Immune and immunohistochemical-related responsive patterns of metoclopramide drug-induced hyperprolactinemia

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Abstract

Immunological and immunohistochemical influences patterns of prolactin (PRL) hypersecretion (hyperprolactinemia, HPRL) have been elucidated in the last decade. Here, we aimed to determine the immune and immunohistochemical-related response patterns of metoclopramide drug (MCP)-induced HPRL on the immune peripheral blood (PB) cells and cytoskeletal vimentin filament protein of adrenal gland in addition to investigate the phenotypic modification of MCP-induced HPRL to the spleen T-lymphocytes surface antigenic markers (CD4 and CD8). For this purpose, elevated PRL levels (HPRL) were established in male Swiss albino mice by intraperitoneally (i.p.) injection of MCP at a dose of 2.2 mg/kg each day for 4 treatment cycles: 2, 4, 7 and 10 weeks. As a result, MCP-induced HPRL resulted in significant increases in total number of WBC and absolute numbers of lymphocytes 2 weeks post MCP treatment. Additionally, phenotypic expression of CD4+ and CD8+ recorded a significant increase, especially at the end of week 2 of MCP exposure. Moreover, there was increment immunoreactivity to vimentin antigen in the adrenal gland of MCP-induced HPRL mice analyzed at the end of week 2 and week 4 from MCP treatment. To conclude, HPRL, induced by MCP, led to immunological perturbations in the immune PB cells and spleen lymphocytes surface markers (CD4+ and CD8+) that were time-dependent. Besides, MCP-induced HPRL caused immunohistochemical alterations in vimentin protein in adrenal gland. Therefore, MCP should not be used for long duration, and must be used under medical supervision.

Keyword: MCP, HPRL, Perturbations, Immune cells, CD4+, CD8+, Vimentin

1. Introduction

Prolactin (PRL), a neuroendocrine peptide hormone produced by the pituitary gland may play an important role in the interaction between the neuroendocrine and the immune system [1, 2]. PRL is known to have a double activity, functioning not only as a hormone, but also as a cytokine that can selectively stimulate some functions of cellular immunity [3]. The physiological control of PRL secretion is mainly inhibitory, and is done through the activation of dopamine 2 (D2) receptors in the pituitary gland. In contrast, blockade of these receptors increases serum PRL levels [3, 4, 5]. MCP, an anti-emetic medication, is D2 antagonist drug and a hyperprolactinemia (HPRL) induced-agent whose mechanism of action is given by the blockade of D2 receptors in the chemoreceptor trigger zone, located outside the blood-brain barrier resulted in increases in peripheral blood (PB) immunological compartments [4, 5, 6].

Functional abnormalities of PB cells have been described in patients with HPRL [7, 8, 9]. PRL receptors have been identified recently on PB cells and spleen T and B lymphocytes that may be involved in the regulation of humoral and cell-mediated immunity, and that one effect of anti-emetic drug on immune function may be due to its ability to inhibit the effects of PRL action on these lymphocytes, thereafter, a number of researches have focused on the potential immunomodulatory roles of PRL [10]. The administration of anti-emetic drugs such as MCP resulted in significant changes in the numbers of immune-related cells [i.e. splenocytes, bone marrow (BM) cells) and PB cells (i.e. leucocytes and lymphocytes)] [11, 12]. Rovenszký et al. [3] reported that the release of PRL had a transient stimulating effect on theophylline sensitive T lymphocytes, suggesting an enhanced activity of T suppressor lymphocytes. Treatment with anti-emetic drugs for long time revealed significant increase in the relative number and percentage of T-lymphocytes surface markers (i.e. CD4 and CD8) [12]. Vimentin is a type of intermediate filament (IF) protein that is expressed in mesenchymal cells [13]. Vimentin is responsible for integrity of the cytoplasm and stabilizing cytoskeletal interactions [14]. HPRL in guinea pigs activated vimentin that was evaluated in pathologically changed tissue specimens [15, 16].
Administration of dopamine antagonists, such as MCP induces a dramatic increase in serum PRL level (HPRL) for duration of several treatment cycles may be resulted in immunological s and immunohistochemical influences. Therefore, the present studies were conducted to determine the influence of experimentally MCP-induced HPRL in adult male Swiss mice on the level of PB immune compartments (leucocytes and lymphocytes), BM cells and splenocytes and to explore whether it had: 1) phenotypic modification in the expression of spleen T-lymphocytes associated surface markers (i.e. CD4 and CD8) and 2) immunohistochemical alterations in the intermediate vimentin filament protein of the adrenal gland.

