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Myelopeptide-5 Abolishes Virus-Induced Immunosuppression by Restoring T Cell Functions

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Myelopeptide-5 (MP-5; synthetic analog of endogenous low-molecular-weight peptide Val-Val-Tyr-Pro-Asp) neutralized the immunosuppressive effects of antiviral vaccines (influenza, measles, and measles/parotitis) and stimulated their immunogenicity by restoring functional activity of T cells, suppressed by the viruses. Specific binding of MP-5 to $CD4^+$ lymphocyte (its target cell) was studied using [^{3}H]MP-5 (dissociation constant 2.03×10^{-7} M).

Key Words: *immunosuppression; myelopeptides; vaccination; immunocorrection*

T cells play an important role in the immune response, specifically, in the development and formation of antiviral immunity. Virus entry into the body triggers the nonspecific defense factors: inflammatory reaction, interferons, antiviral inhibitors, natural killers, macrophages, *etc.* [8,12,13]. Nonspecific defense factors in cooperation with inflammation mediators can destroy virus-infected cells [14]. If this does not happen and the viruses multiply, the specific stage of immunity is unfolded: virus-neutralizing antibodies are produced by B cells and regulatory T lymphocytes (T-helpers, T-suppressors, cytotoxic lymphocytes) and effector T lymphocytes are activated [11]. In the presence of IL-12 and IFN-γ, CD4 lymphocytes differentiate into Th1 cells, start producing and releasing IL-2, IFN-γ, tumor necrosis factor, and determine the cellular type of specific immune response [5,7]. Therefore, normal functioning of T cells is of priority importance for antiviral immune response.

However, the functions of T cells can be disturbed in disease, because many viruses can multiply and destroy immune cells or suppress their functions, which leads to immunosuppression and can promote transformation of acute infection into chronic form. In some cases, viruses are transformed and thus escape the neutralizing effects of specific mechanisms of immune defense. This phenomenon, so-called antigen drift, is well studied for influenza virus [10].

The resistance to viral infections is stimulated by vaccination, use of interferons and their inductors, immunomodulators and chemical drugs. However, antiviral vaccination can be ineffective because of peculiar effects of the viruses on the immune system: the formation of strong specific immunity is accompanied by immunosuppression. The most characteristic manifestation of virus immunosuppression is decreased functional activity of T cells and of IL-2 production [3]. Interferons and their inductors, or drugs with lower specificity compared to vaccines can be used in cases, when the

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vaccine is not available or it is useless because the infection has already taken place [9].

The use of endogenous immunomodulators, which have no side effects attracts special interest as the method preventing virus-induced immunosuppression in virus infection and vaccination. One of them are myelopeptides (MP) isolated from porcine bone marrow culture supernatant, which were characterized and synthesized. They maintain the stability of the immune status due to immunocorrective activity towards certain target cells, binding to them through ligand-receptor interactions [4]. It was shown that MP-5 (synthetic analog of endogenous low-molecular-weight Val-Val-Tyr-Pro-Asp peptide) restored functional activity of T cells, suppressed by tumor cell metabolites or measles virus by stimulating the production of endogenous IL-2 and the expression of IL-2 receptors suppressed under the effects of these factors [1].

We studied the capacity of MP-5 to prevent virus-induced immunosuppression caused by influenza A and B, measles, and measles/parotitis vaccines and evaluated some aspects of MP-5 binding to the target cell.

MATERIALS AND METHODS

Myelopeptide-5 was synthesized by the classical peptide chemistry method at Institute of Experimental Cardiology. The preparation was added to T cell culture in concentrations of 10^{-6} and 10^{-7} g/ml. Bindings of MP-5 to the target cell was evaluated using [^{3}H]MP-5. The conditions of label introduction in MP-5 were determined using a tritium-protium mixture (1:1) as described previously [6]. In order to obtain [^{3}H]MP-5, Val-Val-Tyr-(3,4-dihydro)Pro-Asp was reduced in the presence of palladium catalysts with dimethylformamide as the solvent. The reaction mixtures were analyzed and purified by HPLC: A) 5% methanol-phosphate buffer (pH 2.8); B) methanol (gradient 0→50% B for 15 min) in a C_{18} AQ column (4.6×150 mm), retention time 11.1 min for Val-Val-Tyr-(3,4-dihydro) Pro-Asp and 11.7 min for [^{3}H]MP-5 (Val-Val-Tyr-[3,4-^{3}H]Pro-Asp). The yield of [^{3}H]MP-5 reached 70-75%, molar radioactivity >20 Ci/mmol, and radiochemical purity 96-98%.

The following vaccines were used in experiments on virus-induced immunosuppression: influenza allantoic intranasal live dry vaccine (LIV), live measles v(LMV), parotitis vaccine (LPV), and divaccine containing measles and parotitis components (LMV+LPV; Microgen).

