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Lenalidomide alone or lenalidomide plus dexamethasone significantly inhibit IgG and IgM *in vitro*...A possible explanation for their mechanism of action in treating multiple myeloma

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Abstract

Lenalidomide (len) is an analog of thalidomide (thal), and both are used in the treatment of a diverse group of medical conditions. A common finding in this group is the detection of immunoglobulin in skin lesions, or high levels of immunoglobulin or myeloma protein in serum and urine. While their mechanism(s) of action is not known, the drugs are noted for their ability to modulate monocyte, lymphocyte, and natural killer cell functions; suppression of immunoglobulin synthesis could offer an explanation for their effectiveness in treating multiple myeloma (MM).

Our objective was to determine if, on an equimolar basis, len or dexamethasone (dex) could affect pokeweed (PWM)-induced synthesis of IgG, IgM and IL-2. When peripheral blood mononuclear cells were stimulated with PWM, len surpassed thal in suppressing IgM and IgG, and enhancing IL-2. Dex enhanced IgG, and suppressed IL-2. When the stimulated cells were treated with len (an effective promoter of IL-2 and suppressor of IgM and IgG) plus dex (an effective suppressor of IL-2 and enhancer of IgG), the net result was suppression of IgM and IgG.

The synthesis of IgM and IgG by putative PWM-stimulated B cell blasts is significantly blocked by len. This suggest that the B-lymphocyte is a targeted cell for len, and that suppression of the synthesis of IgG and IgM could provide an explanation for the mechanism by which len effectively treats MM.

Keywords

Thalidomide; Lenalidomide; Dexamethasone; IgM; IgG

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1. Introduction

Lenalidomide (len) is a structural derivative of thalidomide (thal), and both drugs are being used to treat a variety of diverse medical conditions [1,2]. Their mechanism(s) of action is not known, and their anti-inflammatory and immunomodulatory properties offer multiple sites where they may act.

Singhal and colleagues [3] were the first to show that as having *in vivo* efficacy in the treatment of multiple myeloma (MM), and this prompted a large phase II study. The primary end-point of this trial was paraprotein response. A response of >25% reduction in paraprotein was recorded in 27 patients (32%), and reductions of >90% of paraprotein in serum and urine were documented in 8 patients. Using len/dexamethasone (dex) as initial therapy for myeloma, a decrease in serum monoclonal protein by 50% or greater was reported in 31 of 34 patients [4]. These findings suggest that the drugs may affect the synthesis of myeloma protein.

Long before its use in MM, thal was known to be an effective treatment for erythema nodosum leprosum (ENL). This inflammatory condition occurs in multibacillary leprosy patients, and is thought to be, in part, an immune complex-mediated Type III hypersensitivity [5,6]. Today, in addition to treating ENL and MM, thal is used to treat Waldenström's macroglobulinemia, cutaneous lesions of systemic lupus erythematosus, Schnitzler's syndrome, and light chain (AL) amyloidosis. A common finding in these medical conditions is the detection of immunoglobulin in the skin lesions, or high levels of immunoglobulin or myeloma protein in serum and urine. Suppression of antibody synthesis by thal or len may offer an explanation for their effective treatment of MM and other conditions where over-production of immunoglobulin or light chains of immunoglobulin is involved.

Pokeweed (PWM) promotes polyclonal differentiation of B cells into antibody-secreting cells, and it has been used to determine the effect of drugs on antibody synthesis [7]. We used this bioassay to see if thal, len or dex could affect the synthesis of IgM, IgG, IL-2 and IL-6.

2. Materials and methods

2.1. Thalidomide, lenalidomide, dexamethasone

Thal and len were synthesized to greater than 99% purity in the laboratory of NG. Len is an analog of thal, in which the phthalimide moiety is modified by removing oxygen on the number 3 carbon and adding an amino (NH₂) group at the number 4 carbon (Fig. 1). They were dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI-1640. The final concentration of DMSO in the cultures was 0.08% volume/volume. Dex (Sigma, MO) was dissolved in pyrogen-free distilled water, and diluted in RPMI-1640.

