Alterations in IL-6, IL-8, GM-CSF, TNF-α, and IFN-γ Release by Peripheral Mononuclear Cells in Patients with Active Vitiligo

Hsin-Su Yu,* Kee-Lung Chang,† Chia-Li Yu,‡, Hui-Fang Li,* Meng-Tse Wu,* Chieh-Shan Wu,* and Ching-Shuang Wu§

*Departments of Dermatology, and †Biochemistry, and §School of Technology for Medical Sciences, Kaohsiung Medical College, Kaohsiung, and ‡Department of Medicine, Veterans General Hospital-Taipei, National Yang Ming University School of Medicine, Taipei, Taiwan

The purpose of this study was to clarify the relationship between the cellular and humoral immune components in the pathogenesis of vitiligo vulgaris. By using cytokines as indicators of peripheral mononuclear cell (MNC) function, we compared the effects of phytohemagglutinin (PHA) and purified IgG on MNCs derived from patients suffering from active vitiligo with those from normal controls. The results revealed (i) a significant increase in spontaneous production of IL-6 and IL-8 in patients; (ii) PHA, purified IgG from patients (IgG-anti-MC), or IgG from normal controls (N-IgG) induced a significant increase in IL-6 but diminished GM-CSF, TNF-α, and IFN-γ release in patients; and (iii) IgG-anti-MC brought about a significantly higher stimulatory effect on IL-1β and IFN-γ production than N-IgG in normal controls. Immunologically, IL-6 can enhance melanocyte ICAM-1 expression, which may increase leukocyte–melanocyte attachment and cause melanocyte damage in vitiligo. A decrease in GM-CSF (an intrinsic growth factor for melanocyte) production may retard recovery from vitiligo by checking the proliferation of surviving melanocytes. A significant decrease in TNF-α and IFN-γ production may partially explain the reduced inflammatory reaction in vitiliginous lesions. That IgG-anti-MC stimulates an increase in IL-1β and IFN-γ production in controls suggests that IgG-anti-MC may play a role in melanocyte destruction mediated by monocytes. ***Key words: cytokine/vitiligo vulgaris/IgG anti-melanocyte antibodies. J Invest Dermatol 108:527–529, 1997***

Functional melanocytes in patients with vitiligo vulgaris disappear from involved skin by a mechanism(s) that has yet to be identified. An immunologic hypothesis is currently advanced as a possible pathogenesis of vitiligo. Evidence supporting this hypothesis includes (i) vitiligo is occasionally associated with certain autoimmune disorders (Zauli *et al*, 1986; Hegedus *et al*, 1994), (ii) presence of circulating organ-specific autoantibodies (Cunliffe *et al*; 1968; Harsoulas *et al*, 1978; Betterle *et al*, 1985), (iii) occurrence of melanocyte-specific antibodies (Naughton *et al*, 1983a, 1983b), and (iv) aberrations in the proportion of T-cell subpopulations (Soubiran *et al*, 1985; Grimes *et al*, 1986; D'Amelio *et al*, 1990; Mozzanica *et al*, 1990; Hann *et al*, 1993; Al-Fouzan *et al*, 1995). Of these, the presence of anti-melanocyte antibodies and imbalance in peripheral mononuclear cell (MNC) subsets may play particularly important roles in the pathogenesis of vitiligo. Anti-melanocyte antibodies were found to induce melanocyte damage in *vitro* by a complement-mediated mechanism and antibody-dependent cellular cytotoxicity (Norris *et al*, 1988). Although alterations in MNC subpopulations, especially in T lymphocytes, have been the subject of intensive investigations, only a few publications dealt with changes in MNC function in vitiligo patients (Mozzanica *et al*, 1989; Abdel-Naser and Ludwig, 1992; Mozzanica *et al*, 1992; Taher-Uz-Zaman *et al*, 1992). In this study the spontaneous and phytohemagglutinin (PHA)- and anti-melanocyte antibody-stimulated production of cytokines on MNCs were studied to advance our understanding of the roles of MNCs in the pathogenesis of vitiligo.

Manuscript received September 23 1996; revised December 19, 1996; accepted for publication December 30, 1996.