In order to evaluate the proliferation level, T cell were isolated from donor peripheral blood by centrifugation in Ficoll-urograffin density gradient (1.077 g/cm^3). CD4$^+$ lymphocytes were extracted from the common fraction by negative immunomagnetic separation. The cells were stimulated with phytohemagglutinin (PHA) in the suboptimal dose of 2.5 μg/ml and incubated in complete RPMI-1640 in a 96-well plate (2×10^5 cells/well). Virus-containing vaccine and MP-5 were added into some wells simultaneously with PHA. After 68 h, [^{3}H]thymidine was added to the wells (50 μl; 1 μCi/well), the plates were incubated for 4 h, and frozen. After defrosting the cells were transferred onto a cellulose filter with a harvester. Radioactivity of each sample was evaluated on an LKB scintillation counter (Sweden).

In vivo experiments were carried out on albino rats (150 g) and guinea pigs (200 g). The animals were intramuscularly immunized in doses used for vaccination in humans: at least 1000 TCD_{50} (cytotoxic dose) of measles virus and at least 20,000 TCD_{50} of parotitis virus per animal. Titers of anti-measles antibodies in guinea pig blood were evaluated *in vitro* by neutralization of the cytopathogenic effect of vaccine viruses on days 14, 28, and 56 after vaccination. Serum concentration of antibodies to parotitis virus in rats was evaluated by neutralization test and by EIA using measles screen test system with antibody conjugates to guinea pig IgG. The results of vaccination were compared by calculating the geometric mean of reciprocal antibody titers ($\log_2$ 1/titer).

The level of IL-2 receptor expression (CD25) on T cells was evaluated using isotype antibodies: mouse IgG2a (Immunotech & Coulter Com.). Fluorescence was measured on a flow cytofluorometer (Beckman Coulter XL).

Complete saturation isotherms were analyzed by nonlinear regression using PRISM 4.0 software (Graphpad Software Inc.). The results were processed using Statgraphics software.

RESULTS

Anti-influenza vaccine (LIV) contains influenza A (H1N1, H3N2) and B viruses, isolated from virus-containing chicken embryo allantoic fluid. The protective effect of LIV usually starts 3 weeks after vaccination, but it is paralleled by reduction of T cell functional activity (immunodepression). The effect of MP-5 on influenza virus-induced suppression was evaluated by changes in the proliferation of PHA-stimulated donor T cells (Table 1). Lyophilized LIV, diluted 32-, 48-, and 64-fold, was used in the experiments.

The LIV doses used in the study suppressed mitogen-induced proliferation of T cells to 20-56%

TABLE 1. Effect of MP-5 on LIV Immunosuppressive Effect (*M*±*m*)

Group		PHA+LIV		PHA+LIV+MP-5, 10^{-6} g/ml	
		cpm	%	cpm	%
Control		4 895±124			
PHA		29 860±912	100		
LIV	1:32	5863±412	20±7	15 441±1390	52±9
	1:48	14 224±998	48±7	25 132±2770	84±11
	1:64	16 576±830	56±5	28 569±1718	96±6

(Table 1). MP-5 significantly elevated (to 52%) the proliferative response of cells reduced to 20% by influenza viruses and virtually normalized it (96%) in cases when it was reduced to 56% (Table 1).

The expression of IL-2 receptor (CD25) on PHA-stimulated human peripheral blood T cells preincubated with LIV (1:64) and MP-5 for 48 h was studied by flow cytofluorometry (Table 2).

The results showed that in the presence of MP-5 the content of $CD25^+$ cells increased to the normal level after suppression induced by LIV, this indicating recovery of IL-2 receptor expression suppressed by the viruses. Hence, MP-5 prevented the immunosuppressive effect of LIV and stimulated the immunogenic activity of this vaccine.

Previous *in vitro* experiments showed that MP-5 restored functional activity of T cells, suppressed by measles virus by stimulating the reduced production of endogenous IL-2 and the expression of IL-2 receptor [1]. Now we studied the antisuppressive effect of MP-5 *in vivo*. Guinea pigs were intramuscularly injected with LMV (1000 TCD_{50}). In parallel with this, experimental animals were injected with MP-5 (0.01 mg/kg). Antimeasles antibodies were titered in the neutralization test on days 14, 28, and 56 after vaccination (Table 3).

On day 14 of the experiment, antibody level after parallel injections of LMV and MP-5 was significantly higher than in the control (Table 3). It is obvious that MP-5 "protected" the animals from the suppressive effect of measles virus at the initial stage after vaccination by directing the immune response to antibody production. Later, when the immune system of animals could spontaneously control the virus-induced immunosuppression, the difference in antibody production in experimental and control animals was leveled.

TABLE 2. Effect of MP-5 on IL-2 Receptor Expression

Mixtures	$CD25^+$ cells, %
T cell control	4.2
PHA	34.6
PHA+LIV (1:64)	20.2
PHA+LMV+MP-5, 10^{-6} g/ml	33.4
PHA+LMV+MP-5, 10^{-7} g/ml	35.2

Divaccine containing measles (LMV) and parotitis (LPV) components is widely used in modern clinical practice [2]. The immunomodulating effect of MP-5 on the production of antibodies to parotitis virus was studied on albino rats immunized with monoLPV or LMV+LPV divaccine. The results of immunization were evaluated *in vitro* by EIA and neutralization test.

