Clin. exp. Immunol. (1982) **48,** 443–488.

Immunoregulatory T cell subpopulations in patients with scleroderma using monoclonal antibodies

E. C. KEYSTONE, C. LAU, D. D. GLADMAN, S. WILKINSON, P. LEE & A. SHORE
Rheumatic Disease Unit, Department of Medicine, University of Toronto, Wellesley Hospital, Women's College Hospital, Department of Pediatrics, Sick Children's Hospital and Ortho Pharmaceutical Corporation

(Accepted for publication 13 November 1981)

SUMMARY

Twenty-eight patients with scleroderma were compared with 22 healthy age-matched subjects. Monoclonal antibodies were used to detect the whole T cell population (OKT3), T helper cells (OKT4), and T suppressor/cytotoxic cells (OKT8) by indirect immunofluorescence on isolated peripheral blood mononuclear cells. A subset of scleroderma patients (i.e. 30% or eight of 28 patients) exhibited an elevated ratio of OKT4/OKT8 cells which could be accounted for, mainly by a reduction in OKT8 cells compared with controls. The scleroderma patients with an elevated OKT4/OKT8 ratio tended to be younger, have a shorter disease duration and more extensive skin involvement than patients with a normal OKT4/OKT8 ratio. There was no correlation with the presence of autoantibodies, drug therapy, or HLA-DR type. In order to further determine whether this imbalance in immunoregulatory cell subpopulations was specific for scleroderma, we further studied 16 patients with psoriatic arthritis but without manifest autoimmunity and delineated a similar subset of patients with an elevated OKT4/OKT8 cell ratio (i.e. 38% or six of 16 patients). The results demonstrate similar immunoregulatory T cell imbalances in patients with scleroderma and psoriatic arthritis. These findings suggest that numerical imbalances in lymphocyte subpopulations may not be specific for autoimmune disorders.

INTRODUCTION

Scleroderma is a multisystem disorder characterized by excessive B cell reactivity as evidenced by a hypergammaglobulinemia and autoantibody formation. The mechanism(s) accounting for the hyperreactive B cell response is unclear. Current concepts have implicated defective T suppressor cell function (Waldmann & Broder, 1977). Although antigen specific suppressor cell activity in patients with scleroderma has been shown to be normal (Keystone *et al*, 1981), recent evidence has suggested an immunoregulatory imbalance in patients with scleroderma (Inoshita *et al.*, 1981).

An alternative approach to the study of immunoregulatory activity in patients with scleroderma is the quantification of T cells subserving regulatory function. Recently, monoclonal antisera specific for distinct T cell surface determinants have been developed, capable of reacting with these immunoregulatory T cells (Reinherz *et al.*, 1979a). The present study, therefore, was designed to quantify immunoregulatory T cell subpopulations in patients with scleroderma.

Correspondence: Dr E. Keystone, Suite 655, Wellesley Hospital, 160 Wellesley Street East, Toronto, Ontario M4Y 1J3, Canada.

0009-9104/82/0500-0443$02.00

MATERIALS AND METHODS

Patient selection. Twenty-eight patients with scleroderma, 23 females and five males with a mean age of 55·1 years (range 26–71 years), and a mean disease duration of 7·5 years (range 1–25 years) were studied. All patients fulfilled the preliminary diagnostic criteria for scleroderma (Subcommittee for scleroderma, 1980) and were followed in an on-going clinical study in the Rheumatic Disease Unit of the Wellesley Hospital. Three patients had the CREST syndrome. A standard clinical and laboratory protocol was carried out on all patients. Treatment included prednisone (10 mg or less) in eight patients, penicillamine in two, and cimetidine in six. Patients receiving cytotoxic agents were excluded. No patient was azotemic.

The extent of disease was quantified by allocating points for the organ systems involved, according to the technique of Hughes *et al.* (1977).

