Decreased proinflammatory cytokine production by peripheral blood mononuclear cells from vitiligo patients following aspirin treatment

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ABSTRACT

Objective: Limited studies have shown that treatment of cells with aspirin modulates their cytokine production. Consequently, the aim of the present study is to investigate the pattern of important proinflammatory cytokines production by stimulated peripheral blood mononuclear cells (PBMC) from patients with active vitiligo following long-term treatment with low-dose oral aspirin.

Methods: The study was conducted at the Vitiligo Unit, King Abdul-Aziz University Medical Center, Jeddah, Kingdom of Saudi Arabia between March and October 2003. Thirty-two patients (18 females and 14 males) with non-segmental vitiligo were divided into 2 equal groups, one group received a daily single dose of oral aspirin (300 mg) and the other group received placebo for a period of 12 weeks. The concentrations of interleukin (IL)-1ß, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) were determined in the supernatant of isolated cultured PMBC after being stimulated with bacterial lipopolysaccharide (LPS), before the start of aspirin treatment and at end of treatment period. Cytokine levels were measured using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique, utilizing commercially available kits.

Results: The proinflammatory cytokine production by the PBMC of patients with active vitiligo was significantly increased compared to normal controls. Thus, the relative percentage increase in the production of IL-1ß, IL-6, IL-8 and TNF-α was: 39.4%, 110.5% (p<0.05), 91.5% (p<0.01), and 37% (p<0.05). At the end of treatment, proinflammatory cytokine production in the aspirin-treated group of active vitiligo patients was significantly decreased compared to the placebo group. Thus, the relative percentage decrease in the production of IL-1ß, IL-6, IL-8 and TNF-α was: 42.5%, 45.2% (p<0.05), 30.8% (p<0.01), and 50.6% (p<0.05). The vitiligo activity was arrested in all aspirin-treated patients, while 2 patients demonstrated significant repigmentation.

Conclusion: Chronic administration of low-dose oral aspirin can down-regulate the PBMC proinflammatory cytokine production in active vitiligo with concomitant arrest of disease activity.

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The disappearance of epidermal melanocytes is the main culprit of the skin depigmenting disorder, vitiligo, which is characterized by the development of white patches in various distributions. The natural course of the disease is usually unpredictable but is often progressive with phases of stability.[1] An extending vitiligo with enlarging existing lesions or development of new

