

IMPACT OF DIFFERENT CONCENTRATIONS OF HUMAN RECOMBINANT GROWTH HORMONE ON T LYMPHOCYTES

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The aim of the present study is to evaluate the effects induced by increasing concentrations of human recombinant growth hormone on T lymphocytes. Ten healthy volunteers and twelve subjects with symptomatic allergies were enrolled in the study. Peripheral blood mononuclear cells and purified T lymphocytes were cultured in the presence of graded concentrations of growth hormone. Following appropriate *in vitro* stimulations, the proportion of apoptotic T cells, the percentage of activated T lymphocyte subpopulations, the phytohemagglutinin responsiveness and the Th2 response were assessed by flow cytometry analysis. Moreover, in order to evaluate the phosphoinositol-3-kinase signaling pathway involvement, cells were also analyzed after treatment with LY294002. The treatment with different concentrations of growth hormone did not influence the activation pattern of un-stimulated T lymphocytes. On the contrary, growth hormone was able to modify the CD38/HLA-DR co-expression of T cells activated with phytohemagglutinin. A different response was observed when samples obtained from healthy donors and from subjects with symptomatic allergies were analysed. Moreover, growth hormone treatment was able to increase the Th2 response in the samples obtained from healthy donors only. The results of the present study strongly support the hypothesis that growth hormone administration may play an important role in conditions of impaired/activated immune systems. The observation that growth hormone administration at high doses may reverse its effects and that it may promote a Th2-oriented response have significant clinical implications when considering the use of this hormone for artificially enhancing the physical performances of healthy athletes.

Studies carried out almost 80 years ago suggested that growth hormone (GH) may regulate an array of physiological processes, including haematopoiesis and the immune response (1). Further evidence for the existence of a functional link between the endocrine and the immune systems were subsequently obtained from studies demonstrating that both lymphocyte development and function were impaired in Snell

dwarf mice, an animal model displaying both a congenital hypopituitarism and severe cell-mediated as well as humoral immunodeficiency (2, 3). More recently, growing evidence has indicated the existence of a bidirectional relationship between the neuro-endocrine system and immune function. The presence of cell surface receptors for GH on different lymphocyte subpopulations (4) suggested that,

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in addition to the endocrine effects, this hormone may have immunomodulatory properties. Indeed, a relationship between GH and the immune system was demonstrated in several *in vivo* and *in vitro* studies. Conceivably, it was described that GH may improve immune response in pharmacologically immune-depressed mice (5), that GH is able to increase thymic mass and circulating naïve CD4⁺ T cells in adults infected with human immunodeficiency virus type 1 (HIV-1) (6) and that GH has the ability to enhance immune reconstitution after bone marrow transplantation in irradiated or immunodeficient hosts (7, 8). Moreover, it has been demonstrated that the treatment with GH significantly reduces post-irradiation lethality in human peripheral blood lymphocytes through an increased expression of the anti-apoptotic gene *bcl-2* (9). When focusing on T cells, it was described that GH stimulates the proliferation of normal and leukemic T lymphocytes (10, 11), increases the activity of cytotoxic T cells in mixed lymphocyte cultures (12), stimulates Th1-type immune responses (13) and promotes human T-cell adhesion and migration (14). In spite of all of those experimental data, in humans, no obvious immune deficit has been found in GH-deficient patients (15, 16) and it is still not clear whether the described experimental GH effect on T cells should be considered directly as the result of GH binding on T cells or as an indirect effect (17). Taken together, those observations suggest the possibility that GH may serve as an adjunct in immune reconstitution of certain immunodeficient hosts and may also facilitate multilineage reconstitution in individuals with haematopoietic deficiencies but the direct effect of the hormone on normal peripheral blood T cells maybe limited. To date, no studies have dealt with the possible effects of supra-therapeutic doses of recombinant human growth hormone (rhGH) on T lymphocytes, in particular when doping abuse is concerned. In this particular setting, doses of the hormone up to 10 times higher than physiological concentrations are often used with the aim of artificially enhancing the physical performances of young healthy subjects. The aim of the present study is to evaluate the effects induced by different concentrations of rhGH on both peripheral blood mononuclear cells (PBMC) and purified T lymphocytes. The possibility that the hormone may

alter the efficiency of the cellular processes involved in the maintenance of the integrity of functional immunity (e.g. activation, apoptosis and Th2 response) was an integral part of the investigation. Moreover, in order to investigate whether different T lymphocyte functional states may modify the response to rhGH treatment, cells obtained from subjects with symptomatic allergies were analyzed in the same conditions and their behavior was compared to that observed in the samples obtained from healthy subjects.