Controls. Controls consisted of 22 healthy volunteers with a mean age of 52·3 years (range 22–70 years) comparable to the patient group. A second disease control group consisted of 16 patients with psoriatic arthritis (eight females and eight males) with a mean age of 43·2 (range 17–71 years). The mean duration of psoriasis and arthritis was 21·3 years (range 5–61 years) and 12·8 years (range 1–43 years) respectively. None were receiving gold, chloroquine, imuran, or PUVA at the time of study.

Labelling of mononuclear cells with monoclonal antibodies (OKT3, OKT4, OKT8). Human peripheral blood mononuclear cells were isolated from heparinized blood of donors by Ficoll-Hypaque density gradient centrifugation (Ficoll-Hypaque, Pharmica Fine Chemicals, Piscataway, New Jersey). The lymphocyte fraction was collected, washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI at a concentration of 5×10^6 cells/ml. Five microlitres of various monoclonal antibodies (OKT3, OKT4, OKT8) (50 μg/ml) (kindly supplied by Ortho Pharmaceutical Corporation, Raritan, New Jersey) were added to 200 μl of the cells (in some cases, the monoclonal antibodies were added to cell pellets containing approximately 10^6 cells). The labelling was done for 30 min at 4°C. The cells were then washed two times with 2 ml of RPMI and resuspended in 100 μl of 1/20 dilution of fluorescein thioisocyanate labelled goat anti-mouse IgG (Meloy Labs., Mississauga, Ontario) in RPMI. After 30 min of incubation at 4°C, the cells were washed two times with 2 ml of RPMI and resuspended in 100 μl. The percentage of fluorescent positive cells was determined using a Leitz fluorescent microscope.

DR typing. The microcytotoxicity test of Terasaki *et al.* (1978) was used with Terasaki B cell typing trays to detect DR antigens.

Statistical methods. Statistical analysis of the differences between groups was determined by the Student's *t*-test or χ^2 analysis and the level of significance set at 0·05.

RESULTS

Studies of OKT markers in the scleroderma group revealed no significant differences between the patients and healthy controls in the percentage ($\pm$s.d.) of OKT3 cells (78·3 $\pm$ 8·6 vs 76·1 $\pm$ 11·5, respectively), OKT4 cells (60·3 $\pm$ 14·3 vs 57·7 $\pm$ 10·3, respectively), or OKT8 cells (28·6 $\pm$ 11·6 vs 30·1 $\pm$ 7·5, respectively) (Fig. 1). It is notable that the patient group exhibited a much wider scatter of data than controls for the OKT4 and OKT8 cell markers. The OKT4/OKT8 ratio in the patient group (2·5 $\pm$ 1·2) was higher than that of the healthy controls (1·9 $\pm$ 0·4), but the difference was of borderline significance ($0{\cdot}05 < P < 0{\cdot}10$) (Fig. 2).

A subgroup of patients with scleroderma (eight of 28 or 30%) exhibited a numerical imbalance of immunoregulatory cells as denoted by an elevated OKT4/OKT8 ratio greater than 2 standard deviations (s.d.) above the mean ratio of the healthy controls. Of the eight patients with elevated ratios, five exhibited a significant reduction in the percentage of OKT8 cells (Table 1). Two additional patients, with normal OKT4/OKT8 ratios, exhibited an increased percentage of OKT4 cells. All patients with an elevated ratio demonstrated a percentage of OKT8 cells at least 1 s.d. below the normal mean. Indeed, the percentage OKT8 cells in those with an elevated ratio was

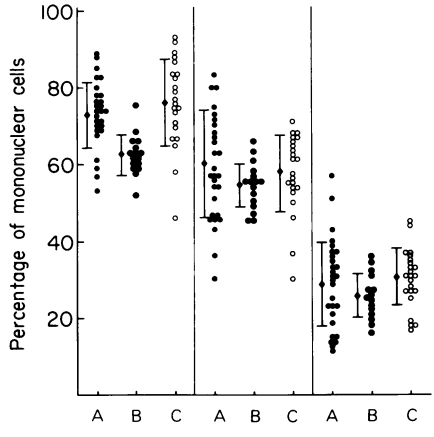

Fig. 1. Percentage of mononuclear cells bound by OKT3 antisera (left hand panel), OKT4 antisera (centre panel) and OKT8 antisera (right hand panel). Patients with scleroderma (A). Patients with psoriatic arthritis (B) while normals (C). The mean percentage for each group is represented by a ♦ and the bars represent ± 1 s.d.

