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Changes in T, B, and NK Lymphocyte Subsets during and after Normal Pregnancy

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Running head: Lymphocyte subsets during and after pregnancy.

ABSTRACT

PROBLEM: Pregnancy affects the maternal immune system and the clinical course of maternal diseases. Here we report the changes in the detailed lymphocyte subsets of helper T cells, suppressor T cells, $CD5^+$ B cells, T cell receptor (TCR) $\alpha\beta$-positive T cells (T$\alpha\beta$ cells), TCR$\alpha\beta$-negative T cells (T$\gamma\delta$ cells) and others during and after pregnancy through to one year postpartum, and discuss the significance of the changes.

METHOD: The absolute numbers of helper T cells, suppressor T cells, cytotoxic T cells, TCR$\alpha\beta$-negative T cells (T$\gamma\delta$ cells), $CD5^-$ B cells, $CD5^+$ B cells, and NK cell subsets were examined by two-color flow cytometry in peripheral blood from 51 healthy non-pregnant women, 106 healthy pregnant women, and 148 healthy postpartum women.

RESULTS: In early pregnancy, the numbers of suppressor T cells and NK cells with strong cytotoxity (NK^{+++} cells) increased, and the number of cytotoxic T cells decreased. In late pregnancy, the helper T cell and NK^{+++} cell numbers decreased. T$\alpha\beta$, $CD5^-$ B and $CD5^+$ B cells decreased during pregnancy. After delivery, helper T cells and cytotoxic T cells increased from 1 to 4 months postpartum, and suppressor T cells increased at 7 months postpartum. TCR$\alpha\beta$-negative T cells increased at 4 to 10 months postpartum. Both $CD5^-$ B and $CD5^+$ B cells decreased further at 1 month postpartum, but $CD5^+$ B cells increased markedly at 7 to 10 months postpartum.

CONCLUSIONS: These data indicate that 1) early increases of suppressor T cells and NK^{+++} cells during pregnancy may be related to the mechanism to accept or reject the fetus in early pregnancy, respectively; 2) late decreases of helper T cells and NK^{+++} cells may be related to the maintenance of pregnancy; 3) postpartum increases of helper T cells, cytotoxic T cells, TCR$\alpha\beta$-negative T cells (T$\gamma\delta$ cells) and $CD5^+$ B cells may be related to the postpartum aggravation of autoimmune diseases, and 4) the immunological effects of pregnancy remains until about one year after delivery.

INTRODUCTION

Pregnancy affects the maternal immune system and the clinical course of maternal diseases. Conversely, the maternal immune system also affects the maintenance of pregnancy, i.e, the survival of the fetus in utero.[1-3] There are many reports of decreases in some systemic parameters of maternal immunity during pregnancy that may be related to the survival of the fetus in utero.[1] During pregnancy, the numbers of peripheral lymphocytes, large granular lymphocytes (LGL), T, B, NK cells, T cell subsets (CD4$^+$ cells and CD8$^+$ cells), and activated T cells are decreased,[4-7] although the proportion of peripheral LGL increases at the first trimester.[7] The proliferative activity of peripheral lymphocytes in response to T cell mitogens and the cytotoxic activities of peripheral K cells[9] and NK cells[10] are also decreased. Furthermore, the serum levels of total IgG, IgM, and IgA,[11] and the levels of autoantibodies[12] are also decreased. Increased susceptibility to viral infection, virus reactivation,[13] and amelioration of autoimmune diseases[14] may also be evidence of decreased immunological activity of the mother during pregnancy.

In the postpartum period, in the contrast, there are reports suggesting increased activity of maternal immunity. The numbers of peripheral lymphocytes, LGL, T and NK cells,[4, 7] the cytotoxic activities of K and NK cells,[9, 10] and the serum levels of total IgG[11] are increased in the postpartum period. The aggravation of autoimmune diseases[15-17] also suggests the increased immunological activity of the mother.

Although there are many reports on lymphocyte subsets during pregnancy and a few reports in the postpartum period as noted above, there are few reports on more specific subsets of T, B, and NK cells examined by two-color flow cytometry.[18] In this study, therefore, we examined more detailed subsets of T, B, and NK cells, which are considered to have specific functions during pregnancy and in the postpartum period, in order to clarify the effects of pregnancy on the maternal immune system and on the clinical course of maternal diseases, such as infectious diseases and autoimmune diseases.

SUBJECTS AND METHODS

Subjects

The subjects studied were 51 healthy non-pregnant women (mean age: 31.2 ± 7.1 years); 106 healthy pregnant women, 38 in the first trimester (7-13 weeks; 29.5 ± 4.6 years), 30 in the second trimester (19-25 weeks; 29.8 ± 4.8 years), 38 in the third trimester (30-38 weeks; 29.1 ± 4.8 years); and 148 healthy postpartum women (30 at 1 month postpartum [29.9 ± 3.8 years], 35 at 4 months postpartum [28.6 ± 4.7 years], 33 at 7 months postpartum [28.7 ± 4.6 years], 27 at 10 months postpartum [30.4 ± 5.3 years], 23 at 13 months postpartum [29.1 ± 5.0 years]. The mean ages of the groups were not significantly different. Samples of peripheral venous blood were taken at 2:00-3:00 P.M. None of the subjects was receiving any medication, and all were nonsmokers. Subjects with associated complications, which were judged by clinical examination and general laboratory tests on pregnant women, and those with subclinical autoimmune diseases, which were judged by serological tests, including anti-nuclear antibody, rheumatoid factor, and anti-thyroid-microsomal antibody, were excluded from this study.

Monoclonal Antibodies

Fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibodies, anti-TCRα/β-1, anti-CD4, anti-CD8, anti-CD19, and anti-CD57 (Becton Dickinson, Mountain View, CA); and phycoerythrin (PE)-conjugated monoclonal antibodies, anti-CD3, anti-CD62L, anti-CD11b, anti-CD5, and anti-CD16 (Becton Dickinson) were used.

Differential Leukocyte Counts

Leukocyte and lymphocyte counts were obtained with an automated leukocyte differential system, Total Hematology Management System (THMS) H-6000 (Technicon Co., Tarrytown, NY), based on principles of cytochemistry, electrooptical measurement, and signal logic processing.

Lymphocyte Subsets

Lymphocyte subsets shown in Table 1 were analyzed by flow cytometry with the FITC-conjugated and PE-conjugated monoclonal antibodies described above.[19] Samples of 100 µl of EDTA-treated whole blood were washed once to exclude the effect of plasma, and incubated for 30 min at 4°C with 20 µl of FITC-conjugated and PE-conjugated monoclonal antibodies. Then the samples were hemolysed and fixed with lysing reagent (FACS™ lysing solution; Becton Dickinson). They were then washed once and subjected to two-color flow cytometry in a FACScan™ with an auto-compensation system (Becton Dickinson) to determine the percentage of each lymphocyte subset in total lymphocytes. The absolute numbers of these lymphocyte subsets in whole blood were calculated as the products of the percentages of each lymphocyte subset and the absolute lymphocyte number, which were obtained with a Total Haematology Management System H-6000 (Techniton).

Statistics

Data were analyzed by Student's t-test, or the Mann-Whitney U-test when variances were unequal. Probability values less than 5% were considered significant.

RESULTS

Lymphocyte subpopulations (Fig. 1)

The number of lymphocytes decreased during pregnancy, and increased after delivery from 4 to 10 months postpartum. The numbers of $CD3^+$ T cells and $CD19^+$ B cells showed similar changes to the lymphocyte number.

T cell subpopulations

The number of $CD3^+TCR\alpha/\beta-1^+$ TCR$\alpha\beta$-positive T cells (T$\alpha\beta$ cells) (Fig. 1) decreased during pregnancy, but was not higher in the postpartum period than that in non-pregnant women. In contrast, the number of $CD3^+TCR\alpha/\beta-1^-$ TCR$\alpha\beta$-negative T cells (T$\gamma\delta$ cells)[20] (Fig. 2) did not change during pregnancy, but increased after delivery from 4 to 10 months postpartum. The number of $CD4^+$ cells (Fig. 1) decreased during pregnancy, but was not higher in the postpartum period than that in non-pregnant women. The number of $CD8^+$ cells (Fig. 1) showed no significant change during and after pregnancy.

T cell subsets (Fig. 2)

The number of $CD8^+CD11b^-$ cytotoxic T (T$_C$) cells[21] decreased in the first trimester of pregnancy, and increased at 4 months postpartum. The number of $CD4^+CD62L^-$ helper T (T$_H$) cells, which have major helper functions,[22] decreased in the third trimester, and increased at 4 months postpartum. In contrast, the number of $CD8^+CD11b^+$ suppressor T (T$_S$) cells[23] increased in the first trimester of pregnancy, and increased again at 7 months postpartum. The number of $CD4^+CD62L^+$ inducer T (T$_I$) cells, which have weak helper and inducer functions,[22, 24] decreased during pregnancy, but was not higher in the postpartum period than that in non-pregnant women (Fig. 1), as well as that of $CD4^+$ cells.

B cell subsets (Fig. 3)

The number of $CD19^+CD5^-$ conventional B ($CD5^-B$) cells decreased during pregnancy and at 1 month postpartum, and returned to the non-pregnant level. The number of $CD19^+CD5^+$ B ($CD5^+B$) cells decreased during pregnancy and at 1 month postpartum, but increased at 7 to 10 months postpartum.

NK cell subsets (Fig. 4)

The number of $CD16^+CD57^-$ NK (NK+++) cells, which were reported to have strong cytotoxic activity,[25, 26] increased in the first trimester of pregnancy, decreased in the third trimester of pregnancy, and returned to the non-pregnant level after delivery. The number of $CD16^+CD57^+$ NK (NK++) cells, which have medium cytotoxic activity,[25, 26] decreased in the third trimester of pregnancy, and returned to the non-pregnant level after delivery. The number of $CD16^-CD57^+$ NK (NK+) cells, which have weak cytotoxic activity,[25, 26] showed no significant change during pregnancy, but increased after delivery from 1 to 4 months postpartum.

DISCUSSION

In this study, we clarified that (1) a decrease in peripheral lymphocytes during pregnancy was caused by decreases in both T and B cells, (2) an increase in peripheral lymphocytes in the postpartum period was caused by increases in both T and B cells, (3) a decrease in T cells during pregnancy was caused by a decrease in T$\alpha\beta$ cells, especially CD4$^+$ T cells, (4) an increase in T cells in the postpartum period was caused by an increase in TCR$\alpha\beta$-negative T cells [TCR$\alpha\beta$-negative T cells represent T$\gamma\delta$ cells, because of a significant correration between the percentages of CD3$^+$TCRα/β-1$^-$ cells and CD3$^+$TCRγ/δ-1$^+$ cells[20]], (5) a decrease in B cells during pregnancy was caused by decreases in both CD5$^-$ B and CD5$^+$ B cells, and (6) an increase in B cells in the postpartum period was caused by an increase in CD5$^+$ B cells. Furthermore, we found the characteristic changes of fine lymphocyte subsets during and after pregnancy, which may explain part of the mechanisms for the maintenance of pregnancy, the increased susceptibility to viral infection during pregnancy,[13] postpartum aggravation of autoimmune diseases,[15-17] and so on.

In early pregnancy, peripheral NK^{+++} cells, which have strong NK cytotoxicity,[25, 26] increased, which is consistent with our previous findings that the proportion of LGL and the activity of NK cells are higher in early pregnancy than in non-pregnant controls.[7, 10] An excessive increase of NK^{+++} cells in early pregnancy may cause recurrent spontaneous abortions, because the NK cell activity in recurrent aborters is higher than that in normal pregnant women in early pregnancy.[27, 28]

We also found that suppressor T (T$_S$) cells increased in early pregnancy. This may be related to our previous finding that the ratio of serum concentration of soluble CD8 to the number of CD8$^+$ cells is increased in the first and second trimester of pregnancy.[29] Furthermore, this is partially compatible with previous reports that short-lived suppressor cell activity and the proportion of CD4$^+$CD45RA$^+$ T suppressor/inducer cells are increased during pregnancy.[18, 30] This increase of T$_S$ cells seems to cause a decrease of cytotoxic T (T$_C$) cells and the following decreases of helper T (T$_H$) cells,

CD5⁻ B cells and probably CD5⁺ B cells during pregnancy, because the immune system is considered to be regulated by the balance between T_H cells and T_S cells.[31] An increase of NK⁺⁺⁺ cells in early pregnancy may also be related to decreases of T_C cells and B cells, because NK cells kill or suppress activated B cells and dendritic cells that function as antigen-presenting cells and suppress both the generation of immunoglobulins and T_C cells.[32, 33]

The decrease of T_C cells in early pregnancy observed in the present study supports the previous finding that cytotoxic T cell activity is suppressed during pregnancy,[34] and suggests that a decrease of T_C cells is also important for the maintenance of pregnancy and the pregnancy-induced amelioration of autoimmune diseases, in which T_C cells are considered to be the main effector for tissue destruction.[35, 36] We found that CD5⁺ B cells decreased progressively and markedly during pregnancy. This finding is consistent with a report by Bhat et al.[37] CD5⁺ B cells are known to be increased in autoimmune disease and to produce autoantibodies.[38, 39] Therefore, this decrease of CD5⁺ B cells during pregnancy may explain the amelioration of autoimmune diseases, especially those in which autoantibodies play an important role in the pathogenesis.[40, 41] A decrease in CD5⁺ B cells during pregnancy may be useful for the acceptance of the fetal allograft and successful outcome of a pregnancy, because the proportion of peripheral CD5⁺ B cells in women with recurrent spontaneous abortion of immunological etiology is higher than that in controls.[42] The conventional CD5⁻ B cells were also observed to be decreased during pregnancy in the present study. This explains the decreases in serum levels of IgG, IgM, and IgA during pregnancy.[11]

In contrast to early pregnancy, NK⁺⁺⁺ cells decreased in late pregnancy, which seems to be a useful adaptation to help ensure fetal survival. NK⁺⁺ cells, which have moderate NK cytotoxicity,[25, 26] were also decreased during this period. These findings are also consistent with our previous reports that LGL number and NK cell activity decrease in late pregnancy,[7, 10] and may explain the increased susceptibility to viral infection and virus reactivation during pregnancy.[13]

Decreases in the numbers of total lymphocytes, T cells, and B cells, and the above-mentioned characteristic changes in T, B, and NK lymphocytes were observed during pregnancy. These changes may be partially caused by the effects of pregnancy or pregnant-associated hormones on thymus[43] and bone marrow,[44] showing progressive reduction of thymic size and cellularity, and inhibition of T and B cell development at the precursor level. Furthermore, pregnancy-associated cytokines such as GM-CSF,[45] TGFβ,[46] interferon-τ,[47] IL-10,[47] and TJ6,[48] which are produced by the interaction between the fetus and the immune system at the maternal-fetal interface, may also be related to these changes, especially increases in NK^{+++} cells and T_S cells, and a decrease in T_C cells in early pregnancy, although the precise mechanism is not clear.