MATERIALS AND METHODS

Donors

Ten healthy volunteers (4 females and 6 males, age range 20-35 years) and twelve subjects with symptomatic allergy (7 females and 5 males, age range 22-38 years) were enrolled in the study. None of the subjects was receiving medication and no abnormal levels of blood pressure, no abnormal liver or renal function, no history of diabetes nor cardiovascular diseases were found. Moreover, all volunteers were free from endocrine disorders and were not receiving any chronic treatment. The subjects were instructed to abstain from caffeine, alcohol and drug consumption and to refrain from any strenuous physical activity for 24 hours before the examination which consisted of a blood sampling in the morning (08:00 h, after an overnight fast) and in a medical evaluation including familial and personal medical history, a detailed sportive personal history and a complete physical examination. The study was designed in agreement with the Declaration of Helsinki and was approved by the local Ethics Committee. All subjects volunteered for the study and gave their informed consent.

Cell preparation

PBMC were obtained by density gradient centrifugation (Ficoll Hypaque) of heparinized venous blood. Human T lymphocytes were obtained from PBMC as follows: monocytes were extensively removed to prevent clearance of apoptotic cells by phagocytosis. The carbonyl-iron method and the lysosomotropic compound L-leucine methyl ester were used to deplete monocytes, as previously reported (18). T lymphocytes were isolated by resetting with sheep erythrocytes at 29°C for 1 hour to exclude the majority of CD3⁻, CD2⁺ rosette-forming cells. Cells forming rosettes were isolated from non-rosetting cells by density gradient centrifugation. Cells were counted microscopically, and their viability, as determined with the trypan blue exclusion dye test, was always >98%. Both PBMC and T lymphocytes were

cultured at $1 \times 10^6/\text{ml}$ in flat-bottomed microtitre plates at 37°C in a humidified atmosphere of 5% CO_2 in air. The standard culture medium was RPMI 1640 (GIBCO, Milan, Italy) containing 10% heat inactivated FCS (GIBCO, Milan, Italy), penicillin 100U/mL, streptomycin 100 $\mu\text{g}/\text{mL}$ and glutamin 2mmol/L. At the indicated time, cells were counted and their viability determined with the trypan blue exclusion dye test (cell culture conditions resulted positive when the viability was $>80\%$). They were then divided into aliquots for subsequent analysis.

Cell treatment

Cells were treated with graded concentrations (200ng/ml, 400ng/ml, 600ng/ml) of rhGH and then evaluated after 2, 4, 6, 8, 12 and 24 h of incubation. To evaluate spontaneous apoptosis, PBMC and T lymphocytes were incubated at $1 \times 10^6/\text{ml}$ in RPMI + 1% FCS for 24, 48 h in 5% CO_2 . To evaluate triggered apoptosis, PBMC and T cells were incubated in RPMI + 10% FCS at 37°C for 24, 48 h in 5% CO_2 in the presence of 1.5 $\mu\text{L}/\text{mL}$ methylprednisolone (MP). For the evaluation of the phytohemagglutinin (PHA) responsiveness, cells were stimulated with 5 $\mu\text{g}/\text{mL}$ PHA (PHA-M, Sigma, St. Louis, MO) for up to 72 h at 37°C in 5% CO_2 . To investigate the phosphoinositol-3-kinase (PI3K) signaling pathway involvement, cells were incubated at $1 \times 10^6/\text{ml}$ in RPMI + 10% FCS at 37°C for 15 minutes in the presence of 3 $\mu\text{L}/\text{mL}$ of the quercetin derivative LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one). After three washes in phosphate-buffered saline (PBS), cells were incubated at the selected conditions.

Antibodies and reagents

The following antibodies were used: anti-CD3 (FITC-conjugated), anti-CD4 (APC-h7-conjugated), anti-CD8 (v450-conjugated), anti-CD45 (v500-conjugated), anti-CD38 (APC-conjugated), anti- HLA-DR (Per CP- cy 5.5-conjugated), Anti- CD 25 (PE-cy7-conjugated) all from Becton Dickinson (Milan, Italy) and anti-CD 294 (PE-conjugated) from Mylteni, Bologna, Italy.

To determine the proportion of apoptotic cells, Annexin-V binding assay was performed using the Annexin-V kit (BD Pharmingen). Cells were washed and re-suspended in binding buffer prior to incubating with Annexin-V (5 μL) and propidium iodide (5 μL) for 15 minutes at room temperature in the dark. Samples were analyzed within one hour of staining.

Flow cytometry analyses

Flow cytometry analysis of surface antigens was performed on intact cells. After washing with the staining medium (PBS + 2% FCS and 0.01% sodium azide), both PBMC and T lymphocytes were incubated with the appropriate antibodies for 20 minutes in the dark.

After washing with PBS cells were analyzed with a fluorescence-activated cell sorter (FACS; FACSCanto II, Becton Dickinson) or a "FACS Calibur cytometer Becton Dickinson (BD, San Jose, CA, USA).

BD FACS 7-Color Setup Beads Sensitivity, determined with the setup beads, measures the ability to resolve a dimly stained population from unstained cells. This sensitivity measure takes into account both the separation of the populations and the broadness of the negative population. The high quality of BD FACSCanto™ II allowed to exclude several variables influencing the results of the multiple samples analysis.

