Aspirin reduces serum anti–melanocyte antibodies and soluble interleukin–2 receptors in vitiligo patients

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ABSTRACT

Objective: Increased serum levels of certain immunologic markers including immunoglobulin G (IgG) anti-melanocyte/ vitiligo antibodies (V-IgG) and soluble interleukin-2 receptors (sIL-2R) are associated with augmented humoral and cellular immunity involved in melanocyte cytotoxicity during the active phase of non-segmental vitiligo. Recent reports have shown that aspirin possesses a wide range of immunomodulatory and antioxidant properties. Therefore, the aim of the present study is to investigate the effect of long-term treatment of vitiligo patients with low-dose oral aspirin on serum V-IgG activity and sIL-2R concentration.

Methods: The present study was carried out at the Vitiligo Unit, King Abdul-Aziz University Medical Center, Jeddah, Kingdom of Saudi Arabia between March and October 2003. Eighteen female and 14 male patients with a recent onset of non-segmental vitiligo were divided into 2 equal groups. One group received a daily single dose of oral aspirin (300 mg) and the second group received only placebo for a period of 12 weeks. Serum V-IgG activity and sIL-2R concentration were determined before and at the end of treatment period. The V-IgG activity was measured using cellular enzyme-linked immunosorbent assay (ELISA) following incubation of IgG antibodies with an adult cultured melanocytes. Serum sIL-2R concentration was measured using the highly sensitive quantitative sandwich ELISA utilizing a commercially available kit.

Results: As expected, the serum V-IgG activity and sIL-2R concentration of the active vitiligo patients (0.81 ± 0.23 optical density (O.D.), 1428 ± 510 pg/ml) were significantly increased compared with that of controls (0.27 ± 0.1 O.D., 846 ± 312 pg/ml; p<0.05, p<0.01). Aspirin-treated vitiligo patients showed significant decrease in serum V-IgG activity and sIL-2R concentration (0.32 ± 0.08 O.D., 756 ± 216 pg/ml) compared with that of placebo-treated patients (0.83 ± 0.19 O.D., 1327 ± 392 pg/ml; p<0.01).

Conclusion: Low-dose oral aspirin treatment of active vitiligo patients can cause significant reduction in the acute serum immunologic markers of T cell activation, V-IgG activity and sIL-2R concentration with concomitant arrest of disease activity.

Vitiligo is a common, acquired depigmenting disorder characterized by a selective loss of melanocytes from the cutaneous epidermis. Although the etiology of vitiligo remains unclear, abnormal immune responses, which have been frequently observed in vitiligo patients, led to the suggestion of an autoimmune mechanism involvement that may explain its pathogenesis. The autoimmune hypothesis gained support from the observation that vitiligo is frequently associated with other disorders that have an autoimmune origin including autoimmune polyglandular syndrome type I and 2, insulin-dependent diabetes mellitus, autoimmune thyroid disease (Grave’s disease) and


