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Immunological Status in Patients with Lower Limb Amputation Due to Peripheral Arterial Disease before and after Comprehensive Rehabilitation

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ABSTRACT

The immunological status before and after a comprehensive rehabilitation program was studied. Seven persons (4 males, 3 females, mean age 71.4 years) after lower limb amputation due to peripheral arterial disease (PAD) were subject to standard comprehensive rehabilitation program for amputees of four-week duration, which included training in activities of daily living, daily exercise of various types, training of crutch-assisted gait and use of leg prosthesis, and mild transcutaneous electrical stimulation. Before and after rehabilitation, peripherial blood was collected and the number and ratio of white blood cells were determined and analysed for the expression of cell surface antigens (CD3, CD4, CD8, CD19, CD25, CD69), cytokines (IFN-gamma, IL-4) and phagocytosis/oxidative killing functional tests. Due to strict patient selection criteria excluding serious accompanying disease, immunological parameters were within normal limits already before rehabilitation. After rehabilitation, an increase in oxidative burst was observed in monocytes and neutrophil granulocytes, but statistically significant only in monocytes. The expression of CD69 molecules by T cells and monocytes was significantly increased, as well as the expression of IL-4 by T cells. A significant decrease in the ratio of CD4 to CD8 cells was also found, but not a clinically critical one. It can therefore be concluded that the comprehensive rehabilitation treatment in patients with lower limb amputation due to PAD led to some – prevailingly positive – immunological changes, which were consistent with the patients' improved physical condition and clinical status.

Key words: *rehabilitation, physical therapy modalities, peripheral vascular disease, amputees, immune system processes*

Introduction

About 80–90% of all lower limb amputations result from peripheral arterial disease (PAD), the main cause being atherosclerosis and its complications. The incidence of dysvascularity (poor circulation) increases with age and the presence of co-morbidities such as diabetes[1]. Atherosclerosis of lower extremities can lead to critical ischaemia and to the amputation of a leg. Amputation is one of the most dramatic surgical interventions resulting in serious impairment and disability, especially if both lower extremities are amputated.

The number of amputations rises with the aging of a population[2]. There are marked differences in the overall incidence of both first and all amputations between different regions[3]. The risk of contralateral amputation is ever present[4]. Amputations among the elderly people represent an increasingly important challenge from the medical and the socio-economic point of view[2].

In most patients, functioning worsens after amputation, whereby old age, co-morbidities and the level of amputation seem to be significant related factors. The loss of lower limb results in prolonged physical consequences for the patient, frequent ones being progressive flexion contracture of the knee or hip, and loss of gait pattern. Especially in the elderly, this can lead to reduced physical activity or even the disuse syndrome with deterioration of the general health status. Complications due to immobility arise in different organ systems (musculoskeletal, cardiovascular, respiratory, etc.) and can be very deleterious[5].

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Immobility also weakens resistance against infectious diseases, whereby the organism's biological defence is regulated by the immune system. In addition, proper immunological condition plays a very important role in successful outcome of rehabilitation.

Limb amputation should be seen as a condition that calls for an adequate rehabilitation program in order to return the amputee to a higher functional level. Modified physical status is accompanied by psychosocial sequelae[6]. Therefore, the aim of rehabilitation after lower limb amputation is to improve physical, psychological and social functioning[7]. Comprehensive rehabilitation in patients after lower limb amputation requires a team approach. The overall satisfaction of the patient with the therapy is also very important[8]. Maximum independence in abilities of daily living for as long as possible with independence of mobility within and outside the home should be preserved or restored.

Immune response in people with some other locomotor disabilities after different kinds of treatment and activity has been addressed before, although seldom[9–11]. However, we could not find any published study on the possible effect of a comprehensive rehabilitation program on the immune response in patients after amputation of lower limb due to PAD. Hence, the objective of our study was to explore whether a comprehensive rehabilitation program, if consistently performed, could result in improvement of immunological status, or whether it might prove to have any other effect upon immune response in such patients.

Materials and Methods

Study design

We conducted a single-group observational study. The immunological status of the patients with lower limb amputation due to PAD was assessed before starting a comprehensive rehabilitation program and after completing it, as detailed below.