Reprint requests to: Dr. Yu, Hsin-Su, Department of Dermatology, Kaohsiung Medical College, 100 Shih Chuan First Road, Kaohsiung, Taiwan 80708.

Abbreviations: IgG-anti-MC, IgG anti-melanocyte antibodies; N-IgG, purified IgG from normal controls; MNC, mononuclear cell.

MATERIALS AND METHODS

Selection of Patients Twelve patients with vitiligo of non-segmental-type were selected in this study. All patients had developed newly depigmented lesions within the previous 3 mo; that is, they were suffering from active vitiligo (Moellmann *et al*, 1982). None of the patients had any known autoimmune disorders such as Hashimoto's thyroiditis, Grave's disease, pernicious anemia, or insulin-dependent diabetes mellitus. An equal number of age- and sex-matched healthy individuals served as controls for the present study. None of the participants had received medical treatment in the preceeding 3 mo.

Purification of IgG from Serum of Vitiligo Patients with Anti-Melanocyte Antibodies Serum from patients with active vitiligo and normal controls was collected for purification. The fraction II (IgG) obtained from Sephacryl S-300 gel filtration was further absorbed into the protein A-Sepharose 4B (Pharmacia LKB, Uppsala, Sweden). Sepharose-conjugated IgG was eluted with sodium acetate buffer at pH 2.5 and immediately neutralized to pH 7.2 with 1 N NaOH. After adequate dialysis against distilled water, the IgG was lyophilized (Yu *et al*, 1989a). The protein concentration of purified IgG was determined by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). IgG preparations were found to be free

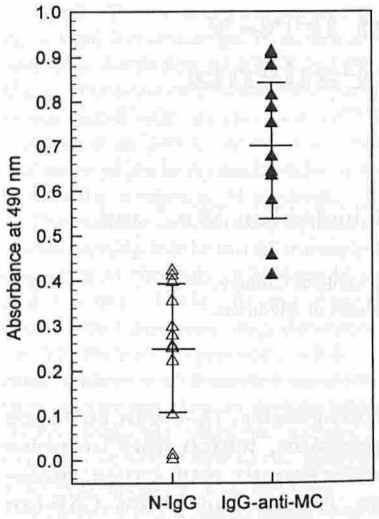

Figure 1. Melanocyte-binding activity of IgG is elevated in vitiligo. With melanocytes as substrate, the optical density at 490 nm as detected by cellular ELISA in purified IgG from active vitiligo (IgG-anti-MC) was significantly higher than that in normal individual IgG (N-IgG) (n = 12).

of contaminants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels (Laemmli, 1970).

Melanocyte Culture and IgG Anti-Melanocyte Antibody Detection Normal adult human foreskin was employed for melanocyte culture. Melanocytes were isolated and maintained in culture as described (Kao and Yu, 1991). Second-passage cells were used for IgG anti-melanocyte antibody (IgG-anti-MC) detection. The individual purified IgG was measured for anti-melanocyte activity by cellular enzyme-linked immunosorbent assay (ELISA) as reported (Yu *et al*, 1993). Six samples containing the highest titer of IgG-anti-MC were then used in the following experiments.

Isolation of Peripheral MNCs and Preparation of MNC Supernatants Peripheral MNCs were isolated from heparinized venous blood of patients and normal individuals by centrifuging at 300 × *g* for 30 min over a Ficoll-Hypaque cushion (specific gravity, 1.077) as reported (Yu *et al*, 1989b). The cell concentration was adjusted to 2×10^6 cells per ml in 10% fetal bovine serum in RPMI 1640 medium (GIBCO, Gaithersburg, MD). Fifty microliters of MNCs were placed in triplicate microwells. Then, 0.02 ml of 10% fetal bovine serum in RPMI 1640 medium (spontaneous), PHA (5 μg per ml, Sigma, St. Louis, MO), IgG-anti-MC (100 μg per ml) or normal IgG (N-IgG) (100 μg per ml), and 0.13 ml of 10% fetal bovine serum in RPMI 1640 medium was added to the microwells. The mixture was incubated at 37°C in 5% CO_2/95% air for 24 h. After incubation, the mixture was centrifuged at 2000 rpm for 10 min and the cell-free supernatants were collected and stored at −20°C until cytokine determination.

