

**Vascular Endothelial
Cell Dysfunction
In Systemic Sclerosis.**

**Submitted for the Degree of Doctor Of Philosophy
of The University of Sheffield**

By

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ABSTRACTS

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THESIS SUMMARY

Systemic sclerosis is a connective tissue disease of unknown aetiology, characterized by extensive vascular damage and an increased deposition of collagen. In addition, various immunological abnormalities have also been described in patients with the disease.

This thesis has provided strong evidence in support of an immunologically mediated vascular pathogenesis in systemic sclerosis. This has been demonstrated by the occurrence of cytotoxicity of human vascular endothelial cells following co-culture with patient sera and normal peripheral blood mononuclear cells. Further characterization of the serum factor capable of causing endothelial cytotoxicity revealed that the responsible serum factor was monomeric IgG and was capable of binding to vascular endothelial cells, findings which are consistent with the cytotoxic factor being an antibody directed against the vascular endothelium.

Further in vivo evidence of endothelial cell damage was demonstrated by the occurrence of elevated plasma levels of von Willebrand factor antigen in patients with systemic sclerosis. In addition, the presence of immune abnormalities including a high incidence of immune complexes and the occurrence of anticardiolipin antibodies was shown, thus emphasizing the autoimmune nature of systemic sclerosis.

Finally, prostacyclin production by cultured vascular endothelial cells was investigated, during both 15 minute and 72 hour exposure to sera from patients with systemic sclerosis. In contrast to previous studies, no significant changes were detected between patient and control sera in their effect on endothelial cell prostacyclin release.

ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
cAMP	Cyclic adenylate monophosphate
DAB	Diaminobenzidene
DEAE (sepharose)	Diethylamino ethyl (sepharose)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethane sulfonic acid
K (cell)	Killer (cell)
NK (cell)	Natural killer (cell)
PBS	Phosphate buffered saline
PBM	Peripheral blood mononuclear cell
SEM	Standard error of the mean
SD	Standard deviation
SLE	Systemic lupus erythematosus
TGF β	Transforming growth factor type beta
TNF	Tumour necrosis factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 Systemic sclerosis

1.1.1 History

Systemic sclerosis is a disorder characterized by increased deposition of collagen in the skin and viscera, including the kidney, lungs, heart and gastro-intestinal tract. This developing fibrosis is associated with a widespread vasculopathy. Systemic sclerosis may exist in a mild form, but progressive visceral involvement, resulting in cardiac failure, hypertension, renal disease, pulmonary complications and intestinal malabsorption can develop (Rodnan 1979).

The first convincing description of systemic sclerosis was published by Carlo Curzio in 1753. By 1837 there were eight such cases in the literature and the condition was termed "Sclerodermie". In 1898 Osler wrote "Scleroderma is one of the most terrible of human ills. Like Tithanus to wither slowly, and like him to be beaten down and maimed and wasted until one is literally a mummy, encased in an evershrinking, slowly contracting skin of steel, is a fate not pictured in any tragedy, ancient or modern."

Although many patients were known to die soon after the onset of skin changes, the systemic nature of the disease was not appreciated until the twentieth century. Eventually, it was generally confirmed that scleroderma was the clinical manifestation of a systemic disease, and in 1945 Goetz proposed that the term scleroderma be replaced with progressive systemic sclerosis (PSS). Many authors, however, have chosen to use the term "generalized scleroderma" or "systemic sclerosis" (SS) because the course of the disease may show a variable pattern and is not necessarily progressive in all cases. While some patients show a rapid progressive decline once visceral involvement

occurs, other cases show remission of certain components of the disease , eg Raynaud's phenomenon, whilst other organs are uninvolved, remain stable or progress (LeRoy 1981).

1.1.2 Epidemiology

Systemic sclerosis is an uncommon disease with an incidence of 4.5-12 new cases per million population per year (Kurland et al 1969; Medsger & Masi 1971, 1979). The female to male ratio in systemic sclerosis is about 3:1. and the average age of onset is 40.3 years, with 85% of cases occurring between 20-60 years (Medsger 1985; Gonzale 1959). Significant racial differences have not been observed and different geographical regions do not show an altered incidence of systemic sclerosis. (Medsger and Masi 1971; 1978). Reports of familial systemic sclerosis remain limited and there is insufficient evidence to determine whether systemic sclerosis is an inheritable disorder (Medsger 1985).

Various conflicting reports have been published regarding associations between systemic sclerosis and HLA system antigens. There appear to be weak associations which may reflect a correlation with progression rather than susceptibility to systemic sclerosis (Black et al 1985a). The discrepancies noted in such studies may be due to regional and/or ethnic differences in patient populations.

1.1.3 Clinical and pathological features of systemic sclerosis

1.1.3.1 General

Patients with systemic sclerosis show fibrosis, atrophy, inflammation and vascular defects as principle pathological features (Campbell and LeRoy 1975). The fibrosis may be diffuse or patchy, with atrophy occurring as an end stage process. Perivascular mononuclear inflammatory infiltrates are frequently observed early on in the

disease and the vascular lesion has been described as the major determinant of patient survival (Norton and Nardo 1970; D'Angelo et al 1969).

Most patients with systemic sclerosis present with either Raynaud's phenomenon or thickening of the skin on the hands or fingers. Raynauds's phenomenon, which consists of episodic digital ischaemia provoked by cold and emotion, is seen in approximately 90% of patients with systemic sclerosis and may antedate other features by months or years (Rodnan 1963). Patients may also present with rheumatic complaints with pain and stiffness of the joints and muscles, whilst others may develop myositis or visceral manifestations.

1.1.3.2 Cutaneous Involvement (Plates 1, 2, 3 & 4)

Three stages of cutaneous involvement can be seen in systemic sclerosis: (1) oedematous; (2) indurative and sclerotic; and (3) atrophic. In the oedematous phase, the skin appears taut and the fingers "sausage like". Next, in the indurative phase, the skin becomes hard and stiff and bound to the underlying structures. These changes may appear in the fingers, dorsa of the hands, forearms, face and sometimes the trunk. Finally in the atrophic phase, involved fingers become fixed in a claw-like position, and facial telangectasia may appear. Eventually, about 3-15 years after the onset of systemic sclerosis, the involved dermis may begin to soften and become pliable.

1.1.3.3 Visceral Involvement

The Kidney

Renal disease and hypertension are the major causes of death in patients with systemic sclerosis. Renal involvement usually follows earlier cutaneous abnormalities. Its presentation may take several forms, such as the onset of malignant hypertension followed by acute



Plate 1

The face in systemic sclerosis showing restriction of the mouth opening, with the development of radial furrowing and a beak-like nose.



Plate 2

The hands in systemic sclerosis, showing acrosclerotic changes, areas of depigmentation and an ulcerated calcinotic lesion.



Plate 3

The hands in systemic sclerosis showing acrosclerotic changes and digital ischaemia with resulting absorption of terminal phalanges and the loss of digits from previous gangrene.

loss of renal function and often death within one month (Scleroderma Renal Crisis). A second group may show progressive renal failure occurring over several months, and a third presentation may be with proteinuria and the development of azotaemia. Pathological findings in "Scleroderma Kidney" include focal vascular lesions consisting of intimal hyperplasia and fibrinoid necrosis of the interlobular and smaller arteries.

The Lung (Plates 5 & 6)

Pulmonary involvement is frequently seen in patients with systemic sclerosis. Abnormal pulmonary function tests and radiographic evidence of fibrosis usually precede dyspnoea. Faulty gas exchange as revealed by an abnormal carbon monoxide transfer factor, together with a decrease in vital and total lung capacity, due to fibrosis, may occur. The commonest histological findings are interstitial fibrosis and thickening of the alveolar septa, as well as pulmonary vascular lesions.

The Heart

Myocardial fibrosis and characteristic arterial lesions are the principle features of sclerodermatous involvement of the heart with resulting ECG abnormalities, arrhythmias and conduction disturbances and in severe cases, left ventricular failure. Accompanying pulmonary arteriolar lesions and fibrosis can place an added burden on the right ventricle leading to the development of cor pulmonale. Pericarditis with effusion has also been reported.

The Gastro-Intestinal Tract (Plate 7)

Oesophageal involvement and dysfunction is very common in systemic sclerosis. Diminished or absent peristalsis due to collagen deposition in the lamina propria and submucosa with atrophy of the muscularis leads to dysphagia with heartburn. Similar involvement of

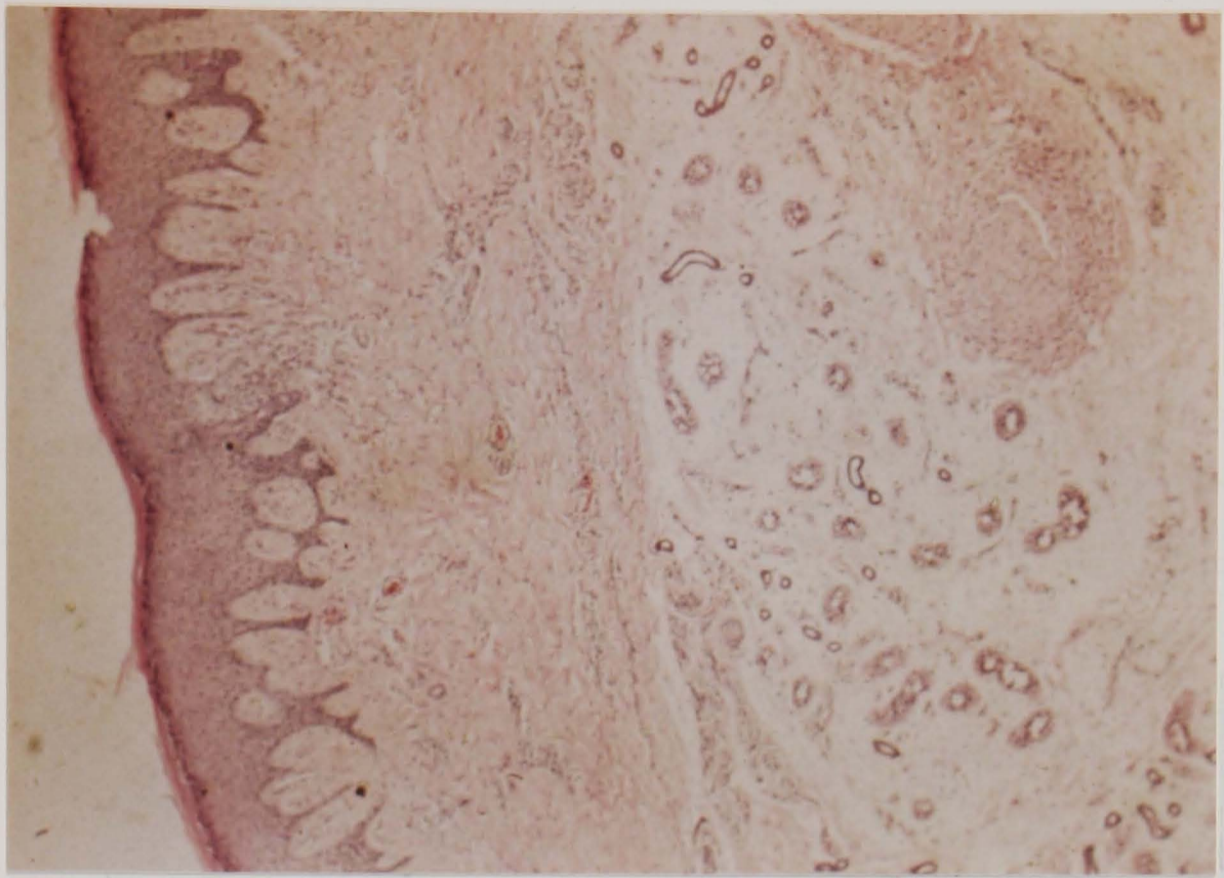


Plate 4

Skin in systemic sclerosis. The dermis is thickened due to excessive collagen deposition and a small artery in the subcutaneous tissues shows sub-intimal proliferative changes.

Haematoxylin and eosin x 40

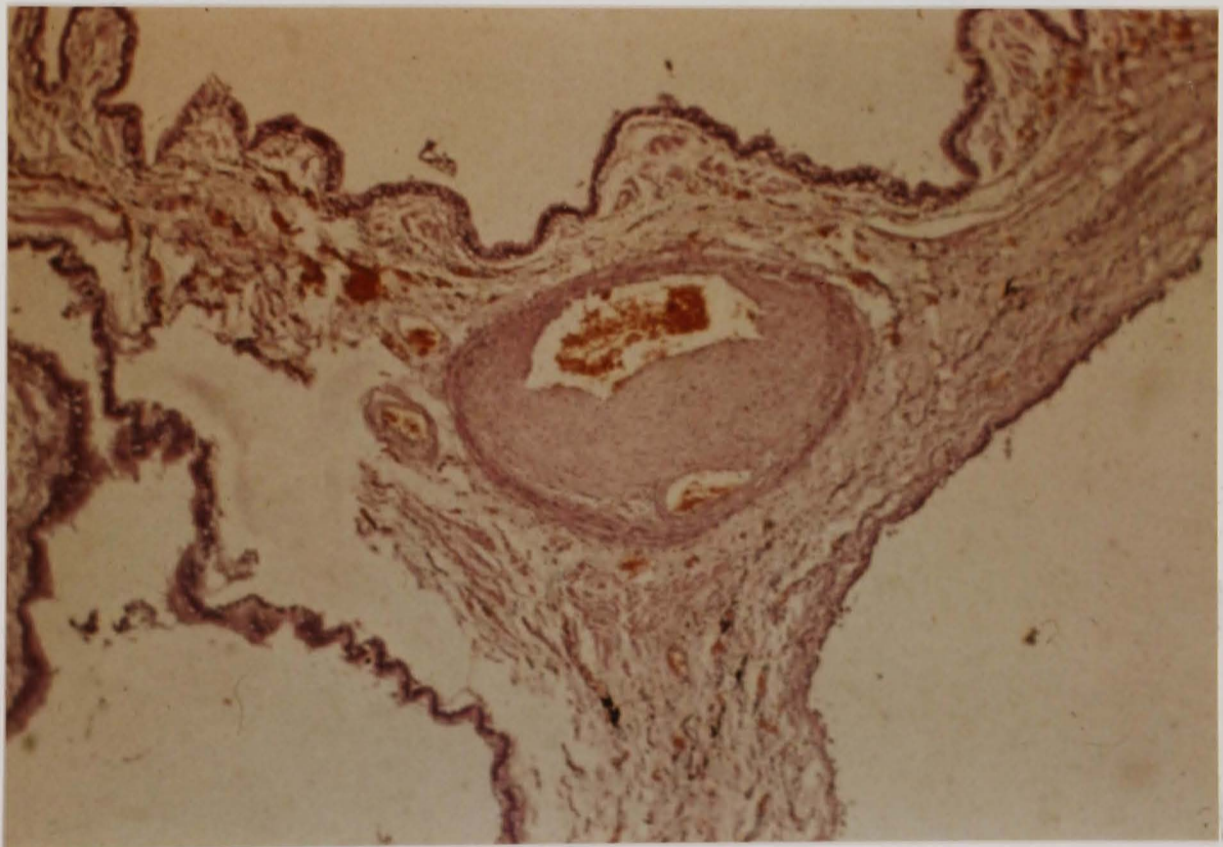


Plate 5

Lung in systemic sclerosis. Thickening of the inter alveolar septa due to excessive collagen deposition. A pulmonary artery shows marked sub-intimal proliferation.

Haematoxylin and eosin x 60

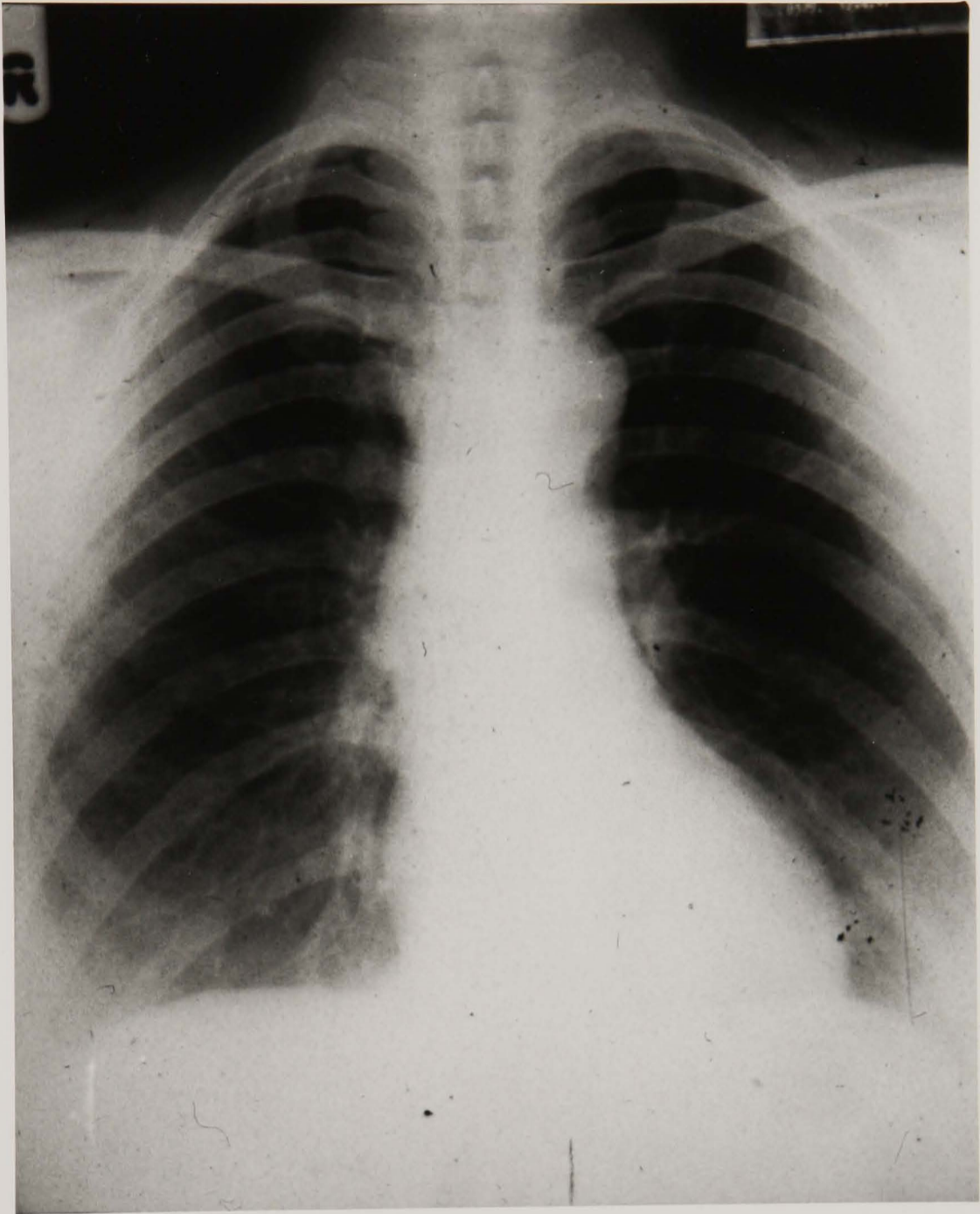


Plate 6

Chest xray from a patient with systemic sclerosis showing reticular shadowing in the lower part of the lung fields, due to pulmonary fibrosis.



Plate 7

Barium follow through examination in a patient with systemic sclerosis and malabsorption, showing multiple areas of stricture formation (and saculation) in the small bowel, due to sclerodermatous involvement.

the remainder of the gastro-intestinal tract may produce symptoms of bloating with alternating constipation and diarrhoea. Small bowel involvement can lead to bacterial colonisation and overgrowth which may contribute to malabsorption.

1.1.3.4 Vascular Involvement (Plate 8)

Raynaud's phenomenon and telangiectasia are the most obvious clinical features of the extensive vascular disease found in patients with systemic sclerosis. The resulting digital ischaemia may lead to areas of infarction in the finger tips with atrophy, recurrent infections and even gangrene.

Pathologically, the smaller arteries of 150-500 μ diameter, show the major abnormalities, whilst the large and medium sized are less involved (Campbell and LeRoy 1975; Norton and Nardo 1970). The major abnormality is thickening of the sub-intimal layer of arterioles, leading to narrowing or occlusion of the lumen. The internal elastic lamina remains intact and the media is relatively normal or slightly thin. The adventitia possesses a fibrous cuff around the artery, in which mononuclear cell infiltrates may be seen (Campbell and LeRoy 1975).

These characteristic vascular abnormalities of subintimal sclerosis and fibrinoid deposits are most frequently described in the pulmonary, renal and digital arteries and arterioles, but are not confined to these areas and have been reported in almost every part of the arterial bed. As these vascular lesions have not been restricted necessarily to areas of fibrosis, some authors have suggested that they represent the earliest lesion, preceding the development of sclerosis (Beigelman et al 1953; Campbell and LeRoy 1975; Norton and Nardo 1970; Jayson 1983,1984; Postlethwaite and Kang 1984). The occurrence of Raynaud's phenomenon months or years before the onset of

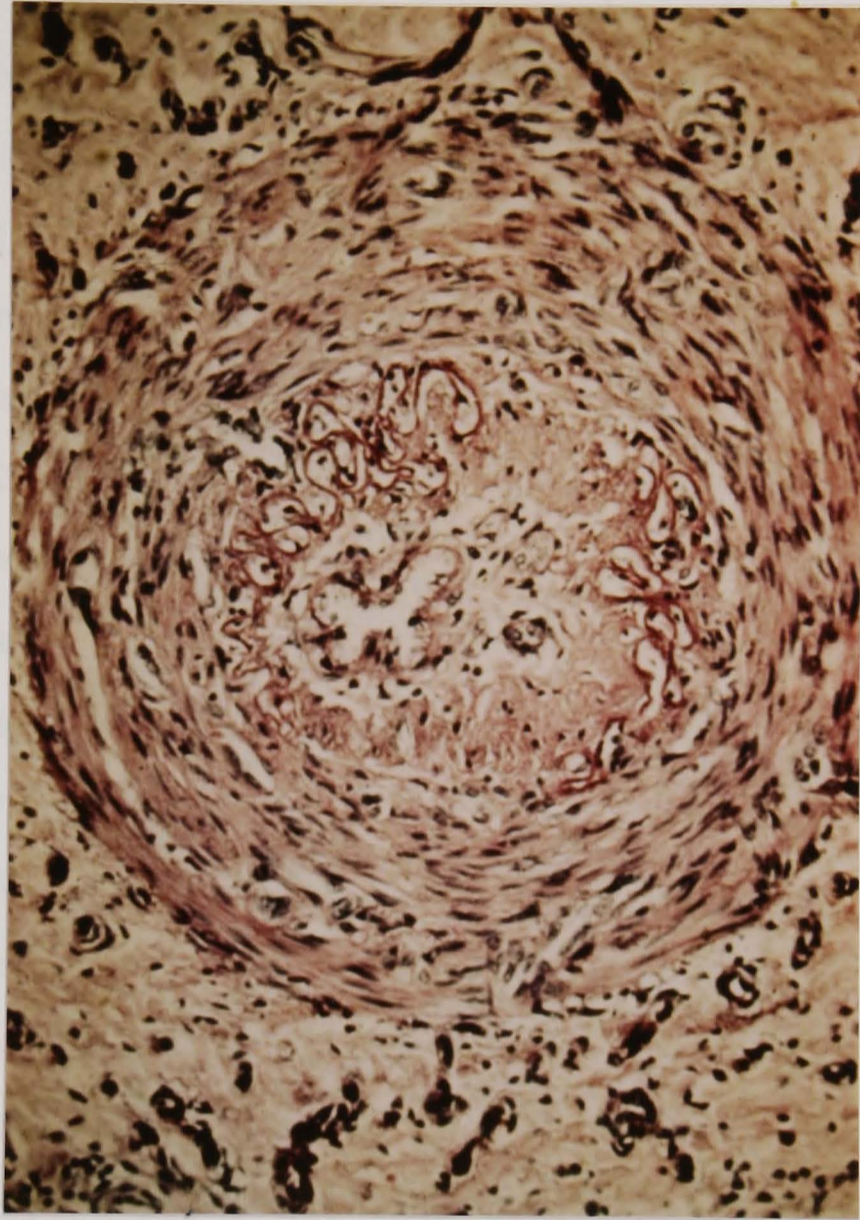


Plate 8

Artery in systemic sclerosis showing sub-intimal proliferation resulting in marked reduction of the lumen.

Haemotoxylin and eosin x 100

other clinical features adds further evidence in favour of the vascular bed being the primary site of injury in systemic sclerosis. Fibrosis could then occur as a result of alterations induced during vascular damage.

The capillary bed is also widely affected in systemic sclerosis. Using in vivo capillary microscopy, Maricq et al (1980) observed enlarged and deformed capillary loops, surrounded by relatively avascular areas, in the nailfolds of 82% of patients with systemic sclerosis. Capillary numbers are severely reduced, resulting in extreme devascularisation. (Statham and Rowell 1986; Ranft et al 1987; Wong et al 1988). The remaining capillaries become dilated with thickened walls and endothelial cells may become swollen or necrotic.

Electron microscopic studies of normal skeletal muscle in patients with systemic sclerosis have also revealed marked capillary basement membrane changes as well as a decrease in the number of capillaries (Norton et al 1968). Thickening and multilayering of capillary basement membranes was also noted in electron micrographs of labial salivary gland and skin biopsies (Camilleri et al 1984; Fleischmajer et al 1976).

In addition to morphological defects, patients with systemic sclerosis also exhibit functional abnormalities of the vascular system. These have been demonstrated by arteriography, capillary blood flow studies, radioactive tracer clearance, calorific heat loss and studies of tissue oxygenation (Maricq 1988).

1.2 The pathogenesis of systemic sclerosis

The pathogenesis of systemic sclerosis is poorly defined. However, immunological, and vascular abnormalities with altered regulation of fibroblast function could be important. It is probable that no single factor accounts for all the features of this disorder and evidence will be presented showing that considerable interaction between these three areas is involved in the pathogenesis of systemic sclerosis.

1.2.1 Connective tissue involvement

In considering the pathogenesis of systemic sclerosis, the role of connective tissue must not be omitted. Systemic sclerosis is a disease which is characterised by over-production of collagen. This process results in the deposition of dense acellular connective tissue which irreversibly impairs the function of involved organs. The structure and function of connective tissue is determined by proteins such as collagen, fibronectin and proteoglycans, and the cells producing these substances. The synthesis, degradation and deposition of these proteins is altered in systemic sclerosis, but whether this occurs as a primary or secondary disease process is unresolved.

Accelerated in vitro growth of normal and scleroderma fibroblasts by a mitogenic factor present in the sera of patients with systemic sclerosis has been described (Potter et al 1985). In addition, an increase in the biosynthetic activity of fibroblasts associated with sclerodermatous lesions has been shown to result in increased production of collagen and glycosaminoglycans (Buckingham et al 1985).

The presence of activated cells synthesising connective tissue may be due to direct stimulation of cells in the area of the lesion. Alternatively, chronic suppression of less active cells may occur, resulting in "selective" accumulation of more actively synthetic

cells. In favour of this second hypothesis, Kahaleh and LeRoy (1986) showed the selective appearance, in vitro, of a high collagen producing subset of fibroblasts as a result of prolonged culture in sera from patients with systemic sclerosis. Thus they deduced that a mitogenic factor in these sera may alter the phenotype of fibroblasts, resulting in the appearance of a population of fibroblasts producing high amounts of collagen.

As well as increased production of collagen, a decrease in its breakdown, for example by collagenase, may occur in systemic sclerosis (Brady 1975). In addition, reduced production of inhibitory mediators may allow mitogenic factors to induce unrestricted fibroblast proliferation (Duncan et al 1984).

Various substances, such as lymphokines, monokines and various platelet derived factors, have been shown to stimulate fibroblast proliferation. Thus, vascular endothelial cell damage, however produced, could result in platelet aggregation following the exposure of collagen in damaged vessel walls. The resulting release of platelet derived factors could then act on the exposed subendothelium and initiate fibroblast proliferation with an increase in collagen biosynthesis, leading to fibrosis. Transforming Growth Factor β ($TGF\beta$) is released during thrombin induced degranulation of platelets (Assoian and Sporn 1986) and has been shown to stimulate collagen and fibronectin formation in cultures of fibroblasts (Roberts et al 1986; Ignatz & Massague 1986). In addition, $TGF\beta$ is a chemoattractant for human monocytes and has been shown to stimulate production, by monocytes, of biologically active mediators which enhance fibroblast growth (Wahl et al 1987). Tumour Necrosis Factor α ($TNF\alpha$) has also

been shown to stimulate the growth of cultured fibroblasts and can stimulate endothelial cells to produce Interleukin 1 which can also act as a fibroblast mitogen (Nawroth et al 1986; Schmidt et al 1982).

Thus it appears that there is a complex interplay between several different cell types and their secretory products, disturbances of which, for example by endothelial cell injury, could lead to the fibrosis which is such a characteristic feature of systemic sclerosis.

1.2.2 Immune pathogenesis

An immune basis for the pathogenesis of systemic sclerosis is suggested by the occurrence of features shared with other diseases of autoimmune aetiology. Thus, occasionally, systemic sclerosis may show "overlap" or clinical association with SLE, polymyositis, or Sjogren's syndrome (Tuffanelli & Winkelmann 1962). Marked autoantibody formation and abnormalities of cell-mediated immunity are features of all these diseases and are also prevalent in systemic sclerosis (Maddison 1988; Postlethwaite & Kang 1988).

The appearance of scleroderma-like skin disease in some recipients of HLA-identical allogeneic bone marrow transplants with graft-versus-host disease, would suggest that systemic sclerosis may be an autoimmune disease (Clements et al 1985). Patients with graft-versus-host disease show many cutaneous, pulmonary, renal, musculoskeletal and vascular abnormalities which are strikingly similar to those seen in systemic sclerosis (Clements et al 1985; Herzog et al 1980). Graft-versus-host disease, whether in the human transplantation patient, or when experimentally induced in the rat, thus offers a model for the further investigation of the immune abnormalities in systemic sclerosis (Stastny et al 1963)

The perivascular infiltrates of lymphocytes, plasma cells and macrophages frequently found in the skin and other affected tissues provide further histological evidence for an immune pathogenesis of systemic sclerosis.

1.2.2.1 Humoral immune abnormalities

Numerous investigators have demonstrated humoral immune abnormalities in systemic sclerosis. These include the presence of hypergammaglobulinemia, cryoglobulinemia, a wide range of autoantibodies including, antinuclear and antinucleolar antibodies, rheumatoid factor, antibodies directed against collagen, mitochondria, lymphocytes and endothelium, and, in addition, raised levels of circulating immune complexes (Bernstein et al 1982; Rodnan 1979; Mackel et al 1982; Fregeau et al 1988; Pruzanski et al 1982; Hashemi et al 1987 & Hughes et al 1983)

(i) Autoantibodies

It has long been recognised that anti-nuclear antibodies are present in the sera of patients with systemic sclerosis. The reported frequencies have shown a wide range from 40-90% (Tan 1982). These variations are probably due to the use of different methods of detection. In more recent studies, the use of cultured cells as substrates and the avoidance of certain fixatives such as methanol and ethanol, has revealed that anti-nuclear antibodies occur in approximately 95% of patients with systemic sclerosis (Bernstein et al 1982; Moroi et al 1980)

Bernstein et al (1982) have demonstrated, by indirect immunofluorescence, anti-nuclear and/or anti-nucleolar antibodies in the sera of 97% of patients with systemic sclerosis, using cultured HEP-2 cells as substrate. As in SLE, various fluorescent patterns can be seen, reflecting the diversity of anti-nuclear antibodies present.

Typical nuclear staining patterns in systemic sclerosis are centromere, fine and coarse speckles, diffusely grainy, homogeneous and nuclear dots. Three nucleolar staining patterns occur:- speckled; homogeneous; and clumpy; (Bernstein et al 1982).

The centromere pattern of staining is present in 31% of patients with systemic sclerosis (Moroi et al 1980). The responsible autoantibody has been shown, by immuno-electron microscopy, to be reactive with the inner and outer plates of the kinetochore (Brenner et al 1981). Clinical studies have revealed that the antikinetochores antibody occurs in patients with the CREST syndrome, characterised by calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly and telangiectasia (Steen et al 1984; Fritzler et al 1980; Chen et al 1985).

The use of rabbit and calf thymus extracts and immunodiffusion analysis has led to the identification of various precipitating antibodies in systemic sclerosis. Douvas et al (1979) showed that precipitating antibodies to a 70,000 Kd soluble extractable nuclear antigen known as Scl 70, are highly specific for systemic sclerosis. However, this antibody is only present in 20-25% of patients with systemic sclerosis, and it correlates with the diffusely grainy pattern of nucleoplasmic staining. The Scl-70 antigen has since been shown, by using SDS PAGE and immunoblotting with anti Scl-70 positive sera, to be topoisomerase 1, with a molecular weight of 100,000 Kd (Shero 1987).

Antibodies to Scl-70 are more frequent in patients with diffuse systemic sclerosis than those with the CREST syndrome, and they appear to identify those patients with more severe skin, joint and lung involvement (Powell et al 1984). Livingston et al (1987), demonstrated the occurrence of Scl-70 in patients with generalised

disease and they showed an association between HLA-DR2 and Scl-70 positive patients, suggesting a genetic-serologic clinical subset of patients.

In addition to Scl-70 and antikinetochores antibodies which are specific for systemic sclerosis, other precipitating antibodies may occur in this disease. Anti RNP, anti Ro and anti La, which may commonly occur in SLE, may also be seen in patients with systemic sclerosis (Harmon 1985; Bernstein et al 1984). Patients with systemic sclerosis/polymyositis overlap syndromes show various specific autoantibody systems, including anti Ku and PM-Scl (Mimori et al 1981; Reichlin et al 1985). These and other antigen-antibody systems occurring in overlap syndromes await biochemical characterisation (Maddison 1988)

Auto-antibodies directed against other antigens have also been detected in patients with systemic sclerosis and antibodies to type I, III and IV collagen have been detected (Mackel et al 1982, Black et al 1985b). In addition, antibodies directed against smooth muscle cells and cell surface and nuclear antigens on cultured rat embryonic fibroblasts have also been detected (Kitridou et al 1974, Brentnall et al 1982). Furthermore, recent studies have produced evidence that the sera of patients with systemic sclerosis also contain antiendothelial cell antibodies (Hashemi et al 1987; Baguley et al 1987a ; Byron et al 1987)

Thus, it is evident that considerable reactivity towards autologous cellular antigens occurs in systemic sclerosis. Although the cause of the vascular damage present in systemic sclerosis is unknown, it is possible to hypothesize that an antibody response

towards autologous endothelium may be partly responsible. Exposure of the basement membrane following endothelial injury could then initiate a further antibody response to autologous collagen.

(ii) Immune Complexes

Immune Complexes are formed by the non-covalent binding of antigen and antibody. They are normally cleared from the circulation either by interaction with Fc Receptors on circulating or organ associated phagocytic cells, or via activation of the complement system, followed by their phagocytic removal. Complexes which are not removed from the circulation may deposit on vessel walls, activate various effector systems and subsequently induce tissue damage.

Immune complexes have been reported in many connective tissue diseases including SLE and Sjogrens syndrome and immune complex deposits have been described in scleroderma kidney (McGiven et al 1971). The presence of variable levels of circulating immune complexes in patients with systemic sclerosis has been demonstrated by numerous authors. This variability has been explained by the use of different immune complex assays, which detect complexes by different physical or immunological properties (Siminovich et al 1982). For instance the Raji cell method detects immune complexes which will bind to Fc receptors on the Raji cells whereas the C1q binding assay detects complexes containing immunoglobulin which is capable of fixing complement. Thus, in any study on the levels of circulating immune complexes, it is important that several different assay systems are employed in the determination. However, an alternative explanation for widely varying results may be differences in the patient sub-groups studied (French et al 1985).

Low levels of immune complexes have been reported by various authors. Siminovich et al (1982), using fluid and solid phase C1q binding assays, C1 activation and the fluid phase conglutin assay, detected immune complexes in only six out of forty one patients with systemic sclerosis. In a smaller study of eight patients, Solling et al (1979), tested C1q binding, polyethylene glycol precipitable light chain determinants and a complement consumption assay. They showed only one patient to be positive in any two of the three assays. O'Loughlin et al (1980), found no evidence of elevated immune complexes in ten patients with systemic sclerosis using the C1q binding method.

In contrast to these studies, high levels of circulating immune complexes have been demonstrated when generally larger samples have been investigated. An early study by Pisko et al (1979), using the Raji cell radio immunoassay, showed that seven of sixteen patients had elevated levels of circulating immune complexes. Swierczynska et al (1984), using an immunoelectrophoretic method of determination, showed that eighteen of twenty nine of patients with systemic sclerosis had elevated levels of immune complexes.

Later studies have combined various methods of immune complex determination. Cunningham et al (1980) used five different methods: decreased haemolytic complement, C1q precipitins, cryoglobulins, Raji cell radioimmunoassay and C1q binding. Twelve of twenty patients (60%) showed immune complexes by at least one method of determination. Similar levels of immune complexes were demonstrated by Hughes et al (1983) using fluid phase C1q binding, K cell inhibition and Raji cell radioimmunoassay. Twenty of thirty four (58.5%) showed a positive result in one or more tests. Chen et al (1984) studied forty one patients with systemic sclerosis and found

that nineteen (46%) showed immune complexes by either C1q or Protein A binding assays. A large study of ninety two patients with systemic sclerosis was carried out by Seibold et al (1982). Abnormal results were found in forty three patients (47%), using Raji cell radio-immunoassay, agarose gel electrophoresis and C1q binding assays. Haustein and Herman (1985) examined seventy four patients for circulating immune complexes by polyethylene glycol precipitation and C1q binding, 31% of patients were again positive in both tests.

The Raji cell determination for immune complexes has produced the largest number of positive tests: Haustein and Herman (1985) 78%; Seibold et al (1982) 72%; Pisko et al (1979) 44%; Cunningham et al (1980) 55%; and Hughes et al (1983) 38%. It is possible that antilymphocytic antibody can give rise to false positive results in the Raji cell radioimmunoassay (Anderson & Stillman 1980). However, other methods of immune complex determination in which the presence of anti lymphocytic antibodies will not produce false positive results, have shown significantly elevated levels of circulating immune complexes in patients with systemic sclerosis.

Several authors have reported an association between organ involvement and the presence of circulating immune complexes. An association between elevated immune complexes and abnormal echocardiograms was found by Pisko et al (1979). Hughes et al (1983) demonstrated that the presence of immune complexes in patients with systemic sclerosis correlated with extensive visceral involvement and Siminovich et al (1982) showed that there was a significant correlation between immune complexes and lung involvement in systemic sclerosis. This pulmonary association was also noted by Seibold et al (1982). The immune complex positive patients studied by Chen et al

(1984) had higher frequencies of kidney, heart and muscle involvement and digital ulceration, compared to those with no detectable immune complexes.

Thus, there is good evidence that patients with systemic sclerosis have increased levels of circulating immune complexes, which may well be formed by the union of autoantibodies and released cellular antigens. A relationship between immune complexes and precipitating antibodies to Scl 70, nRNP, Ro and La in the sera of patients with systemic sclerosis has been demonstrated (French et al 1985). The relevance of these immune complexes to the pathogenesis of systemic sclerosis remains unclear. However, there is evidence that they may be deposited in the walls of diseased blood vessels, and they also have the potential to interact with Fc receptors on platelets and effector cells (Penttinen 1977). Hence, immune complexes may be partly responsible for some of the pathological features of systemic sclerosis.

(iii) Cytokines

In addition to the direct effect of the cells of the immune system, various cytokines secreted from these cells may play a vital role in the pathogenesis of systemic sclerosis. Mononuclear cell infiltrates are frequently seen in the skin and it is possible therefore that monokines and lymphokines released during cell mediated immune reactions may be important in the fibrotic processes occurring at these sites in patients with systemic sclerosis. In addition, antigen or mitogen stimulation of mononuclear cells may release monokines and lymphokines which have been shown to be capable of modulating fibroblast function, such as collagen and collagenase production and fibroblast chemotaxis.

Postlethwaite and Kang (1985) have described a lymphocyte derived chemotactic factor for fibroblasts, and a mononuclear cell derived mitogenic factor for fibroblasts. In addition, these authors have demonstrated that Interleukin-1 from monocytes stimulates collagenase production and that fibroblast derived collagen production can be stimulated by a lymphokine. Further support for this concept has been presented by Perlsh and Fleischmajer (1985) who showed that normal cultured fibroblasts showed increased synthesis of collagen following exposure to mononuclear cells from patients with systemic sclerosis. Such an effect was not seen with mononuclear cells obtained from normal and control donors.

More recent studies have shown a decreased production of Interleukin-1 by peripheral blood mononuclear cells of patients with systemic sclerosis compared to those from normal controls (Whicher et al 1986, Sandborg et al 1986). Sandborg et al have also demonstrated increased production, by mononuclear cells in systemic sclerosis, of an interleukin-1 inhibitor which has fibroblast stimulating activity.

The precise mechanisms involved in cytokine regulation of the cellular immune system and their effect on fibroblast activities remain unresolved. Further investigation is required before their role in the fibroblast proliferation and excess collagen production seen in patients with systemic sclerosis can be defined. However, it appears likely that cytokines contribute in some way to the pathogenesis of this disease.

1.2.2.2 Cellular immune abnormalities

As cellular immune abnormalities are common in SLE and other autoimmune diseases, several investigators have studied the cellular immune system in patients with systemic sclerosis. Thus, it has been found that patients have decreased numbers of peripheral T lymphocytes

but normal levels of circulating B lymphocytes (Hughes et al 1976; Bakke et al 1985). The reason for the reduction in T cell number remains unknown, but may be due to the accumulation of these cells in the skin and viscera, especially in perivascular areas (Whiteside et al 1985a).