Subjects

The study included 7 persons (4 men, 3 women) after amputation of one lower limb. Their average age was 71.4 years, the youngest patient being 58, the oldest 84 years old. In all of them, the cause of amputation was atherosclerotic PAD and the resulting critical ischemia and gangrene dictating limb amputation. A below-knee amputation was performed in 5 and an above-knee amputation in 2 patients. In all cases, the remaining lower limb showed a symptomatic PAD form (stage II Fontaine). The selection criteria excluded any serious accompanying disease which could influence patient's immunity. None of the selected patients had any form of diabetes mellitus. They suffered from no other serious intercurrent diseases, complications on the amputation stump or wound infections. In four patients, antithrombotic therapy consisted of a daily dose of 100 mg of acetyl salicylic acid; one patient was on a combination of 200 mg of dipyridamol and 25 mg of acetyl salicylic acid; and one had anticoagulation therapy by means of varfarin according to a personal scheme. The remaining medicamentous therapy included mainly antihypertonic drugs and sedatives.

The patients were admitted to the rehabilitation program on average 5.1 months after the amputation (minimum 2.5, maximum 9 months). They were then already beyond the acute post-surgery phase, their general status was stable and the amputation stump had healed. The patients were selected for the study provided that they were not suffering from any intercurrent conditions that might affect their immune status. Due to ethical constraints, the study employed a single-group within-subject observational design.

Rehabilitation program

The patients were subject to a standard program of comprehensive rehabilitation for amputees at the University Rehabilitation Institute in Ljubljana, of which the main goal was to fit them with leg prostheses and train them to walk. The program consisted of training in activities of daily living, regular daily exercises for strengthening the stump muscles (mainly strengthening of the knee extensors in the below-knee amputees and of the hip extensors in the above-knee amputees, in view of preventing knee and hip flexion contractures), general fitness exercises, and exercises for maintaining the range of motion of single joints also for the remaining leg. Special attention was dedicated to the training of crutch-assisted gait and use of suitable leg prosthesis. The program was adjusted in such a way as to avoid overfatigue.

To increase blood circulation of the remaining leg, low-frequency transcutaneous electrical stimulation was applied to the posterior aspect of the calf gently stimulating underlying triceps surae muscle (gastrocnemius-soleus)[12]. The intensity of the stimulation was adjusted for each patient just above the motor excitation threshold, thus evoking slight contractions of the stimulated muscles. When applying electrical stimulation, all safety measures and contraindications to neuromuscular electrical stimulation were strictly respected[13,14]. The patients experienced no discomfort during the electrical stimulation sessions.

The rehabilitation program lasted 5 days a week (from Monday to Friday) with rest at Saturday and Sunday, for the period of 4 weeks, for a total of 20 treatment days. All the patients included into the study also had psychological support regarding acceptance of disability and motivation for taking part in rehabilitation activities.

Determination of immune response

Determination of potential changes in immune response was focused on three white blood cell groups: lymphocytes, neutrophil granulocytes and monocytes. The number and ratios of blood cells were determined and their function investigated. Lymphocytes were analyzed for the expression of cell surface antigens (CD3, CD4, CD8, CD19, CD25, CD69), cytokines interferon γ (IFNγ)

and interleukin 4 (IL-4). Attention was dedicated also to the phagocytic potential of neutrophils and monocytes. In addition to special immunological blood cell tests, routine hematological determinations of erythrocytes, thrombocytes and leucocytes were carried out, with emphasis on the differential white blood cells count.

Blood samples

The blood cells to be examined and analyzed were taken from the peripheral blood obtained from the cubital vein. Prior to starting the treatment but on the same day of the first sessions in the morning, fasting blood samples were taken for laboratory tests (using BD Diagnostics Vacutainer Plastic tubes): 2 heparin-containing test tubes, each with a 7 ml blood sample for special immunological tests, and 1 EDTA-containing tube with a 2 ml blood sample for hematology determinations. The same blood samples were taken for the same tests after completion of the 4 weeks of treatment (in the morning following the last sessions on the previous day).

Blood count

Usual blood count was performed to determine the number of white cells per L of blood in the sample. Differential count was done to acquire information on the percent distribution of specific white blood cells. Both data were used to calculate the absolute number (AN) of a specific white cells population for a single individual.