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atrophic gastritis. Moreover, the therapeutic response of vitiligo to various immunosuppressants such as steroids, psoralen with ultraviolet A radiation (PUVA), indirectly supports the autoimmune mediated process of depigmentation. Importantly, autoantibodies are detected in sera of vitiligo patients, which can induce in vitro damage to melanocytes by complement-mediated and antibody-dependent cellular cytotoxicity. Characterization of these autoantibodies demonstrate that they belong to the IgG class with IgG1, IgG2 and IgG3 subclasses, hence, the name vitiligo IgG (V-IgG). The autoantibodies are heterogeneous in that they react with multiple antigens not only expressed on melanocytes. Despite their heterogeneity, they apparently can cause selective destruction of melanocytes in vitiligo. This may be explained by the greater sensitivity of melanocytes to toxic or immune-mediated injury than any other epidermal cell type or the preferential expression of these antigens on melanocytes that make them more susceptible to selective destruction. Vitiligo autoantibodies (V-IgG) is most commonly directed against cell surface antigens, 35 KDa, 40-45 KDa, 75 KDa, and 90 KDa. Some of these antigens appear to be common tissue antigens, whereas others (35 KDa and 90 KDa) are preferably expressed on melanocytes. Autoantibodies against melanocyte-specific proteins tyrosinase, tyrosinase related protein 1 (TRP-1), TRP-2, pmel 17, and transcription factor SOX 10 have been demonstrated. However, autoantibodies to tyrosinase and pmel 17 are of low frequencies, reflecting their minor role in the autoimmune response in vitiligo. Recently, the surface receptor, melanin-concentrating hormone receptor 1 (MCHR1) was detected as an autoantibody target in 16.4% of vitiligo sera. The involvement of activated peripheral and cutaneous infiltrating T lymphocytes in melanocytotoxicity has been suggested as an important pathomechanism in vitiligo. Peripheral circulating CD8+, cytotoxic T lymphocytes (CTL) specific for a melanocyte-specific differentiation antigen, Melan A and tyrosinase have been demonstrated in a significant number of vitiligo patients; the numbers of which correlate with disease activity. Moreover, these blood-derived Melan A recognizing T cells express the skin-homing receptor, cutaneous lymphocyte-associated (CLA) antigen. Immunohistochemical studies of the perilesional skin in generalized vitiligo demonstrate the presence of activated inflammatory T cells, mainly CD4+ and CD8+ in the dermal and epidermal infiltrate. These cells express activation molecules such as, major histocompatibility complex (MHC) II (human leukocyte antigen [HLA-DR]), CLA antigen, IL-2R (CD 25) and interferon-γ (IFN-γ). All of these findings advocate the important role of autoreactive and melanocyte-specific T lymphocytes in the induction of melanocytotoxicity in vitiligo. Many of the actions of immune competent T cells are primarily mediated through cytokines, and several reports have demonstrated the presence of these molecules in the peripheral blood circulation and cutaneous infiltrates of vitiligo patients. Pro-inflammatory cytokine production by peripheral blood mononuclear cells (PBMC) from patients with active vitiligo was shown to be significantly increased. Furthermore, an alteration in the antioxidants levels of PBMC was reported in vitiligo patients. Interleukin-2 (IL-2) is a cytokine that seems to play an important role in vitiligo pathogenesis. It is secreted primarily by the T helper lymphocytes, which in turn stimulate the production of IL-2R on the T cell surface. The soluble form of IL-2R (sIL-2R) is then released into the serum during immune response. The serum levels of sIL-2R are increased in many dermatoses such as chronic plaque psoriasis, cutaneous T cell lymphoma (CTCL) and vitiligo. Moreover, sIL-2R is significantly increased in tissue fluids taken from lesional skin in patients with active non-segmental vitiligo. There exists a direct positive relationship between serum levels of sIL-2R and disease activity in non-segmental vitiligo, hence, its increased levels might be taken as an acute immunologic marker in active vitiligo patients.

Aspirin is a classical nonsteroidal anti-inflammatory agent (NSAID) that exerts its therapeutic effect, at least partly by the irreversible inhibition of cellular cyclooxygenases (COX). Particularly, the inhibition of COX-2, the isoform expressed during inflammation, which seems to play a pivotal role in the release of inflammatory cytokines, prostaglandins and proteases that believed to be responsible for cell apoptosis. In addition, aspirin possesses many antioxidant properties through various mechanisms and can also exert immunomodulatory effect on different cell types. Recently, low-dose oral aspirin treatment of patients with active vitiligo significantly increases the antioxidant status of PBMC and causes significant decrease in the proinflammatory cytokine production by these cells. Therefore, the present study aims to investigate the effect of low-dose (300 mg) oral aspirin treatment of active vitiligo patients on the serum activity of V-IgG and sIL-2R concentration, the 2 important immunologic markers of vitiligo disease activity.

Methods.Patients' selection, preparation, and aspirin treatment. The present work was carried out at the Vitiligo Unit, King Abdul-Aziz University (KAU) Medical Center, Jeddah, Kingdom of Saudi Arabia, between March to October 2003. Thirty-two
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adult vitiligo patients were selected for this study. These patients were newly diagnosed and subsequently, referred to the Vitiligo Unit. The great majority of 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. 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 active in the past 6 weeks and +3 active in the past 3 months, with skin photo types 4 or 5, according to Fitzpatrick’s classification. The controls were 32 age-and gender-matched healthy subjects with a mean age of 25.9 years (range 18-40 years) and had no positive family history of vitiligo. Clinical examination and laboratory investigations were carried out on both the patients and control subjects to exclude any major systemic disease such as inflammatory manifestations (for example chronic infections and autoimmune disorder), neoplasm, hematological, liver or kidney disease and diabetes. Furthermore, all volunteers were non-smokers and had no peptic ulcer, 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 treatment nor PUVA phototherapy. However, 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 dispersible aspirin (The Boots Company, Nottingham, United Kingdom) 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.