In the postpartum period, characteristic changes in T, B, and NK cell subsets suggesting an enhancement of immune reaction were observed. These changes may be caused by a rebound of pregnancy-induced immune suppression.[14, 15] At 1-4 months postpartum, NK^+ cells increased. This is consistent with our previous reports that LGL and $CD57^+$ NK cells increase at 4 months postpartum.[4, 7] Furthermore, T_C cells and TCRαβ-negative T cells (Tγ/δ cells) also increased from 1 to 4 months postpartum, and an increase in the latter continued until 10 months postpartum. Increases in these cytotoxic cells represent an enhancement of cell-mediated cytotoxicity at 1-4 months postpartum, and may be useful for the defense against postpartum infection. However, this increase of cytotoxic activity may cause the aggravation of autoimmune diseases. Indeed, rheumatoid arthritis and Hashimoto's disease, in which T_C cells and TCRαβ-negative T cells (Tγ/δ cells) are considered to be the main effector cells for tissue destruction,[20, 35, 49] are frequently aggravated at 1-4 months postpartum.[15, 50, 51] In contrast, $CD5^+$ B cells showed a marked increase in the postpartum period, showing the peak between 7 and 10 months postpartum. $CD5^+$ B cells, which produce autoantibodies,[38] increase in autoimmune diseases, especially in the active stage of Graves' disease,[39] which is caused by anti-TSH receptor autoantibodies. Graves' disease frequently develops or relapses in the postpartum period,[51] especially between 4 and 12 months postpartum. Therefore, a postpartum increase of $CD5^+$ B cells may cause the

onset or the aggravation of autoimmune diseases, in which autoantibodies are important for the pathogenesis, such as Graves' disease.[41]

T_H cells increased at 4 months postpartum, which was accompanied by an increase in T_C cells and followed by an increase in B cells ($CD5^+$ B cells). However, this enhancement of immune reaction in the postpartum period was transient. The increase in T_S cells at 7 months postpartum observed in this study may suppress the enhancement and stabilize maternal immune system. Very surprisingly, however, recovery of the maternal immune system to the non-pregnant level took about one year after delivery. In addition to the marked characteristic changes in subsets of T, B, and NK lymphocytes during and after normal pregnancy, it is important for considering maternal physiology to pay attention to the very long-term effect of pregnancy on the maternal immune system.

ACKNOWLEDGMENT

REFERENCES

1. Gleicher N and Siegel I: Pregnancy and the immune state. *In*: Autoimmune endocrine disease. New York, John Wiley & Sons, 1983, pp.225-45.

2. Clark DA, Arck PC, Jalali R, Merali FS, Manuel J and Chaouat G: Psycho-neuro-cytokine/endocrine pathways in immunoregulation during pregnancy. Am J Reprod Immunol 1996;35:330-7.

3. Robertson SA, Seamark RF, Guilbert LJ and Wegmann TG: The role of cytokines in gestation. Crit Rev Immunol 1994;14:239-92.

4. Iwatani Y, Amino N, Tachi J, Kimura M, Ura I, Mori M, et al.: Changes of lymphocyte subsets in normal pregnant and postpartum women: Postpartum increase of NK/K (Leu 7) cells. Am J Reprod Immunol Microbiol 1988;18:52-5.

5. Plum J, Thiery M and Sabbe L: Distribution of mononuclear cells during pregnancy. Clin Exp Immunol 1978;31:45-9.

6. Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R, et al.: Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. J Immunol 1985;135:3172-7.

7. Iwatani Y, Amino N, Kabutomori O, Kaneda T, Tanizawa O and Miyai K: Peripheral large granular lymphocytes in normal pregnant and postpartum women: decrease in late pregnancy and dynamic change in the puerperium. J Reprod Immunol 1989;16:165-72.

8. Kim HS, Degiannis D, Raskova J and Raska KJ: Cyclosporine A and prednisolone inhibit lectin- and alloantigen-induced release of sCD8: correlation with proliferative responses. Clin Immunol Immunopathol 1991;60:27-39.

9. Asari S, Iwatani Y, Amino N, Tanizawa O and Miyai K: Peripheral K cells in normal human pregnancy: decrease during pregnancy and increase after delivery. J Reprod Immunol 1989;15:31-7.

10. Hidaka Y, Amino N, Iwatani Y, Kaneda T, Mitsuda N, Morimoto Y, et al.: Changes in natural killer cell activity in normal pregnant and postpartum women: increases in the

first trimester and postpartum period and decrease in late pregnancy. J Reprod Immunol 1991;20:73-83.

11. Amino N, Tanizawa O, Miyai K, Tanaka F, Hayashi C, Kawashima M, et al.: Changes of serum immunoglobulins IgG, IgA, IgM, and IgE during pregnancy. Obsete Gynecol 1978;52:415-20.

12. Amino N, Kuro R, Tanizawa O, Tanaka F, Hayashi C, Kotani K, et al.: Changes of serum anti-thyroid antibodies during and after pregnancy in autoimmune thyroid disease. Clin Exp Immunol 1978;31:30-7.

13. Sakamoto K, Greally J and Gilfillan RF: Epstein-Barr virus in normal pregnant women. Am J Reprod Immunol 1982;2:217-21.

14. Amino N and Miyai K: Postpartum autoimmune endocrine syndromes *In*: Autoimmune endocrine disease New York, John Wiley & Sons, 1983, pp.247-72.

15. Amino N, Mori H, Iwatani Y, Tanizawa O, Kawashima M, Thuge I, et al.: High prevalence transient post-partum thyrotoxicosis and hypothyroidism. N Engl J Med 1982;306:849-52.

16. Garsenstein M, Pollak VE and Karl RM: Systemic lupus erythematosus and pregnancy. N Engl J Med 1962;267:165-9.

17. Oka M: Effect of pregnancy on the onset and course of rheumatoid arthritis. Ann Rheum Dis 1953;2:227-9.

18. Matthiesen L, Berg G, Ernerudh J and Håkansson L: Lymphocyte subsets and mitogen stimulation of blood lymphocytes in normal pregnancy. Am J Reprod Immunol 1996;35:70-9.

19. Stites DP: Clinical laboratory methods for detection of cellular immunity *In*: Basic & clinical immunology East Norwalk, Prentice-Hall International Inc., 1994, pp.195-215.

20. Iwatani Y, Amino N, Hidaka Y, Kaneda T, Ichihara K, Tamaki H, et al.: Decreases in $\alpha\beta$ T cell receptor negative T cells and CD8 cells, and an increase in CD4^{+} CD8^{+} cells in active Hashimoto's disease and subacute thyroiditis. Clin Exp Immunol 1992;87:444-9.

21. Clement LT, Dagg MK and Landay A: Characterization of human lymphocyte subpopulations: Alloreactive cytotoxic T-lymphocyte precursor and effector cells are phenotypically distinct from Leu 2$^+$ suppressor cells. J Clin Immunol 1984;4:395-402.

22. Gatenby PA, Kansas GS, Xian XY, Evans RL and Engleman EG: Dissection of immunoregulatory subpopulations of T lymphocytes within the helper and suppressor sublineages in man. J Immunol 1982;129:1997-2000.

23. Landay A, Gartland GL and Clement LT: Characterization of a phenotypically distinct subpopulation of Leu-2$^+$ cells that suppresses T cell proliferative responses. J Immunol 1983;131:275761.

24. Damle NK and Engleman EG: Antigen-specific suppressor T lymphocytes in man. Clin Immunol Immunopathol 1989;53:S17-S24.

25. Lanier LL, Le AM, Phillips JH, Warner NL and Babcock GF: Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 1983;131:1789-96.

26. Abo T, Miller CA and Balch CM: Characterization of human granular lymphocyte subpopulations expressing HNK-1 (Leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. Eur J Immunol 1984;14:616-23.

27. Coulam CB, Stephenson M, Stern JJ and Clark DA: Immunotherapy for recurrent pregnancy loss: Analysis of results from clinical trials. Am J Reprod Immunol 1996;35:352-9.

28. Higuchi K, Aoki K, Kimbara T, Hosoi N, Yamamoto T and Okada H: Suppression of natural killer cell activity by monocytes following immunotherapy for recurrent spontaneous aborters. Am J Reprod immunol 1995;33:221-7.

29. Watanabe M, Iwatani Y, Hidaka Y, Mitsuda N and Amino N: Changes in soluble CD4 and CD8 proteins in healthy pregnant and postpartum women. Am J Reprod Immunol (in press).

30. Gastilla JA, Vargas ML, Garcia-Trtosa C, Garcia-Pachero M, Gonzalez-Gomez F and Garcia-Olivares E: Short-lived suppressor cell activity during normal human pregnancy. J Reprod Immunol 1990;18:139-45.

31. Imboden JB: T lymphocytes & natural killer cells *In*: Basic & clinical immunology East Norwalk, Prentice-Hall International Inc., 1994, pp.94-104.

32. Gilbertson SM, Shah PD and A. RD: NK cells suppress the generation of LYT-2$^+$ cytolytic T cells by suppressing or eliminating dendritic cells. J Immunol 1986;136:3567-71.

33. Brieva JA, Targan S and Stevens RH: NK and T cell subsets regulate antibody production by human in vivo antigen-induced lymphoblastoid B cells. J Immunol 1984;132:611-5.

34. Nakamura N, Miyazaki K, Kitano Y, Fujisaki S and Okamura H: Suppression of cytotoxic T-lymphocyte activity during human pregnancy. J Reprod Immunol 1993;23:119-30.

35. Feldmann M, Brennan FM and Maini RN: Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996;14:397-440.

36. Tomer Y and Davies TF: Infections and autoimmune endocrine disease. Baillieres Clin Endocrinol Metab 1995;9:47-70.

37. Bhat NM, Mithal A, Bieber MM, Herzenberg LA and Teng NNH: Human CD5$^+$ B lymphocytes (B-1 cells) decrease in peripheral blood during pregnancy. J Reprod Immunol 1995;28:53-60.

38. Hayakawa K and Hardy RR: Normal, autoimmune, and malignant CD5+ B cells: The LY-1 B lineage? Ann Rev Immunol 1988;6:197-218.

39. Iwatani Y, Amino N, Kaneda T, Ichihara K, Tamaki H and Tachi J: Marked increase of CD5$^+$ B cells in hyperthyroid Graves' disease. Clin Exp Immunol 1989;78:196-200.

40. Willcox N: Myasthenia gravis. Curr Opin Immunol 1993;5:910-7.

41. Volpé R: Graves' Disease - Pathogenesis *In*: The Thyroid Philadelphia, J.B. Lippincott Company, 1991, pp.648-57.

42. Kwak JY, Beaman KD, Gilman-sachs A, Ruiz JE, Schewitz D and Beer AE: Up-regulated expression of CD56$^+$, CD56$^+$/CD16$^+$, and CD19$^+$ cells in peripheral blood lymphocytes in pregnant women with recurrent pregnancy losses. Am J Reprod Immunol 1995;34:93-9.

43. Clarke AG and Kendall MD: The thymus in pregnancy: the interplay of neural, endocrine and immune influences. Immunol Today 1994;15:545-51.

44. Medina KL, Smithson G and Kincade PW: Suppression of B lymphopoeisis during normal pregnancy. J Exp Med 1993;178:1507-15.

45. Athanassakis I, Bleackley RC, Paetkau V, Guilbert L, Barr PJ and Wegmann TG: The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. J Immunol 1987;138:37-44.

46. Clark DA, Falbo M, B. RR, Banwatt D and Stedronska-Clark J: Active suppression of host-vs graft reaction in pregnant mice. J Immunol 1988;141:3833-40.