Flow cytometry

White blood cells were stained with specific monoclonal antibodies directed against different CD markers. Three color analyses were performed concurrently to determine the population of cells under investigation and their function. The following reagents were used: monoclonal antibodies against markers CD3 (BD, CyChrome CD3, 555334), CD4 (BD, CyChrome CD4, 555348), CD8 (BD, CyChrome, 555368), CD19 (BD, CyChrome CD19, 555414), CD25 (BD, CD25 FITC, 345796), and CD69 (BD, CD69 PE, 341652). For the detection of intracellular interleukin 4 (IL-4) and interferon γ (IFNγ) cells T helper 1 – T helper 2 (Th1 – Th2) detection kit (BD, 550749) was used; blood was stimulated for 6 hours with ionomycin/PMA and the inhibitor of exocytose breferdin A. At least 1000 gated cells were analyzed for each test and signals from the two light scatters and the four fluorescence parameters were analyzed.

Phago-burst test

Phagocytosis and oxidative burst capacity was measured using Phago-burst test (Orphegen Pharma, 341060+341058, Heidelberg, Germany). Whole blood test samples were divided into six plastic test tubes (BD, Falcon, 353052) in volume of 100 µl. All the tubes were held in ice bath during the preparation of the test. The test reagents were added as follows: in the first tube FITC labeled *E. coli* (phagocytosis control – FK); in the second FITC labeled *E. coli* (phagocytosis test – FT); in the third wash solution and 123 dihydrorhodamin (wash solution control – WS); in the fourth unlabeled stimulating opsonised E. coli and 123 dihydrorhodamin (*E. coli* stimulated test – EC); in the fifth fMLP and 123 dihydrorhodamin (fMLP stimulated control – FMLP); and in the sixth PMA (phorbol myristate acetate) and 123 dihydrorhodamin (PMA stimulated test – PMA). Tube 1 was incubated at 0°C (FK) and all others at 37°C for 10 minutes. The cells were then lysed with lysing solution and washed three times with wash solution. DNA of live cells was stained with propidium iodide. Measuring was performed using flow cytometer (BD, FACSort, USA) equipped with argon-ion laser of 488 nm excitation wavelength.

Ethical approval

The participating patients were acquainted with the purpose of the study and signed an informed consent statement. The study was approved by the Research Ethics Committee of the University Rehabilitation Institute, Republic of Slovenia.

Statistics

Descriptive statistics were calculated for all the measured parameters. Because of the small sample size and the exploratory nature of the study, differences between pre- and post-treatment values were tested both using paired t-test and exact Wilcoxon signed-rank matched-pairs test. For the same reasons, confidence intervals for the differences were estimated with only 90% coverage and based on bootstrap (with 1000 resamples). Similarly, no adjustment for multiple testing was performed, so all the differences were taken as statistically significant if $p<0.05$. Statistical analyses were performed using SPSS for Windows 15.0.1.1 software (SPSS Inc., Chicago, IL, 2007).

Results

The tested immunological parameters were within normal limits already before the beginning of the rehabilitation program. After the program, there were no statistically significant changes in the percent and absolute number of any of the investigated cell populations, but there were changes in distribution and characteristics of the investigated white blood cells. These changes are summarized in Table 1. Below, the results are presented in more detail.

Number of CD3+, CD4+, CD8+ and CD19+ cells was not statistically significantly changed after the rehabilitation program (data not reported in Table 1). There was a statistically significant decrease in the ratio of CD4 to CD8 cells from 3.19 to 2.39 on average ($p=0.020$ from t-test).

TABLE 1
CHANGES IN IMMUNE RESPONSIVENESS BEFORE AND AFTER FOUR-WEEK COMPREHENSIVE REHABILITATION IN SEVEN SUBJECTS WITH UNILATERAL LOWER LIMB AMPUTATION