Collection of blood samples and melanocyte culture. In a single occasion, fasting venous blood samples (10 ml) from control subjects and vitiligo patients were collected in plain tubes. Immediately, sera were isolated and divided into 2 lots. One lot was stored at -20°C used later for antibodies detection, and the other lot was kept at -80°C to be used for sIL-2R analysis.

Fresh adult skin biopsies from abdominoplasty provided by the Eed Clinic, Jeddah, KSA were cleaned of excess subcutaneous tissue, cut into small pieces (5 x 5 mm) and incubated with 0.25% trypsin-EDTA solution (Sigma-Aldrich, Saint Louis, MO, USA) at 4°C overnight. The tissue pieces were gently vortex-mixed for 30 seconds to produce epidermal cells were pelleted by centrifugation at 500×g for 10 minutes and resuspended in keratinocyte serum-free medium (Life Technologies Ltd, Paisley, United Kingdom) as described previously.14 plated on to plastic 10 cm² Petri dishes, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed 24 hours following the primary seeding, and then changed every 2 days. Approximately 7-10 days later, the semiconfluent melanocytes were incubated with 0.25% trypsin- EDTA solution at 37°C for 5 min, harvested with 5% fetal calf serum (Sigma-Aldrich), centrifuged and the pellet was resuspended in the serum-free medium and reincubated as described above. The second-passage cells were used for IgG anti-melanocyte antibody detection.

Measurement of serum anti-melanocyte antibodies activity and sIL-2R concentration. The serum samples of controls and patients were affinity purified with the Enchant Protein G IgG purification kit (Pall Life Sciences, Ann Arbor, MI, USA) according to manufacturer’s instructions. The resulting purified IgG antibodies were used for subsequent experiment. The anti-melanocyte activity of the purified IgG was measured by cellular ELISA as described previously. The cultured adult melanocytes were subcultured into chamber slides and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 hours. The cells were fixed in 100% ice-cold methanol for 3 min, washed 3 times with phosphate-buffered saline (PBS) and immediately incubated with 100 µg/ml of purified V-IgG or normal IgG (N-IgG) at 37°C for 30 min. Subsequently, the slides were washed 3 times with PBS, and 100 µl of rabbit antiserum (1:50 diluted) was added for 30 min at room temperature. The unbound rabbit antiserum was washed 3 times with PBS, then the slides were incubated with a pig antirabbit antibody for one hour at room temperature followed by a rabbit peroxidase antiperoxidase complex Z0113 (Duko, Carpinteria, CA, USA). The slides were read at 490 nm with an ELISA reader, and the relative titer of anti-melanocyte antibodies was expressed as an optical density. Each sample was carried out in duplicate.

The frozen (-80°C) serum samples of controls and patients were thawed once and used for sIL-2R determination. The serum concentration of sIL-2R was measured for each sample by the highly sensitive quantitative ELISA technique using a commercially available kit (Quantikine, R&D Systems, Minneapolis, MN, USA). The assay employs a monoclonal antibody specific for sIL-2R that has been precoated into a microplate. The development of color due to final step of the
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### Table 1 - Serum anti-melanocyte antibodies (V-IgG) activity and interleukin-2 receptors (sIL-2R) concentration following long-term treatment of vitiligo patients with low-dose oral aspirin.

<table>
<thead>
<tr>
<th>Immunologic markers</th>
<th>Normal control (n=32)</th>
<th>Active vitiligo, aspirin-treated group (baseline, n=16)</th>
<th>Active vitiligo, aspirin-treated group (n=16)</th>
<th>Active vitiligo, placebo group (baseline, n=16)</th>
<th>Active vitiligo, placebo group (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-anti-MC (O.D. 490 nm)</td>
<td>0.27 ± 0.1</td>
<td>0.81 ± 0.23*</td>
<td>0.32 ± 0.08*</td>
<td>0.79 ± 0.18</td>
<td>0.83 ± 0.19</td>
</tr>
<tr>
<td>sIL-2R (pg/ml)</td>
<td>846 ± 312</td>
<td>1428 ± 510**</td>
<td>756 ± 216**</td>
<td>1388 ± 406</td>
<td>1327 ± 392</td>
</tr>
</tbody>
</table>

Data are mean ± SEM and were compared by the Mann-Whitney U-test. * - 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, O.D. - optical density, MC - melanocyte.