Statistical analyses

Database management and all statistical analyses were performed using the Statistica 6 for Windows software package (Statsoft Inc., Tulsa, OK, USA). Statistical comparisons were performed using the Student's *t*-test. In consideration of the physiological inter-subject variability, data were normalized by dividing all values by the highest value in their set and the normality was assessed by the Wilk-Shapiro's test. The *chi*-square test and the Friedman's ANOVA were used to determine whether there were differences among the different conditions tested. If a significant difference was present a Dunn's post hoc test was used to locate the difference. Levels of statistical significance were always set to $p < 0.05$.

RESULTS

Susceptibility to apoptosis

Susceptibility to spontaneous apoptosis was investigated in both PBMC and purified T lymphocytes after 24 and 48 hours of incubation in RPMI + 1% FCS at 37°C . Percentages of apoptotic cells were significantly lower in the samples treated with rhGH 200ng/ml when compared to the untreated sample ($p = 0.001$). This anti-apoptotic effect was maintained over a 48 hour-incubation period ($p < 0.001$) (Fig. 1). On the contrary, higher concentrations of rhGH, namely 400 and 600 ng/ml, were not able to reduce the entity of spontaneous apoptosis (Fig. 1). Susceptibility to triggered apoptosis was investigated using MP 1.5 $\mu\text{mol}/\text{L}$ in the presence of increasing concentrations of rhGH. As shown in Fig. 1, different concentrations of rhGH did not reduce MP induced apoptosis.

T cell activation

CD38, HLA/DR and CD25 expression was analyzed in both PBMC and purified T lymphocytes