	Before rehabilitation		After rehabilitation		p		90% BCI for mean difference	
	$\overline{X}$ (SD)	Median	$\overline{X}$ (SD)	Median	t-test	EWMPT	Lower limit	Upper limit
CD4:CD8	3.19 (2.09)	2.20	2.39 (1.64)	1.86	0.020	0.016	0.43	1.20
CD3/69	3.12 (1.37)	3.73	5.25 (1.11)	5.12	0.033	0.063	–3.25	–0.92
CD4/69	3.06 (1.57)	2.92	4.87 (2.68)	3.34	0.017	0.016	–2.66	–0.99
CD8/69	4.70 (1.76)	5.21	8.47 (3.16)	8.54	0.030	0.031	–5.92	–1.85
ncIL-4/CD4	0.06 (0.03)	0.06	0.10 (0.05)	0.10	0.038	0.063	–0.07	–0.02
EC Neutro	81.8 (20.3)	93.6	96.6 (3.3)	97.5	0.080	0.125	–33.20	–7.08
EC Mono	66.61 (32.34)	86.22	87.70 (7.28)	84.32	0.049	0.063	–48.30	–14.00
CD69 Neutro	6.37 (3.24)	5.27	10.51 (5.75)	7.80	0.171	0.078	–9.17	–0.91
CD69 Mono	4.13 (2.50)	3.49	13.42 (7.28)	11.28	0.005	0.016	12.85	–6.35

CD4:CD8 – CD4/CD8 index; CD3/69 – percentage of CD3+ lymphocytes expressing simultaneously CD69 molecule; CD4/69 – percentage of CD4+ lymphocytes expressing simultaneously CD69 molecule; CD8/69 – percentage of CD8+ lymphocytes expressing simultaneously CD69 molecule; ncIL-4/CD4 – number of CD4+ cells synthesising IL-4; EC Neutro – percentage of neutrophils executing oxidative bursts; EC Mono – percentage of monocytes executing oxidative bursts; CD69 Neutro – percentage of neutrophils expressing CD69 molecule; CD69 Mono – percentage of monocytes expressing CD69 molecule; EWMPT – exact Wilcoxon matched-pairs signed-rank test; BCI – bootstrap-based confidence interval for mean before rehabilitation minus mean after rehabilitation

Before beginning of the rehabilitation, 3.12% (SD 1.37%) of CD3+ cells (1.1×10^9 lymphocytes/L) expressed CD69 antigen on average, compared to 5.25% (SD 1.11%) of CD3+ cells that were positive after the rehabilitation program. The change was statistically significant (p=0.033 from *t*-test). From 0.71×10^9 lymphocytes/L expressing CD4 molecule, 3.06% (SD 1.57%) were activated (CD69+) at the beginning of the study on average, compared to 4.87% (SD 2.68%) at the end of study. This change was also statistically significant (p=0.017 from t-test). CD8+ cells were also activated: at the beginning of the study, 4.70% (SD 1.76%) of the CD8+ lymphocytes (of which were 0.33×10^9 lymphocytes/L on average) expressed CD69 molecule, while 8.47% (SD 3.16%) did so at the end of study. This change was also statistically significant (p=0.030 from t-test).

The rehabilitation did not affect the expression of the CD25 (IL-2R) molecule (data not reported in Table 1) on CD3+, CD4+ or CD8+ cells.

Expression of intracellular production of IFNγ was not changed in any of the observed population (data not reported in Table 1), but there was a significant change in the production of the IL-4 in the population of the CD4+ cells. At the beginning of the study, on average 0.06 (SD 0.03) $\times10^9$ CD4+ cells/L synthesized IL-4, compared to 0.10 (SD 0.05) $\times10^9$/L after the study (p=0.038 from t-test).

We observed increased oxidative burst in neutrophil granulocytes and monocytes. This activity was statistically significant in monocytes (p=0.049 from t-test), but not quite in neutrophils (p=0.080 from t-test). There was no change in phagocytic activity of both kinds of cells (data not reported in Table 1). Monocytes also expressed increased density of CD69 molecules after rehabilitation (p=0.005 from t-test).

To summarize, the ratio of CD4 to CD8 cells was significantly decreased; an increase in oxidative killing was observed in monocytes and neutrophil granulocytes (especially in monocytes), together with increased expression of CD69 molecules by T cells and monocytes, as well as the expression of IL-4 by T cells.

Discussion

The objective of the study was to examine the possible effect of a comprehensive rehabilitation program on immune response of patients after amputation of a lower limb due to PAD. The results demonstrate that the status of the immune system was within the normal limits already before the start of the rehabilitation program. Nevertheless, after four weeks of comprehensive rehabilitation some noteworthy changes were observed. The first arising question is whether the observed changes may indicate some beneficial systemic effects that may result in better immune protection.