Functional activities of T cells from patients with systemic sclerosis have also been studied, often with conflicting results. T cell responses to phytohaemagglutinin have been reported as depressed (Hughes et al 1977) and normal (Alarcon-Segovia et al 1985; Whiteside et al 1985b). These discrepancies are probably due to the purity of cell preparations and variations in the patient populations studied.

Immunoregulatory function has also been studied in these patients, by measuring T Helper and T Suppressor cell functions. T Helper cell function has been shown to be elevated in studies utilising pokeweed mitogen stimulated B cells (Alarcon-Segovia 1985). This may be due to a decrease in suppressor cell numbers, as is suggested by studies which have used monoclonal antibodies directed against the CD4 and CD8 markers of T helper and T suppressor/cytotoxic cells (Whiteside et al 1983; Keystone et al 1982). However the use of these markers to distinguish helper and suppressor populations of T cells remains controversial.

Patients with systemic sclerosis have been shown to have cellular immunity to various autoantigens including collagen, fibroblasts and muscle cells and other undefined antigens. A proliferative response of lymphocytes from patients with systemic sclerosis has been demonstrated following exposure to connective tissue, muscle, skin, and normal human and chick type I collagen (Currie et al 1970; Kondo et al 1979; Stuart et al 1976). Furthermore, lymphocytes from patients with systemic sclerosis have been found to be cytotoxic to

cultured fibroblasts, epithelial cells and myocytes (Currie et al 1970). However, Kondo et al (1979) failed to confirm these observations. Wright and colleagues also showed no cytotoxicity against epithelial and fibroblastic cell lines. These authors studied spontaneous and phytohaemagglutinin induced cytotoxicity and observed a decrease in cytotoxicity against Chang liver cells which correlated with disease severity (Wright 1979 & Wright et al., 1978, 1979, 1982).

However, of greater pathological interest is the ADCC produced by normal human peripheral blood mononuclear cells in the presence of sera obtained from patients with systemic sclerosis. This type of cytotoxicity has been demonstrated against target cells obtained from various cell lines, and more recently against vascular endothelium, with sera from patients with both systemic sclerosis and SLE (Penning et al 1983, 1984a, 1984b, 1984c, 1985; Wright et al 1983). It is likely that this mechanism is responsible for some of the vascular damage which occurs in patients with systemic sclerosis. In this thesis the ADCC against vascular endothelium, occurring in patients with systemic sclerosis, will be further characterised with the aim of identifying the serum factor responsible for inducing cytotoxicity.

In addition to abnormalities of mononuclear cell mediated immunity, recent work has suggested that polymorphonuclear neutrophils may play a role in the pathogenic mechanisms occurring in systemic sclerosis. Oxygen radicals released from stimulated neutrophils have been shown to damage the vascular endothelium in vitro (Weiss et al 1981; Sacks et al 1978; Varani et al 1985). Maslen et al (1987) have demonstrated that "resting" neutrophils from patients with systemic sclerosis secrete increased levels of peroxide in vitro, compared to normal control cells. An increase in the basal chemiluminescence activity of neutrophils from patients with systemic sclerosis has also

been shown (Czirjak et al 1987). It is possible, therefore, that the impaired function of neutrophils thus demonstrated in patients with systemic sclerosis could give rise to some of the vascular damage that is a prominent feature of this disease. In addition, neutrophils possess receptors for the Fc region of IgG and therefore could act as effector cells mediating tissue damage by ADCC.

1.2.3 The vascular hypothesis as a pathogenic mechanism

Vascular abnormalities are a prominent feature in the pathology of systemic sclerosis. It is well established that proliferative intimal arterial lesions and obliterative microvascular defects are common in the disease. The distinctive vascular pathology occurring in systemic sclerosis, together with the frequent occurrence of perivascular mononuclear infiltrates, have lead several authors to hypothesise that the vascular system is the primary site of injury in systemic sclerosis (Campbell and LeRoy 1975; Norton and Nardo 1970). Further support of this hypothesis comes from the frequent early occurrence of Raynaud's phenomenon in up to 98% of patients with systemic sclerosis (Rodnan 1963; Rowell 1988).

Numerous authors have looked for evidence of vascular dysfunction in systemic sclerosis. Most of these studies have been directed towards the vascular endothelium. Endothelial cell damage, and defects in the production and release of various endothelial cell products, such as von Willebrand factor antigen and prostacyclin, are possible indicators of mechanisms giving rise to vascular injury in systemic sclerosis.

1.2.3.1 Von Willebrand factor antigen and its related activities

Von Willebrand factor antigen is produced by endothelial cells and elevated levels of this factor have been proposed as a marker of endothelial cell damage (Woolf et al 1987; Nusinow et al 1984). Raised plasma levels have been demonstrated following vascular injury and in a variety of clinical conditions often in association with vascular disease. These diseases include diabetes mellitus with proliferative retinopathy, severe atherosclerosis, and renal failure (Coller et al 1978; Green et al 1978; Warrel et al 1979).

Raised circulating levels of von Willebrand factor antigen have been observed in patients with systemic sclerosis, as has ristocetin co-factor, which is the functionally active component of Factor VIII (Kahaleh et al 1981; Pagano et al 1986; Lee et al 1985). However, it has been suggested that von Willebrand Factor antigen may participate in acute phase responses so great care is needed in interpreting studies based solely on measurements of the factor VIII complex alone (Paleolog et al 1987).

1.2.3.2 Disorders of the coagulation system

Abnormalities of the coagulation system may well play a role in the development of lesions in systemic sclerosis. Endothelial cell damage, however it is caused, may be one factor leading to platelet aggregation, through the exposure of collagen in blood vessel walls. This would result in the release of β thromboglobulin, transforming growth factor β and serotonin, which would then be free to act on the exposed subendothelium. In addition, arachidonic acid, cAMP and endoperoxides may also be released following platelet degranulation and these could be utilised in the production of prostacyclin by endothelial cells. Increased circulating levels of platelet aggregates and β thromboglobulin have been detected in patients with

systemic sclerosis, confirming some degree of in vivo platelet activation (Kahaleh et al 1982). β Thromboglobulin has been shown to inhibit the production of prostacyclin in cultured bovine endothelium (Hope et al 1979). Since prostacyclin is a potent platelet disaggregating agent, inhibition of its release could further contribute to the vascular endothelial cell dysfunction occurring in patients with systemic sclerosis.

In addition, plasminogen activator, a substance which is important in the initiation of fibrinolysis, is derived mainly from vascular endothelial cells. Endothelial cell damage may therefore be followed by a fall in plasminogen activator levels and a corresponding reduction in fibrinolysis leading to fibrin deposition. Impairment of fibrinolysis has been demonstrated in patients with systemic sclerosis by measurements of the euglobulin clot lysis time (Holland & Jayson 1985; Kahaleh & Leroy 1985). Prolongation of this time indicates a decrease in fibrinolytic activity, and possibly reflects a reduction in the release of plasminogen activator. Holland & Jayson (1985) also showed increased levels of plasma fibrinogen in patients with systemic sclerosis. Thus, impairments of the fibrinolytic system in systemic sclerosis offers additional indirect support for the occurrence of vascular endothelial damage in the pathogenesis of this disease.

1.2.3.3 Endothelial cell injury

The widespread occurrence of vascular lesions in patients with systemic sclerosis, together with ultrastructural studies of affected endothelium, showing swelling, separation of endothelial cell tight junctions and detachment of cells, has led many authors to examine the effect of sera from patients on endothelial cell growth and viability in vitro.

Kahaleh et al (1978,1979) were the first investigators to describe specific endothelial cell cytotoxic activity, as assessed by depressed tritiated thymidine incorporation and a dye exclusion microcytotoxicity assay. This in vitro endothelial cell injury was blocked by protease inhibitors, and immunoglobulin and complement did not appear to be essential for its activity. Levels of Kahaleh's endothelial cell specific cytotoxic activity fluctuated and did not always correlate with disease activity. Furthermore, when serum from one patient was tested on endothelial cells derived from eight different primary cultures, the degree of inhibition of tritiated thymidine uptake varied from 10-91% indicating differences in endothelial cell susceptibility to this toxic factor. A more recent report by Kahaleh et al (1988) describes the presence of lymphotoxin in the sera of some patients with systemic sclerosis, and they suggest that endothelial cell injury may be mediated by activated T lymphocytes through the production of lymphotoxin.

Since this original report, various authors have carried out similar studies with conflicting results. Shanahan and Korn (1982) found that serum-mediated endothelial cell cytotoxicity occurred in only 7% of twenty eight sera from patients with systemic sclerosis, shown by an inhibition of tritiated thymidine incorporation, or ⁵¹chromium release, in cultured human umbilical vein endothelial cells. These authors also found that cytotoxicity of endothelial cells occurred in other connective tissue diseases such as SLE and rheumatoid arthritis, and it lacked specificity in that it also occurred with fibroblasts. A similar lack of target cell specificity was observed by Cohen et al (1983) who showed that 40% of sera from patients with systemic sclerosis were cytotoxic towards pulmonary arterial and human umbilical vein endothelial cells, foreskin

fibroblasts and neuroblastoma cells. Meyer et al (1983) also described lack of disease and target cell specificity by the cytotoxic factor occurring in the sera of patients with systemic sclerosis which was originally described as being specific for endothelial cells (Kahaleh et al 1978,1979). Cohen et al (1983) demonstrated inhibition of tritiated thymidine incorporation in fibroblast as well as human umbilical vein endothelial cell cultures, by sera from seven of thirty patients with systemic sclerosis. The cytotoxic serum factor was also demonstrated in patients with SLE, mixed connective tissue disease, Raynaud's phenomenon and rheumatoid arthritis. A more recent report by Deicher et al (1987) described a small molecular weight endothelial cell cytotoxic activity in the sera of patients with systemic sclerosis, which consisted of leukotriene B₄ bound to a 5 kd carrier. These authors have since described an endothelial cytotoxic factor which was capable of reducing endothelial cell migration and fibronectin production in vitro, and when the factor was injected into rabbits it caused a significant increase in serum levels of von Willebrand factor antigen (Drenk & Deicher 1988). However, Summers et al (1984) found no evidence of cytotoxicity in sera from patients with systemic sclerosis, using both tritiated thymidine incorporation and cellular ATP levels to assess cell growth. These conflicting observations have been put into context by a more recent suggestion that the cytotoxic factor may be an artifact produced by the oxidation of lipoproteins occurring during the storage of serum (Blake 1985).

In contrast to any possible protease dependent serum-mediated endothelial cell cytotoxicity, Penning et al (1984a,b) reported that IgG containing fractions of sera from patients with systemic sclerosis were capable of producing cytotoxicity of cultured human umbilical vein endothelial cells, when co-cultured with normal peripheral blood

mononuclear cells. Cytotoxicity of this nature, which was first described by Moller in 1965, is known as antibody dependent cellular cytotoxicity (ADCC). The ADCC described by Penning et al (1984a) tended to occur in patients with both circulating immune complexes and antibodies to nuclear and cytoplasmic antigens, and it correlated with extensive visceral disease.

Further evidence suggesting the occurrence of immune mediated vascular damage in systemic sclerosis, is the recent finding of antibodies directed against the vascular endothelium. These antibodies have been detected by various techniques in patients with various autoimmune diseases, including systemic sclerosis and SLE, and it is possible that they may be responsible for the ADCC reported by Penning et al (1984a). Cormane et al (1979) eluted antiendothelial cell antibodies from the circulating lymphocytes of patients with systemic sclerosis and immunoblotting techniques have revealed reactivity against endothelial cell nuclei in the sera of 22% of these patients (Alderuccio et al 1986). More recently, antiendothelial cell antibodies have been detected in the sera of patients with systemic sclerosis by an enzyme linked immunoabsorbent assay (ELISA) (Hashemi al 1987; Baguley et al 1987a; and Byron et al 1987). In addition, antiendothelial cell antibodies have also been detected in sera from patients with SLE (Shingu & Hurd 1981; LeRoux et al 1986; Cines et al 1984).

Thus it is well established that antiendothelial cell antibodies occur in autoimmune disease and it is possible that they may play an important role in the pathogenesis of the vascular lesions of systemic sclerosis.

1.3 Summary

Although the pathogenesis of systemic sclerosis is undefined, a vast amount of evidence suggests that interaction of immunological abnormalities with the vascular system is important in producing the fibrosis which is such a prominent feature of the disease.

Whilst cellular immune abnormalities have been demonstrated in systemic sclerosis, their role in the pathogenesis of the disease is unclear. However, the vascular lesion occurring in systemic sclerosis is a prominent feature and is likely to result from endothelial cell damage. Various humoral abnormalities occurring in systemic sclerosis could be responsible for this endothelial cell damage. A diversity of autoantibodies and elevated levels of immune complexes are known to occur in this disease. Both autoantibodies, especially antiendothelial cell antibodies, and immune complexes could initiate endothelial cell damage, either by activation of the complement system, or by interaction with Fc receptor bearing cells to produce ADCC.

Endothelial cell damage, however it is caused, would result in exposure of collagen in the subendothelium, which could then initiate platelet activation and aggregation. Moreover, altered endothelial cell metabolism due to cell damage could cause an imbalance of prostacyclin levels which would predispose to further aggregation of platelets. Following aggregation, factors of platelet origin would be able to act on the exposed subendothelium to initiate fibrosis. In addition, other substances such as cytokines, released from perivascular infiltrates, could also be important in stimulating the fibrosis found in systemic sclerosis.

1.4 Systemic lupus erythematosus

In this thesis, SLE will be studied in parallel with systemic sclerosis as an additional disease group. Klemperer et al (1942) originally classified SLE and systemic sclerosis together as diffuse collagen diseases. These two diseases frequently show overlap features and common clinical manifestations, such as fibrinoid necrosis in blood vessels and the frequent involvement of internal organs. They also show similar immunological abnormalities with a high incidence of both autoantibodies of various types, and circulating immune complexes. While SLE has been described as "the autoimmune disease par excellence" (Burnet 1976) evidence has accumulated in the last ten years that systemic sclerosis has as florid immunological abnormalities comparable with those occurring in SLE.

It is possible therefore, that common pathogenic mechanisms may be responsible for some of the shared features of the two diseases, a possibility that provides a case for studying systemic sclerosis and SLE in parallel.

1.5 Aims of the present study

Systemic sclerosis is a connective tissue disease characterised by extensive vascular damage (Norton & Nardo 1970). The endothelium has been described as grossly abnormal in patients with systemic sclerosis, at sites of vascular lesions and endothelial cells may appear thickened and separated from one another with a decrease in sites of cell to cell contact. In addition, some blood vessels may exhibit areas which have been totally denuded of endothelial cells.

In addition to the occurrence of vascular defects in patients with systemic sclerosis, various immunological abnormalities have also been reported. These include abnormalities in the cellular immune system, and the occurrence of immune complexes and autoantibodies (Maddison 1988; Postlethwaite & Kang 1988). Systemic sclerosis therefore shows many of the features exhibited by the related autoimmune connective tissue disease of SLE, in which pathogenic mechanisms are clearly defined.

The aim of this study, therefore, was to determine possible ways in which endothelial damage might be produced and thus lead to the development of vascular disease in systemic sclerosis.

In this study, ADCC of endothelial cells was investigated in an in vitro system, to confirm the previous findings of Penning et al (1983, 1984a, 1984b). It is likely that ADCC may be an important pathogenic mechanism accounting for the vasculopathy observed in patients with systemic sclerosis. Further characterisation of the ADCC mediating serum factor was then carried out and linked with experiments to determine if an antibody directed against the endothelium was responsible for mediating ADCC in patients with systemic sclerosis.

To confirm the occurrence of endothelial damage, in vivo plasma levels of von Willebrand factor antigen, which is released by damaged endothelium, were determined in patients with systemic sclerosis (Woolf et al 1987; Nusinow et al 1984).

In addition, the presence of other autoimmune phenomenon capable of affecting endothelium, ie circulating immune complexes and anticardiolipin antibodies have been investigated. Both these immunological abnormalities are found in patients with SLE and their occurrence in systemic sclerosis would further emphasize the autoimmune nature of this disease.

Finally, the synthesis of prostacyclin by cultured endothelial cells, following incubation with sera from patients with systemic sclerosis was investigated to determine if a prostacyclin imbalance might be responsible for the increase in platelet activation which has been shown to occur in patients with systemic sclerosis.

CHAPTER 2

VASCULAR ENDOTHELIAL CELL CULTURE

2.1 Materials

2.1.1 Sterilization procedures

All tissue culture procedures were performed in a class II laminar flow microbiological safety cabinet. Aseptic technique was observed for all manoeuvres. All glassware, water, gelatin solutions, millipore filters and Gilson pipette tips were sterilized by autoclaving for 20 minutes, at 120°C and 15 psi. Pasteur pipettes and instruments were sterilized by heat treatment at 160°C for 120 minutes. Heat sensitive solutions such as sodium bicarbonate and collagenase were sterilized by passage through a millipore filter, 0.22 µm pore size. Culture media were sterility tested prior to use by placing 1ml into 10mls thioglycolate solution and incubating the mixture at 37°C for 24 hours.

2.1.2 Glass distilled water

All reagents and media for tissue culture use were made up in "Ultra Pure" water. Glass distilled water was purified in an Elga water purification system and sterilized by autoclaving.

2.1.3 Tissue culture media

Minimum Essential Medium (MEM) used for the collection of cords was obtained in the "AUTOPOW" form and made up as follows:-

MEM (AUTOPOW)	9.59g
Ultra Pure Water	940ml
Sodium Bicarbonate (22g/l)	40ml
1 M HEPES Buffer	20ml
Penicillin	10 ⁵ units
Streptomycin	100mg
Fungizone	2.5mg

M199 for the culture of human umbilical vein endothelial cells was obtained as a 10X concentrate and made up as follows:-

M199 (10X concentrate)	100ml
Ultra Pure Water	790ml
Sodium Bicarbonate (22g/l)	100ml
L-Glutamine (200mM)	10ml
Penicillin	10 ⁵ units
Streptomycin	100mg
Fungizone	2.5mg

2.1.4 Conditioned medium

B16 conditioned medium was used as a growth supplement for human umbilical vein endothelial cells grown in T75 flasks. A mouse melanoma cell line (B16) was grown in T75 flasks and fed every three days with M199 and 10% foetal calf serum. The spent medium was harvested, filtered and stored at -40°C.

2.1.5 Normal human serum

Normal human AB serum used for the growth of human umbilical vein endothelial cells was obtained at monthly intervals from the Blood Transfusion Service, Sheffield. Clotted blood was separated aseptically and the serum was heat inactivated at 56°C for 45 minutes. Serum was stored at -80°C in 5ml aliquots.

2.1.6 Tissue culture ware

All tissue culture ware used for the growth of human umbilical vein endothelial cells was of tissue culture grade. Primary cultures were grown in T25 and T75 Nunc flasks. Cytotoxicity assays were performed in Nunc 96 well flat bottomed microtitre plates. For prostacyclin assays, Falcon 16mm, 24 well plates were used.

All tissue culture ware was gelatin coated prior to use. 15, 5, 0.5 and 0.1 ml of 0.2% gelatin was added to each T75 or T25 flask, and each well of 24 or 96 well plates respectively, and left to coat at 4°C overnight. Excess gelatin solution was rinsed out with M199 immediately before use.

2.2 Methods

2.2.1 The isolation and culture of human umbilical vein endothelial cells

Umbilical cords from normal vaginal deliveries were placed into sterile bottles, containing 150ml MEM, immediately following delivery. Cords were stored at 4°C for up to 24 hours prior to use.

Umbilical cords of less than 20cm were discarded. Clamped ends and traumatised regions of the cord were cut off and any excess blood remaining was removed by gently massaging the cord along its length. The vein was cannulated with a 14G plastic cannula, and firmly secured with a silk ligature. The vein was washed free of any remaining blood by perfusing with M199 until all traces of blood had been removed. The uncannulated end of the cord was then ligated and a stopper was secured in place over the cannula.

The vein was then inflated with a 0.2% solution of type IV collagenase in M199, and was incubated for ten minutes at 37°C in a humidified atmosphere of 95% air, 5% carbon dioxide. The digested layer of endothelial cells was collected into sterile universal containers and a further 10-15 ml of M199 was flushed through the vein to collect any dislodged cells. The resulting cell suspension was centrifuged at 500g for five minutes at 20°C. The cell pellet was resuspended in M199 and washed twice. Finally, the endothelial cells were suspended in either 5 or 20 mls M199 containing 20% normal human AB serum and placed in a T25 or T75 flask. The human umbilical vein endothelial cells were cultured in a Flow incubator, at 37°C in a humidified atmosphere of 95% air, 5% carbon dioxide.

Twenty four hours after isolation, the human umbilical vein endothelial cells were fed with M199 and 20% AB normal human serum and were then fed every two days until they reached confluence. Human umbilical vein endothelial cells grown in T75 flasks were supplemented with 20% B16 conditioned medium.

2.2.2 Passaging of human umbilical vein endothelial cells

Once the human umbilical vein endothelial cell monolayer had reached confluence, the cells were passaged and plated out at the required cell density. The intact cell monolayer was washed once with M199 and then incubated with a 0.2% solution of type IA collagenase in M199 for 10 minutes at 37°C. The human umbilical vein endothelial cells became detached from the substratum in clumps and were dispersed to a single cell suspension by treating the spun cell pellet with 0.025% EDTA in PBS for 10 minutes at 37°C. Following this second digestion, the cells were centrifuged at 500g for five minutes and washed twice with M199 containing 10% AB normal human serum, to inhibit further enzyme action. The human umbilical vein endothelial cells were counted and tested for viability using a trypan blue dye exclusion test. Cell number was corrected to the required density and the suspension seeded into 96 or 24 well plates.

2.2.3 Culture of human umbilical artery endothelial cells

The isolation of umbilical artery endothelial cells was performed as for the isolation of vein endothelial cells, with the cannulation of an artery in place of the vein.

Isolates obtained from umbilical arteries were seeded into gelatin coated T25 flasks, and fed with M199 supplemented with 20% normal human AB serum and 20% B16 conditioned medium. Passaging of arterial endothelial cells was identical to the method for venous endothelial cells.

2.2.4 Identification of human umbilical endothelial cells

Staining for von Willebrand factor antigen

Identification of endothelial cells in culture was carried out by staining for von Willebrand factor antigen by the direct immuno-peroxidase method. Primary cultures of human umbilical endothelial cells were passaged and seeded onto gelatin coated plastic cover slips situated in the wells of 24 well plates. When confluent, the endothelial cells were fixed in ice cold ethanol for 10 minutes. Then the coverslips were mounted on glass slides with DPX mountant, and the cells were rehydrated with PBS. Peroxidase labelled anti human von Willebrand factor antigen antibody was diluted 1/25 with PBS and the endothelial cell monolayers on coverslips were coated with this antibody and incubated for one hour in a humidified chamber at room temperature. Following this incubation the monolayers were washed three times with PBS with a five minute incubation at each wash. Following the third wash, the substrate, diaminobenzidine (DAB), was made up in PBS at 1mg/ml and 1ul of hydrogen peroxide (30%) added immediately prior to use. The endothelial cell monolayers on coverslips were then incubated for ten minutes with the substrate and hydrogen peroxide. Control coverslips on which cells were incubated with peroxidase labelled rabbit anti-mouse immunoglobulin in place of von Willebrand factor antigen antibody, and DAB alone were also set up. After incubation with the substrate the monolayers were washed once with PBS and then counterstained with haematoxylin by immersion for ten minutes followed by rinsing in tap water. Finally, the endothelial cell monolayers on coverslips were dehydrated in ethanol then xylene and remounted on glass slides with DPX mountant.

2.3 Results

2.3.1 In vitro growth of human umbilical endothelial cells

Primary cultures of human umbilical endothelial cells were established from the majority of umbilical cords processed. However, some cultures failed to become established and reach confluence. Cord length appeared to be a factor determining the successful growth of human umbilical vein endothelial cells. Insufficient cells were isolated from shorter cord lengths and the resulting plating density was too low for successful growth of human umbilical endothelial cells under these conditions.

2.3.2 In vitro morphology of human umbilical endothelial cells

Freshly isolated human umbilical vein endothelial cells occurred as small clusters of cells, which rapidly attached to the vessel substratum. Small colonies of cells were visible spreading out over the surface of the vessel soon after the initial attachment. These small colonies increased in size as the cells multiplied. Such growing endothelial cells were of uniform appearance with an elongated shape. Each cell possessed a single nucleus containing prominent nucleoli, with surrounding peripheral cytoplasm (plate 9). By approximately four days, depending upon the initial plating density, a confluent monolayer of human umbilical vein endothelial cells was obtained (plate 10). Confluent monolayers consisted of closely opposed polygonal shaped cells, which had stopped dividing, and overlapping of cells was not observed. Contamination of cultures with other cell types was rarely noticed. However, any cultures containing small numbers of fibroblasts were discarded.

Arterial endothelial cell isolates from umbilical cords appeared as clumps of cells similar to those obtained from umbilical cord veins. The arterial endothelial cells quickly adhered to the

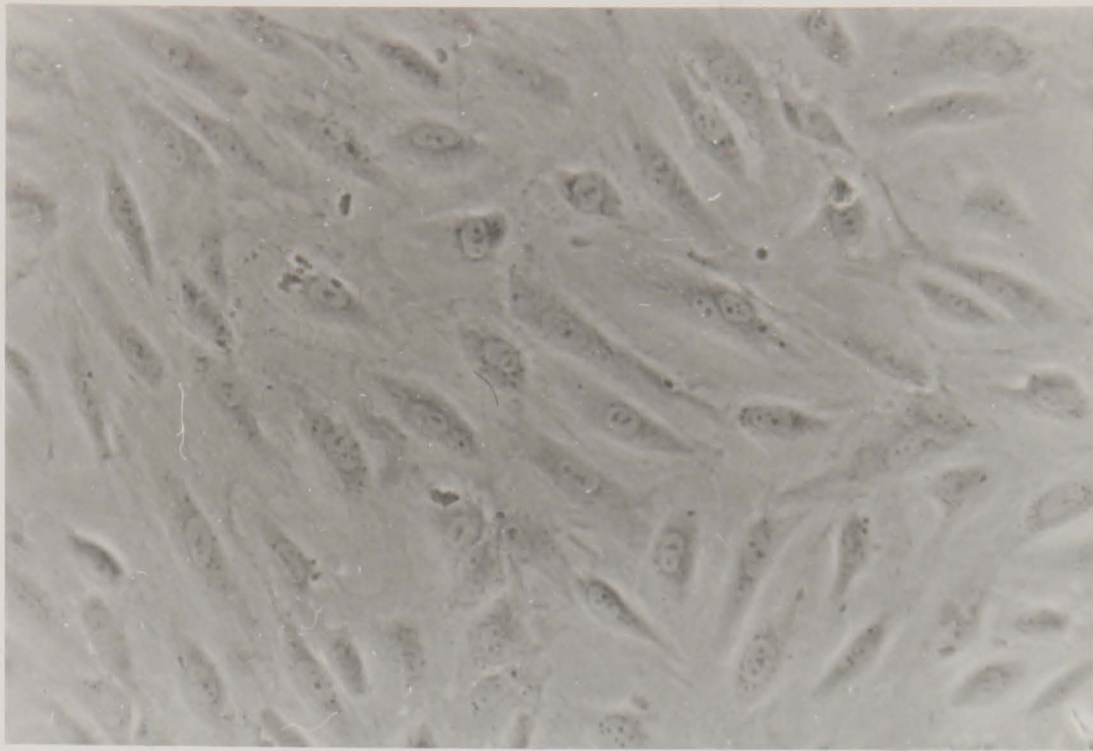


Plate 9

A semi confluent monolayer of a primary culture of human umbilical vein endothelial cells. Each cell pocesses a single nucleus with several prominent nucleoli. Phase contrast x 200

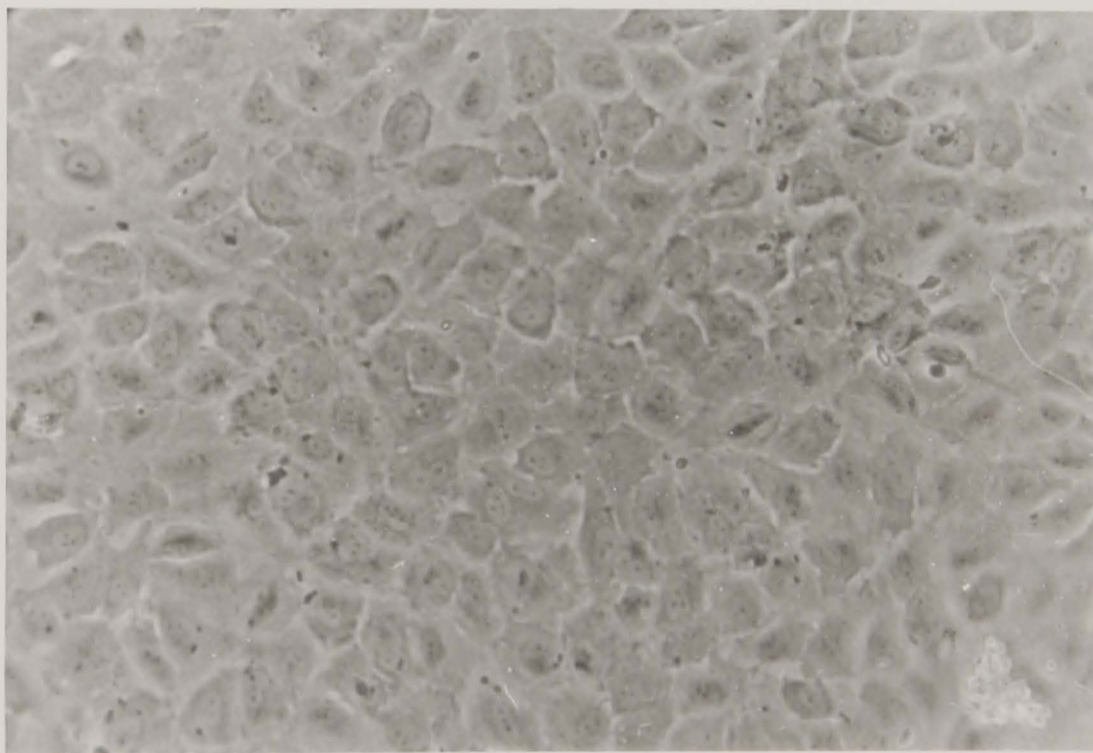


Plate 10

A confluent monolayer of a primary culture of human umbilical vein endothelial cells, showing the typical cobblestone appearance and contact inhibition. Phase contrast x 200

substrate and began to divide. However, arterial endothelial cells did not take on the elongated appearance shown by venous cells at the semi confluent stage. Instead arterial cells remained cobblestone morphology throughout their period of growth. Once confluent, arterial and venous monolayers exhibited similar morphology.

Staining for von Willebrand factor antigen

Human umbilical endothelial cells which had been incubated with peroxidase labelled anti human von Willebrand factor antigen, followed by substrate and hydrogen peroxide, showed a distinct staining pattern. Granular perinuclear staining which is characteristic for von Willebrand factor antigen was observed. In contrast, control endothelial cells stained with peroxidase labelled rabbit anti-mouse immunoglobulin showed no such staining (Plates 11, 12 & 13).

Prostacyclin production

Cultures of human umbilical vein endothelial cells consistently demonstrated the release of prostacyclin as detected by radioimmunoassay of its stable metabolite, 6-keto-PGF_{1α}(see chapter 4).

Plate 11

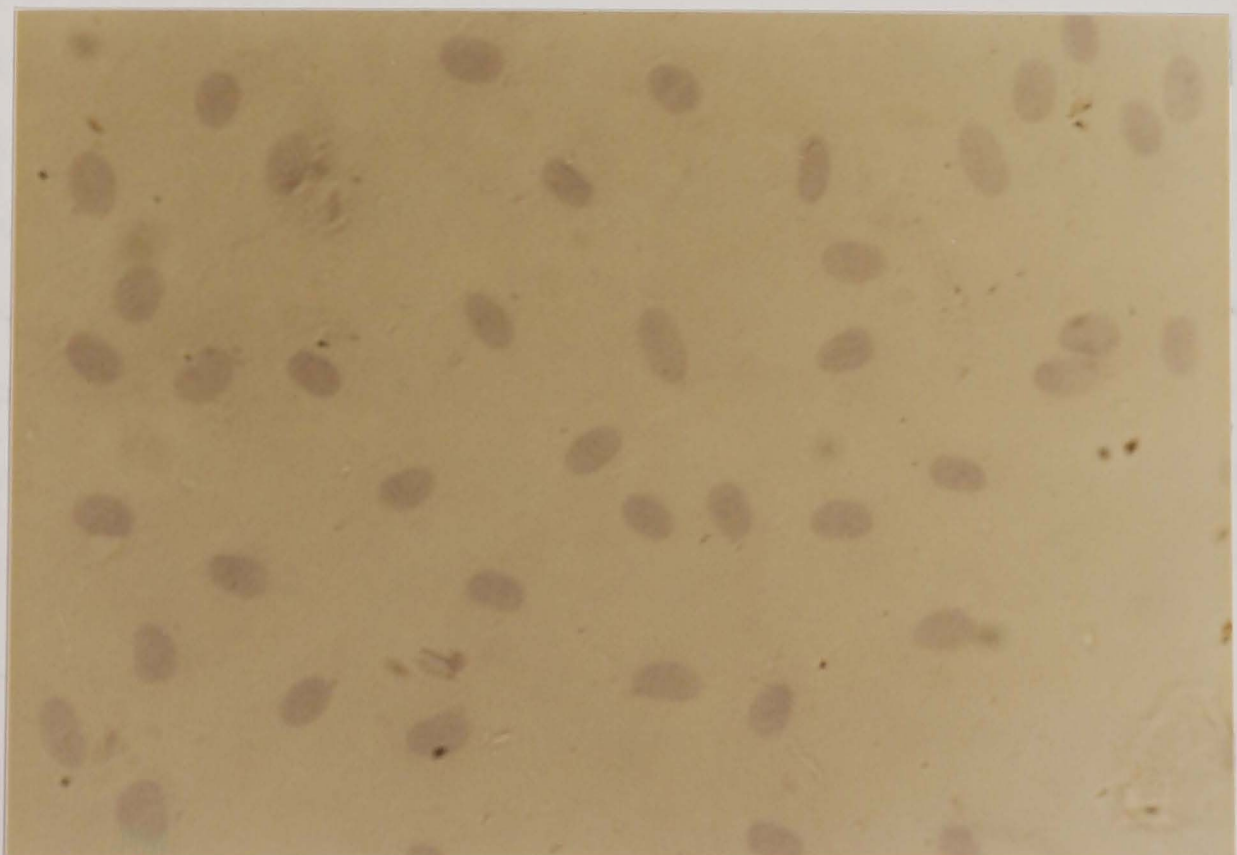
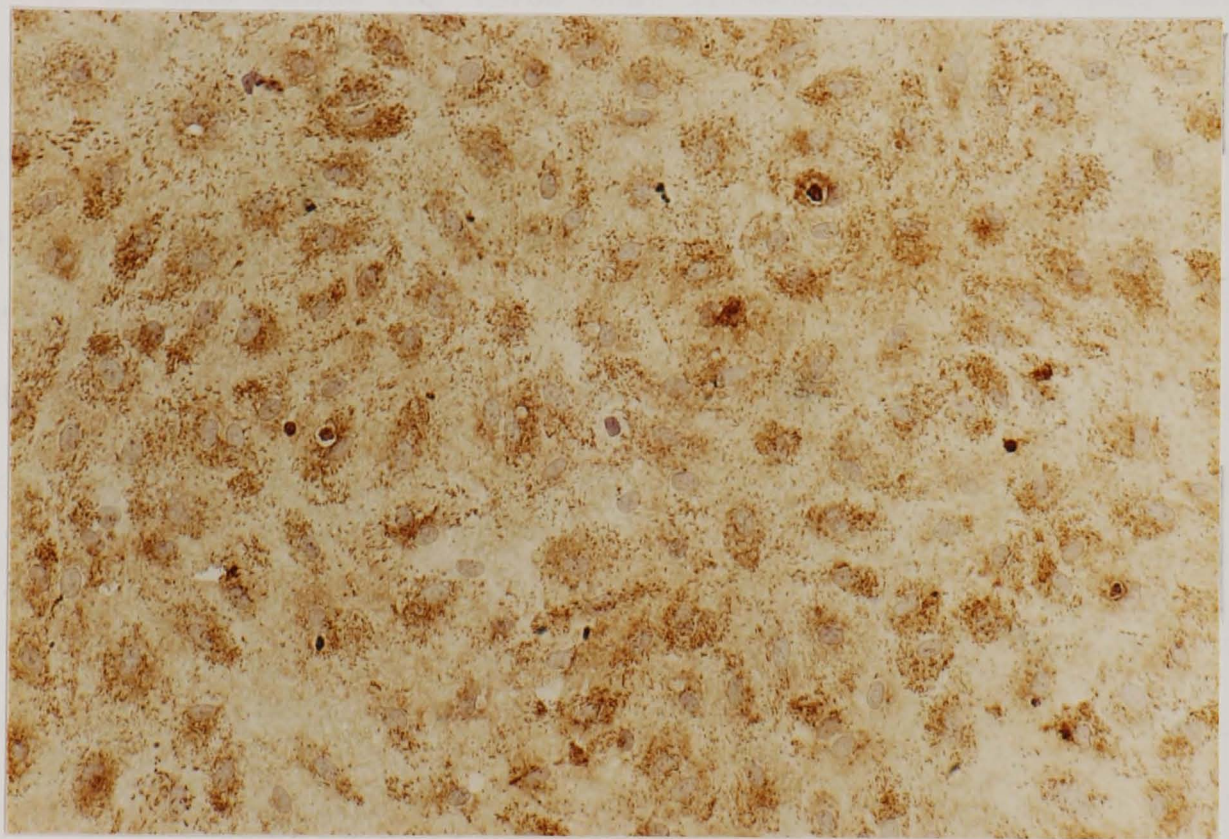
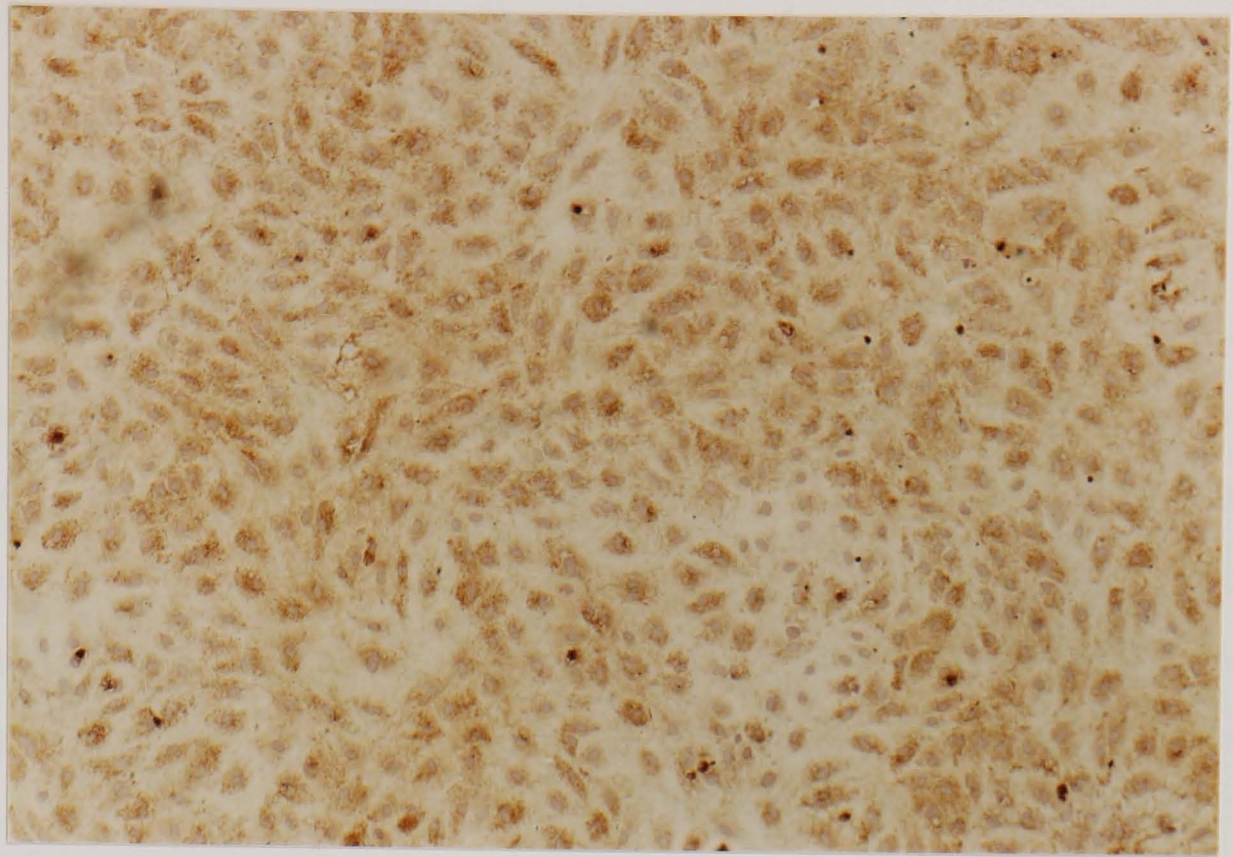
Immunoperoxidase staining of a monolayer of human umbilical vein endothelial cells with peroxidase labelled monospecific antisera directed against von Willebrand factor antigen. A dark brown, perinuclear granular staining pattern is shown. (x50)

Plate 12

Immunoperoxidase staining of a monolayer of human umbilical vein endothelial cells with peroxidase labelled monospecific antisera directed against von Willebrand factor antigen. As above, dark brown, perinuclear granular staining of the antigen is observed. (x100)

Plate 13

Control staining of a monolayer of human umbilical vein endothelial cells, with peroxidase labelled rabbit anti-mouse immunoglobulin. No dark brown, granular staining is visible. (x100)



2.4 Discussion

Endothelial cells line the entire vascular system of the body, where they provide a nonthrombogenic surface to which platelets and other blood cells will not adhere. Endothelial cells grow as a monolayer, both in vitro and in vivo, thus enabling the vessels they line to maintain a patent vessel lumen. Various metabolically active substances are synthesized and released by endothelial cells, and these give them their procoagulant, antiplatelet, anticoagulant and fibrinolytic properties (see table 1.).

