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Antibody-dependent and phytohaemagglutinin-induced lymphocyte cytotoxicity in systemic sclerosis

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SUMMARY

Cell-mediated cytotoxicity was examined in thirty-seven patients with systemic sclerosis using both whole blood and purified peripheral blood mononuclear cells (PBM) to measure antibody-dependent (ADCC) and phytohaemagglutinin (PHA) induced lymphocyte cytotoxicity to ^{51}Cr-labelled Chang liver cells. In twenty-three mildly affected patients, ADCC and PHA-induced cytotoxicity did not differ from that found in control populations. By contrast, fourteen patients severely affected by extensive visceral disease showed reductions in both ADCC and PHA-induced cytotoxicity which were more marked in whole blood assays ($P<0{\cdot}001$) than in those performed with PBM ($P<0{\cdot}05$). The addition of patient's sera to control cytotoxicity assays suggested that blocking or suppressive serum factors could only account for some of the disproportionate reduction in whole blood cytotoxicity which, in the main, must be due to a lack of circulating effector cells. These results are in agreement with previous findings of reduced numbers of circulating thymus-dependent lymphocytes in patients with severe disease, a defect of cell-mediated immunity that may result from the chronic antigenic stimulation of an autoimmune disease process.

INTRODUCTION

Systemic sclerosis (SS) is a distinctive member of the connective tissue diseases which may show clinical association or 'overlap' with other disorders in the group, such as systemic lupus erythematosus (SLE) (Tuffanelli & Winkelmann, 1962) Sjögren's syndrome (Shearn, 1960) or polymyositis (Walton & Adams, 1958). The disease, like SLE, has a high incidence of autoantibodies (Beck *et al.*, 1963; Alarcón-Segovia *et al.*, 1975) and has also been reported to show evidence of cellular autoimmunity (Currie, Saunders & Knowles, 1971). In earlier studies we found both a deficiency of circulating thymus-dependent (T) lymphocytes (Hughes *et al.*, 1976) and impairment of the lymphocyte transformation response to phytohaemagglutinin (PHA) in severely affected patients with visceral involvement (Hughes *et al.*, 1977). In the present report the continued investigation of cell-mediated immunity (CMI) in the disease, using antibody-dependent (ADCC) and PHA-induced lymphocyte cytotoxicity for Chang liver cells, has once more produced evidence of abnormalities in patients with extensive visceral disease.

MATERIALS AND METHODS

Patients. Thirty-seven patients with SS (thirty-two women, five men; mean age 55·2± 12·9 years) were studied. Five of these patients were receiving treatment with immunosuppressive drugs (prednisolone, penicillamine or azathioprine). Raynaud's phenomenon and acrosclerosis were constant features and all patients were assessed for the extent of systemic

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involvement by the disease using previously described investigational criteria (Hughes *et al.*, 1977) to allocate points and so produce a 'disease score' for each patient. On this basis patients with SS were divided into categories of mild (disease score 0–5; twenty women, three men; mean age 56·9± 11·8 years) and severe (disease score 6–17; twelve women, two men; mean age 52·3± 14·6 years) disease.

Controls. Three groups of controls were used. The first consisted of fifty-one normal healthy volunteers (twenty-four women, twenty-seven men; mean age 39·9± 14·7 years). The second group of selected elderly controls comprised eighteen subjects (eight women, ten men; mean age 57·3± 3·4 years). The third group consisted of seventeen hospital in-patients (twelve women, five men; mean age 53·0± 11·1 years), who were suffering from non-immunological disease (cardiovascular diseases, bronchitis and peptic ulcer).

Target cells. Chang liver cells (Flow Laboratories, Irvine Scotland) were grown in 75 cm^2 glass culture bottles using Eagle's basal medium supplemented with 10% foetal bovine serum, non-essential amino acids, penicillin 100 u/ml, streptomycin 100 μg/ml and amphotericin B 2·5 μg/ml. Single cell suspensions were obtained by treating monolayers with 0·1% trypsin (Sigma Chemical Co., London) in calcium-magnesium-free Hanks's balanced salt solution with 20 mM HEPES buffer pH 7·2 for 10 min at 37°C.

