calculated. The dominant peaks in this cross-spectrum correspond to IS frequencies F1(0.02) and F2(0.22). According to the ISM concept, these two frequency components represent information that characterize interaction between these V_H3 chains and gp120(MN). The cross-spectrum between VIP and V_L(L43498) chain of human anti-VIP autoantibody, which efficiently binds and hydrolyzes VIP (Tyutyulkova et al., 1996), is also characterized by two dominant peaks which correspond to frequencies F1(0.02) and F2(0.22). This indicates that interaction between gp120 and SAg-binding V_H3 chains and between VIP and V_L(L43498) chains is determined by the same information which is encoded in primary structures of these proteins. This result suggests that some of anti-VIP antibodies could be targeted to region of HIV-1 gp120, which encompasses SAg. It is of note that the C2 domain (positions 252–272), which plays an important role in gp120 SAg binding to normal human immunoglobulins (Karray & Zouali, 1997), is located very close to the C2 domain encompassing peptide NTM (residues 278–297).

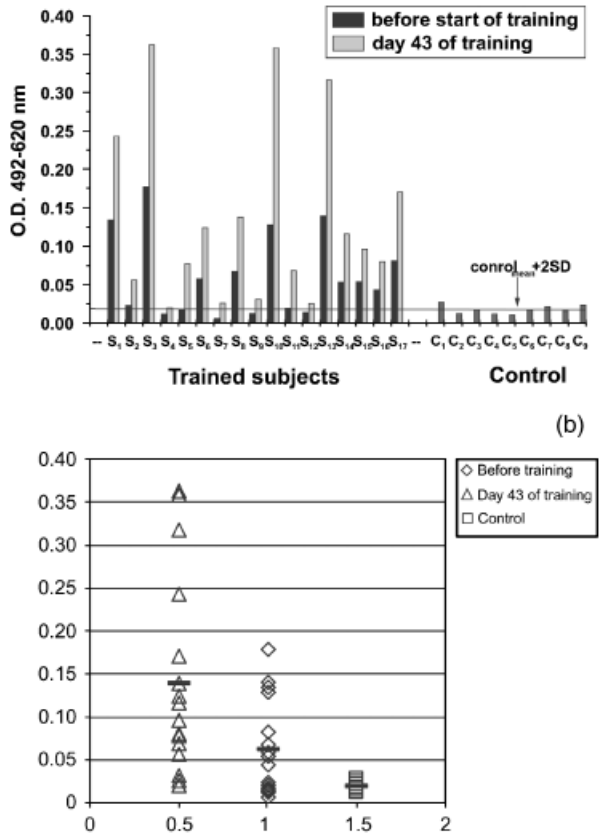

Fig. 1. (a) Reactivity of sera of well-trained healthy (HIV-negative) subjects with HIV-1 gp120-derived peptide NTM1, collected before start of training period and after 43 days of training. (b) The absorbance values obtained for sera of trained individuals and control (ordinate). Antibodies recognizing peptide NTM1 are significantly more prevalent in serum samples collected on day 43 of training compared with sera collected before the start of the training period ($OD2_{mean}/OD1_{mean} = 2.21$; $P = 0.0212$, $U = 85$) and compared with control ($OD2_{mean}/OD_{cont.(mean)} = 7.53$; $P = 0.0001$, $U = 5$).

Discussion

The results from the present study support three conclusions. First, physical training with a predominant aerobic component significantly increased reactivity of all analyzed sera with peptide NTM1. Second, sera of 58.8% trained subjects, collected before start of the training cycle, showed high reactivity ($OD > OD_{control} + 2SD$). This percentage is remarkably higher than previously reported percentage ($\sim$ 5%) of normal healthy subjects, but we have previously reported well-trained subjects whose sera showed NTM reactivity above this threshold (Veljkovic et al., 2001). This implies that regular physical exercise has a long lasting effect on production of the natural antibodies recognizing peptide NTM1. Third, reactivity of sera of some well-trained subjects which was very low at the beginning of the training period (S2, S4, S5, S7, S9, S11, and S12) still significantly increased (see Fig. 1(a)), confirming that the immune system of these individuals has the capability for production of NTM1-reactive antibodies after stimulation by aerobic exercise training.