markedly reduced (16·7 ± 4·3%) relative to that observed in the remainder of the scleroderma population studied (33·6 ± 9·9%) ($P < 0{\cdot}001$). No significant difference in the percentage of OKT4 cells was observed between the patients with an elevated (62·5 ± 10·6%) and normal ratios (57·6 ± 15·2%) ($P > 0.05$).

Since a recent study from this laboratory demonstrated an increased incidence of HLA-DR 5 in patients with scleroderma (Gladman *et al.*, 1981), DR typing was carried out on all patients in the present study. There was no difference in the frequency of DR antigens tested between patients with and without an elevated OKT4/OKT8 ratios (data not shown).

A variety of clinical measures were used in an attempt to determine whether patients with an elevated OKT4/OKT8 ratio constituted a unique subset within the scleroderma population. Although no statistical differences were evident, patients with an elevated OKT4/OKT8 ratio tended; (a) to be younger (mean age ± s.d.) (48·3 ± 15·3 vs 57·8 ± 11·7 years, respectively), (b) to have

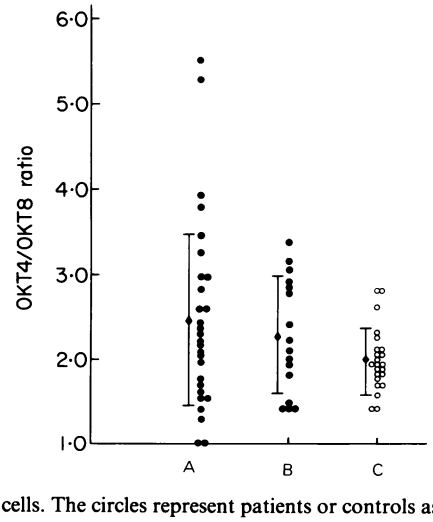

Fig. 2. Ratio of OKT4/OKT8 cells. The circles represent patients or controls as per Fig. 1. The mean ratio for each group is represented by a ♦ and the bars represent ± 1 s.d.

Table 1. Scleroderma patients exhibiting imbalances in immunoregulatory cell subpopulations

Patient	OKT4 (%)	OKT8 (%)	OKT4/OKT8 ratio
O.F.	54	19	2·9(↑)†
E.B.	36(↓)*	14(↓)	2·6
C.L.	47	14(↓)	3·4(↑)
G.B.	59	15(↓)	3·9(↑)
J.W.	72	13(↓)	5·5(↑)
J.T.	57	15(↓)	3·8(↑)
P.M.	63	12(↓)	5·3(↑)
T.A.	68	23	2·9(↑)
H.L.	80(↑)	23	3·5(↑)
D.R.	84(↑)	35	2·4
S.G.	80(↑)	31	2·6
M.N.	30(↓)	37	0·8(↓)
A.H.	43	51(↑)	0·8(↓)
Normals ($n=22$)	57·7 ± 10·3‡	30·1 ± 7·5	2·0 ± 0·4

* (↓) indicates value ⩽2 s.d. below normal mean.
† (↑) indicates value ⩾2 s.d. above normal mean.
‡ indicates ± 1 s.d.

a shorter disease duration (4·3 ± 2·4 vs 8·8 ± 7·5 years, respectively) and (c) to have higher skin scores (2·4 ± 0·9 vs 1·8 ± 1·2, respectively) and lower disease scores (6·0 ± 3·5 vs 7·6 ± 3·6, respectively) than patients with normal OKT4/OKT8 ratios. There were no differences between the two groups in the frequency of autoantibodies (antinuclear antibody and rheumatoid factor) or hypergammaglobulinemia. There was no correlation between the OKT4/OKT8 ratio and any particular drug regimen. Two of the eight patients with elevated OKT4/OKT8 ratios were receiving less than 5·0 mg of prednisone on a daily basis. Two of the remaining five patients with immunoregulatory abnormalities were receiving less than 10 mg of prednisone. Four of 15 patients with no immunoregulatory abnormalities were receiving 10 mg or less of prednisone and two were receiving pencillamine.