Effector cells. A comparison of cytotoxic effector mechanisms in patients and controls was made using both heparinized (20 u/ml) whole blood (200 μl) and purified peripheral blood mononuclear cells (PBM). PBM were isolated from 20 ml of defibrinated venous blood after a preliminary sedimentation of red blood cells by plasma gel (Uniscience Ltd., Cambridge, England). The resulting leucocyte-rich serum was centrifuged at 1000 *g* for 20 min on Ficoll–Triosil and the interface PBM harvested, washed in Eagle's basal medium and adjusted to 5×10^5 cells/ml.

Rabbit anti-Chang serum. This was prepared by initially immunizing rabbits intradermally with 10^7 Chang cells in Freund's complete adjuvant, followed 3 weeks later by the intravenous injection of a further 10^7 Chang cells in 1·0 ml phosphate buffered saline pH 7·2. Serum was harvested 3 weeks after the second injection and showed cytotoxic activity for Chang cells which was confined to the 7S fraction.

Cytotoxicity: method. Target cells damage was assessed by the release of ^{51}Cr from labelled Chang cells. Trypsinized and washed Chang cells (2×10^6) were incubated with 100 μCi ^{51}Cr sodium chromate (Radiochemical Centre, Amersham; sp. act. 100–350 μCi/μgCr) in 2·0 ml Eagle's basal medium, with 20% foetal bovine serum and 20 mM HEPES buffer pH 7·2, for 40 min at 37°C. The cells were then washed three times and adjusted to a final concentration of 2×10^4 cells/ml in Eagle's basal medium with non-essential amino acids and 10% foetal bovine serum.

Cytotoxicity tests were performed in 2·0 ml aliquots of Eagle's basal medium with 10% foetal bovine serum containing 2×10^4 ^{51}Cr-labelled Chang cells and either 5×10^5 PBM (effector:target ratio, 25:1) or 200 μl heparinized whole blood. Triplicate cultures were incubated for 18 hr at 37°C in 5% CO_2 95% air and then centrifuged at 320 *g* for 10 min. Aliquots (1·0 ml) were withdrawn and the percentage ^{51}Cr release was calculated from the activity in both supernatant and residue, and the results expressed as:

$$\text{percentage specific cytotoxicity} = \frac{\text{experimental percentage } ^{51}\text{Cr release} - \text{baseline percentage } ^{51}\text{Cr release}}{\text{maximum percentage } ^{51}\text{Cr release} - \text{baseline percentage } ^{51}\text{Cr release}} \times 100$$

where baseline ^{51}Cr release was determined by incubating Chang cells alone and maximum ^{51}Cr release was obtained by lysis of Chang cells with saponin (BDH Chemicals Ltd., Poole, England).

Cytotoxicity: types of assay. (1) ADCC of both PBM and whole blood in patients and controls was determined by the addition to the basic culture system of rabbit anti-Chang serum at a dilution of 10^{-5}, this being the minimum dilution at which the antiserum failed to produce cytotoxic effects with added complement. (2) PHA-induced cytotoxicity was similarly assayed by the addition of PHA 0·1 μg/ml (KO547, Burroughs Wellcome Ltd., Beckenham, England) to the basic culture system, this concentration of PHA being one that produced slightly suboptimal cytotoxicity when tested on a dose-response basis.

Serum addition experiments. In order to test for the presence of possible blocking or inhibitory factors, 100 μl serum from twenty-three patients with SS were added to otherwise standard triplicate assays for ADCC and PHA-induced cytotoxicity, using PBM from one of three normal controls. The effect produced by aliquots of serum from thirty-five normal controls was similarly examined. All blood samples for these experiments were allowed to clot at room temperature and the harvested serum deep frozen and stored at −70°C within 3–4 hr of bleeding.