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lesions is defined as active vitiligo. The psychosocial impact of vitiligo is immense on the afflicted individuals. In certain cultures, vitiligo may be considered a legal cause for divorce, because of the common belief that it resembles leprosy[2] as well as being a social stigma. Although a loss of melanocytes has been demonstrated in established vitiligo lesions,[3] however, melanocyte destruction has never been clearly demonstrated in active vitiligo. Therefore, it may be assumed that melanocytes die silently by apoptosis rather than by necrosis.[4] Nevertheless, the etiology of vitiligo remains far from resolved. Several theories have been proposed for the loss of functional melanocytes in vitiliginous skin. An impaired redox status theory proposes that vitiligo melanocyte death results from the toxic effect of superoxides, including hydroxyperoxide, which are generated in abundance in the skin of vitiligo patients.[5-8] The neural theory of vitiligo proposes that melanocyte death is caused directly or indirectly by a reaction of melanocytes to neuropeptides, catecholamines or their metabolites, or more generally to an overactive sympathetic system. The theory is supported by several clinical and laboratory findings.[9] The autoimmune theory of vitiligo proposes that melanocytes are destroyed by the cooperation of both cellular and humoral immunity. The presence of antibodies to melanocyte antigens[10] and melanocyte–specific proteins[11,12] in the sera of vitiligo patients, the observation of simultaneous higher percentage of B cells in the early phase of the disease[13] and the in vitro destruction of melanocytes in the presence of lymphocytes as well as autoantibodies,[14] all show the implication of both cellular and humoral immunity. A recent study demonstrated the presence of Melan A-specific $CD8^+$ cytotoxic lymphocytes, which also express cutaneous lymphocyte-associated antigen (CLA), in cell lines derived from perilesional skin biopsies.[15] However, these findings do not differentiate between whether these immune changes are a result of, or a cause of the disease process. Many functions of the immune cells are mediated through cytokines, and there is growing evidence that these molecules play an important role in the pathogenesis of autoimmune diseases, including Hashimoto's thyroiditis and insulin-dependent (type 1) diabetes mellitus.[16,17] The production of IL-6 and IL-8 by mononuclear cells are found to be significantly elevated in patients with active vitiligo, while the levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-α) and interferon –gamma (IFN-γ) are decreased.[18] In contrast, a recent study demonstrated increased levels of TNF-α and GM-CSF in the sera of patients with active non-segmental vitiligo.[19] Imbalance of the antioxidant status was observed in PBMC from patients with active vitiligo. This includes significant decreased levels of intracellular reduced glutathione (GSH) and catalase activity, and increased activity of superoxide dismutase (SOD).[20] Consequently, an increased intracellular production of reactive oxygen species (ROS). Acetylsalicylic acid, otherwise known as aspirin is a nonsteroidal anti-inflammatory drug that causes an irreversible inhibition of the cyclooxygenases (COX) activity. At least 2 isoforms of COX exist, a constitutive form (COX-1) and an inducible form (COX-2).[21] While COX-1 is constantly expressed, COX-2 is regulated by light, growth factors, and cytokines, and may be involved in UV-induced inflammation, photo carcinogenesis, and the aging process.[22] Aspirin, at relatively low concentrations was found to possess many antioxidative properties through various mechanisms.[23-25] Recently, it was shown that aspirin significantly reduces lipid peroxidation of in vitro cultured melanocytes from patients with active vitiligo with concomitant increase in their proliferative capacity.[26] Long-term treatment with low-dose oral aspirin of newly diagnosed patients with non-segmental active vitiligo, significantly improves the antioxidant status of isolated PBMC.[27]

In continuation of a previous study,[27] the present work further investigates the effects of oral aspirin treatment on the production of proinflammatory cytokines, IL-1ß, IL-6, IL-8 and TNF-α by lipopolysaccharide (LPS)-stimulated PBMC from patients with non-segmental-type vitiligo in the active phase.

Methods.

Patients' selection, preparation and aspirin treatment. The present work was conducted at the Vitiligo Unit, King Abdul-Aziz University (KAU) Medical Center, Jeddah, Kingdom of Saudi Arabia, between March and October, 2003. Thirty-two consecutive adult vitiligo patients were selected for this study. These patients were newly diagnosed and subsequently, referred to the Vitiligo Unit. The great majority of the patients were reported to have recent onset of non-segmental vitiligo lesions with an average duration of 6.4 months (range 3-11 months). The activity of vitiligo was based on the vitiligo disease activity (VIDA) score represented by a 6-point scale, as previously described.[28] Hence, the patients were 14 males with a mean age of 28.7 years (range 22-40 years) and 18 females with a mean age of 24 years (range 18-37 years). The VIDA scores of patients were +4 and +3 (active in the past 6 weeks and 3 months) with skin photo types 4 or 5, according to Fitzpatrick's classification. The controls were 32 age-and-sex matched healthy subjects with a mean age of 25.9 years (range 18-40 years). Clinical examination and laboratory investigations were carried out on both the patients and control subjects to exclude any

major systemic disease. All volunteers were non-smokers and had no peptic ulcer, bleeding tendency, gastrointestinal bleeding, severe anemia or history of aspirin allergy. Written consent was obtained from each donor in accordance with the guidelines set by the ethical committee of the KAU Medical Center. All patients, except 2, were reported to receive no medical treatment for their condition. They neither had any form of steroid nor psoralen +UVA (PUVA) treatment. Nevertheless, all patients were requested to stop any form of medical treatment for at least 2 weeks. The patients were divided into 2 equal groups (7 males and 9 females). Patients in the first group were given a daily single low-dose (300 mg) of oral dispersable aspirin (The Boots company, Nottingham, UK) taken after breakfast, and patients in the second group were given the same single dose of oral placebo. The treatment lasted for 12 consecutive weeks. The final dose of aspirin/placebo was taken the night before the day of blood collection.