It is only since the 1970s that investigators have been able to routinely isolate, identify and maintain vascular endothelial cells in culture. In the first half of the twentieth century, workers unsuccessfully attempted to grow endothelial cells in vitro using the technique of cell outgrowths from explants. However, limited cell growth and overgrowth by fibroblasts and smooth muscle cells inhibited their studies. In 1963 trypsin perfusion of vessels was performed. Maruyama (1963) carried out trypsin perfusion of human umbilical cord veins, and obtained cells that had the morphological appearance of endothelial cells. However, these cells failed to replicate, they could not be passaged and they degenerated after 21 days in culture. At the same time, Pomerat and Slick (1963) isolated endothelial cells from rabbit aortas using trypsin perfusion. Their cells could be serially passaged, but later passaged cells had the appearance of smooth muscle cells or fibroblasts. The major problems faced by workers attempting to culture endothelial cells was overgrowth by contaminating smooth muscle cells and fibroblasts, and the positive identification of endothelial cells. In 1973, Jaffe et al successfully isolated and cultured endothelial cells from human umbilical veins, by

Table 1

Molecules involved in haemostasis which are synthesised by human umbilical vein endothelial cells (Bull 1988).

1. Adhesive Proteins

Von Willebrand factor antigen

Thrombospondin

Fibronectin

Collagens type IV, V? and III

Sulphated Proteoglycans

2. Coagulation Proteins

Von Willebrand factor antigen

Factor V

Thromboplastin

3. Anti Coagulant Proteins

Anti thrombin III

Thrombomodulin

Protein S

4. Fibrinolytic Proteins

Plasminogen activators _ Tissue & urokinase type

5. Prostaglandins

Prostacyclin (PGI₂)

Prostaglandin E₂

Prostaglandin F_{2a}

Thromboxane A₂

perfusion with collagenase. These authors also demonstrated the presence of von Willebrand factor antigen on these cells which is now widely used as a unique marker for endothelial cells (Jaffe 1973).

The method of Jaffe et al (1973) for the isolation of human umbilical vein endothelial cells involves the use of collagenase rather than trypsin. Collagenase selectively digests the collagen component of the subendothelial cell basement membrane thus reducing contamination by smooth muscle cells and fibroblasts from the internal elastic lamina. In addition, collagenase is non-toxic to cells in culture. Thus, collagenase offers a more controlled method of endothelial cell isolation compared with trypsin, which nonspecifically digests all protein and may destroy cell surface antigens (Ryan & Ryan 1983).

In addition, in his isolation procedure, Jaffe used undamaged sections of cord, which had not been clamped during delivery, as these damaged areas of cord were more likely to produce contamination by smooth muscle cells and fibroblasts. The method of isolation of human umbilical vein endothelial cells originally documented by Jaffe has since been adopted by several workers and a technique adapted from this method was used in the present study. 0.2% type IV collagenase was successfully used for the perfusion, with an incubation period of 10 minutes at 37°C. This digestion period was used since longer times resulted in further digestion of the subendothelial cell layer with an increased risk of smooth muscle cell and fibroblast contamination.

Endothelial cells have also been isolated from bovine aortas using a similar technique to that described for human umbilical vein endothelial cells (Booyse al 1975). However, cloning techniques are required in order to obtain populations of pure bovine aortic endothelial cells (Jaffe 1984). Various methods have also been adopted

for the culture of endothelial cells from a variety of animals and sites, including human iliac arteries; human and bovine pulmonary arteries and veins; bovine saphenous veins; porcine aortas, inferior vena cava and pulmonary veins; rabbit aortas and marginal vessels of the ear; guinea pig aortas and portal veins; and canine jugular veins (Jaffe 1984). In addition, several groups have obtained transformed endothelial cells. In early studies, various properties of the endothelium, such as monolayer morphology, Wiebel-Palade bodies, and the synthesis of von Willebrand factor antigen were not retained in these transformed cells, and thus they did not provide adequate model systems for the study of normal endothelial cell functions. However, a recent report by Faller et al (1988) describes the immortalisation of human endothelial cells by murine sarcoma viruses, without morphological transformation, a technique which could be used in future studies.

More recently, investigators have been attempting to isolate and maintain microvessel endothelial cells in vitro. It appears that the growth requirements for these cells differ from those of the larger arteries and veins and depend upon the site that vessels are obtained from. Although some workers have succeeded in isolating human microvascular endothelial cells, no major studies have been carried out using these cells (Folkman et al 1979; Davison et al 1980, 1981; Sherer et al 1980; Marks et al 1985; Vinters et al 1987)

Systemic sclerosis is a disease which shows marked vascular abnormalities. The involved vessels are the smaller arteries and arterioles, and the capillary bed. Thus it would perhaps be relevant to study the effects of serum from patients with systemic sclerosis on capillary endothelium. However, the maintenance of these cells in vitro has proved very difficult and contamination with fibroblasts has

been a major problem. In this laboratory, attempts have been made to culture capillary endothelial cells from human lung, obtained from surgical specimens, and human foetal skin, lung and brain. Although the isolation of capillary endothelial cells has been achieved from these sites, maintenance and growth has been inhibited by the contamination of microvessel cultures with fibroblasts. Capillary endothelial cells from omental tissue have also been isolated and cultured in this laboratory using the method of Kern et al (1983) However, we were not satisfied with the endothelial origin of these cells and therefore this source was not pursued.

In this study, endothelial cells were derived from human umbilical veins. There are various advantages in using umbilical cords as a source of endothelial cells. Cords are easily obtained and a supply of over 500 umbilical cords was available for this study. The vein is an unbranched vessel and is easily cannulated at both ends, thus making the flushing through of washing medium, addition of enzyme and collection of effluents a relatively easy task. However, various uncontrollable foetal and maternal factors may affect the growth and biological activity of endothelial cells thus obtained. For instance large variations in the production of prostacyclin have been recorded from endothelial cells obtained from different cords. Factors such as foetal stress, maternal anaesthesia and maternal smoking habits may all affect the viability, growth characteristics and activity of isolated endothelial cells. Thus, caution must be used in making direct comparisons of endothelial cells derived from different cords.

The low mitotic index of human umbilical vein endothelial cells in vitro has prevented long term culture of these cells (Maciag et al 1984). Most workers have been unable to cultivate endothelial cells

beyond the second or third passage and later passaged cells appear to have lost their functional properties. For example, prostacyclin production diminishes when endothelial cells are serially passaged (Ager et al 1979).

Serum is a necessary growth requirement for human umbilical vein endothelial cells. Foetal calf serum was used in early studies on the growth of human umbilical vein endothelial cells. However, foetal calf serum has been shown to cause a relative inhibition of endothelial cell growth (Summers et al 1984) and therefore human serum has since been adopted by many investigators and concentrations up to 50% have been shown to be mitogenic (Jaffe 1984). In the present study 20% human AB serum was used for the growth of human umbilical vein endothelial cells in vitro as this serum concentration adequately supported endothelial cell growth.

It is widely accepted that human umbilical vein endothelial cells are much more fastidious in culture than endothelial cells derived from large animal vessels. Bovine and porcine aortic endothelial cells can be maintained in vitro for long periods of time with relative ease. However this is not true of human umbilical vein endothelial cells and therefore workers have been searching for a factor, not present in serum, which will support serial passaging of human umbilical vein endothelial cells whilst maintaining their in vivo characteristics.

Early studies showed that fibroblast growth factor, derived from bovine neural tissue, enhances the proliferation of human umbilical vein endothelial cells (Gospodowicz et al 1978). Maciag et al (1979) later identified the reactive fraction of fibroblast growth factor which causes this stimulation of proliferation, which is known as endothelial cell growth factor. The use of endothelial cell growth

factor with fibronectin coated plates has enabled workers to maintain human umbilical vein endothelial cells in vitro for fifteen to twenty one passages or up to twenty seven population doublings. In the presence of endothelial cell growth factor the requirement for serum is diminished, and Maciag et al (1981) reported the growth of human umbilical vein endothelial cells at plating densities as low as 1.25 cells per square centimeter. Heparin appears to diminish the requirement for endothelial cell growth factor (Thornton et al 1983). It probably works by stabilising the structure of the endothelial cell growth factor molecule.

It is likely that factors of platelet origin are required for endothelial cell growth since serum is a necessary requirement and impure preparations of platelet derived growth factor have shown proliferative effects on human umbilical vein endothelial cells. However, these cells do not possess receptors for platelet derived growth factor and further investigation is required before the role of this factor in the growth of endothelial cells is known (Heldin et al 1981; Bowen-Pope & Ross 1982).

Endothelial cells themselves have been shown to synthesize and release growth factors which promote fibroblast and smooth muscle cell growth and one of these has been shown to bind to endothelial cell surfaces (DiCorleto et al 1983; Gajdusek 1984). However, whether endothelial derived growth factor stimulates the proliferation of endothelial cells is uncertain.

In this study, the growth of human umbilical vein endothelial cells to first passage was accomplished on gelatin coated plastics, with 20% normal human serum, without the addition of growth factors. Thus, although it appears that external growth factors are important

for the long term propagation of human umbilical vein endothelial cells, in short term culture, morphological and functional properties can be retained without the addition of such factors.

In addition to growth factors, it is evident that substances produced by endothelial cells and incorporated into their extracellular matrix and subendothelial basement membrane, are important in the maintenance of an intact monolayer, and may be important in cellular attachment, spreading, migration and proliferation. Hence, such substances are often used as substrates to aid the growth of endothelial cells in vitro. Elastin, laminin, fibronectin, collagens, thrombospondin and sulphated proteoglycans are all synthesized by endothelial cells and incorporated into their basement membrane (Bull 1988). Several of these substances have been used to coat tissue culture plastics to facilitate cell growth.

2.4.1 Identification of human umbilical endothelial cells

In this study the criteria used for defining cultures as endothelium were:-

1. Morphological appearance at the light microscope level
2. Positive staining for von Willebrand Factor antigen
3. The production of prostacyclin

The simplest way of identifying endothelial cells is by their morphological appearance as seen by phase contrast microscopy. In culture, human umbilical vein endothelial cells grow as a monolayer of closely opposed polygonal shaped cells with an oval, centrally located nucleus. The human umbilical vein endothelial cells grown for this study possessed this typical "cobblestone" appearance. Once a confluent monolayer is obtained endothelial cells in vitro exhibit

"density" or "contact inhibition" growth characteristics; ie they cease to proliferate once a compact monolayer has been formed. This inhibition of proliferation is thought to be due to cell to cell contact (Wieser et al 1985). However, the subendothelial cell basement membrane may also be important in the control of proliferation.

In contrast to endothelial cells in vitro, fibroblasts differ morphologically since they appear as long spindle shaped cells, which when confluent grow close together in parallel arrays with whorling and multiple overlapping layers. Smooth muscle cells have a typical "hill and valley" appearance in vitro, with areas of multiple layers of cells interspersed with areas of lower cell density (Jaffe 1984). Thus, smooth muscle cells and fibroblasts can be distinguished from human umbilical vein endothelial cells by their morphological appearance.

Von Willebrand factor antigen is the major component of the factor VIII complex found in plasma. Von Willebrand factor antigen may be measured directly or functionally as ristocetin co-factor. Since the early 1970s it has been known that endothelial cells contain von Willebrand factor antigen. In 1973 Jaffe et al demonstrated its synthesis and release by human umbilical vein endothelial cells in vitro. Von Willebrand factor antigen is either incorporated into the subendothelial cell basement membrane or it is released and complexed to factor VIII procoagulant molecules in plasma. Immunoelectron microscopic studies have localized von Willebrand factor antigen to the Weibel-Palade bodies, which may act as a processing or storage site (Wagner et al 1982). Endothelial cells from a variety of sites and species have been shown to synthesize and secrete von Willebrand factor antigen in vitro, with the exception of porcine endothelial

cells. Thus, its detection offers a method for the identification of endothelial cells. The positive staining for von Willebrand factor antigen has been widely used as a unique marker for endothelial cells and was one of the criteria used for the positive identification of human umbilical endothelial cells cultured in this study. Human umbilical endothelial cells grown in vitro showed characteristic granular perinuclear staining when the direct immuno-peroxidase technique of staining for von Willebrand factor antigen was carried out (Plates 11, 12 & 13).

Another characteristic of endothelial cells in vitro is the synthesis and release of prostacyclin. Prostacyclin is produced by endothelial cells following the oxidative reduction of arachidonic acid, which is mobilized from membrane phospholipids. It is the most potent disaggregator of platelets known and its production by the vascular endothelium gives blood vessels their non-thrombogenic properties. In this study prostacyclin release by endothelial cells in vitro was detected by radioimmunoassay of its stable metabolite, 6 keto PGF_{1α}. Fibroblasts and smooth muscle cells have also been shown to release prostacyclin in vitro (Baenziger et al 1979; Ali et al 1980). However the levels obtained from these cell types are much lower than those produced by endothelial cells. The levels of prostacyclin produced by human umbilical vein endothelial cells which were measured in this study were compatible with measurements carried out by other workers on similar cells.

An additional characteristic feature of endothelial cells is the presence of Weibel-Palade bodies. First demonstrated by Weibel and Palade, these are tubular cytoplasmic organelles which are unique to endothelial cells. Identifiable by electron microscopy, these structures have been described in endothelial cells derived from a

variety of sites and species. Typically, they occur as rod shaped cytoplasmic components containing bundles of tubules enclosed by a tightly fitting membrane (Weibel and Palade 1964). Various functional roles have been suggested for Weibel-Palade bodies, the most recent being that they are a site of storage of von Willebrand factor antigen (Wagner et al 1982). Although Weibel-Palade bodies may not always be seen in cultured endothelial cells, when present they serve as a useful marker for this cell type.

2.4.2 The endothelial cell surface

The vascular endothelium provides a vast surface which is in contact with all constituents of the blood. Thus, the surface antigens expressed on endothelial cells have been extensively studied, especially by workers in the field of renal transplantation, since vascular lesions are a prominent feature in patients exhibiting allograft rejection.

HLA A,B, and C antigens have all been demonstrated on the surface of cultured human umbilical vein endothelial cells (Moraes and Stastny 1977; Neppart et al 1984). However, the expression of HLA DR antigens on human endothelial cells is less well defined. Using eleven different monomorphic monoclonal antibodies against human DR antigens, Stastny and Nunez (1984) failed to demonstrate HLA DR antigens on human umbilical vein endothelial cells. This failure to detect HLA DR antigens on endothelial cells was also reported by Neppert et al (1984). However, HLA DR antigens have been demonstrated, by immunofluorescent techniques, on capillary walls of kidney vessels in glomeruli and around tubuli, but not in the walls of larger vessels, and they were not found in the walls of capillaries and larger vessels of umbilical cord and placenta (Scott et al 1981). In addition, using a complement dependent cytotoxic method of

detection, HLA DR antigens have been detected on human umbilical vein endothelial cells (Hirschberg et al 1980 and 1984). Thus, it appears that endothelial cells from different sites and different sized vessels may differ in their expression of HLA DR antigens, and the method of detection employed may be critical. In addition, the activation state of endothelial cells may be important. For instance, Pober et al (1986) demonstrated the inducible expression of HLA DR antigens by activated T cells or immune interferon.

Receptors for the Fc component of immunoglobulin and the activated third component of the complement cascade are also inducible on cultured bovine aortic endothelial cells. Under "normal" conditions, these cells do not possess receptors for Fc and C3 but these can be induced following exposure to white cell lysates or infection with influenza or cytomegalovirus. (Ryan et al 1981, 1983).

In addition to HLA antigens, antigens unique to the surface of endothelial cells have also been demonstrated. Using an immunofluorescent technique, Lindquist and Osterland (1971) showed specific staining of vascular endothelium by human sera from patients with a variety of diseases including connective tissue diseases. Sera from kidney transplant recipients obtained following allograft rejection have been shown to react with endothelial cells (Moraes and Stastny 1977; Stastny and Nunez 1984; Cerilli et al 1977, Cerilli & Brasile 1980). Stastny and Nunez also demonstrated reaction of these sera with monocytes, hence the responsible antigens have been termed endothelial-monocyte antigens. Antiendothelial cell antibodies have also been eluted from lymphoid cell membranes (Cormane et al 1979) and sera and IgG from patients with SLE have been shown to bind to human umbilical vein endothelial cells (Shingu and Hurd 1981; LeRoux et al 1986; Cines et al 1984). More recently, using an ELISA,

antiendothelial cell antibodies have been detected in the sera of patients with systemic sclerosis (Hashemi et al 1987; Baguley et al 1987a; Byron et al 1987)

In addition to the expression of surface antigens, endothelial cells also exhibit important accessory cell functions. Hirschberg et al (1980, 1984) demonstrated that endothelial cells can substitute for macrophages as antigen presenting cells to sensitized T lymphocytes and this property involves self HLA DR determinants. Endothelial cells produce interleukin 1 which is important for the triggering and proliferation of T lymphocytes (Miossec et al 1986)

Thus endothelial cells play an important role in the response of the immune system. They have been shown to express molecules of the Class II MHC and they exhibit accessory cell functions. In addition, endothelial cells produce interleukin 1 in vitro. Hence, as a result of these properties and their distribution throughout the blood and lymph system, endothelial cells may be involved in various immune reactions occurring both in the healthy and diseased individual.

CHAPTER 3

ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY
OF VASCULAR ENDOTHELIAL CELLS IN SYSTEMIC SCLEROSIS
AND SYSTEMIC LUPUS ERYTHEMATOSUS

3.1 Introduction

Antibody dependent cellular cytotoxicity (ADCC) is an immune mechanism in which target cell specific antibody interacts with immune effector cells enabling them to mediate target cell lysis. ADCC was first described in 1965 by Moller et al and then by Perlmann and Perlmann (1970). The latter authors showed lysis of chicken erythrocytes in the presence of peripheral blood lymphocytes, and target cell specific antisera. Since these initial observations, ADCC has been described in a variety of allogeneic and xenogeneic assay systems. It is complement independent and occurs at antisera titres much lower than those required for complement mediated cytotoxicity (Perlmann and Holm 1969; Perlmann & Perlmann 1970).

Although ADCC has been recognised for some time, the precise nature of the effector cells involved and the mechanism of cytotoxicity are not fully established. However, for effective ADCC there are three requirements (Zigheboim and Gale 1974) :-

1. An effector cell with a surface receptor, capable of interacting effectively with the exposed binding site on the Fc region of the antibody molecule (Fc receptor).
2. Target cell specific antibody.
3. An intact Fc region of the antibody molecule.

3.1.1 Effector cell populations in ADCC

Monocytes, macrophages, polymorphonuclear leukocytes and a heterogeneous subpopulation of lymphocytes have all been shown to mediate ADCC (Sarmay et al 1985; Ralph 1984; Johnson et al 1985; Shen

et al 1987; Richer et al 1983). It appears that the nature of the effector cell mediating ADCC may vary with differing target cells (Nelson et al 1976; Kovithavongs et al 1975). Different antibodies directed against the same target cell may preferentially cause activation of different effector cells (Richter et al 1983). The concentration of antibody molecules coating the target cell may be another determining factor in the nature of the effector cell mediating ADCC, as may the class of immunoglobulin (Kurlander et al 1978; Zoller et al 1983).

In general, it appears that lymphocytes are most effective at mediating ADCC of nucleated targets such as allogeneic lymphocytes and tumour cell lines, whereas monocytes are more effective at killing anucleated erythrocytes. However chicken erythrocytes, which are nucleated, are equally well lysed by monocytes and lymphocytes (Greiss 1984).

The subpopulation of lymphoid cells effective in ADCC make up part of the null cell population of lymphocytes. They have been classed together with natural killer (NK) cells, and may be the same population of cells. These have been termed large granular lymphocytes. The ADCC mediating cells have been called killer (K) cells and make up approximately 5% of peripheral blood lymphocytes (Maclennan et al 1976). They possess receptors for the Fc region of immunoglobulin, are non-phagocytic and non-adherent and do not possess surface membrane immunoglobulins or receptors for complement (Maclennan et al 1976; Bean et al 1976).

In addition to the type of effector cell determining ADCC reactions, its level of activation is also important. Various factors known to modify biological responses may also alter the effector cell's ability to mediate ADCC. For example, γ interferon has been

shown to both inhibit and enhance ADCC by different populations of effector cells against various targets (Ortaldo et al 1987; Catalona et al 1981). However, α interferon and interleukin 1 have both been shown to act as potent stimulators of ADCC (Ortaldo et al 1987). It is possible that such factors may exert their effects by altering the expression of Fc receptors on effector cells as it has recently been shown that δ interferon induces an increase in the expression of FcR1 and FcR III (see later) on monocytes and neutrophils (Buckle et al 1988).

The requirement, in ADCC, for an interaction between target specific antibody and the Fc receptor on effector cells is demonstrated by the inhibition of ADCC seen in the presence of heat aggregated IgG and soluble immune complexes (Panayi et al 1977; Lustig and Bianco 1976). Recent studies centred on the nature of the Fc receptor have identified three different types, termed FcRI, II and III. These different receptors have differing affinities for IgG and can be identified with specific monoclonal antibodies. As different Fc receptors are present on different effector cells, the type of receptor involved in ADCC depends on the nature of the effector cell in the system under study. Gergely (1988) demonstrated the occurrence of FcRIII on human K cells. He also examined the IgG domain specific sites on this receptor which are important in ADCC. The FcRIII on K cells is shown to have a CH2 and CH3 domain specific binding site. The CH2 specific site is important both in receptor binding of antibody and mediating target cell destruction, whilst the CH3 specific site only has antibody binding capability (Sarmay et al 1985; Gergely 1988). ADCC induced by monocytes, however, has been shown to occur via

FcR1 (Shen et al 1986). Further work is required before the precise antibody binding sites on the Fc receptors of effector cells can be determined for all ADCC reactions.

3.1.2 Antibody nature in ADCC

Early studies demonstrating ADCC involved antibody of IgG class as the sensitizing agent (MacLennan et al 1969). Further investigation of the role of antibodies of the IgM class as mediators of ADCC have, however, produced conflicting results. ADCC by IgM antibodies has not been demonstrated against human erythrocytes and chicken erythrocytes (Holm et al 1974; Richer et al 1983; Lustig and Bianco 1976). However, IgM induced ADCC has been detected in a systems using human effector cells and sheep erythrocytes as target cells (Fuson and Lamon 1977). In a comparison of IgM and IgG induced cytotoxicity, Fuson et al (1978) showed that pre-incubation of lymphocytes with antibody facilitated IgM induced ADCC, but had little effect on ADCC mediated by IgG. In contrast, these authors showed that cytotoxicity produced by freshly isolated peripheral blood lymphocytes against IgM sensitized target cells was significantly lower than that produced by IgG sensitized target cells.

Zoller et al (1982) also demonstrated IgM mediated ADCC with highly purified monoclonal antibodies against sheep erythrocytes by murine and human effector cells, but found that much greater concentrations of antibody of IgM class were necessary to induce ADCC than were required with IgG antibody. In a later study, these authors studied the nature of the effector cells mediating ADCC induced by antibodies of the IgG and IgM classes against sheep erythrocyte target cells (Zoller et al 1983). Although the populations of effector cells mediating ADCC by the two different classes appeared to overlap, they

differed in their relative killing capacity. Although a 10x greater IgM concentration was used compared to the IgG class antibody, a lower percentage of targets coated with IgM were killed.

It appears that although the antibody class mediating ADCC is mainly IgG, IgM has also been shown to be capable of inducing this type of killing. However, the concentration of antibody required in IgM induced ADCC is much higher and the killing is less efficient. It is also possible that different populations of effector cells may be used in ADCC induced by IgG and IgM classes of antibody.

In systems where IgG acts as the class of antibody capable of mediating ADCC, the effect of antibody subclass has also been investigated. Early studies measured the inhibition of an ADCC system by the various IgG subclasses, on the assumption that should a particular subclass inhibit ADCC, then antibodies of the same subclass are those mediating ADCC. Inhibition of lysis of human and chicken erythrocytes by human effector cells has been shown with IgG1 and IgG3, but not with IgG2 and IgG4 subclasses of antibody (Holm et al 1974; Spiegelberg et al 1976; Urbaniak & Greiss 1980). In a study where IgG monoclonal antibodies against various murine cell surface antigens were examined in ADCC reactions with human effector cells, IgG antibody of the IgG2a and IgG3 subclasses was the most effective in mediating lysis, while IgG2b and IgG1 were less and least effective respectively (Sears and Christiaansen 1985). However, different monoclonal antibodies of the same subclass showed large variations in their ability to induce ADCC, thus implicating the importance of other factors such as antibody affinity towards the target cell. This is illustrated by a recent study by Gergely (1988) which revealed that although the IgG1 and IgG3 subclasses of antibody both bound to the Fc receptors on human K cells, only the IgG1 subclass was capable of

mediating lysis of human erythrocytes by K cells. IgG of different subclasses has been shown to exhibit variable binding affinities for FcRI, the order of binding being IgG1>IgG3>IgG4>>IgG2 (Unkeless et al 1988). Thus the differing abilities of the various subclasses to mediate ADCC may be due to the variability in their binding affinities for Fc receptors.

Thus it appears that several factors influence ADCC reactions. These include the type of effector cell, its state of activation and the number and type of Fc receptors expressed. Antibody class and subclass and its affinity towards the target cell may also be important factors. The target cell and its susceptibility to lysis is also an important controlling factor. For instance, the density and distribution of target cell surface antigens may affect antibody binding and the nature of the effector cell capable of mediating lysis and the degree of lysis. Interspecies variations are also important factors. Differences in ADCC have been shown when target cells, antibodies and effector cells from different species are tested (Zigelboim and Gale 1974). Thus, it is of crucial importance that homologous systems are used in the study of ADCC in human disease (Urbaniak 1978).

3.1.3 Mechanisms occurring in ADCC

The mechanisms occurring in K cell mediated ADCC remain undefined. However, it is possible that specific antibody must first combine with the appropriate antigen on the target cell before combining with Fc receptors on effector cells. Thus, the target cell specific antibody acts as a bridge allowing cytolytic cells bearing Fc receptors, to recognize and lyse target cells. Once the effector cell has bound to the target cell by way of Fc receptor binding to target cell bound antibody, it becomes activated and ready for the delivery

of its lethal hit, i.e. it becomes programmed for target cell lysis. Following the activation process, target cell destruction ensues. The steps involving programming for lysis and target cell death were originally thought to be similar for both K cell mediated ADCC and lysis by cytotoxic T lymphocytes and NK cells (Sears and Christiaansen 1985). However, recent work has shown that although the perforin pathway of cell lysis (see below) is calcium dependent, many cytotoxic T lymphocytes remain cytotoxic even in calcium free conditions, thus indicating another or other mechanisms of cytolysis which are calcium independent (Young and Liu 1988b; Clark 1988).

The perforin mechanism of cytolysis occurs following conjugation between target and effector cells. The latter undergo cytoplasmic rearrangement resulting in secretion of a pore forming protein, called perforin, or cytolyisin, which is present in granules. Perforin monomers released from these granules polymerise to produce tubular complexes which then insert into the target cell membrane, forming channels which are a necessary component of the lethal hit. Channel formation by these perforin monomers is not enough to kill the target cell, although it is not known whether soluble lytic factors from effector cells enter target cells by way of these membrane channels.

However, the mode of target cell death occurring in ADCC appears to involve the mechanism of apoptosis rather than necrosis, which occurs in complement mediated lysis (Duvall and Wyllie 1986). Apoptosis involves early degradation of nuclear material with distinct patterns of DNA fragmentation (Cohen et al 1985). The effector cells appear to be "immune" to their own cytolytic mediators and may possibly kill more than one target cell. However, other experiments

have suggested that under certain circumstances, cytotoxic T lymphocytes are equally susceptible to lysis when recognised by other cytotoxic T lymphocytes as targets (Young and Liu 1988a).

In contrast to the above mechanism of ADCC involving recognition of target cells via specific antibody previously bound to the target cell surface, is a mechanism involving "arming" of effector cells, thus rendering them cytotoxic to target cells. Greenberg and Shen (1973) demonstrated effector cell binding of preformed complexes of antibody and target cell antigen via Fc receptors. They suggest that, providing excess antibody is present in the complex, the effector cell will acquire specificity through free binding sites on the antibodies in the complex, thereby allowing specific recognition of target cell antigen, followed by specific cytotoxicity.

3.1.4 ADCC and its role in disease states

Although there is no firm evidence that ADCC occurs in vivo, various roles have been proposed for this potentially important immune mechanism, including immunological responses towards tumours and virally infected cells (Sears and Christiaansen 1985; Gonik et al 1985). ADCC of vascular endothelial cells has also been implicated in allograft rejection and, with relevance to this study, could be one of the immunological mechanisms functioning in autoimmune diseases (Hirschberg et al 1975)

The pathological features of many autoimmune diseases could well be mediated, at least in part, by ADCC. Some of the various autoantibodies found in autoimmune diseases may be capable of interacting with a patient's K cell population to produce autologous tissue injury. A number of reports have demonstrated a factor, present in the serum of patients with autoimmune diseases, capable of mediating ADCC of various target cells when co-cultured with effector

cells from normal human donors. Thus, IgG from patients with rheumatoid arthritis has produced ADCC of Chang liver target cells (MacLennan et al 1969; Panayi 1976). In another study, IgG anti-DNA antibodies from patients with SLE caused destruction of DNA coated chicken erythrocytes by normal lymphocytes (Quismorio et al 1976). Norris et al (1984) showed ADCC of nonhistone nuclear antigen coated target cells induced by autoantibodies such as RNP, Sm and SS/A Ro from patients with SLE. In addition, ADCC towards human target cells by normal human peripheral blood mononuclear cells in the presence of sera from patients with SLE and systemic sclerosis, has been demonstrated by Penning et al (1983, 1984a, 1984b, 1984c, 1985) and Wright et al (1983) and will be further characterised in this study. In further support of ADCC as a pathogenic mechanism occurring in autoimmune diseases, is the occurrence of perivascular mononuclear cell infiltrates in the blood vessels of these patients (Campbell & LeRoy 1975). These occur in regions of tissue damage and it is feasible that autoantibodies and mononuclear cells could interact at these sites and thus inflict tissue injury by ADCC.

Although a number of studies have demonstrated ADCC mediated by sera from patients with various autoimmune diseases, reduced ADCC against Chang liver cells has also been reported by the effector cell populations obtained from these patients (Wright et al 1979; Cooper et al 1978). However, Cooper et al (1978) failed to show a reduction in ADCC against chicken erythrocytes by effector cells from the same patients, thus indicating a different population of effector cells mediating ADCC against the 2 different targets. Other workers have, however, demonstrated reduced ADCC towards antibody coated chicken erythrocytes by effector cells from patients with SLE compared with those obtained from normal controls (Feldmann et al 1976). In another

study, sera from patients with SLE were shown to suppress ADCC towards sensitised chicken erythrocytes, compared with sera from normal controls (Zvalfler & Bluestein 1979). The suppressive activity was shown to co-elute with IgG and the authors suggested that the suppression of ADCC was due to a direct effect of antibody combining with membrane antigens of the effector cell population.

Thus, the reduced levels of ADCC reported by various authors in autoimmune diseases, could be caused either by blocking of effector cell Fc receptors, by antibody or immune complexes, or by a generalised suppression of cellular immunity occurring in these patients. Alternatively, the effector cells mediating ADCC could be sequestered in tissues as a result of their involvement in damaged tissue, thereby depleting the circulating blood of this population of cells

3.1.5 In vitro measurement of cytotoxicity

The detection, characterisation and quantitation of ADCC can only be carried out in vitro. Various methods have been employed in the measurement of cytotoxicity. These involve either visual methods, where target cell damage is assessed microscopically, or biochemical or radioisotope based techniques.

Visual determinations of cytotoxicity, which usually involve cell counting, can be criticised in that they are very subjective. A biochemical marker, the release of lactate dehydrogenase, acts as a sensitive indicator of cell lysis, and its measurement can be used in a more objective assessment of cytotoxicity. However, in this study, cytotoxicity was measured by radioisotopic release from prelabelled target cells. A radioisotope is easily measured, it is released in a

soluble form by lysed cells and it is easily separated from residual cells and cell debris. Thus, radioisotopes offer an excellent method for the measurement of cytotoxicity.

Several radioisotopes have been utilised in cytotoxicity assays. There are several requirements for an isotope used in such studies:

1. The isotope should show minimal toxicity towards both target and effector cell populations.
2. The isotope should be evenly distributed among target cells, regardless of their metabolic activity or stage in the cell cycle.
3. The release of isotope into the supernatant should only occur following irreversible damage to the labelled cell.
4. The isotope should be rapidly and readily released by target cells.
5. Once released, isotope should not be reutilised by target or effector cells.

⁵¹Chromium matches these requirements and hence was used for target cell labelling in this study. ⁵¹Chromium has been widely used as a routine label in studies of the survival of erythrocytes in patients with haemolytic disease. In 1961 Goodman showed that ⁵¹chromium release could serve as a precise indicator of antibody and complement induced lysis of nucleated cells. This work was confirmed by Sanderson (1965) and Wigzel (1965). Later, ⁵¹chromium was used as

a label for the determination of cell mediated lysis of cultured cells in monolayer (Holm and Perlmann 1967). As a result of these initial observations, $^{51}\text{chromium}$ has been widely used in target cell labelling in cytotoxicity assays.

Chromium labelling is usually carried out using sodium $^{51}\text{chromate}$. The chromate ion $^{51}\text{CrO}_4$ will passively diffuse into the cell cytoplasm where it is non-covalently bound to proteins and other cell constituents. Generally the label is retained within the target cells, with only about 15-30% spontaneous release, during a 24 hour culture period. Most of this probably comes from spontaneously damaged or dying cells (Holm & Perlmann 1967). However, once the cells are lysed, >90% of the total radioactivity is released (Holm and Perlmann 1967). In addition to its relatively low spontaneous release, $^{51}\text{chromium}$ is non-toxic to target and effector cells even at the high concentrations used in cytotoxicity assays.

One of the principle features of $^{51}\text{chromium}$ is that released label from lysed target cells is not reused (Holm & Perlmann 1967; Bunting et al 1963). The reason for this is uncertain but two possible explanations have been put forward. The first suggests that the hexavalent ion $^{51}\text{CrO}_4$ is reduced to trivalent $^{51}\text{CrO}_3$ which cannot traverse the cell membrane (Rajam and Jackson 1958). The second explanation suggests that $^{51}\text{chromium}$ is released from target cells in a stable form, covalently bound to small protein fragments to which cell membranes are impermeable (Ronai 1969).

Thus $^{51}\text{chromium}$ is commonly used for in vitro studies of cytotoxicity. It is ideally suited for this use because of its low toxicity, low levels of spontaneous release and its rapid uptake and release by target cells. $^{51}\text{Chromium}$ is incorporated via passive

diffusion and therefore its uptake is not dependent upon cellular metabolic activity. Finally, released ^{51}Cr is not reused by target or effector cells.

Hence, ^{51}Cr offers a very sensitive index of cell damage and was used in this study to measure the direct serum cytotoxicity and ADCC of human umbilical vein endothelial cells caused by sera from patients with systemic sclerosis and SLE.

3.2 Materials and methods

3.2.1 Patient and control groups

Systemic sclerosis

48 patients with systemic sclerosis (39 women; 9 men; mean age (\pm SD) 54.5 ± 15.8 years) were investigated for the ability of their sera to produce cytotoxicity of vascular endothelial cells, either directly or in co-culture with peripheral blood mononuclear cells. Raynaud's phenomenon and acrosclerosis were constant features in all the patients who had been investigated by a standard protocol (Table 2) which enabled points to be awarded for cutaneous and visceral involvement and so produce a disease score for each patient (see appendix IV). On this basis, the patients were sub-divided into categories of "severe disease" (disease score ≥ 6 ; 19 women; 4 men; mean age (\pm SD) 57.4 ± 16.9 years) and "mild disease" (disease score ≤ 5 ; 21 women; 4 men; mean age (\pm SD) 52.0 ± 14.6 years). 5 of these patients were being treated with prednisolone, supplemented in 3 cases (all with "severe disease") with azathioprine. Nifedipine and captopril were used to treat an additional 9 and 8 patients respectively. None of the patients were receiving treatment with penicillamine.

Systemic lupus erythematosus

49 patients with SLE (41 women; 8 men; mean age (\pm SD) 44.1 ± 13.6) years were similarly examined for the ability of their sera to cause cytotoxicity of vascular endothelial cells either directly or indirectly. The principle clinical features of these patients, who all had positive tests for antinuclear and anti DNA antibodies, are shown in appendix V. All but one of these patients were being treated with prednisolone the dose ranging from 2.5mg to 30mg daily. In addition, 12 patients were receiving further treatment with

Table 2

Criteria of systemic involvement in patients with Systemic Sclerosis.

System involved	Criteria	Disease score (points allotted) for involvement)
Skin	Sclerosis - Face	1
	Hands	1
	Trunk	1
Gut	Radiological changes	3
Lungs	Radiological changes &/or abnormal CO transfer factor	3
Heart	ECG changes	3
Kidneys	Proteinuria &/or creatinine clearance <60 ml/min	3
Other	Sjogren's syndrome, Polymyositis	3

azathiaprine, 4 with cyclophosphamide, 8 with warfarin and 4 with chloroquine.

Normal controls

The control group consisted of 44 normal healthy adults, 34 women; 10 men; mean age (\pm SD) 36.3 \pm 11.2 years.

3.2.2 Serum samples

Serum samples were obtained from blood which was collected by venepuncture and allowed to clot at 37°C, to avoid the loss of cryoprecipitable proteins. The blood was centrifuged at 1,500g for 20 minutes at 37°C, the serum removed aseptically and aliquoted into sterile glass bijoux. Serum samples were stored at -80°C and were not reused once thawed.

3.2.3 Isolation of effector cell populations for use in ADCC assays

For ADCC assays effector cells were obtained from one control donor (CH) for all assays, to minimize any variation that could arise between different donors.

To determine the viability of isolated effector cells, a trypan blue dye exclusion test was carried out. 10 μ l of 0.4% trypan blue was gently mixed with 40 μ l peripheral blood mononuclear cells in M199 and an aliquot of this was placed in a haemocytometer. The number and viability of effector cell populations was then determined under a light microscope. Populations of effector cells with viability <90% were discarded.

3.2.3.1 Peripheral blood mononuclear cells

Mononuclear cells have a lower density than erythrocytes and granulocytes and remain at the top of a separation fluid with a density of 1.077 g/ml, following centrifugation of whole blood (Boyum 1968).

Procedure

Effector cells for use in ADCC assays were obtained from heparinised (20 units/ml of preservative free heparin) peripheral blood, collected by venepuncture with a wide bore (19G) needle. 5ml heparinised blood was diluted with an equal quantity of sterile PBS and was then layered onto 10ml "Lymphoprep" separation fluid in a glass universal container. This preparation was then centrifuged at 1000g for 20 minutes at 20°C. The resulting separated peripheral blood mononuclear cells were present as a visible band of cells at the sample/medium interface. These cells were carefully aspirated from the interface using a glass pasteur pipette. The peripheral blood mononuclear cells were then washed in M199 and centrifuged at 500g for 10 minutes at 20°C. Following this first wash, the peripheral blood mononuclear cells were twice washed in M199 and centrifuged at 350g for 5 minutes. Finally, the peripheral blood mononuclear cells were resuspended in HEPES buffered M199, counted and tested for viability using a trypan blue dye exclusion test. The cell suspension was adjusted to the required concentration with HEPES buffered M199. For ADCC assays the peripheral blood mononuclear cells were used at a concentration of 2.5×10^6 viable cells/ml.

3.2.3.2 Monocytes

The average density of monocytes is lower than that of lymphocytes, but because the densities of the two cell types overlap, separation using a density gradient results in cross contamination of the two different populations of cells. However, if the osmolality of the gradient medium is appropriately adjusted whilst the density is kept constant, as in "Nycodenz monocytes", the separation of monocytes from lymphocytes can be achieved. If the osmolality is increased, the cells expel water, shrink and increase in density. Lymphocytes are

more sensitive than monocytes in this respect, and will therefore sediment further during centrifugation, resulting in a band of monocytes at the top of the gradient.

Procedure

Blood was collected by venepuncture and 9ml was placed into a glass universal containing 1ml 2.7% w/v EDTA (pH 7.4). 3ml of this was layered onto 3ml "Nycodenz monocytes" and the preparation was centrifuged for 15 minutes at 600g and 20°C. The resulting band of monocytes at the sample/medium interface were harvested using a glass pasteur pipette and washed three times with M199. The final monocyte suspension was counted and tested for viability, then adjusted to 2.5×10^5 cells/ml. The purity of monocyte preparations were assessed using the non-specific esterase method, which specifically stains monocytes (Yam et al 1971).

3.2.3.3 Monocyte depletion

Two methods, which use either the adherent or phagocytic properties of monocytes, were employed to deplete these cells from peripheral blood mononuclear effector cell populations.

