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THE STUDY ON THERAPEUTIC EFFICACY OF INOSINE PRANOBEX IN CHILDREN

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Many studies *in vitro* and *in vivo* have shown immunomodulating and antiviral activities of inosine pranobex. The objective of this research was to examine the potential beneficial effects of inosine pranobex (Groprinosin) on the immune system in children with cellular immunodeficiency as a prophylaxis of recurrent infections, mainly of viral origin. Inosine pranobex (50 mg/kg b.w./day) in divided doses was given to the group of 30 children aged 3–15 years for 10 days in 3 following months. Clinical and immunological investigations were carried out before and after the treatment. Statistically significant increase in CD3T lymphocytes number ($p=0.02$) and in this CD4T lymphocytes number ($p=0.02$) as well as statistically significant improvement of their function ($p=0.005$) evaluated with the blastic transformation method were found. These laboratory findings were parallel to clinical benefits.

INTRODUCTION

The use of non-specific immunostimulants occupies an important place in the current development of immunotherapy. Non-specific immunostimulation has progressed from traditional herbal medicines which have many active substances – through microbially derived substances, endogenous compounds as cytokines, and thymic extracs – to chemically-defined drugs with selective effects on different components of the immune system [Siwicki & Skopińska-Różewska, 2003].

Thymic extracts display immunopharmacologic activity which does not induce new thymus-dependent T lymphocytes but augments the functions of existing T cells. The last two decades have been rich in immunotherapy with the entry of recombinant (r) cytokines into clinical use. With international licensing approval of rIFN-α, r-IFN-γ, rIL-2, and colony-stimulating factors (granulocyte and granulocyte-macrophage rCSFs), there has been an increase in clinical immunotherapy to treat neoplastic and infectious diseases [Hadden, 1993]. Following a long history of experimental use in many different diseases, levamisole became the first chemically-defined, orally-active immunostimulant to be licensed (USA) for clinical use.

Inosine pranobex, the next one, is an inosine-salt complex licensed in 63 countries as an antiviral, immunomodulating drug. Inosine pranobex is a synthetic compound formed from the p-acetamido benzoate salt of N-N dimethylamino-2-propanol and inosine at a 3:1 molar ratio. It has a thymomimetic immunopharmacology that supports a pro-host action to increase cellular immune defences. When applied *in vitro*, it induces T-cell differentiation and promotes functions such as proliferation, cytotoxicity and interleukin production and action. It also promotes macrophage and granulocyte function. *In vivo* these actions translate into enhanced cellular immune responses in infected or cancer-bearing individuals, following burns, or in age-related immunodeficiencies [Campoli-Richards *et al.*, 1986; Milano *et al.*, 1991; Flo *et al.*, 1994; Marton *et al.*, 2003].

Results of many studies indicated beneficial clinical effects of inosine pranobex in several infections and diseases, including mucocutaneous Herpes simplex infections, zoster, subacute sclerosing panencephalitis, influenza, genital warts, and type B viral hepatitis. Still, some studies have been preliminary in nature and deficient in the reporting of their results. One must therefore conclude that while inosine pranobex may prove to be a valuable and innovative therapy for a number of infections and diseases for which no satisfactory therapy exists, further studies in additional numbers of patients are required.

While the inosine pranobex is orally active and relatively nontoxic, its immunorestorative effects are mild to moderate – this is very important and valuable especially for children whose immune system is still developing.

Therefore, the aim of our study was to examine potential beneficial effects of inosine pranobex (Groprinosine) on the immune system and clinical condition of children with mild cellular immunodeficiency and recurrent infections, mainly of viral origin.

MATERIALS AND METHODS

The study was carried out on the group of 40 children aged between 3 and 15 years: 30 children who were direct-

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ed to the Immunology Outpatient Clinic of Mother and Child Institute due to recurrent respiratory inflammations and laboratory confirmed mild cellular immunodeficiency, and 10 healthy children at the same age (waiting for small surgery correction).

Thirty children were qualified according to the inclusion and exclusion criteria.

The criteria for the inclusion:

1. Age of children: 3–15 years, recurrent infections of respiratory tract, difficult to cure - in medical interview.
2. Laboratory confirmed defect of cellular immunity (decreased total number and/or percentage of lymphocytes T, defect of their function, phagocytosis defect).
3. To the exclusion of immunostimulators and corticosteroides in period of 6 months before investigation.
4. To the exclusion of immunotherapy or vaccination in the time of 6 months before the investigation.
5. To the exclusion of antibiotics during last 2 months.

The criteria for the exclusion:

1. To fail in conditions of criteria for the inclusion in the points 1 to 5.
2. Renal insufficiency, gout, urolithiasis, hypersensitivity to the drug, actual infection.

Children qualified for the treatment (based on the criteria of including and excluding) were given Inosine Pranobex (Groprinosin) 50 mg/kg b.w. daily in divided doses for ten days in three following months. After the treatment, all children were observed for 12 months in the Immunology Outpatient Clinic.