2.2. Cell culture

Blood was collected from donors at the Blood Bank of Our Lady of the Lake Hospital, and the Medical Oncology Clinic in Baton Rouge, Louisiana. The peripheral blood mononuclear cells (PBMC) were isolated and suspended in RPMI-1640 supplemented with 2 mM

glutamine, Hepes, and 20% FCS. The PBMC at 1×10^5 cells/well were incubated in: (1) a volume of 200 μ l; (2) with 5% CO₂ in humidified air at 37 °C; (3) and stimulated with PWM. The cultures were treated with len, thal or dex at 15 μ M each or len plus dex (len/dex). The supernatant was harvested after 6 days for assessment of immunoglobulin, IL-2 or IL-6. The cells were assayed after 24 h or on day 6 for reduction of a tetrazolium dye, or day 7 for the incorporation of [³H]thymidine. To determine if len prevented the release of IgG, we assayed for secreted IgG in the supernatant, and what was retained in the cytosol of PWM stimulated cells.

2.3. Assessment of IgM, IgG

Human IgM and IgG were detected by an enzyme-linked immunosorbent (ELISA) assay [8]. Rabbit anti-human IgM- μ -chain or goat anti-human IgG-Fc γ -chain (Sigma-Aldrich) was adsorbed onto plastic. Culture supernatant was incubated with the capture antibodies, and washed. Peroxidase conjugated to goat anti-human IgM (Fc μ fragment specific), or to rabbit anti-human IgG (Fc γ fragment specific) was added. The plates were incubated for 1 h, and TMB ELISA reagent (Pierce) was added. The reaction was stopped with acid. From a standard curve using known concentrations of IgM (myeloma) and IgG (Pierce), the concentrations in ng/ml of IgM or IgG in the cultures were determined using software KC4 Signature Ver. 3.4, Rev 2.1 (Bio-Tek Instruments Inc. Vt).

2.4. Assessment of IL-2, IL-6

ELISA kits (Pierce/Endogen) were used to quantify IL-2 and IL-6 in culture supernatants.

2.5. Assay for viability, metabolic activity and proliferation

Cells from 24 h cultures were assayed for viability by exclusion of trypan blue. In 24 h and 6 day cultures, cellular metabolic activity was assayed by reduction of the tetrazolium compound 3-(4,5-di-methylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) (Celltiter 96 TM aqueous Non-Radioactive Cell Proliferation Assay, Promega). To assay for proliferation, the cells were pulsed on day 6 with 0.5 μ Ci/well of [Methyl-³H]-Thymidine (Perkin Elmer Life and Analytical Science, Shelton, Ct., USA), and harvested after 16–18 h. The disintegrations per minute (DPMs) were determined using a Beckman/Coulter LS6500 scintillation counter.

2.6. Statistical methods

A one-way Analysis of Variance (ANOVA) with a Dunnett's post test was performed using GraphPad Prism version 5, GraphPad Software, (San Diego, CA).

3. Results

3.1. Comparison of len and thal on the ability of PBMC stimulated with PWM to synthesize IgM, IgG and IL-2

The concentration of IgM or IgG or IL-2 in the supernatant of 6 day cultures from 4 donors was standardized and expressed as % of Control (Fig. 2). Compared to the control cultures stimulated with 10 μ g/ml of PWM, len at 15 μ M reduced IgM to 26% of control, reduced

IgG to 18% of control, and enhanced IL-2 to 419% of control. Thal at 15 μ M reduced IgM to 58% of control, reduced IgG to 88% of control, and enhanced IL-2 to 149% of control.