47. Chaouat G, Assal MA, Martal J, Raghupathy R, Elliot J, Mosmann T, et al.: IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-τ. J Immunol 1995;154:4261-8.

48. Beaman K, Angkachatchai V and Gliman-Sachs A: TJ6: The pregnancy-associated cytokine. Am J Reprod Immunol 1996;35:338-41.

49. Iwatani Y, Hidaka Y, Matsuzuka F, Kuma K and Amino N: Intrathyroidal lymphocyte subsets, including unusual $CD4^+$ $CD8^+$ cells and $CD3^{lo}TCR\alpha\beta^{lo/-}CD4^-CD8^-$ cells, in autoimmune thyroid disease. Clin Exp Immunol 1993;93:430-6.

50. Scott JR and Ward K: Autoimmune disease in pregnancy. Immunol Allergy Clin North Am 1990;10:119-31.

51. Amino N: Postpartum Thyroid disease In: Advances in Perinatal Thyroidology New York, Plenum Press, 1991, pp.167-180.

Figure Legends

Figure 1:

Changes in absolute numbers of peripheral lymphocytes, T cells, B cells, $CD4^+$ T cells, $CD8^+$ T cells, TCR$\alpha\beta$-positive T (T$\alpha\beta$) cells, and inducer T (T$_I$) cells during and after pregnancy.

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (compared with non-pregnant control)

Figure 2:

Changes in absolute numbers of TCR$\alpha\beta$-negative T (T$\gamma\delta$) cells, helper T (T$_H$) cells, suppressor T (T$_S$) cells, and cytotoxic T (T$_C$) cells during and after pregnancy.

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (compared with non-pregnant control)

Figure 3:

Changes in absolute numbers of $CD5^-$ B cells and $CD5^+$ B cells during and after pregnancy.

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (compared with non-pregnant control)

Figure 4:

Changes in absolute numbers of NK cell subsets during and after pregnancy.

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (compared with non-pregnant control)

Table 1   Lymphocyte subsets examined in this study

Subsets	Major population
CD3$^+$	T cells
CD3$^+$TCRα/β-1$^+$	TCR$\alpha\beta$-positive cells (T$\alpha\beta$ cells)
CD3$^+$TCRα/β-1$^-$	TCR$\alpha\beta$-negative T cells (T$\gamma\delta$ cells)
CD4$^+$	helper/inducer T cells (TH/I)
CD4$^+$CD62L$^+$	inducer T cells (TI)
CD4$^+$CD62L$^-$	helper T cells (TH)
CD8$^+$	suppressor/cytotoxic T cells (Ts/c)
CD8$^+$CD11b$^+$	suppressor T cells (Ts)
CD8$^+$CD11b$^-$	cytotoxic T cells (Tc)
CD19$^+$	B cells
CD5$^+$CD19$^+$	CD5$^+$ B cells
CD5$^-$CD19$^+$	CD5$^-$ B cells
CD16$^-$CD57$^+$	NK cells with weak cytotoxity (NK$^+$)
CD16$^+$CD57$^+$	NK cells with medium cytotoxity (NK^{++})
CD16$^+$CD57$^-$	NK cells with strong cytotoxity (NK^{+++})

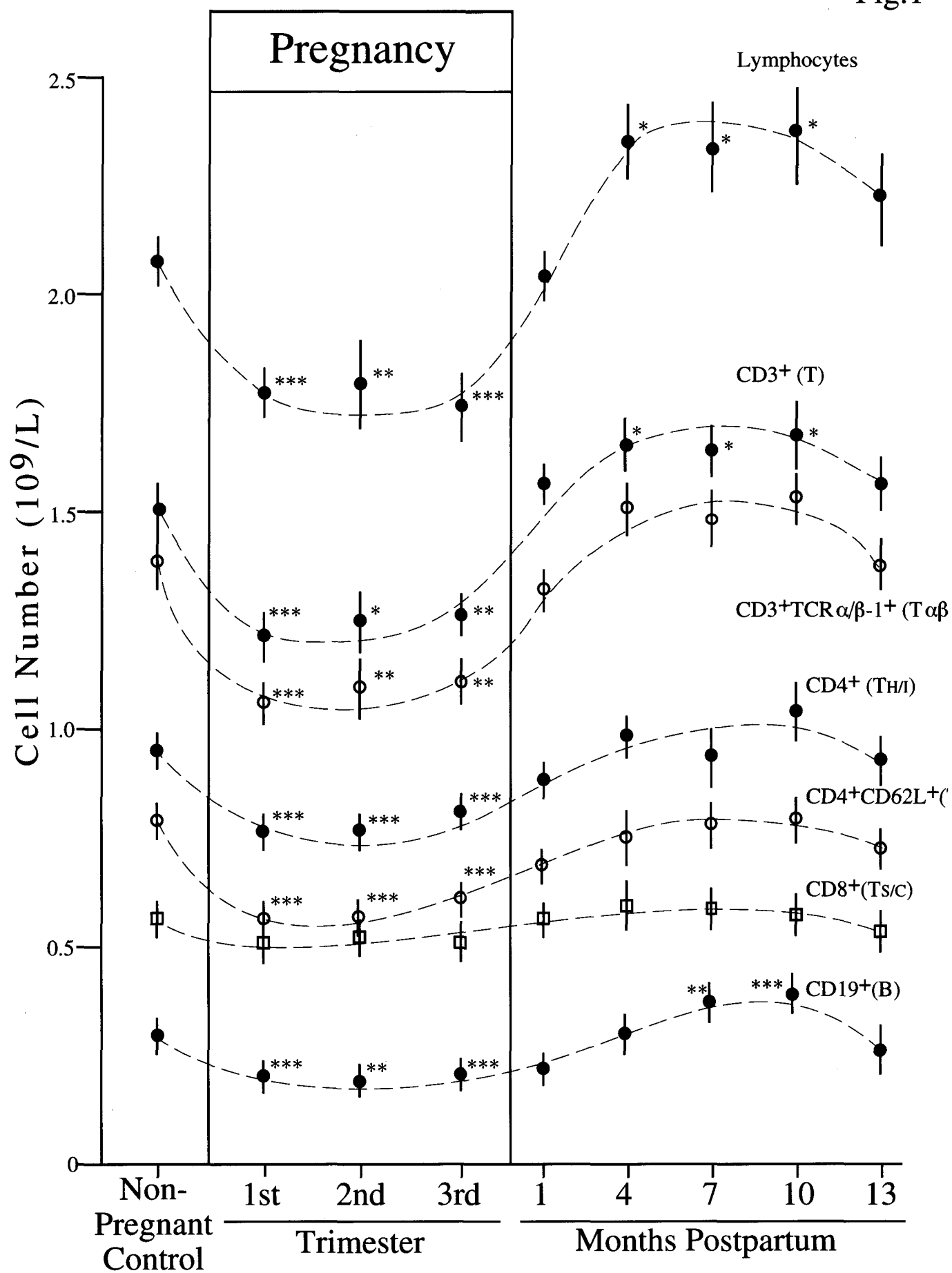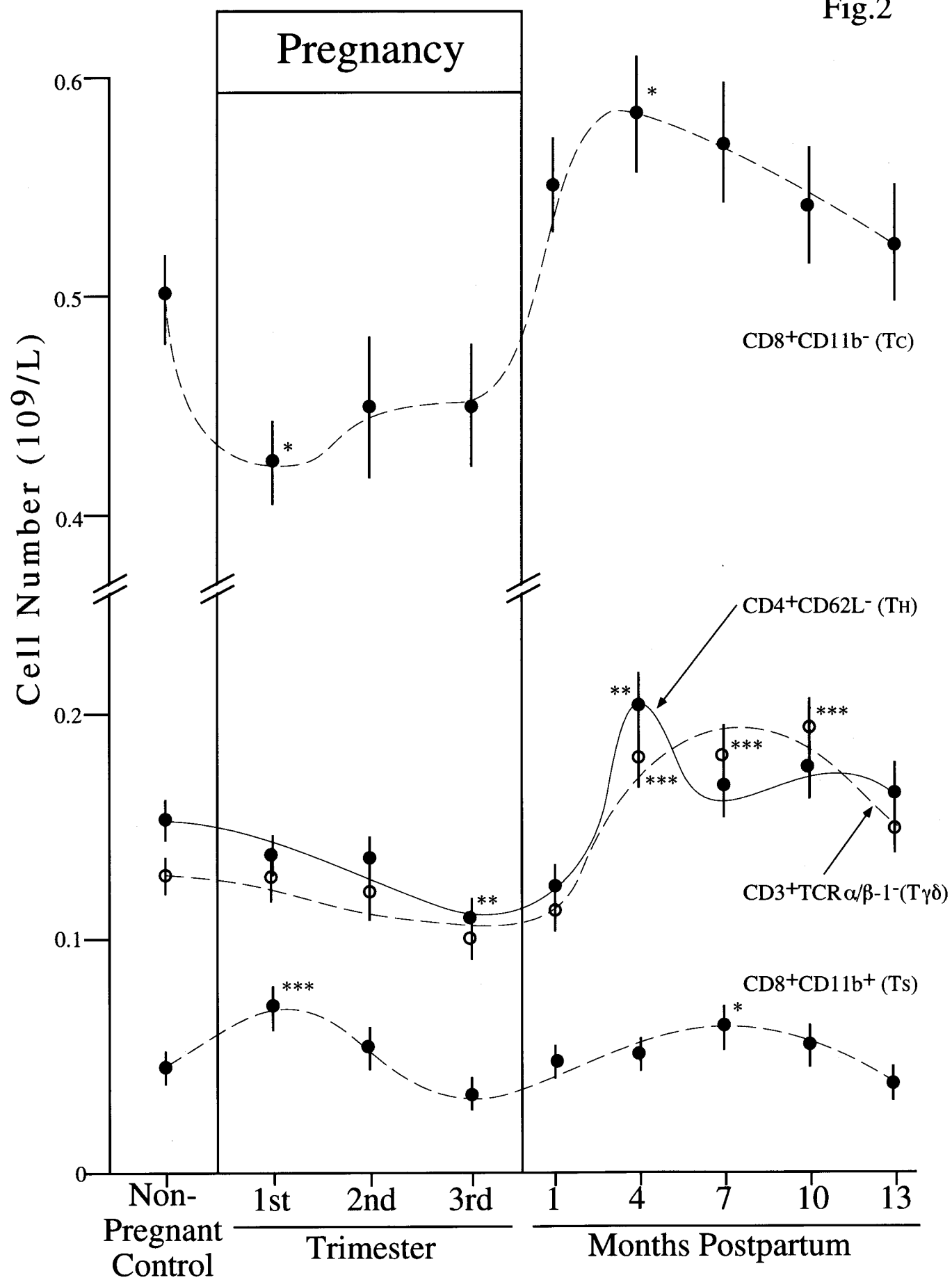TCR$\alpha\beta$: T cell receptor α and β chains
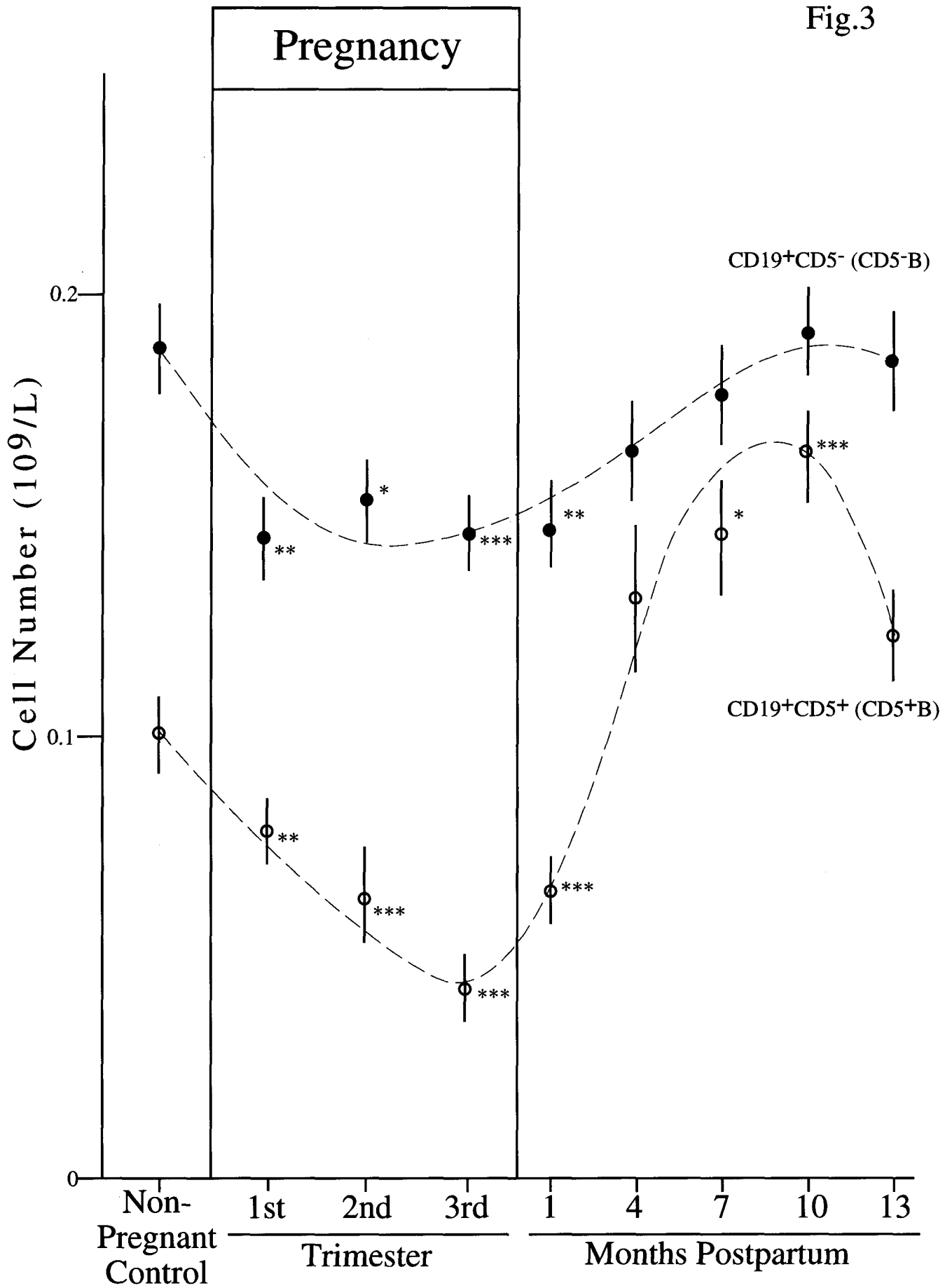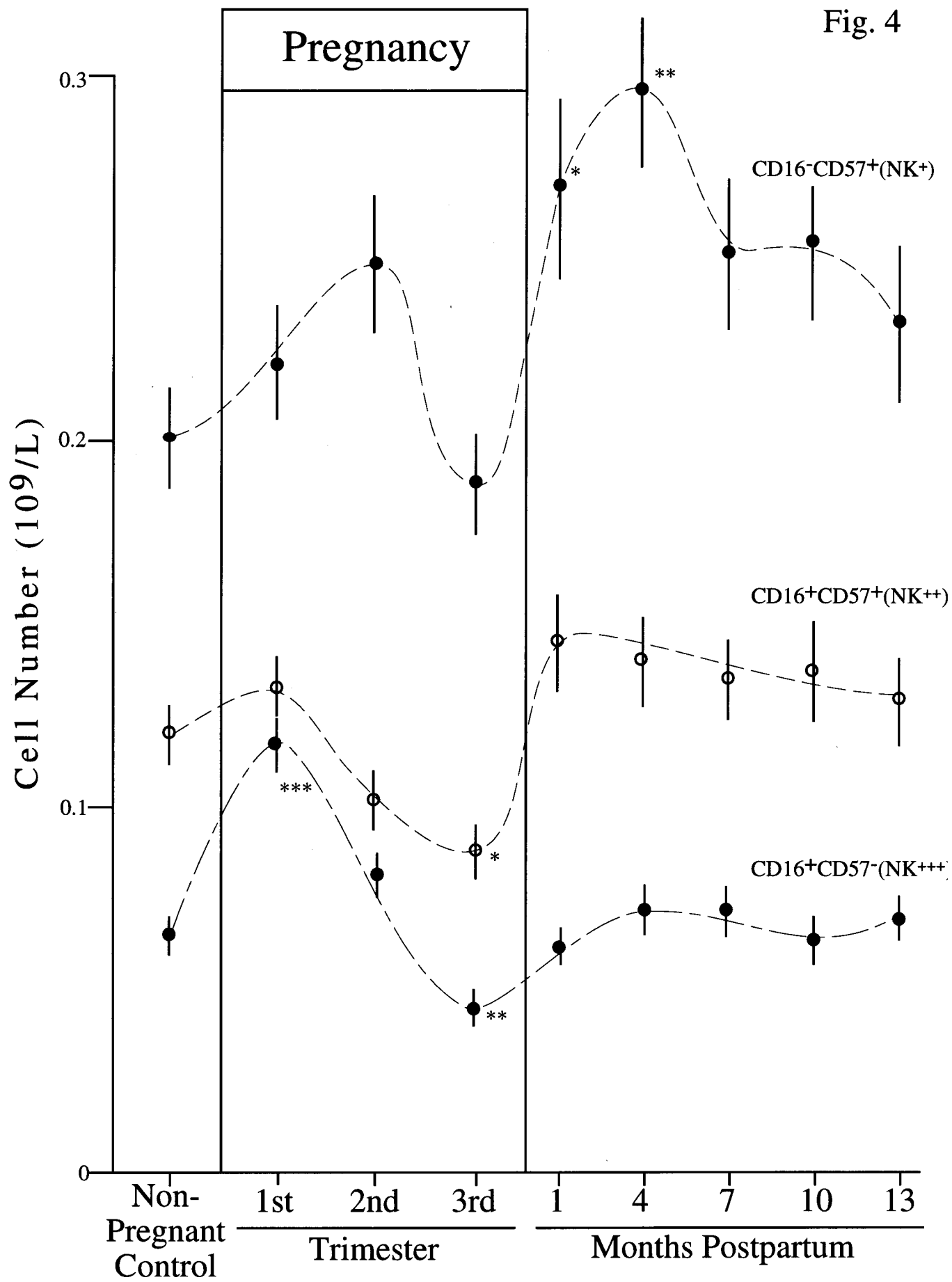
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参考論文

