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Immunomodulatory Role of Active Vitamin D *(Vitamin D٣)* In Bronchial Asthma

A thesis submitted for partial fulfillment of Master degree
in Internal Medicine
By

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Introduction

Bronchial asthma is an allergic disorder that depends on many cytokines as interleukins ٤ & ٥ which are responsible for the allergic inflammatory response in asthma ***(Wills-Karp, ١٩٩٩)***. One of the strategies in managing such a disease is to induce synthesis of interleukin ١٠ *(Th٢ dependent cytokine)*.

The immunological role of IL-١٠ was exclusively studied, demonstrating its inhibitory effect on cytokine secretion from allergen-specific Th٢ cells. Such study achieved a marvelous therapeutic target in allergic asthma ***(Lee et al., ٢٠٠٢)***.

Kang et al., ١٩٩٨ and Hara et al., ٢٠٠١ studied the in vitro effects in inducing IL-١٠ secreting cells by IL-١٢ and IL-١٣.

Dr. Barrat & collegues studied the active form of vitamin D *(Vitamin D٣)* or ١,٢٥ dihydroxy-cholecalciferol in cell culture. They explained its immunomodulatory role through direct stimulation of IL-١٠ synthesis ***(Barret et al, ٢٠٠٢)***.

Also, ***Emmanuel et al,*** ٢٠٠٦ had demonstrated that **VITAMIN D٣** in vitro overcame ligand-induced down regulation of glucocorticoid receptors by CD_4^+ T-cells in patients with bronchial asthma.

Aim of the Work

Thus, the aim of this work is to study the therapeutic potential of vitamin D٣ for asthma whether steroid dependent or not and revealing its possible clinical relevance which can offer an additional strategy in the management of bronchial asthma.

Patients and Methods

This study has been carried out on forty non smoker patients with allergic asthma. All of them had positive skin test to one or more common environmental or food antigens. They are taking inhaled or oral corticosteroids with different degrees of severity of their asthma & twenty healthy individuals as a control group. Their ages range from ١٦ to ٦٠ years. The patients were selected from those admitted in the inpatient units or those followed up in the allergic clinic of Ain shams university hospital. The patients were classified into three groups:

Group I (A): twenty patients received ٠,٥ µg oral vitamin D_3 daily for one month.

Group II (B): twenty patients did not receive such treatment.

Group III (C): The control group (twenty healthy individuals) received ٠,٥ µg oral vitamin D_3 daily for one month.

Methods:

Patients were subjected to the following:

١) Full history taking, especially being nonsmoker & taking inhaled or oral corticosteroids with different degrees of severity of their asthma.
٢) Clinical examination.
٣) Skin prick test.
٤) Pulmonary function test (FEV_1/FVC ratio).
٥) Serum Calcium, IL-١٠ & IL-٤ by (ELISA) before &after treatment.

Exclusion criteria:

١) Patients with non-atopic asthma

٢) Patients who are smoking
٣) Patients not taking oral or inhaled corticosteroids

Classification of asthma severity by clinical features before & after treatment: (Osborne et al., ٢٠٠٥)

Step ١: Mild intermittent

- Symptoms are ≤ twice per week.
- Brief exacerbations.
- Nocturnal symptoms are not > twice per month.
- Asymptomatic with normal lung function between exacerbations.
- FEV_1 and PEF ≥ ٨٠٪ of predicted.
- PEF variability < ٢٠٪.

Step ٢: Mild persistent

- Symptoms > twice per week but less than once per day.
- Exacerbations may affect activity.
- Nocturnal symptoms are ≥ twice per month.
- FEV_1 and PEF ≥ ٨٠٪ of predicted.
- PEF variability ٢٠-٣٠٪.

Step ٣: Moderate persistent

- Daily symptoms.
- Exacerbations are ≥ twice per week and affect activity.
- Nocturnal symptoms are > once per week.
- Daily use of short-acting β_2-agonist.
- FEV_1 and PEF ٦٠-٨٠٪ of predicted.
- PEF variability > ٣٠٪.

Step ٤: Severe persistent

- Continuous symptoms.
- Frequent exacerbations.
- Frequent nocturnal asthma symptoms.
- Limitation of physical activities.
- FEV_1 and PEF ≤ ٦٠٪ of predicted.
- PEF variability > ٣٠٪.

The presence of just *one* feature within a given category is sufficient to place a patient in that category. Thus, an individual should be assigned to the most severe grade in which any feature occurs.