2. Materials and methods

2.1 Mice

Male Swiss albino mice (20±2.8 g in weight) were purchased from Animal House of National Research Center, Giza, Egypt. Mice were randomly divided into 4 treatment groups in addition to control group (n=5/group). All mice were handled and kept in a specific pathogen-free facility and all the experimental protocols were approved and carried out in accordance with the approval of the Institutional Animal Ethics Committee of Egyptian National Research Center, Cairo, Egypt and in accordance with recommendation of the proper care and use of laboratory animals, regulated by Faculty of Science, Tanta University, Tanta, Egypt.

2.2 Induction of hyperprolactinemia

Metoclopramide drug (MCP), 4-amino-N(2-diethylaminoethyl)-5-chloro-2- methoxy-benzamide (Sigma Chemicals Co., St. Louis, Mo., USA) was dissolved in phosphate-buffered saline (PBS) fresh each day at a concentration of 0.5 mg/ml. For HPRL induction, 100 µl of the drug solution was intraperitoneally (i.p.) injected into mice at a final dose of 2.2 mg/kg [17] daily for 4 treatment cycles: 2, 4, 7 and 10 weeks. Control animals were i.p. injected with an equal volume of sterile PBS. At the last day of each timepoint cycle, mice were euthanized by cervical dislocation at fasting state. Prior to the sacrificing, blood samples were collected from retro-orbital plexus for immunological analyses. Spleen and tibia and femur bones were aseptically removed.

2.3 Hematological analysis

Heparinized blood parameters were proceeded for hematological analysis using an automated instrument (VetScan HM2™ Hematology System, Abaxis®, Union City, CA), including leucocytes (WBC) (10^3/mm³) and their differential absolute number of neutrophils (10^3/mm³).

2.4 Cell suspensions preparation

Spleen and tibia and femur bones were aseptically removed and placed individually in a 60 mm × 15 mm petri dish with phenol red-depleted RBMI-1640 media (Gibco BRL, Grand Island, NY). Single-cell suspension and count of spleen and BM cells were prepared as described previously by Lutz et al. [18] and Diaz-Montero et al. [19]. Briefly, splenocytes were isolated by dissociating spleen on 60 µm mesh Seives screens (Sigma, St. Louis, MO) and lysing of RBC was carried out with ACK (Ammonium-Chloride-Potassium) buffer. Splenocytes and thymocytes were washed and diluted in supplemented RBMI-1640 media. Tibia and femur were extracted aseptically and the BM cells were flushed from tibia and femur of mice. BM cells suspension was subjected to ACK buffer for the depletion of RBC then washed and diluted in RBMI-1640 media. Splenocytes, thymocytes and BM cells viability and count analysis had been done by the trypan blue exclusion method using a hemocytometer.

2.5 Flow cytometry analysis

Spleen cell suspension was prepared and counted using a hemocytometer with trypan blue dye exclusion as described previously by Lutz et al. [18] and Diaz-Montero et al. [19]. Briefly, cell suspension of spleen T-lymphocyte surface phenotypes were established with anti- mouse CD4 and anti- mouse CD8 (eBioscience, San Diego, CA, USA). T- lymphocytes were stained with the indicated conjugated monoclonal anti-mouse CD4 and CD8 antibodies (10 µL/10^6 cells) and incubated cells for 30 minutes at room temperature in the dark and chill on ice for 1 min. The cells were washed twice with PBS and resuspended in 0.3 ml of PBS supplemented with 0.5% BSA and 0.02% sodium azide. Cells were then washed and acquired by Partec flow cytometer (Sysmex-Partec Company, Germany). The CD4+ and CD8+ cell subsets analyses were phenotypically detected using FlowJo software (TreeStar, Ashland, OR, USA).

2.6 Immunohistochemical study of vimentin protein

Immunohistochemical analysis of vimentin protein was processed according to Hus et al. [20]. Briefly, Pieces of mice adrenal glands were fixed in 10% neutral buffered formalin for 24 hrs. The specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylene, embedded in paraffin wax and sectioned at 5µ thicknesses. Paraffin sections were used for
immunohistochemical analysis using avidin-biotin method to express vimentin. Briefly, monoclonal antibody against vimentin was used Avidin-biotin immunoperoxidase technique is applied in which a biotinylated secondary antibody reacts with peroxidase conjugated streptavidin molecules. Color reaction was developed by using diamino – benzidine gives a brown color. Hematoxylin was used for counterstaining.