1. Adherence to Plastic

This method removes all adherent cells from the effector cell population. A suspension of peripheral blood mononuclear cells in 10ml M199 was incubated in a T75 flask for 60 minutes at 37°C. Non-adherent cells were vigorously washed from the flask with M199 and centrifuged for 5 minutes at 350g. The adherent cell depleted preparation of peripheral blood mononuclear cells was counted, tested for viability and resuspended in M199 at the appropriate cell concentration.

2. Treatment with Carbonyl Iron Powder.

This method removes actively phagocytic and adherent cells. Peripheral blood mononuclear cells were incubated for 30 minutes at 37°C on 90mm petri dishes in 10ml M199, containing 10mg carbonyl iron powder. The non-adherent cells were vigorously washed off the petri dish with M199. The resulting preparation of non-adherent cells, which included macrophages containing phagocytosed carbonyl iron particles, was layered onto "lymphoprep" and centrifuged at 1000g for 20 minutes at 20°C. While macrophages containing ingested carbonyl iron particles sedimented through the separation gradient, the phagocyte depleted cells which formed a band at the interface were collected, washed and resuspended in M199.

3.2.4 **⁵¹Chromium release cytotoxicity assays**

⁵¹Chromium release assays were used in this study to measure the cytotoxicity of human venous or arterial umbilical endothelial cells caused by sera from patients with systemic sclerosis and SLE, either alone or in conjunction with peripheral blood mononuclear cells.

Cytotoxicity assays were performed in situ in microtitre plates using human venous or arterial endothelial cells, at first passage, as target cells. Endothelial cells were seeded at 10,000 cell/well into flat-bottomed, gelatin coated 96 well microtitre plates, and incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. After 48 hours the human umbilical vein endothelial cells were confluent and ready for radiolabelling prior to their use as target cells in cytotoxicity assays

3.2.4.1 **Radiolabelling of endothelial cell monolayers**

100µl M199 + 20% normal human AB serum containing 0.5 µci ⁵¹chromium, in the form of sodium chromate, was added to each well which contained a confluent monolayer of endothelial cells. The

microtitre plate was then incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air, for a further 18 hours. After this labelling period the medium above the endothelial cell monolayer was discarded and the cells were carefully washed 3 times to remove any excess ⁵¹chromium.

3.2.4.2 Assay conditions

Triplicate assay wells were set up for each particular set of conditions and the final volume in each well was adjusted to 200µl with HEPES buffered M199.

Control conditions for each individual assay consisted of:-

1. Baseline or spontaneous ⁵¹chromium release
M199+ 5% normal human AB serum.

2. Maximal ⁵¹chromium release
100µl saponin and 100µl M199.

3. Spontaneous cell mediated cytotoxicity (NK activity)
M199 + 5% normal human AB serum and 2.5x10⁵ peripheral blood mononuclear cells

The test wells consisted of :-

1. Direct serum induced cytotoxicity
M199 + 5% test serum

2. Antibody dependent cellular cytotoxicity
M199 + 5% test serum + 2.5x10⁵ peripheral blood mononuclear cells

Once all the control and test wells were set up, the microtitre plate was incubated for 18 hours at 37°C. Following incubation, the microtitre plate was centrifuged at 200g for 10 minutes at 15°C and 100µl supernatant was transferred from each well to a supernatant tube. 100µl formic acid was then added to each well and the contents of each well were transferred to a residue tube (each well being scraped to assure the total removal and transfer of all cellular material).

The supernatant and residue tubes were then counted in pairs for 4 minutes/tube, in an Intertechnique CG 30 gamma spectrometer. The spectrometer was equipped with an Epson RX-80 computer, programmed to calculate the percentage ⁵¹chromium release and the percentage specific cytotoxicity (see below)

3.2.4.3 Calculation of cytotoxicity

The percentage ⁵¹chromium release (CR) and the percentage specific cytotoxicity (SC) were calculated by the method of Gale and McLennan (1976):-

$$\%^{51}\text{Cr Release} = \frac{(\text{supernatant cpm} - \text{bg}) \times 2}{(\text{supernatant cpm} - \text{bg}) + (\text{residual cpm} - \text{BG})} \times 100$$

Where cpm = counts per minute

bg = back ground cpm

The percentage specific cytotoxicity (% SC) was determined to compensate for interassay variation in baseline and maximal isotopic release:-

$$\% \text{ SC} = \frac{(\text{Experimental } \% \text{ } ^{51}\text{Cr release} - \text{baseline } \% \text{ } ^{51}\text{Cr release})}{(\text{Maximal } \% \text{ } ^{51}\text{Cr release} - \text{baseline } \% \text{ } ^{51}\text{Cr release})} \times 100$$

For each serum tested in ADCC assays, the "excess percentage specific cytotoxicity" (excess % SC) was determined from the percentage specific cytotoxicity of the serum only tubes and the percentage specific cytotoxicity of the serum + peripheral blood mononuclear cell co-culture tubes:-

$$\text{Excess } \% \text{ SC} = [\% \text{SC (S+PBM)}] - [\% \text{SC (S)} + \% \text{SC (PBM)}]$$

Where S = serum

PBM = peripheral blood mononuclear cells

3.2.4.4 Statistical analysis of data

Levels of "excess % specific cytotoxicity" obtained in patient and control groups were statistically compared using the Wilcoxon Mann-Whitney test (Seigel et al 1956). In addition, as 95% of sera from controls produced levels of "excess % specific cytotoxicity" <8%, levels of "excess % specific cytotoxicity" >10% were considered to be "positive" values. Further statistical analysis of the proportions of sera from patients and controls producing "excess % specific cytotoxicity" of >10% was then also made, using the chi-squared test for association (Norusis 1988).

3.2.4.5 Cytotoxicity in the presence of a source of complement

In addition to direct serum induced cytotoxicity with 5% serum and ADCC studies, experiments were carried out to determine the effects of 20% serum on cultured human umbilical vein endothelial

cells, either alone or in the presence of a source of complement (normal rabbit serum). Control wells were set up as previously described and test wells consisted of :-

1. M199 + 20% test serum
2. M199 + 20% test serum + 12.5% normal rabbit serum

3.2.5 Pre-incubation studies

Pre-incubation studies were performed with human umbilical vein endothelial cells to determine whether the factor, present in patient serum, capable of mediating ADCC would preferentially bind to target and/or effector cells. These pre-incubation studies were performed in conjunction with a standard direct serum induced cytotoxicity and ADCC test, ie for one particular serum, all these tests were carried out on human umbilical vein endothelial cells derived from a single cord

3.2.5.1 Serum pre-incubation of target cells

Confluent human umbilical vein endothelial cell monolayers in microtitre plates were labelled with 51 chromium as previously described. Following the 18 hour labelling period, the microtitre wells for preincubation were washed once with HEPES buffered M199. For each particular serum under observation, 6 wells were pre-incubated, with 5% test serum in M199, for 1 hour at 37°C in a humidified atmosphere of 5% CO₂/95% air. Following this pre-incubation period, the endothelial cell monolayers were washed 3 times with HEPES buffered M199. 3 of the wells were set up to test for any direct cytotoxic effects. To these wells, 5% normal human AB serum in HEPES buffered M199 was added. The other 3 wells were set up to test for ADCC. To these wells, 5% normal human AB serum and 2.5×10^5 peripheral blood mononuclear cells in HEPES buffered M199 were added. The necessary controls, ie baseline, maximal 51 chromium release and spontaneous cell mediated cytotoxicity were also set up in triplicate.

The microtitre plate was then incubated for a further 18 hours at 37°C and processed as outlined previously.

3.2.5.2 Serum pre-incubation of effector cells

Peripheral blood mononuclear cells were isolated as previously described. Following the third wash, they were counted, tested for viability and resuspended at 2.5×10^6 viable cells / ml, in HEPES buffered M199. 5% test sera was then added to 2.5×10^6 peripheral blood mononuclear cells and the volume adjusted to 2ml with HEPES buffered M199. This mixture was then incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO₂/air and gentle mixing every 20 minutes. Following this pre-incubation period, the peripheral blood mononuclear cells were washed with M199. The resuspended cells were counted and the concentration re-adjusted to 2.5×10^6 viable cells / ml. These preincubated peripheral blood mononuclear cells were then cultured with ⁵¹chromium labelled human umbilical vein endothelial cells with 5% normal human AB serum in microtitre plates and the assay performed as usual.

3.2.6 Serum titration studies

In order to determine the lowest dilution of serum at which ADCC could be detected, the serum of a patient with SLE and 2 patients with systemic sclerosis, who had previously consistently demonstrated cytotoxicity against human umbilical vein endothelial cells in co-culture studies, were titrated down to one part serum in one million parts M199. Triplicate assay wells were set up and 10 µl of the following dilutions of serum, 5% normal human AB serum and M199 were added. The various dilutions of serum were tested both alone and in the presence of peripheral blood mononuclear cells. The dilutions of serum tested in co-culture studies were :- 1, 1/2, 1/5, 1/10, 1/102, 1/103, 1/10⁴, 1/10⁵, 1/10⁶.

3.2.7 Column chromatography of serum samples

Column chromatography was performed on serum samples by gel filtration on an "ULTROGEL" column, using PBS (pH 7.2). An upward flow of 20ml per hour was maintained with a peristaltic pump, and separation was performed at 4°C. 3ml of serum was separated and fractions of 7ml were collected following passage through a UV monitor measuring optical density at 280nm. The fractions were then pooled to give 10 pooled fractions covering the elution profile as shown in fig 1.

The pooled fractions were then concentrated to a volume of 1ml using an "Amicon" concentrator with a membrane of 30,000 MW cut off and a maximum operating pressure of 70 psi. Concentrated fractions were then aliquoted and stored at -40°C prior to use. In cytotoxicity assays, 20 µl of each pooled fraction was used per well together with 10 µl normal human AB serum.

The presence of IgG and IgM in the pooled fractions were determined by double and radial immunodiffusion (see appendices I & II).

3.2.8 Isolation of IgG from serum samples

The isolation of IgG from patient and control sera was performed by the techniques of ion exchange chromatography and affinity chromatography.

3.2.8.1 Ion exchange chromatography

Principle

In ion exchange chromatography, proteins bind electrostatically to an ion exchange matrix which has an opposite charge to the protein. The affinity of the protein for the exchanger depends upon its charge density, and unbound substances are washed from the exchanger using a column volume of starting buffer. The bound protein is eluted by

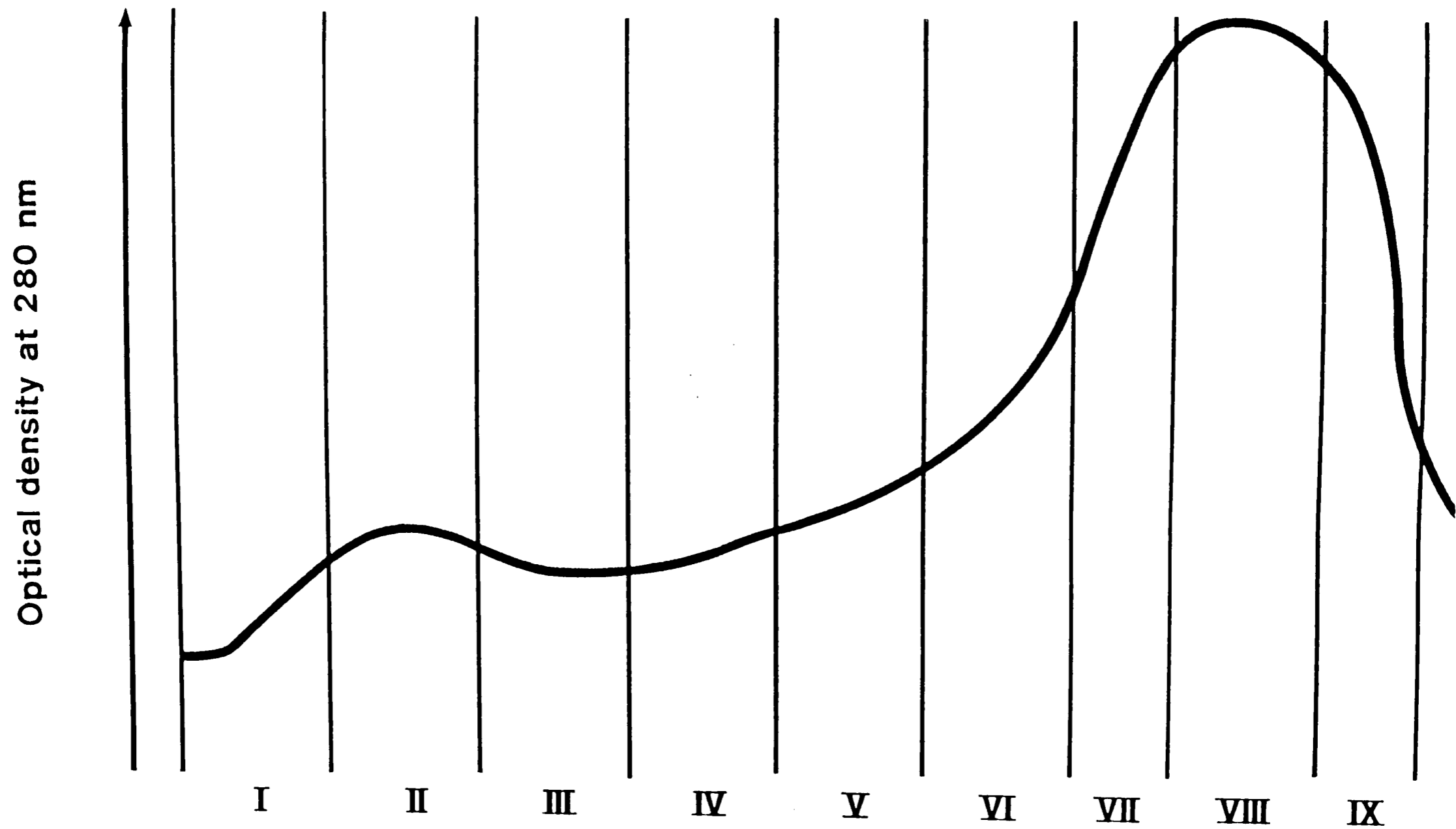


Figure 1

A typical elution profile of serum following passage through an ULROGEL column, showing the 10 pooled fractions which were concentrated prior to use in ADCC assays.

either increasing the ionic strength, or else by altering the pH of the buffer. An eluting buffer of increasing ionic strength causes competition between buffer ions and proteins for the charged groups on the ion exchanger. If the pH is altered, then when the isoelectric point of the bound protein is reached, its net charge becomes zero and it no longer binds to the exchanger.

Procedure

An anion exchanger (DEAE cellulose) was used in this study for the separation of serum immunoglobulins. 1ml serum, to be separated, was dialysed against 20mM phosphate buffer (pH 8.0) with three changes of buffer. The dialysed sample was then loaded onto the ion exchange column and approximately 30, 10ml fractions were eluted with 20mM phosphate buffer. Under these conditions, the IgG elutes straight through and all other serum components bind to the column. The bound protein was eluted with 30, 10ml fractions of 200mM phosphate buffer (pH 8.0). The column was re-equilibrated to 20mM prior to the next separation.

The IgG containing fractions were detected by UV absorbance at 280nm. The pooled fractions were then dialysed against 3 changes of PBS, and concentrated to a volume of 1ml in an "AMICON" concentrator with a membrane of 30,000 MW cut off and a maximum operating pressure of 70 p.s.i. IgG preparations were aliquoted and stored at -40°C prior to use. IgG content was determined by radial immunodiffusion (see Appendix II). For cytotoxicity assays, the amount of IgG / well was 0.1 mg (which is equivalent to 10 µl of 10 mg/ml).

3.2.8.2 Affinity chromatography

Principle

Affinity chromatography is a form of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed to a specific ligand which is immobilised on an insoluble support, such as sepharose. For the isolation of IgG from serum, the ligand is staphylococcal protein A. IgG binds to this via its Fc receptors and all other serum components will pass through the affinity column, bound IgG is subsequently eluted. Protein A binds human IgG of subclasses 1,2 and 4 but not 3 (Hudson & Hay 1980)

Procedure

The staphylococcal protein A / sepharose column was washed with about 20ml stripping buffer (0.1M sodium ethanoate pH 5.0) and was then equilibrated with 20ml 10 mM Tris buffer (pH 7.4). Following equilibration, the serum sample to be separated was loaded onto the column and the unbound serum components were eluted with a further 20 ml of Tris buffer. The IgG remaining bound to the column was then eluted with 0.1 M glycine buffer (pH adjusted to 3.0 with HCl). The IgG containing fractions were determined by absorbance at 280 nm and fractions were pooled and dialysed with three changes of PBS, then concentrated to 1 ml in an Amicon concentrator, and the IgG content determined as previously described. IgG was aliquoted and stored at -40°C.

3.3 Results

3.3.1 Basic parameters of the assays

3.3.1.1 Baseline ⁵¹chromium release

The baseline or spontaneous ⁵¹chromium release from cultured human umbilical vein endothelial cells, occurring over 18 hours, was $24.37\% \pm 0.36\%$ (mean \pm SEM; n = 126 experiments). From cultured arterial endothelial cells, the baseline ⁵¹chromium release was $25.74\% \pm 1.97\%$ (mean \pm SEM; n = 4 experiments).

3.3.1.2 Maximal ⁵¹chromium release

The maximal ⁵¹chromium release determined by the addition of saponin, was $94.48\% \pm 0.33\%$ (mean \pm SEM; n = 126 experiments) from human umbilical vein endothelial cells, and $87.91\% \pm 4.26\%$ (mean \pm SEM; n = 4 experiments) from arterial endothelial cells.

3.3.1.3 Spontaneous cell mediated cytotoxicity

The specific cytotoxicity caused by spontaneous cell mediated cytotoxicity, following co-culture with normal human peripheral blood mononuclear cells and human umbilical vein endothelial cells, was $13.16\% \pm 0.59\%$ (mean \pm SEM; n = 89 experiments). For arterial endothelial cells the spontaneous cell mediated cytotoxicity was $13.94\% \pm 2.18\%$ (mean \pm SEM; n = 4 experiments).

3.3.1.4 Effector donor variation

Peripheral blood mononuclear cells derived from different donors showed varying levels of spontaneous cell mediated cytotoxicity (table 3). For routine use, peripheral blood mononuclear cells obtained from a single control donor (CH) were used.

Table 3

Percentage specific cytotoxicity (SC) of human umbilical vein endothelial cells due to spontaneous cell mediated cytotoxicity produced by peripheral blood mononuclear cells obtained from different donors.

Donor	%SC (mean \pm SEM)	n(expts)
PH	16.21 \pm 1.79	4
NL	19.44	2
JM	18.77	2
CH	13.16 \pm 0.59	89

3.3.1.5 Effector cell to target cell ratio

In co-culture studies, the level of cytotoxicity increased as the number of effector cells increased (see fig 2). For routine use 2.5×10^5 peripheral blood mononuclear cells per well were used as the best compromise between adequate levels of cytotoxicity and availability of the effector cells.

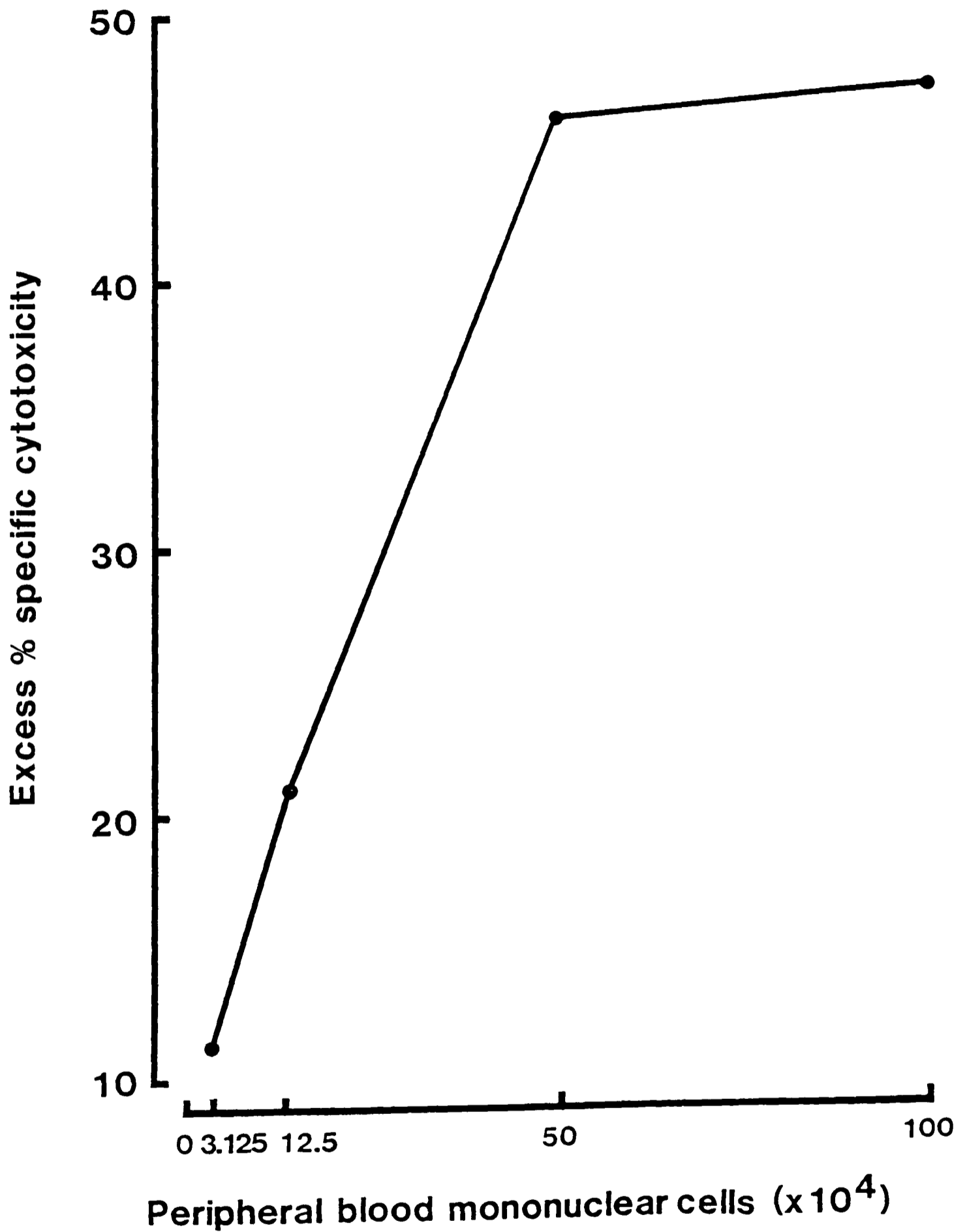


Figure 2
Excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with increasing amounts of normal peripheral blood mononuclear cells and serum from a patient with systemic sclerosis which had previously exhibited ADCC of human umbilical vein endothelial cells.

3.3.1.6 Serum concentration causing maximal ADCC

In preliminary ADCC assays, serum was added at a concentration of 5%. To determine if this was the optimal amount of serum required for maximal ADCC to occur, serum concentrations ranging from 2.5% to 50% were tested in co-culture experiments with 2.5×10^5 peripheral blood mononuclear cells / well. The results are illustrated in fig 3.

A serum concentration of 5% was maintained for future routine testing, since maximal ADCC was observed with this concentration.

3.3.1.7 Intra and inter assay variation

Intra-assay variation observed in ADCC assays was low. In a series of 12 baseline release wells within the same assay, the value of 51 chromium release was $20.24\% \pm 0.36\%$ (mean \pm SEM) with a coefficient of variation of 6.69%.

However, inter-assay variation was much wider. The baseline 51 chromium release showed a coefficient of variation of 16.68% (n=126 experiments) and an even wider variation was observed between values of spontaneous cell mediated cytotoxicity derived from assays performed on separate occasions, the coefficient of variation being 42.16% (n=89 experiments).

3.3.2 Clinical studies

3.3.2.1 Effect of co-culture of human umbilical vein endothelial cells with serum and peripheral blood mononuclear cells

In this study, 48 patients with systemic sclerosis were examined for cytotoxicity against cultured human umbilical vein endothelial cells by co-culture with patient sera and normal human peripheral blood mononuclear cells. Sera derived from 10 of 48 patients demonstrated "excess % specific cytotoxicity" of $>10\%$; ie approximately 20% of patients with systemic sclerosis exhibited ADCC

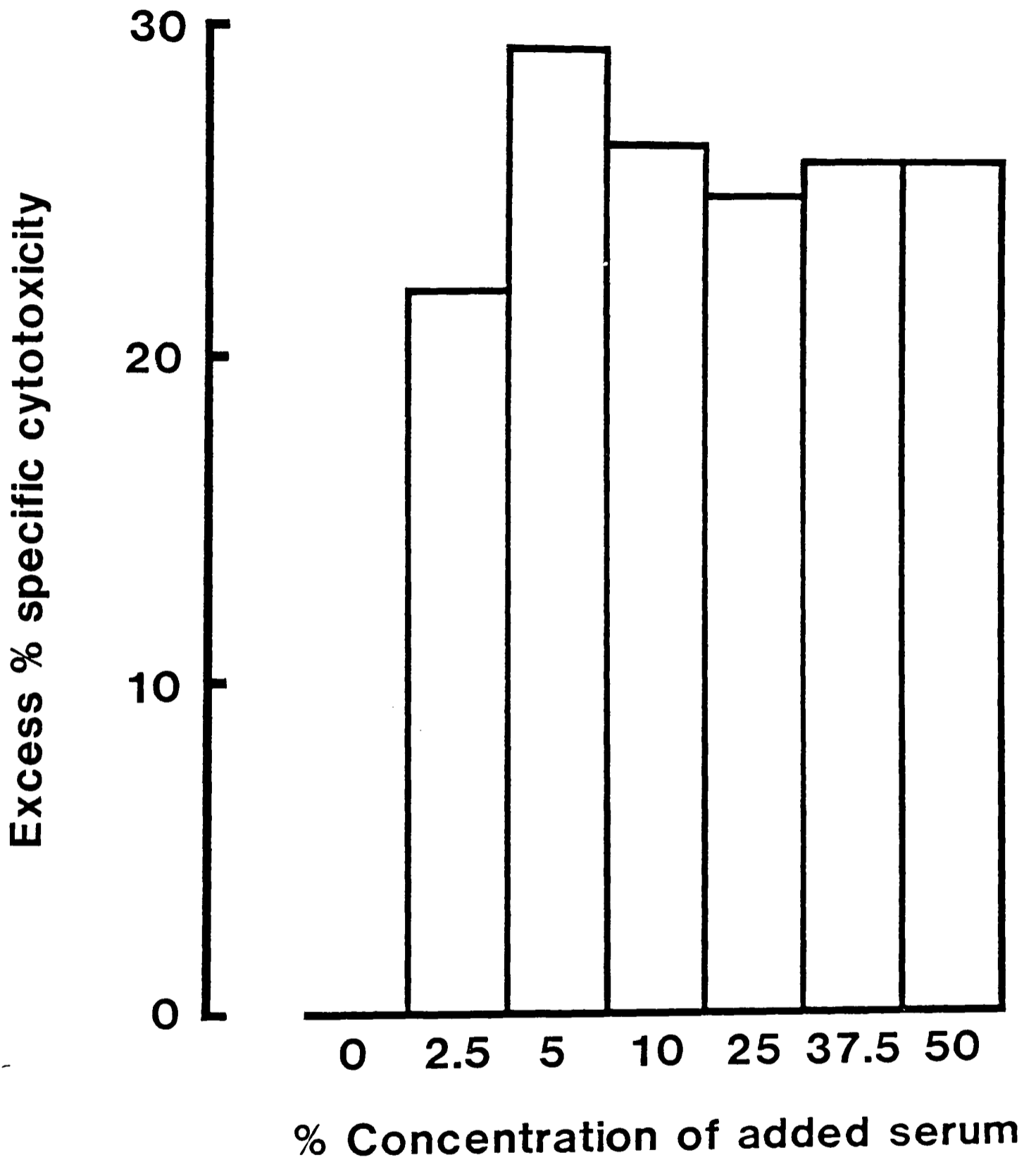


Figure 3

The effect of varying the % concentration of added serum, on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and serum from a patients with systemic sclerosis.

against cultured human umbilical vein endothelial cells (see fig 4). All 48 patients were serially examined and successive bleeds were performed on 3-10 occasions over a period of 2 years. Of the 10 patients exhibiting ADCC, 7 showed cytotoxicity for each successive bleed, whereas in 3 patients, the cytotoxic effect was not observed in later bleeds.

In contrast to the above results obtained from patients with systemic sclerosis, only 2 out of 44 sera obtained from normal control donors showed "excess % specific cytotoxicity" of >10%. ie <5% of sera from normal control donors exhibited ADCC against cultured human umbilical vein endothelial cells (see fig 4).

When the levels of "excess % specific cytotoxicity" obtained following co-culture of sera from patients with systemic sclerosis and normal controls, were statistically compared using the Wilcoxon Mann-Whitney test, no significant difference was observed ($p = 0.075$) due to the disproportionately large number of nil values in both groups. However, when a comparison of the proportion of sera from patients with systemic sclerosis demonstrating "excess % specific cytotoxicity" of >10% was statistically compared to the proportion of control sera demonstrating "excess % specific cytotoxicity" of >10% a significant difference between the 2 populations was observed ($p < 0.025$).

In addition to the group of patients with systemic sclerosis 49 patients with SLE were also observed for their effects on the cytotoxicity of human umbilical vein endothelial cells following co-culture with patient serum and normal peripheral blood mononuclear cells. 7 patients with SLE exhibited increases in "excess % specific cytotoxicity" of >10%. ie 14% of patients with SLE exhibited ADCC against cultured human umbilical vein endothelial cells (see fig 4).

When studied serially, 4 patients showed cytotoxic effects when up to 14 successive bleeds were tested. 2 patients were not tested on more than one occasion and in one patient the cytotoxic effect was no longer demonstrated by serum obtained from successive bleeds. When compared using both statistical tests, the SLE and control groups did not differ significantly.

Comment

Sera from approximately 20% of patients with systemic sclerosis and 14% patients with SLE demonstrated enhanced cytotoxicity of cultured human umbilical vein endothelial cells following co-culture with normal human peripheral blood mononuclear cells. The majority of these patients also exhibited enhanced cytotoxicity when examined serially over a period of 2 years. In contrast, <5% of sera from normal control donors exhibited cytotoxicity against human umbilical vein endothelial cells.

3.3.2.2 Effect of co-culture of human umbilical artery endothelial cells with serum and peripheral blood mononuclear cells

28 patients with systemic sclerosis were examined for cytotoxicity against arterial endothelial cells following co-culture with sera and normal peripheral blood mononuclear cells. Sera from 6 patients exhibited ADCC; ie approximately 20% of patients with systemic sclerosis showed cytotoxicity towards cultured arterial endothelial cells following co-culture with normal peripheral blood mononuclear cells (see fig 5). However, the levels of "excess % specific cytotoxicity" of arterial endothelial cells obtained following co-culture with sera from patients with systemic sclerosis and normal controls, were not significantly different as determined by

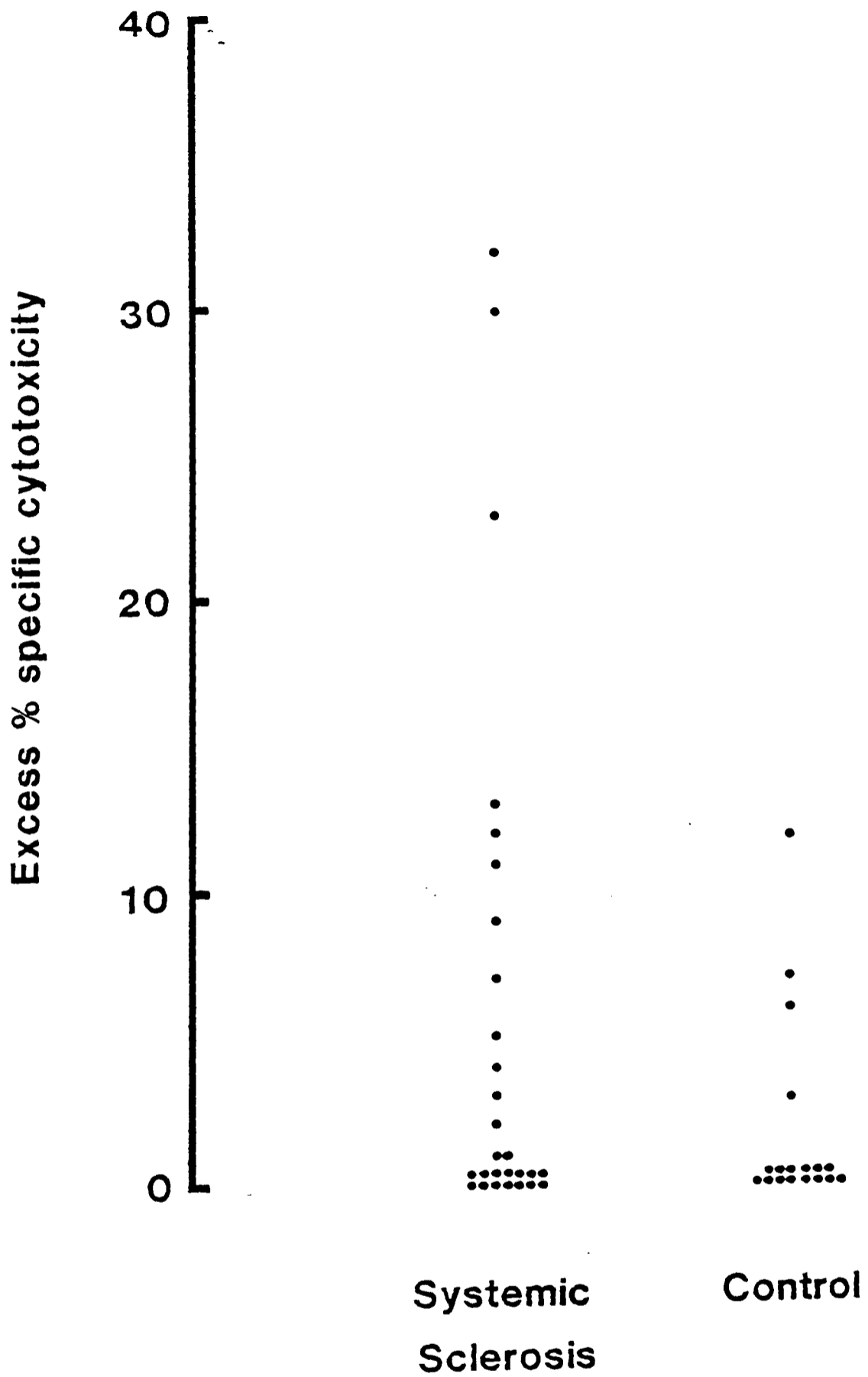


Figure 5
 Excess % specific cytotoxicity of human umbilical artery endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and sera from patients with systemic sclerosis or normal controls.

the Wilcoxon Mann-Whitney and chi-squared tests.

Sera from 3 patients that had previously demonstrated ADCC against venous endothelial cells failed to cause cytotoxicity of arterial targets. These 3 sera correlated with those those which had lost ADCC activity in serial bleeds. In addition, 6 sera derived from patients with SLE which had previously shown ADCC towards human umbilical vein endothelial cells were also tested for their effect against cultured arterial endothelial cells. 5 of the 6 sera once more, showed "excess % specific cytotoxicity" of >10%. In contrast, only 1 of 18 sera derived from normal control donors showed enhanced cytotoxicity towards arterial endothelial cells. The results obtained following the co-culture of human umbilical artery endothelial cells with serum from patients with systemic sclerosis and normal peripheral blood mononuclear cells are summarized in fig 5.

Comment

Approximately 20% of sera derived from patients with systemic sclerosis were cytotoxic towards cultured human arterial endothelial cells following co-culture with normal human peripheral blood mononuclear cells. These sera were also cytotoxic against human umbilical vein endothelial cells.

3.3.2.3 Direct serum induced cytotoxicity of human umbilical endothelial cells

All the serum samples tested in co-culture with peripheral blood mononuclear cells and either venous or arterial cells were also examined for a direct serum induced cytotoxicity against the same targets. No evidence of venous or arterial endothelial cell

cytotoxicity was found with 5% serum from patients with systemic sclerosis or SLE.

Further studies were carried out, in 19 patients with systemic sclerosis, 15 patients with SLE and 19 normal controls, to determine the effects of 20% serum on cultured venous endothelial cells either alone or in the presence of normal rabbit serum as a source of complement. Once again, no increase in 51 chromium release was observed following exposure to 20% serum either alone or with added complement.

Comment

No evidence of direct serum induced cytotoxicity of venous or arterial endothelial cells was demonstrated by 5% sera obtained from patients with systemic sclerosis and SLE. In addition, cytotoxicity against venous endothelium by 20% serum, either alone or in the presence of complement, was not detected.

3.3.2.4 Effect of serum pre-incubation with target cells

No increase in "excess % specific cytotoxicity" was observed following the pre-incubation of human umbilical vein endothelial cells with serum from normal control donors. However, when endothelial cells were pre-incubated with sera from patients with systemic sclerosis, 7 sera consistently demonstrated increased "excess % specific cytotoxicity" of >10%. These sera had previously produced cytotoxicity against against human umbilical vein endothelial cells by co-culture with peripheral blood mononuclear cells. Sera from 3 patients with systemic sclerosis, which had previously produced ADCC, did not produce cytotoxicity following pre-incubation with target cells. These samples correlated with those which had lost ADCC activity in serial bleeds. In addition, 3 sera derived from patients with SLE also produced cytotoxicity following pre-incubation of target cells. (see fig 6 and table 4)

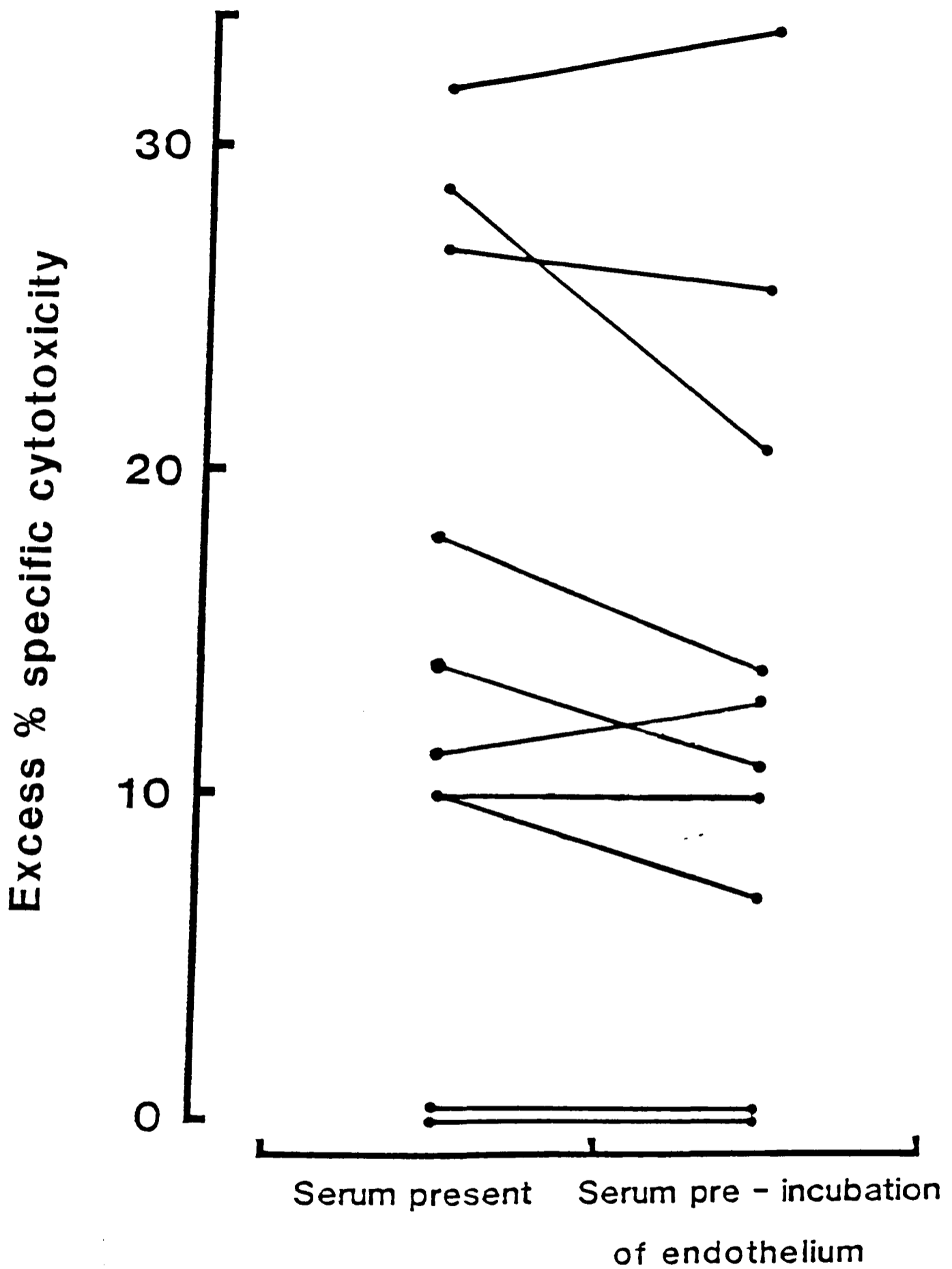


Figure 6

The effect of serum pre-incubation of target cells on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal human peripheral blood mononuclear cells and sera from 10 patients with systemic sclerosis, who had previously demonstrated enhanced cytotoxicity of endothelial cells in co-culture experiments.