In order to further determine whether these abnormalities observed were specific for scleroderma, a disease control population of 16 patients with psoriatic arthritis was studied. Compared to the healthy controls, the psoriatic patients exhibited a significant reduction in the percentage of OKT3 cells (62·4 ± 5·1 vs 76·1 ± 11·5) ($P < 0·001$) and OKT8 cells (24·7 ± 5·8 vs 30·1 ± 7·5) ($P < 0·05$). Like the scleroderma group, the psoriatic patients had an elevated OKT4/OKT8 ratio (2·3 ± 0·2) relative to the control group (1·9 ± 0·4) which was of borderline significance ($0·05 < P < 0·10$). Again, (as in the scleroderma group), a subgroup of psoriatic patients (six of 16 or 38%) exhibited a significantly elevated ratio of OKT4/OKT8 cells. All of the psoriatic patients with an elevated ratio had a percentage of OKT8 cells at least 1 s.d. below the normal mean. The percentage (± s.d.) of OKT8 cells in the psoriatic patients with an elevated ratio was significantly reduced (19·8 ± 2·6%) relative to the remainder of the patient group (28·7 ± 4·2%) ($P < 0·05$). The results thus demonstrate that the imbalance in immunoregulatory cell subpopulations in a subgroup of scleroderma patients is not unique to this disorder.

DISCUSSION

The recent development of heterologous monoclonal antibodies has enabled a more comprehensive quantification of T cell subpopulations (Reinherz *et al.*, 1979a). The OKT3 marker recognizes the

total population of mature T cells (Reinherz *et al.*, 1979b) while subpopulations of T cells, namely T helper cells are recognized by OKT4 antisera (Reinherz *et al.*, 1979c) and suppressor/cytotoxic cells by OKT8 antisera (Reinherz *et al.*, 1980).

We previously demonstrated a lower mean absolute lymphocyte count for patients with scleroderma (1653 ± 125 cells/mm^3) compared with controls (2339 ± 151 cells/mm^3) (Baron *et al.*, 1981). The present study demonstrates that a substantial subgroup of patients with scleroderma exhibit a numerical imbalance in immunoregulatory cells characterized by a reduction in the percentage of OKT8 T-suppressor/cytotoxic cells. Moreover, the subset of patients with a low percentage of OKT8 cells had an even lower absolute lymphocyte count (1414 ± 293 cells/mm^3). This data suggests that most PSS patients have an absolute reduction in OKT8 cells while a subgroup have in addition a reduced proportion of OKT8 cells.

Our data is consistent with a recent study by Inoshita *et al.* (1981) who demonstrated a reduction in T suppressor cells. The T suppressor and helper cells in that study were delineated by the presence of Fc receptors for IgG (Tγ cells) and IgM (Tμ cells) respectively. In contrast to their findings, however, Gupta *et al.* (1979) reported an increase in Tγ cells and a decrease in Tμ cells. The reasons for the discrepancy remain unclear.

The mechanism for the loss of OKT8 cells is unclear. Although lymphocytotoxic antibodies were demonstrated in some of the patients studied, there was no correlation between the presence of the antibodies and reduction in OKT8 cells (unpublished observations). The possibility of anti-lymphocyte antibodies blocking determinants was also considered. Since there was not always a relative increase in OKT4 cells to account for the decrease in OKT8 T cells in the six patients with reduced OKT8 cells (Table 1), this possibility could not be excluded in some of the patients (Morimoto *et al.*, 1980).