The results of Inosine Pranobex (Groprinosin) treatment were estimated using special clinical scale as well as immunological laboratory evaluation. The clinical scale includes such parameters as: the amount of recurrent infections, degree of exacerbation, time and amount of cure with antibacterial drugs during the last 2 years before this treatment in comparison with 12 months after its discontinuation.

Immunological investigations of healthy children were carried out once. Their results were normal and used as additional actual, referential values. The results were statistically analysed using the T-test for paired sample comparison data in Statgraphics Plus Program ver. 2.1.

The following laboratory examinations were performed before and after the treatment:

Lymphocytes T – rosettes test (used as a screening test). Cells were isolated on density gradient and mixed with equal volume with suspension (1%) of sheep red blood cells, centrifuged and incubated overnight in a refrigerator (4°C). After incubation, T rosettes were fixed using paraformaldehyde solution and counted under light microscope. A cell coated by three erythrocytes or more was identified as T lymphocyte. Absolute number (cells/mm^3) of T lymphocytes by counting rosettes, leucocytosis and percentage of lymhocytes in the blood smears was evaluated.

Lymphocytes blast transformation test. Lymphocytes from heparinized blood were cultured in RPMI 1640 medium with gentamycin, autologous serum and PHA in humidity, 37°C and 5% CO_2 atmosphere (CO_2-incubator – ASSAB) for 3 days. Then, the cells were fixed in a mixture of acetic acid with ethanol and blast cells were counted.

Cytometric analysis of peripheral blood lymphocytes subclasses using an EPICS XL flow cytometer by Beckmann Coulter. Monoclonal antibodies were provided by Caltag Laboratories and Uti-Lyse Erythrocyte Lysing Reagent was purchased in DacoCytomation. The evaluation of peripheral blood lymphocytes subclasses using monoclonal antibodies by flow cytometry was measured. Monoclonal antibody (10 μL) was added to a polystyrene tube (CD45/CD14, IgG1/IgG2a, CD3/CD19, CD4/CD8, CD3/CD16+56, CD3/HLA-DR, respectively), then 100 μL of anticoagulated (EDTA) whole blood was added to each sample and mixed. A non-reactive monoclonal antibody of the same isotype, and conjugated with the same fluorochrome, as negative control was used. Following a 30-min incubation, 100 μL of the reagent A (Uti-Lyse Erythrocyte Lysing Reagent) was added to each sample and vortex. After a 15-min incubation at room temperature in the dark, 1 mL of the reagent B (Uti-Lyse Erythrocyte Lysing Reagent) was added and samples were analysed.

Endocytosis of neutrophils (with latex). Full heparinized venous blood was incubated with suspension of polystyrene latex beads for 30 min at a temperature of 37°C. At the end of incubation, the mixture was gently stirred, smears were done and stained by May Grunwald and Giemza. Polymorphonuclear cells capable of endocytosis which engulfed over three particles of latex beads were counted.

NBT test. Spontaneous nitro blue tetrazolium test was performed. Heparinized venous blood was incubated in equal volume with NBT solution in 37°C. The incubation spanned for 15 min. After gently stirring, smears were obtained and the percentage of NBT positive cells (polymorphonulcear with formazan particles) was counted.

Chemiluminescence of neutrophils. The activity of neutrophils (oxidative metabolism) was evaluated using the chemiluminescence test. The intensity of the chemiluminescence is proportional to the amount of reactive oxygen forms in the sample. A substance amplifying cellular chemiluminescence (bioluminescence) was luminol. Heparinized peripheral blood was diluted with Hanks solution in the ratio of 1:4 and emission of impulses (cpm) was measured in a Beckman LS scintilation counter at Single Photon Monitor Programme. Oxidative metabolism was stimulated by fMLP. After putting the sample into scintilation counter and softening the spontaneous biolumnescence (background bioluminescence), the chemiluminescence stimulator- fMLP was added. After reaching the pick of free radicals production – the maximal number of impulses (cpm_{max}) – cells activity decreased.

The results obtained were expressed as a stimulation index counted according to the following formula:

$$IS = (cpm_{max} - cpm_{bkd}) / cpm_{bkd}$$

where: cpm_{max} – the number of impulses (counts per minute – cpm) during chemiluminescence peak, and cpm_{bkd} – the number of impulses of spontaneous chemiluminescence (background).

IgG, IgA, IgM concentration in serum. The quantitative immunoglobulines' concentrations were determined in the serum samples using Dade Behring Turbitimer. The required amount of reagent was 500 μL for each determination and 20 μL (IgG), 50 μL (IgA), 200 μL (IgM) diluted with isotonic saline samples (1:21 → 50 μL serum + 1000 μL saline). Proteins present in the human serum sample formed immune complexes with the specific antibodies of the reagent (antiserum). The turbidity generated in the reaction was measured photometrically. The results were estimated by comparing the obtained values with the reference values determined at Dade Behring. The outcomes are given in IU/mL – international units.