Table 4

The effect, on endothelial cytotoxicity, of pre-incubating target and effector cells with sera from patients with systemic sclerosis capable of producing ADCC of human umbilical vein endothelial cells.

Patient	Target cell pre-incubation	Effector cell pre-incubation	Co-culture (target & effector cells)
1	+	-	+
2	-	-	+ (-)*
3	+	-	+
4	-	-	+ (-)*
5	+	+	+
6	+	-	+
7	+	-	+
8	+	-	+
9	-	-	+ (-)*
10	+	-	+

(+) = "Excess % specific cytotoxicity > 10

(-) = "Excess % specific cytotoxicity < 10

(*) = Serum samples which have lost cytotoxic activity in successive bleeds

3.3.2.5 Effect of serum pre-incubation with effector cells

Sera from 10 patients with systemic sclerosis which had previously produced cytotoxicity in co-culture experiments were also examined in studies in which the effector cells were pre-incubated with serum. In contrast to pre-incubation with target cells, only one serum showed enhanced cytotoxicity of venous endothelium following preincubation with effector cells. (see fig 7 and table 4)

Comment

These results established that the cytotoxicity inducing factor present in the sera of patients with systemic sclerosis was capable of binding to human umbilical vein endothelial cells; a finding consistent with it being an antibody directed against the vascular endothelium. In the one patient that binding to effector cells occurred a different factor, such as fragments of IgG or a cytokine, may be involved.

3.3.2.6 Effect of serum titration

The effects of serial dilutions of serum from 1 patient with SLE and 2 patients with systemic sclerosis in co-culture experiments are illustrated in fig 8. Enhanced cytotoxicity was observed with serum dilutions up to $1/10^6$ with serum from the patient with SLE. ie cytotoxicity of human umbilical vein endothelial cells following co-culture with peripheral blood mononuclear cells was observed with serum concentrations as low as 5×10^{-6} %. Serum from patients with systemic sclerosis exhibited "excess % specific cytotoxicity" of $>10\%$ when serum was diluted 1/10 and 1/2, ie 0.5% and 2.5% final concentration.

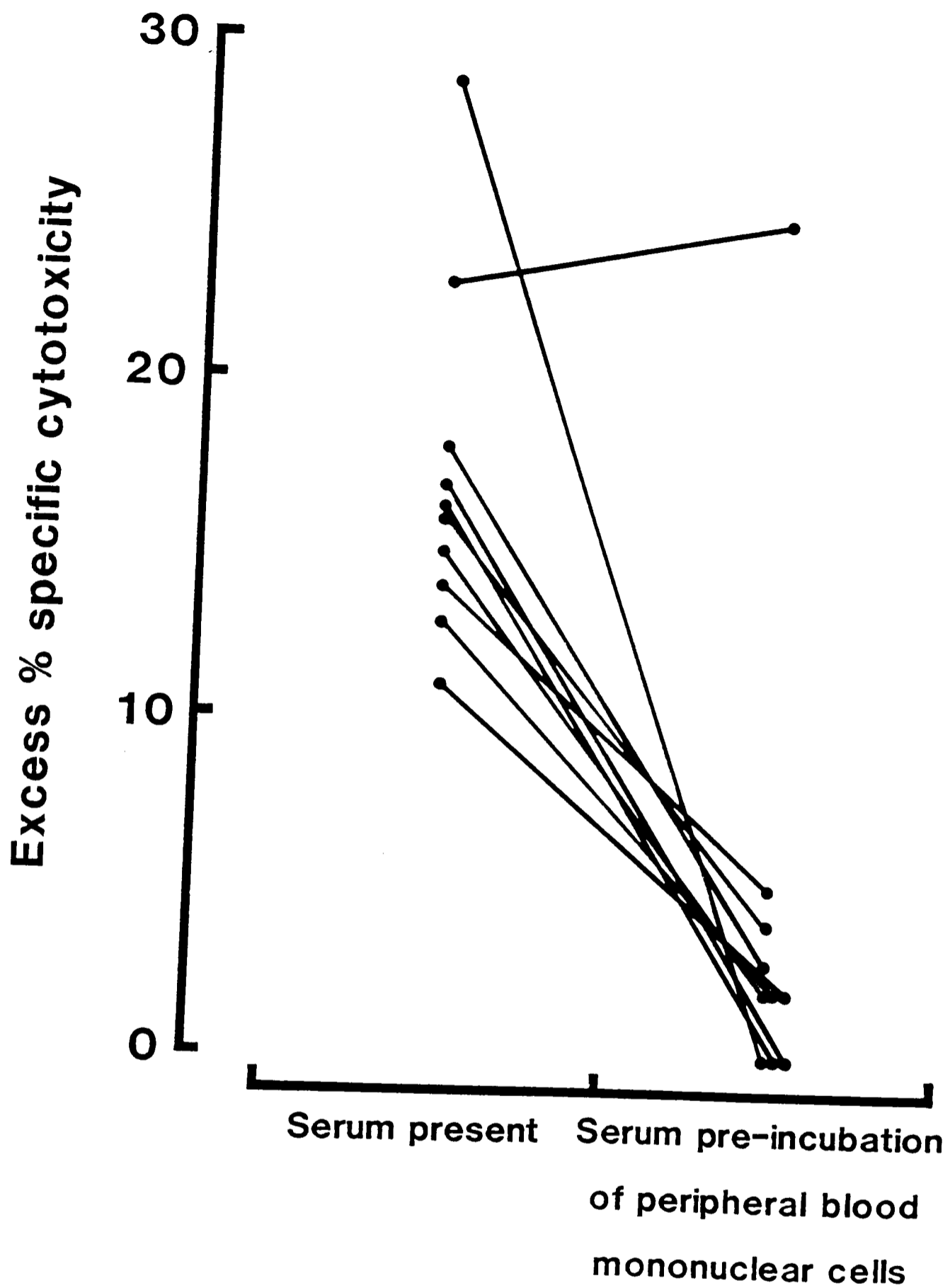


Figure 7

The effect of serum pre-incubation of effector cells, on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and sera from 10 patients with systemic sclerosis, who had previously demonstrated enhanced cytotoxicity of endothelial cells in co-culture experiments.

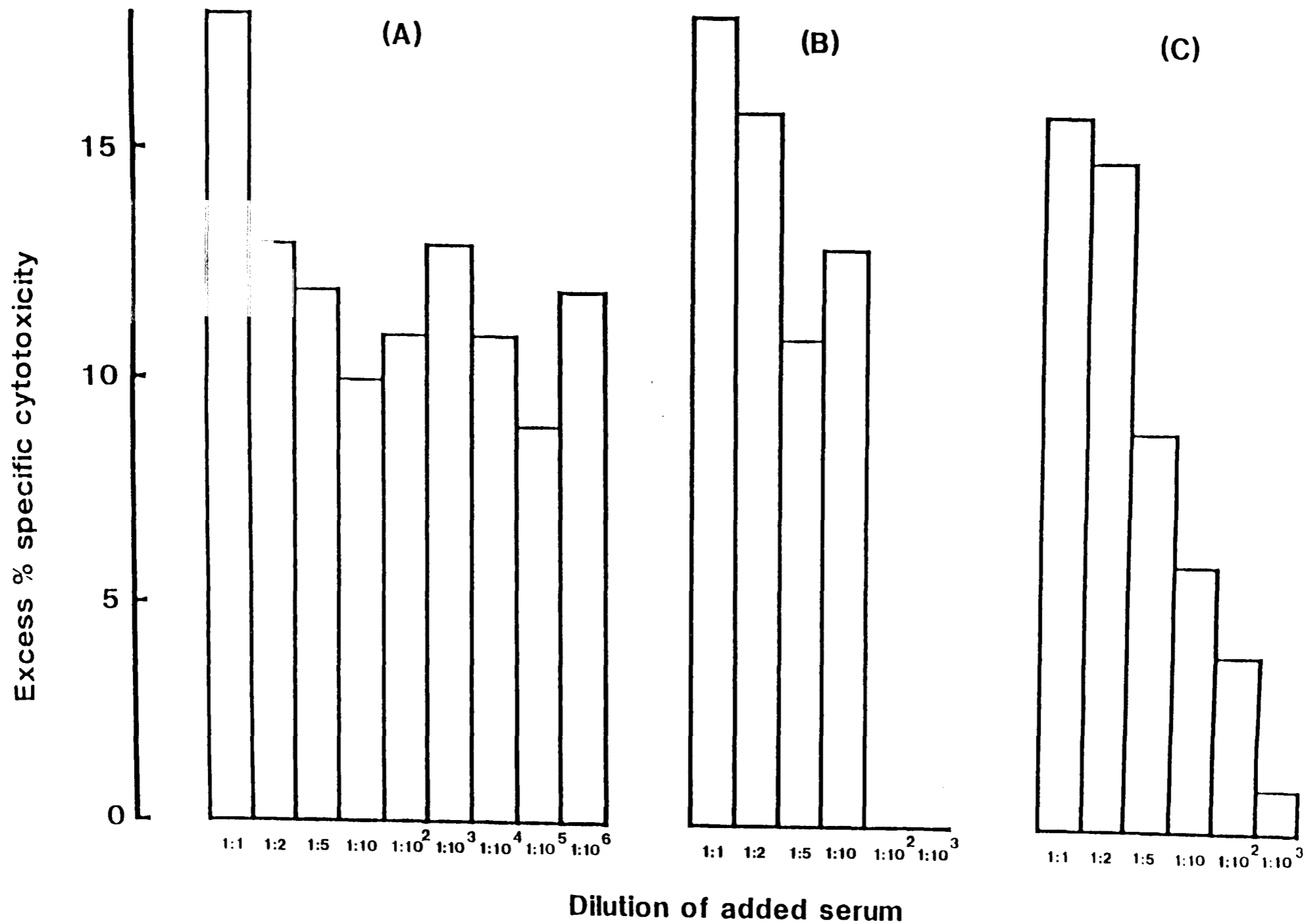


Figure 8

The effect of serum dilution, on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and sera from 1 patient with SLE (a), and 2 patients with systemic sclerosis (b,c).

Comment

Enhanced cytotoxicity towards cultured human umbilical vein endothelial cells following co-culture with serum and peripheral blood mononuclear cells was observed with serum dilutions as low as $1/10^6$. This observation is consistent with the view that the mechanism of cytotoxicity occurring in co-culture experiments is ADCC since very low titres of antibody are sufficient to produce this type of cytotoxicity. In addition, these results indicate variable titres of the antibody mediating ADCC of endothelial cells, which could be responsible for the fluctuating results obtained in the serial studies.

3.3.2.7 Effect of depletion of effector cell populations for use in co-culture studies with human umbilical vein endothelial cells

Adherence to plastic

The effect of the plastic adherence depletion of effector cells on "excess % specific cytotoxicity" following co-culture with sera from patients with systemic sclerosis and cultured human umbilical vein endothelial cells is shown in fig 9. An enhanced cytotoxicity was observed in 6 of the 7 cases.

Comment

The enhanced cytotoxicity observed in these 6 cases implied the enrichment of the remaining population of effector cells following adherent cell depletion, and indicate that the population of effector cells mediating cytotoxicity against human umbilical vein endothelial cells was non-adherent.

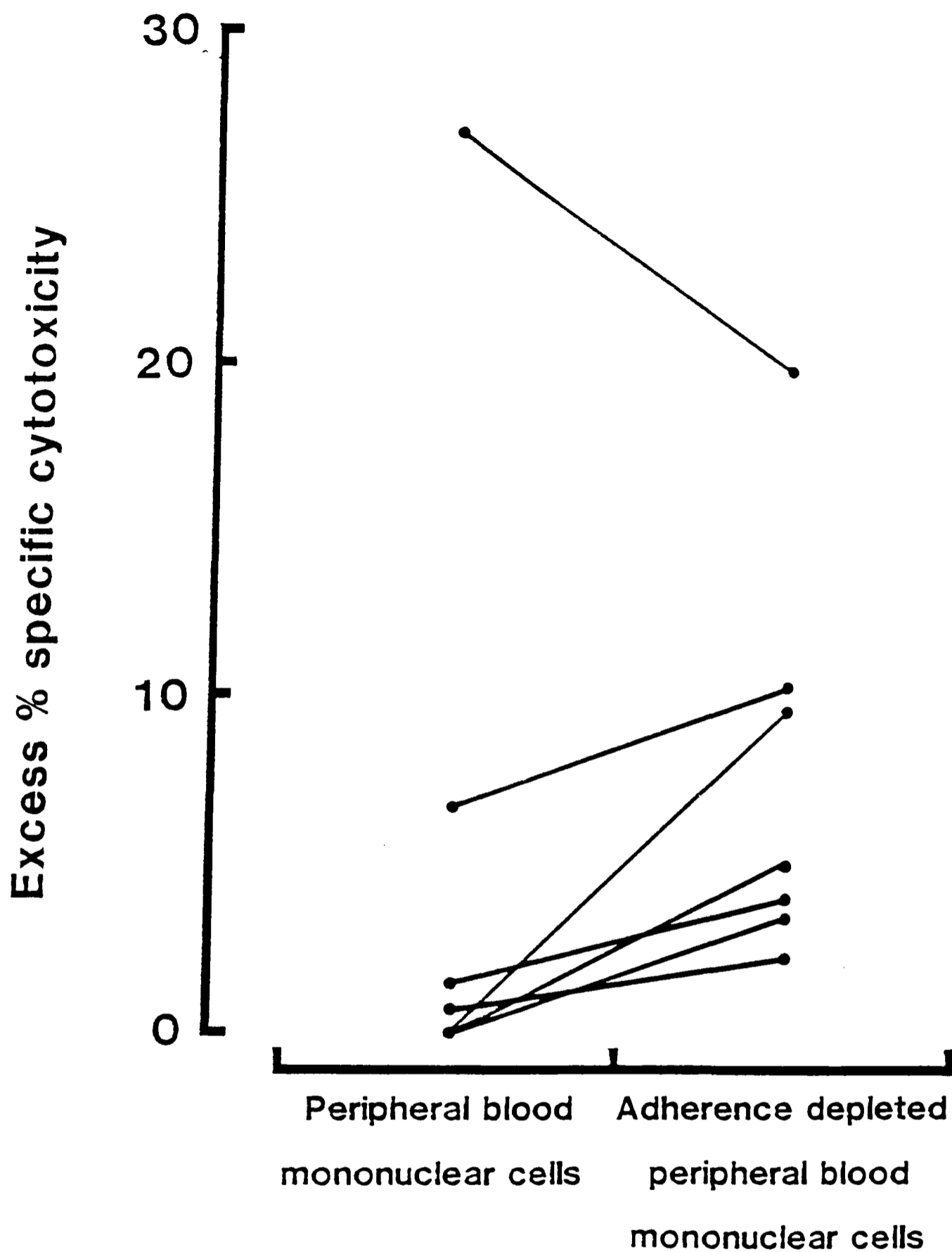


Figure 9

The effect of adherent cell depletion on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and sera from 7 patients with systemic sclerosis.

Treatment with carbonyl iron powder

The effect of depletion of peripheral blood mononuclear cells by treatment with carbonyl iron powder on "excess % specific cytotoxicity" following co-culture with sera derived from patients with systemic sclerosis is illustrated in fig 10. In all 3 cases, enhanced cytotoxicity was observed.

Comment

These results again suggested that the population of effector cells causing cytotoxicity was non-adherent and also non-phagocytic.

3.3.2.8 Effect of pure monocytes as effector cells co-culture studies with human umbilical vein endothelial cells

When monocytes were isolated, and used at a concentration of 2.5×10^4 cells / well, as effector cells in co-culture studies with sera from patients with systemic sclerosis and human umbilical vein endothelial cells, the "excess % specific cytotoxicity" was zero for each sera examined (see fig 11).

Comment

These results confirmed those of the adherence depletion and phagocytosis studies, and provided firm evidence that monocytes were not the responsible effector cells mediating cytotoxicity towards human umbilical vein endothelial cells in co-culture studies.

3.3.2.9 Effect of column chromatography of sera for use in co-culture studies with human umbilical vein endothelial cells

The results of ULTROGEL fractionation of sera capable of inducing enhanced cytotoxicity of human umbilical vein endothelial cells following co-culture with normal human peripheral blood mononuclear cells are shown in figs 12, 13, 14, 15 & 16. Sera from 4 patients with systemic sclerosis and from one patient with SLE (Fig 15) were fractionated and 10 pooled fractions collected. The IgG levels were

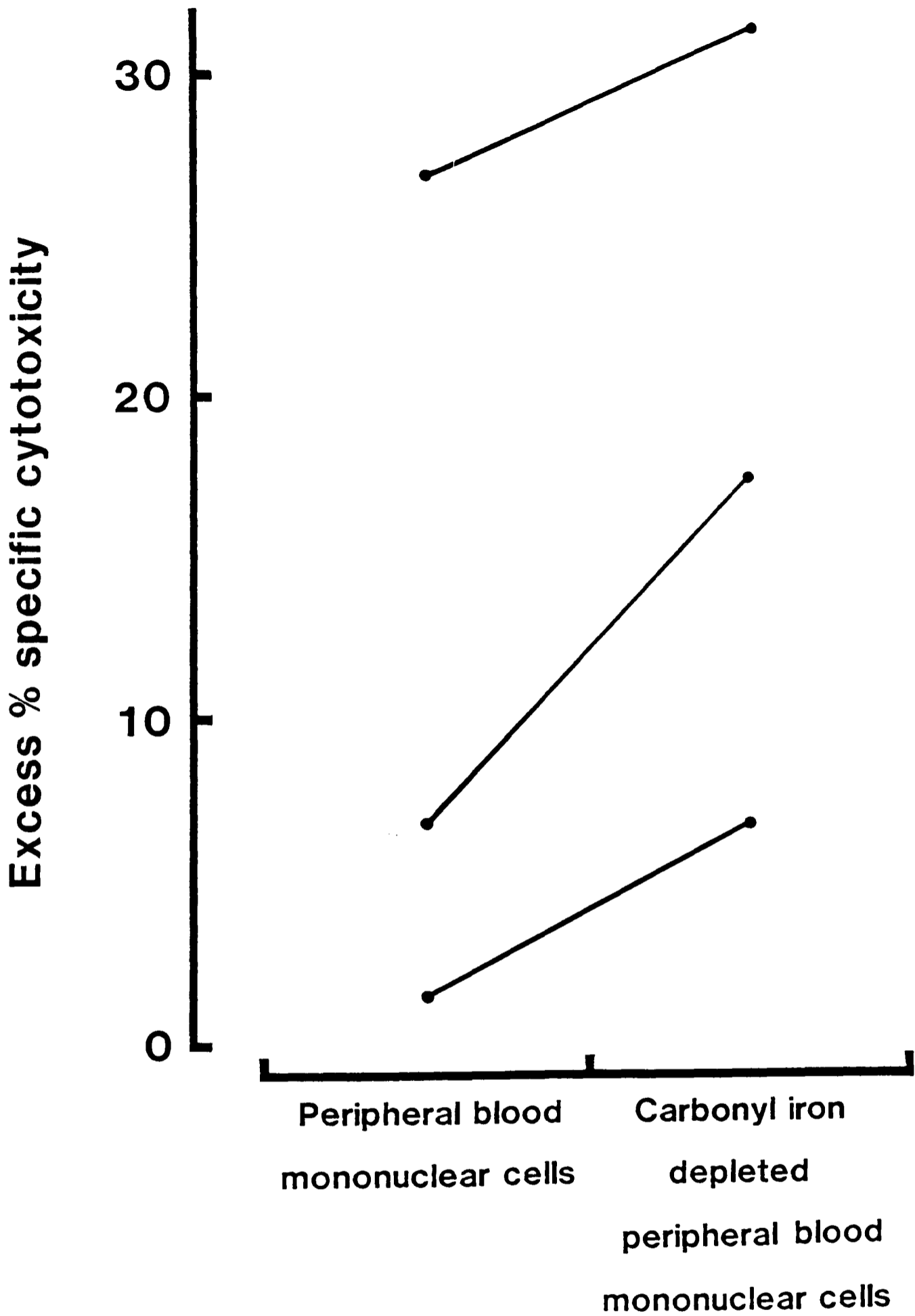


Figure 10

The effect of carbonyl iron depletion on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and sera from 3 patients with systemic sclerosis.

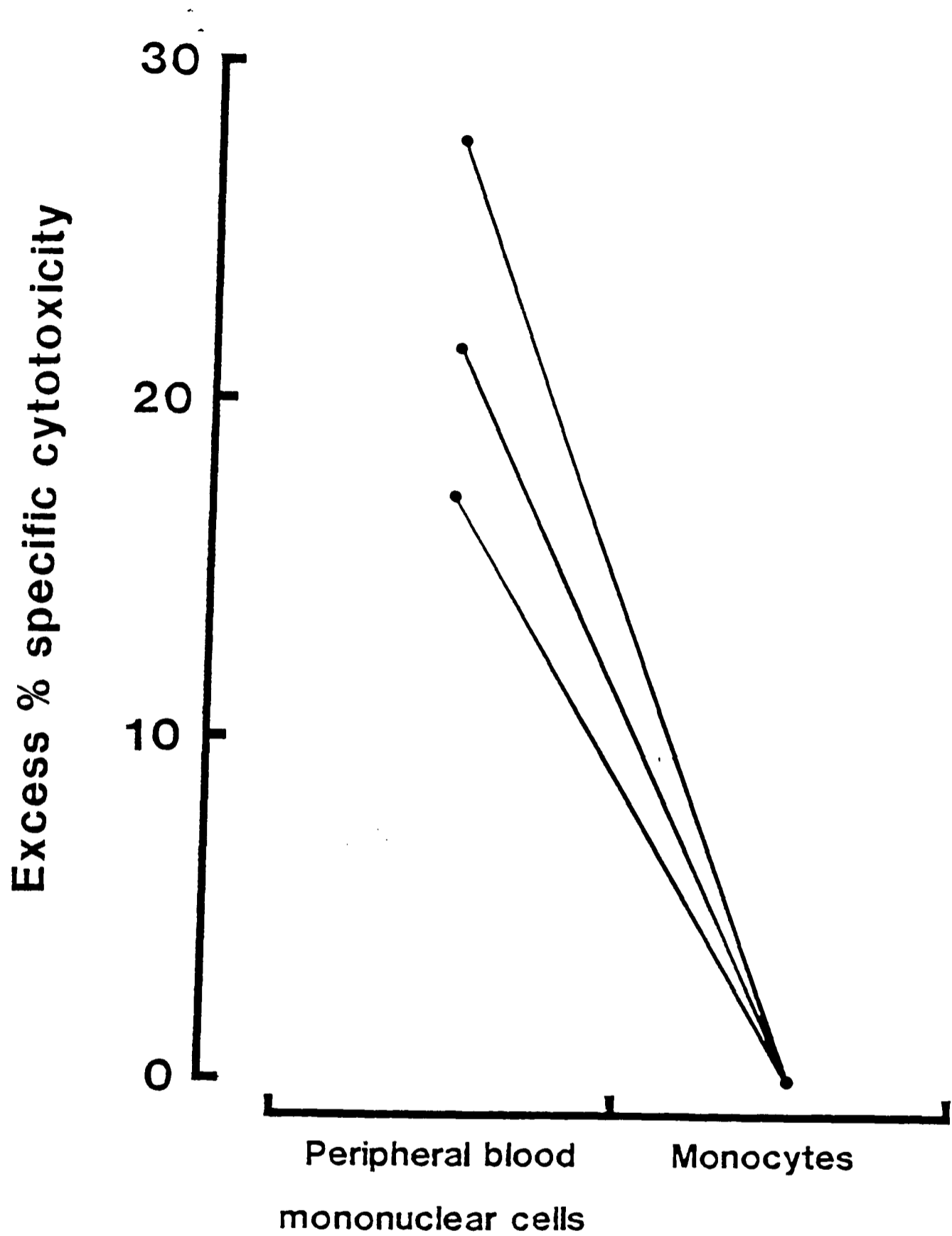


Figure 11

The effect of pure monocytes as effector cells, on the excess % specific cytotoxicity of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and sera from 3 patients with systemic sclerosis.

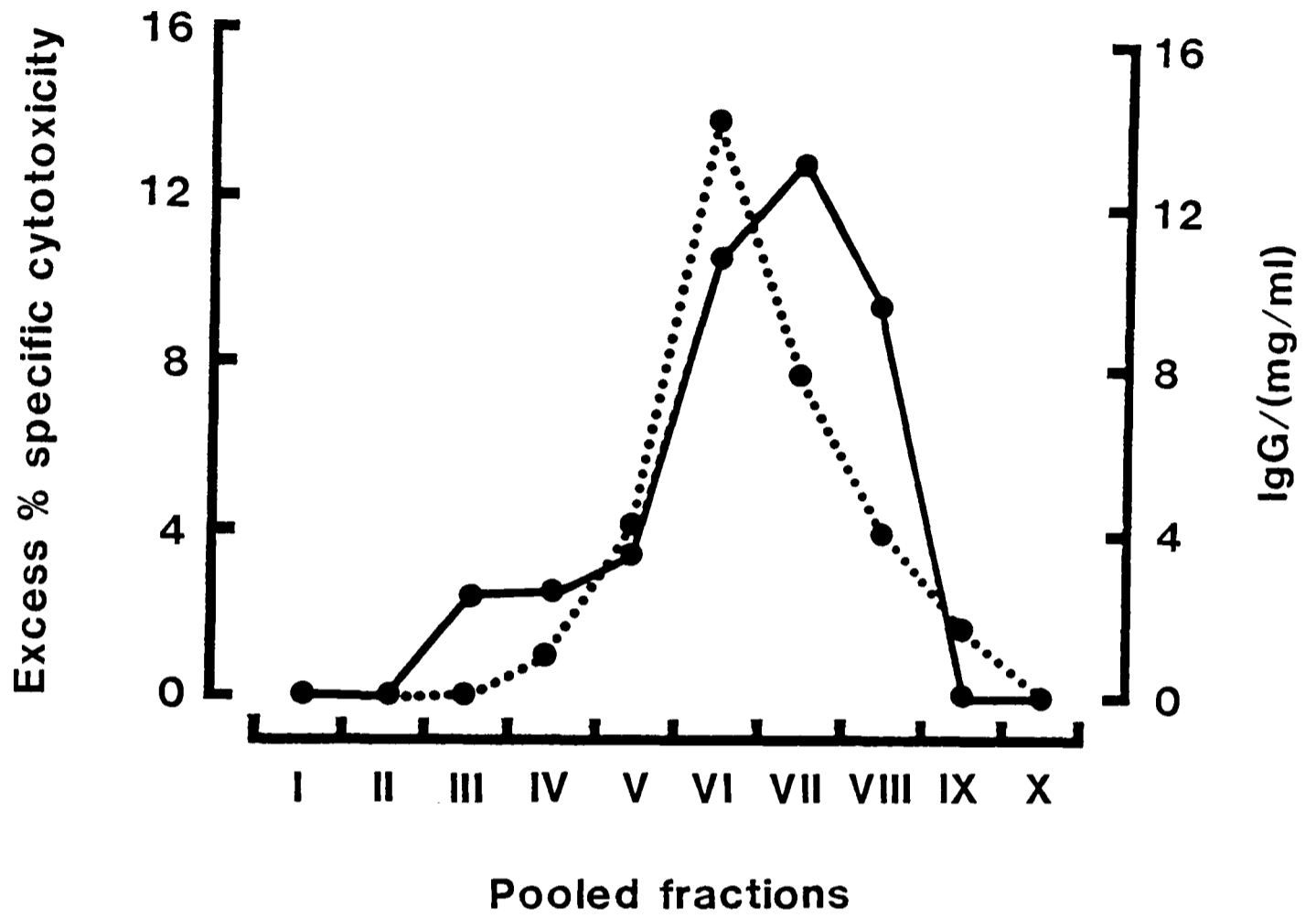


Figure 12

Excess % specific cytotoxicity (—) of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and pooled ULTROGEL fractions of sera from a patient with systemic sclerosis. The IgG content of each fraction is also illustrated (..).

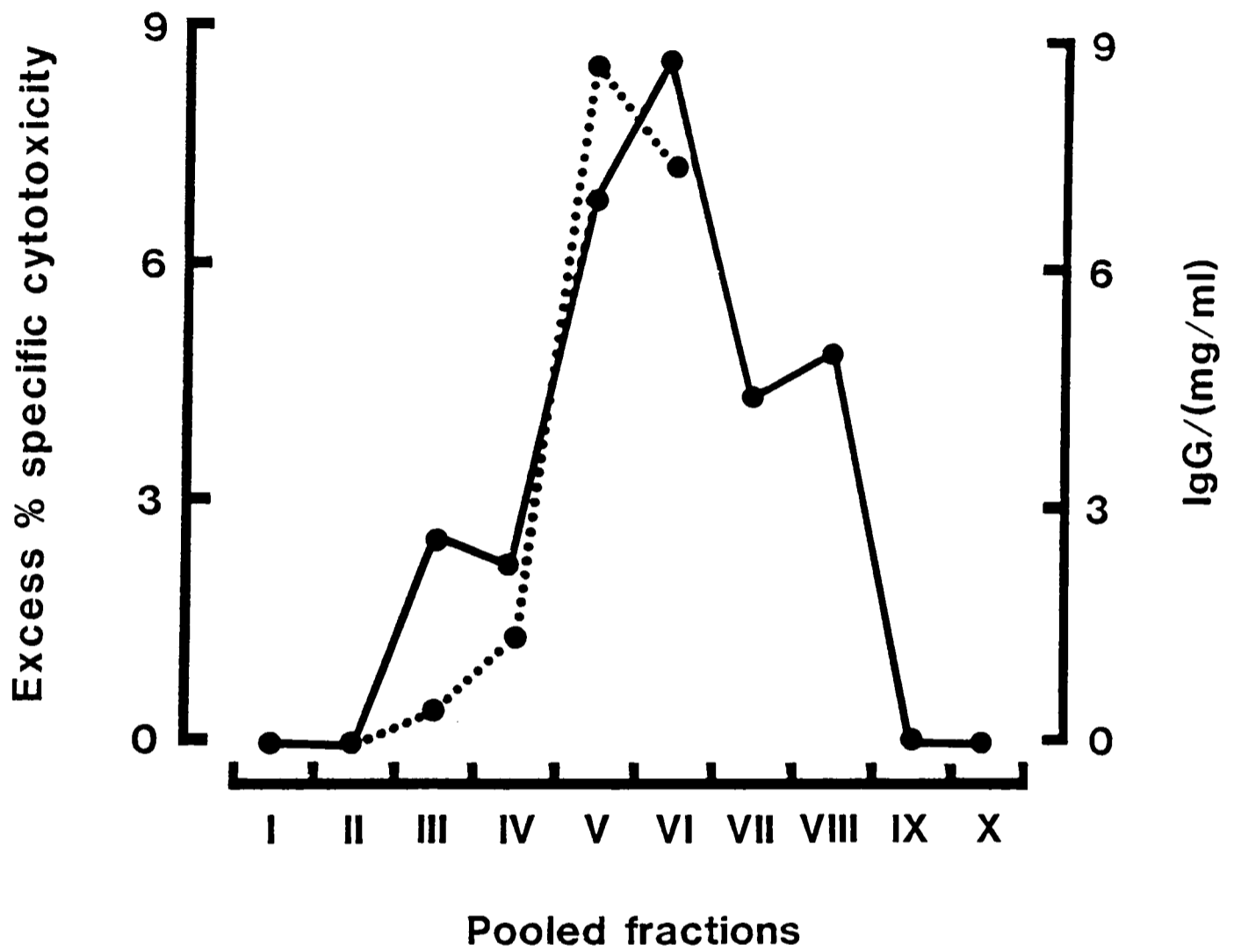


Figure 13

Excess % specific cytotoxicity (—) of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and pooled ULTROGEL fractions of sera from a patient with systemic sclerosis. The IgG content of each fraction is also illustrated (..).

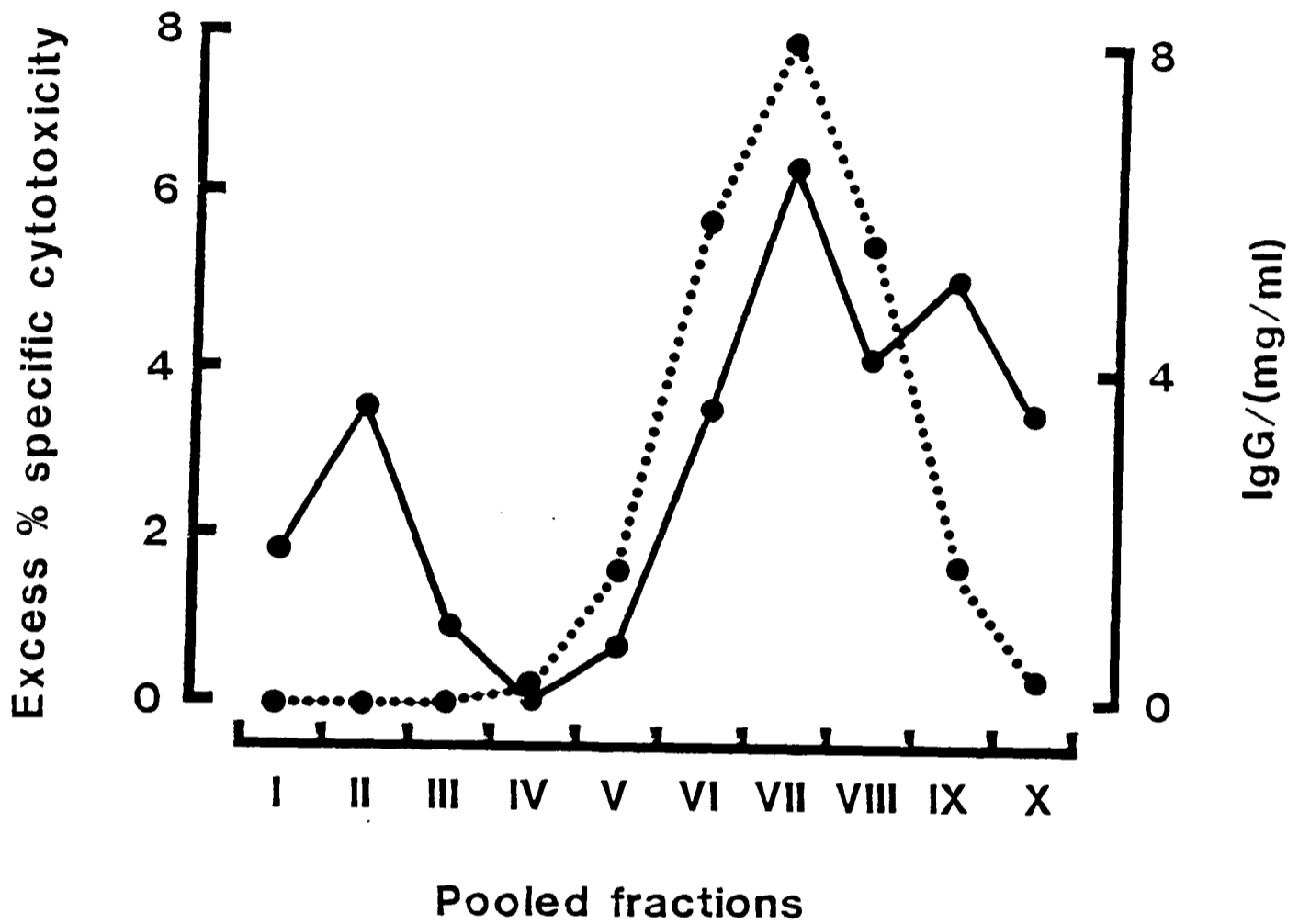


Figure 14

Excess % specific cytotoxicity (—) of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and pooled ULTROGEL fractions of sera from a patient with systemic sclerosis. The IgG content of each fraction is also illustrated (...).

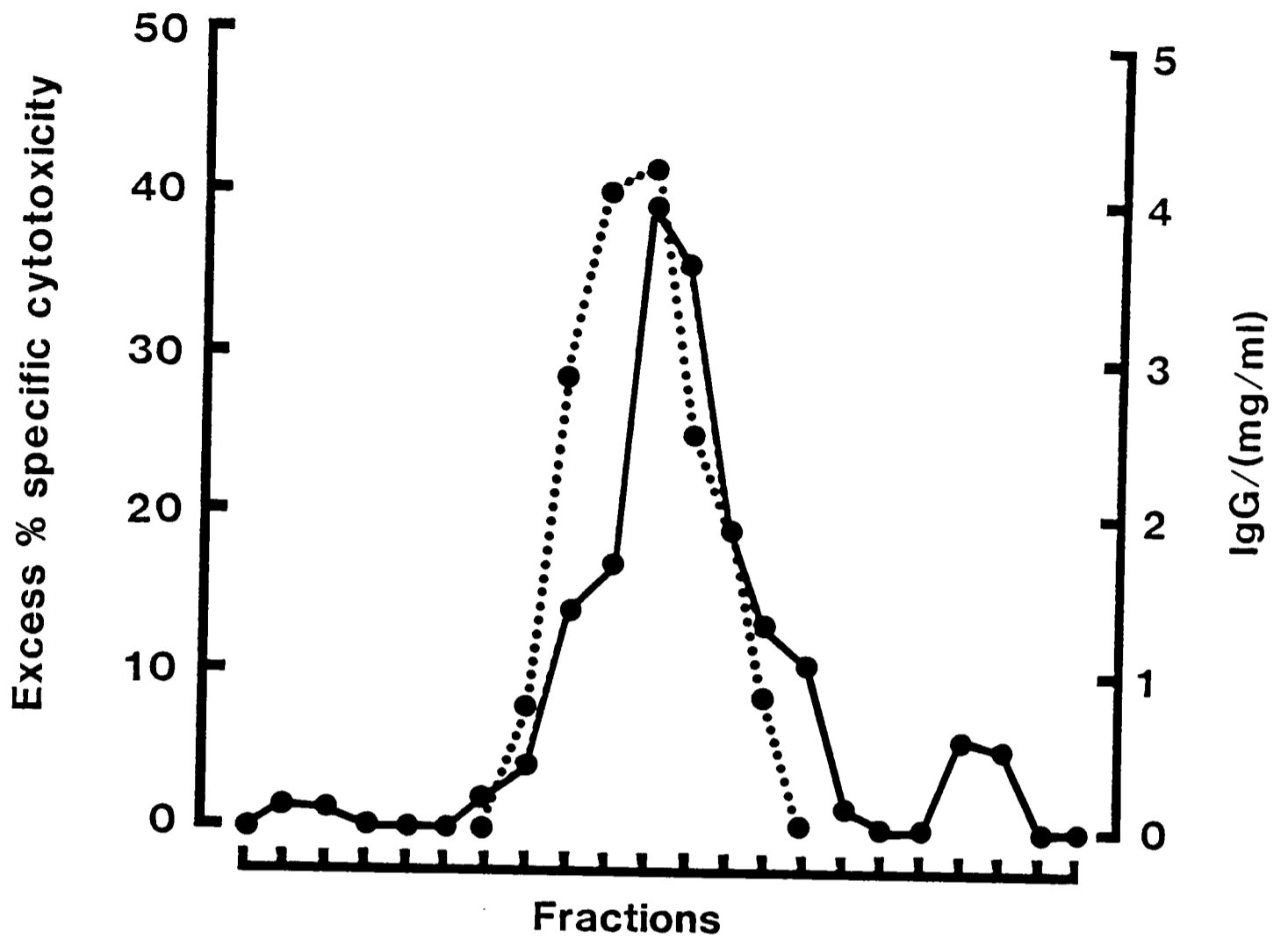


Figure 15

Excess % specific cytotoxicity (—) of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and pooled ULTROGEL fractions of sera from a patient with systemic sclerosis. The IgG content of each fraction is also illustrated (...).

