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Modulating Effects of the Synthetic Thymic Dipeptide Pidotimod on the Immune System in the Aging Rat

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Abstract: We have investigated the *in vivo* and *in vitro* effect of pidotimod, a synthetic thymic-derived drug, on the immune response in young (2 month-old), and aging (24 month-old) Sprague-Dawley rats. We studied the effect of different doses of pidotimod on the responsiveness of both cultured peripheral blood lymphocytes and splenocytes to mitogenic stimuli, as well as on interleukin-2 production by peripheral blood lymphocytes after stimulation with interleukin-1 and phytohemagglutinin. Treatment with pidotimod *in vivo* as well as *in vitro* resulted in an increase of tritiated thymidine incorporation in both mitogen-stimulated lymphocytes and splenocytes from 24 month-old rats. Production of IL-2 from lymphocytes of 24 month-old rats was significantly increased in groups of animals treated with pidotimod. On the other hand, treatment with pidotimod did not influence the responsiveness of 2 month-old rat lymphocytes to mitogens, nor affected IL-2 production. Our results suggest a possible specific modulatory activity of pidotimod on the aging immune system.

Aging is associated with changes of the immune system, characterized by decreased response of specific lymphocyte subclones to mitogenic stimuli (Staiano-Coico *et al.* 1984), increased levels of circulating immunocomplexes (Delafuente 1985) and impaired immune-neuroendocrine feedback (Bernardini *et al.* 1992). Such decreased proliferative capability of lymphocytes has been proposed to be partially due to diminished lymphocyte responsiveness to soluble mediators of the immune response (Staiano-Coico *et al.* 1984; Gillis *et al.* 1981). In addition, the ratio mature/immature T-cells decreases with aging (Gupta & Good 1981). In this line, the thymus is functionally deficient in aging (Fabris *et al.* 1989).

3-L-Pyroglutamyl-L-thiazolidine-4-carboxylic acid (pidotimod) is a thymic factor-derived dipeptide drug with low toxicity (>8 g/kg/intraperitoneally), which, consistently with other thymic factors (Ahmed *et al.* 1979), has been shown to possess immunomodulatory properties (Barchielli & Coppi 1990; Coppi & Mailland 1990; Iurato *et al.* 1990).

We have investigated possible immunostimulatory properties of pidotimod in the aging rat. In this light, we studied effects of subchronic treatment with pidotimod on peripheral blood lymphocyte and splenocyte proliferation in aging (24 month-old), compared to young (2 month-old) male Sprague-Dawley rats. Specifically, we have studied the capability of immunocytes of aging animals to respond to mitogens such as concanavalin A and phytohaemagglutinin, after treatment with pidotimod. In addition, we have measured levels of interleukin-2 from *ex vivo* lymphocyte cultures of aging rats treated with pidotimod.

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Materials and Methods

Animals. Two-month-old (150–220 g) or 24 month-old (450–500 g) male Sprague-Dawley rats (Charles River, Calco, Italy) were housed for 3 days in standard animal cages, in a temperature-controlled (24±1°) environment, maintained on a 12 hr light-dark cycle. Standard laborators chow and tap water were available *ad libitum*.

Animals were housed 4 per cage and treated intraperitoneally for a week with pidotimod (100 or 400 mg/kg daily). Control rats received Dulbecco's phosphate buffered saline composed by 2.0 g/l KCl, 2.0 g/l KH_2PO_4, 80.0 g/l NaCl, and 21.60 g/l $Na_2HPO_4 \cdot 7H_2O$ (D-PBS, pH 7.4, Gibco, Grand Island, NY, U.S.A.).

Animal studies were conducted in accord with the highest standards of humane care. At the end of the experiments, all animals were sacrificed and examined at autopsy to exclude the presence of gross pathology and pituitary macroadenomas. In addition, the thymus of animals of different groups of treatment was excised and weighted.

Chemicals. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.), unless otherwise specified.

Human recombinant interleukin-1α was a generous gift from Dr. Peter Lomedico, Hoffmann La Roche (Nutley, NJ, U.S.A.). The protein was purified from *E. coli* and consists of the carboxy-terminal 155 amino acid human interleukin-1α precursor. Activity of interleukin-1 was 2×10^8 units/ml; protein concentration was 0.68 mg/ml (by amino acid analysis) and specific activity was 3×10^8 units/mg; endotoxin was 0.5 EU/ml (LAL). Interleukin-1 was dissolved in 50 mM K-PO_4, 0.1 M NACl buffer (pH 6.5).