Results. Serum anti-melanocyte antibodies activity and sIL-2R concentration of vitiligo patients. By using the methanol-fixed adult cultured melanocytes as substrates in cellular ELISA, the optical density (O.D.) at 490 nm for a quantitative measure of anti-melanocyte activity of purified IgG, was significantly higher in V-IgG (0.81 ± 0.23) than N-IgG (0.27 ± 0.1) (p<0.05), a percentage increase of 200% (Table 1). This result agrees with previously reported results that show significant increase in anti-melanocyte activity of V-IgG. In a separate experiment using the indirect immunofluorescence method, V-IgG demonstrated positive reaction with methanol-fixed melanocytes, located mainly in the cytoplasm of the cells. The mean serum levels of sIL-2R in the patients with active vitiligo was significantly higher than that of controls (1428 ± 510; 846 ± 312, p<0.01), a percentage increase of 68.8% (Table 1) and, consistent with previously reported results.

Effect of aspirin treatment on serum anti-melanocyte antibodies activity and sIL-2R concentration of vitiligo patients. The long-term (12 weeks) treatment with low-dose oral aspirin of vitiligo patients resulted in a significant decrease of anti-melanocyte activity in V-IgG, compared to placebo-treated vitiligo patients (0.32 ± 0.08; 0.83 ± 0.19, p<0.01), a percentage decrease of 61.4% (Table 1). Similarly, aspirin treatment of vitiligo patients produced significant decrease in serum concentration of sIL-2R when compared with placebo-treated patients (756 ± 216; 1327 ± 392, p<0.01), a percentage decrease of 43% (Table 1).

In parallel with the decrease in the serum immunologic markers, V-IgG activity and sIL-2R concentration, there was a significant improvement in the clinical course of vitiligo, namely an arrest of disease activity in the aspirin-treated patients. No new vitiliginous macules appeared during treatment and one-month of follow-up. Whereas, placebo-treated vitiligo patients continued to show no abatement in the disease activity.