Measurement of Cytokines in MNC Supernatants by ELISA The concentration of cytokines in the culture supernatants derived from 1×10^6 MNCs per ml after a 24-h incubation with medium, PHA, IgG-anti-MC, or N-IgG was measured by commercially available ELISA test kits. The ELISA kits include interleukin (IL)-1β, IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (Research and Develop System, Minneapolis, MN), and we followed the manufacturer's instruction. The results were presented as mean ± SD. Unpaired Student's t test was used to analyze the statistical differences between patients and controls. A difference of $p < 0.05$ was considered as statistically significant.

RESULTS

Anti-Melanocyte Activity of Purified IgG from Active Vitiligo Patients Is Significantly Higher Than That from Controls Purified IgG from serum of 12 patients with active vitiligo was assessed for melanocyte-binding activity by cellular ELISA. By using methanol-fixed melanocytes as substrate, the optical density at 490 nm, a measure of purified IgG, was significantly higher for vitiligo patients (0.70 ± 0.16) than for the controls (0.25 ± 0.14) ($p < 0.05$, **Fig 1**).

A Significant Increase in Spontaneous Production of IL-6 and IL-8 in Patients with Active Vitiligo The spontaneous release of IL-6 and IL-8 was significantly higher in patients than in healthy controls (IL-6, 150.6 ± 32.5 pg per ml *vs.* 104.7 ± 15.9 pg per ml; IL-8, 1412.2 ± 857.7 pg per ml *vs.* 198.7 ± 31.6 pg per ml; $p < 0.05$). In contrast, differences in the spontaneous IL-1β, GM-CSF, TNF-α, and IFN-γ release were not significant **(Table I)**.

PHA, IgG-anti-MC, and N-IgG Induced a Higher IL-6 Production but Lower GM-CSF, TNF-α, and IFN-γ Release in the Patient Group as Compared with the Control Group As demonstrated in **Table I**, PHA, IgG-anti-MC, or N-IgG induced a significant increase in IL-6 production, and a decrease in GM-CSF, TNF-α, and IFN-γ release were found in patients with active vitiligo as compared with controls. Moreover, IgG-anti-MC induced significantly higher IL-1β production in controls than in patients ($p < 0.05$). PHA- or N-IgG-stimulated IL-1β production was similar between patients and normal controls. An additional interesting finding was that IgG-anti-MC in controls had a significantly higher stimulatory effect on IL-1β ($p < 0.005$) and IFN-γ ($p < 0.05$) production than did N-IgG.

DISCUSSION

Melanocytic cytotoxicity of immune mechanisms in vitiligo is a complex process involving effector–target attachment via pairs of adhesive molecules, activation of leukocyte receptors by target antigens and other cell-surface markers, and triggering of cytolysis (Martz, 1987; Norris, 1990; Morelli and Norris, 1993). Al Badri *et al* (1993) reported increased expression of intercellular adhesion

Table I. PHA, IgG-anti-MC, N-IgG Induced Higher IL-6 Production but Lower GM-CSF, TNF-α, and IFN-γ Release in the Patient Group (Pt) Compared to Controls (NC)[a]