Changes in Soluble CD4 and CD8 Proteins in Healthy Pregnant and Postpartum Women

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PROBLEM: We examined physiological changes in serum levels of soluble CD4 (sCD4) and soluble CD8 (sCD8) during pregnancy and 1–12 months postpartum to study changes in the maternal immune system during and after pregnancy.

METHOD: The serum concentrations of sCD4 and sCD8 were measured by enzyme immunoassay in the sera separated from blood samples withdrawn from healthy women in the 1st, 2nd, and 3rd trimesters of pregnancy and 1,4,7,and 10–12 months postpartum (n=182) and healthy non-pregnant women (n=25), and in 90 of the women, the changes in sCD4 and sCD8 were compared with changes in the number of peripheral CD4^{+} and CD8^{+} cells measured by flow cytometry.

RESULTS: The serum concentration of sCD4 decreased throughout pregnancy, from the first trimester, and recovered gradually after delivery. The serum concentration of sCD8 did not change significantly during or after pregnancy compared to the concentration in the non-pregnant controls, but the concentration 1 month postpartum was significantly higher than that in the 3rd trimester. The numbers of CD4^{+} and CD8^{+} cells decreased during pregnancy but did not change significantly after delivery. Interestingly, the ratio of the serum sCD4 level to the number of CD4^{+} cells decreased and the ratio of the sCD8 level to the number of CD8^{+} cells increased in the first and second trimesters of pregnancy, but these ratios were within the normal range from the third trimester of pregnancy to 10–12 months postpartum.

CONCLUSIONS: Decreases in serum sCD4 concentration and in the ratio sCD4/CD4^{+} cells, and an increase in the ratio sCD8/CD8^{+} cells may be important factors in the immunological changes that occur during pregnancy.

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INTRODUCTION

Pregnancy affects the maternal immune system.[1] It has been suggested that local immunosuppression in utero is important for the maintenance of pregnancy, that is, for the survival of the fetus in utero.[2-5] However, many studies have reported of decreases in some systemic parameters of maternal immunity during pregnancy, although these findings are still controversial.[6-22] During pregnancy, for example, there are decreases in numbers of peripheral lymphocytes; large granular lymphocytes (LGL); T, B, and NK cells; T cell subsets (CD4, CD8); and activated T cells.[6-9]; the proliferative activity of peripheral lymphocytes in response to mitogen[10]; the cytotoxic activity of peripheral K cells[11] and NK cells[12]; serum levels of total IgG, IgM, and IgA;[13] and decreases in the levels of autoantibodies.[14] Increased suscepti-

bility to viral infection and virus reactivation,[15] and amelioration of autoimmune disease[16,17] may also be evidence of decreased maternal immunological activity during pregnancy.

In the postpartum period, in contrast, the increased numbers of peripheral lymphocytes, LGL, T cells, and NK cells,[6,9] the increased cytotoxic activity of K and NK cells, the increased levels of serum IgG,[13] and the exacerbation of autoimmune disease[23-25] suggest increased immunological activity in the mother. There are, however, few reports of studies on the immune system in this period.

Recently, it has been reported that many proteins lead a dual existence as both membrane-bound and soluble isoforms.[26] Membrane-bound CD4 and CD8 proteins on $CD4^+$ and $CD8^+$ cells, both of which are very important for immune regulation, are required for interaction with antigen-presenting cells or target cells to induce specific immune responses.[27] Soluble isoforms of CD4 and CD8 have been measured in various diseases and have been suggested to play some immunological role,[28-32] but the precise functions of these molecules are still unknown.

In this study, we examined the serum levels of soluble CD4 (sCD4) and soluble CD8 (sCD8) during and after pregnancy to clarify the changes that take place in the maternal immune system during and after pregnancy.

SUBJECTS AND METHODS

Subjects

Eight groups of healthy women were studied. Group 1 consisted of 36 subjects in the first trimester of pregnancy (7–13 weeks; age, 21–37 years; mean age, 28.0 years), group 2 consisted of 29 subjects in the second trimester (19–25 weeks; 22–39 years; 28.8 years), and group 3, 29 subjects in the third trimester (30–38 weeks; 23–37 years; 29.5 years). Groups 4, 5, 6, and 7 were postpartum; group 4 consisted of 29 subjects 1 month postpartum (age, 19–39 years; mean, 30.5 years), group 5, 22 at 4 months postpartum (20–38 years; 27.2 years), group 6, 15 at 7 months postpartum (20–35 years; 26.5 years), and group 7, 22 at 10–12 months postpartum (21–39 years; 29.8 years). The eighth group consisted of 25 healthy non-pregnant women as controls (age, 21–45 years; mean, 30.2 years). The mean ages of the groups were not significantly different. None of the subjects was receiving any medication at the time of the study and none had any associated complications or autoimmune disease, assessed by clinical and laboratory examinations, including serological tests for antinuclear antibody, rheumatoid factor, and anti-thyroid microsomal antibody. Blood samples were collected and sera were separated and stored at -70°C until use.

sCD4 and sCD8 Assay

Soluble CD4 and CD8 were examined with commercially available enzyme immunoassay kits for sCD4 and sCD8 (Cellfree CD4 test kit and Cellfree CD8 test kit, T Cell Diagnostics, Inc., Cambridge, MA), employed according to the manufacturer's instructions. Both of these assays are sandwich ELISA, using two monoclonal antibodies, the second being peroxidase-conjugated. Briefly, 96-well microplates were coated with murine monoclonal antibodies to CD4 and CD8, respectively. After the coating, the coating solution was discarded and the plates were incubated for 2 hours at 37°C with 300 μl of blocking solution per well, followed by four washings. Sera, at final dilutions of 1:3 (for sCD4) and 1:9 (for sCD8) were applied to the plate, and the plates were incubated at 37°C for 2 hours and for 90 minutes, respectively. After four washings, the plate was incubated with horseradish peroxidase (HRP)-conjugated murine monoclonal antibodies directed against different epitopes of human CD4 or CD8. After the removal of unbound HRP-conjugate by washing, o-phenylenediamine was added to each well and the plates were incubated for 30 min at room temperature. The reaction was stopped by the addition of 2 N H_2SO_4 and the absorbance was measured at 490 nm. The results, expressed as U/ml, were determined from standard curves obtained with six standard CD4 samples (ranging from 0 to 160 U/ml) and five standard CD8 samples (ranging from 0 to 2,000 U/ml).

T Cell Subsets

T cell subsets were analyzed in 10–15 subjects from each group of pregnant and postpartum women (87 subjects in all) and in 20 of the non-pregnant controls. Analysis was carried out with FITC-conjugated anti-CD4 (Leu-3a) and phycoerythrin (PE)-conjugated anti-CD8 (Leu-2a) antibodies (Becton Dickinson, Mountain View, CA) and with a FACScan (Becton Dickinson, Mountain View, CA).[33] Briefly, 100 μl samples of EDTA-treated whole blood were incubated for 30 minutes at 4°C with 10 μl of FITC- or PE-conjugated monoclonal antibodies, with shaking at 10 minute intervals. The samples were then hemolyzed and fixed with FACS lysing solution (Becton Dickinson, Mountain View, CA). They were then washed once and subjected to two-color flow cytometry to determine the percentages of each lymphocyte subset in the total lymphocyte population. Absolute counts of lymphocyte subsets in whole blood were calculated as the products of the percentages of each lymphocyte subset and the absolute lymphocyte count obtained with an automated leukocyte differential system, Total Hematology Management System H-6000 (Technicon, Tarrytown, NY).

Statistics

The statistical significance of differences in the amounts of sCD4 and sCD8 molecules, and in percentages of T cell subsets was evaluated by the Mann-Whitney U-test, because of unequal variances. Correlations were established using Pearson's correlation coefficient.