2.7 Statistical analysis
All results were represented as mean ± SE. Statistical comparisons between groups were made using the paired Student’s t test and a one-way analysis of variance (ANOVA) as part of an SPSS software package (v.16.0 for Windows, 2007; SPSS, Inc., Chicago, IL). p values ≤ 0.05 considered significant.

3. Results
HPRL induced by daily i.p. injection of MCP at a dose of 2.2 mg/kg into male Swiss albino mice for 2 or 10 weeks resulted in no significant increase in the total number of WBC (Fig. 1A) and absolute number of lymphocytes monocytes (Fig. 1B, when compared with prospective values in the mice group with normal PRL (received no MCP). Noticeably, there was significant decreases in the total number of WBC (Fig. 1A) and absolute numbers of lymphocytes (Fig 1B) evaluated at the end of week 10 treatment cycle when compared with the prospective values analyzed at the end of week 2 treatment cycle.

Comparing to their values in prospective MCP-untreated groups, HPRL induced by MCP injection at a dose of 2.2 mg/kg resulted in significant increase in the total number of BM cells when analyzed at week 2 treatment cycle and at week 4 treatment cycle and resulted in non-significant increase in their values when evaluated at week 10 treatment cycle (Fig. 2B). Markedly, HPRL induced by MCP injection at a dose of 2.2 mg/kg led to significant decrease in the total number of BM cells evaluated at the end of week 4 treatment cycle and at the end of week 10 treatment cycle when compared with their values at the end of week 2 treatment cycle.

Fig 1: Effect of hyperprolactinemia (HPRL) on immune cells of peripheral blood (PB). Mice were intraperitoneally (i.p) administrated once daily with saline phosphate buffer (PBS) or 2.2 mg/kg metoclopramide (MCP) at two different treatment cycles: 2 and 10 weeks. At the last day of each detected treatment cycle, blood samples were with-drawn from the intra-orbital retro-bulbar plexus to determine the total numbers of WBC (A) and the absolute numbers of lymphocytes (B). Data were represented as mean ± SE (n= 5). *p ≤ 0.05.
Contrarily, i.p. invocation of 2.2 mg/kg MCP for 10 weeks resulted in significant increase in the total number of BM cells when compared with their values at the end of week 4 treatment cycle (Fig. 2B). As shown in Figure 2A, i.p. inoculation of 2.2 mg/kg MCP resulted in significant increases in the total numbers of splenocytes investigated at weeks 2, 4 and 10 post-MCP injections when compared to their prospective values in MCP-untreated mice (control). Comparing to their values in week 2 treatment cycle, HPRL induced by MCP injection at a dose of 2.2 mg/kg resulted in non-significant decreases the total number of splenocytes when evaluated at the end of week 4 and week 10 post MCP treatment (Fig. 2A).

![Figure 2A](image)

**Fig 2A:** Effect of hyperprolactinemia (HPRL) on the total numbers of immune-related cells [splenocytes and bone marrow (BM) cells]. Mice were intraperitoneally (i.p.) administrated once daily with saline phosphate buffer (PBS) or 2.2 mg/kg metoclopramide (MCP) at three different treatment cycles: 2, 4 or 10 weeks. At the last day of each detected treatment cycle, mice were sacrificed by cervical dislocation and BM cells were flushed from tibia and femur and splenocytes were removed aseptically. The total numbers of splenocytes (A) and BM cells (B) were prepared and counted using a hemocytometer with trypan blue dye exclusion. Data were represented as mean ± SE (n= 5). *p ≤ 0.05.

In the next series of experiments, we investigated the phenotypic modifications resulted from MCP-induced HPRL on the expression of the spleen T lymphocytes-associated surface markers (CD4). As shown by flow cytometric analysis in figure 3, administration of MCP for 2 or 10 weeks resulted in an increase in the expression of CD4+ as compared to their percentage in control (median value of 33.92 % and 38.69 %, respectively, versus 23.94% and 30.45%, respectively). On the contrary, CD4+ expression could not be efficiently induced by MCP injection when we compared with control group for 4 weeks (median of 21.75% versus 28.76%) (Fig. 3).