Cytokine (pg/ml)	N = 12	Spontaneous Release	PHA (5 μg per ml)	IgG-anti-MC (100 μg per ml)	N-IgG (100 g per ml)
IL-1β	NC	88.7 ± 16.3[b]	657.2 ± 6.2	1054.6 ± 105.3[c]	792.3 ± 145.9[g]
	Pt	94.8 ± 18.1	673.8 ± 91.9	915.4 ± 134.0	756.1 ± 142.1[g]
IL-6	NC	104.7 ± 15.9[c]	572.1 ± 385.3[d]	809.2 ± 251.4[c]	827.8 ± 142.3[c]
	Pt	150.6 ± 32.5	1275.6 ± 427.8	1225.6 ± 288.4	1068.3 ± 231.5
IL-8	NC	198.7 ± 31.6[c]	5818.7 ± 1531.7	6621.7 ± 1570.2	6144.2 ± 1308.6
	Pt	1412.2 ± 857.7	6397.1 ± 1777.7	7104.9 ± 2303.4	6696.0 ± 1932.3
GM-CSF	NC	23.6 ± 51.6	1293.7 ± 97.4[e]	1269.4 ± 104.3[e]	1446.9 ± 212.7[e]
	Pt	33.4 ± 10.1	118.9 ± 84.8	115.0 ± 75.0	141.2 ± 105.7
TNF-α	NC	20.1 ± 51.1	1212.5 ± 502.9[e]	1255.0 ± 668.3[e]	1186.4 ± 541.3[c]
	Pt	37.4 ± 29.9	291.1 ± 137.5	268.5 ± 126.3	254.8 ± 127.6
IFN-γ	NC	54.3 ± 7.5	592.1 ± 66.3[d]	964.1 ± 117.5[d]	651.5 ± 105.8[df]
	Pt	73.9 ± 27.0	249.0 ± 108.6	267.4 ± 64.9	264.2 ± 110.3

[a] The mononuclear cells (1×10^6 cells per ml) obtained from patients with active vitiligo and from controls were incubated with medium (spontaneous release), PHA, IgG-anti-MC purified from active vitiligo, or N-IgG at 37°C for 24 h. The cell-free supernatants were measured for respective cytokines by ELISA kit.

[b] Value denotes mean ± SD. [c] $p < 0.05$; [d] $p < 0.01$; [e] $p < 0.001$, when comparing controls with patients; [f] $p < 0.05$; [g] $p < 0.005$, when comparing controls stimulated with IgG-anti-MC or N-IgG (using unpaired Student's t test).

molecule 1 (ICAM-1) on melanocytes in active vitiligo lesions. ICAM-1 is necessary for leukocyte–melanocyte attachment that develops into immunologic cytotoxicity (Morelli and Norris, 1993). Many cytokines such as IFN-γ, TNF-α, TNF-β, IL-1, IL-6, and IL-7 can induce the expression of cell surface ICAM-1 on melanocytes (Yohn *et al*, 1990, Kirnbauer *et al*, 1992). In this study, IL-6 production of MNCs was found to be significantly increased in patients with vitiligo. It is not inconceivable that this cytokine not only enhances melanocyte ICAM-1 expression, which may trigger leukocyte–melanocyte attachments, but also induces polyclonal B-cell activation, increasing autoantibody production and causing melanocyte damage in vitiligo. In addition, increased IL-8 production by patients activated monocytes may be able to attract polymorphonuclear neutrophils (Yoshimura *et al*, 1987) and T lymphocytes (Larsen *et al*, 1989) to the sites of lesions, amplifying inflammatory reactions and, therefore, facilitating melanocytic cytotoxicity.

Another interesting finding in this study is a decrease in the production of GM-CSF, TNF-α, and IFN-γ by MNCs in patients with active vitiligo. GM-CSF had been reported as an intrinsic factor for melanocyte growth (Imokawa *et al*, 1996). A significant decrease in GM-CSF release in active vitiligo may retard the proliferation of surviving melanocytes and recovery from vitiligo. TNF-α has been implicated as a fundamental inflammatory mediator (Vlassara *et al*, 1988; Tracey *et al*, 1989) and IFN-γ as an immunomodulator (Trinchieri *et al*, 1985). A significant decrease in TNF-α and IFN-γ production by mononuclear cells in active vitiligo may partially explain the reduced inflammatory reaction and cell infiltration (Moellmann *et al*, 1982; Kao and Yu, 1990) in lesions.

By using cultured melanocytes as a substrate, cellular ELISA revealed a higher optical density for IgG-anti-MC as compared to N-IgG. On the other hand, IgG-anti-MC and N-IgG exhibited a potent stimulatory effect on cytokine production by MNCs both in patients with active vitiligo and in normal individuals. This may indicate that MNCs *per se* are not the unique target cells for IgG-anti-MC. IgG-anti-MC, however, had a significantly higher stimulatory effect on IL-1β and IFN-γ production than did N-IgG in normal controls. Since IFN-γ stimulates IL-1β production by monocytes and major histocompatibility complex class I and class II expression on many cells, IgG-anti-MC may play a role in the inflammatory and immunologic processes of melanocyte destruction mediated by monocytes.