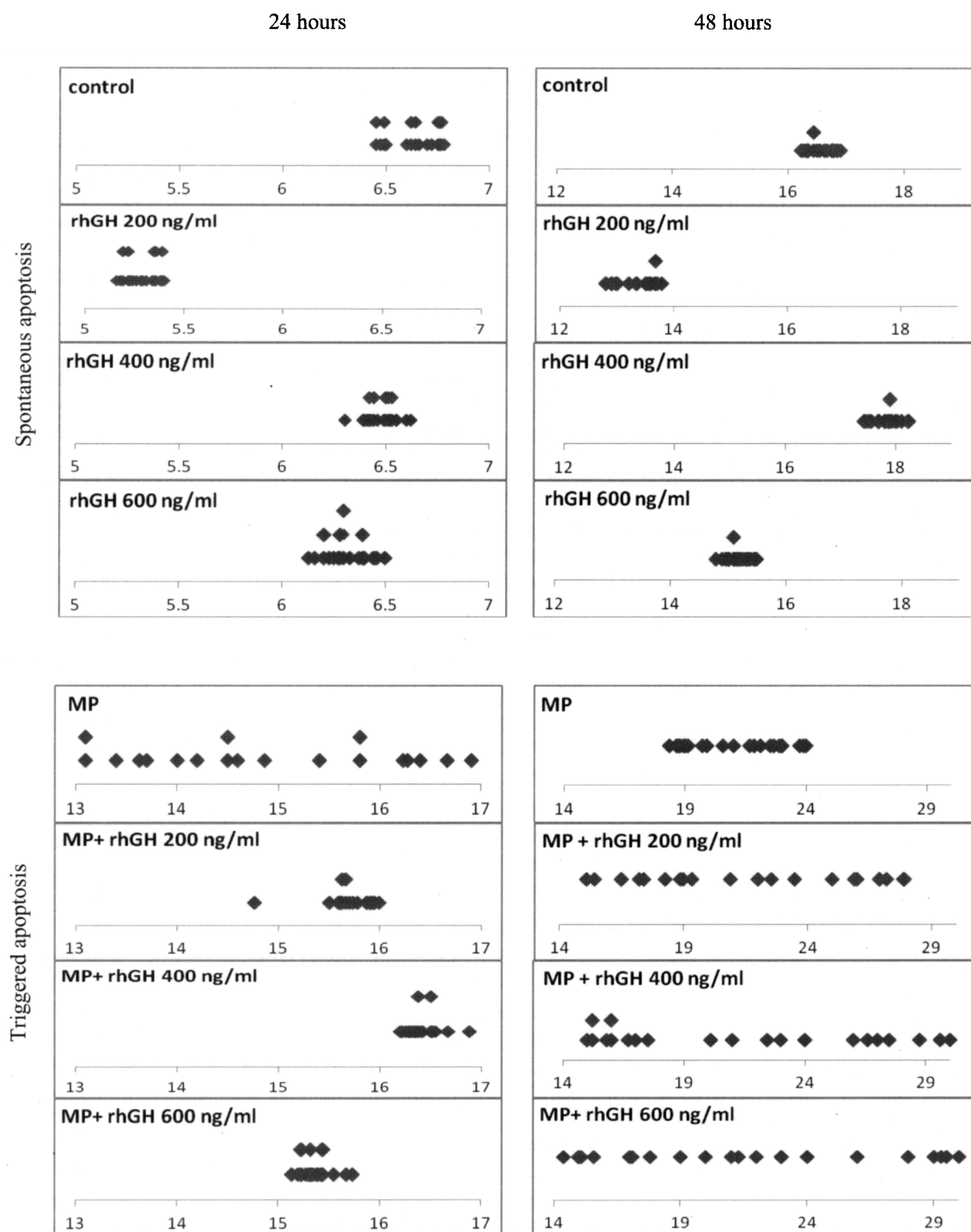
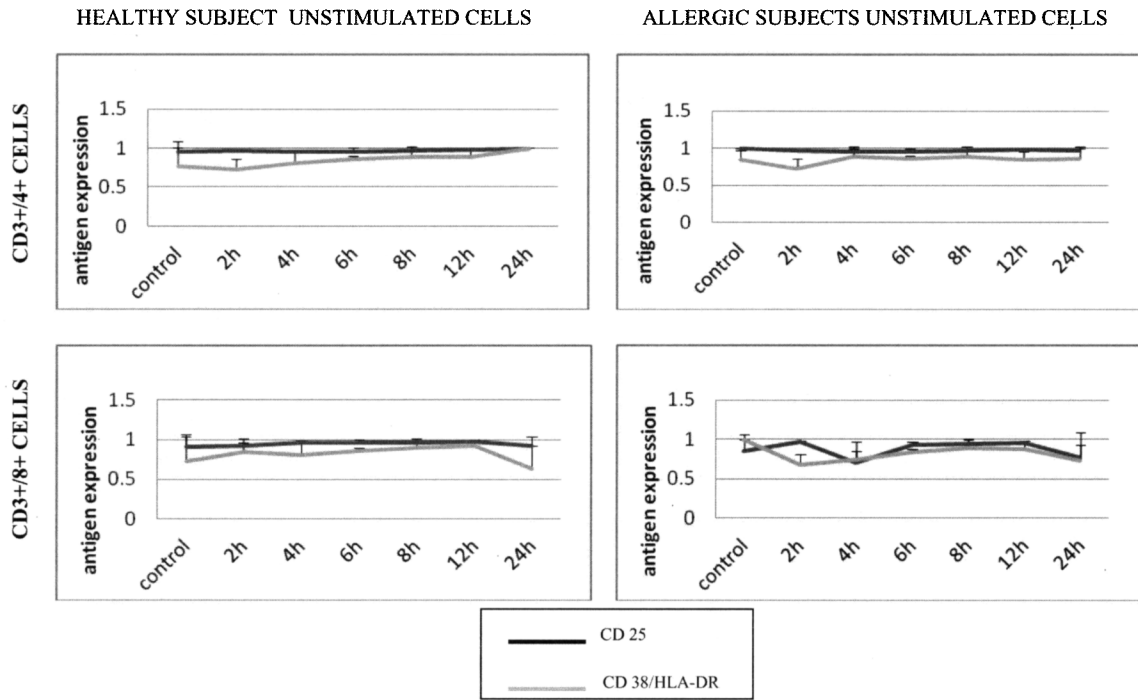


Fig. 1. Dot plot representations of susceptibility to apoptosis. For spontaneous apoptosis cells were incubated for 24 and 48 hours in RPMI + 1% FCS. For triggered apoptosis cells were incubated for 24 and 48 hours in RPMI + 10% FCS in the presence of MP 1.5 $\mu\text{mol/L}$.

