

Effects of Thalidomide on Polymorphonuclear cell functions *in vitro*

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Abstract : Effects of different concentrations of thalidomide were studied on the Polymorphonuclear (PMN) cell functions *in vitro*. The PMN cells were derived from three different species *viz.* human volunteers, rats and rabbits.

In phagocytosis assay, opsonized *candida* cells were co-incubated with PMN cells in presence of different concentrations of thalidomide. The average number of *candida* associated with PMN cells at the end of incubation was taken as phagocytic index. Further, the PMN cells were lysed using sodium deoxycholate and percentage of viable *candida* cells were determined to evaluate the effect of thalidomide on intracellular killing capacity of the PMN cells.

The effect thalidomide on a bacterial peptide FMLP (*formyl-metionyl-leucyl-phenylalanine*) induced PMN cell chemotaxis was studied by 'under agarose chemotaxis assay' method. PMN cells were incubated with different concentrations of thalidomide in the wells on an agarose plate which surrounded the central well containing FMLP. Thalidomide was found to suppress FMLP induced chemotaxis of PMN cells and increase the phagocytosis and intracellular killing of *candida albicans* in a dose dependent manner.

The results of this preliminary investigation show that thalidomide exerts similar effects on the *in vitro* PMN cells of different species. Recently thalidomide has been re-introduced in the market for treatment of type-2 lepra reactions in leprosy patients owing to its anti-inflammatory and immunomodulatory effects. As observed in this study, thalidomide suppresses the PMN cell chemotaxis and exerts stimulatory effect on the PMN cell phagocytosis as well as intracellular killing capacity. These effects of thalidomide on PMN cells correlate with its therapeutic efficacy in the leprosy patients.

Key words: Thalidomide, PMN cells, FMLP, chemotaxis, phagocytosis.

Introduction and Experimental:

Thalidomide, the worst known teratogen of history was introduced as a sedative 1950s. It was extensively prescribed as a treatment for morning sickness. Due to multiple reports on its teratogenic effects, it was removed from markets within 5 years of its introduction. Recently, this drug has re-emerged as a salvage therapy for many immune system related diseases that are resistant to conventional immunosuppressive therapies [1].

The most well accepted mechanism of action of thalidomide is inhibition of the synthesis of a proinflammatory cytokine, TNF- α [2]. However, many clinical studies as well as *in vitro* studies show that TNF- α inhibitory effect of thalidomide is not consistent and depends upon the patient's immune status [3] or the

stimulants used for TNF- α release as well as the target cells used in the *in vitro* assays [4].

Thalidomide possesses anti-inflammatory effects in both acute and chronic inflammation. Preincubation of PMN cells with thalidomide has been reported to inhibit their chemotaxis induced by the chemotactic factor generated by interacting normal human serum with bovine gamma globulin-antibovine-gamma globulin immune complexes [5]. In case of monocytes and lymphocytes, thalidomide has been found to increase random migration of these cells. Further, it was found to decrease the chemotaxis of mononuclear cells in response to FMLP and IL-8 [6]. Thalidomide has been reported to interact with protein kinase- C involved in the process of kinesis of these cells. This effect of thalidomide has been proposed to

be involved in its anti-inflammatory effect in chronic inflammatory conditions [6].

Certain effects of thalidomide like teratogenesis are known to be species specific. It induces teratogenesis in selectively in human beings and primates while rats are resistant. Rabbits are sensitive to teratogenicity but at higher doses as compared to humans [7]. The sleep inducing doses also appear to be considerably different across different species [8]. However, whether variation exists in different animal species in immunomodulatory effects of thalidomide has not been systematically evaluated. In present study, the effect of thalidomide on polymorphonuclear cell function from human volunteers, rats and rabbits has been evaluated to investigate its effect on PMN cell function in these species.

Chemicals used:

Agarose, Fetal Calf serum, RPMI 1640, May Grunwald stain, Giemsa stain, Minimum essential medium (10 X), Agarose for gel electrophoresis and fetal calf serum (Himedia lab pvt. Ltd., Mumbai, India) Dextran (Molecular weight- 2-5 lacs; Pharmacea, Denmark), Phosphate buffered Saline ($P^H = 7.3$, 0.1 M), FMLP and DMSO (tissue culture grade, Sigma Aldrich Inc. USA), Thalidomide (Dabur Research Foundation, India), Heparin, Sterile deionized water, Sterile saline solution.

Blood sample collection:

Blood samples were collected from human volunteers, rats and rabbits. Healthy rats and rabbits were chosen from institutional animal house. The protocol for blood collection was approved by institutional ethical committees.

Each 2 ml sample collected from the volunteers and the animals was immediately processed for separation of PMN cells using 6% dextran solution. The separated cells were washed thrice with Phosphate buffered saline (PBS) by centrifugation and the final cell pellet obtained was suspended in PBS at a density of 1×10^6 cells/ml.