It was shown that the divaccine LMV component caused immunosuppression modulating the production of antibodies to not only measles, but also parotitis virus and reducing their level by 21-26% compared to vaccination with LPV alone. According to neutralization test and EIA, MP-5 virtually did not modify the production of antibodies to parotitis virus after immunization with LPV monovaccine. However, in vaccination with the LMV+LPV divaccine, when the production of anti-parotitis antibodies was reduced because of suppression induced by the measles component, MP-5 restored it to the normal level. Hence, MP-5 exhibited an immunocorrective effect and stimulated antibody production to antigens of both viruses (measles and parotitis).

In previous studies, binding of human peripheral blood T cell $CD4^+$ subpopulation to MP-5-FITC was evaluated and the dissociation constant was calculated ($Kd=2.28\times10^{-7}$ M) [1]. In contrast

TABLE 3. Effect of MP-5 on the Formation of Antimeasles Antibodies in Guinea Pigs According to Neutralization Test (*M*±*m*; *n*=16 per Vaccination Period)

Day after vaccination	LMV (control)	LMV+MP5
14	3.83±0.38	5.28±0.28*
28	5.42±0.64	5.92±0.32
56	6.78±0.48	6.82±0.44

Note. *$p<0.05$ compared to the control.

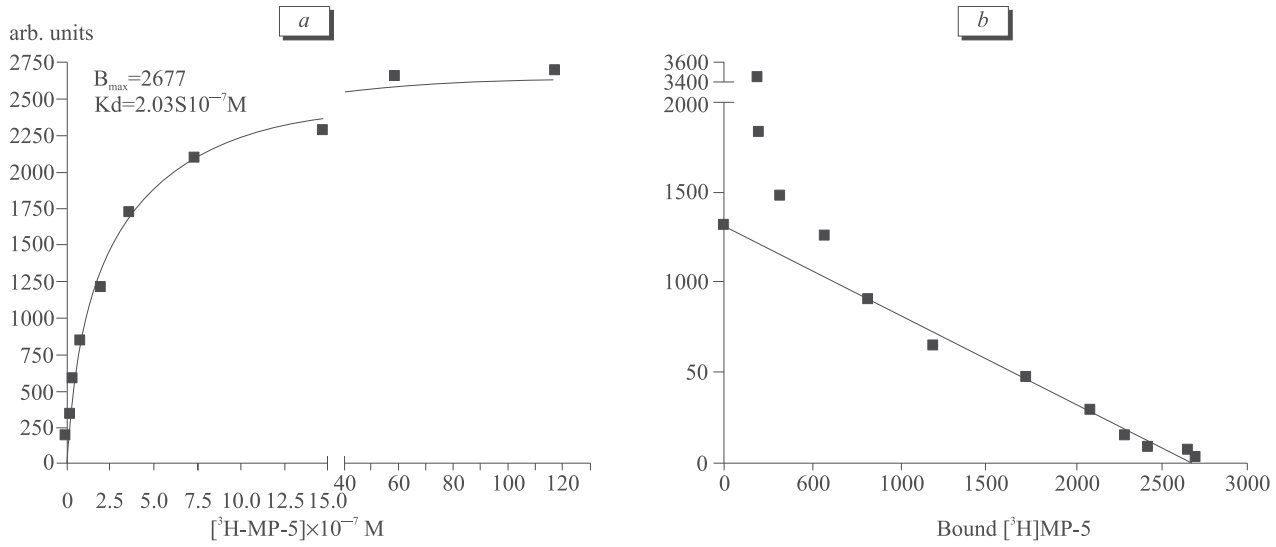

Fig. 1. Specific [^{3}H]MP-5 binding to human peripheral blood CD4$^+$ lymphocytes (*a*) and proportion of bound to free [^{3}H]MP-5 (*b*). *b*: ordinate: proportion of bound to free [^{3}H]MP-5.

to the fluorescent label, which can modulate spatial structure of the peptide molecule, radioactive label has no effect of this kind, and [^{3}H]MP-5 is in fact quite identical to MP-5. In this study human peripheral blood T cell CD4$^+$ subpopulation was incubated with different concentrations of [^{3}H]MP-5 for 45 min. After centrifugation at 500*g* for 7 min, the supernatant was carefully removed and a similar volume of buffer was added. The cells were transferred onto a Whatman cellulose filter for 24 h and their radioactivity was measured on a liquid scintillation counter. Complete saturation isotherm for [^{3}H]MP-5 with CD4$^+$ lymphocytes (Kd=2.03×10^{-7} M; Fig. 1) is presented.

The FITC- and [^{3}H]MP-5 saturation curves proved to be similar, the Kd values calculated from these curves were close (2.28×10^{-7} M and 2.03×10^{-7} M), which confirms the existence of a mechanism of specific binding of MP-5 to CD4$^+$ lymphocytes.

Hence, MP-5 specifically binds to CD4$^+$ lymphocytes, restores functional activity of human peripheral blood T cells suppressed by various factors, prevents virus-induced immunosuppression, thus increasing the immunogenic activity of antiviral vaccines without adverse effects on the organism.

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