Patients with psoriatic arthritis were chosen as controls since, like scleroderma, there is both skin and joint involvement in a patient population with a low level of disability. Moreover, psoriatic patients are classically seronegative with little propensity to exhibit autoimmune phenomena. The abnormalities detected in the psoriatic arthritis patients were similar to the scleroderma population indicating a lack of specificity for the immunoregulatory T cell imbalance observed. This lack of specificity is supported by a recent study demonstrating reduced suppressor cell numbers (with an elevated OKT4/OKT8 cell ratio) in patients with multiple sclerosis (Bach *et al.*, 1980).

The authors wish to thank the Ortho Pharmaceutical Corporation for supplying the monoclonal antisera and Miss N. Sheridan for her excellent secretarial assistance.

REFERENCES

BACH, M.A., TOURNIER, E., PHAN-DUNH-TUY, F., BACH, J.F., CHATENOID, L., MARTIN, G. & DEGOS, J.D. (1980) Deficit of suppressor T cell in active multiple sclerosis. *Lancet*, **ii**, 1221.

BARON, M., KEYSTONE, E.C., GLADMAN, D.D., LEE, P., POPLONSKI, L. & CANE, D. (1981) Lymphocyte subpopulations and reactivity to mitogens in patients with scleroderma. *Clin. exp. Immunol.* **46**, 70.

GLADMAN, D.D., KEYSTONE, E.C., BARON, M., LEE, P., CANE, D. & MERVERT, H. (1981) Increased frequency of HLA DR5 in progressive systemic sclerosis (Scleroderma). *Arthrit. Rheum.* **24**, 854.

GUPTA, S., MALAVIGA, A.N., RAJAGOPALAN, P. & GOOD, R.A. (1979) Subpopulations of human T lymphocytes. IX. Imbalance of T cell subpopulations in patients with progressive systemic sclerosis. *Clin. exp. Immunol*, **38**, 342.

HUGHES, P., HOLT, S., ROWEL, N.R., ALLONBY, I.D. & DODD, J.K. (1977) The relationship of defective cell-mediated immunity to visceral disease in systemic sclerosis. *Clin. exp. Immunol.* **28**, 233.

INOSHITA, R., WHITESIDE, T., RODNAN, G. & TAYLOR, F.H. (1981) Abnormalities of T lymphocyte subsets in patients with progressive systemic sclerosis (PSS, scleroderma) *J. Lab. clin. Med.* **97**, 264.

KEYSTONE, E.C., GLADMAN, D.D., BARON, M., LEE, P. & POPLONSKI, L. (1981) Antigen-specific suppressor cell activity in patients with scleroderma. *J. Rheumatol.* **8**, 747.

MORIMOTO, C., REINHERZ, E.L., SCHLOSSMAN, S.F., SCHUR, P.H., MILLS, J.A. & STEINBERG, A.D. (1980) Alterations in immunoregulatory T-cell subsets in active systemic lupus erythematosus. *J. clin. Invest.* **66**, 1171.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1979a) Separation of functional subsets of human T cells by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, **76**, 4061.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1979b) A monoclonal antibody

with selective reactivity with functionally mature and all peripheral human T cells. *J. Immunol.* **123,** 1312.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1979c) Further characterization of the human inducer T cell subset defined by a monoclonal antibody. *J. Immunol.* **123,** 2894.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1980) A monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset previously defined by a hetero-antiserum TH2. *J. Immunol.* **124,** 1301.

SUBCOMMITTEE FOR SCLERODERMA CRITERIA OF THE AMERICAN RHEUMATISM ASSOCIATION DIAGNOSTIC AND THERAPEUTIC CRITERIA COMMITTEE (1980) Special article—preliminary criteria for the classification of systemic sclerosis (scleroderma): *Arthrit. Rheum.* **23,** 581.

TERASAKI, P.I., BERNOCO, D., PARK, M.S., OZTURK, G. & IWAKI, Y. (1978) Microdroplet testing for HLA-A, -B, -C, and -D Antigens. *Am. Soc. clin. Path.* **69,** 103.

WALDMANN, T.A. & BRODER, S. (1977) Suppressor cells in the regulation of the immune response. *Prog. clin. Immunol.* **3,** 155.