RESULTS

Changes in sCD4 and sCD8 Concentrations

The serum concentrations of sCD4 were significantly lower in the first, second, and third trimesters of pregnancy [n=36, 14.2 ± 2.2 (S.E.) U/ml, $P<0.0001$; n=29, 15.4 ± 4.2 U/ml, $P<0.0001$; and n=29, 11.1 ± 1.6 U/ml, $P<0.0001$, respectively] than in the non-pregnant controls (n=25, 44.1 ± 6.1 U/ml) (Fig. 1a). The serum concentration of sCD4 1 month postpartum (n=29, 27.9 ± 4.2 U/ml,) was significantly higher ($P<0.001$) than the concentration in the third trimester of pregnancy, but was still significantly lower ($P<0.05$) than in the non-pregnant controls. The sCD4 concentrations 4, 7, and 10–12 months postpartum (n=22, 31.9 ± 4.9 U/ml; n=15, 32.5 ± 4.4 U/ml; and n=22, 41.4 ± 5.8 U/ml, respectively) were not significantly different from the concentration in the non-pregnant controls.

The serum concentration of sCD8 was not significantly different either during or after pregnancy compared with the concentration in the non-pregnant controls (n=25, 227.5 ± 22.4 U/ml) (Fig. 1b). However, the concentration of sCD8 1 month postpartum (n=29, 243.7 ± 15.9 U/ml, $P<0.01$) was significantly higher than that in the third trimester (n=29, 187.0 ± 9.6 U/ml).

The sCD4/sCD8 ratio was significantly lower during pregnancy (first trimester: n=36, 0.079 ± 0.015, $P<0.0001$; second trimester: n=29, 0.080 ± 0.020, $P<0.0001$; and third trimester: n=29, 0.063 ± 0.009, $P<0.0001$) and 1 month postpartum (n=29, 0.129 ± 0.023, $P<0.01$) than in the non-pregnant controls (n=25, 0.217 ± 0.027). There was no significant difference between the sCD4/sCD8 ratios in non-pregnant controls and in women 4, 7, or 10–12 months postpartum.

Changes in Absolute Count of CD4+T Cells and CD8+ T Cells

The absolute counts of peripheral CD4+ T cells were significantly lower in the first and second trimesters (n=12, 549.1 ± 42.2, $P<0.001$; n=14, 510.3 ± 65.6, $P<0.005$, respectively) than in the non-pregnant controls (n=20, 854.4 ± 56.2). The absolute counts of CD8+ T cells were lower in the second trimester of pregnancy(n=14, 389 ± 44.9, $P<0.05$) than in the non-pregnant controls (n=20, 473.5 ± 36.5) (Fig. 2).

The ratio of CD4+cells/CD8+cells was not significantly different during pregnancy and postpartum compared with that in the non-pregnant controls (n=20, 1.51 ± 0.02).

Correlation Between Soluble Proteins and T Cell Subsets

There was a significant correlation between the serum concentration of sCD4 and the absolute number of peripheral CD4+ T cells in 107 women (20 non-pregnant, 38 pregnant, and 49 postpartum) (r=0.392, $P<0.001$), but there was

no significant correlation between the concentration of sCD8 and the absolute number of CD8+ T cells. There was no correlation between the concentration of sCD4 (or sCD8) and the percentage of CD4+ (or CD8+) T cells in the total lymphocyte population.

Changes in sCD4/CD4+ Cells and sCD8/CD8+ Cells

The ratio of the serum sCD4 level to the number of CD4+ cells in peripheral blood (sCD4/CD4+ cells) was significantly lower during pregnancy (first trimester, n=12, [0.6 ± 0.3(S.E.)]×10^{-2}, $P<0.0001$; second trimester, n=14, [0.4 ± 0.2]×10^{-2}, $P<0.0001$; and third trimester, n=14, [0.4×0.1]×10^{-2}, $P<0.0001$) than in non-pregnant controls (n=20, [3.3 ± 0.7]×10^{-2}); the ratio had partially recovered 1 month postpartum (n=15, [1.8 ± 0.5]×10^{-2}, NS) (Fig.3). The ratio then declined slightly from 1 to 7 months postpartum and had completely recovered 10–12 months postpartum (n=13, [2.4 ± 0.4]×10^{-2}, NS) (Fig.3).

The ratio of sCD8/CD8+ cells was significantly higher in the first and second trimesters of pregnancy (first trimester, n=12, [6.3 ± 1.3(S.E.)]×10^{-1}, $P<0.05$; second trimester, n=14, [6.6 ± 1.4(S.E.)]×10^{-1}, $P<0.05$) than in non-pregnant controls (n=20, [4.0 ± 0.1]×10^{-1}) and had recovered to the normal level in the third trimester (Fig.3). The ratio of sCD8/CD8+ cells was then slightly, but not significantly, increased 1 month postpartum (n=15, [4.8 ± 0.5]×10^{-1}, NS), declining slightly from 1 to 7 months postpartum, and slightly increasing again from 7 to 10–12 months postpartum (n=13, [4.9 ± 0.7]×10^{-1}, NS) (Fig.3).

DISCUSSION

Soluble CD4 and CD8 are the soluble counterparts of membrane-bound CD4 and CD8, and are shed from CD4+ and CD8+ T cells, respectively.[26] These membrane-bound proteins are important for T cells to recognize specific antigens presented by antigen-presenting cells or target cells, and to induce specific immune responses.[27] However, the functions of sCD4 and sCD8 have not been yet clarified. Many clinical findings for sCD4 and/or sCD8 have been reported in various diseases[28–32,34–37]; however, to our knowledge, there are no reports on sCD4 and sCD8 during and after pregnancy.

Serum levels of sCD4 are increased in autoimmune diseases, such as rheumatoid arthritis,[29] systemic lupus erythematosus,[30] and Sjögren syndrome,[34] and in viral infections, such as measles[35] and human immunodeficiency virus (HIV) infection.[36] In rheumatoid arthritis, interestingly, serum levels of sCD4 were correlated positively with levels of soluble interleukin-2 receptor (sIL-2R), and fell preceding clinical improvement.[29] These reports suggest that increased levels of serum sCD4 reflect increased immunological activity or abnormalities.

In this study, we found that serum levels of sCD4 were decreased during pregnancy. The effect exerted by hemodi-
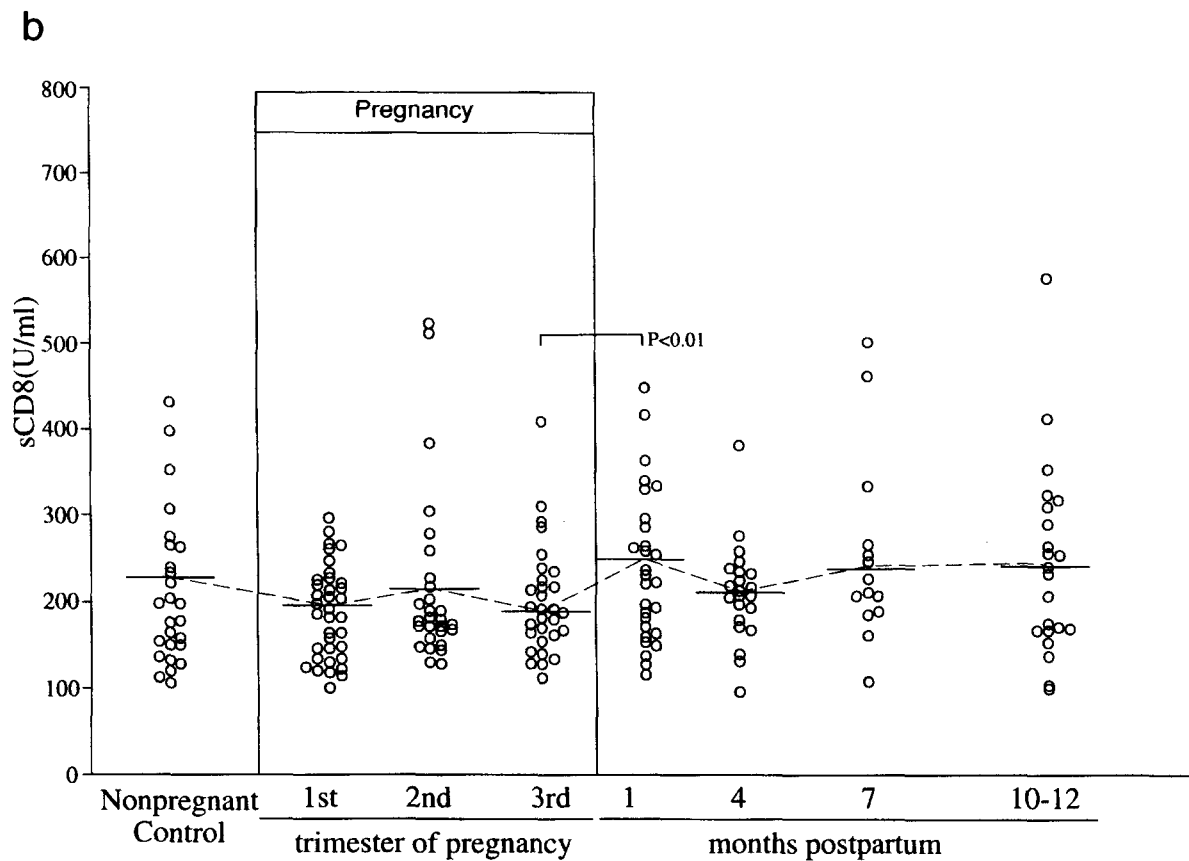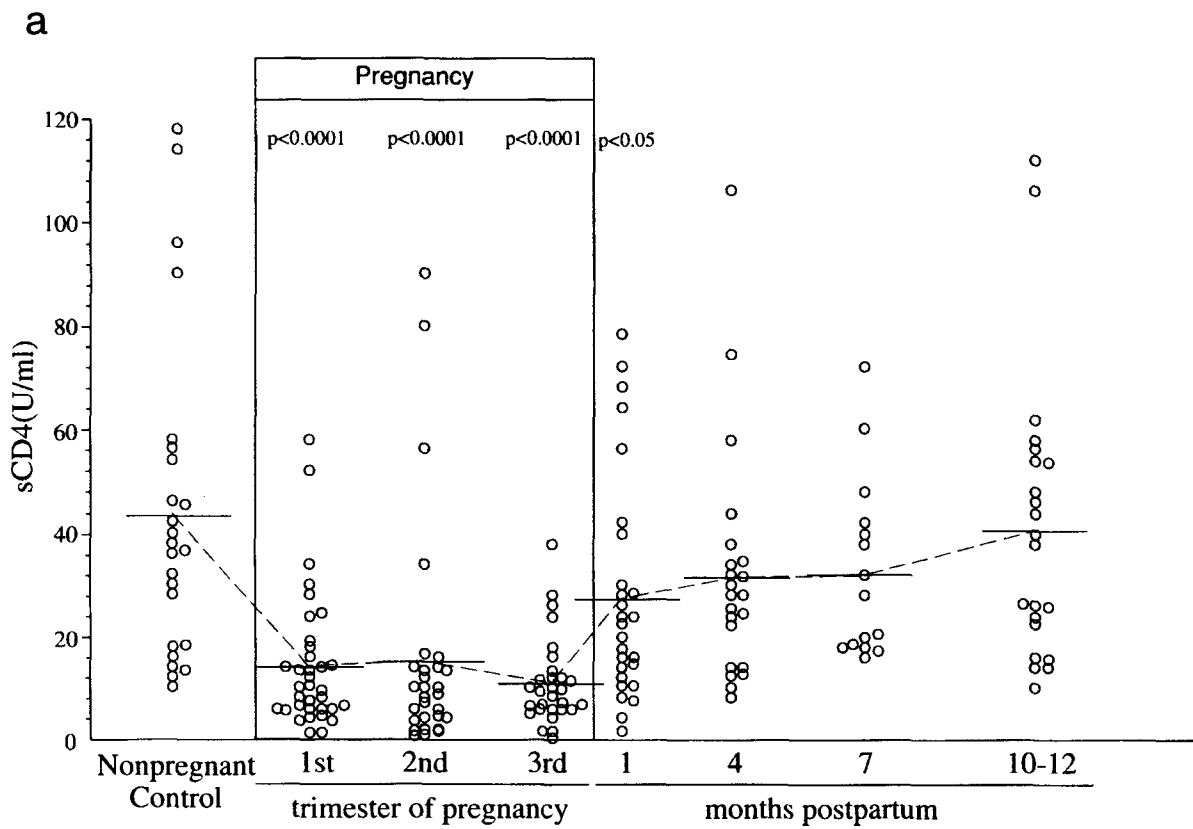
a