Pidotimod was from Poli Industria Chimica (Milano, Italy). Pidotimod has been previously shown to be active at doses ranging from 100 to 700 mg/kg (Barchielli & Coppi 1990; Chiarenza *et al.* 1994).

Lymphocyte cultures. All animals were sacrificed by decapitation. Peripheral trunk blood was collected in sterile haparinized tubes.

Spleens were removed and mechanically dissociated to a single cell suspension. Cell preparations were then stratified onto a Ficoll-Hypaque gradient (Pharmacia, Piscataway, NJ, U.S.A.) and centrifuged 15 min. at 3000 r.p.m. Resulting buffy coats were then washed twice in phosphate buffered saline (Gibco), cells counted and plated in 96-well U-bottom plastic microplates (Costr, Cambridge, MA, U.S.A.), at a density of 2×10^5/100 µl/well in RPMI 1640 medium (Gibco) supplemented with 2 g $NaHCO_3$, 1 ml/1000 ml L-glutamine, 50 µg/ml gentamicin, and 10% foetal calf serum (Gibco). Cell proliferation was measured following the classic mitogenic assay (Reite *et al.* 1981). Concanavalin A or phytohemagglutinin were added at concentrations of 1 µg and 5 µg/ml, respectively. Cells were incubated for 72 hr at 37°, in 5% CO_2-saturated atmosphere. Tritiated thymidine (^{3}H-thymidine, New England Nuclear, Florence, Italy) was added to cultures (1 µCi/well; volume: 20 µl/well) for a 6 hr incubation. Cells were then harvested onto fiberglass filters (Skatron, Norway), which were heath-dried and successively placed in scintillating vials with 3.5 ml of scintillating solution (Instagel, Beckman). Radioactivity (d.p.m.) was counted 2 min. in a β-counter. Each sample was run in triplicate. All cultures were repeated at least twice.

Interleukin-2 production by peripheral blood lymphocytes. After separation, periheral blood lymphocytes were plated at a density of 5×10^5/well/ml in a 48-well plate (volume: 1 ml). Interleukin-1α (10 nM) (Dinarello 1987), or phytohemagglutinin (5 µg/ml) were then added to cultures and incubated for 24 hr. Thereafter, media were separated by centrifugation (1,800 rpm) and frozen at −80° until assayed for interleukin-2. Interleukin-2 was measured by means of a specific radioimmunoassay developed in our laboratory. Briefly, 100 µl of culture media were incubated (4°) with 100 µl of a 1:40,000 specific anti-interleukin-2 serum, that shows no significant cross-reaction for other cytokines in RIA buffer, containing sodium phosphate, sodium EDTA, sodium azide, normal rabbit serum, and aprotinin (pH 7.4). Forty-eight hr later, 100 µl of rat [^{125}I] interleukin-2 (Sigma Immuno Chemicals, Milano, Italy; specific activity: 20–50 Ci/µg) (3,000 cpm) were added, and incubated at 4° for 24 hr. Separation of bound from free was achieved by incubating each tube (16–18 hr at 4°) with 50 µl of goat anti-rabbit serum, diluted 1:5 with assay buffer containing 1% BSA. The tubes were centrifuged at 2,500 rpm for 20 min., the supernatants aspirated, and pellets counted for 2 min. in a τ-counter. All standards were assayed in triplicate. Media samples were assayed in duplicate. The mean of non-specific binding was 15.0 (S.E. 5.0) %. Sensitivity (ED_{90}) of the assay was 6.0 (S.E. 1.5) pg/assay tube (60 pg/ml of sample). Interassay coefficient of variation was 6.94%.

In vitro experiments. Peripheral blood lymphocytes and splenocytes from young and aging rats were prepared and plated as previously described. Cells were then incubated for 72 hr at 37°, in 5% CO_2-saturted atmosphere with graded concentration of pidotimod (ranging from 1 µM to 1 mM) or combination of pidotimod (100 and 400 µM) and concanavalin A (1 µg/ml) or phytohaemagglutinin µg/ml). Peripheral blood lymphocytes and splenocytes proliferation was evaluated by measuring ^{3}H-thymidine incorporation rate as described above.

Statistical analysis. Analysis of all results was performed by one-way analysis of variance (ANOVA), followed by a Duncan test.

Results

In vivo studies.

We assessed the efficacy of intraperitoneal treatment with pidotimod by measuring ^{3}H-thymidine incorporation rate in peripheral blood lymphocytes and splenocytes from young and aging rats.

Proliferation of either peripheral blood lymphocytes (table 1A&B) and splenocytes (table 1C) of 2 month-old rats was similar in both treated or untreated rats.