No statistically significant changes were found in the percent and absolute number of any of the investigated cell populations. However, we observed a moderate decrease in the CD4/CD8 index. Decrease in this index can be seen in some severe infections, but in a much more pronounced form. Otherwise, the CD4-to-CD8 ratio decreases also during exercise, reflecting the greater increase in CD8+ lymphocytes than CD4+ lymphocytes[15].

We observed increased expression of CD69 molecules by T cells after the rehabilitation program. The CD69 activation molecule is a phosphorylated 28 to 32-kDa disulfide-linked homodimer that is rapidly induced after lymphocyte activation. CD69 is not present on the surface of peripheral blood resting T cells. Activation of protein kinase C (PKC) by stimulation of the TCR/CD3 or by phorbol esters directly induces CD69 expression by T cells. CD69 induces IL-2 and IFNγ gene expression and initiates T cell

proliferation. CD69, as a marker of an early T cell activation, is usually associated with Th1 cell differentiation and cytotoxic T cell response against pathogens. Activated T cells are important for the cellular immune response, therefore we may expect that stimulation of CD69 expression is beneficial for immune defense.

We also observed increased oxidative burst in monocytes, and monocytes expressed also increased density of CD69 molecules, pointing to the premise that these cells may be activated due to our rehabilitation program. As the increased oxidative burst was achieved in experiments using opsonized E. coli, we may presume that phagocytic immune defense was also improved. The data suggest that after the comprehensive rehabilitation program, our patients have additionally achieved a better non-specific immune protection.

On the other hand, we also found that the treatments increased the proportion of IL-4 positive T cells. IL-4 is released by Th cells of the Th2 subtype and affects a range of cells, including lymphocytes, monocytes, and polymorphonuclear cells. IL-4 induces naive Th cells to differentiate into Th2 cells and is particularly active on resting and active B cells[16]. On resting B cells, and macrophages, IL-4 increases major histocompatibility complex (MHC) class II expression. On activated B cells, proliferation and differentiation is stimulated and an antibody class switch is induced. IL-4 appears necessary for the early production of IL-12; lack of IL-4 production causes altered resistance to infection, defective antibody response and defective cytotoxic T lymphocyte (CTL) generation. Secretion of IL-12 by antigen presenting cells requires exposure to IL-4[17].

Therefore, answering the first arising question, i.e., whether observed immunological changes after rehabilitation have produced some beneficial systemic effects in our patients, we may conclude that several positive aspects of immune response have indeed been found, except for a moderate decrease of the CD4/CD8 ratio. The improved parameters include better immune cell activation, cytokine secretion, phagocytosis and intracellular killing.

The second question is whether the observed immunological changes may be explained as specific effects of different procedures included into the comprehensive rehabilitation program. We observed an increased T cell activation and Th1 response, which can be attributed to exercise-induced effects, since muscle work per se is a well-known enhancer of Th1 immune response. As an increased CD69 expression is associated with Th1 T cell differentiation and cytotoxic T cell response against pathogens, the observed increased expression of CD69 molecules by T cells may also be associated with increased muscle work.

A number of studies have shown that physical exercise can lead to redistribution and modification of the circulating cells of the immune system[18]. Strenuous exercise decreases the percentage of circulating Th1 lymphocytes and prolonged strenuous exercise is followed by a temporary functional immune impairment[19]. The intensity and duration of exercise may be a critical element in determining whether there is a positive or negative effect on the immune response[20]. Regular moderate exercise training improves specific Th1 reaction also in healthy elderly patients[21]. Moderate exercise can increase immune function and decrease risk of infection[22].