3.2. IgM, IgG, IL-2 and IL-6 secreted by PBMC stimulated with PWM and treated with thal, len, dex or len/dex

The concentration of IgM, IgG, IL-2 and IL-6 from 6 day cultures stimulated with PWM, and treated with the test compounds is shown in Table 1. Len consistently enhanced IL-2, and consistently suppressed IgM and IgG. With thal, there was a similar effect with enhancement of IL-2, and depressed synthesis of IgM. Dex suppressed IgM in 2 of 5 donors, and significantly enhanced IgG in all 5 donors. It suppressed IL-2 in 3 of 3 donors. Interestingly, when PWM-stimulated cells were treated with 15 μ M len (a promoter of IL-2 and suppressor of IgM and IgG) plus 15 μ M dex (an effective suppressor of IL-2 and enhancer of IgG), the net result was suppression of IgM and IgG and enhancement of IL-2. In all of the cultures treated with len alone or len/dex, IL-2 was significantly enhanced. IL-6 was significantly suppressed in 3 of 3 donors cells treated with len/dex.

3.3. Titration of len

The cells from two blood donors were significantly suppressed in their ability to synthesize IgM and IgG when treated with len at 15 μ M and 1.5 μ M, but not at 0.15 to 0.00015 μ M. As determined by incorporation of [3 H] thymidine, len at 1.5 μ M suppressed proliferation of 6 day PWM-stimulated cells. The data from one donor is illustrated in (Fig. 3). In another donor (BB51), the PWM-stimulated cells in culture for 24 h and treated with 15 μ M len, were also suppressed (33% of control) [mean of control 4908 DPM vs 3787 DPM, $p > 0.01$, Dunnett]. Cells cultured from donor BB 52 for 6 days were inhibited in their ability to incorporate [3 H] thymidine (30% of control) [mean of control 8436 DPM vs. 2560, $p < 0.001$, Dunnett]. Len also suppressed IgM and IgG in 6 day cultures from donors BB 51 and BB 52. Len's ability to suppress PWM-induced synthesis of IgM and IgG is associated with a decrease in cell proliferation.

3.4. Titration of len/dex

Len and dex were titrated to determine an end point for an effective combination in suppressing IgG. When compared to PWM-stimulated cultures (Control), suppression of IgG by len was canceled when cells were treated with len at 0.2 μ M/dex at 15 μ M (Fig. 4).

3.5. Metabolic activity of the test drugs

The suppression of IgG and IgM by len was not caused by a significant decline in cell viability. The cytoplasm in 85–90% of the cells from 24 h cultures treated with the test compounds alone or in combination did not stain with trypan blue (data not shown). Mitochondrial dehydrogenase activity in metabolically active cells from 24 h and 6 day cultures was determined by reduction of the tetrazolium salt (MTS). The cells stimulated with PWM and treated with the test compounds were bioactive, and reduced MTS. The data from one of four individuals is illustrated in Fig. 5.

3.6. Secretion of IgG by len

The suppression of IgG by len was not caused by retention of IgG in the cytosol of cells following stimulation with PWM. Len at 15 μ M inhibited de novo synthesis of IgG. Data from one of three donors is illustrated in Fig. 6.

4. Discussion

Our primary objective was to determine if thal, len or dex could affect PWM-induced B cell synthesis of IgG and IgM. Thal was assayed at 15 μ M (4.0 μ g/ml). This concentration is within the range of concentrations (0.9 to 5.0 μ g/ml) of thal detected in the plasma of healthy individuals in kinetic studies [9], as well as healthy individuals dosed with 100 mg of thal every 6 h for 24 h [10]. Len was assayed at 15 μ M (4.1 μ g/ml), and dex was assayed at 15 μ M (6.0 μ g/ml).

Len surpassed thal in suppressing IgG and IgM, while dex enhanced the synthesis of IgG. When the PBMC were stimulated with PWM and treated with len/dex, IgG and IgM were significantly suppressed. When len/dex was titrated to determine an effective concentration in suppressing IgG, len's ability was lost when the cells were treated with 0.2 μ M len/15 μ M dex.