Lymphocyte subpopulations. T lymphocytes were estimated by the formation of E-rosettes with SRBC as described previously (Hughes *et al.*, 1977). B lymphocytes were detected by immunofluorescence staining of surface immunoglobulin (Papamichail, Brown & Holborow, 1971) using fluorescein-conjugated goat anti-human immunoglobulin serum (Hoechst Pharmaceuticals, Hounslow, England).

Statistical method. The cytotoxicity data were analysed by the Mann–Whitney ranking test (Siegel, 1956). The relationship between cytotoxicity in whole blood and PBM assays was examined by regression analysis, while comparison of lymphocyte subpopulations in patient and control groups was made with Student's *t*-test.

RESULTS

Antibody-dependent cytotoxicity

Patients with severe SS had reduced ADCC in both whole blood ($P = 0{\cdot}001$) and PBM assays ($P<0{\cdot}05$) when compared with normal controls, whereas the corresponding cytotoxic responses of mildly affected patients, elderly controls and those with non-immunological disease showed no such reduction (Fig. 1). There was a good correlation for ADCC between whole blood and PBM assays in both

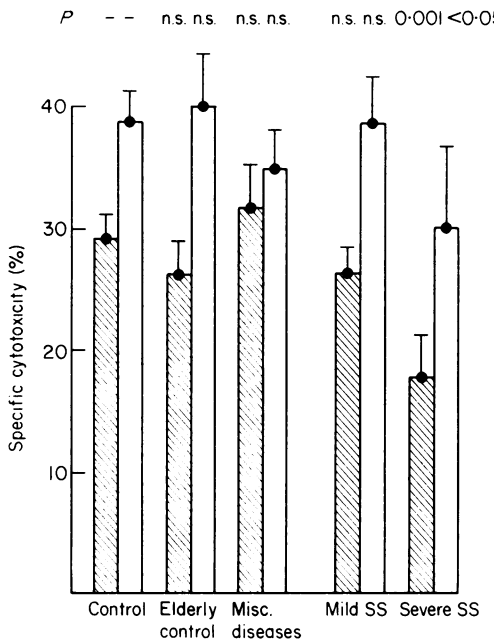

FIG. 1

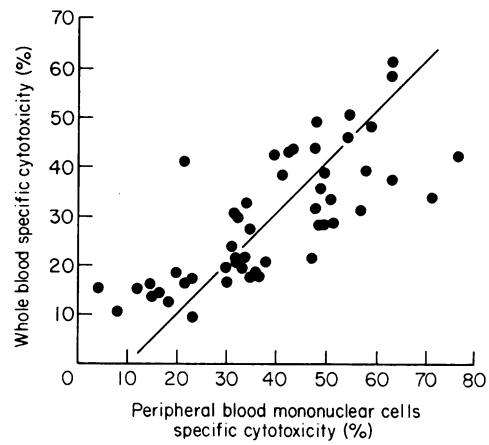

FIG. 2

FIG. 1. Antibody-dependent lymphocyte cytotoxicity (mean ± s.e.m.) for Chang liver cells (20,000/ml) using both whole blood 200 μl (▨) and peripheral blood mononuclear cells, 500,000/ml (□) in patients with mild ($n = 23$) and severe ($n = 14$) systemic sclerosis (SS), miscellaneous diseases ($n = 17$), and elderly ($n = 18$) and normal controls ($n = 51$).

FIG. 2. The relationship between antibody-dependent lymphocyte cytoxicity for Chang liver cells (20,000/ml) using both whole blood (200 μl) and peripheral blood mononuclear cells (500,000/ml) in normal controls. ($r = 0{\cdot}75$, $P< 0{\cdot}001$).

normal controls ($r = 0{\cdot}75$, $P<0{\cdot}001$, Fig. 2) and patients with mild SS ($r = 0{\cdot}64$, $P<0{\cdot}001$, Fig. 3), but this relationship was no longer observed in severely affected patients ($r = 0{\cdot}43$, n.s., Fig. 4) due to a proportionately greater reduction of ADCC in the whole blood system.