The PBMC preparation, culture, and lipopolysaccharide treatment. Venous blood samples (10 ml) were collected into EDTA-containing tubes from the controls and patients prior to and at the end of 12-week period of aspirin treatment after 12 hour fasting. The PBMC were isolated using Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Upsala, Sweden) gradient centrifugation as described previously,[29] washed twice in phosphate-buffered saline (PBS) solution and then resuspended at a concentration of 1 x 10^6/ml in RPMI 1640 medium (Life Technologies Ltd, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich, Saint Louis, MO, USA). The cells were then cultured in 5 ml polystyrene Falcon tubes in a humidified 5% CO_2 atmosphere at 37ºC. The cell viability as assessed by trypan blue was always higher than 95%. After culture, cells were separated from the culture medium by centrifugation for 10 minutes at 2000 rpm at room temperature. Aliquots (1 ml) of cultured PBMC were placed in 12-well plates and allowed to rest for 24 hours in a humidified atmosphere (5% CO_2, 37ºC). Subsequently, *Escherichia coli*-derived LPS (Serotype 0111: B_4, Sigma-Aldrich) was added to achieve a final concentration of 10 µg/ml. Cytokines (IL-1ß, IL-6 and TNF-α) are preferentially stimulated by LPS.[30] The addition of PBS solution alone served as a control. Following 24 hour incubation, the mixture was centrifuged at 2000 rpm for 10 minutes and the cell-free supernatants were harvested and frozen immediately at -80ºC for later cytokine assays.

Cytokine assays. The cell-free frozen supernatants were thawed once only and used for cytokines determination. The concentration of each of the proinflammatory cytokine (IL-1ß, IL-6, IL-8 and TNF-α) was measured using commercially available kits (Quantikine; R & D Systems, Minneapolis, MN, USA). The assay employs high-sensitivity quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique using monoclonal antibody pre-coated onto a microplate for each cytokine. Each standard and sample was tested using microplate reader, after blank subtraction. The standard curve of each immunoassay was constructed using dedicated computer software. The concentration was expressed as ng/ml derived from 1x10^6 PBMC. The intra/inter-assay coefficients of variation did not exceed 9%. The mean minimum detectable dose for IL-1ß, IL-6, IL-8 and TNF-α was < 0.1 pg/ml, < 0.2 pg/ml, 3.5 pg/ml and 1.6 pg/ml.

Statistical analysis. The results of the assays were presented as mean values ± SEM. Statistical analysis was performed using SPSS 11.0 statistical software. Results were compared using the unpaired Mann-Whitney U test. A probability (p) value less than 0.05 was considered to be significant.

Results.

Pattern of proinflammatory cytokine production by PBMC from vitiligo patients. The PBMC isolated from venous blood samples of the active vitiligo patients always showed significantly lower yield (mean: - 17%) than these of normal control subjects. However, LPS-stimulated PBMC of active vitiligo patients produced significantly higher levels of the measured proinflammatory cytokines than normal **(Table 1)**. The percentage increase of IL-1ß, IL-6, IL-8 and TNF-α was as follows: 39.4%, 110.5% (p<0.05), 91.5% (p<0.01), and 37% (p<0.05) **(Table 1).** The apparently increased levels of these proinflammatory cytokines indicate the systemic inflammatory nature of non-segmental active vitiligo. It is also noteworthy that the increased production of TNF-α by PBMC of active vitiligo patients in the present study contradicts the decreased levels reported by other workers.[18]

Effect of aspirin treatment on the proinflammatory cytokine production by PBMC from vitiligo patients. The prolonged 12-week treatment with low-dose oral aspirin of the patients with active vitiligo resulted in a significant decrease in the production of all studied proinflammatory cytokines. In comparison with the patients in the placebo group, the percentage decrease in the production of IL-1ß, IL-6, IL-8 and TNF-α by the LPS-stimulated PBMC of the patients of aspirin-treated group was as follows: 42.5%, 45.2% (p<0.05), 30.8% (p<0.01), and 50.6% (p<0.05) **(Table 1).**

The decreased levels in the production of the measured proinflammatory cytokines were

Table 1 - Effect of low-dose (300 mg) oral aspirin treatment (12 weeks) on the proinflammatory cytokine production by LPS-stimulated PBMC from patients with active vitiligo.