Discovery of natural antibodies recognizing the V3 loop of gp120 in sera of healthy HIV-negative individuals, which could efficiently neutralize HIV-1 (Metlas et al., 1999a, b, 2007), and natural anti-VIP antibodies recognizing the C-terminus of the C2 domain of gp120 in sera of HIV patients (whose presence strongly correlates with the disease progression; Veljkovic et al., 2001, 2004; Djordjevic et al., 2007), suggests that the immune system possesses some natural capabilities for control of the HIV disease. These antibodies could provide new therapeutic approaches that would increase the defense capacity of the immune system against HIV.

Because of a possible important role of natural NTM/NTM1 reactive antibodies in control of the HIV disease progression, it will be necessary to define the mode of action of these antibodies. Based on accumulating evidence of functional importance of the C2 domain encompassing peptides NTM and NTM1 (reviewed in Veljkovic et al., 2003; Veljkovic & Metlas, 2004), it has been suggested previously that natural antibodies recognizing this region of gp120 could block interaction with CD4, CCR5, and CXCR4 receptors (Veljkovic & Metlas, 2004), restore the immune network (Metlas & Veljkovic, 2004) and block replication and integration of HIV stimulated by VIP receptors (Branch et al., 2002; Bokaei et al., 2007). In this manuscript we have presented results that indicate that natural anti-HIV antibodies could also block the SAg-like activity of HIV-1 gp120.

HIV infection is characterized by accelerated apoptosis and progressive loss of B cells (Titanji et al., 2006). It has been suggested that these abnormalities are related to the property of gp120 to act as a superantigen for V_H3^+ B cells (Berberian et al., 1994; Zouali, 1995). Because V_H3^+ antibodies play an important role in protection, their under-expression may contribute to acceleration of HIV disease progression (Juompan et al., 1998). Karray and Zouali (1997) demonstrated that SAg binding requires contributions of residues from C2 domain (positions 252–272). This domain is very close to the region encompassing peptides NTM (positions 278–297) and NTM1 (positions 282–291). Our ISM analysis of SAg-binding human immunoglobulins and natural anti-VIP immunoglobulin, together with previously reported binding of anti-VIP antibodies by peptides NTM and NTM1 (Metlas et al., 1991; Veljkovic et al., 2003; Djordjevic et al., 2007), point out a possibility that binding of natural anti-VIP antibodies to the C-terminus of the C2 domain could sterically block access of immunoglobulins to the SAg site on HIV-1 gp120. Thus, the NTM/VIP-reactive natural antibodies could prevent

depletion of V_H3^+ B cells and in this way contribute to control of the HIV disease progression.

Despite the numerous studies aimed at explaining the specific immune response to exercise, conflicts exist in the results obtained so far from such studies. Regular and moderate exercise has been reported to have several favorable effects on immunological functions (Sharp & Kouledakis, 1992; Simonson, 2001; Gleeson, 2007). On the other hand, some studies have reported that various immune cell functions are temporarily impaired following acute bouts of prolonged, continuous heavy exercise (Nieman, 1994; Mackinnon, 1999; Ronsen et al., 2001). These data point out that safety aspect of aerobic exercise is an important issue that needs to be addressed in future studies, particularly in HIV-infected individuals with compromised immune system.

Perspectives

Increased level of natural autoantibodes induced by aerobic exercise, which recognize peptide NTM1 derived from HIV-1 gp120, may have two beneficial effects. First, in HIV-negative individuals, these antibodies can bind HIV particles in circulation and prevent them from reaching their target cells, thereby reducing the risk of infection and decreasing transmission of the disease. Second, in HIV-positive individuals increased level of NTM1-recognizing antibodies could slow-down HIV disease progression and reconstitute the damaged immune system (Neurath et al., 1990; Veljkovic et al., 2001, 2004; Metlas & Veljkovic, 2004; Djordjevic et al., 2007). Accordingly, aerobic exercise might be an important, inexpensive, non-toxic, and widely available frontline defense and adjunctive therapy against HIV/AIDS. For this reason, further studies of stimulation by aerobic exercise of natural autoantibodies that are protective against HIV need to be conducted in HIV-positive patients, as well as in HIV-negative patients. These studies should be carefully designed in order to avoid possible adverse effects, especially in individuals with compromised immune system.

Key words: adjunctive therapy, AIDS, physical activity, immune system.

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