Phytohaemagglutinin-induced cytotoxicity

Patients with severe SS similarly showed reductions in PHA-induced cytotoxicity in both whole blood ($P<0{\cdot}001$) and PBM assays ($P = 0{\cdot}02$) when compared with normal controls, in contrast to the normal responses of both mildly affected patients and the other two control groups (Fig. 5). There was, again, a correlation for PHA-induced cytotoxicity between whole blood and PBM assays in both normal controls ($r = 0{\cdot}40$, $P<0{\cdot}001$, Fig. 6) and patients with mild SS ($r = 0{\cdot}72$, $P<0{\cdot}001$, Fig. 7), but severely affected patients showed a loss of this relationship ($r = 0{\cdot}42$, n.s., Fig. 8) due, once more, to a proportionately greater reduction of cytotoxicity in the whole blood assay.

Serum addition experiments

The effect of sera from patients with severe and mild SS and normal controls on ADCC and PHA-

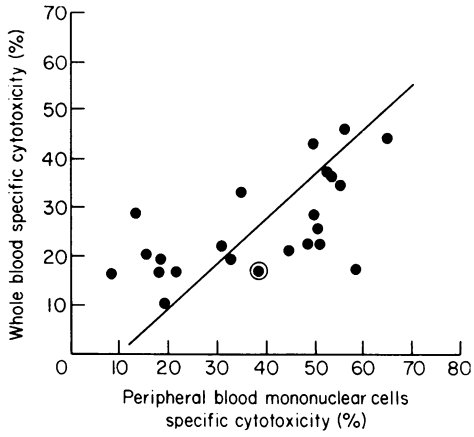

FIG. 3

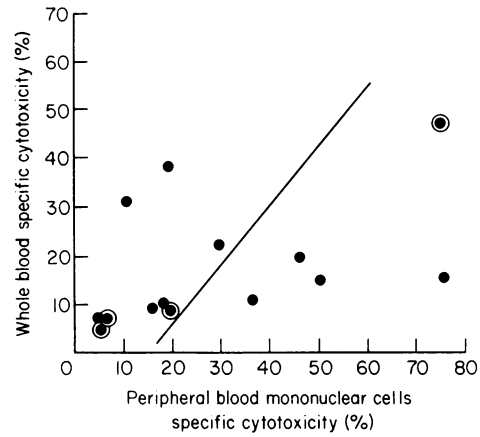

FIG. 4

FIG. 3. The relationship between antibody-dependent lymphocyte cytotoxicity for Chang liver cells (20,000/ml) using both whole blood (200 μl) and peripheral blood mononuclear cells (500,000/ml) in patients with mild systemic sclerosis. ($r = 0{\cdot}64$, $P < 0{\cdot}001$). (◉) Patient on immunosuppressive drugs.

FIG. 4. The relationship between antibody-dependent lymphocyte cytotoxicity for Chang liver cells (20,000/ml) using both whole blood (200 μl) and peripheral blood mononuclear cells (500,000/ml) in patients with severe systemic sclerosis. ($r = 0{\cdot}44$, P = n.s.) (◉) Patient on immunosuppressive drugs.