![Figure 2B](image)
Fig 3: Phenotypic modifications induced by effect of hyperprolactinemia (HPRL) on the expression of the spleen T lymphocytes-associated marker (CD4). Mice were intraperitoneally (i.p.) treated daily with saline phosphate buffer (PBS) or 2.2 mg/kg metoclopramide (MCP) at three different treatment cycles: 2, 4 or 10 weeks. At the last day of each detected treatment cycle, the splenocytes were stained with anti-CD4 monoclonal antibody (CD4 mAb) to measure the CD4 expression on T lymphocytes by using flowcytometry. In all plots, histograms represent the staining level of the control (A) or MCP-treated mice (B: week 2 post-MCP treatment, C: week 4 post-MCP treatment; D: week 10 post-MCP treatment). The amount of the rightward shift of the filled histograms on the x-axis reflects the intensity of the staining, while on the y-axis the number of cells is reported.

Fig 4: Phenotypic modifications induced by Effect of hyperprolactinemia (HPRL) on the expression of the spleen T lymphocytes-associated marker (CD8). Mice were intraperitoneally (i.p.) treated daily with saline phosphate buffer (PBS) or 2.2 mg/kg metoclopramide (MCP) at three different treatment cycles: 2, 4 or 10 weeks. At the last day of each detected treatment cycle, the
splenocytes were stained with anti-CD8 monoclonal antibody (CD8 mAb) to measure the CD8 expression on T lymphocytes by using flow cytometry. In all plots, histograms represent the staining level of the control (A) or MCP-treated mice (B: week 2 post-MCP treatment, C: week 4 post-MCP treatment; D: week 10 post-MCP treatment). The amount of the rightward shift of the filled histograms on the x-axis reflects the intensity of the staining, while on the y-axis the number of cells is reported.

The influence of MCP-induced HPRL on the expression of CD8+ was observed as illustrated in Figure 4. The phenotypic analysis of CD8+ at the end of week 2 post MCP exposure was higher (median of 24.62%) versus control (19.58%) (Fig. 4). CD8+ expression could not be efficiently induced by MCP exposure for 4 or 10 weeks (median of 13.02% versus 18.41%, and 19.47% versus 21.12% of control) (Fig. 4).

In the present study, vimentin immunostaining detection shows that avidin-biotin immunoperoxidase analysis reveals that the adrenal glands of PBS-treated mice express normal vimentin antigen immunoreactivity at the trabeculae periphery of the cortical cells and in blood vessel walls as a weak brown filamentous color (Fig. 5A). The treatment of mice with MCP at a dose of 2.2 mg/kg daily for 2 or 4 weeks showing an obvious increase of vimentin antigen immunoreactivity at the trabeculae periphery of the cortical cells, in medullary cells, in the blood sinusoids and in the capsule (Figs. 5B and 5C). On the other hand, the treatment of mice with MCP daily for 7 or 10 weeks showing an obvious decrement of vimentin antigen immunoreactivity at the trabeculae periphery of the cortical cells, in medullary cells, in the blood sinusoids and in the capsule (Figs. 5D and 5E).

**Fig 5:** Vimentin antigen immunoreactivity features in the adrenal gland of metoclopramide (MCP)-induced hyperprolactinemic mice. (A) Section in the adrenal gland of PBS-treated mice showing normal weak vimentin antigen immunoreactivity (arrows) at the trabeculae periphery of the cortical cells zona glomerulosa (ZG), zona fasciculate (ZF) and zona reticularis (ZR); (B) and (C): Sections in the adrenal gland of mice treated with MCP (2.2 mg/kg) for 2 and 4 weeks, respectively showing an obvious increase of vimentin antigen immunoreactivity (arrows) at the trabeculae periphery of the cortical cells of ZG, ZF and ZR and marked increase of vimentin antigen immunoreactivity in medullary cells (m) and blood sinusoids (b.s). The capsule (Ca) also expressing strong immunostaining to vimentin antigen immunoreactivity. (D) and (E): Sections in the adrenal gland of mice treated with MCP (2.2 mg/kg) for 7 and 10 weeks, respectively showing marked reduction of vimentin antigen immunoreactivity (arrows) at the trabeculae periphery of the cortical cells of ZG, ZF and ZR and reduction vimentin antigen immunoreactivity in the connective tissue periphery of medullary cells (M) and blood sinusoids (b.s). The capsule (Ca) is also seen with weak immunostaining to vimentin antigen immunoreactivity. Line at right lower corner, 6.25 µm.
4. Discussion
The present study was conceived to explore whether MCP-induced HPRL had any immunological perturbations on the immune-related cells or/and immunohistochemical changes in the expression of vimentin filament protein of adrenal gland. The results here revealed that HPRL induced by MCP (2.2 g/kg) led to immunological changes in the PB immune cells and phenotypic modifications in the expression of spleen-T lymphocytes CD4 and CD8. Besides, HPRL caused immunohistochemical alterations in the immunostaining expression of adrenal gland vimentin filament protein. The positive correlation between PRL levels and the immunological and immunohistochemical indices in MCP-induced HPRL animals was clearly apparent in the experimental mice that were treated with MCP (2.2 mg/kg). This observation is in agreement with previous reports from other laboratories [21, 22].