GROUP A



GROUP B

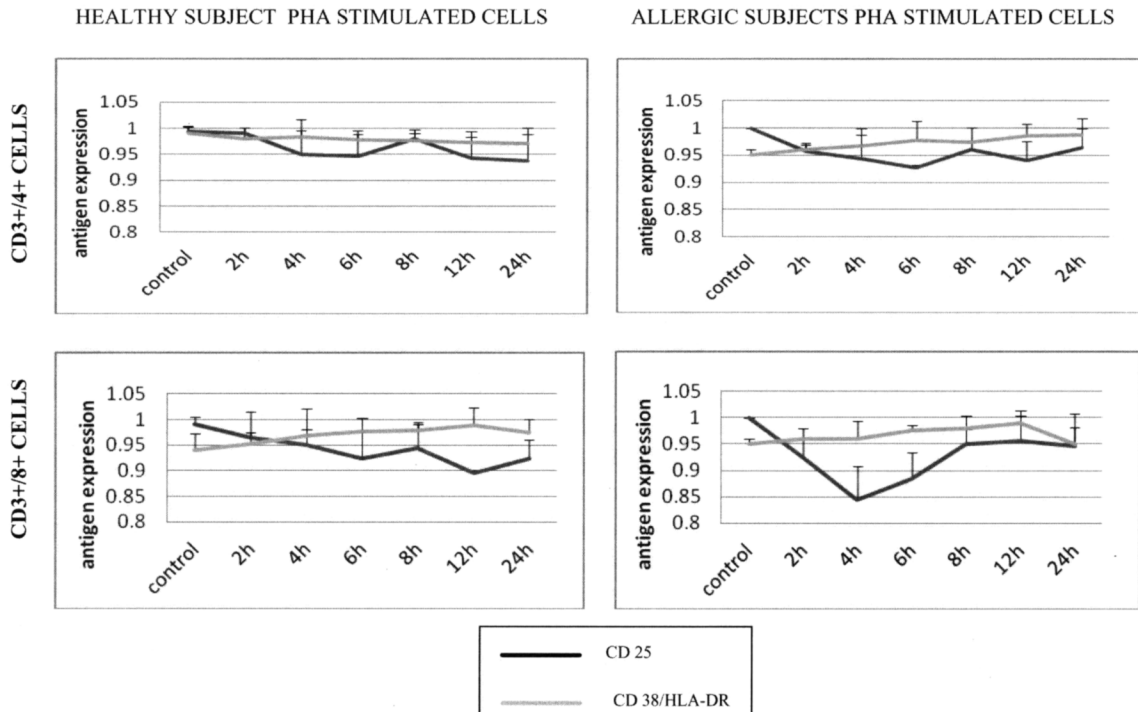


Fig. 2. CD38/HLA-DR co-expression and CD25 expression on CD3+/4+ and CD3+/8+ cells obtained from healthy subjects and from subjects with symptomatic allergies after 2, 4, 6, 8, 12 and 24 hours of incubation in the presence of rhGH 200 ng/ml (group A) and after 2, 4, 6, 8, 12 and 24 hours of incubation in the presence of PHA 5 μ l/ml and rhGH 200 ng/ml (group B).