The effect of the test compounds on PWM-induced synthesis of IL-2 played a significant regulatory role in the synthesis of IgG and IgM. Enhancement of IL-2, especially by len, resulted in a significant suppression of IgM and IgG. With thal, there was a similar effect with enhancement of IL-2 and depressed IgM. Dex suppressed IL-2, and enhanced IgG. When len/dex were assayed, the net result was suppression of IgM and IgG, and enhancement of IL-2. Len neutralized the enhancing effect of dex on the synthesis of IgG, and it neutralized the suppressive effect of dex on IL-2.

Thal acts as a co-stimulant and can enhance the synthesis of IL-2 [11]. This occurs when PBMC are stimulated with Con-A, SEA, purified protein derivative or anti-CD3. Len also promoted the synthesis of IL-2 when cells were stimulated for 18 h with Con-A (4-fold increase) or SEA (1.9-fold increase) (data not shown). The cell most responsive to mitogen or anti-CD3 stimulation in the presence of thal is CD4(+) [12,13]. Len also promotes CD4-cell secretion of IL-2 [14]. In one study, in which T cells were stimulated with anti-CD3, 1 μ M len promoted the maximum synthesis of IL-2, but when len/dex (10 nM) was used, IL-2 was suppressed [15]. Our findings are in partial agreement with this finding. Len alone promoted the synthesis of IL-2; however, the combination of len/dex did not suppress IL-2. Dex's ability to inhibit IL-2 did not supersede len's ability to promote the synthesis of IL-2. This contradicts the finding of Gondi, et al., and could be explained by differences in the protocol. We used PWM (which activates T and B cells), a mixed population of mononuclear cells, and higher concentrations of len and dex.

Len or dex did not have an effect on the synthesis of IL-6. However, when used in combination, IL-6 was suppressed. IL-6 has a role in the treatment of MM. As a prognostic factor, patients whose IL-6 serum concentration at the time of diagnosis was less than 7 pg/ml showed a significantly longer survival than patients whose IL-6 was 7 pg/ml or higher

[16]. The predominant source of IL-6 in MM patients appears to be bone marrow, and the level of IL-6 has a positive correlation with bone marrow plasmacytosis. *In vitro* IL-6 promotes the synthesis of immunoglobulin (Ig) [17], and the synthesis of Ig by PWM-stimulated B cell blasts is significantly blocked by anti-IL-6 [18].

When initially dissolved in an aqueous acid saline solution, thal (15 μ M) enhanced PWM (10 μ g/ml)-induced synthesis of IL-2, had no effect on IL-6, and suppressed IgM and IgG [8]. In this study, when we dissolved thal in DMSO, it did not suppress IgG. This difference could be related to the use of DMSO.

Promoting the synthesis *in vitro* of Ig by corticosteroids like dex has been reported [19, 20]. Enhancement of Ig synthesis by corticosteroids may be the *in vitro* equivalent to its effect *in vivo*. In contrast to the enhancement *in vitro* of PWM induced Ig synthesis by corticosteroids is the *in vivo* finding of suppressed synthesis of Ig in the bone marrow following treatment with corticosteroids. The synthesis of Ig by bone marrow is inhibited by high-doses of corticosteroids for both short [21] and prolonged courses [22] of administration. Cooper et al., [20] suggest that corticosteroids may have two separate effects on Ig synthesis. One occurs in the bone marrow, and is impaired by corticosteroid. The other effect is on peripheral lymphoid tissues (blood, tonsil, spleen, lymph node) whose production is unaffected or even enhanced by corticosteroids.