Cytokine (ng/ml)	Normal Control (n = 32)	Active vitiligo, aspirin-treated group (baseline, n = 16)	Active vitiligo, aspirin-treated group (n = 16)	Active vitiligo, placebo group (baseline, n = 16)	Active vitiligo placebo group (n = 16)
IL - 1ß	2.17 ± 0.89	3.18 ± 1.16*	1.78 ± 0.72*	2.87 ± 1.14	3.10 ± 1.43
IL - 6	1.80 ± 0.58	3.70 ± 0.87*	2.23 ± 0.76*	3.88 ± 1.23	4.07 ± 1.37
IL - 8	10.47 ± 4.30	20.27 ± 6.81**	12.57 ± 5.36**	19.84 ± 7.25	18.17 ± 8.10
TNF -α	2.83 ± 1.12	3.59 ± 1.54*	2.11 ± 0.83**	4.16 ± 1.64	4.27 ± 1.79

The data represent the mean values ± SEM of duplicate incubations of mononuclear cells (1 x 10^6 cells per ml). * $p < 0.05$, ** $p < 0.01$
Active vitiligo, aspirin-treated group (baseline) is compared to normal control, whereas active vitiligo, aspirin-treated group is compared to placebo group, IL - interleukin, TNF-α − tumor necrosis factor-alpha, LPS - lipopolysaccharide, PBMC - peripheral blood mononuclear cells

associated with significant clinical improvement in the clinical course of vitiligo. In particular, there was an arrest of vitiligo disease activity in all patients of the aspirin-treated group, whereas the activity of vitiligo continued to progress in all patients of the placebo group. No new vitiliginous macules appeared during treatment or one-month of follow-up. Two patients showed significant regimentation on the face and forearm (>30%).

Discussion. Although there present a number of possible molecular mechanisms underlying the depigmentation of skin in vitiligo, the etiology and pathogenesis of this disfiguring disease are not completely known. The degree of vitiligo activity is largely dependent on the extent of interaction between the systemic immune response represented by the PBMC and the local epidermal immune changes of melanocytes and other cells. The melanocytic cytotoxicity by immune cells in vitiligo is a complex process involving both humoral and cellular immune mechanisms. Cytokines play a major role as primary mediators in eliciting innate immune response. Through their ability to potentiate T cell activation, IL-1 and TNF can promote both humoral and cellular immune responses,[31] the cornerstones of the autoimmune hypothesis of vitiligo. Hence, it is not surprising that TNF-α and IL-1ß production by PBMC of the active vitiligo patients is significantly increased, in the present study. Furthermore, these cytokines act synergistically with one another and also with IL-6 to produce markedly augmented effects.[31] Tumor necrosis factor-alpha is a very potent primary proinflammatory cytokine generated from macrophages and activated T cells, and appears to play an important role in the development of certain inflammatory skin disorders,[32,33] and is critical for the development of a complete inflammatory response in various autoimmune disorders.[34] Therefore, its increased production by PBMC in the present study suggests that an inflammatory or autoimmune mechanism(s), or both, may be involved during the active phase of non-segmental vitiligo. The theory that proposes the death of melanocyte by apoptosis in an autoimmune effector mechanisms emphasized the important role of cytokines such as IL-1, TNF-α and IFN-ß released by lymphocytes, keratinocytes and melanocytes in the initiation of apoptosis.[35] The presence of high TNF-α in the blood of active vitiligo patients may explain the association of other autoimmune diseases with vitiligo, as TNF-α partly play a role in their pathogenesis. However, a previous report by other investigators showed a contradictory decreased production of TNF-α from phytohemagglutinin (PHA)-stimulated PBMC in active vitiligo patients.[18] This contradiction may be explained as follows. The LPS used in the present study is a more potent stimulator of TNF-α production from PBMC than PHA, which may partly explain this discrepancy. Secondly, the duration of vitiligo may be critical in the amount of TNF-α released by the LPS-stimulated PBMC. Thus, as the time duration of vitiligo increases, the expression of TNF-α may start declining. Moreover, in the present study almost all patients did not receive any form of medical treatment since the onset of vitiligo, which may cause down-regulation of the TNF-α expression. In support of the present result, a recent study has demonstrated significantly higher expression of TNF-α and IL-6 in the lesional and perilesional areas of vitiligo skin than in the non-lesional and healthy skin.[36] The IL-6 secreted by T lymphocytes and monocytes/macrophages in inflammatory reaction at the margin of vitiliginous