Before being submitted to a comprehensive rehabilitation program, our patients were inactive and did not walk. In order to facilitate their gait by a prosthesis, they had to undertake mobility and strengthening exercises, and above all engage in the training of gait, all of which is associated with muscle activity. It can be said that this therapeutic program included mild to moderate exercise. Regarding physical modalities, they received only mild therapeutic electrical stimulation evoking slight contractions of posterior calf muscles to increase blood circulation in the remaining leg which caused them no distress or other discomfort. It is less believable that the performed electrical stimulation would have any effect on immune response, but we can not exclude it. The electrical stimulation used in our patients was relatively weak regarding amplitude of electrical pulses. Otherwise in clinical rehabilitation practice, electrotherapy can be used to stimulate various tissues and organs, mostly aimed at improving abnormal motor patterns[23]. Hence, it is not known what the immune response would be (i.e., better or worse) using electrical stimulation for other purposes, in case of application at different frequencies and/or higher amplitude. This problem remains open, indicating the need for further research.

A broader issue is whether the natural course would bring improvement of immunological condition in the studied patients. This seems highly unlikely, because they had been in a stationary state for a relatively long period before starting the inpatient rehabilitation. Furthermore, it is known that exercise training improves functional status in patients with PAD[24]. Hence, it must have been the 4-week rehabilitation program that brought about the changes manifested as clinical improvement.

For older persons with amputation due to vascular disease, the time required for medical care and hospitalization becomes more and more significant[25]. One should take into account that our patients were mostly elderly persons in whom the response to certain therapeutic measures can be different than in persons of less advanced age. Besides, our study group included a small number of subjects (as further elaborated below), so we can consider this investigation as a purely exploratory one. Nevertheless, it is important to note that after the rehabilitation program, the physical condition and the clinical status of all our patients improved, and they all met their rehabilitation goals.

The major potential limitation of our study lies in the sample size. The seven persons are a small number, especially with regard to the large number of amputations due to PAD. However, among such patients, who are mainly elderly, there are very few who do not suffer either from chronic accompanying infections or from diabetes mellitus or other diseases that highly affect the immunological status. Therefore, it was difficult to find the patients meeting the inclusion criteria that we had devised in order to be able to reach a clear conclusion.

Conclusions

Our observational study indicates that a comprehensive rehabilitation program performed in patients with lower limb amputation due to PAD produced increased responsiveness of the immune system and natural resistance. The improved immunological parameters include better immune cell activation, cytokine secretion, phagocytosis and intracellular killing. Which treatment within the program contributed (most) to the achieved results remains an open question; the prevailingly favorable immune response may also result from the rehabilitation treatment as a whole.

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IMUNOLOŠKI STATUS KOD BOLESNIKA S AMPUTIRANIM DONJIM UDOVIMA ZBOG PERIFERNE ARTERIJSKE BOLESTI PRIJE I NAKON SVEOBUHVATNE REHABILITACIJE

SAŽETAK

Istražen je imunološki status prije i nakon programa sveobuhvatne rehabilitacije. Sedam osoba (4 muškarca, 3 žene, prosječne starosti 71,4 godine), nakon amputacije donjih udova zbog periferne arterijske bolesti (PAD), bilo je podvrgnuto programu standardne sveobuhvatae rehabilitacije u trajanju od četiri tjedna, što je uključivalo obuku u svakodnevnom životu, svakodnevne vježbe raznih vrsta, obuka hoda potpomognutim štakama i korištenje nogu protezom te blagim transkutanim elektrostimulacijama. Prije i nakon rehabilitacije, uzorkovana je periferna krv i određen je broj i omjer bijelih krvnih stanica te su analizirani na testove ekspresije površinskih antigena (CD3, CD4, CD8, CD19, CD25, CD69), citokine (IFN-gama, IL-4) i fagocitoze. Zbog strogih kriterija odabira bolesnika, koji isključuju teške popratne bolesti, imunološki parametri bili su u granicama normale već prije rehabilitacije. Nakon rehabilitacije, zabilježeno je povećanje oksidativnog praska kod monocita i neutrofilnih granulocita, ali statistički značajno samo u monocita. Ekspresija CD69 molekula T-stanica i monocita značajno je povećana te ekspresija IL-4 pomoću T stanica. Također je uočeno značajno smanjenje omjera CD4 i CD8 stanica, ali nije klinički kritično. Stoga se može zaključiti da je sveobuhvatan tretman rehabilitacije u bolesnika s amputacijom donjih ekstremiteta zbog PAD-a dovelo do nekih – pretežito pozitivnih – imunoloških promjena, koje su u skladu s poboljšanim fizičkim stanjem i kliničkim statusom pacijenata.