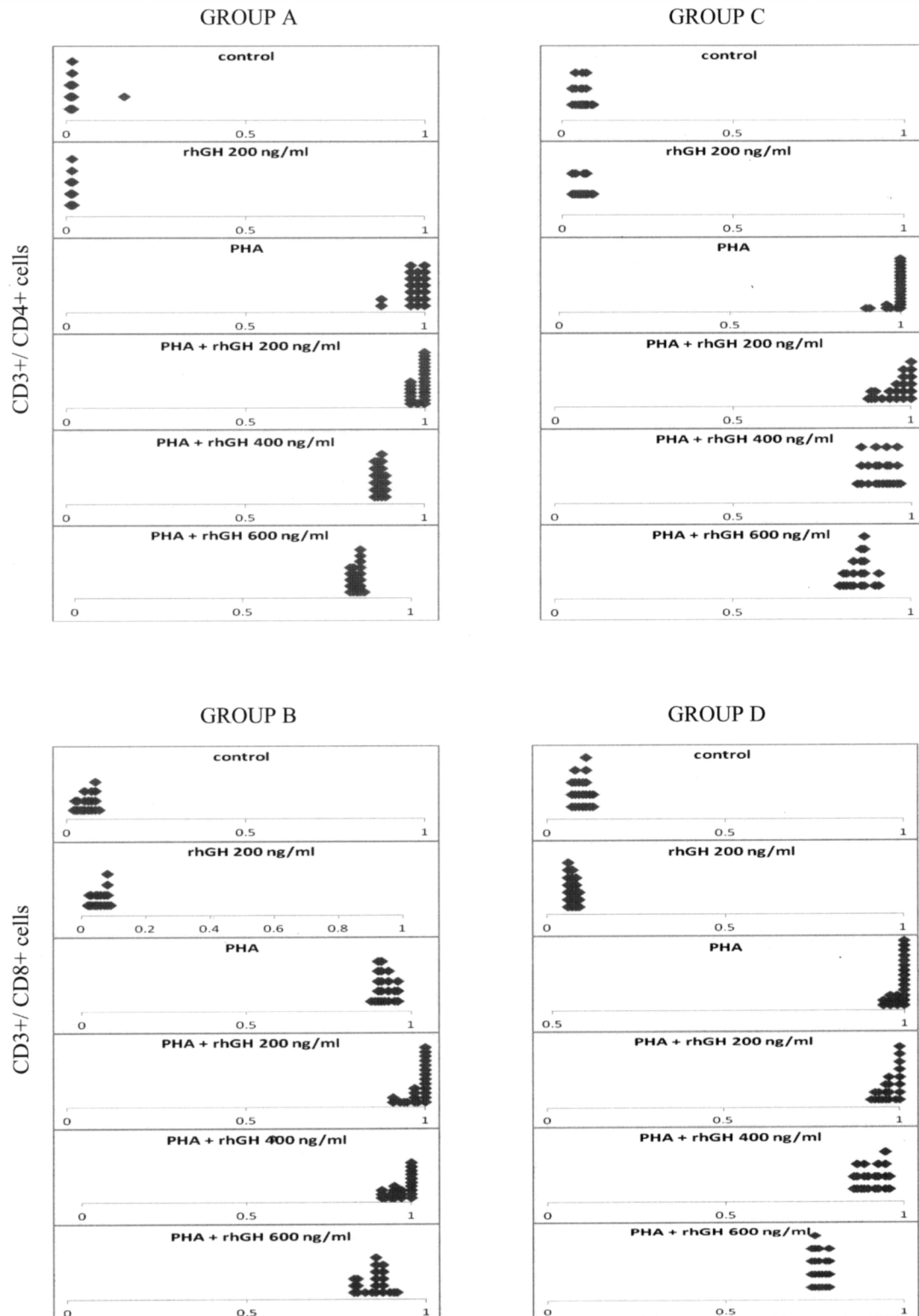


Fig. 3. CD38/HLA-DR co-expression on CD3+/CD4+ (column A) and CD3+/CD8+ (column B) cells obtained from healthy subjects after 24 hours of incubation in the presence of PHA and grading concentrations of rhGH. CD38/HLA-DR co-expression on CD3+/CD4+ (column C) and on CD3+/CD8+ (column D) cells obtained from subjects with symptomatic allergies after 12 hours of incubation in the presence of PHA and grading concentrations of rhGH.

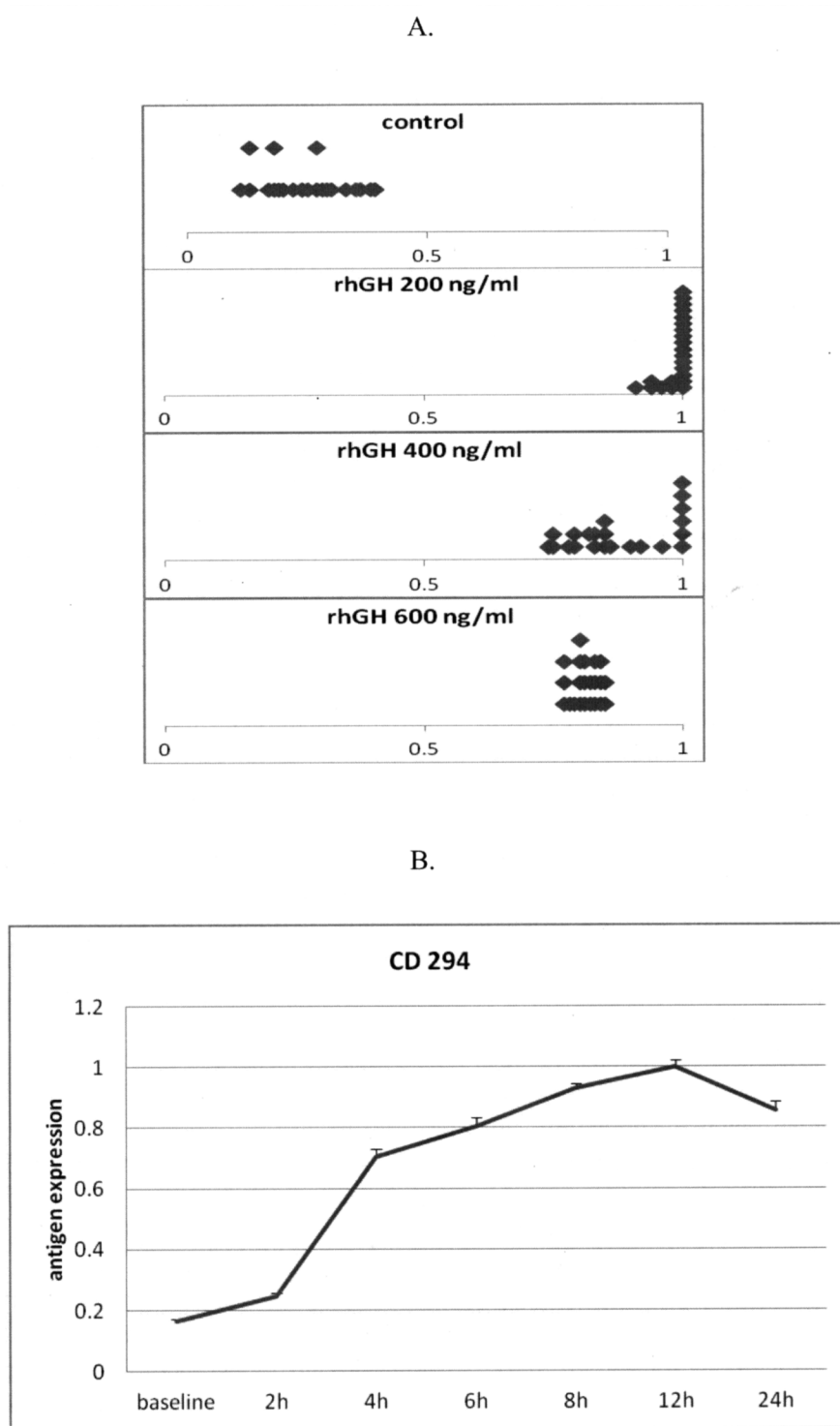


Fig. 4. CD294 expression in healthy subjects after 12 hours of incubation in the presence of grading concentrations of rhGH (A). CD294 expression in healthy subjects after 2, 4, 6, 8, 12 and 24 hours of incubation in the presence of 200 ng/ml of rhGH (B).