b

Fig. 1. Changes in serum concentrations of sCD4 (a) and sCD8 (b) during and after pregnancy.
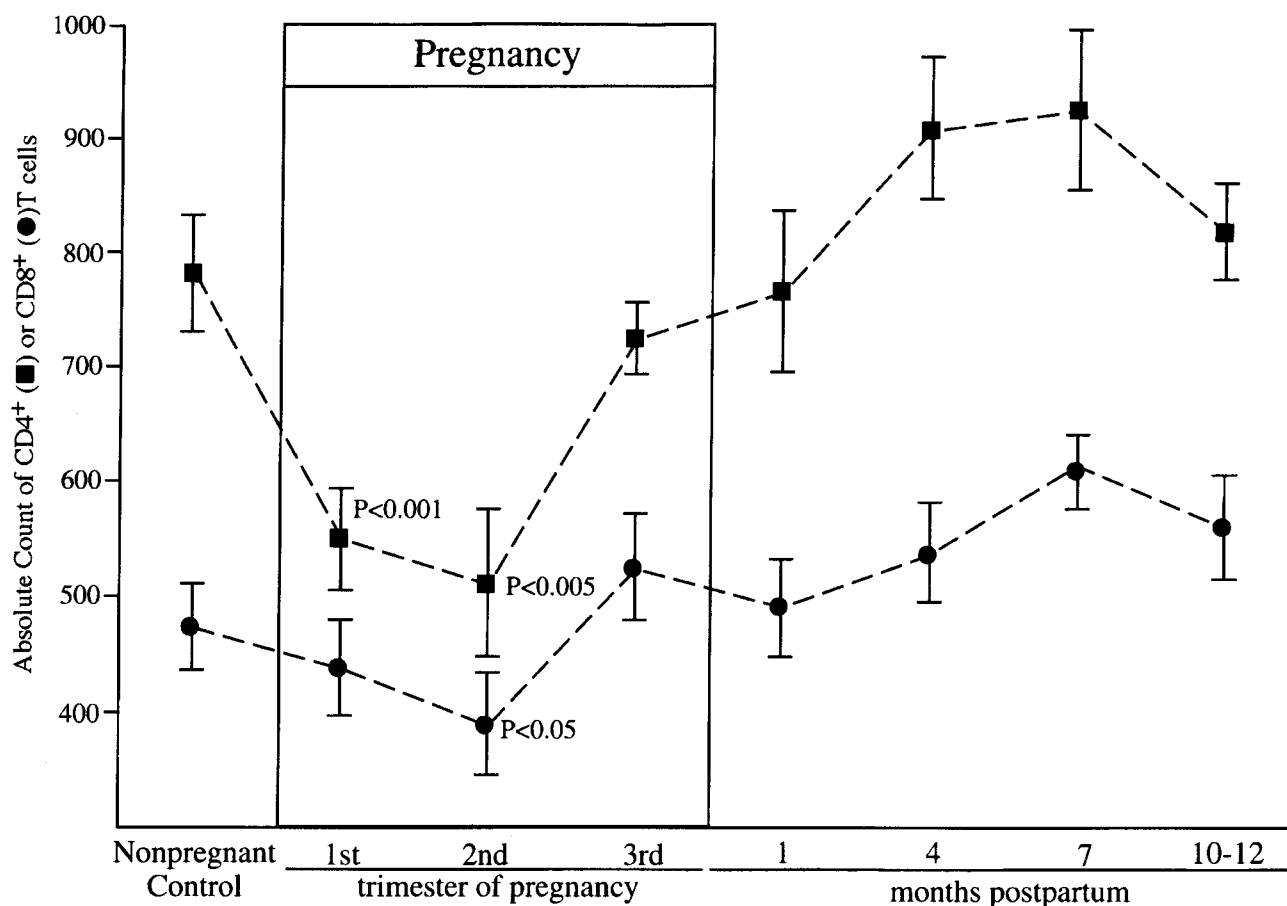
Fig. 2. Changes in absolute count of CD4+ and CD8+ T cells during and after pregnancy.

lution on the decrease of sCD4 during pregnancy does not appear to be important, since we found that the level of sCD4 was about 70% lower during pregnancy than the level in the non-pregnant controls; in contrast with the reported 10–20% decrease in the level of hemoglobin[38] and the approximately 30% decrease in albumin[39] brought about by hemodilution during pregnancy. The level of sCD4 increased rapidly after delivery and recovered to the non pregnant level 4 months postpartum. The decrease of sCD4 during pregnancy indicates that maternal immunological activity may be suppressed during pregnancy, as has been shown in previous studies.[40–42] However there are some reports showing that systemic suppression is not necessary for pregnancy.[43,44]

Weak correlation between the serum levels of sCD4 and the numbers of peripheral CD4+ cells suggests that the decrease in serum sCD4 levels during pregnancy is caused, in part, by a decrease in the number of CD4+ cells. We also found that the ratio sCD4/CD4+ cells was decreased during pregnancy. This indicates that the shedding of sCD4 from CD4+ cells is decreased during pregnancy, and that the decrease in serum sCD4 levels during pregnancy is due

mainly to this decrease. Furthermore, it has also been suggested that the activity of CD4+ T helper/inducer cells is decreased during pregnancy.[29]

Soluble CD8 levels are increased in systemic lupus erythematosus[30] and in insulin-dependent diabetes mellitus,[37] whereas, on the other hand, sCD8 is decreased in rheumatoid arthritis[28] and in Sjögren syndrome.[34] Although serum levels of sCD8 show various changes in autoimmune disease, levels are increased in infectious diseases such as EBV-induced infectious mononucleosis,[31] measles,[32] and HIV infection.[36] We found no correlation between the serum levels of sCD8 and the absolute number of peripheral CD8+ cells, but it has been reported that the levels of sCD8 were positively correlated with the number of peripheral HLA-DR+ CD8+ T cells.[31,45] It has also been reported that the release of sCD8 occurs at an early stage in the activation of cytotoxic T cells.[10] Thus, it is suggested that serum levels of sCD8 reflect the activity of cytotoxic T cells.

In this study, we found that sCD8 was increased significantly in 1 month postpartum compared to the level in the 3rd trimester of pregnancy, suggesting that cytotoxic T
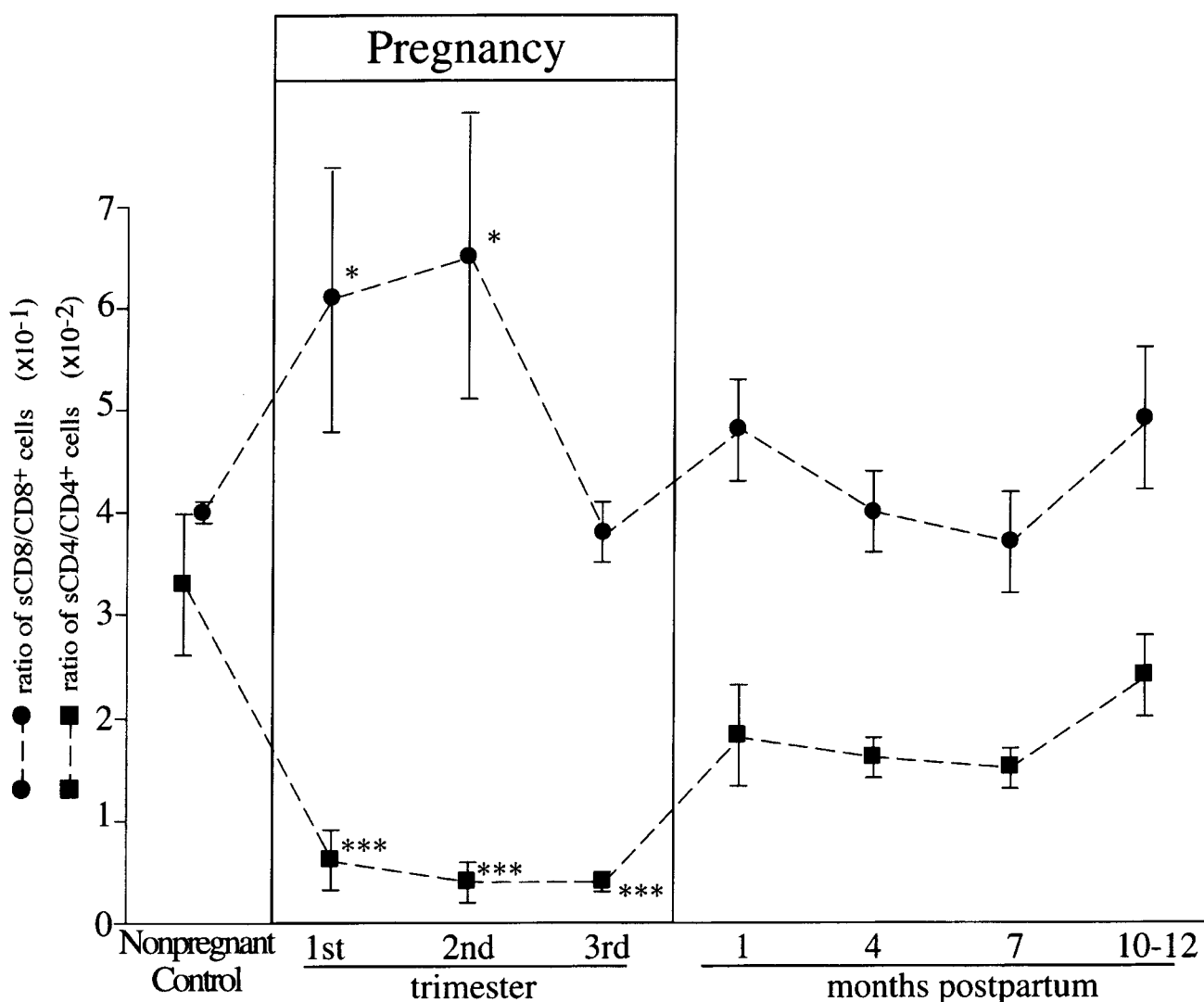
Fig. 3. Changes in the ratios of sCD4/CD4⁺ cells and sCD8;/CD8⁺ cells during and after pregnancy. Closed symbols and vertical bars indicate means ± S.E. *$p<0.05$; ***$p<0.0001$, compared with non-pregnant controls.

cells may be activated 1 month postpartum. This possibility is supported by clinical findings showing that destructive exacerbation of autoimmune diseases, such as rheumatoid arthritis and autoimmune thyroid disease, occurs 1–3 months postpartum.[25,46] Interestingly, the density of CD8 molecules on CD8⁺ cells was reported to be decreased during the exacerbation of autoimmune thyroid disease,[47] suggesting an increase in the shedding of CD8 molecules from CD8⁺ cells. The relative increase in the ratio of sCD8/CD8⁺ cells 1 month postpartum may also lend support to this idea, although it was not significant.

It is very interesting that the ratios of sCD4/CD4⁺ cells and sCD8/CD8⁺ cells changed in an opposite manner in the first and second trimesters of pregnancy, whereas both ratios changed in a parallel fashion from the third trimester of pregnancy throughout the postpartum period to

10–12 months postpartum (Fig. 3). In the light of the correlation between the activities of CD4⁺ or CD8⁺ cells and the release of sCD4 or sCD8, respectively,[29,31,45] the increase in the ratio of sCD8/CD8⁺ cells and the marked decrease in the ratio of sCD4/CD4⁺ cells in the first and second trimesters of pregnancy suggests that suppressor/cytotoxic T cells (CD8⁺ cells) are activated and stimulated to release sCD8, and that helper/inducer T cells (CD4⁺ cells) are suppressed, reducing the release of sCD4. It has been reported that, in non-pregnant women, the release of sCD8 increases during the early stage of cytotoxic T cell activation.[10] However, it is possible that suppressor T cells may be activated in the first and second trimesters of pregnancy, since cytotoxic T cell activity is suppressed during pregnancy.[48] If suppressor T cells are so activated, then this opposite change in the ratios of sCD4/CD4⁺ cells and

sCD8/CD8⁺ cells during early and middle pregnancy may be related to the maintenance of pregnancy. In any case, these different changes in the ratios of sCD4/CD4⁺ cells and sCD8/CD8⁺ cells between the first and second and the third trimester of pregnancy indicate that maternal immune dynamics are very different between early and middle pregnancy and late pregnancy.

The ratio of CD4⁺ T cells to CD8⁺ T cells (CD4/CD8 cell ratio) is often used as a marker of immunological activity,[49] but this cell ratio was not significantly changed during pregnancy.[6] However, we found that the serum sCD4/sCD8 ratio decreased markedly during pregnancy and recovered 4 months postpartum, showing changes similar to those in serum sCD4 levels. Considering the increased susceptibility, during pregnancy, to viral infection and virus reactivation,[15] and the amelioration of autoimmune disease,[16,17] these findings suggest that, during pregnancy, serum sCD4 levels and the sCD4/sCD8 ratio, as well as the ratios of sCD4/CD4⁺ cells and sCD8/CD8⁺ cells, may be more useful as clinical indices of some types of immunological activity than the CD4/CD8 cell ratio.