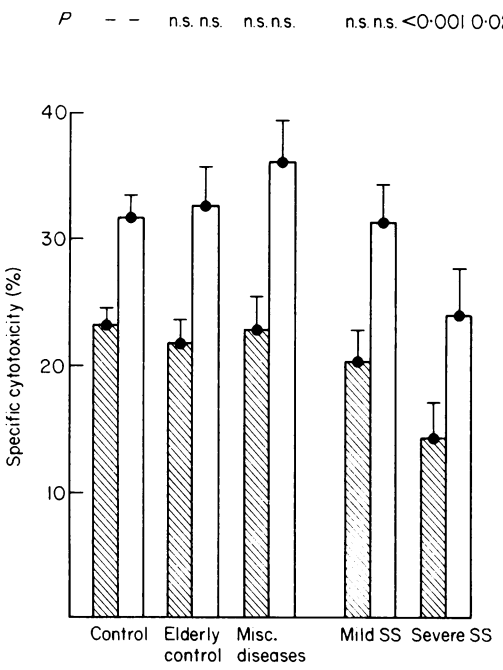

FIG. 5

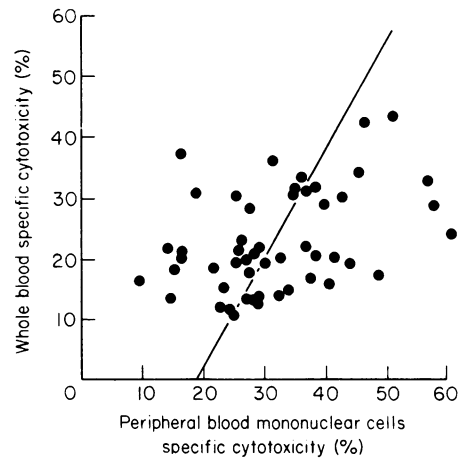

FIG. 6

FIG. 5. Phytohaemagglutinin-induced lymphocyte cytotoxicity (mean ± s.e.m.) for Chang liver cells (20,000/ml) using both whole blood, 200 μl (▨) and peripheral blood mononuclear cells, 500,000/ml (□) in patients with mild ($n = 23$) and severe ($n = 14$) systemic sclerosis (SS), miscellaneous diseases ($n = 17$), and elderly ($n = 18$) and normal ($n = 51$) controls.

FIG. 6. The relationship between phytohaemagglutinin-induced lymphocyte cytotoxicity for Chang liver cells (20,000/ml) using both whole blood (2000 μl) and peripheral blood mononuclear cells (500,000/ml) in normal controls. ($r = 0{\cdot}40$, $P < 0{\cdot}001$).

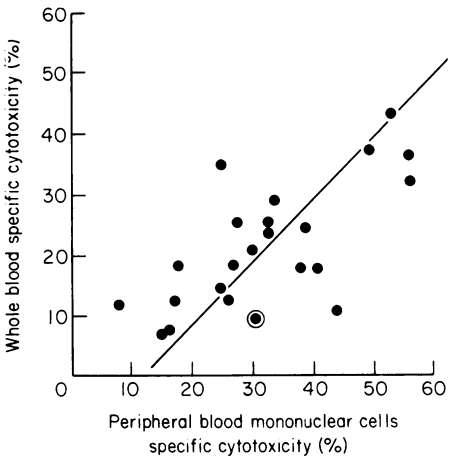

FIG. 7

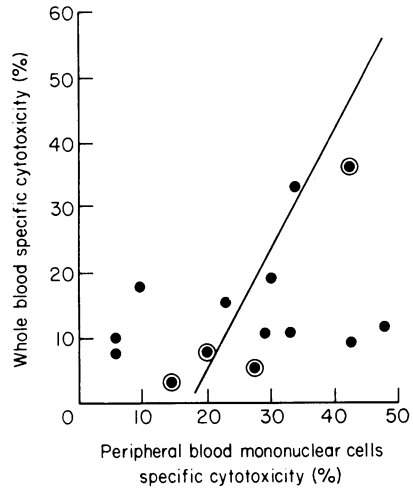

FIG. 8

FIG. 7. The relationship between phytohaemagglutinin-induced lymphocyte cytotoxicity for Chang liver cells (20,000/ml) using both whole blood (200 μl) and peripheral blood mononuclear cells (500,000/ml) in patients with mild systemic sclerosis. ($r = 0{\cdot}72$, $P < 0{\cdot}001$) (●) Patient on immunosuppressive drugs.

FIG. 8. The relationship between phytohaemagglutinin-induced lymphocyte cytotoxicity for Chang liver cells (20,000/ml) using both whole blood (200 μl) and peripheral blood mononuclear cells (500,000/ml) in patients with severe systemic sclerosis. ($r = 0{\cdot}42$, P = n.s.) (●) Patient on immunosuppressive drugs.