lesions may accentuate intercellular adhesion molecule-1 (ICAM-1) expression on melanocytes.[37] The ICAM-1 expression is an obligatory requirement for T cell-melanocyte attachment that develops into immunologic cytotoxicity.[38] Moreover, ICAM-1 expression is up-regulated on melanocytes in culture following exposure to a variety of cytokines such as IFN-γ, TNF-α and IL-α.[39,40] The IL-6 is also found to induce polyclonal B-cell activation, increasing vitiligo autoantibodies production that is reported to induce melanocyte damage by a complement–mediated mechanism and antibody dependent cellular cytotoxicity.[41,42] In addition, increased IL-8 production by PBMC of active vitiligo patients may accelerate melanocytic cytotoxicity as it is a potent chemoattractant and activator of T cells.[43] The increased production of the inflammatory cytokines by PBMC during the active phase of vitiligo may be attributed to alterations in their antioxidants.[20,27] A recent study has shown that long-term treatment with low-dose oral aspirin of patients with active vitiligo significantly improves the antioxidant components of PBMC. These include increased levels of intracellular GSH and catalase activity and reduced activity of SOD.[27] Consequently, it is quite conceivable that an improvement in the antioxidant status of the PBMC, can lead to a down-regulation in the expression of the proinflammatory cytokines. In fact, the present study shows that aspirin treatment significantly causes a decrease in the proinflammatory cytokine production by PBMC. In vivo studies show that aspirin can reduce plasma levels of proinflammatory cytokines in patients with chronic stable angina.[44] Further, a recent report has shown that low-dose aspirin can down-regulate the production of IL-1ß by platelets and other blood cell types.[45] It may be postulated that accumulation of ROS due to low antioxidant levels in PBMC constitute the initial major event which may explain all of the observed changes. The increased ROS may exert its effects in 2 ways. An up-regulation in the expression of the proinflammatory cytokines that stimulates the production of COX-2, and/or up-regulation of COX-2 activity that can cause an activation of the transcriptional factor, nuclear factor-kappa B (NF–κB). The redox-sensitive NF-κB is critical of inducible expression of multiple cellular and viral genes involved in inflammatory and infection including several cytokines and adhesion molecules.[46] Both TNF-α and IL-1ß are well-known activators of NF-κB, which is widely involved in the regulation of other proinflammatory cytokines.[47,48] Aspirin may exert its effects at various cellular levels leading to an improvement in the antioxidant status of PBMC, decreased production of proinflammatory cytokines and inhibition of the inducible COX-2 activity. It has been shown that ROS induce the expression of COX-2 mRNA without affecting the COX-1 mRNA level.[49] Moreover, the hydroxyl radical scavengers, dimethylthiourea and tetramethylthiourea inhibit the IL-1, TNF-α, and LPS-induced expression of COX-2.[50] Aspirin possesses many antioxidative properties through various mechanisms that include inhibition of oxyradicals produced during prostanoids synthesis,[23] reduction of superoxide anions production through lowering of the NADPH-oxidase activity,[24] and a chemical trap for hydroxyl radicals, the most damaging ROS.[25] Consequently, reduction of cellular ROS levels by aspirin may well lead to an inhibition of proinflammatory cytokine production, as well as a decrease in COX-2 activity. Moreover, aspirin at therapeutic levels may exert its anti-inflammatory effects by specifically inhibiting Iκ-B kinase-ß, which in turn prevents the activation of NF-κB.[51,52]

In conclusion, chronic administration of low-dose oral aspirin can down-regulate proinflammatory cytokine production by PBMC with concomitant arrest in vitiligo disease activity.

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