REFERENCES

1. Gleicher N, and Siegel I. Pregnancy and the immune state. In: Autoimmune endocrine disease. John Wiley and Sons, New York, 1983, pp 225–245.
2. Sargent IL. Maternal and fetal immune responses during pregnancy. Exp Clin Immunogenet 1993; 10:85–102.
3. Hunt JS. Immunobiology of pregnancy. Curr Opin Immunol 1992; 4:591–596.
4. Arkwright PD, Redman CWG. Syncytiotrophoblast membrane glycoprotein components block lymphocyte proliferation by interfering with an early event in their activation. In Chaouat, Mowbray, Cellular and molecular biology of the maternofetal relationship Paris, Colloque INSERM, 1991, pp 227–235.
5. Lea RG, Flanders KC, Harley CB, Manuel J, Banwatt D, Clark DA. Release of a transforming growth factor (TGF)-β2-related suppressor factor from postimplantation murine decidual tissue can be correlated with the detection of a subpopulation of cells containing RNA for TGF-b2. J Immunol 1992; 148:778–787.
6. Iwatani Y, Amino N, Tachi J, Kimura M, Ura I, Mori M, et al. Changes of lymphocyte subsets in normal pregnant and postpartum women: Postpartum increase of NK/K (Leu 7) cells. Am J Reprod Immunol Microbiol 1988; 18:52–55.
7. Plum J, Thiery M, Sabbe L. Distribution of mononuclear cells during pregnancy. Clin Exp Immunol 1978; 31:45–49.
8. Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R, et al.. Soluble interleukin 2 receptors are relased from activated human lymphoid cells in vitro. J Immunol 1985; 135:3172–3177.
9. Iwatani Y, Amino N, Kabutomori O, Kaneda T, Tanizawa O, Miyai K. Peripheral large granular lymphocytes in nor-
mal pregnant and postpartum women: decrease in late pregnancy and dynamic change in the puerperium. J Reprod Immunol 1989; 16:165–172.
10. Kim HS, Degiannis D, Raskova J, Raska KJ. Cyclosporine A and prednisolone inhibit lectin- and alloantigen-induced release of sCD8: correlation with proliferative responses. Clin Immunol Immunopathol 1991; 60:27–39.
11. Asari S, Iwatani Y, Amino N, Tanizawa O, Miyai K. Peripheral K cells in normal human pregnancy: Decrease during pregnancy and increase after delivery. J Reprod Immunol 1989; 15:31–37.
12. Hidaka Y, Amino N, Iwatani Y, Kaneda T, Mitsuda N, Morimoto Y, et al. Changes in natural killer cell activity in normal pregnant and postpartum women: Increases in the first trimester and postpartum period and decrease in late pregnancy. J Reprod Immunol 1991; 20:73–83.
13. Amino N, Tanizawa O, Miyai K, Tanaka F, Hayashi C, Kawashima M, et al.. Changes of serum immunoglobulins IgG, IgA, IgM, and IgE during pregnancy. Obsete Gynecol 1978; 52:415–420.
14. Amino N, Kuro R, Tanizawa O, Tanaka F, Hayashi C, Kotani K, et al. Changes of serum anti-thyroid antibodies during and after pregnancy in autoimmune thyroid disease. Clin Exp Immunol 1978; 31:30–37.
15. Sakamoto K, Greally J, Gilfillan RF. Epstein-Barr virus in normal pregnant women. Am J Reprod Immunol 1982; 2:217–221.
16. Amino N, Miyai K. Postpartum autoimmune endocrine syndromes. In Autoimmune endocrine disease. John Wiley and Sons, New York, 1983, pp 247–272.
17. Persellin RH: The effect of pregnancy on rheumatiod arthritis. Bull Rheum Dis 1976; 27:922–927.
18. Glassman AB, Bennett CE, Christopher JB, Self S. Immunity during pregancy: Lymphocute subpopulations and mitogen responsiveness. Ann Clin Lab Sci 1985; 15:357–362.
19. Toder V, Nebel L, Cleicher N. Studies of natural killer cells in pregnancy. I. Analysis at the single cell level. J Clin Lab Immunol 1984; 14:123–127.
20. Gregory CD, Shan LP, Lee H, Scott IV, Golding PR. Cytotoxic reactivity of human natural killer (NK) cells during normal pregnancy: A longitudinal study. J Clin Lab Immunol 1985; 18:175–181.
21. Sridama V, Pacini F, Yang SL, Moawad A, Reilly M, DeGroot LJ. Decreased levels of helper T cells. Apossible cause of immunodeficiency in pregnancy. N Engl J Med 1982; 307:352–356.
22. Maroulis GB, Buckley RH, Younger JB. Serum immunoglobulin concentrations during normal pregnancy. Am J Obstet Gynecol 1971; 109:971–976.
23. Amino N, Mori H, Iwatani Y, Tanizawa O, Kawashima M, Thuge I, et al.. High prevalence transient post-partum thyrotoxicosis and hypothyroidism. N Engl J Med 1982; 306:849–852.
24. Garsenstein M, Pollak VE, Karl RM. Systemic lupus erythematosus and pregnancy. N Engl J Med 1962; 267:165–169.
25. Oka M. Effect of pregnancy on the onset and course of rheumatoid arthritis. Ann Rheum Dis 1953; 2:227–229.
26. Ehlers MRW, Riordan JF. Membrane proteins with soluble counterparts: Role of proteolysis in the release of transmembrane proteins. Biochemistry 1991; 30:10065–10074.
27. Imboden JB. T lymphocytes and natural killer cells In Basic and Clinical Immunology East Norwalk, CT, Appleton and Lange, 1994, pp 94–104.
28. Symons JA, Wood NC, di GF, Duff GW. Soluble CD8 in

patients with rheumatic diseases. Clin Exp Immunol 1990; 80:354–359.

29. Symons JA, McCulloch JF, Wood NC, Duff GW. Soluble CD4 in patients with rheumatoid arthritis and osteoarthritis. Clin Immunol Immunopathol 1991; 60:72–82.

30. Sawada S, Hashimoto H, Iijima S, Tokano Y, Takei M, Shida M, et al. Immunologic significance of increased soluble CD8/CD4 molecules in patients with active systemic lupus erythematosus. J Clin Lab Anal 1993; 7:141–146.

31. Tomkinson BE, Brown MC, IP SH, Carrabis S, Sullivan JL. Soluble CD8 during cell activation. J Immunol 1989; 142:2230–2236.

32. Griffin DE, Ward BJ, Jauregui E, Jhonson RT, Vaisberg A. Immune activation in measles. N Engl J Med 1989; 320:1667–1672.

33. Iwatani Y, Amino N, Kaneda T, Ichihara K, Tamaki H, Tachi J. Marked increase of CD5+ B cells in hyperthyroid Graves' disease. Clin Exp Immunol 1989; 78:196–200.

34. Sawada S, Sugai S, Iijima S, Takei M, Paredes E, Hayama T, et al.. Increased soluble CD4 and decreased soluble CD8 molecules in patients with Sjogren's syndrome. Am J Med 1992; 92:134–140.

35. Griffin DE, Ward BJ. Differential CD4 T cell activation in measles. J Infect Dis 1993; 168:275–281.

36. Reddy MM, Vodian M, Grieco MH. Elevated levels of CD4 antigen in sera of human immunodeficiency virus-infected populations. J Clin Microbiol 1990; 28:1744–1746.

37. Cesare ED, Previti M, Ingemi MC, Bagnato GF, Cucinotta D. High serum levels of soluble CD8 in insulin-dependent diabetes. Clin Exp Immunol 1994; 95:283–286.

38. Pritchard JA, Hunt CF. A comparison of the hematologic responses following the routine prenatal administration of intramuscular and oral iron. Surg Gynecol Obstet 1958; 106:516.

39. Mendenhall HW. Serum protein concentrations in pregnancy: I. Concentrations in maternal serum. Am J Obstet Gynecol 1970; 106:388.

40. Billington WD. Maternal immune response to pregnancy. Reprod Fertil Dev 1989; 1:183–191.

41. Feinberg BB, Gonik B. General precepts of the immunology of pregnancy. Clin Obstet Gynecol 1991; 34:3–16.

42. Beckerman KP. Reproduction & the immune system In Basic & Clinical Immunology East Norwalk, CT, Appleton and Lange, 1994, pp 552–568.

43. Clark DA, Slapsys R, Croy BA, Krcek J, Rossant J. Local active suppression by suppressor cells in the decidua: A review. Am J Reprod Immunol 1984; 5:78–83.

44. Chaouat G, Monnot P. Systemic active suppression is not necessary for successful allopregnancy. Am J Reprod Immunol 1984; 6:5–8.

45. Agostini C, Pizzolo G, Zambello R, Trentin L, Siviero F, Vinante F, et al. Shedding of the soluble form of the CD8 complex by CD8+/HLA-DR+ cells in HIV-1-infected patients. AIDS 1991; 5:813–819.

46. Amino N. Postpartum Thyroid disease In Advances in perinatal thyroidology. Plenum Press, New York, 1991, pp 167–180.

47. Iwatani Y, Amino N, Mori H, Asari S, Izumiguchi Y, Kumahara Y, et al. T lymphocyte subsets in autoimmune thyroid diseases and subacute thyroiditis detected with monoclonal antibodies. J Clin Endocrnol Metab 1983; 56:251–254.

48. Nakamura N, Miyazaki K, Kitano Y, Fujisaki S, Okamura H. Suppression of cytotoxic T-lymphocyte activity during human pregnancy. J Reprod Immunol 1993; 23:119–130.

49. Stites DP. Clinical laboratory methods for detection of cellular immunity. In Basic and clinical immunology East Norwalk, CT, Appleton and Lange, 1994, pp 195–215.

参考論文

CASE REPORT

Euthyroid Graves' disease showing no thyroid abnormalities except positive thyroid-stimulating antibody (TSAb): two case reports

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Abstract. Watanabe M, Iwatani Y, Kashiwai T, Iijima T, Fujikado T, Amino N (Departments of Laboratory Medicine, Clinical Laboratory Science and Ophthalmology, Osaka University Medical School, Suita, Japan). Euthyroid Graves' disease showing no thyroid abnormalities except positive thyroid-stimulating antibody (TSAb): two case reports (Case report). *J Intern med* 1995; **238**: 379–84.

We report two cases of euthyroid Graves' disease in women who had ophthalmopathy without previous history of hyperthyroidism. Enlargement of extraocular muscles was observed by magnetic resonance imaging (MRI). The patients had no thyroid enlargement and their serum concentrations of free T_4, free T_3, and TSH were normal. The sera were negative for antithyroid microsomal and thyrogobulin antibodies, and anti-TSH receptor antibodies measured by radioreceptor assay. T_3 suppression test results were normal. Only thyroid-stimulating antibody (TSAb) measured by sensitive bioassay was positive. These findings indicate that sensitive TSAb is the most useful laboratory test in the diagnosis of euthyroid Graves' disease.

Keywords: euthyroid Graves' disease, ophthalmopathy, TSAb.

Introduction

Graves' thyrotoxicosis is caused by thyroid-stimulating antibody (TSAb), and is often associated with ophthalmopathy consisting of a combination of symptoms and signs such as proptosis, lid retraction, extraocular muscle dysfunction, diplopia, eyelid edema, and optic neuropathy. However, the cause of the ophthalmopathy is still unknown. Some patients with Graves' ophthalmopathy never develop hyperthyroidism and some even have associated hypothyroidism [1, 2]; these types of the disease are termed euthyroid Graves' disease [3, 4] and hypothyroid Graves' disease, respectively. These patients often have small goitre and/or positive antithyroid antibodies indicating autoimmune thyroid abnormality [3, 4]. In this report, we describe two patients with nongoitrous euthyroid Graves' disease in whom laboratory data, such as TSH binding inhibitor immunoglobulins (TBII), antimicrosome antibodies (McAb), antithyroglobulin antibodies (TgAb), and T_3-suppression test, were all normal but in whom thyroid-stimulating antibody was positive.

Case report

Patient 1

A 40-year-old woman was referred to our clinic in May, 1993, because of diplopia and pain of the right eye. She was euthyroid and showed no symptoms suggestive of hyperthyroidism. Thereafter, she was treated with prednisolone (5–10 mg day^{-1}) until July, 1993.

Physical examination revealed that her height was 172 cm and weight was 60 kg. Her blood pressure was 110/60 mmHg, and pulse rate 72 beats min^{-1} with regular rhythm. Graefe's sign and lid retraction were observed in the right eye. Right hypotropia at the primary position and disturbance of right eyeball movement at the upper lateral view were observed. The proptosis was 21 mm for the left eye and 23 mm for the right, with a baseline of 104 mm, measured with a Hertel's ophthalmometer (normal, less than 18 mm). The thyroid gland was not palpable. No abnormalities of the heart, chest or abdomen were detected, and the deep tendon reflexes were normal.

Swelling of both the right lateral and right inferior rectus muscles were detected by magnetic resonance imaging (MRI) (Fig. 1). Laboratory studies revealed mild hypercholesterolaemia but no abnormality of urinalysis results (data not shown). Thyroid function studies revealed euthyroidism (Table 1). The serum TSH concentration was normal, and antithyroid autoantibodies (McAb, TgAb, and TBII) were all negative. TBII was measured by a commercial kit of radioreceptor assay (TRAb 'Cosmic'; Cosmic Co., Tokyo, Japan). Her thyroid uptake of ^{123}I was suppressed after 7-day treatment with 75 µg day^{-1} of T$_3$. The only positive laboratory test related to the thyroid was TSAb (508%; normal range less than 140%) in serum. Thus, her illness was diagnosed as euthyroid Graves' disease.

To improve the limitation of eye movement, we applied external radiotherapy and intend to perform surgical recession of the extraocular muscle.