On the other hand, response of 24 month-old rat peripheral blood lymphocytes (tables 1A&B) and splenocytes (table 1C) to phytohaemagglutinin and concanavalin A was greater in animals treated with pidotimod than in untreated animals.

In aging animals treated with pidotimod, in both interleukin-1 and phytohaemagglutinin stimulated peripheral blood lymphocyte cultures, production of interleukin-2 was significantly greater than in controls, whereas a similar treatment did not influence the robust response to mitogens of 2 month-old rat lymphocytes (fig. 1; panels A and B).

No differences were observed in peripheral blood lymphocytes and splenocytes number between 2 month-old and 24 month-old rats in untreated and pidotimod-treated groups on the basis of the percent count. In addition, in both latter groups, thymic weight was significantly different (* < 0.0001; data not shown).

Table 1.

Effects of the intraperitoneal treatment with pidotimod (PT) (100 and 400 mg/kg for 1 week prior to the test) on ^{3}H-thymidine incorporation (dpm × 1000) in peripheral lymphocytes (PBL) and splenocytes (SPL) incubated with ConA or PHA. * $P < 0.05$ vs vehicle (ANOVA, followed by Duncan test). n = 8 each experiment

A

Cells	Basal	ConA alone	PT 100+ConA	PT 400+ConA
2 month-old rat PBL	3.255±0.30	67.110±3.71	71.371±2.29	66.972±3.881
24 month-old rat PBL	2.846±0.426	40.110±4.081	54.598±7.76*	56.22±2.767*

B

Cells	Basal	PHA alone	PT 100+PHA	PT 400+PHA
2 month-old rat PBL	3.066±0.553	74.819±2.571	66.412±3.941	70.316±4.402
24 month-old rat PBL	3.293±0.528	38.493±2.733	48.256±4.16*	58.307±2.97*

C

Cells	Basal	ConA alone	PT 100+ConA	PT 400+ConA
2 month-old rat SPL	3.00±0.100	154.909±6.9	158.50±8.0	148.8±6.2
24 month-old rat SPL	3.50±0.750	103.00±2.40	110.0±11.20	132.0±2.60*

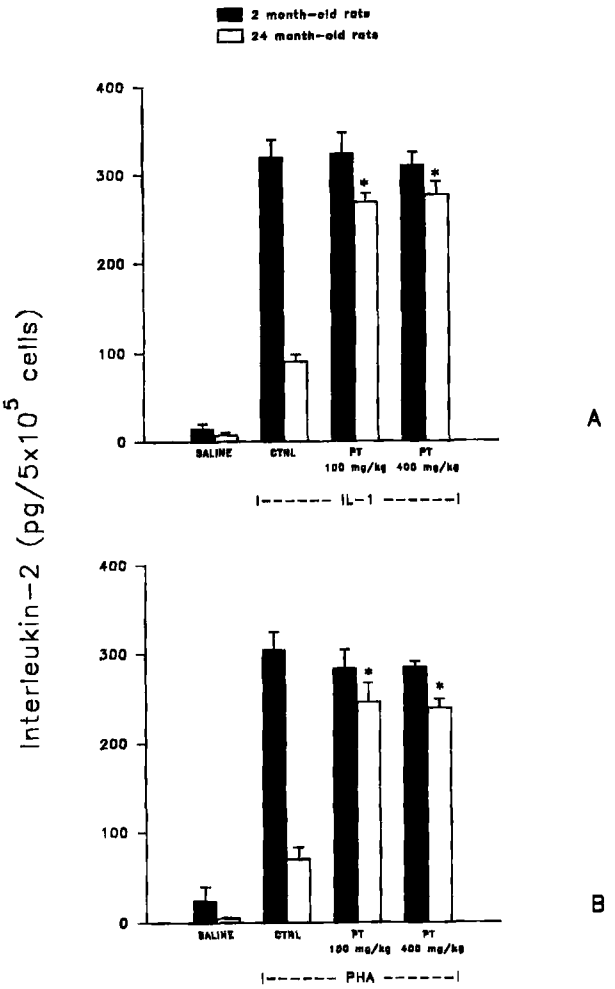

Fig. 1. Effects of the intraperitoneal treatment with pidotimod (PT) (100 and 400 mg/kg for 1 week prior to the test) on interleukin-1 (IL-1)- (panel A) and phytohaemagglutinin (PHA)-stimulated (panel B) interleukin-2 production by young and old rat peripheral blood lymphocytes. * $P<0.05$ vs controls (ANOVA, followed by Duncan test). Vertical bars are the mean ± S.E. n = 8.

General incidence of gross pathology and pituitary macroadenomas in all the experiments was zero (data not shown).