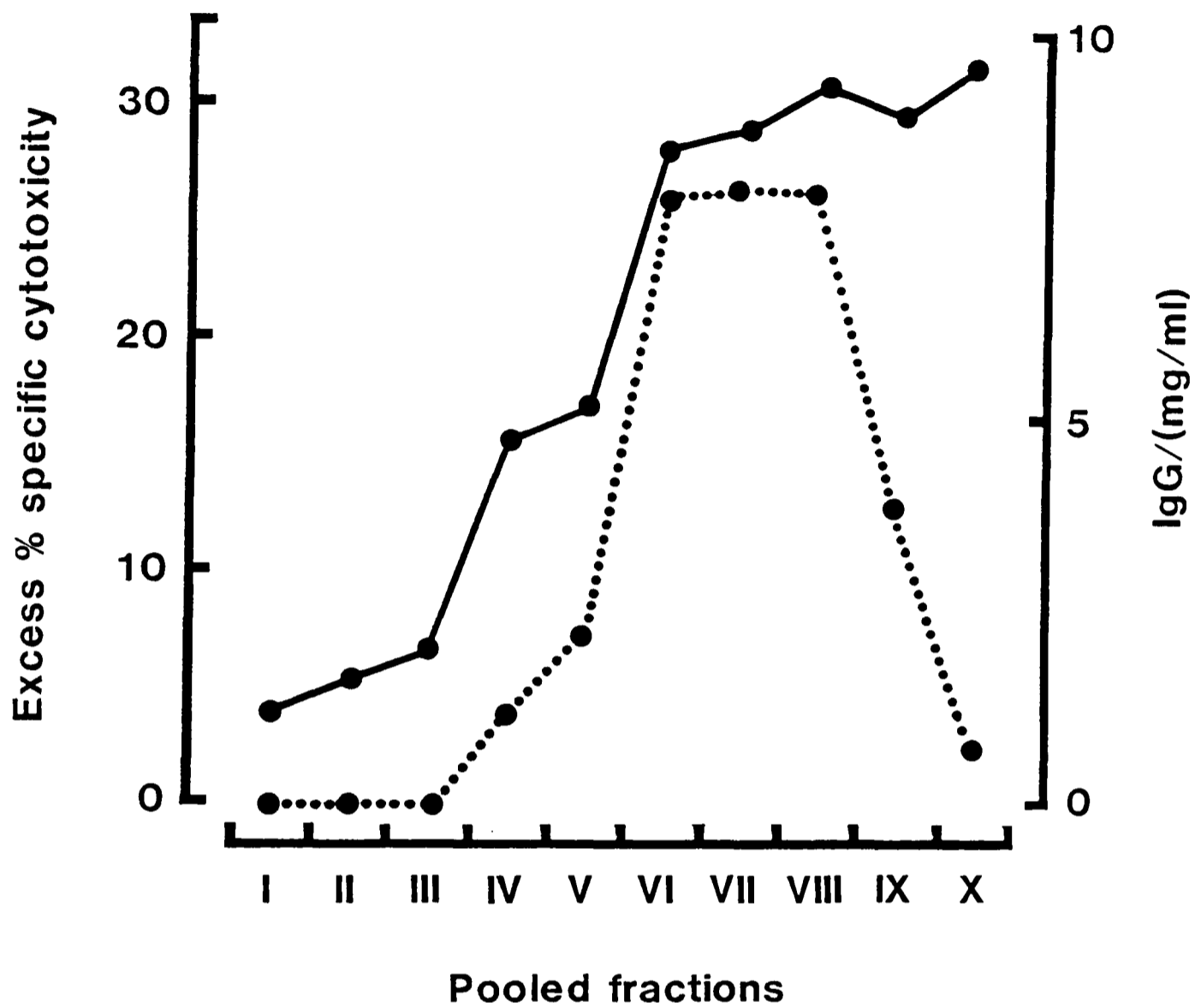


Figure 16

Excess % specific cytotoxicity (—) of human umbilical vein endothelial cells produced by co-culture with normal peripheral blood mononuclear cells and pooled ULTROGEL fractions of sera from a patient with systemic sclerosis. The IgG content of each fraction is also illustrated (...).

determined for each fraction and these are also illustrated in figs 12-16. The "excess % specific cytotoxicity" produced by the fractions correlated with the IgG levels present in these fractions for 4 of the 5 fractionated sera. In one case, the elevated "excess % specific cytotoxicity" continued in fractions beyond the peak of IgG (Fig 16). Further fractionation of different bleeds obtained from this patient were carried out and the same effect was observed on 3 separate occasions. (This serum was the only one capable of sensitizing effector cells in pre-incubation studies.) In addition to the fractionation of patient sera, serum samples obtained from normal controls were also fractionated in the same way. None of these fractions produced ADCC of endothelial cells.

Comment

ULTROGEL fractionation of sera capable of inducing cytotoxicity against human umbilical vein endothelial cells demonstrated that the cytotoxicity-inducing activity paralleled the IgG content of fractions and was consistent with the idea that the factor was an antibody directed against the vascular endothelium.

3.3.2.10 Effect of IgG preparations in co-culture studies with human umbilical vein endothelial cells

In addition to the fractionation of sera using ULTROGEL column chromatography, IgG was also prepared, by DEAE-sepharose and staphylococcal protein A column chromatography, and the resulting samples of IgG were tested in co-culture studies with human umbilical vein endothelial target cells. The results obtained are shown in table 5.

As illustrated, 7 of 10 patients who had previously exhibited enhanced cytotoxicity of human umbilical vein endothelial cells, also demonstrated cytotoxicity by IgG preparations obtained by DEAE

Table 5

The effect, on endothelial cytotoxicity, of IgG prepared by DEAE sepharose and Staphylococcal protein A column chromatography, from sera of patients with systemic sclerosis capable of producing ADCC of human umbilical vein endothelial cells.

Patient	DEAE	Protein A	Co-culture (serum & effector cells)
1	-		+
2	+	+	+
3	+		+
4	+		+
5	+	+	+
6	-	-	+
7	+		+
8	+		+
9	-		+
10	+		+

(+) = "Excess % specific cytotoxicity" > 10

(-) = "Excess % specific cytotoxicity" < 10

separation. 3 sera were also separated on a protein A column and the IgG obtained tested in co-culture experiments. 2 of the 3 showed "excess % specific cytotoxicity of >10%. IgG preparations were also prepared from 9 normal control sera, and these all failed to produce ADCC of cultured endothelial cells.

Comment

These results provided further evidence that the serum factor causing enhanced cytotoxicity of human umbilical vein endothelial cells in co-culture with peripheral blood mononuclear cells was an antibody of the IgG class.

3.4 Discussion

3.4.1 Basic parameters of the assays

3.4.1.1 Baseline and maximal ⁵¹chromium release

The spontaneous release of ⁵¹chromium from labelled cells in vitro is known as the baseline ⁵¹chromium release. It is mainly the result of target cell death occurring during the incubation period. The rate of ⁵¹chromium release occurring spontaneously varies considerably with different cell types, and target cells showing high baseline values may not be used in such assays. The mean baseline release from human umbilical vein endothelial cells observed in this study was 24.37% over the 18 hour incubation period. Variation was observed between individual assays with extreme values of 16.59% and 32.96%. This variation is probably due to the robustness and variability of endothelial cell cultures derived from different donors. Clearly, the number of target cells spontaneously dying over 18 hours may vary from culture to culture.

Values for baseline release reported by other authors compare favorably with the value obtained in this study, and values of spontaneous ⁵¹chromium release of 15-30% have been previously been described (Perlmann and Holm 1969).

The mean value obtained in this study for maximal ⁵¹chromium release was 94.48%. This figure represents the total amount of ⁵¹chromium released from targets following cellular lysis. A value <100% is usually obtained due to the fact that a small amount of label remains bound to cell debris following lysis, and hence is not released into the supernatant (Heberman et al 1976). Previously reported values for maximal release of ⁵¹chromium from dead cells are in the range of 80-95% (Wigzell 1965; Holm and Perlmann 1967)

3.4.1.2 Spontaneous cell mediated cytotoxicity

Spontaneous cell mediated cytotoxicity is the process of destruction of target cells by normal lymphocytes in the absence of any sensitizing agent. The responsible effector cells are natural killer (NK) cells, ie cells which can spontaneously kill certain susceptible target cells in a manner which is not MHC restricted. NK cells possess receptors for the Fc region of IgG and hence may also be capable of mediating ADCC. In fact NK cells and K cells may consist of the same, or an overlapping sub-population of cells within the peripheral blood mononuclear cell population.

The specificity of lysis by NK cells is demonstrated by the fact that some targets are resistant to killing by NK cells. It is not known, however, whether susceptible cells share a common determinant or whether multiple subsets of NK cells exist, all with different target cell specificities.

In this study, the mean value obtained for spontaneous cell mediated cytotoxicity towards cultured human umbilical vein endothelial cells was 13.16% specific cytotoxicity. Although in general there was good correlation in the levels of spontaneous cytotoxicity between assays (as illustrated by a SEM of 0.59%) occasional large fluctuations were observed. The reasons for these variations is uncertain. However, one possible explanation for this variability could be that altered ratios of different subpopulations of effector cells are isolated on each occasion. Each assay was performed on endothelial cells derived from a different umbilical cord. It is also possible, therefore, that variations in the susceptibility to lysis of endothelial cells obtained in this way, may be an additional factor accounting for the wide range of values obtained for spontaneous cell mediated cytotoxicity. A particular determinant on the target cell

surface may determine whether cellular lysis by NK cells occurs. Thus, the absence or presence of such a determinant, or its altered frequency or distribution, may result in the exhibition of differing susceptibilities to lysis of endothelial cells obtained from different donors.

3.4.1.3 Effector cell donor variation

The spontaneous cell mediated cytotoxicity observed with effector cells obtained from different donors, showed little variation. The observed variation may be due to higher numbers of competent cytotoxicity mediating cells occurring in the effector cell population. In order to eliminate any variation arising from different effector cell donors, a single donor (CH) was used for all routine studies.

3.4.1.4 Effector cell to target cell ratio

The number of cells capable of mediating ADCC within the mononuclear population is likely to be very small (MacLennan et al 1976). Hence there is a requirement for an excess of effector cells in order that significant target cell lysis may occur. To determine the optimal number of peripheral blood mononuclear cells to use in cytotoxicity assays, ADCC produced by varying numbers of effector cells, in combination with a standard number of target cells, was assessed.

The results obtained showed that, for a given number of target cells, ADCC increased as the number of effector cells increased, an effect which began to plateau when the number of peripheral blood mononuclear cells per well was 5×10^5 .

Maximal ADCC was observed when the number of peripheral blood mononuclear cells per well was $5-10 \times 10^5$. The yield of peripheral blood mononuclear cells obtained in these studies prohibited the use of this amount of effector cells and so, as a compromise, the number of effector cells used was 2.5×10^5 per well.

3.4.1.5 Intra and inter assay variation

In the present study, the intra-assay variation observed between triplicate assay wells was relatively low and the values obtained for baseline $^{51}\text{chromium}$ release were relatively consistent between successive assays. Hence, cytotoxicity assays were performed with reproducible accuracy. However, some what greater levels of variation were observed between values of spontaneous cell mediated cytotoxicity which were obtained in assays performed on separate occasions. In addition, the levels of ADCC obtained for a particular serum sample tested on separate occasions was variable. This type of variation is not due to any controllable factor, and is an inherent problem in cytotoxicity assays of this nature.

A major source of this inherent variability could be the number of competent effector cells present in any particular sample. A different composition in the effector cell population may arise from alterations in the way in which peripheral blood mononuclear cells recirculate between the blood and tissues, in vivo. Blood collected on different occasions may contain different amounts of the various subpopulations of effector cells (Frietas & Desousa 1976). In addition to the composition of the effector cell population, and the nature of the cells present, the activation state of these cells may be important in determining the level of spontaneous cell mediated cytotoxicity (Ralph 1984).

The various values obtained for the "excess % specific cytotoxicity" following co-culture of target and effector cells and an individual serum sample may also be a result of the different composition of effector cell populations. The number and distribution of Fc receptors may be another important factor, as discussed earlier in the introduction to this chapter.

Target cell heterogeneity could be another factor responsible for this inherent variability. The variation in values obtained for spontaneous cell mediated cytotoxicity may be explained by differing frequencies of the target cell surface determinants responsible for inducing NK cell mediated cytotoxicity. Target cell heterogeneity could also be an important factor in determining levels of ADCC. Cell surface antigens involved in the binding of specific antibody, may be present at different densities on human umbilical vein endothelial cells obtained from different umbilical cords. Such a variation in specific antigen densities could be yet another important factor accounting for the variable levels of ADCC observed.

3.4.2 Clinical studies

3.4.2.1 Co-culture studies

Investigations concerning the possibility of ADCC as a pathogenic mechanism in human autoimmune diseases remains limited. The probable reason for the small number of such studies is the complex nature of ADCC assays, which require the co-culture of two separate populations of cells. However, IgG from patients with rheumatoid arthritis, capable of enhancing cytotoxicity towards Chang liver cells in co-culture studies has been described (MacLennan et al 1969; Panayi 1976). In addition, antilymphocytic antibodies from patients with SLE have been shown to induce ADCC (Kumagi et al 1981). Penning et al

(1984a) described a serum factor in 18% of patients with systemic sclerosis and 45% of patients with SLE, that was capable of enhancing the cytotoxicity of a variety of established human target cells when co-cultured with normal human peripheral blood mononuclear cells.

More relevant to what may occur in vivo, however, is the use of primary cultures of vascular endothelial cells as targets. The endothelium is in intimate contact with both the sensitizing antibody and circulating effector K cells, and thus could readily become a target in vivo. Moreover, the use of vascular endothelium as a target in ADCC assays is particularly relevant in systemic sclerosis, since vascular lesions are a major feature of the disease.

In this study, endothelial cells were isolated and grown in vitro for use as target cells in cytotoxicity assays. Serum from approximately 20% of patients with systemic sclerosis showed enhanced cytotoxicity of both venous and arterial endothelium, following co-culture with normal human peripheral blood mononuclear cells. In addition, 14% of serum from patients with SLE showed a similar enhancement of cytotoxicity against human umbilical vein endothelial cells.

Similar results were obtained by Penning et al (1984a, 1984b, 1985) who showed enhanced cytotoxicity of human umbilical vein endothelial cells following co-culture with peripheral blood mononuclear cells by sera obtained from 25% and 9% of patients with systemic sclerosis and SLE respectively.

The present study has established, through the use of IgG preparations obtained by the methods of ion exchange and affinity chromatography, that IgG is the serum factor responsible for mediating this ADCC of vascular endothelial cells. Further confirmation was provided by column chromatography which revealed that the responsible

serum factors causing cytotoxicity were confined to IgG containing fractions. However, the fractionation of serum obtained from one patient showed that cytotoxicity continued beyond the IgG containing fractions. In this patient it is possible that IgG fragments may also be responsible for mediating ADCC. It is interesting to note, that serum obtained from this patient was the only serum which caused cytotoxicity following preincubation of effector cells. In all other sera examined, no such effect was observed. A possible explanation for this effect is that immunoglobulin fragments may be capable of "arming" K cells in a manner similar to that described by Greenberg & Shen (1973). Alternatively, a non-immunoglobulin serum component, such as a cytokine, may have caused the activation of peripheral blood mononuclear cells during the preincubation period.

The majority of sera which demonstrated ADCC of cultured human umbilical vein endothelial cells, were capable of binding to target cells, and enhanced cytotoxicity of these cells was observed following preincubation of the target cell monolayer. These results indicate that the IgG capable of mediating ADCC, binds to the target cell prior to the occurrence of cellular lysis. This evidence supports the idea that the serum factor is an antibody directed against the vascular endothelium. Antiendothelial cell antibodies have previously been described by alternative methods, such as ELISA, and have been demonstrated in patients with systemic sclerosis, with an incidence similar to that of the ADCC found in this study (Hashemi et al 1987; Baguley et al 1987a; Byron et al 1987). It thus seems probable that ADCC of human umbilical vein endothelial cells demonstrated in this study was mediated by antiendothelial cell antibodies.

In addition to cytotoxicity towards cultured human umbilical vein endothelial cells, this study has also demonstrated a similar cytotoxicity towards human umbilical arterial endothelial cells. Sera obtained from patients with both systemic sclerosis and SLE, that produced enhanced cytotoxicity of venous endothelial cells, also caused enhanced cytotoxicity of arterial endothelial cells. It has been suggested that endothelial cells of venous and arterial origin exhibit different cell surface molecules. However, the antiendothelial cell antibody that is present in the sera of patients with systemic sclerosis and SLE and which mediates ADCC must, on the evidence of these results, be directed against an antigen common to the surface of both venous and arterial human umbilical endothelium.

The demonstration of ADCC against arterial target cells is of major pathological significance, as the characteristic vascular lesion of systemic sclerosis principally affects arteries. Although the observations made in this study occurred in vitro, it is also possible that a similar cytotoxic mechanism could also occur in vivo, if arterial endothelial cells exhibit the same antigenic determinants as the cultured endothelial cells used in this study. The vascular defects which are a common feature of systemic sclerosis could thus be explained by an antibody directed against the vascular endothelium, which is capable of binding to endothelial cells. K cells could then mediate endothelial cell lysis by binding to antibody via their Fc receptors, and thus contribute to the development of the vascular lesions which are characteristic of the disorder.

3.4.2.2 Direct serum induced cytotoxicity of human umbilical endothelial cells

One of the pathogenic mechanisms that was initially proposed for systemic sclerosis was the presence of a circulating factor directly cytotoxic for vascular endothelium. The first investigators to describe such a circulating endothelial cell specific cytotoxic factor were Kahaleh et al (1978, 1979) who reported that sera from 60% of patients with systemic sclerosis caused diminished ^3H thymidine incorporation by cultured human umbilical vein endothelial cells. Cohen et al (1983) later reported a similar cytotoxic factor in the sera of patients with systemic sclerosis, but they failed to show specificity towards endothelial cells. Shanahan and Korn (1982) also showed a lack of target cell specificity and in addition, they found cytotoxic activity in sera from patients with other connective tissue diseases, an observation which was also reported by Meyer et al (1983).

In contrast to these reports, this study has failed to demonstrate any direct serum induced cytotoxicity of endothelial cells by sera from patients with systemic sclerosis. This observation is, however, supported by the work of Summers et al (1984) who also failed to demonstrate the presence of an endothelial cytotoxic factor in sera obtained from patients with systemic sclerosis.

The conflicting observations reported in the present study may be explained by the different types of assays used in the assessment of cytotoxicity. The work of Kahaleh et al (1978, 1979); Cohen et al (1983) and Shanahan and Korn (1982) was carried out using ^3H thymidine incorporation studies, whereas the experiments performed in this study involved ^{51}Cr chromium release assays for the assessment of endothelial cell cytotoxicity. The former assay gives an indication of cell

growth by measuring DNA synthesis, whereas ^{51}Cr chromium release assays measure cellular lysis. It is possible that the earlier reports in fact described an endothelial cell growth inhibitory factor as opposed to a cytotoxic factor. Penning (1984a) also demonstrated the absence of an endothelial cytotoxic factor in the sera of patients with systemic sclerosis as assessed by ^{51}Cr chromium release. However when these sera were tested in ^3H thymidine uptake studies, inhibition of uptake was revealed.

Variations in the patient subpopulations studied may be another factor explaining the conflicting reports on direct serum induced endothelial cell cytotoxicity. Previous investigators have shown an incidence of serum cytotoxic factor in patients with systemic sclerosis that has varied from 60% to 7% (Kahaleh et al 1979; Shanahan and Korn 1982; Cohen et al 1983). It is possible therefore, that different sub-populations of patients, showing differing degrees of severity of disease, may be a cause for the varying and conflicting incidence of this serum induced cytotoxicity. Finally, it has been proposed that the endothelial cell cytotoxic factor may be an artifact produced by oxidation occurring during the storage of serum (Blake et al 1985); an explanation that would also explain the conflicting results obtained in the earlier studies. All serum samples obtained for the present study were treated in a standard manner, stored at -80°C and thawed samples were not reused. In addition, all new serum samples were tested prior to long periods of storage to minimize the development of oxidative artifacts.

3.4.2.3 Serum titration studies

One of the features characteristic of ADCC is the fact that very low titres of antibody are capable of producing this type of cytotoxicity. The amount of antibody capable of mediating ADCC is much lower than that required to produce complement mediated lysis (Perlmann & Holm 1969; Perlmann & Perlmann 1970).

Serum dilutions were carried out in the present study and 1:2; 1:10; and 1:10⁶ were the lowest serum dilutions capable of mediating cytotoxicity by the 3 different sera investigated. The varying titres of serum required for ADCC to occur with samples derived from patients, may be due to different amounts of specific antibody present in the serum sample. In addition antibody affinity may be important. High affinity antibody may allow greater numbers of effector cells to participate in ADCC, since more antibody will bind to antigenic determinants on target cells, and hence more Fc sites will be available for effector cell binding. Differences in affinity may be due to different antibody subclasses taking part in the ADCC reaction, and the involvement of different Fc receptors which show varying binding affinities for IgG (Hogg, 1988).

However, the amount of serum required to mediate ADCC was low, providing further evidence that the mode of cytotoxicity is probably ADCC. In addition, heat inactivation of test serum did not cause a decrease in ADCC (data not presented) thus further eliminating the possibility of complement mediated lysis.

3.4.2.4 Effector cell populations

The nature of the effector cell mediating ADCC may be variable. It appears that the nature of the target cell, and the sensitizing antibody are important factors, as discussed in the introduction to this chapter. The major effector cell capable of mediating ADCC is

thought to be the K cell, an Fc receptor bearing, non-thymus dependent lymphocyte (Bean et al 1976). However, monocytes, polymorphonuclear neutrophils and macrophages have also been shown to mediate ADCC in some in vitro systems (Richter et al 1983; Shen et al 1987; Ralph 1984; Johnson et al 1985). In this study, depletion studies were performed in an attempt to determine the nature of the effector cells mediating the ADCC of vascular endothelium. Preparations of peripheral blood mononuclear cells were depleted by plastic adherence, or phagocytosis of carbonyl iron, and the remaining purified cell preparations were then used in cytotoxicity assays.

Following the adherent cell depletion of peripheral blood mononuclear cells by incubation on plastic petri dishes, a rise in ADCC was observed. This effect was also seen following treatment of peripheral blood mononuclear cells with carbonyl iron. These results indicate that the effector cells responsible for mediating the ADCC described in this study, were unlikely to be macrophages or monocytes.

In order to obtain further proof that monocytes were not capable of mediating ADCC, pure preparations of this cell type were isolated from a suspension of peripheral blood mononuclear cells. These were then used as effector cells in ADCC assays. No cytotoxicity was detected, adding further evidence that monocytes were not the effector cells required for ADCC to occur in the assay system under observation. In addition, pure polymorphonuclear neutrophils were isolated and in preliminary experiments, these cells also failed to produce ADCC of cultured endothelial cells (data not presented).

Hence the effector cells responsible for mediating the ADCC of vascular endothelium, which have previously been described as non-B, non-T and to possess Fc receptors (Penning 1984a), are neither plastic adherent nor phagocytic, and are unlikely, therefore, to be either monocytes or macrophages.

CHAPTER 4

PROSTACYCLIN SYNTHESIS BY HUMAN
UMBILICAL VEIN ENDOTHELIAL CELLS

4.1 Introduction

4.1.1 The discovery of prostacyclin

In 1975 Moncada and colleagues discovered a substance in the vessel wall which they called PGX and which was later termed prostacyclin or PGI₂ (Moncada et al 1976a). These workers discovered that this compound relaxed vascular strips in vitro and acted as a very potent inhibitor of platelet aggregation. Further characterization of this compound showed that it caused vasodilation in vivo, was the most potent inhibitor of platelet aggregation known and possessed antithrombotic properties (Armstrong et al 1977; Higgs et al 1978; Ubatuba et al 1979).

4.1.2 Sites of synthesis of prostacyclin

Early studies of prostacyclin were performed on arterial microsomes prepared from homogenates of whole arterial walls (Bunting et al 1976) or with arterial and venous strips (Raz et al 1977; Johnson et al 1976). Moncada and co-workers (1977) demonstrated the differential formation of prostacyclin in the layers of rabbit aorta, the intimal layer being the major site of synthesis, with the media and adventitia showing progressively diminishing synthetic capacity.

Weksler et al (1977) were the first to demonstrate the synthesis of prostacyclin from human umbilical vein and bovine aortic endothelial cells in vitro. Other types of endothelial cells have since been shown to produce prostacyclin in vitro, including porcine endothelial cells and also microvascular endothelial cells (McIntyre et al 1978; Gerritsen 1987). However, whereas prostacyclin is the major product of arachidonic acid metabolism in endothelial cells derived from large blood vessels, a study performed by Charo et al (1984) demonstrated that cultured endothelial cells derived from dermal microvessels in human new born foreskin, release PGF₂ and PGE₂

in greater quantities than PGI₂, which was only released in relatively small amounts. Although fibroblasts and smooth muscle cells also synthesize prostacyclin, the levels of prostacyclin released by endothelial cells is much greater (Ali et al 1980; Baenziger et al 1979). Prostacyclin is also produced by renal glomerular cells, gastric mucosal cells, macrophages and mesothelial cells (Hassid et al 1979; Whittle et al 1980; Humes et al 1977 & Herman et al 1979). However, the vascular endothelium appears to be the major site of prostacyclin synthesis in the body (Schorr 1985).

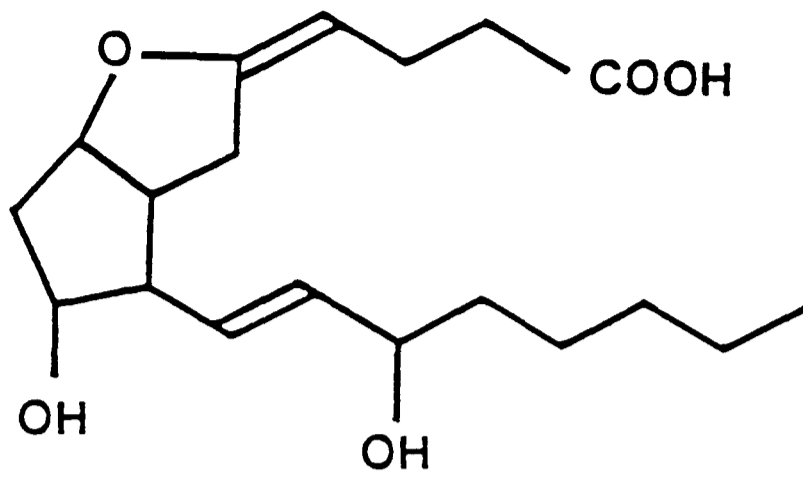
4.1.3 Structure of prostacyclin

Prostacyclin represents a member of the group of compounds known as eicosanoids, which includes prostaglandins (PG), thromboxanes (TX), leukotrienes (LT) and other oxygenation products of polyunsaturated 20-carbon unsaturated fatty acids, in particular arachidonic acid. The chemical structures of prostacyclin and the stable product of its hydrolysis are illustrated in Fig 17.

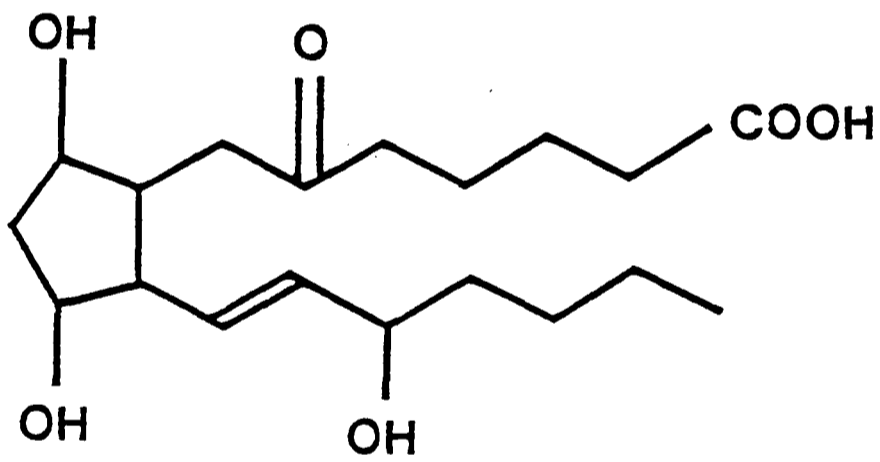
4.1.4 Biosynthesis of prostacyclin (Fig 18)

The initial step in the biosynthesis of prostacyclin involves the release of arachidonic acid via the activation of phospholipase (primarily phospholipase A₂). Arachidonic acid is either derived from dietary linoleic acid or is ingested as a constituent of the diet. After absorption from the gut, it is esterified and occurs in the body as a component of cell membrane phospholipids and other complex lipids.

A wide variety of physical, chemical and hormonal factors may activate the enzyme phospholipase A₂. Once the fatty acids are released they may follow two pathways:



PROSTACYCLIN PGI₂



6 - KETO - PGF_{1α}

Figure 17

The structure of prostacyclin (PGI₂) and its stable metabolite, 6-keto-PGF_{1α}.

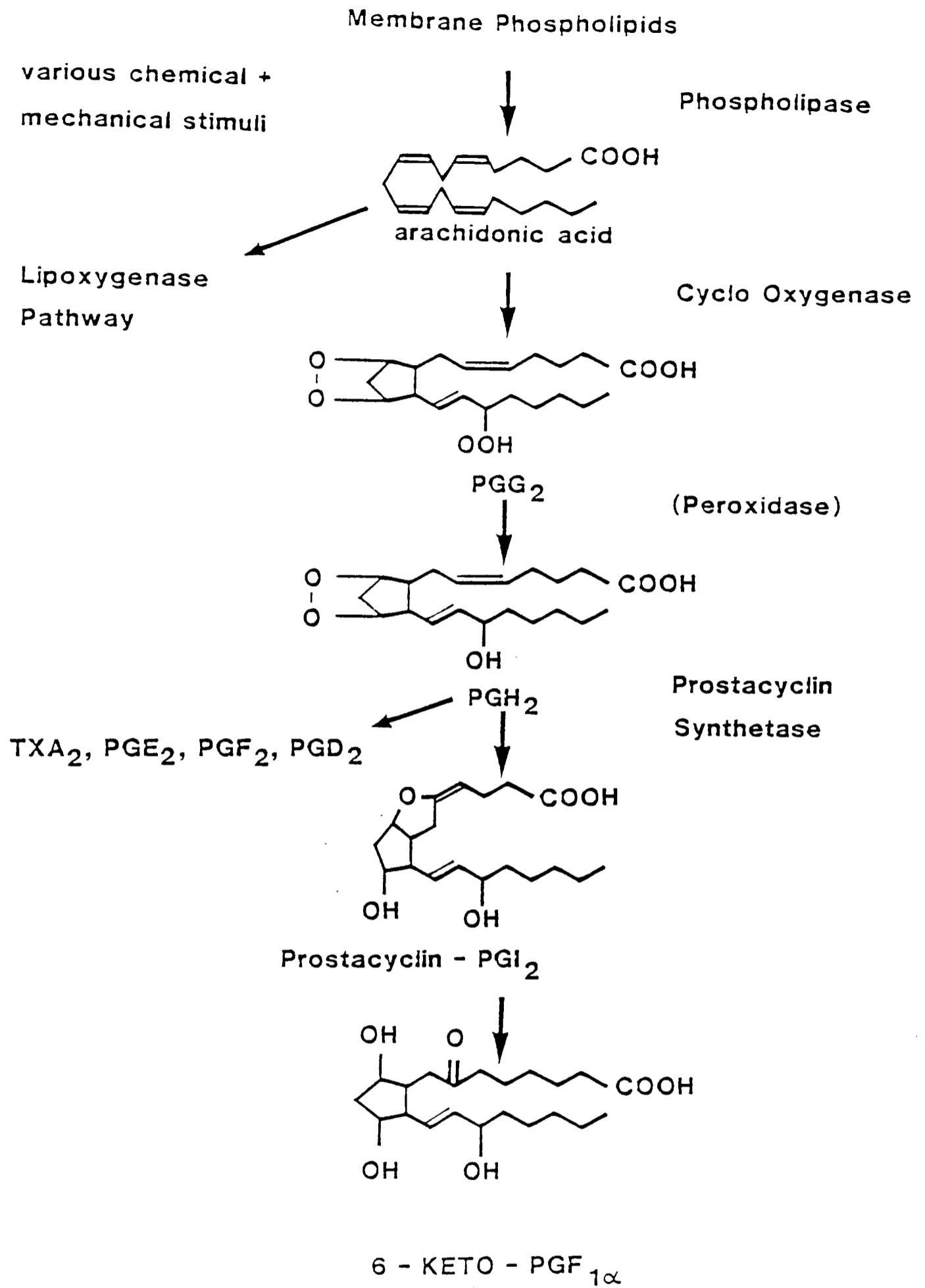


Figure 18

The cyclooxygenase pathway, showing the conversion of arachidonic acid to prostacyclin.

The cyclo-oxygenase pathway

This gives rise to the prostaglandins and thromboxanes.

The lipo-oxygenase pathway

This gives rise to the leukotrienes and other unsaturated hydroxy acids.

The pathway followed in vivo varies according to the tissue and species. In the cyclo-oxygenase pathway, the first product is an unstable cyclic endoperoxide derivative (PGG₂) which can proceed either spontaneously, or via the action of a peroxidase, to PGH₂. PGH₂ is the common intermediate for TXA₂, PGD₂, PGE₂, PGF₂ and PGI₂. Prostacyclin synthetase converts PGH₂ into prostacyclin, which is unstable under physiological conditions and is rapidly hydrolysed to a stable compound, 6-keto-PGF_{1α}.

Various substances act as inhibitors of prostaglandin synthesis, such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS) which are very potent inhibitors. These compounds work by preventing the formation of prostaglandin endoperoxides. Anti-inflammatory steroids appear to reduce prostaglandin synthesis by decreasing the availability of substrate to cyclo-oxygenase, or by blocking the efflux of prostaglandins from their site of synthesis.

The release and metabolism of arachidonic acid can be initiated by numerous factors including nerve stimulation, neurotransmitters (eg norepinephrine), neuropeptides (eg somatostatin), various hormones (eg bradykinin), hyperosmolar solutions and mechanical strain. Deformation of the cell membrane, as caused by stretching of a blood vessel, for instance during inflation of the lung and contraction of

the intestine, may also cause increased prostaglandin formation. In addition, so may tissue damage for example by trauma, hypoxia and breakdown of lysosomes.

4.1.5 Metabolism of prostacyclin

Under physiological conditions of pH and temperature, prostacyclin is an unstable molecule, with a half life in the blood of 3 minutes, due to its rapid hydrolysis to a stable product, 6-keto-PGF_{1α} (Gryglewski & Moncada 1987). Circulating levels of prostacyclin are low (approximately 0.5 pmol/ml, 18 pg/ml) although plasma levels of 6-keto-PGF_{1α} ranging from <50 pg/ml to 300 pg/ml have been reported (Haslam & McClenaghan 1981; Greaves & Preston 1982). Prostacyclin is more stable at alkaline pH and in the cold.

Prostacyclin and 6-keto-PGF_{1α} are both enzymatically degraded in vivo by 15-OH and 9-OH prostaglandin dehydrogenase and by delta-13,14 reductase, to yield a 13,14-dihydro-15-oxo-PG product which has a longer half life (8-16 minutes) in the systemic circulation than the primary prostaglandins.

Unlike PGE₁ and PGE₂, prostacyclin is not taken up by the lung transport system, and is therefore not metabolized by these enzymes, following one passage through the circulation. The major clearance route is probably via the liver and kidney. There are several urinary metabolites of prostacyclin including 2,3-dinor-6-keto-PGF_{1α} and 6,15 diketo,13,14 dihydro,2-3 dinor PGF_{1α} (Myatt et al 1981). In addition, it is possible that prostacyclin and 6-keto-PGF_{1α} are metabolized by blood vessels, platelet and hepatic 9-OH prostaglandin dehydrogenase to an active, stable metabolite, 6-keto-PGE₁, thus implying the recycling of inactive products (Wong et al 1980).

Since prostacyclin is not readily inactivated following passage through the lungs, it has been described as a circulating hormone

(Moncada et al 1978). However, its biological instability suggests that it is more likely to act locally.

4.1.6 Biology of action of prostacyclin

Prostacyclin is the most potent inhibitor of platelet aggregation known. In contrast thromboxane, which is also derived from arachidonic acid, exerts opposing effects in that it has potent proaggregatory and vasoconstrictor properties. Platelets are the major site of synthesis of thromboxane, as endothelial cells are for prostacyclin. Thus, a balance between these two substances maintains normal blood flow and control of haemostatic responses to injury. (See Fig 19).

The biological effects of prostacyclin are mediated via its stimulatory effects on the cellular adenylate cyclase system, with resulting elevation in cellular levels of cAMP (Gorman et al 1977; Tateson et al 1977). In this respect, prostacyclin is 10x more potent a stimulator of platelet cAMP than PGE₁ (Gorman et al 1977; Best et al 1977). In addition, the accumulation of cAMP caused by prostacyclin occurs more quickly and is longer lasting than that mediated by PGE₁ (Gorman et al 1977). As well as stimulating levels of cAMP in platelets, prostacyclin has also been shown to exhibit this effect in endothelial cells (Hopkins & Gorman 1981). When cAMP levels are high, prostacyclin production falls thus endothelial cells may regulate their biosynthesis of this compound by means of a feedback inhibition mechanism.

The stimulation of cAMP induced by prostacyclin has been studied in the platelet. Intracellular Ca²⁺ ions are required for phospholipase activation, aggregation and the release reaction. The formation of thromboxane A₂ in activated platelets results in the release of Ca²⁺ from bound intracellular sites. The calcium ions thus

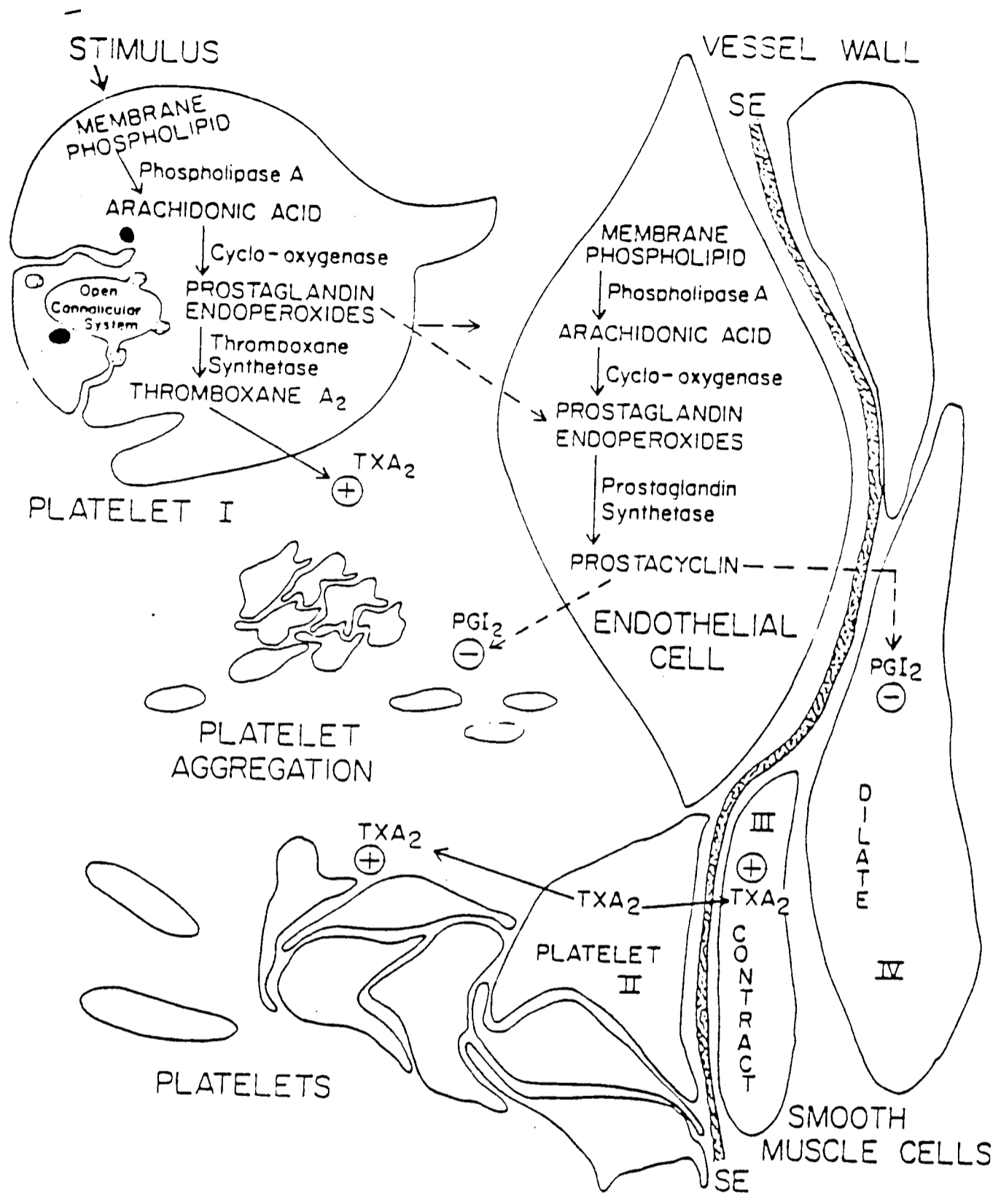


Figure 19 (Barnhart & Chen 1978)

The balance between thromboxane A₂ and prostacyclin. "Thromboxane A₂ provokes platelet aggregation and has a vasoconstrictor role on smooth muscle (III) in the vessel wall. Prostacyclin, a potent inhibitor of platelet aggregation, also produces vasodilation in smooth muscles (IV) of the vessel wall."

(+) = positive influence
 (-) = negative influence
 SE = subendothelium

released bind to calmodulin, and this calcium-calmodulin complex stimulates phospholipase A₂ activity and also activates myosin kinase.

Myosin is phosphorylated by the active kinase enzyme and is then able to interact with actin, resulting in platelet contraction and the release reaction (Hathaway & Adelstein 1979). Prostacyclin causes elevated levels of cAMP which results in Ca²⁺ sequestration with a resulting fall in phospholipase activation; thus less arachidonic acid is available for thromboxane and prostaglandin synthesis. Elevated levels of cAMP results in the activation of a cAMP-dependent protein kinase that phosphorylates myosin-kinase. Hence myosin phosphorylation decreases, the actin-myosin interaction declines and the release reaction is inhibited. Thus prostacyclin is able to inhibit platelet activation at various metabolic levels (Weksler 1982).

Prostacyclin is also able to reduce platelet adhesion. However, much greater concentrations of prostacyclin are required for adhesion to be inhibited (Moncada et al 1976b). When the vessel is mildly injured and the levels of prostacyclin in the vicinity are low, platelets will adhere but aggregation will be inhibited. Hence the process of wound healing is initiated without thrombus formation. Adherent platelets could provide the endothelial cells with endoperoxides which could then be used in further synthesis of prostacyclin. Experiments have demonstrated that platelet endoperoxides are in fact "stolen" by endothelial cells for conversion to prostacyclin (Bunting et al 1976; Moncada et al 1977; Marcus et al 1980; Chesterman et al 1986)

Additional actions of prostacyclin on platelets include the prevention of platelet shape change, and inhibition of the expression of procoagulant activity (PF₃) and fibrinogen receptors (Ehrman & Jaffe 1980; Harsfalvi et al 1980; Hawiger et al 1980). Thus platelet

aggregation is readily inhibited by prostacyclin. Aggregation induced by all known aggregating agents, including thrombin, can be inhibited by prostacyclin, as can the secretion of vasoactive mediators which are released during aggregation (Bunting et al 1981).