after 2, 4, 6, 8, 12 and 24 h of incubation in RPMI + 10% FCS at 37°C in the presence of increasing concentrations of rhGH. As shown in Fig. 2, following incubation with rhGH 200ng/ml no evidence of cell activation was detected on either CD3+/CD8+ or CD3+/CD4+ lymphocyte subpopulations. Similar results were obtained by incubating the cells in the presence of rhGH 400ng/ml and 600ng/ml. Even in the samples obtained from subjects with symptomatic allergies, different concentrations of rhGH were not able to significantly increase the expression of the tested antigens

In order to assess the effect of rhGH in conditions of activation of the immune system, CD38, HLA-DR and CD25 expression was analyzed in both PBMC and purified T lymphocytes after 2, 4, 6, 8, 12 and 24 hours of incubation in RPMI + 10% FCS + PHA 5µl/ml at 37°C in the presence of increasing concentrations of rhGH. CD25 expression was unaffected by rhGH treatment in all the tested conditions (Fig. 2). On the contrary, CD38/HLA-DR co-expression displayed a different behavior when samples obtained from healthy donors and from subjects with symptomatic allergies are concerned. In the former samples, CD3+/CD4+ lymphocyte subpopulation showed a trend indicative for a dose-dependent decrease of the CD38/HLA-DR co-expression after 24 hours of incubation even if data did not reach a statistical significance (Fig. 3). On the contrary, in the CD3+/CD8+ lymphocyte subpopulation, rhGH treatment induced an increase in the CD38/HLA-DR co-expression at lower concentrations, namely 200ng/ml, after 24 hours of incubation ($p=0.038$). At higher concentrations, namely 600ng/ml, the hormone induced a decrease of the CD38/HLA-DR co-expression ($p=0.026$) (Fig. 3). In the samples obtained from subjects with symptomatic allergies, a statistically significant dose-dependent decrease of the CD38/HLA-DR co-expression was evident in the CD3+/CD4+ lymphocyte subpopulation ($p=0.02$ for 200ng/ml, $p=0.02$ for 400ng/ml and $p=0.012$ for 600ng/ml) and in the CD3+/CD8+ lymphocyte subpopulation ($p=0.038$ for 200ng/ml, $p=0.008$ for 400ng/ml and $p=0.012$ for 600 ng/ml) after 12 hours of incubation (Fig. 3).

Th2 subpopulation analysis

In order to assess the impact of rhGH upon Th2

lymphocytes, cells were incubated for 2, 4, 6, 8, 12 and 24 hours in RPMI + 10% FCS at 37°C in the presence of increasing concentrations of rhGH. In the samples obtained from healthy donors, a statistically significant increase of the expression of the surface antigens CD294 was observed after 4 hours of incubation ($p=0.036$). CD294 expression constantly increased till 12 hours of incubation and then started to decline (Fig. 4). This increase in CD294 expression was mostly evident when cells were incubated in the presence of rhGH 200 ng/ml ($p=0.038$). At higher concentrations of rhGH this increase was less evident (Fig. 4). Cells obtained from subjects with symptomatic allergies showed discordant results. In some cases a slight reduction of the CD294 expression was evident following rhGH stimulation but this observation was not confirmed statistically (data not shown).

Role of PI3-K

To evaluate the PI3K signaling pathway involvement, cells were incubated in RPMI + 10% FCS at 37°C for 15 minutes in the presence of 3µl/ml of the quercetin derivative LY294002 before the appropriate *in vitro* stimulation. This treatment did not affect the cell response to rhGH treatments in all of the tested conditions. In particular, LY294002 was not able to inhibit the observed CD294 increased expression and the modification of the CD38/HLA-DR co-expression following rhGH treatments (data not shown).

DISCUSSION

The results of the present study showed that different concentrations of rhGH induced different responses on T lymphocytes. Moreover, the effects induced by rhGH were not uniform when T lymphocytes obtained from healthy donors and those obtained from subjects with symptomatic allergies are concerned. Thus, suggesting that the dose of rhGH as well as the T lymphocyte functional state play a significant role when considering the effect of the hormone on the immune system.