Skin Prick Test: ***(Slavin and Reisman, ٢٠٠١)***

Skin test for the detection of specific IgE antibodies to *inhalant allergens:* (House dust, mixed moulds, mixed pollen, mite, tobacco, Candida, straw, feathers, cat hair, goat, hay dust, rabbit, hair, wool, pigeon, book dust) and *food allergens:* (Fish, milk, wheat, maize, cacao, egg) was done .

Allergens were used in the diagnostic work- up of allergy. The following approaches were followed for skin testing:

١. Selection of high quality extracts of appropriate concentrations.

٢. Positive and negative controls were included.

٣. Performance of tests on normal skin: evaluation for dermographism.

٤. Recording results at proper time (١٥-٢٠ minutes). Skin prick test was used as it is more specific than intradermal

testing (i.e., less false positive cases). The prick test is widely accepted because it is quicker, less painful, has better specificity, less likely to cause systemic reactions, and less expensive than intradermal testing**.**

Spirometry: (Shim & Williams, ٢٠٠٣).

The most reliable way to determine reversible airway obstruction is with **spirometry**, a test that measures the amount of air entering and leaving the lungs. This simple test can be performed in the physician's office.

Spirometry uses a measuring device called a **spirometer** that is connected by a flexible tube to a disposable cardboard mouthpiece. The patient exhales and inhales deeply, then seals his or her lips around the mouthpiece and blows as forcefully and for as long as possible until all the air is exhaled from the lungs.

Ideally, the patient should exhale for at least ٦ seconds. The spirometer measures the amount of air exhaled and the length of time it took to exhale it. The amount of air exhaled in the first second, expressed as "FEV_1," is measured and compared to the total amount exhaled. If the amount exhaled in ١ second is disproportionately low to the total exhaled, the patient has an obstruction.

Spirometry is very much a patient effort-dependent test that requires the understanding and cooperation of patients for accurate and reproducible test results. The test can be performed with either closed-circuit spirometry or an open-circuit system. Both are equally reliable and have some different advantages and disadvantages. The closed circuit allows more accurate measurement of the functional

residual capacity (FRC), but because the patient breathes into the machine, maintaining good hygiene is a concern. The minimum number of respiratory loops needed is three, and the best FEV_1 and forced vital capacity (FVC) of the three test values should be recorded and used for interpretation. The maximum recommended number for consecutive testing is eight, because studies have shown that patient fatigue becomes a significant factor after more than eight trials.

Traditional spirometry measures lung volumes using a water-sealed chamber. It is expressed as change in lung volume over time. A more modern method using an electronic device measures flow rate as a function of lung volume during inspiration and expiration.

Indications

Diagnostic

- Evaluate presence of pulmonary disease.
- Evaluate symptoms of dyspnea, cough, or wheezing.
- Screen for high-risk individuals (e.g., smokers or those with occupational exposures).
- Preoperative evaluation.

Monitoring

- Assess response to bronchodilator therapy.
- Follow progression of present pulmonary disease.
- Assess for adverse reactions to drugs that may have pulmonary side effects (e.g. amiodarone).
- Assess pulmonary dysfunction in response to environmental exposures.

Criteria for Acceptable Spirometry

Criteria for acceptable spirometry from the American Thoracic Society are *(a)* a smooth continuous exhalation for > ٦ sec demonstrating a *plateau*, defined as no change in the volume for at least ١ sec during expiration; *(b)* a satisfactory start of test without hesitation or false start; *(c)* lack of artifacts such as coughing, glottic closure, early termination of exhalation, or obstructed mouth piece; and *(d)* properly calibrated equipment without leak.

Criteria for Reproducibility

The reproducibility of lung function tests are important not only for following the longitudinal trends but also to ensure that such test can be compared from one test center to the another. These criteria are that (a) the largest FVC should be within ٠,٢ L of the second largest FVC value, and (b) the largest FEV, should be within ٠,٢ L of the second largest $FEV_{١}$. Additional spirograms (maximum of eight) should be obtained to assure that the above criteria are met in at least three of the maneuvers.

Frequency

Follow-up spirograms are used mainly for monitoring purposes and can be repeated at the physician's discretion. For asthma patients, guidelines recommend an initial diagnostic spirogram and at least one annually for management of therapy.