Patient 2

A 38-year-old woman was referred to our clinic in September, 1991, because of diplopia, limitation of right eyeball movement, and right eyelid oedema. In August, 1990, an ophthalmologist at our hospital had not detected any organic abnormalities except enlargement of the bilateral medial rectus muscle examined by MRI (Fig. 2). The patient was euthyroid and had no symptoms suggestive of hyperthyroidism. Thereafter, she had been treated with prednisolone (10–20 mg day^{-1}).
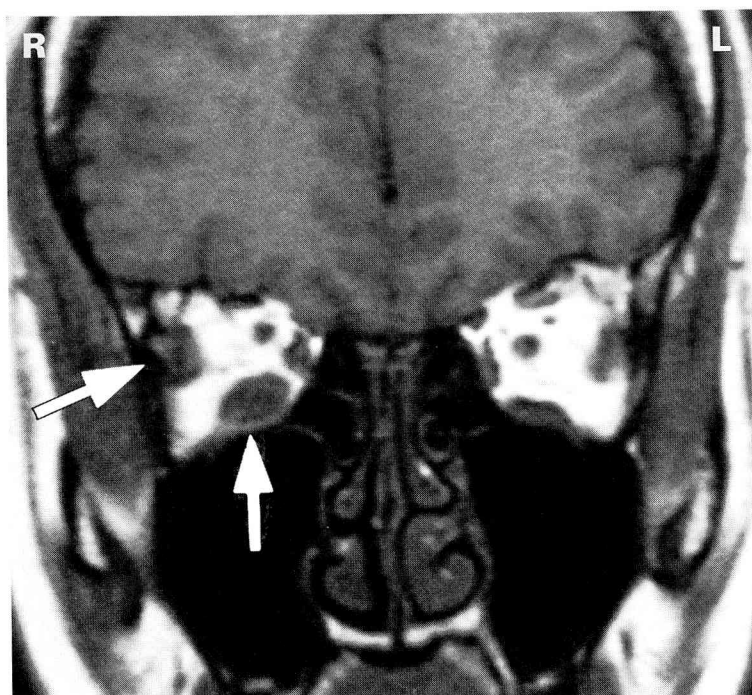

Fig. 1 Orbital MRI showing swellings of right lateral and inferior rectus muscles (indicated by arrows) in patient 1.

Table 1 No thyroid abnormalities except that of TSAb were detected in either patient

	Patient 1	Patient 2	Normal range
Free T$_4$	1.2 ng dL^{-1}	1.1 ng dL^{-1}	0.8–1.4 ng dL^{-1}
Free T$_3$	3.1 pg dL^{-1}	5.0 pg dL^{-1}	2.8–5.8 pg dL^{-1}
TSH	1.8 µU mL^{-1}	1.6 µU mL^{-1}	0.6–3.7 µU mL^{-1}
TBII	8%	5%	< 12%
McAB	Negative	Negative	Negative
TgAb	< 0.3 U mL^{-1}	< 0.3 U mL^{-1}	< 0.3 U mL^{-1}
Tg	13.8 ng mL^{-1}	12.3 mg mL^{-1}	< 45 ng mL^{-1}
T$_3$ suppression test	11.4 → 5.3%	18.5 → 6.5%	
TSAb	508%	240%	< 140%

Fig. 2 Orbital MRI showing swelling of bilateral medial rectus muscles (indicated by arrows) in patient 2.

Physical examination revealed that her height was 162 cm and weight was 45.2 kg. Her blood pressure was 146/82 mmHg. and pulse rate 72 beats min^{-1} with regular rhythm. Graefe's sign and lid retraction were observed in the right eye. Disturbance of right eyeball movement was observed in right lateral view

Fig. 3 Limitation of eye movement on right lateral view in patient 2. Disturbance of right eyeball movement is seen.
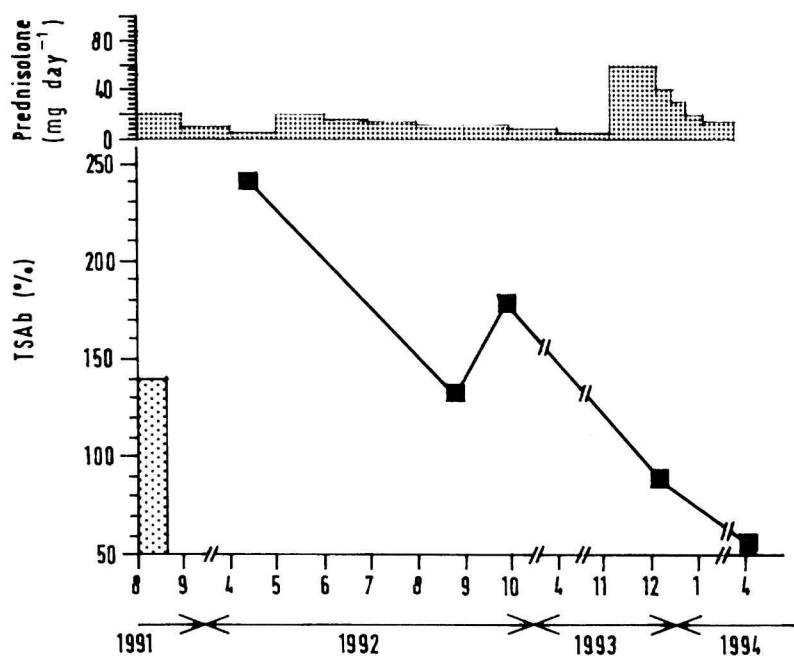

Fig. 4 Changes of TSAb activity and the dose of prednisolone in patient 2. The TSAb activities changed in parallel with the severity of her eye symptoms.

(Fig. 3). However, the proptosis was 16 mm for both eyes with a baseline of 102 m. The thyroid was not palpable. No abnormalities of the heart, chest or abdomen was detected. The deep tendon reflexes were normal.

Laboratory studies revealed slight leukocytosis due to the effect of prednisolone treatment, and normal urinalysis values (data not shown). Biochemical tests disclosed hypercholesterolaemia and low level of creatine kinase (data not shown). Thyroid function studies revealed euthyroidism and no thyroid abnormalities except TSAb, which showed activity of 240% (Table 1). Thus, her illness was diagnosed as euthyroid Graves' disease.

The symptoms were kept under good control with prednisolone (5–20 mg day^{-1}). However, in November, 1993, the right-eye symptoms intensified and oedema of the left eyelid was noted. Therefore, we increased the dose of prednisolone to 60 mg day^{-1} and applied external radiotherapy to the bilateral

extraocular muscle for 2 weeks (total 20 Gy) in December 1993. The changes in the patient's TSAb activities during the course of treatment are shown in Fig. 4. The TSAb activities changed in parallel with the severity of her eye symptoms.

Discussion

Ophthalmopathy, which is one of the extrathyroidal components of Graves' disease, sometimes emerges in euthyroid patients with no history of thyrotoxicosis. (euthyroid Graves' disease). The pathogenesis of the ophthalmopathy in Graves' disease, euthyroid or otherwise, is still unknown. Because lymphocytic infiltration is observed in the retro-orbital tissue, it has been suggested that an autoimmune reaction against the retro-orbital tissue is the cause of Graves' ophthalmopathy [3–5].

The hyperthyroidism in Graves' disease is caused by autoantibodies against TSH receptor, which stimulates the production of thyroid hormone by thyroid epithelial cells. TBII, measured by a kit of radioreceptor assay (TRAb 'Cosmic') can be detected in about 95% of patients with Graves' disease. With a more sensitive bioassay, TSAb is found in almost all patients with Graves' disease [1]. Consistent with this, distribution analysis has revealed exophthalmos in almost all patients with Graves' disease [6]. Patients with euthyroid Graves' disease often show some abnormalities upon thyroid examination [4]. The reported incidence of various thyroid abnormalities is as follows: presence of thyroid enlargement, 24–40%; low TSH, 14–23%; no or low response of TSH to TRH, 40–63%; high thyroid uptake of ^{123}I, about 20%; negative T_3-suppression test, 30–73%; positive TgAb, 11–67%; positive McAb, 18–60%; positive TBII, 31–36%; and positive TSAb, 43–87% [1, 7–12]. Amongst these abnormalities, that of TSAb can be detected most often in euthyroid Graves' disease. We recently detected TSAb in the serum of 93% of our patients with euthyroid Graves' disease by using an ultrasensitive assay for TSAb we have developed, which detects 0.1 μU mL^{-1} of bTSH and is one thousand times more sensitive on the basis of the bTSH than the TBII assay ([13, 14], and unpublished data). This incidence was almost the same as that in patients with hyperthyroid Graves' disease. Furthermore, this TSAb assay was useful in the prediction of the onset of Graves' disease after delivery [14]. However, it was reported that in

a few hyperthyroid Graves' disease patients, neither radioreceptor assays nor bioassays could detect any antibodies [15]. Subsequent to radioiodine therapy, TSH receptor antibodies appeared in almost all of their patients. Considering all of this, a local production of TSAb within the thyroid gland, and possibly in retro-orbital tissue, is important in the patients with Graves' disease associated with ophthalmopathy. Moreover, follow-up study is essential in these patients.

Our patients showed no goitre, with euthyroidism, normal TSH concentration, normal ^{123}I uptake, normal T_3 suppression, and negative McAb, TgAb, and TBII. However, their ocular symptoms and the enlargement of the extraocular muscles demonstrated by MRI were typical findings in Graves' ophthalmopathy. Furthermore, TSAb activities were positive as measured with out ultrasensitive assay. Thus, we diagnosed them as having euthyroid Graves' disease. Whilst there are many reports regarding this disease, this is the first report to our knowledge of patients with euthyroid Graves' disease showing no thyroid abnormalities other than positive TSAb.

It was recently reported that TSH receptors are present on retro-orbital tissue [16]. Therefore, TSH-receptor-antibody-stimulating thyroid cells (TSAb) might also stimulate or interact with retro-orbital tissue, causing ophthalmopathy. Indeed, the change in TSAb activity almost paralleled the extent of ophthalmopathy in our patients. Furthermore, we found that the TSAb activity was also related to the degree of ophthalmopathy in patients with hypothyroid Graves' disease, who present Graves' ophthalmopathy and TSAb, but are hypothyroid because of the practical destruction of the thyroid [2]. Given these considerations, TSAb might be important in the development of Graves' ophthalmopathy.

The mechanism by which euthyroid condition was maintained in our patients, even though they had positive TSAb, is unknown, but it might be that the TSAb activity was not high enough to cause hyperthyroidism or because of the presence of a suppressive mechanism against TSAb stimulation. Alternatively, an antigenic epitope on the TSH receptor recognized by TSAb in euthyroid Graves' disease may differ from that in usual Graves' disease, or there may be other differences between the two forms of the disease.

In any event, the measurement of TSAb in patients

who have ophthalmopathy without other thyroid abnormalities is essential to accurate diagnosis.

References

1 Kasagi K, Konishi J, Arai K, Misaki T, Iida Y, Endo K, Torizuka K. A sensitive and practical assay for thyroid-stimulating antibody in Graves' disease. *Ann Intern med* 1986; 93: 28–32.

2 Tamaki H, Amino N, Iwatani Y, Miyai K. Improvement of infiltrative ophthalmopathy in parallel with decrease of thyroid-stimulating antibody (TSAb) activity in two patients with hypothyroid Graves' disease. *J Endocrinol Invest* 1989; 12: 47–53.

3 Bahn RS, Heufelder AE. Pathogenesis of Graves' ophthalmopathy. *N Engl J Med* 1993; 329: 1468–75.

4 Weetman AP. Update. Thyroid-associated ophthalmopathy. *Autoimmunity* 1992; 12: 215–22.

5 Volpé R. Immunology of the thyroid. In: Volpé R, ed. *Autoimmune Disease of the Endocrine System.* Boca Raton: CRC Press, 1990; 187–99.

6 Amino N, Yuasa T, Yabu Y, Miyai K, Kumahara Y. Exophthalmos in autoimmune thyroid disease. *J Clin Endocrinol Metab* 1980; 51: 1232.

7 Zakarija M, McKenzie JM, Bsnovac K. Clinical significance of assay of thyroid-stimulating antibody in Graves' disease. *Ann Intern Med* 1980; 93: 28–32.

8 Kasagi K, Hidaka A, Misaki T, Miyamoto S, Takeuchi R, Sakahara H, Sasayama S, Iida Y, Konishi J. Scintigraphic findings of the thyroid in euthyroid ophthalmic Graves' disease. *J Nucl Med* 1994; 35: 811–17.

9 Tamai H, Nakagawa K, Ohsako N, Fukino O, Takahashi H, Matsuzuka F, Kuma K, Nagataki S. Changes in thyroid function in patients with euthyroid Graves' disease. *J Cli Endocrinol Metab* 1980; 50: 108–12.

10 Teng CS, Smith BR, Clayton B, Evered DC, Clark F and Hall F Thyroid-stimulating immunogloburins in ophthalmic Grave disease. *Clin Endocrinol* 1977; 6: 207–11.

11 Munro RE, Lamki L, Row VV, Volpé R. Cell-mediated immunit in the exophthalmos of Graves' disease as demonstrated by th migration inhibition factor (MIF) test. *J Clin Endocrinol Meta* 1973; 37: 286–92.

12 Rapoport R, Greenspan FS, Filetti S, Pepitone M. Clinica experience with a human thyroid cell bioassay for thyroic stimulating immunoglobulin. *J Clin Endocrinol Metab* 1984 58: 332–8.

13 Tamaki H, Amino N, Kimura M, Miyai K. Ultrasensitiv bioassay of thyrotropin. *Clin Chem* 1989; 35: 1252–3.

14 Hidaka Y, Tamaki H, Iwatani Y, Tada H, Mitsuda N, Amino N Prediction of postpartum onset of Graves' thyrotoxicosis b measurement of thyroid stimulating antibody in early pre nancy. *Clin Endocrinol* 1994; 41: 15–20.

15 Ilicki A, Gamstedt A, Karlsson FA. Hyperthyroid Grave: disease without detectable thyrotropin receptor antibodie: *J Clin Endocrinol Metab* 1992; 74: 1090–94.

16 Heufelder AE, Bahn RS. Evidence for the presence of functional TSH-receptor in retroocular fibroblasts fror patients with Graves' ophthalmopathy. *Exp Clin Endocrin* 1992; 100: 62–7.

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