As well as being capable of preventing platelet aggregation, prostacyclin is also able to disaggregate previously clumped platelets, both in vitro and in vivo (Ubatuba et al 1979). Prostacyclin inhibits thromboxane generation and the release of platelet arachidonic acid via the elevation of cAMP levels. Inhibition of clot retraction and a prolonged bleeding time is reported in patients receiving prostacyclin infusions (Ubatuba et al 1979; O'Grady et al 1980).

Prostacyclin also has vasodilatory properties (Weksler 1982). It can affect erythrocyte deformability in patients with systemic sclerosis and Raynaud's phenomenon, and via its effects on cAMP, alter polymorphonuclear leukocyte functions and adherence to the endothelium (Dowd et al 1981; Boxer et al 1980). "Cytoprotective" activity is also exhibited by prostacyclin for example its anti-ulcer actions on the gastric mucosa (Robert 1979).

Thus prostacyclin has a diversity of effects on platelets, haemostasis and the vascular system. These effects are finely balanced with levels of thromboxane, which exerts opposing actions. When an imbalance occurs between these 2 compounds, various pathological states may follow (see later).

4.1.7 Regulation of prostacyclin biosynthesis

Endothelial cells are the major site of prostacyclin synthesis. However, the regulation of prostacyclin synthesis by these cells is poorly understood, although it appears that a close interaction with other cell types and their products is important, in particular with

platelets and platelet derived substances.

The rate limiting step in the synthesis of prostacyclin is the availability of free substrate. Arachidonic acid present in cultured endothelial cells is obtained from plasma or medium and cannot be synthesized by endothelial cells (Sivarajan et al 1984; Spector et al 1981, 1983). However arachidonic acid formed in other cells, for example platelets, polymorphonuclear cells and vascular smooth muscle cells, may be transferred to endothelial cells for the synthesis of prostacyclin (Schorr 1985). Thus, an endogenous supply of substrate can exist in vivo. Endoperoxides can also be obtained from other cell types for the synthesis of prostacyclin, in particular platelet derived endoperoxides may be used by endothelial cells in the synthesis of prostacyclin (Bunting et al 1976; Moncada et al 1977; Marcus et al 1980; Chesterman et al 1986).

Resting endothelial cells do not synthesize prostacyclin and a stimulus is required to initiate a response. The prostacyclin thus released is all newly synthesized as it is not stored by endothelial cells (Schorr 1985)

Serum and plasma both stimulate prostacyclin release from cultured endothelial cells (Seid et al 1983). Serum may affect prostacyclin production by providing an exogenous source of arachidonic acid, or else by hydrolysing cellular lipids. Factors present in serum generated during the clotting process, for example platelet derived growth factor, may also be important in inducing the synthesis of prostacyclin by endothelial cells (Coughlin et al 1980; Seid et al 1983a; Ritter et al 1982; 1983) although this finding has been disputed (Callahan et al 1986; Poggi et al 1983).

Thrombin was one of the first substances shown to stimulate prostacyclin synthesis in cultured endothelial cells (Weksler et al 1978). These authors proposed that thrombin stimulation of prostacyclin may occur in vivo to limit thrombus formation at sites of vascular injury. In addition, endothelial cells were shown to become refractory to thrombin stimulation (Weksler et al 1978). The mechanism of thrombin induced stimulation is unclear. Thrombin binding sites have been demonstrated on endothelial cells although the nature of these sites has not been established (Awbrey et al 1979). Thrombin stimulation of prostacyclin is species dependent and is not seen in bovine endothelial cells (Goldsmith et al 1981).

Various other stimuli may induce the synthesis of prostacyclin by cultured endothelial cells, some of which are listed in table 6. They include chemical and mechanical mediators, drugs and sublethal immunological injury. C5a des arg, leukocytes, C5b-8 activated neutrophils and a heterologous antibody towards endothelial cells have all been shown to act as stimulators of prostacyclin production (Rampart et al 1985; Harlan & Callahan 1984; Goldsmith & McCormick 1984; Suttorp et al 1987). Interleukin 1, α interferon and a lymphocyte derived factor have also been shown to induce prostacyclin synthesis in cultured endothelial cells, as have endotoxin and phorbol diester (Rossi et al 1985). Mechanical stress factors such as those induced by blood flow have also been shown to initiate a prostacyclin response (Frangos et al 1985; van Grondelle et al 1984; McIntire et al 1987).

Several studies concerned with the production of prostacyclin by cultured endothelial cells have presented conflicting data. It appears that the endothelial cell donor and the in vitro culture conditions used, are important variables. Weksler et al (1977)

Table 6

Exogenous activators of prostacyclin synthesis in human umbilical vein endothelial cells (Bull 1988).

1. Enzymes

Trypsin

Thrombin

2. Vasoactive peptides

Bradykinin

Angiotensin II

Histamine

Adrenaline

DDAVP

3. Delayed activators

Phorbol esters

Endotoxin

Interleukin-1

4. Mechanical stimulus

Hypoxia

Physical assault

Others

Calcium ionophore A23187

High density lipoprotein

Hydrogen peroxide (low concentrations)

demonstrated a two-fold increase in prostacyclin production by bovine aortic endothelial cells compared with human umbilical vein endothelial cells. Within a particular species, arteries produce greater amounts of prostacyclin compared with veins (Johnson 1980). Sex may also have an effect , and serial passaging of endothelial cells results in a reduction in levels of prostacyclin release (Maggi et al 1980; Johnson 1980; Goldsmith et al 1984).

In addition, cell density is also important. Studies have demonstrated that prostacyclin production is greatest in the exponential phase of growth compared with confluent monolayers of human umbilical vein and bovine endothelial cells, and there is a fall in prostacyclin levels as the density of cell growth increases (Ali et al 1980; Evans et al 1984). However, this has been contradicted by Eldor et al (1983) who demonstrated an increased capacity to synthesize prostacyclin following the formation of a contact inhibited cell monolayer of bovine aortic endothelial cells. Arachidonic acid levels in the cell may be important in determining the amounts of prostacyclin synthesized, thus the concentration of arachidonic acid in the culture medium may be crucial (Spector et al 1983; Hong et al 1979).

The various agents capable of stimulating prostacyclin formation and release, may act by altering cellular cAMP levels. Catecholamines and prostacyclin itself can cause an elevation of cAMP levels, so that released prostacyclin could enhance its own synthesis. However, increased amounts of cAMP may also inhibit arachidonic acid release with a resulting fall in prostacyclin synthesis due to a lack of the precursor (Adler et al 1981). Other stimulatory agents may also modify endothelial cell cAMP levels. Thus, this mechanism may be a common pathway for the control of prostacyclin synthesis. Ca^{2+} levels

are important in the stimulation of prostacyclin synthesis and a Ca^{2+} influx may occur following the stimulation of endothelial cells (Hassid 1982). In addition, prostacyclin production in response to serum is inhibited by TMB-8 (an antagonist of intracellular calcium mobilisation) and W7, a calmodulin antagonist (Seid et al 1983b). However, more work remains to be done in order to fully establish the complex mechanisms occurring in the regulation of prostacyclin biosynthesis by the vascular endothelium.

4.1.8 Prostacyclin in Disease States

Prostacyclin synthesis by vascular tissue may be altered in various disease states. A decrease in prostacyclin production is seen in diseases characterised by acute or chronic endothelial cell injury, or by lipid deposition in the vessel wall. Conversely, increased prostacyclin production is associated with diseases where there is a bleeding tendency and defective platelet function (Weksler 1982)

Altered levels of prostacyclin synthesis have been described in atherosclerosis, and aortas from atherosclerotic rabbits produce enhanced amounts of prostacyclin in vitro (Voss et al 1983). In addition, sera from hyperlipidemic subjects caused increased synthesis of prostacyclin from cultured human umbilical vein endothelial cells (Tremoli et al 1985). Mechanically de-endothelialised rabbit aortas, which mimic injured vessels, produce markedly reduced levels of prostacyclin, thus indicating the requirement of an intact endothelial cell monolayer for prostacyclin synthesis (Eldor et al 1981).

Prostacyclin production is also altered in diabetes. Johnson et al (1979; 1981) reported reduced vascular levels of prostacyclin in diabetes, as did Patel et al (1982) who measured the levels of 6-

keto-PGF_{1α} in supernatants from cultured human umbilical vein endothelial cells incubated in the presence of sera from diabetic patients.

Foetal and placental tissues have been shown to generate much greater levels of prostacyclin than vessels from normal adults (Remuzzi et al 1979; Terragno et al 1978; 1979). However, placental and foetal vessels obtained during pre-eclampsia and eclampsia produce diminished amounts of prostacyclin in vitro (Remuzzi et al 1980a; Downing et al 1980)

Sera from patients with haemolytic uremic syndrome fail to stimulate prostacyclin synthesis in cultured endothelial cells to the same extent as normal sera (Remuzzi et al 1980b). It has been postulated that these sera lack a stimulatory factor for prostacyclin synthesis. In patients with thrombotic thrombocytopenic purpura, a factor causing endothelial cell damage, which could interfere with normal prostacyclin production, has been described (Burns et al 1981). Other authors have described an abnormal rate of prostacyclin degradation in patients with thrombotic thrombocytopenic purpura (Chen et al 1981; Wu et al 1982). In addition, a plasma factor has been described in patients with uremia, which stimulates prostacyclin synthesis from cultured bovine endothelial cells.

Nicotine has been shown to alter the production of prostacyclin by cultured endothelial cells. In one study by Bull et al (1985) prostacyclin synthesis by rat aortic rings was significantly decreased after 7 days continuous subcutaneous infusion of nicotine. In addition, the synthesis of prostacyclin by cultured endothelial cells obtained from placental umbilical cords of mothers who smoked, was markedly reduced (Busacca et al 1984)

The IgG fraction obtained from plasma containing the lupus anticoagulant, was shown to inhibit prostacyclin release by bovine endothelial cells and rings of rat aorta (Carreras et al 1981; 1982). This patient had experienced recurrent thrombosis and repeated spontaneous abortions, although she did not have SLE. However, plasma from two patients with SLE were found to inhibit prostacyclin release from rabbit aorta (McVerry et al 1980). In another study, cultured porcine endothelial cells released less prostacyclin when incubated with sera from patients with SLE and systemic sclerosis compared to that released following stimulation with normal control sera (Seid et al 1982).

However, reports on altered production of prostacyclin in patients with systemic sclerosis remain limited. Belch et al (1984) found elevated levels of endogenous 6-keto-PGF_{1α} in 15 patients with systemic sclerosis compared with normal controls. Sera from patients with systemic sclerosis has been shown to inhibit the synthesis of prostacyclin by cultured human umbilical vein endothelial cells (Rustin et al 1987a). However, in another study, prostacyclin production by cultured endothelial cells was the same following incubation with sera from patients with systemic sclerosis and normal control sera (Evans et al 1984). Thus, conflicting data exists regarding the effects of sera from patients with systemic sclerosis on prostacyclin synthesis by cultured endothelial cells. Since prostacyclin production is crucial for the maintenance of vascular integrity, further investigations of these serum-induced effects on cultured human umbilical vein endothelial cells were performed as an additional aspect of this study.

4.2 Measurement of prostacyclin levels

Principle

Prostacyclin has a very short half life and is rapidly converted to a stable metabolite, 6-keto-PGF_{1α}. Thus, for the determination of prostacyclin release, 6-keto-PGF_{1α} was measured by means of a radio-immunoassay. Tritiated 6-keto-PGF_{1α} and 6-keto-PGF_{1α} present in the sample under analysis, compete for binding sites on antibody directed against the 6-keto-PGF_{1α} molecule. Unbound 6-keto-PGF_{1α} is removed by spining with charcoal, and the amount of labelled 6-keto-PGF_{1α} remaining bound to the antibody is measured by liquid scintillation counting. The level of radioactivity is proportional to the amount of 6-keto-PGF_{1α} present in the test sample, and by reference to a standard curve, the amount of 6-keto-PGF_{1α} expressed as pmol/100 μl can be determined.

Procedure

Test samples (100μl) were mixed with an equal volume of tritiated 6-keto-PGF_{1α} and antibody, and incubated for 16-20 hours at 4° C. Tritiated 6-keto-PGF_{1α} was previously diluted in 100 μl tris gelatin buffer (pH 7.4) to give approximately 3000 counts per minute, and antibody (kindly supplied by Dr M Greaves, Dept Haematology, Royal Hallamshire Hospital, Sheffield), was diluted to give 50% binding in the absence of unlabelled 6-keto-PGF_{1α}. All assay procedures were performed on ice, and duplicate tubes were prepared for each test. A reference standard curve was prepared in parallel by the addition of 0-0.4 pmol/100 μl 6-keto-PGF_{1α} to 100 μl antibody and labelled 6-keto-PGF_{1α}. Non-specific binding was determined in tubes containing standard, labelled 6-keto-PGF_{1α} and tris gelatin buffer in place of antibody; and total counts were assessed by the addition of 500 μl tris gelatin buffer to 100 μl labelled 6-keto-PGF_{1α}.

After overnight incubation at 4°C, 300 µl charcoal suspension was added to each tube, except those used for determining total counts, and mixed. Tubes were then centrifuged at 2000g for 15 minutes at 4°C. 250 µl supernatant was then transferred to scintillant tubes and 2 ml liquid scintillant added. After mixing, samples were counted for 5 minutes in an LKB 1217 Rack-Beta liquid scintillation counter, equipped with a computer programmed to calculate the 6-keto-PGF_{1α} content of each sample, by reference to the standard curve. Results were expressed as pmol 6-keto-PGF_{1α}/100 µl.

Tris gelatin buffer

4.05g sodium chloride

0.90g Tris

0.25g gelatin

500ml distilled water

Sodium chloride and Tris in distilled water were heated to 56°C , prior to the addition of gelatin. The buffer was then cooled and the pH adjusted to 7.4.

Charcoal suspension

0.5g bovine serum albumin

0.5g dextran T 70

0.6g Norit A charcoal

The above were made up to 100 ml in tris gelatin buffer, and the solution well mixed before use.

4.2.1 Characteristics of the radio-immunoassay of 6-keto-PGF_{1α}

The radio-immunoassay performed in this study for the detection of 6-keto-PGF_{1α} had a limit of detection of 50 pg/ml. The intra-assay variation between duplicate determinations within a single assay was <5%. The inter-assay variation of standard curves was <10% (coefficient of variation) [See fig 20]. The cross reactivities of the anti-serum to 6-keto-PGF_{1α}, with available prostaglandins and metabolites were the same as those published by Greaves and Preston (1982) and are listed in table 7.

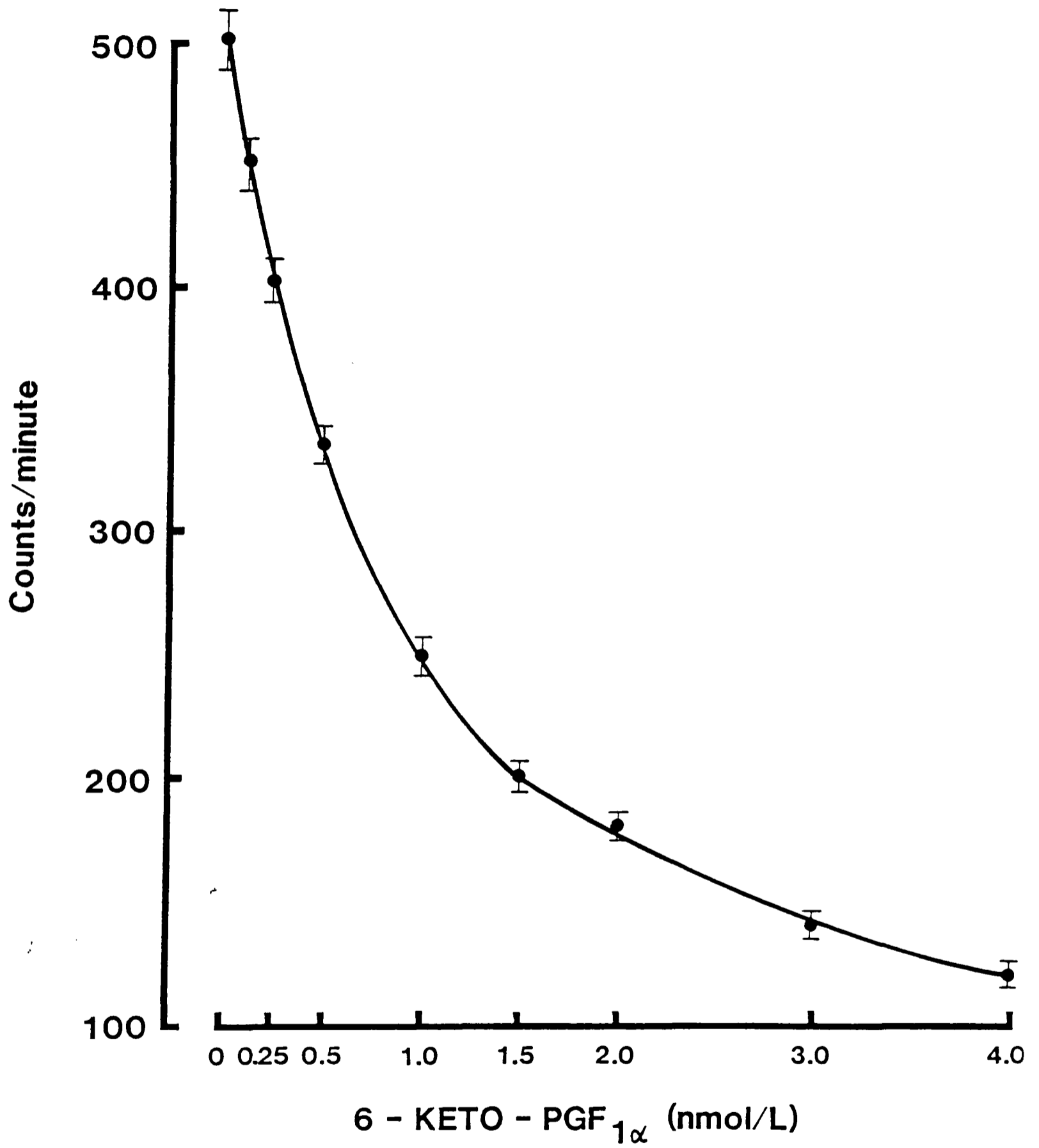


Figure 20

The standard curve of 6-keto-PGF_{1α} determinations by radioimmunoassay (mean ± SEM; n = 13 experiments).

Table 7

The cross-reactivity of the antiserum to 6-keto-PGF_{1α} with available prostaglandins and metabolites, calculated from the mass of prostaglandin required to produce 50% displacement of bound [³H]-6-keto-PGF_{1α} from the binding sites (Greaves and Preston 1982)

	<u>% cross reactivity</u>
6-keto-PGF _{1α}	100.000
13,14-dihydro-6-keto-PGF _{1α}	1.100
PGF _{1α}	0.350
PGD ₂	0.250
PGF _{2α}	0.200
PGE ₁	0.075
13,14-dihydro-6,15-diketo-PGF _{1α}	0.075
6,15-diketo-PGF _{1α}	0.075
13,14-dihydro-PGF _{2α}	0.020
PGA ₂	0.015
PGA ₁	0.004
Thromboxane B ₂	0.004
13,14-dihydro-PGE ₁	0.004
PGB ₁	0.002
13,14-dihydro-PGE ₂	<0.001
13,14-dihydro-15-keto-PGF _{2α}	<0.001
13,14-dihydro-15-keto-PGE ₁	<0.001
Arachidonic acid	<0.001

4.3 Effect of cell density on prostacyclin release from cultured human umbilical vein endothelial cells

4.3.1 Introduction

Various authors have demonstrated altered prostacyclin synthesis by cultured endothelial cells at different cell densities. However, reports of enhanced synthesis during the exponential growth phase (Ali et al 1980; Evans et al 1984), have conflicted with the demonstration of increased production following the formation of a contact inhibited monolayer (Eldor et al 1983). In this study, therefore, experiments were performed in an attempt to clarify these discrepant observations. Prostacyclin release by human umbilical vein endothelial cells per μg protein and per cell was determined over a period of 2-12 days following 15 minute exposure to both 20% normal human AB serum and M199 alone, and also following 24 hour exposure to 20% normal human AB serum.

4.3.2 Materials and methods

4.3.2.1 Effect of protein content on prostacyclin release

Primary cultures of human umbilical vein endothelial cells were isolated and grown to confluence in T25 flasks as previously described. The cells were fed at 24 hours and then subsequently at two day intervals, with M199 supplemented with 20% normal human AB serum. Confluent monolayers of human umbilical vein endothelial cells were passaged and seeded into gelatin coated 24 well plates at 4×10^4 cells per well. Endothelial cells were fed daily during cell growth assays, which were carried out for 12 days, in order to minimize any effects on the synthesis of prostacyclin caused by the depletion of precursors, for example arachidonic acid, from the culture medium.

At 2, 4, 6, 8, 10 and 12 days, the cell supernatant was removed from duplicate wells and retained for analysis of 6-keto-PGF_{1α} content. These samples were used in the determination of prostacyclin release during the previous 24 hours. In addition a 15 minute incubation with either 20% normal human AB serum or M199 alone was also performed in duplicate at 2, 4, 6, 8, 10 and 12 days and the cell supernatants retained for analysis of 6-keto-PGF_{1α} content. Supernatant samples were stored at -40°C prior to analysis of 6-keto-PGF_{1α} content. After the collection of 24 hour and 15 minute stimulation supernatants, human umbilical vein endothelial cells were washed 3 times with PBS and 0.25 ml 1N sodium hydroxide was added to each well. Following an overnight incubation at 37°C in a humidified atmosphere with 5% CO₂, the contents of each well was removed. These samples were then assayed for protein content by the Lowry method (see Appendix III). The amount of prostacyclin released, as determined by 6-keto-PGF_{1α} levels, was expressed per µg protein.

4.3.2.2 Effect of cell number on prostacyclin release

The experiments performed to determine the prostacyclin release per cell from cultures of human umbilical vein endothelial cells were identical to those carried out to assess the prostacyclin release per µg protein. However, prior to the addition of 1N sodium hydroxide, cells were counted under a phase contrast microscope equipped with an eye piece graticule. Two fields were randomly selected and the number of cells in 100 squares were counted. The total number of cells per well was determined using the following formula:

$$\begin{array}{l} \text{Total number} \qquad \qquad \text{Mean number cells/100 squares} \\ \text{cells/well} \qquad = \qquad \text{-----} \qquad \qquad \times \text{ Area of well} \\ \qquad \qquad \qquad \qquad \qquad \qquad \text{Area of 100 squares} \end{array}$$

The amount of prostacyclin released was expressed per cell.

4.3.3 Results

4.3.3.1 Determination of protein

The protein content of human umbilical vein endothelial cells was determined at 2, 4, 6, 8, 10, and 12 days, following an initial plating density of 4×10^4 cells per well. 10 experiments were performed on human umbilical vein endothelial cells derived from different umbilical cords. There was a rise in protein content of cultures in parallel with cell growth from $4.59 \pm 1.03 \mu\text{g}$ (mean \pm SEM) at day 2, to $19.03 \pm 2.73 \mu\text{g}$ at day 12 (fig 21).

4.3.3.2 Effect of protein content on prostacyclin release

Prostacyclin release per μg protein decreased with increasing protein content, following 15 minute stimulation with both 20% normal human AB serum (n=10 experiments) and M199 alone (n=5 experiments) [figs 22 & 23]. However, the amount of prostacyclin released per μg protein was greater from endothelial cells stimulated by 20% serum compared with those stimulated by M199 alone. In addition, the accumulated release of prostacyclin per μg protein over 24 hours showed a similar pattern of production (fig 24).

The amount of prostacyclin released per μg protein varied with human umbilical vein endothelial cells derived from different umbilical cords in both the 15 minute stimulation, and 24 hour accumulation experiments.

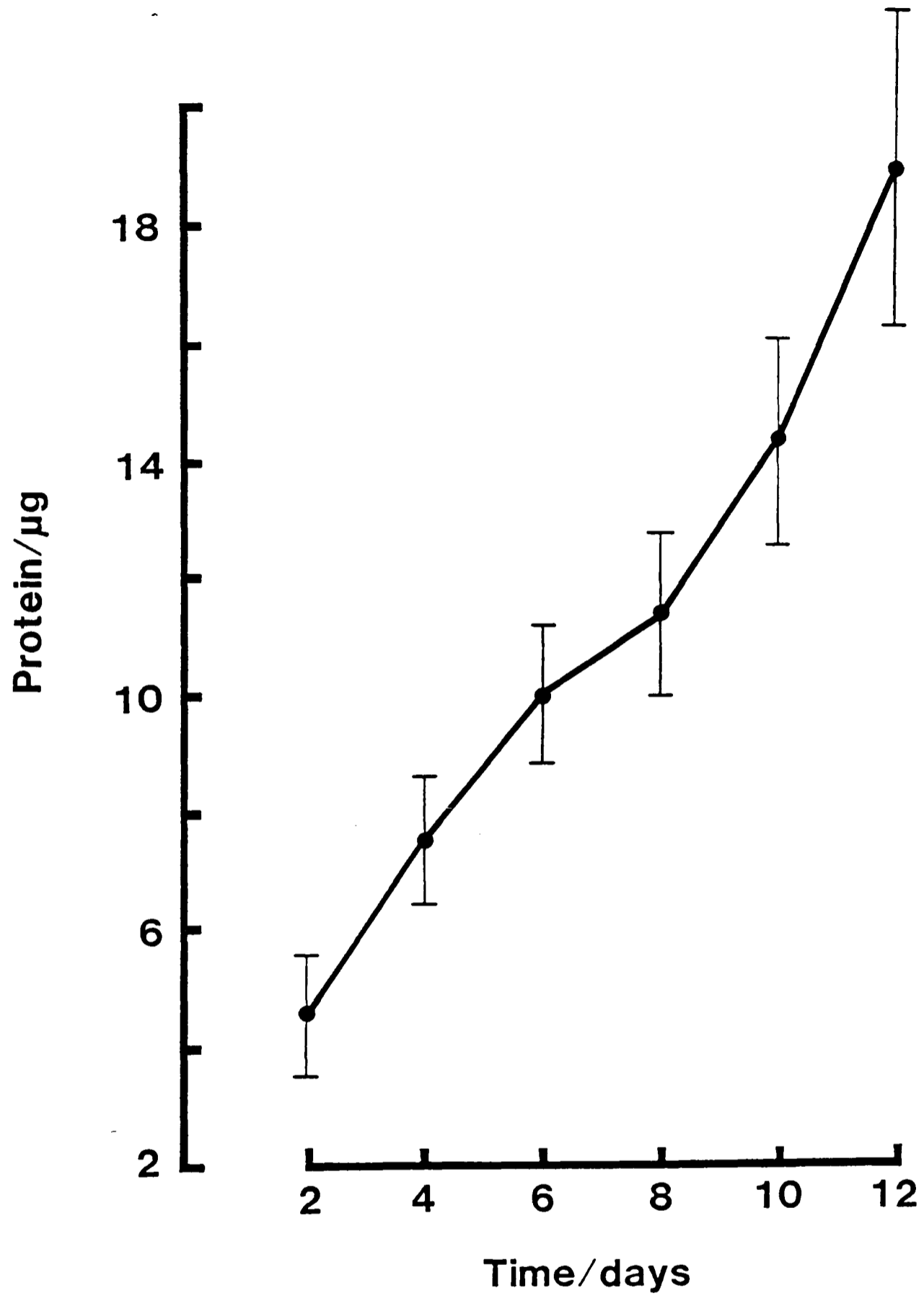


Figure 21

The protein content (mean \pm SEM) of human umbilical vein endothelial cells during continuous culture for 12 days (n=10 experiments).

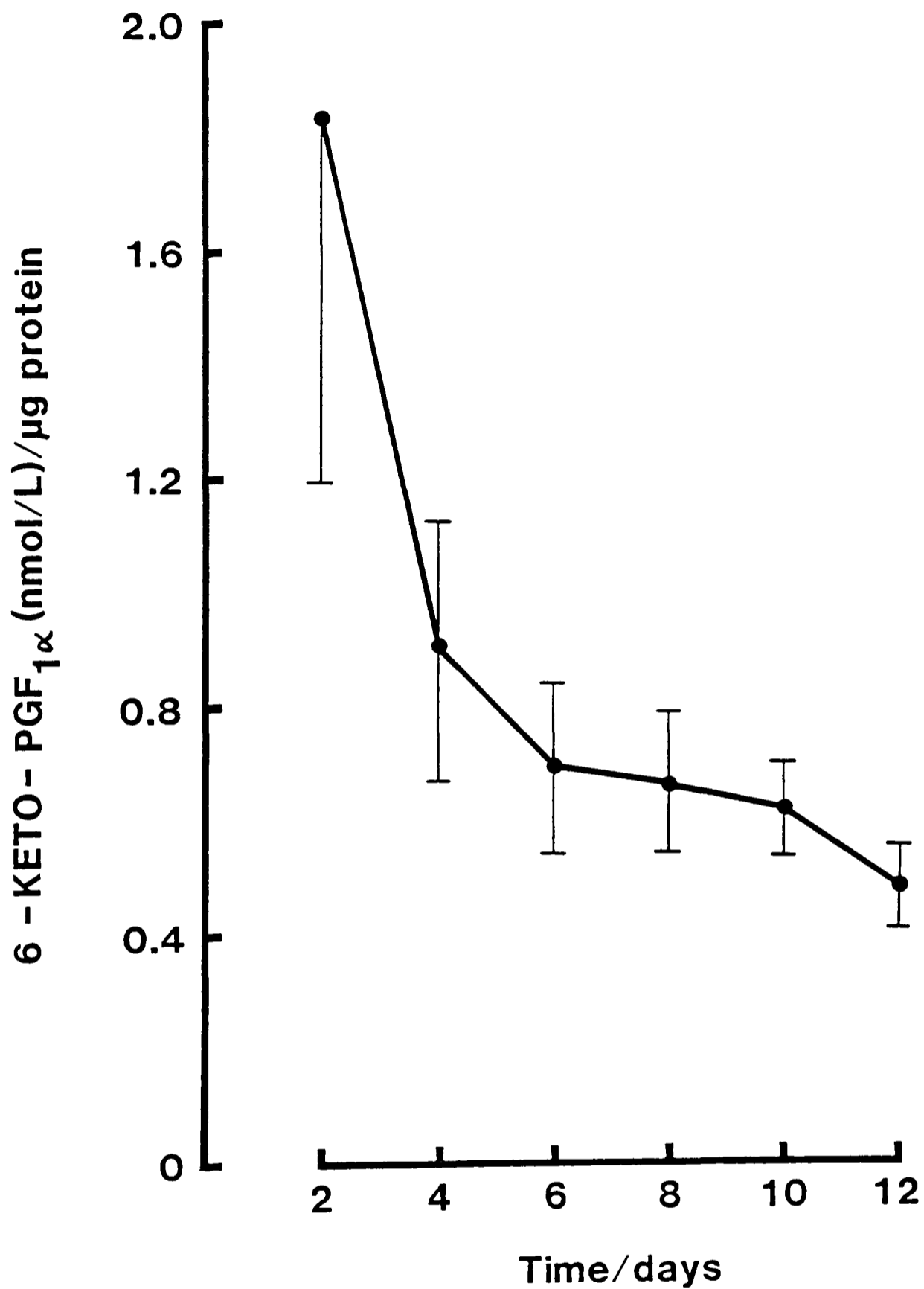


Figure 22

The prostacyclin release / ug protein (mean ± SEM) of human umbilical vein endothelial cells following stimulation by human AB serum for 15 minute periods at intervals during continuous culture for 12 days (n=10 experiments).

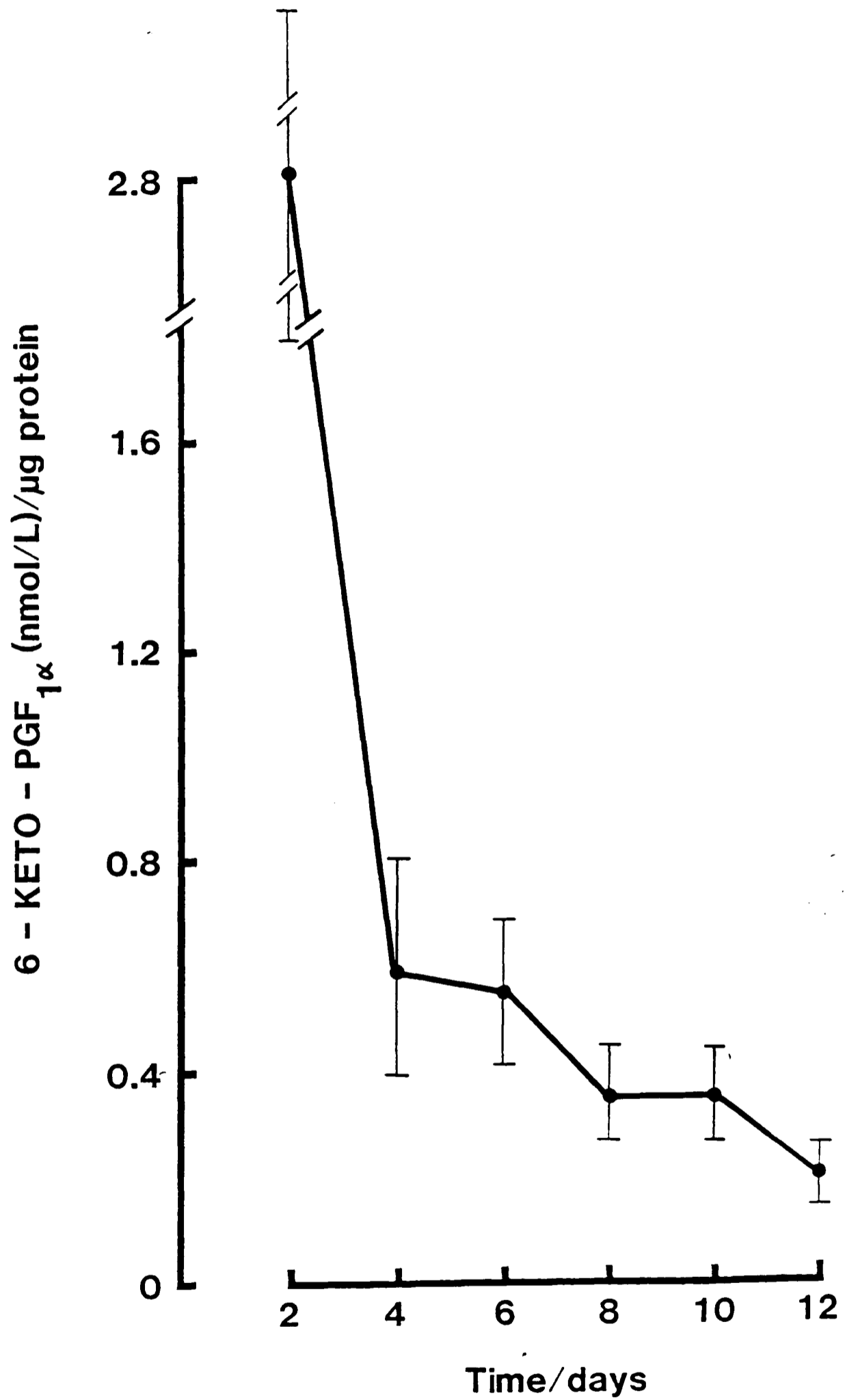


Figure 23

The prostacyclin release / ug protein (mean ± SEM) of human umbilical vein endothelial cells following stimulation by M199 for 15 minute periods at intervals during continuous culture for 12 days (n=5 experiments).

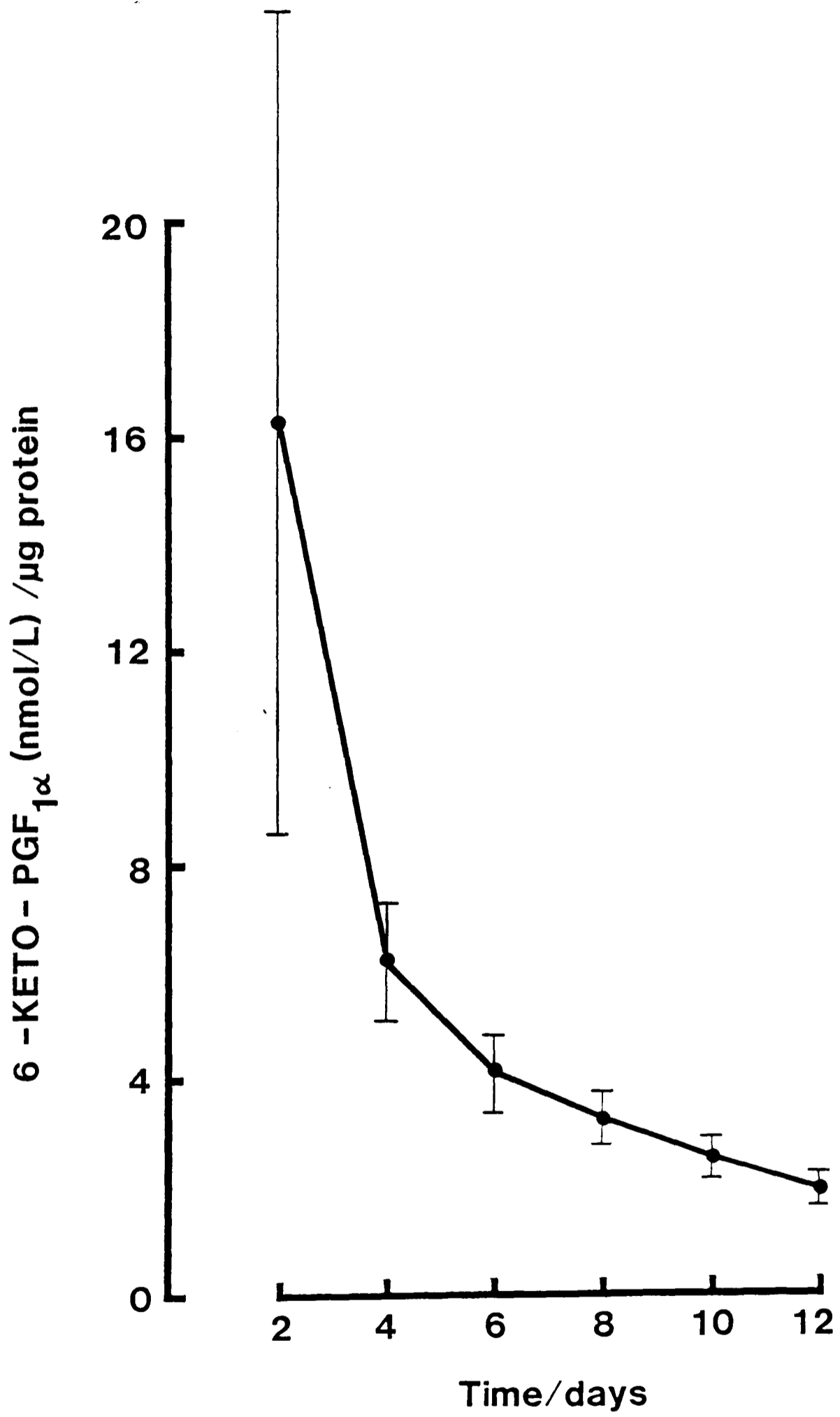


Figure 24

The cumulative prostacyclin release / ug protein (mean ± SEM) of human umbilical vein endothelial cells during 24 hour periods at intervals during continuous culture for 12 days (n = 5 experiments).

4.3.3.3 Determination of cell number

The number of human umbilical vein endothelial cells was determined in duplicate wells at 2, 4, 6, 8, 10, and 12 days (n=5 experiments). An increase in cell number was observed with increasing time (fig 25), although the final cell number at day 12 varied considerably between cultures obtained from different cords.

4.3.3.4 Effect of cell number on prostacyclin release

As in the experiments performed to determine the effect of protein content on prostacyclin release, an inverse relationship was observed between cell number and prostacyclin release (figs 26 & 27). This effect was seen in human umbilical vein endothelial cells during 15 minute exposure to 20% normal human AB serum (n=5 experiments), and in 24 hour accumulation experiments (n=5 experiments).

Comment

The protein content and cell number of cultured human umbilical vein endothelial cells increased with time. In contrast, the amount of prostacyclin released per μg protein and per cell decreased with time during both 15 minute exposure to 20% normal human AB serum and M199 alone, and following 24 hour incubation. The amount of prostacyclin released during 15 minute exposure to 20% serum was greater than that released following exposure to M199 alone. In addition, there was a large variation in the amount of prostacyclin released from human umbilical vein endothelial cells derived from different umbilical cords. Also, the rate of increase of protein and cell number varied with the different cultures of human umbilical vein endothelial cells.