RESULTS AND DISCUSSION

The results of inosine pranobex (Groprinosin) influence on different parameters of children immune system in our study appeared to vary.

The best stimulatory effect, with the statistically highly significant difference, was shown for the number of T lymphocytes and for the results of lymphocyte blast transformation test (LBTT) with PHA (p=0.0001; p=0.005, respectively), (Table 1).

TABLE 1. Comparison of the selected parameters of cellular immunity in children before and after the administration of Inosine Pranobex.

Number of patients n=30		Before treatment		After treatment
Lymphocytes T - rosettes test (cells/μL)	x	1784		2791
	p		0.0001	
Lymphocyte blast transformation test with PHA (% of cells)	x	51.5		77
	p		0.005	
Endocytosis of neutrophils (% of cells)	x	99.0		99.0
	p		0.33	
NBT test (% of cells)	x	1.8		1.2
	p		0.14	
Chemiluminescence of neutrophils (index of stimulation)	x	4.2		3.4
	p		0.88	

Similar results were obtained in flow cytometric analysis (Table 2). The increase in CD3T lymphocytes as well as CD4 helper T lymphocytes number was statistically significant (p=0.02).

The values of other cellular parameters as: CD8 cytotoxic T lymphocytes, CD19 B lymphocytes, CD16 CD56 NK cells, CD3/HLA-DR active T lymphocytes, showed tendency to increase but without statistical significance.

No significant changes were obtained in the values of endocytosis, NBT test, chemiluminescence of neutrophils or in serum concentrations of IgG, IgA and IgM (Tables 1 and 3).

This indicates that the main immunostimulative action of inosine pranobex is connected with the number and function of T lymphocytes. It was also confirmed in studies of *e.g.* Bekesi *et al.* [1987], Wójcik *et al.* [2004], although this last study and others reported also on the stimulation of phagocytosis.

This improvement of cellular immunity induced by inosine pranobex is very important for the prophylaxis of infections, mainly of viral origin, which are so widely spread especially in children.

TABLE 2. Comparison of the selected lymphocyte's markers in children before and after the administration of Inosine Pranobex.

Number of patients n = 20		Before treatment		After treatment
CD3+ **T lymphocytes** (cells/μL)	x	1506		1942
	p		0.02	
CD4+ **helper T lymphocytes** (cells/μL)	x	889		1114
	p		0.02	
CD8+ **cytotoxic T lymphocytes** (cells/μL)	x	547		611
	p		0.27	
CD19+ **B lymphocytes** (cells/μL)	x	453		505
	p		0.22	
CD16+CD56+ **NK cells** (cells/μL)	x	318		338
	p		0.72	
CD3+/HLA-DR **active T lymphocytes** (cells/μL)	x	90		101
	p		0.48	
ratio CD4/CD8	x	1.7		1.9
	p		0.15	

TABLE 3. Changes of serum immunoglobulin concentration in children before and after the administration of Inosine Pranobex.

Number of patients n=30		Before treatment		After treatment
IgG (IU/mL)	x	94.8		101.3
	p		0.23	
IgA (IU/mL)	x	68.7		68.8
	p		0.49	
IgM (IU/mL)	x	106.2		112.7
	p		0.55	

The condition of the examined children was observed to improve after the administration of Groprinosin. We noticed substantial decreases in all main parameters describing the length, heaviness and scores of the illness, namely:

- the amount of recurrent respiratory infections per year decreased by 81.2% (equivalent to 5.31 times less infections than before treatment by Groprinosin);
- an verage degree of exacerbation decreased by 60.3% (2.52 times less);
- the number of antibiotic treatments per year decreased by 93.5% (15.3 times less);
- the number of treatment by other non-antibiotic drug decreased by 78.3% (4.51 times less);
- time length of the illness was decreased by 88.2% (8.44 times less);
- and finally overall score index of clinical symptoms was decreased by 72.5% (3.64 times less).

In medical world literature there are many publications of studies which confirm positive results of inosine pranobex administered in various viral infections, mainly herpes virus infections in humans and animals [Galli *et al.*, 1982; Vainiene *et al.*, 1983; Ochocka *et al.*, 1984;

Tanphaichitra & Srimuang, 1987; Janeczko *et al.*, 1991; Mulcahy *et al.*, 1991; Bijlenga, 2001; Femiano *et al.*, 2001]. This data was partly summarized by O'Neill and Glasky [1987] and in the Clinical, Pharmacological and Toxicological Expert Report on Groprinosin [Siwińska-Gołębiowska & Piekarczyk, 2004].

It is evident that cellular immune dysfunction is common in children, viral infections, aging, cancer, malnutrition. The clinical use of inosine pranobex is the most appropriate as an adjunct to antimicrobial or other drugs, particularly under conditions where T lymphocytes are present but dysfunctional due to pathogen- or tumor--derived immunosuppressive influences.

This was also confirmed by the results of our study presented above.

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