Drug dilutions:

Thalidomide was dissolved in sterile pyrogen free DMSO at concentration of 20 mg/ml. The dilutions of this stock were made in RPMI-1640 medium in such a way that the final concentration of DMSO in the assay mixture was less than 0.05%. All solutions were freshly prepared on the day of experiment. The concentration of thalidomide in the final assay units was adjusted between 0.1 μ g/ml to 10 μ g/ml. This concentration range was selected considering the levels in human plasma after administration of its therapeutic doses [9].

Assay for Phagocytosis and intracellular killing capacity [10, 11,12]:

Candida culture and opsonization:

A twelve hour old culture of candida albicans containing unicellular candida was suspended in PBS. The count was adjusted approximately to 2×10^8 yeast cells/ml. *Candida albicans* were suspended in PBS containing 10% pooled plasma from respective donors for 30 minutes at 37°C (concentration of yeast cells during incubation was 2×10^8 yeast cells/ml.) After opsonization the candida cell suspension was washed with PBS at 4°C and suspended in RPMI medium containing 10% fetal calf serum at concentration of approximately 1×10^8 yeast cells/ml.

Assay for Phagocytosis and intracellular killing capacity:

The final assay mixture contained 100 μ l of PMN cell suspension, 100 μ l Candida suspension and 100 μ l serum from respective species (volunteers, rats and rabbits). The assay mixtures were incubated at 37°C. At the end of 30 minutes incubation, 20 μ l of the assay mixture was taken from each sample and smears were prepared on microscopic slides. The smears were air dried, fixed with methanol for 5 minutes, stained with Giemsa stain (10%) and were observed under oil immersion microscopy. Average number of candida cells engulfed by and associated with PMN cells was counted.

The same assay mixtures were further incubated 30 minutes. At the end of incubation, assay tubes were centrifuged at 2000 rpm for 3 minutes and the supernatant was discarded. To each tube 1 ml 2.5 % Sodium deoxycholate solution was added to break down the PMN cells. To the resultant suspension, 1 ml 0.01% Methylene blue was added. The mixture was further incubated for 5 minutes, a drop of this mixture was taken on microscopic slide and was rapidly observed under light microscope under high power. The stained cells were enumerated per 100 cells as dead candida cells. The average percentage of the dead candida cells was determined in case of each concentration of thalidomide. For each concentration studied i.e. 0.1, 0.4, 1, 2, 5 μ g/ml assay was carried out in triplicates.

PMN cell chemotaxis assay [13, 14,15].

Preparation of Agarose solution:

Agarose (0.24 g) was added to 10 ml sterile deionized water and heat dissolved. After dissolution, it was mixed in equal quantity with 2X minimum essential medium. Before pouring on the slides this mixture was cooled to 56°C. On each microscopic slide (of 50 mm \times 75 mm dimension) exactly 6.0 ml mixture was poured and slides were allowed to cool for 15 min

following which they were kept at 4°C for one hour for proper settling of the agar. With a borer having 4 mm internal diameter, wells were prepared in the settled agarose. With a 4 mm borer well were made in the settled agarose such that, the central well contained FMLP solution in RPMI medium containing 10% FCS while surrounding four wells contained PMN cell suspension along with thalidomide in culture medium (containing 10% FCS) at concentrations ranging from 0.1 to 10 µg/ml. After putting the samples into the respective wells in the agarose, the slides were incubated in humidified atmosphere at 37°C for two hours.

For the PMN cell samples from human beings the concentration of FMLP was 10⁻⁶ M while in case of PMN cells from rat and rabbit it was 10⁻¹⁰ M.

At the end of incubation, slides were removed from the incubator and were fixed in methanol for 30 min followed by fixing in Formaldehyde for 30 min. The agarose gel was removed gently without disturbing the surface of slide. The slides were then stained with 10% Giemsa stain for 20 min, air dried and observed under 10X and 45X powers under microscope. The distance of the leading front of cells towards FMLP as well as on opposite front was determined using digital microscopic system.

Results and discussion:

PMN cell Phagocytosis in vitro:-

As shown in table-1, it was observed that thalidomide increases the average number of phagocytosed candida per PMN cell in dose dependent manner. Also

percentage killing of the engulfed candida was found to increase with increasing concentrations of thalidomide in the assay medium. This effect was observed in the PMN cell samples derived from all the three different species, rats, rabbits and human beings. In our study we found that thalidomide caused dose dependent stimulation of phagocytosis of opsonised *candida* by PMN cells at concentrations studied. This effect was similar in all the three species, however, dose dependent rise in phagocytosis was more pronounced in human and rabbit PMN cells as compared to rat PMN cells.