The results of the present study highlighted that the treatment with different concentrations of rhGH did not induce any modification of the CD38, HLA-DR and CD25 antigen expressions on either CD3+/

CD8+ or CD3+/CD4+ lymphocyte subpopulations. The observation that rhGH did not influence the activation pattern of T lymphocytes reinforces the results of previous studies describing that no clinical signs of immune dysfunction have been observed in humans in spite of severe GH deficiency (19). This conclusion should be considered valid only in a situation of non-stimulated T cells. Indeed, in our hands rhGH treatment of T cells activated with PHA induced different modifications of the CD38/HLA-DR co-expression when compared to samples obtained from healthy donors and from subjects with symptomatic allergies. In the former samples, CD3+/CD4+ lymphocyte subpopulation showed a trend indicative for a dose-dependent decrease of the CD38/HLA-DR co-expression while in the CD3+/CD8+ lymphocyte subpopulation, rhGH treatment induced an increase in the CD38/HLA-DR co-expression at lower concentrations and a decrease of these antigen expressions at higher concentrations. In the samples obtained from subjects with symptomatic allergies, a dose-dependent decrease of the CD38/HLA-DR co-expression was evident in both the CD3+/CD4+ and the CD3+/CD8+ lymphocyte subpopulations. These data strongly support the hypothesis that rhGH may exerts its effects on impaired and/or stimulated T cells but is not able to significantly modify an intact immune system. This observation is supported by several data available in literature. Indeed, it has been extensively demonstrated that the administration of rhGH to aged animals and humans improved immune function (20-24), that GH and Insulin-Like Growth factor-1 (IGF-1) administration accelerate the recovery of lymphopoiesis after cyclosporine treatment (25), that rhGH and IGF-1 administration enhances the immune reconstitution after bone marrow transplantation (8) and that, when administered at the time of immunization, rhGH induces a Th1 cytokine response to HIV-1 envelope protein (13). Moreover, it has been demonstrated that rhGH treatment increases lectin-induced and anti-CD3-induced proliferation (26) as well as the activity of cytotoxic T cells in mixed lymphocyte cultures (12).

The evaluation of the rhGH effect on Th2 subpopulation was carried out analyzing the expression of the prostaglandin D2 (PGD₂) receptor DP-2 (CD294) which has emerged as a consistent,

predictive marker of Th2-cell function, IL-4 production, and Th2 bias (27, 28). In the present study, physiologic doses of rhGH induced an increase of the Th2 subpopulation while higher concentrations of the hormone showed inconsistent responses. Surprisingly, the Th2 increase was evident only in the samples obtained from healthy donors. T cells obtained from subjects with symptomatic allergies showed discordant responses with a trend towards a decrease of the CD294 expression. This observation may be partially explained by the fact that in subjects with symptomatic allergies a Th2 response was already stimulated and that rhGH in such a situation is not able to further increase this response. This hypothesis is supported by the demonstration that, in burned mice, where there is a conversion of Th cell populations from Th1 to Th2 cells, the administration of rhGH leads to a Th1-dominant response (29). Nonetheless, an important caveat that needs to be addressed is the potential role of GH in promoting a Th2-oriented response in healthy subjects, thus potentially dysregulating the immune responses with clinically significant effects.

With regard to the anti-apoptotic effect of GH, the results of the present study confirmed previous observations showing that rhGH treatment reduces spontaneous and triggered T-cell apoptosis through up-regulation of the anti-apoptotic protein Bcl-2 (9, 30, 31). The results of the present study confirm that therapeutic concentrations of rhGH exert anti-apoptotic effect on T cells. Surprisingly, in our study, rhGH treatment did not reduce the MP induced apoptosis. Previous studies showed that, at physiologic doses, rhGH rescued cells from glucocorticosteroid-induced apoptosis. The discrepancy in the results may be explained by the fact that in those experiments, the cells were co-stimulated with immobilized anti-CD3 and anti-CD28 monoclonal antibodies (31). This observation reinforces the hypothesis that rhGH may exert its effects on impaired and/or stimulated T cells but is not able to significantly modify an intact immune system.

When considering GH signalling pathway, it has been demonstrated that GH activates mitogen-activated protein kinase (MAPK) through two pathways, one involving Grb2/Shc/Ras/Raf/MEK/

MAPK and the other one involving the PI3K (32). In the present study, in order to evaluate the PI3K signaling pathway involvement, cells were incubated in the presence of the quercetin derivative LY294002, a specific and cell-permeable inhibitor of PI3K. This treatment did not affect the cell response to rhGH treatments in any of the tested conditions, suggesting that PI3K signalling pathway is not involved in the T cells response to rhGH stimulation.

The results of the present study strongly support the hypothesis that rhGH administration may play an important role in conditions of impaired/activated immune systems. The observation that rhGH administration at high concentrations may reverse its effects and that it may promote a Th2-oriented response have significant clinical implications when considering the use of this hormone for artificially enhancing the physical performances of healthy athletes. The results of the present study strongly support the hypothesis that rhGH abuse has the potential risk of dysregulating the immune system, especially when Th2-mediated disorders are concerned, thus potentially dysregulating the immune responses with clinically significant effects.

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