Discussion. In consistent with previous studies, the present investigations confirm the involvement of humoral and cellular immunity in melanocyte destruction in the active phase of non-segmental vitiligo. A wide spectrum of immunologic markers can be demonstrated in the peripheral circulation and at lesional and perilesional skin of vitiligo patients. However, it remains debatable whether this change in immune response is the primary cause or an epiphenomenon of melanocytic destruction in vitiligo. The detection of increased titer of V-IgG in sera of patients with active vitiligo, clearly demonstrates the role of the humoral immunity in induction of melanocyte damage through complement-mediated mechanism and antibody-dependent cellular cytotoxicity. The increased serum concentration of sIL-2R is a typical example of the involvement of cellular immunity in vitiligo pathogenesis. Therefore, both serum V-IgG activity and sIL-2R concentration can be used as immunologic markers to monitor vitiligo disease activity. A recent report has shown that V-IgG induce HLA-DR and intercellular adhesion.
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molecule-1 (ICAM-1) expression and an increase in interleukin-8 (IL-8) release by melanocytes.\textsuperscript{35} Elevated expression of the MHC class II (represented by HLA-DR antigen) and ICAM-1 augment antigen specific immune-effector-cell attachment and cause melanocyte cytotoxicity. In addition, IL-8 is a strong T cell chemoattractant and activator\textsuperscript{46} and its increased production from melanocytes by V-IgG may specifically attract T lymphocytes to lesional areas of vitiligo skin, hence, amplifying the inflammatory reaction and facilitating melanocytotoxicity.\textsuperscript{45} Moreover, V-IgG stimulate IL-6 production from PBMC,\textsuperscript{28} which induces ICAM-1 expression on melanocytes.\textsuperscript{47} In many important skin dermatoses the activation of T lymphocytes is expressed by sIL-2R and therefore, its increased serum level in patients with vitiligo is usually a reliable marker of acute activation of T cell mediated immunity.\textsuperscript{24,48} In general, the increased systemic and cutaneous levels of proinflammatory cytokine production may advocate the inflammatory nature of vitiligo.\textsuperscript{27,28,42} However, such changes in cytokine production may be attributed to a common single factor, that is the tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)). The TNF-\(\alpha\) is regarded as a dominant regulator of activated inflammatory cascade\textsuperscript{49} and believed to be implicated as a pathogenic component in autoimmune disease.\textsuperscript{46} Further, TNF-\(\alpha\) regulates various lymphocyte functions such as cell proliferation and IL-2R expression.\textsuperscript{51} An increasing evidence suggests that reactive oxygen species (ROS) may function as second messengers in cytokine (IL-1 and TNF-\(\alpha\)) and some growth factor signal pathways that regulate transcription factors such as nuclear factor-kappa B (NF-\(\kappa\)B) and activated protein 1 (AP-1). The AP-1 is activated by several proinflammatory cytokines such as TNF-\(\alpha\) and regulates genes expression involved in inflammatory and immune responses.\textsuperscript{52,53} The NF-\(\kappa\)B has been regarded as a key element in the response of cells to inflammatory stimuli that binds to DNA target sites, where it directly regulates gene expression. These gene targets include proinflammatory enzymes, cytokines, chemokines and cell adhesion molecules.\textsuperscript{55,56} It has been shown that ROS induce the expression of COX-2 mRNA without affecting the COX-1 mRNA level.\textsuperscript{57} In non-segmental vitiligo, there are significant low antioxidant levels, namely intracellular glutathione (GSH) in the PBMC,\textsuperscript{29} and possibly the epidermal melanocytes. Therefore, mechanisms such as trauma, emotional stress, viral infection or UV radiation that precede the onset of vitiligo may lead to an increased production of ROS, that cannot be adequately scavenged by the already existing low antioxidant status, hence, ROS accumulate inside the PBMC and melanocytes of vitiligo patients. On the light of the above, the decreased serum V-IgG titer and sIL-2R concentration by aspirin treatment in the present study may be explained as follows. The anti-inflammatory and immunomodulatory effects of aspirin may be attributed, largely to its ability to reduce the intracellular ROS. This is mainly due to the antioxidant properties of aspirin. In rabbits, aspirin prevents lipopolysaccharide induced rise of hydroperoxides and inactivation of catalase, hence, reducing ROS production mediated by increased activity of NADPH oxidase.\textsuperscript{54} It is also found that aspirin reduces superoxide anions production through lowering of NADPH oxidase activity.\textsuperscript{55} Moreover, aspirin inhibits oXyradicals produced during prostanooids synthesis\textsuperscript{57} and acts as chemical trap for hydroxyl radicals, the most damaging ROS.\textsuperscript{56} It has been suggested that uncoupling of oxidative phosphorylation by salicylates decreases intracellular ATP formation\textsuperscript{57} and consequently, induces the release of adenosine into extracellular fluids in sufficient quantities to exert anti-inflammatory effects. Aspirin can specifically inhibit Ik B kinase-\(\beta\) which prevents activation of NF-\(\kappa\)B pathway\textsuperscript{60,61} and decrease TNF-\(\alpha\) production.\textsuperscript{52} The COX-2, upregulated during oxidative stress due to ROS, and partly is amenable for NF-\(\kappa\)B activation, is classically inhibited by aspirin. In a recent study by the author, aspirin significantly improves the antioxidant status of PBMC, with marked increase in intracellular GSH levels.\textsuperscript{41} A direct effect of aspirin on the humoral immunity has been previously reported.\textsuperscript{46} It has been shown that aspirin inhibits IgG synthesis by up to 50%. It was presumed that NSAIDs including aspirin act as early stage of B cell differentiation that appears to be independent of IL-1 synthesis and early proliferative events.\textsuperscript{83} Collectively, these effects of aspirin reduce the oxidative stress and subsequently, improve the abnormal antioxidant homeostasis. Such improvement in antioxidant homeostasis may down-regulate the enhanced immune responses by decreasing the proinflammatory cytokine production in the active phase of vitiligo, reflected by the arrest of disease activity.

In conclusion, aspirin treatment of patients with active vitiligo can modulate the immunologic factors that cause up-regulation of humoral and cellular immunity involved in melanocyte cytotoxicity. Of these factors, serum V-IgG and sIL-2R, which are considered as immunologic markers of vitiligo disease activity.

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