PMN cell Chemotaxis:

The chemotaxis of PMN cells in response to FMLP was measured in terms distance traversed by the leading front of PMN cells towards the wells containing FMLP. Thalidomide was found to reduce the chemotaxis induced by FMLP in a dose dependent manner in all the three species i. e. rat, rabbit and human beings. Moreover, human PMN cells were found to respond to the concentrations of FMLP as high as 1 X 10⁻⁶ M, whereas the PMN cells obtained from rats and rabbits were highly sensitive to concentrations of FMLP as low as 1X 10⁻¹⁰ M. The distance traversed by PMN cells on the opposite front is due to the random kinesis of the PMN cells. Thalidomide did not affect such random kinesis of the PMN cells.

As shown in table-2, the concentrations of thalidomide corresponding to therapeutic plasma concentrations were found to significantly inhibit the PMN chemotaxis in a dose dependent manner.

Table- 1: Effect of Thalidomide on phagocytosis of *Candida albicans* by PMN Cells and intracellular killing capacity.

| Concentration of thalidomide (µg/ml) | | 0 µg/ml Control | 0.1µg/ml | 0.4µg/ml | 1µg/ml | 2µg/ml | 5µg/ml |
|--------------------------------------|--------------------------|-----------------|----------|----------|--------|--------|--------|
| Human PMN cells | % Phagocytosis | 40 | 16 | 25 | 25 | 36 | 40 |
| | Avg Candida per PMN cell | 5 | 3 | 3 | 4 | 4 | 4 |
| Rat PMN cells | % Phagocytosis | 40 | 15 | 20 | 20 | 38 | 40 |
| | Avg Candida per PMN cell | 4 | 2 | 2 | 2.2 | 2.8 | 3 |
| Rabbit PMN cells | % Phagocytosis | 42 | 15 | 20 | 28 | 30 | 30 |
| | Avg Candida per PMN cell | 5 | 2.5 | 3 | 4 | 4 | 5 |

Table 2: Effect of thalidomide on PMN cell chemotaxis induced by FMLP

| Concentration of thalidomide | | 0 ($\mu\text{g/ml}$) Control | 0.1 ($\mu\text{g/ml}$) | 0.4 ($\mu\text{g/ml}$) | 1 ($\mu\text{g/ml}$) | 2 ($\mu\text{g/ml}$) | 5 ($\mu\text{g/ml}$) | 10 ($\mu\text{g/ml}$) |
|---|--------|-----------------------------------|--------------------------|--------------------------|------------------------|------------------------|------------------------|-------------------------|
| Distance traversed by PMN cells towards FMLP containing wells (μm) | Rat | 85.5 \pm 6.8 | 53.2 \pm 2.9 | 47.0 \pm 2.5 | 44.3 \pm 1.9 | 37.8 \pm 4.5 | 43.8 \pm 3.6 | 12.7 \pm 2.0 |
| | Rabbit | 147.0 \pm 4.0 | 97.0 \pm 3.7 | 82.0 \pm 7.2 | 17.0 \pm 1.9 | 32.0 \pm 3.1 | 15.0 \pm 3.7 | 1.3 \pm 0.71 |
| | Human | 70.9 \pm 5.2 | 45.9 \pm 6.4 | 43.1 \pm 8.0 | 36.4 \pm 5.6 | 29.6 \pm 7.1 | 18.1 \pm 6.7 | 5.8 \pm 4.3 |

Discussion:

The results of our study show that there is no species difference in the effect of thalidomide on in vitro PMN cell function in the PMN cells derived from human beings, rats and rabbits. In case of PMN cells collected from these species, thalidomide was found to increase the phagocytosis of unicellular candida albicans as well as intracellular killing of candida. Thalidomide has been reported to inhibit PMN cell phagocytosis of latex beads at a concentration of 10 $\mu\text{g/ml}$ while it stimulated phagocytosis at a 1 $\mu\text{g/ml}$ [16]. Similarly, thalidomide has been reported to increase superoxide anion release from human PMN cells and monocytes [17]. The increased intracellular killing of candida albicans as observed in our study supports this earlier report.

As per a prior report [6] thalidomide increases random motility of lymphocytes and monocytes of human cells which is due to activation of protein kinase C. It is further stated that it inhibits FMLP induced chemotaxis of monocytes in a concentration dependent

manner. While lack of effect of thalidomide on PMN cell chemotaxis has also been reported [18]. In our study there was no increase in random movement of the PMN cells in presence of thalidomide. This observation is in congruence with an earlier report [5]. It was further found that thalidomide inhibited FMLP induced chemotaxis of PMN cells in a dose dependent manner. This inhibitory effect on chemotaxis was consistent in the PMN cells from all the three species studied.

The efficacy of thalidomide in chronic inflammatory diseases has been proposed to involve its inhibitory effects on lymphocyte and monocytes chemotaxis [6]. However, thalidomide is also reported to inhibit acute inflammation allergic skin diseases, which may involve its action on PMN cells.

Our results emphasize the necessity to consider effects of thalidomide on PMN cell functions while delineating the mechanism of its anti-inflammatory actions.

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