In vitro studies

Pidotimod (1 μM to 1 mM) was able to stimulate peripheral blood lymphocytes (fig. 2, panel A) and splenocytes (fig. 2, panel B) proliferation in a concentration-dependent fashion in both young and aging rats, to a lesser extent than concanavalin A and phytohaemagglutinin (fig. 2, panels A and B).

On the other hand, combination of pidotimod (100 μM and 400 μM) and concanavalin A or phytohaemagglutinin exhibited a synergistic effect on peripheral blood lymphocyte (fig. 3, panel A) and splenocyte (fig. 3, panel B) proliferation in 24 month-old rats as shown by the increase of ^{3}H-thymidine incorporation rate, while had no effect on 2 month-old rat cells (fig. 3, panels A and B).

Discussion

Treatment with pidotimod significantly increased responsiveness of 24 month-old rat peripheral blood lymphocytes and splenocytes to mitogens both *in vivo* and *in vitro*. In addition, interleukin-2 production was significantly increased in cultured cells from aging rats treated with pidotimod.

These results suggest that treatment with pidotimod is able to restore, at least in part, immune function in the aging rat.

It is well known that aging is associated to a decline of immunocyte function (Delafuente 1985). Such age-related deficiency is characterized by diminished responsiveness of

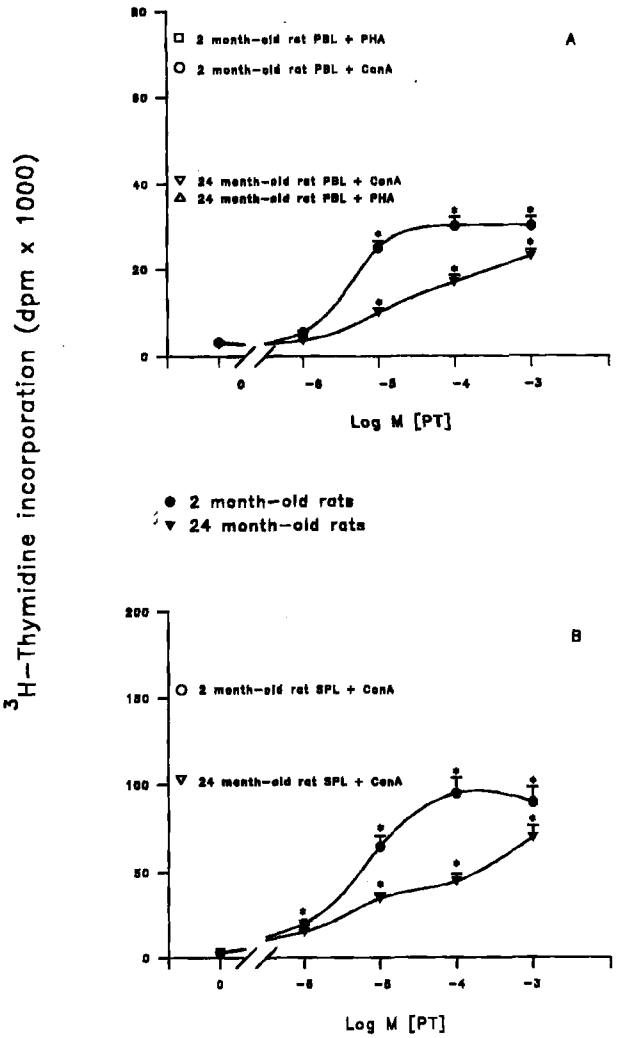

Fig. 2. *In vitro* effects of graded concentrations of pidotimod (PT) (from 1 μM to 1 mM) on peripheral blood lymphocytes (PBL) (panel A), and splenocytes (SPL) (panel B) from (●) 2 month-old and (▼) 24 month-old rats. Hollow symbols show the effects of concanavalin A (ConA) (1 μg/ml) and phytohaemagglutinin (PHA) (5 μg/ml) on ^{3}H-thymidine incorporation (dpm × 1000) in PBL and SPL of 2 month- and 24 month-old rats. * $P<0.05$ vs controls (ANOVA, followed by Duncan test). Vertical bars are the mean ± S.E. n = 8.