HPRL often occurs associated with the use of centrally acting dopamine antagonistic drugs such as MCP [23, 24]. The physiological control of PRL secretion is mainly inhibitory, and is done through the activation of dopamine 2 (D2) receptors in the pituitary gland. In contrast, blockade of these receptors increases serum PRL levels [5]. MCP is an antagonist drug of dopamine receptors whose mechanism of action is given by the blockade of D2 receptors in the chemoreceptor trigger zone, located outside the blood-brain barrier [8, 9].

The present study shows that MCP-induced HPRL recognized to have immunostimulatory properties hence the present findings recorded a significant elevation in the estimated total number of WBC (week 2 and week 10 post MCP exposure), absolute numbers of lymphocytes (2 weeks post MCP injection), absolute number of granulocytes (week 2 and week 10 post MCP treatment), total number of BM cells (2 weeks post MCP inoculation) and total number of splenocytes (2, 4 and 10 weeks post MCP exposure). On the other hand, MCP-induced HPRL induced significant decrease in the level of BM cells count (4 weeks post MCP exposure). These results are in accordance with previous reports which concluded that immune cells activations are crucial in inducing HPRL [10, 11, 25] that stimulates lymphocytes in a dose and timepoint-dependent fashion of MCP exposure. The proliferative response of mouse spleen cells exposed to concanavalin A (Con-A) was significantly potentiated by elevated PRL level [8]. Mukherjee et al. [26] studied the in vitro effect of hypersecretion of PRL on splenocytes from ovariectomized rats and showed that HPRL induced the formation of interleukin-2 cell surface receptors. A variety of mammalian cells possess receptors for PRL, including those of immune cells of PB [11]. Data was accumulated to suggest that drug-induced HPRL may have a physiologic role in the regulation of humoral and cell-mediated immune responses. Specific PRL receptors have been identified on human T-lymphocytes [10, 11, 25]. However, an exhaustive literature search revealed scant information [26] regarding the effects of elevated PRL level on monocytes, granulocytes and neutrophils.

We analyzed a possible effective regulation of PRL elevation on the phenotypic expression of T-lymphocytes subsets CD4 and CD8 of mice with HPRL. There was an increase in the relative percentages of CD4^+ (week 2 and week 10 post MCP exposure) and CD8^+ (week 2 post MCP injection). On the other hand, there was a significant decrease in the relative percentage of CD4^+ (4 weeks post MCP exposure) and CD8^+ (week 4 post MCP treatment). Our results are in accordance with previous report of Gala and Shevach [27] who concluded that the percentage of CD4^+ and CD8^+ cells in the hypoprolactinemic mice was increased 2- to 3fold when compared to untreated-mice (control). Also, Koller et al. [28] showed an increased CD4^+ and CD8^+ ratio in MCP-induced hyperprolactinemic animals. Similarly, hyperprolactinemic patients had higher CD4^+ and CD8^+ counts than patients with normal PRL [29]. Further phenotypic and functional studies are needed to clarify the best condition in which HPRL exerts its immunostimulatory and immunohistochemical effects.

In the present study, the PBS-treated mice express normal weak immunoreactivity vimentin antigen immunoreactivity in the analyzed parts of adrenal gland. Contrary, MCP-hyperprolactinemic mice at a dose of 2.2 mg/kg injected daily for short duration (2 or 4 weeks) show marked increment of vimentin antigen immunoreactivity. However, daily injection for long duration (7 or 10 weeks) show marked decrement of vimentin antigen immunoreactivity. The presence of intermediate filament proteins of the cytoskeleton and vimentin types was evaluated in pathologically changed tissue specimens [15]. Our results are in accordance with Kathleen et al. [10] who reported that HPRL in guinea pigs activated intermediate vimentin filaments protein.

5. Conclusion
In conclusion, MCP caused an increase in PRL levels (HPRL) which in turn led to immunological influences in the murine PB immune (i.e. WBC and lymphocytes) and phenotypic modification in the expression of spleen lymphocytes surface markers (CD4 and CD8) that were time-dependent. Besides, HPRL caused immunohistochemical changes in the cytoskeletal intermediate vimentin filament protein. Therefore, MCP should not be used for long duration, and must be used under medical supervision.
6. References


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