Approximately 50 years ago, thal was identified as an effective treatment for ENL [23]. The inhibition of complement-fixing antibodies was suggested as a mechanism of action [24]. This was based on reports that ENL was an immune complex hypersensitivity, and partially on studies showing that when leprosy patients were treated with thal for ENL, the IgM in the serum of dapsone-and thal-treated patients was significantly less than dapsone-treated patients or healthy staff [25]; and when injected with sheep erythrocytes, mice and rabbits treated with thal were suppressed in IgM plaque-forming cells in their spleens [25] and serum hemagglutinating antibodies [26]. In studies using autoimmune-prone NZB and MRL mice, thal inhibited the usually increased synthesis of IgM in the spleens of NZB mice, and it inhibited IgG in the lymph nodes of MRL mice [27]. These studies suggest that thal down-regulates B cell functions (review by Paravar and Lee) [28]. Today, thal is listed as a treatment for ENL and tuberculosis, aphthous ulcers related to HIV, and MM. The mechanism(s) of action include inhibition of TNF- α or angiogenesis and immune-stimulation [29].

Effective treatment of ENL with thal is associated with immune stimulation [30]. Thal's and len's ability to promote the synthesis of IL-2 and suppress IgM suggest that len may be an effective treatment for ENL. While they are both teratogenic, there are fewer adverse events like, constipation, neuropathy and sedation, when len is used to treat MM. The trend today in the treatment of MM is to replace thal/dex with len/dex, with some suggesting using only len.

Thal as monotherapy is a very effective treatment of ENL. It would be interesting to see if len, as a single agent, would be yet more an effective treatment for ENL. In this PWM model, suppression of the synthesis of immunoglobulin by len is documented. The activated

B-lymphocytes or plasma cell is suggested to be a target for len. The suppression of IgG and IgM by len may be a mechanism by which it effectively treats MM.

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Abbreviations

Thal	thalidomide
Len	lenalidomide
Dex	dexamethasone
len/dex	lenalidomide +dexamethasone
ENL	erythema nodosum leprosum
MM	multiple myeloma
ELISA	enzyme-linked immunosorbent assay

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Structure of thalidomide ($C_{13}H_{10}N_2O_4$, MW 258.23)

Structure of lenalidomide ($C_{13}H_{13}N_3O_3$, MW 259.26)

Fig. 1.
Structure of thalidomide and lenalidomide.

Fig. 2.

The synthesis of IgM, IgG, and IL-2 by PBMC stimulated with PWM and treated with 15 μ M thalidomide or 15 μ M lenalidomide. The concentration of IgM or IgG or IL-2 in 6 day cultures from 4 donors was standardized and expressed as % of Control. The control response was the mean ng/ml (IgM or IgG) or mean pg/ml (IL-2) from 4 to 5 replicate cultures stimulated with 10 μ g/ml of PWM. The % of Control=[(mean from 4 to 5 replicate cultures treated with PWM+drug divided by the control response) $\times$ 100]. The mean of means of the % Control responses is in the rectangle above the horizontal bar (mean) or vertical bars ($\pm$ sem).

Fig. 3.

Titration of lenalidomide for PWM induced synthesis of IgG, IgM and cell proliferation. The cells from three donors were stimulated with 10 μ g/ml of PWM or PWM+len serially diluted to a range 1.5 μ M to 0.00015 μ M. On day 6, the culture supernatant was assayed for IgM and IgG. The cells were re-suspended, pulsed with [3 H] thymidine, and harvested the next day. The scatter dots depict the ng/ml of IgM (3a) or IgG (3b) for each of 6 replicate cultures, or the cellular incorporation of [3 H] thymidine (3c) from 4 replicate cultures. The data from one of the donors is illustrated. Compared with the Control (cultures stimulated with PWM) the cultures stimulated with PWM+1.5 μ M len were suppressed in the synthesis of IgM, IgG, and the incorporation of [3 H] thymidine (* p <0.05, ** p <0.01, *** p <0.0001, Dunnett).

Fig. 4.

Titration of lenalidomide and dexamethasone to determine effective molar concentration of lenalidomide necessary to maintain suppression of IgG. PWM stimulated cells from a blood donor [BB53] were treated for 6 days with len or dex at 15 μM or len or dex diluted to 5.0 μM, 1.0 μM or 0.2 μM. When compared to the PWM stimulated cultures (Control), the suppression of IgG was lost when cells were treated with 0.2 μM len/15 μM dex (***) $p < 0.0001$, Dunnett).