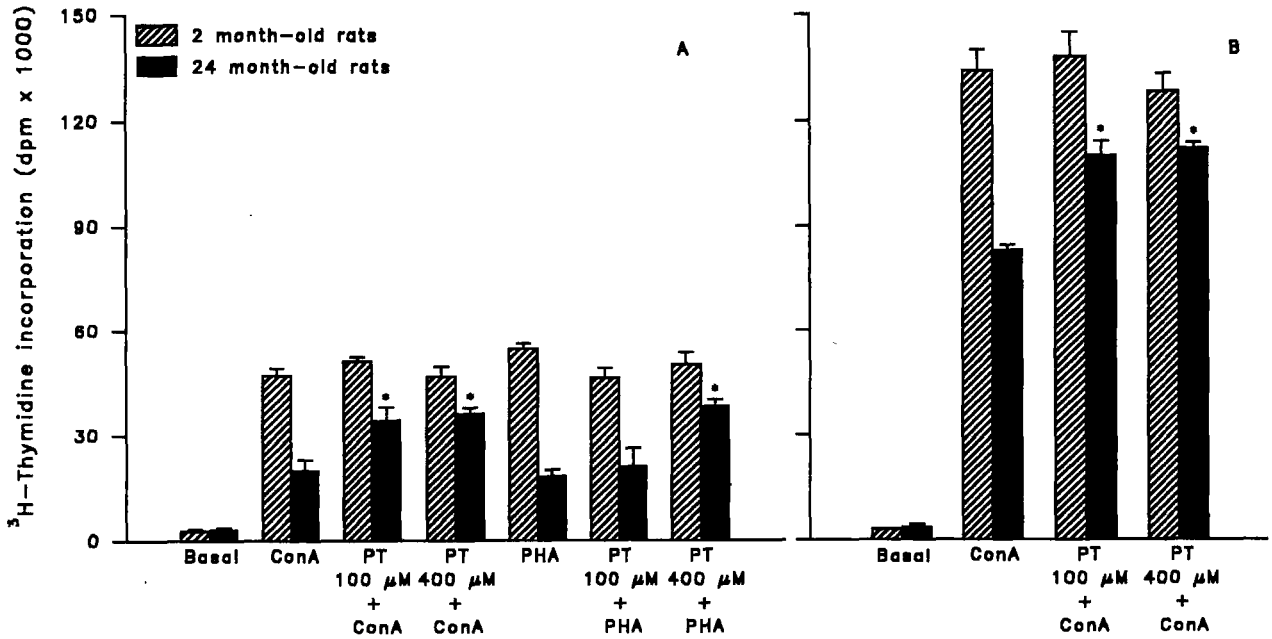

Fig. 3. *In vitro* effect of a combination of pidotimod (PT, 100 and 400 μM) and concanavalin A (ConA) (1 μg/ml) or phytohaemagglutinin (PHA) (5 μM/ml) on peripheral blood lymphocytes (panel A) and splenocytes (panel B) from young and aging rats. * $P<0.05$ vs controls (ANOVA, followed by Duncan test). Vertical bars are the mean ± S.E. $n=8$.

lymphocytes to antigenic challenges (Staiano-Coico *et al.* 1984), as well as by relative incapability of synthesis and/or secretion of humoral factors of the immune response (Staiano-Coico *et al.* 1984), including cytokines and thymic factors. Our results appear consistent to the hypothesis that age-related thymic atrophy represents one of the causes of such decline of immunocompetence, referring to both cell-mediated and humoral thymus-dependent immune responses (Burnet 1979). In this line, pidotimod, a synthetic thymic factor, partially restores the capability of immunocytes to normally proliferate and differentiate, as well as their cytokine synthesis and/or release capability.

The mechanism by which pidotimod enhances the immune response in aging individuals might be primarily due to its direct, specific receptor-mediated action on immunocytes. However, it is not unlikely that the immunostimulating effects of pidotimod represent a consequence of an increased production of interleukin-2. The latter may be coupled to a restored function of the interleukin-2 receptor system, which is also known to be impaired in aging (Powers *et al.* 1987). Third, it is possible that thymic factor-mediated immune response improvement could be explained by the above mentioned effects or, more extensively, by pleiotropic effects of pidotimod. It is plausible to hypothesize that the increase in lymphocyte activity of pidotimod-treated animals could also be due to a relative increase in thymic cellularity and/or restorement of normal thymic factor levels after exogenous administration. In addition, such improvement of the immune response could be due to an increase in lymphocyte sensitivity to thymic factors, as it is deducible from their augmented *in vitro* responsiveness.

Finally, the lack of difference in immune parameters in young rats after treatment with pidotimod supports the subsequential hypotheses that 1) in the intact immune system,homeostatic mechanisms governing the immune response are relatively insensitive to pharmacological manipulation; 2) deficiency of thymic factors in the aging individual may be, at least in part, responsible of the decline of the immune response. The possible, consequent increased sensitivity of the aging immune system to physiological and pharmacological stimuli would therefore account for its augmented responsiveness.

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