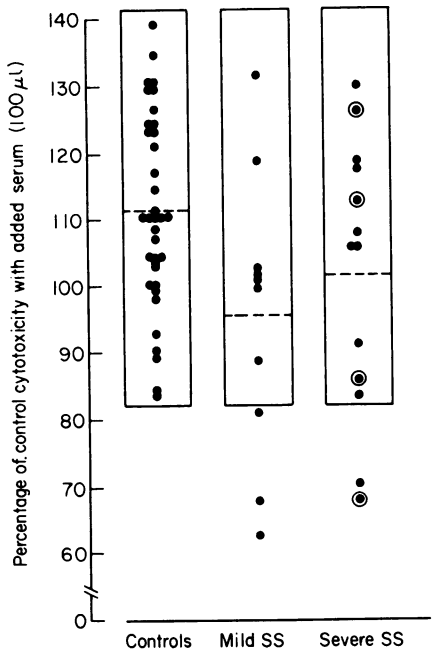

FIG. 9

FIG. 9. The effect of serum (100 μl) from patients with mild and severe systemic sclerosis (SS) and normal controls on antibody-dependent lymphocyte cytotoxicity for Chang liver cells using peripheral blood mononuclear cells from three normal controls. Enclosed area indicates 95% confidence limits for normal control sera. (●) Patient on immunosuppressive drugs.

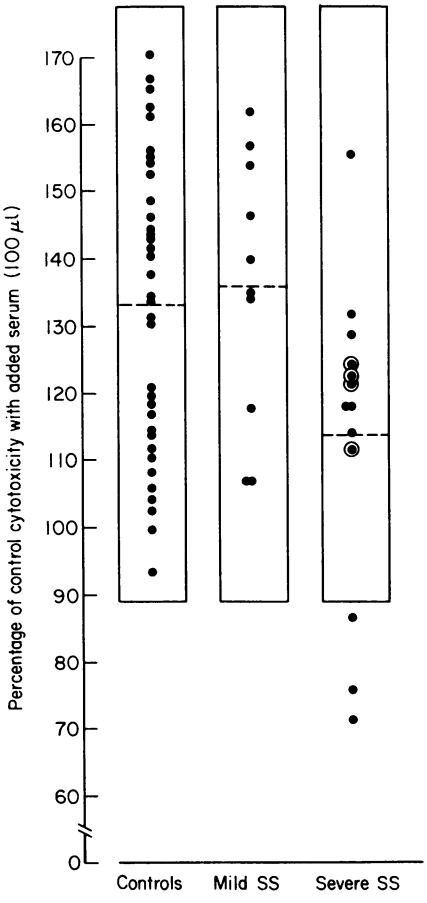

FIG. 10

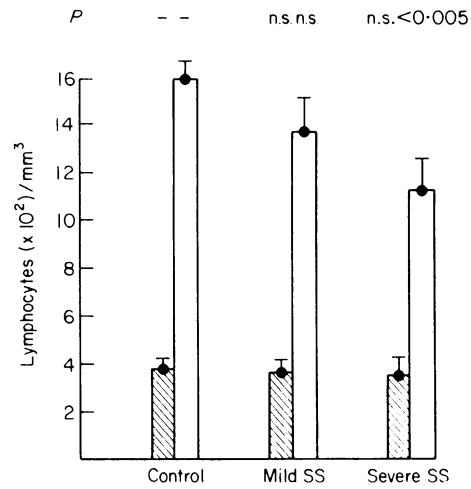

FIG. 11

FIG. 10. The effect of serum (100 μl) from patients with mild and severe systemic sclerosis (SS) and normal controls on phytohaemagglutinin-induced lymphocyte cytotoxicity for Chang liver cells using peripheral blood mononuclear cells from three normal controls. Enclosed area indicates 95% confidence limits for normal control sera. (●) Patient on immunosuppressive drugs.

FIG. 11. Lymphocyte subpopulations (mean ± s.e.m.) in patients with mild ($n = 17$) and severe ($n = 11$) systemic sclerosis (SS) and normal controls ($n = 31$). (▨) B lymphocytes; (□) T lymphocytes.

induced cytotoxicity with normal lymphocytes is summarized in Figs 9 and 10. Three sera, all from patients with severe SS, produced a marked depression of PHA-induced cytotoxicity. By contrast, a marked reduction of ADCC was produced by five sera; two sera came from patients with severe SS (only one of which produced impairment of PHA-induced cytotoxicity) and three sera from mildly affected patients. These results indicate the presence in six patients of serum factors with differing blocking or inhibitory effects in the two assay systems.