An increase in the production of proinflammatory cytokines, that is, IL-6 and IL-8, by the MNCs of active vitiligo patients may play an important role in melanocytic cytotoxicity through the enhancement of effector cell migration and effector–target attachment.

This study was supported by the National Science Council of the Republic of China research grant NSC 83–0412-B-037–005.

REFERENCES

Abdel-Naser MB, Ludwig W-D: Nonsegmental vitiligo: Decrease of the CD45RA+ T-cell subset and evidence for peripheral T-cell activation. *Int J Dermatol* 31:321–326, 1992

Al Badri AMT, Foulis AK, Todd PM, Garioch JJ, Gudgeon JE, Stewart DG, Gracie JA, Goudie RB: Abnormal expression of MHC class II and ICAM-1 by melanocytes in vitiligo. *J Pathol* 169:203–206, 1993

Al-Fouzan A, Al-Arbash M, Fouad F, Kaaba SA, Mousa MA, Al-Harbi SA: Study of HLA class I/II and T lymphocyte subsets in Kuwaiti vitiligo patients. *Eur J Immunogenet* 22:209–213, 1995

Betterle C, Caretto A, De Zio A, Pedini B, Veller-Fornasa C, Cecchetto A, Acccordi F, Peserico A: Incidence and significance of organ-specific autoimmune disorders (clinical, latent or only autoantibodies) in patients with vitiligo. *Dermatologica* 171:419–423, 1985

Cunliffe WJ, Halder RM, Newell DJ: Vitiligo, thyroid disease and autoimmunity. *Br J Dermatol* 80:135–139, 1968

D'Amelio R, Frati C, Fatlorossi A, Aiuti F: Peripheral T-cell subset imbalance in patients with vitiligo and in their apparently healthy first-degree relatives. *Ann Allergy* 65:143–145, 1990

Grimes PE, Ghoneum M, Stockton T, Payne C, Kelly P, Alfred L: T cell profiles in vitiligo. *J Am Acad Dermatol* 14:196–201, 1986

Hann SK, Park YK: Chung KY, Kim HI, Im S, Won JH: Peripheral blood lymphocyte imbalance in Koreans with active vitiligo. *Int J Dermatol* 32:286–289, 1993

Harsoulas P, Kanakouki-Tsakalidis F, Vyzantiadis A, Minas A, Cassimos C: Autoimmunity and vitiligo. *Arch Dermatol* 114:1154–1157, 1978

Hegedus L, Heidenheim M, Gervil M, Hjalgrim H, Hoier-Madsen M: High frequency of thyroid dysfunction in patients with vitiligo. *Acta Dermato-Venereol* (Stockholm) 74:120–123, 1994

Imokawa G, Yada Y, Kimura M, Morisaki N: Granulocyte/macrophage colony-stimulating factor is an intrinsic keratinocyte-derived growth factor for human melanocytes in UVA-induced melanosis. *Biochem J* 313:625–631, 1996

Kao CH, Yu HS: Depletion and repopulation of Langerhans cells in non-segmental type vitiligo. *J Dermatol* 17:287–296, 1990

Kao CH, Yu HS: A study of the effects of phorbol 12-myristate-13-acetate on cell differentiation of pure human melanocytes *in vitro*. *Arch Dermatol Res* 283:119–125, 1991

Kirnbauer R, Charvat B, Schauer K, Kock A, Urbanshi A, Forster E, Neuner P, Assmann I, Luger TA, Schwarz T: Modulation of intercellular adhesion molecule-1 expression on human melanocytes and melanoma cells: Evidence for a regulatory role of IL-6, IL-7, TNF-α and UVB light. *J Invest Dermatol* 98:320–326, 1992

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970

Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K: The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* 243:1464–1466, 1989

Martz E: LFA-1 and other accessory molecules functioning in adhesions of T and B lymphocytes. *Hum Immunobiol* 18:3–37, 1987

Moellmann G, Klein-Angfrer S, Scollay DA, Nordlund JJ, Lerner AB: Extracellular granular material and degeneration of keratinocytes in the normally pigmented epidermis of patients with vitiligo. *J Invest Dermatol* 79:321–330, 1982