Fig. 5.

Effect of the test compounds on for mitochondrial dehydrogenase activity. Cells from cultures incubated for 24 h or 6 days in the presence of the test compounds were assayed for mitochondrial dehydrogenase activity by reduction of the tetrazolium salt, MTS. Compared to cells stimulated with 10 µg/ml of PWM (Control), the cells from the 24 h cultures stimulated with PWM plus the test compounds were bioactive and reduced MTS. Cells from cultures not stimulated with PWM were very active in reducing MTS after 24 h or 6 days. The bars are the mean±SD after 4 h of exposure to MTS. When compared to the PWM stimulated cultures (Control) the ability of reduce MTS in 24 h cultures was enhanced by len, dex and len/dex. After 6 days the cells were in a decline phase of viability (**p<0.01, ***p<0.0001, Dunnett).

Fig. 6.

Effect of len on *de novo* synthesis of IgG. Secreted and intracellular IgG from PWM stimulated cells incubated for 6 days in the presence of len, dex, and len/dex. Decrease levels of IgG were detected in the supernatant (secreted) and cell lysate (intracellular) from all cultures treated with len at 15 μ M. Data from six replicate cultures per treatment (mean $\pm$ sem) from one of three donors is illustrated.

Table 1

Effect of thalidomide, dexamethasone, lenalidomide and lenalidomide + dexamethasone on IgM, IgG, IL-2 and IL-6 secreted by PBMC stimulated with PWM for 6 days.

Donor	Assay	PWM	PWM+thal	PWM+dex	PWM+len	PWM+len+dex
BB45	IgM	210 (14)	181 (8)	94 (6)	35 (10)	6 (2)
	IgG	209 (11)	219 (6)	223 (9)	85 (10)	52 (6)
	IL-2	34 (4)	132 (16)	29 (0.4)	934 (32)	296 (41) [†]
	IL-6	1286 (8)	1217 (8)	1264 (10)	1373 (62)	1148 (10) [†]
BB46	IgM	382 (6)	380 (14)	391 (12)	108 (18)	16 (1) [†]
	IgG	448 (20)	365 (16)	493 (4) [*]	63 (5)	42 (6) [†]
	IL-2	92 (6)	256 (48)	34 (3)	1567 (2)	347 (50)
	IL-6	1900 (19)	1185 (18)	1216 (9)	1289 (9)	1056 (3)
BB47	IgM	26 (4)	15 (2)	42 (2)	10 (0.6)	9 (6)
	IgG	92 (13)	107 (9)	122 (4) [*]	63 (6)	54 (8) [†]
	IL-2	767 (95)	1138 (86)	29 (0.8)	1565 (2) [†]	1417 (23) [†]
	IL-6	1154 (17)	1164 (22)	1179 (11)	1226 (19)	958 (25)
BB 51	IgM	182 (39)	60 (8)	102 (27)	4 (4)	5 (3)
	IgG	186 (12)	ND	393 (63)	46 (63)	43 (29)
BB52	IgM	23 (3)	19 (11)	165 (58)	6 (5)	3 (2)
	IgG	143 (23)	155 (9)	418 (3)	39 (8)	63 (3)

Pokeweed (PWM) 10 µg/ml.

Thalidomide (Thal), dexamethasone (Dex), lenalidomide (Len) @ 15 µM, Len+Dex 15 µM.

IgM, IgG: mean ng/ml (sem) 4–5 replicate cultures.

IL-2, IL-6: mean pg/ml (sem) 4–5 replicate cultures.

Arrow indicates statistically significant suppression ([†]) or enhancement ([†]) vs PWM.

Dunnett's test:

* indicates degree of statistical significance.

p<0.001.

p<0.01
**
p<0.05
*

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