Lymphocyte subpopulations

There was no difference in the numbers of circulating B lymphocytes between the various control and patient sub-groups (Fig. 11). By contrast, patients with SS showed a reduction in the number of circulating T lymphocytes which was most marked in severely affected patients ($P < 0{\cdot}002$).

DISCUSSION

Our investigations have produced further evidence of disordered cellular immune function in SS. Patients severely affected with extensive visceral disease had impaired ADCC and PHA-induced lymphocyte cytotoxicity, irrespective of whether the effector system consisted of whole blood or PBM. The significance of these findings was emphasized by the normal responses present in patients with mild SS and in those suffering from miscellaneous non-autoimmune diseases. The present investigation also illustrates the importance of using both whole blood and PBM as effector systems when studying lymphocyte cytotoxicity in human disease. In controls and mildly affected patients, the two effector systems showed a remarkably good correlation in both assays, even though the whole blood assay inevitably contained a variable number of lymphocytes. The patients with severe disease, however, had a proportionately greater reduction in cytotoxicity in the whole blood assay which could have been due to reduced numbers or impaired function of circulating effector cells, or to the presence of blocking or inhibitory serum factors. The serum addition experiments suggested that serum factors, which could include lymphocytotoxins (Terasaki, Mottironi & Barnett, 1970), immune complexes or even immunosuppressive globulins (Cooperband *et al.*, 1969) were not a major cause of the reduced whole blood cytotoxicity which, presumably, must be due to an effector cell defect. The technically simpler whole blood assay with its variable number of lymphocytes, therefore, proved in practice to be a more sensitive system for revealing abnormalities of CMC than the PBM assay with its harvested and standardized number of lymphocytes.

The cause of the demonstrated defect in cell-mediated cytotoxicity in severe SS remains speculative. While there has certainly been debate as to the type of effector cell(s) responsible for PHA-induced cytotoxicity, a major, if not total, part of such cytotoxicity has been shown to be T lymphocyte-mediated (Wisløff, Frøland & Michaelsen, 1974; Hersey, Edwards & Edwards, 1976). The low values found in the present studies, therefore, may be partly related to the reduced numbers of circulating T lymphocytes found in severely affected patients on this (Fig. 11) and earlier occasions (Hughes *et al.*, 1976; 1977). The low K cell activity, which is also a new finding, may be part of the general depression of CMI, but could also be partly due to the blocking effects of circulating immune complexes (or lymphocytotoxins) or even result from the sequestration of this type of lymphocyte in the tissues (de Sousa *et al.*, 1977). Cytotoxic activity of lymphocytes from patients with SS against cultured muscle, fibroblasts and epithelial cells has certainly been described (Currie *et al.*, 1971), but these findings have never been confirmed nor has the type of lymphocyte participating in any of these reactions been defined. Additional indirect evidence, however, does suggest that cell-mediated immune reactions could be involved in the pathogenesis of the disease. Animals with graft-*vs*-host disease may develop scleroderma-like skin changes (Statsny, Stembridge & Ziff, 1963), while the products of stimulated lymphocytes are said to increase collagen synthesis by cultured fibroblasts (Johnson & Ziff, 1976). In man, patients who have received bone marrow transplants may also develop scleroderma-like skin lesions as a late sequel to earlier episodes of graft-*vs*-host reactions (van Vloten, Scheffer & Douren, 1977; Gratwhol *et al.*, 1977; Fenyk *et al.*, 1978). Moreover, in experimental host-*vs*-graft disease, the chronic antigenic stimulation produced by transplanted spleen cells causes not only hyperglobulinaemia and immune complex formation but also a progressive decline of cellular immune competence (Hard, 1975). In this context, the findings of the present study suggest that further investigation of both cellular cytotoxic mechanisms and humoral factors, such as immune complexes, may improve our understanding of the pathogenesis of SS.

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