Morelli JG, Norris DA: Influence of inflammatory mediators and cytokines on human melanocyte function. *J Invest Dermatol* 100:191s–195s, 1993

Mozzanica N, Frigerio U, Finzi F, Cattaneo A, Negri M, Scaglione F, Fraschini F, Foppa S: T cell subpopulations in vitiligo: A chronobiologic study. *J Am Acad Dermatol* 22:223–230, 1990

Mozzanica N, Frigerio U, Negri M, Tadini G, Villa ML, Mantovani M, Finzi AF: Circadian rhythm of natural killer cell activity in vitiligo. *J Am Acad Dermatol* 20:591–596, 1989

Mozzanica N, Villa ML, Foppa S, Vignati G, Cattaneo A, Diotti R, Finzi AF: Plasma α-melanocyte-stimulating hormone, β-endorphin, met-enkephalin, and natural killer cell activity in vitiligo. *J Am Acad Dermatol* 26:693–700, 1992

Naughton GK, Eisinger M, Bystryn JC: Antibodies to normal human melanocytes in vitiligo. *J Exp Med* 158:246–251, 1983a

Naughton GK, Eisinger M, Bystryn JC: Detection of antibodies to melanocytes by specific immuno-precipitation. *J Invest Dermatol* 81:540–542, 1983b

Norris DA: Cytokine modulation of adhesion molecules in the regulation of immunologic cytotoxicity of epidermal targets. *J Invest Dermatol* 95:111s–120s, 1990

Norris DA, Kissinger PM, Naughton GM, Bystryn JC: Evidence for immunologic mechanism in human vitiligo: Patients' sera induce damage to human melanocytes *in vitro* by complement-mediated damage and antibody-dependent cellular cytotoxicity. *J Invest Dermatol* 90:783–789, 1988

Soubiran P, Benzaken S, Bellet C, Lacour JP, Ortonne JP: Vitiligo: peripheral T-cell subset imbalance as defined by monoclonal antibodies. *Br J Dermatol* 113(suppl 28):124–127, 1985

Taher-Uz-Zaman, Begum S, Waheed MA: *In vitro* assessment of 'T' lymphocyte functioning in vitiligo. *Acta Dermato-Venereol* (Stockholm) 72:266–267, 1992

Tracey KJ, Vlassara H, Cerami A: Cachectin/TNF (tumour necrosis factor) *Lancet* 1:1122–1126, 1989

Trinchieri G, Perussia B: Immune interferon: a pleiotropic lymphokine with multiple effects. *Immunol Today* 6:131–136, 1985

Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A: Cachectin/TNF and IL-1 induced by glucose-modified protein: role in normal tissue remodeling. *Science* 240:1546–1548, 1988

Yohn JJ, Critelli M, Lyons MB, Norris DA: Modulation of melanocyte intercellular adhesion molecule-1 by immune cytokines. *J Invest Dermatol* 90:233–237, 1990

Yoshimura T, Matsushima K, Oppenheim JJ, Leonard EJ: Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL-1). *J Immunol* 139:788–793, 1987

Yu CL, Chang KL, Chiu CC, Chiang BN, Han SH, Wang SR: Alteration of mitogenic responses of mononuclear cells by anti-ds DNA antibodies resembling immune disorders in patients with systemic lupus erythematosus. *Scand J Rheumatol* 18:265–276, 1989b

Yu CL, Chang KL, Hong ST, Chiang BN, Han SH, Wang SR: Defective helper T cell function in IgM rheumatoid factor synthesis in patients with ankylosing spondylitis. *Scand J Rheumatol* 18:43–49, 1989a

Yu HS, Kao CH, Yu CL: Coexistence and relationship of antikeratinocyte and antimelanocyte antibodies in patients with non-segmental-type vitiligo. *J Invest Dermatol* 100:823–828, 1993

Zauli D, Tosti A, Biascs G, Miserocchi F, Patrizi A, Azzaroni D, Andriani G, Di Febo G, Callegari C: Prevalence of autoimmune atrophic gastritis in vitiligo. *Digestion* 34:169–172, 1986.