Values Derived from Spirometry

• FVC: The volume of gas that can be forcefully expelled after maximal inhalation
• FEV_1: The volume measured in the first sec of maximal forced exhalation
• $FEF_{25-75\%}$: maximal mid expiratory flow rate; flow during ٢٥-٧٥٪ of vital capacity
• PEF (peak expiratory flow) or FEF max: maximal airflow rate achieved during expiration.

Obstruction is defined as a ratio less than ٧٠٪ of forced expiratory volume in ١ second (FEV_1) to forced vital capacity (FVC). FEV_1 is normally greater than ٨٠٪ of values predicted by age.

IL-٤ Assay procedure (Lin et al, ١٩٩٥ & Hilton et al, ١٩٩٦)

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-٤ has been pre-coated onto a microtiter plate. Standards and samples are pipetted into the wells, and any IL-٤ present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-٤ is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-٤ bound in the initial step. The color development is stopped and the intensity of the color is measured.

REAGENTS

- *IL-٤ microtiter plate* – ٩٦ well polystyrene microtiter plate (١٢ strips of ٨ wells) coated with a murine monoclonal antibody against IL-٤.
- *IL-٤ Conjugate* - ٢١ ml of polyclonal antibody against IL-٤ conjugated to horseradish peroxidase, with preservative.
- *IL-٤ Standard* - ١٠ ng of recombinant human IL-٤ in a buffered protein base with preservative, lyophilized.
- *Assay Diluent RD١* - ٦ ml of a buffered protein base with preservative. *For serum/plasma samples.*
- *Calibrator Diluent RD٦* - ٢١ ml of animal serum with preservative. *For serum/plasma samples.*
- *Wash Buffer Concentrate* - ٢١ ml of a ٢٥-fold concentrated solution of buffered surfactant with preservative.
- *Color Reagent A* - ١٢,٥ ml ol stabilized hydrogen peroxide.
- *Color Reagent B* - ١٢,٥ ml of stabilized chromogen (tetramethylbenzidine).
- *Stop Solution* - ٦ ml of ٢N sulfuric acid.
- *Plate Covers* - ٤ Adhesive strips.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay. Store samples at ≤ -٢٠ ºC. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow sample to clot for ٣٠ minutes before centrifugation for ١٠ minutes at approximately ١٠٠٠ x g. Remove serum and

assay. Store samples at ≤ -٢٠ ºC. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or citrate as an anticoagulant . A rapid separation of plasma after collection less than ٣٠ minutes, ensures optimal recovery. Remove particulates by centrifugation and assay. Store samples at ≤ -٢٠ ºC. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer – if crystals have formed in the concentrate, warm to room temperature and mix gently until crystals have completely dissolved. Dilute ٢٠ ml of Wash Buffer concentrate into deionized or distilled water to prepare ٥٠٠ ml of Wash Buffer.

Substrate Solution – Color Reagents A and B should be mixed together in equal volumes within ١٥ minutes of use. ٢٠٠ µL of the resultant mixture is required per well.

IL-٤ Standard - Reconstitute the IL-٤ Standard with ٥ ml of Calibrator Diluent RD٦ (for serum/plasma samples). This reconstitution produces a stock solution of ٢٠٠٠ pg/mL. Allow the standard to sit for a maximu of ١٥ minutes with gentle agitation prior to making dilutions.

Pipette ٥٠٠ µL of the appropriate Calibrator Diluent into each tube. Use the stock solution to produce a dilution series. Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (٢٠٠٠ pg/mL). The appropriate Calibrator Diluent serves as the zero standard (٠ pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

١. Prepare all reagents and working standards as directed in the previous sections.
٢. Remove excess microtiter plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
٣. For Serum/Plasma Samples Only: *Add* ٥٠ µL of Assay Diluent RD١ to each well. Assay Diluent RD١ will have a precipitate present. Mix well before and during use.
٤. Add ٢٠٠ µL of Standard or sample per well. Cover with the adhesive strip provided. lncubate for ٢ hours at room temperature.
٥. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (٤٠٠ µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper toweling.
٦. Add ٢٠٠ µL of IL-٤ Conjugate to each well. Cover with a new adhesive strip. Incubate for ٢ hours at room temperature.
٧. Repeat the aspiration/wash as in step ٥.
٨. Add ٢٠٠ µL of Substrate Solution to each well. Incubate for ٢٠ minutes at room temperature.
٩. Add ٥٠ µL of Stop Solution to each well. If color changes don't appear uniform, then gently tap the plate to ensure thorough mixing.
١٠. Determine the optical density of each well within ٣٠ minutes, using a microtiter plate reader set