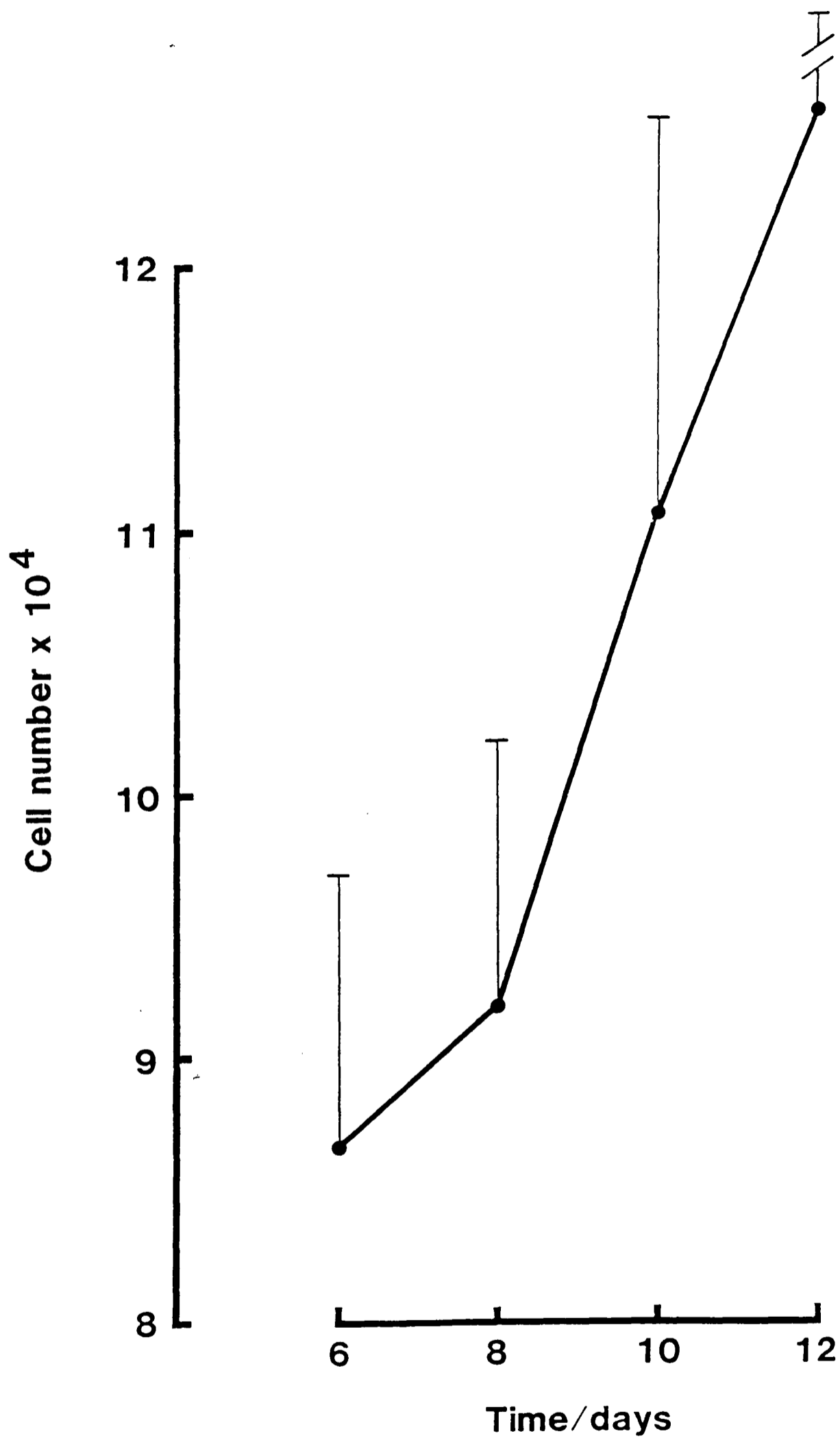


Figure 25

The number (mean \pm SEM) of human umbilical vein endothelial cells at intervals during continuous culture for 12 days (n=5 experiments).

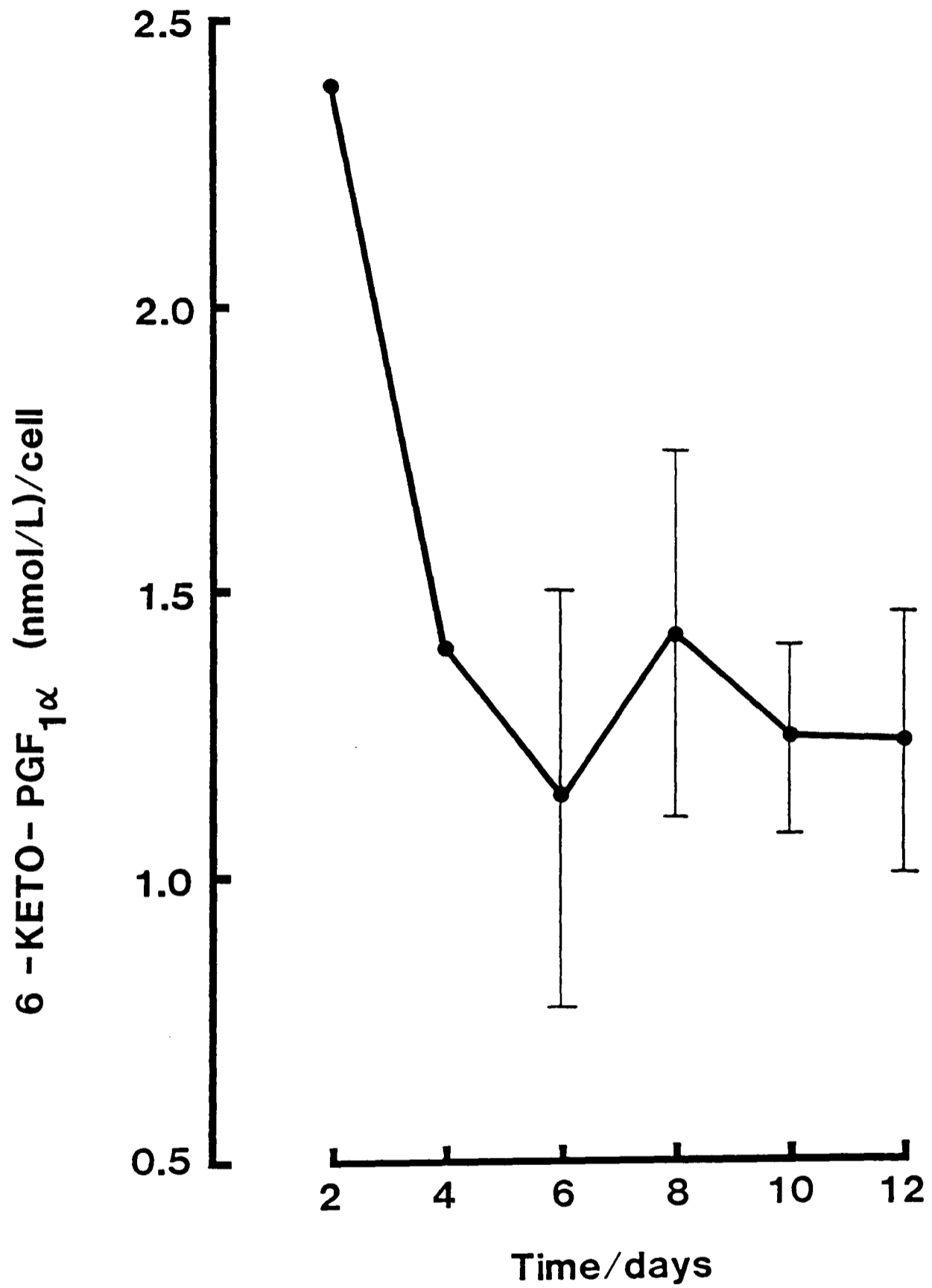


Figure 26

The prostacyclin release / cell (mean \pm SEM) from human umbilical vein endothelial cells following stimulation by 20% human AB serum for 15 minutes at intervals during continuous culture for 12 days (n=5 experiments).

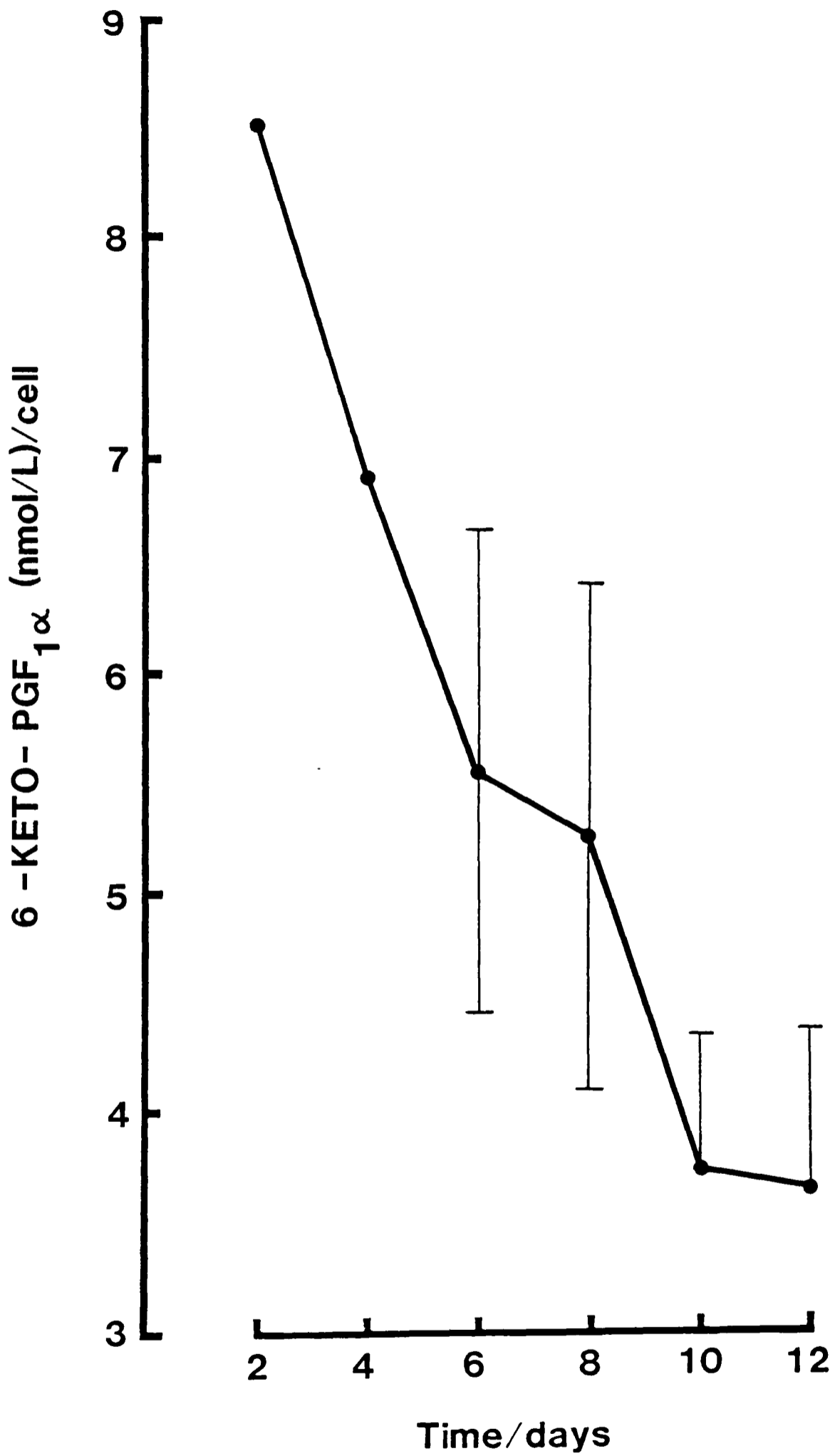


Figure 27

The continuous prostacyclin release / cell (mean ± SEM) from human umbilical vein endothelial cells during 24 hour periods at intervals during continuous culture for 12 days (n=5 experiments).

4.3.4 Discussion

4.3.4.1 Effect of cell density on prostacyclin release

The results obtained from these experiments have clearly demonstrated an inverse relationship between cell density and prostacyclin release from cultured human umbilical vein endothelial cells. This phenomenon was observed when the prostacyclin release was expressed as either per μg protein or per cell. In addition, both 15 minute stimulation of human umbilical vein endothelial cells with either 20% normal human serum or M199 alone, and accumulated 24 hour release of prostacyclin showed this density dependent phenomenon. Thus, one can conclude that actively growing human umbilical vein endothelial cells produce increased amounts of prostacyclin compared with confluent cell monolayers.

A similar density dependent release of prostacyclin from cultured human umbilical vein endothelial cells was demonstrated by Evans et al (1984). These authors analysed spent growth medium obtained at 24 hourly intervals and observed a decline in prostacyclin content with increasing time without, however, correcting their results for cell number or protein content. In addition, Ali et al (1980) demonstrated significantly higher production of all prostanooids, including prostacyclin, during the exponential growth phase of bovine aortic endothelial cells, as compared with confluent monolayers. These effects were observed following the initial seeding of cells at varying densities.

In contrast to the results obtained in this study, and those demonstrated by Evans et al (1984) and Ali et al (1980), are the findings of Eldor et al (1983), who described an increased capacity of bovine aortic endothelial cells to synthesize prostacyclin following the formation of a contact inhibited cell monolayer. However, these

authors determined the stimulated release of prostacyclin per cell following exposure to arachidonic acid, which may account for their differing results, since arachidonic acid may provide substrate for further prostacyclin release, and hence stimulate its production.

A possible reason for the decline in prostacyclin release with increasing cell density, may be the depletion of available substrate for prostacyclin synthesis. Endothelial cells obtain substrate for the synthesis of prostacyclin, in the form of arachidonic acid, mainly from cell membrane phospholipids. It appears that endothelial cells do not synthesize arachidonic acid but must acquire it in a preformed state from exogenous sources (Spector et al 1981; Sivarajan et al 1984). In conditions of high cell density, insufficient arachidonic acid may be available in the culture medium for maximum uptake and subsequent conversion to prostacyclin to occur. Hong et al (1979) analysed the cellular content of arachidonic acid at different growth stages of a mouse fibroblast cell line. They showed that the arachidonic acid per cell diminished with time and the release of prostacyclin from these cells showed a similar rate of decline with time. However, feeding the cells with fresh growth medium stimulated prostacyclin production. In addition, these authors found that the majority of arachidonic and linoleic acid in the culture medium was rapidly (within one day) incorporated into cellular phospholipid. The results of Eldor et al (1983) who, in contrast to the results obtained in this study, demonstrated increased prostacyclin release following the formation of a contact inhibited monolayer (by arachidonic acid stimulation) could thus be explained, since by supplying an exogenous source of substrate, no depletion effects would be likely to occur.

In contrast to the above mechanism, it has been suggested that arachidonic acid is infrequently rate-limiting (Weksler 1987). Instead the release of this substrate may be important. Depletion in the culture medium of various growth factors, following an increase in cell density, could result in diminished activation of the enzymes cyclooxygenase and prostacyclin synthase. Platelet derived growth factor and epidermal growth factor have both been found to stimulate prostaglandin synthesis in fibroblasts and smooth muscle cells by the induction of cyclooxygenase synthesis (Rozengurt et al 1983; Habenicht et al 1985). In addition, platelet derived growth factor has been shown to stimulate prostacyclin synthesis by bovine aortic endothelium, aortic smooth muscle and adrenal capillary endothelium (Coughlin et al 1980). However, this observation is somewhat controversial since Callahan et al (1986) failed to demonstrate the stimulation of prostacyclin synthesis by platelet derived growth factor in human umbilical vein and bovine aortic endothelial cells, as did Poggi et al (1983). In addition, Heldin et al (1981) and Bowen-Pope & Ross (1982) failed to demonstrate the occurrence of receptors for platelet derived growth factor on bovine aortic and human endothelial cells. However, it is possible that this factor could act via a receptor independent mechanism.

Alternatively, rather than the depletion of stimulatory factors of cyclooxygenase and prostacyclin synthase, inhibitory factors may be important. As cell density rises, there is a build up of endothelial cell metabolites which may exert an inhibition on the release of prostacyclin. In support of such a hypothesis, Evans et al (1984) showed decreased prostacyclin release from human umbilical vein endothelial cells grown in endothelial cell conditioned medium, compared with those grown in fresh culture medium. Thus, endothelial

cells may exert a negative feedback mechanism on their release of prostacyclin. The formation of a contact inhibited cell monolayer by human umbilical vein endothelial cells may act as a signal for the diminished synthesis of prostacyclin and in this study, a confluent monolayer was obtained before the end of the experiments.

The differences in endothelial cell capacity to synthesize prostacyclin at different stages of growth, may serve a physiological compensatory mechanism whereby less prostacyclin is released from intact vascular endothelium, compared with growing cells following localized injury. Such growing cells may enhance repair mechanisms and reduce adhesion and thrombosis, by synthesizing increased levels of prostacyclin.

Since the release of prostacyclin varies with stages of cell growth and possibly with availability of substrate, studies on prostacyclin biosynthesis must be strictly standardized in terms of cell density and feeding regimes, in order to reduce any variables arising from these factors. In all experiments performed in this study, human umbilical vein endothelial cells were seeded at a constant initial density and the feeding of cells during experiments was carefully controlled.

4.3.4.2 Serum stimulation of prostacyclin release from human umbilical vein endothelial cells

In experiments performed to observe the effects of cell density on the release of prostacyclin from cultured human umbilical vein endothelial cells 15 minute stimulations with either 20% normal human serum or M199 alone were employed. The release of prostacyclin was much greater following stimulation with 20% normal human serum

compared with M199 alone. This effect was also observed in later experiments performed to observe the effects of sera from patients with systemic sclerosis on the release of prostacyclin.

The stimulation of prostacyclin release by serum has previously been documented (Ritter et al 1982, 1983; Seid et al 1983a). Seid et al (1983a) observed that serum stimulated the release of prostacyclin from pig aortic endothelial cells to a greater extent than plasma and the stimulatory effect of plasma was higher in platelet rich plasma compared with platelet poor plasma. Thus, platelet derived factors appear to be important in the stimulation of prostacyclin release. In addition, these authors showed that isolated degranulated platelets released factors which caused the stimulation of prostacyclin release. These studies were preceded by the work of Coughlin et al (1980) who demonstrated platelet dependent stimulation of prostacyclin synthesis caused by platelet derived growth factor. Ritter et al (1982 & 1983) also described a serum factor formed during coagulation which stimulated the release of prostacyclin from rat aortic rings. However, their factor did not appear to be platelet derived and they suggested that it could be a small fragment cleaved from a coagulation factor such as factor VIII or IX.

In addition to stimulatory factors, serum also contains platelet factors which may be used as substrate in the formation of prostacyclin. As mentioned previously, it appears that endothelial cells must obtain substrate from an exogenous supply (Spector et al 1981, 1983; Sivarjaan et al 1984). Platelets are capable of providing arachidonic acid and/or endoperoxides for this purpose (Bunting et al 1976; Moncada et al 1977; Marcus et al 1980; Chesterman et al 1986).

Thus the stimulation of prostacyclin release caused by serum may also be due to the provision of substrate in the form of platelet endoperoxides.

The stimulatory effects of platelets, by the provision of arachidonic acid, may be important in vivo, at sites of vascular damage. Following endothelial cell damage, platelets adhere to the exposed subendothelium, aggregate and release various factors or provide arachidonic acid. Hence, the stimulation of prostacyclin release by local endothelium may occur in response to these degranulated platelets, thus inhibiting further aggregation of platelets and limiting thrombus formation.

4.3.4.3 Variation of prostacyclin release from cultured human umbilical vein endothelial cells derived from different umbilical cords

In this study, large variations in the release of prostacyclin were observed from human umbilical vein endothelial cells derived from different umbilical cords. For example a 5-6 fold difference in the stimulation of prostacyclin release by aliquots of a standard AB human serum was observed in experiments performed to study the effects of sera from patients with systemic sclerosis on the release of prostacyclin (see later). These variations in prostacyclin synthesis are in agreement with the observations of several other authors working with endothelial cells in a variety of culture systems (Levin 1984). For example, Eldor et al (1983) found a greater than five fold variation in the synthesis of prostacyclin by bovine aortic endothelial cells.

Several factors may contribute to this variability. For instance, the maternal smoking habits of umbilical cord donors may influence the production of prostacyclin from cultured human umbilical

vein endothelial cells. Busacca et al (1984) reported a marked reduction in the capacity of cultured human umbilical vein endothelial cells to produce prostacyclin in cells derived from mild and heavy smokers. In addition, these cells were less able to grow and reach confluence. Smoking during pregnancy thus appears to induce modifications in the enzymes of the prostacyclin synthesis pathway, which persist when they are cultured in vitro. In addition, experiments in animals have demonstrated that exposure of aortic rings to nicotine results in diminished prostacyclin synthesis (Bull et al 1985). Since the age, and particularly, the smoking habits of umbilical cord donors could not be monitored in this study, it is likely that the variability in prostacyclin release from different cultures of human umbilical vein endothelial cells may be thus explained.

Prostacyclin production has been shown to diminish with increased passaging of cells in vitro (Ager et al 1979). However, in this study, all experiments were performed on human umbilical vein endothelial cells at first passage, and therefore this factor cannot account for the variation in prostacyclin release observed in this study.

Mechanical trauma, such as that occurring when growth medium is changed, may provide a stimulus for prostacyclin production. In this study the intra-assay variations were much lower than those observed between assays performed on different cultures of human umbilical vein endothelial cells. Thus it is unlikely that the variation in prostacyclin was due to mechanical trauma since this would be the same between different assays and within an individual experiment.

Finally, the administration of drugs to pregnant mothers and maternal anaesthesia were not recorded and may be a further source of variability between different cultures of human umbilical vein cells. For example, aspirin is known to have a marked inhibitory effect on prostacyclin synthesis (Roth et al 1975).

4.4 Effect of sera from patients with systemic sclerosis on prostacyclin release from cultured human umbilical vein endothelial Cells

4.4.1 Introduction

The various immunological abnormalities, such as immune complexes, antiendothelial cell and anticardiolipin antibodies, detected in the sera of patients with systemic sclerosis, may be capable of causing damage to the vascular endothelium. This could cause alterations in the production and release of prostacyclin (Goldsmith et al 1984). Thus, evidence of a prostacyclin imbalance may serve as an important indicator of altered endothelial cell function. The effect of sera from patients with systemic sclerosis on the release of prostacyclin from cultured human umbilical vein endothelial cells was therefore examined, during both short term (15 minutes) and long term (72 hours) exposures.

4.4.2 Materials and methods

4.4.2.1 Patients and Controls

Sera obtained from 28 patients with systemic sclerosis (24 women, 4 men; mean age 52.3 ± 15.3 years), were compared with sera obtained from 30 normal laboratory controls (22 women, 8 men; mean age 37.3 ± 12.5 years). An AB "standard" serum was also obtained from a single bleed of a normal donor, for use in the calculation of stimulation indices.

4.4.2.2 Endothelial prostacyclin release during exposure to 20% serum for 15 minutes

To determine the effect of sera from patients with systemic sclerosis, on the synthesis of prostacyclin from cultured human umbilical vein endothelial cells, during 15 minutes exposure, cells were incubated with 20% test or control serum. Since the mechanical

trauma arising from the washing of cells may stimulate the production of prostacyclin, a basal level of prostacyclin release, occurring in the presence of M199 alone, was first determined to quantify the basal prostacyclin release. Thus, endothelial cells were allowed to reach a steady state of prostacyclin release prior to the addition of test sera. The basal prostacyclin release was subsequently subtracted from the amount of prostacyclin released following 15 minute incubation with 20% test or control sera.

Human umbilical vein endothelial cells were seeded at a concentration of 10^5 cells / well into 24 well tissue culture plates. The cells were incubated at 37°C for 48 hours, by which time they were confluent and ready for use in prostacyclin assays.

Endothelial cells were washed once with M199, and 1ml M199 was then added to each well. After a 15 minute incubation at 37°C in a humidified atmosphere of 5% CO₂/95% air, 250µl was removed from each well and stored at -40°C prior to determination of the basal 6-keto-PGF_{1α} release. Immediately following the removal of this basal sample, 250µl of stimulatory agent was added to each well, the agent consisting of 200µl patient or control serum and 50µl M199, ie final concentration 20% serum. All tests were performed in triplicate. Following a further 15 minute incubation at 37°C, the entire contents of each well were retained for analysis of 6-keto-PGF_{1α} content.

The 6-keto-PGF_{1α} content of the 20% serum samples used as stimulatory agents were also determined, and the values thus obtained were subtracted from the 6-keto-PGF_{1α} levels in cell supernatants following the 15 minute incubations.

In addition to 20% patient and control sera, human umbilical vein endothelial cells were also incubated with 20% normal human AB serum. This serum was obtained from a single batch, and the values

obtained for prostacyclin release following incubation with aliquots of this human "AB standard" serum were used to determine "stimulation indices". All manipulations between consecutive wells were performed with a standard time interval of 15 seconds per well.

Calculation of Prostacyclin Release

$$\text{Prostacyclin Release} = A - [B + C]$$

where:-

A= 6-keto PGF₁α release following 15 minute stimulation

B= basal 6-keto-PGF₁α release x 0.75

C= 6-keto-PGF₁α content in diluted test sera x 0.25

Calculation of stimulation index

$$\text{Stimulation Index} = \frac{\text{prostacyclin release with test or control sera}}{\text{prostacyclin release with "AB Standard"}}$$

4.4.2.3 Endothelial prostacyclin release during exposure to 20% serum for 72 hours

Primary cultures of human umbilical vein endothelial cells were passaged as previously described, washed and seeded into gelatin coated 24 well plates at a density of 2×10^4 cells per well. The cells were allowed to adhere overnight and then the spent culture medium was removed and replaced with 0.5 ml M199 containing 20% patient or control sera. Within each assay, an additional control of 0.5 ml M199 containing 20% human AB serum as a standard, was also

performed. All tests were performed in triplicate. After 72 hours incubation at 37°C in a humidified atmosphere of 5% CO₂, the medium was removed and stored at -40°C prior to the determination of 6-keto-PGF_{1α} content. The remaining cells were fixed in methanol and stained by the Giemsa method. The stained endothelial cells in each well were then counted in triplicate, using an overhead microscope and 1mm square graticule, and the results expressed as a mean for all 3 wells. 6-keto-PGF_{1α} release over 72 hours was then expressed per cell and stimulation indices calculated as previously described.

4.4.2.4 Statistical analysis of data

All results are expressed as the mean \pm SEM. Comparison between patient and control group responses was made using the Wilcoxon-Mann-Whitney test.

4.4.3 Results

4.4.3.1 Endothelial prostacyclin release during exposure to 20% serum for 15 minutes

Basal prostacyclin release

The mean value obtained for the basal prostacyclin release was 1.729 ± 0.4340 nmol/l (n=11 experiments). While there was relatively little intra-assay variation between individual wells (coefficient of variation = 12-26%), the inter-assay variation was much greater, with human umbilical vein endothelial cells derived from different umbilical cords showing differing capacities to synthesize prostacyclin (coefficient of variation = 78%). However, there was little difference in the values of intra assay variation obtained for different plates of endothelial cells derived from the same umbilical cord (18.83% & 18.97%; 15.54% & 16.77%; 21.61% & 18.46%).

15 minute exposure with 20% "standard AB" serum and M199 alone

The prostacyclin release following 15 minute incubation with the 20% human "standard AB" serum and M199 alone are shown in table 8 and fig 28.

Culture medium	prostacyclin release (nmol/l) (mean \pm SEM)	n (experiments)
M199	0.1582 \pm 0.0513	11
20% Human AB serum	0.4355 \pm 0.0591	11

Table 8

The prostacyclin release (mean \pm SEM) from human umbilical vein endothelial cells in response to exposure to 20% standard AB serum or M199 alone for 15 minute periods.

Thus, the prostacyclin release following stimulation with 20% human serum was greater than that following a 15 minute incubation with M199 alone ($p < 0.01$, student's t test).

As with the results for the basal prostacyclin release, the release following exposure to 20% human AB serum or M199 alone showed large fluctuations when human umbilical vein endothelial cells were derived from different umbilical cords (coefficient of variation =

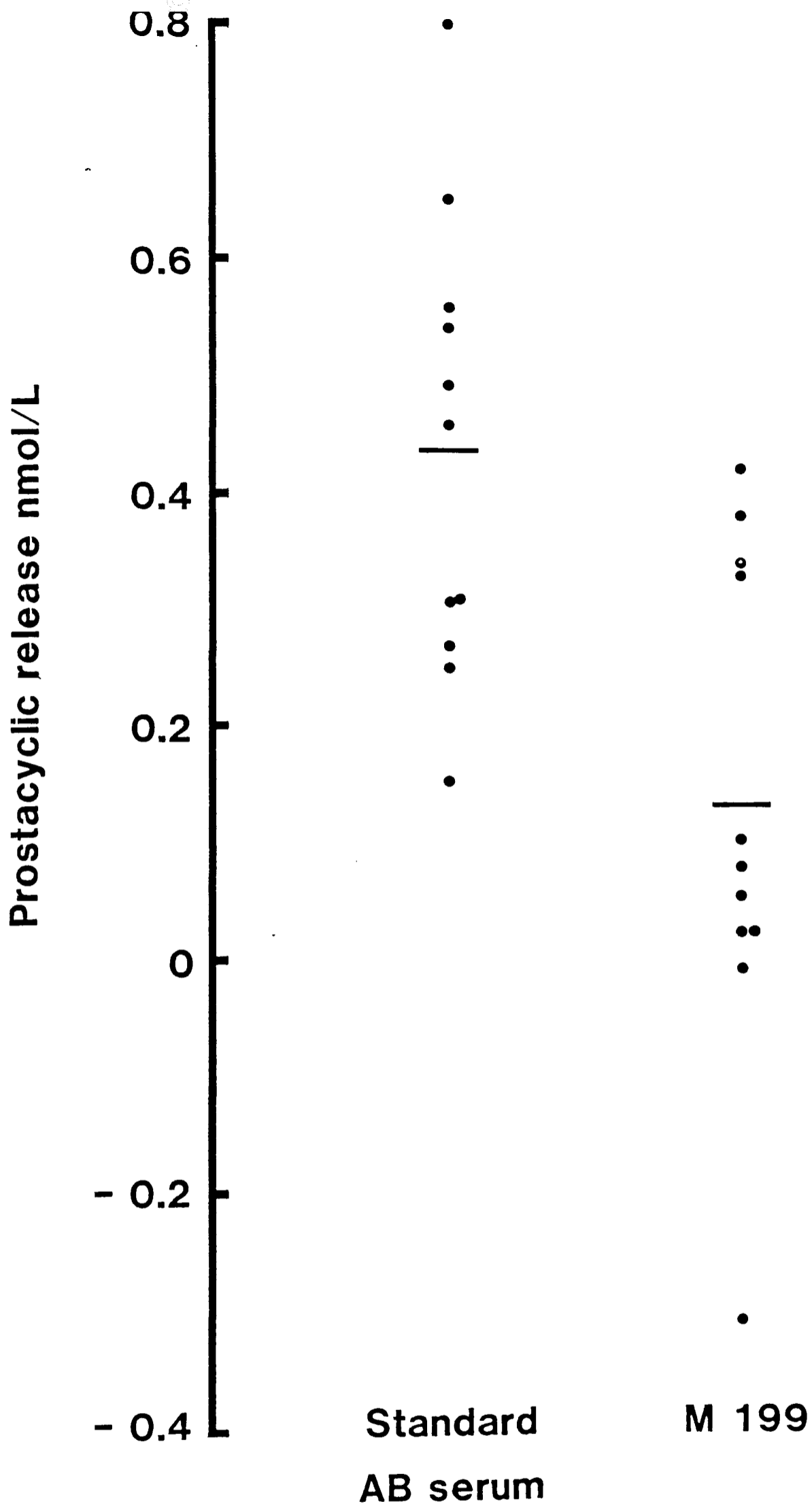


Figure 28

The prostacyclin release from human umbilical vein endothelial cells in response to exposure to 20% standard AB serum or M199 alone for 15 minute periods.

64%).

Endothelial prostacyclin release during 15 minute exposure to 20% serum from patients with systemic sclerosis and controls

As large variations were found in prostacyclin release from endothelial cells derived from different cords, direct comparisons of different assays could not easily be made. To compensate for this variability, the stimulation index was derived (as previously described). The results obtained showed no significant difference between patient and control groups. (table 9; fig 29)

Group	Stimulation index	
studied	(mean \pm SEM)	n
Systemic sclerosis	1.6698 \pm 0.2921	26
Controls	1.5452 \pm 0.1601	25

Table 9

The prostacyclin release from human umbilical vein endothelial cells (mean \pm SEM) in response to exposure to 20% serum from patients with systemic sclerosis and normal controls for 15 minute periods, expressed as a ratio (stimulation index) of that produced by aliquots of a standard 20% AB serum.

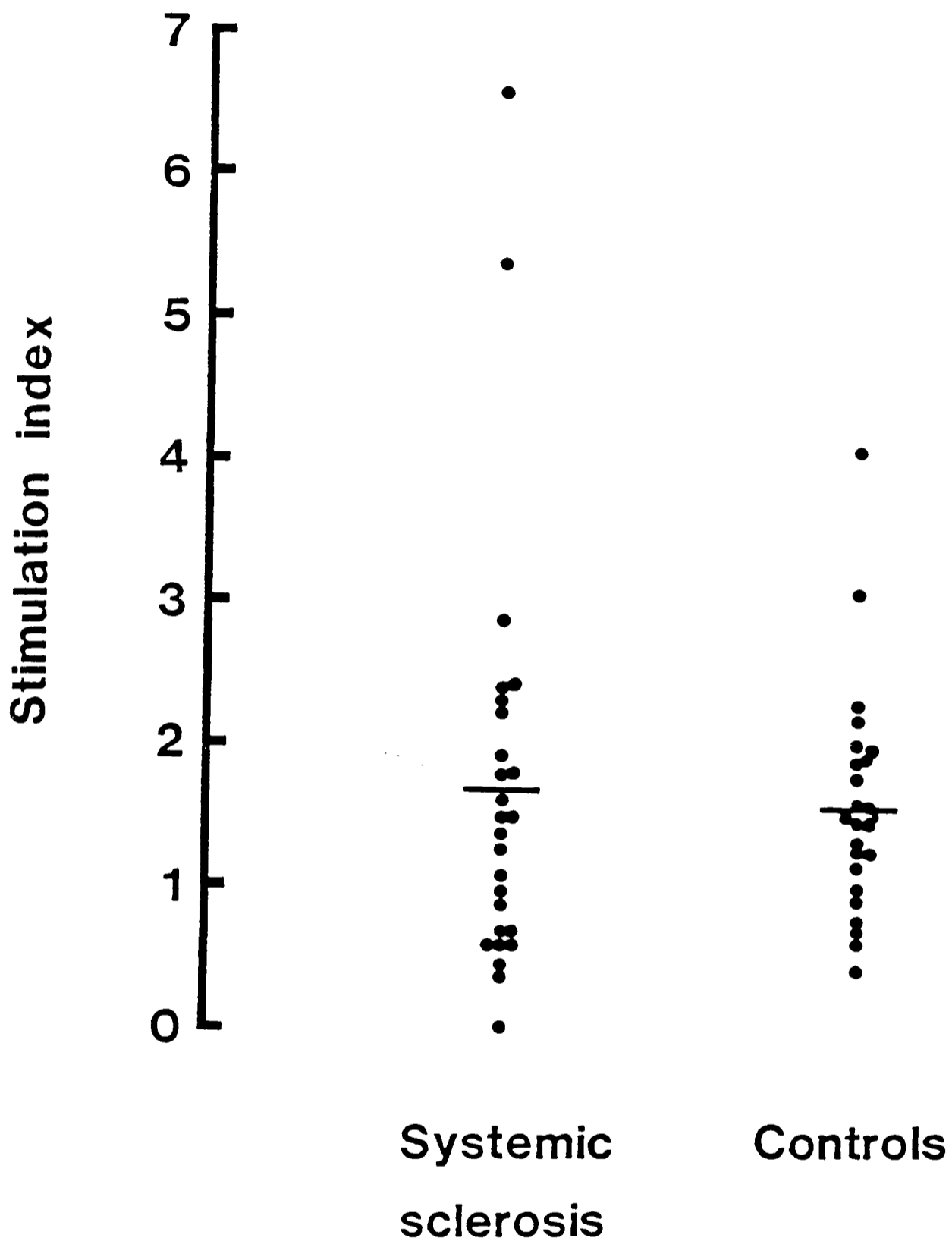


Figure 29

The prostacyclin release from human umbilical vein endothelial cells in response to exposure to 20% serum from patients with systemic sclerosis and normal controls for 15 minute periods, expressed as a ratio (stimulation index) of that produced by aliquots of a standard 20% AB serum.

4.4.3.2 Endothelial prostacyclin release during exposure to 20% serum from patients with systemic sclerosis for 72 hours

As large variations in prostacyclin release from different cultures of human umbilical vein endothelial cells were again observed, stimulation indices were once more calculated. As with the results of 15 minute exposure experiments, no significant difference was observed between patient and control groups (Table 10; Fig 30).

Group	Stimulation index	
studied	(mean \pm SEM)	n
Systemic sclerosis	1.3234 \pm 0.1438	28
Controls	1.8268 \pm 0.2813	30

Table 10

The prostacyclin release from human umbilical vein endothelial cells (mean \pm SEM) in response to exposure to 20% serum from patients with systemic sclerosis and normal controls for 72 hours, expressed as a ratio (stimulation index) of that produced by aliquots of a standard 20% AB serum.

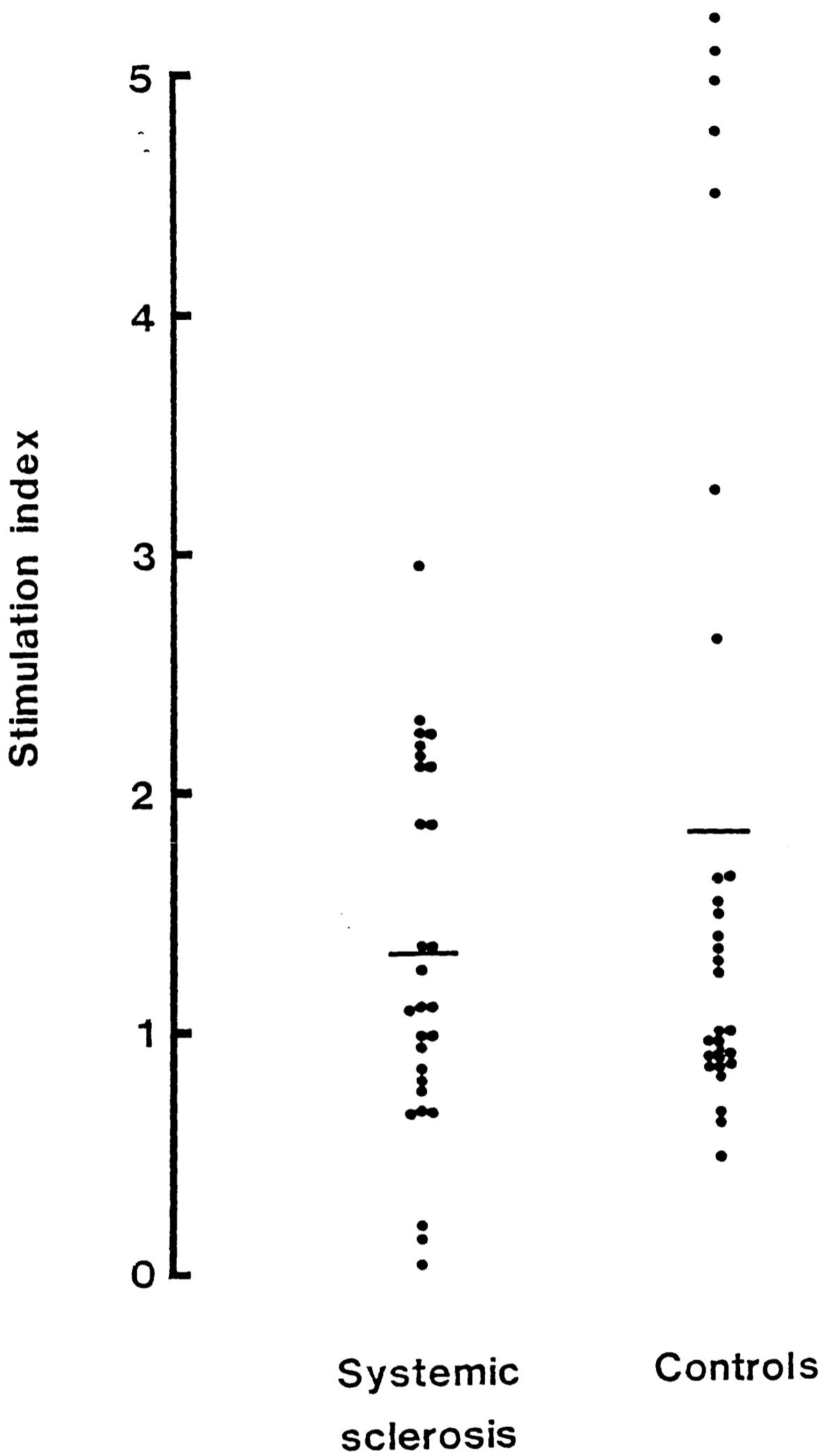


Figure 30

The prostacyclin release from human umbilical vein endothelial cells in response to exposure to 20% serum from patients with systemic sclerosis and normal controls for 72 hours, expressed as a ratio (stimulation index) of that produced by aliquots of a standard 20% AB serum.

4.4.3.3 Prostacyclin levels in sera from patients and controls

The levels of 6-keto PGF_{1α} in serum samples used in 15 minute exposure experiments showed no significant difference between patient and controls (p = NS, student's t test). Table 11; fig 31.

Group studied	Concentration of 6-keto-PGF _{1α} / nmol/L (mean ± SEM)	n
Systemic sclerosis	0.9722 ± 0.1043	20
Controls	0.7773 ± 0.0846	24

Table 11

The levels of 6-keto-PGF_{1α}(mean ± SEM) present in serum from patients with systemic sclerosis and normal controls. (Sera from 6 patients and 1 control contained levels of 6-Keto-PGF_{1α} below the lower limit of detection)

Comment

No difference was observed in the release of prostacyclin from human umbilical vein endothelial cells during 15 minute and 72 hour exposure to sera from patients with systemic sclerosis and normal controls. In addition, the serum levels of prostacyclin did not differ in the 2 groups.

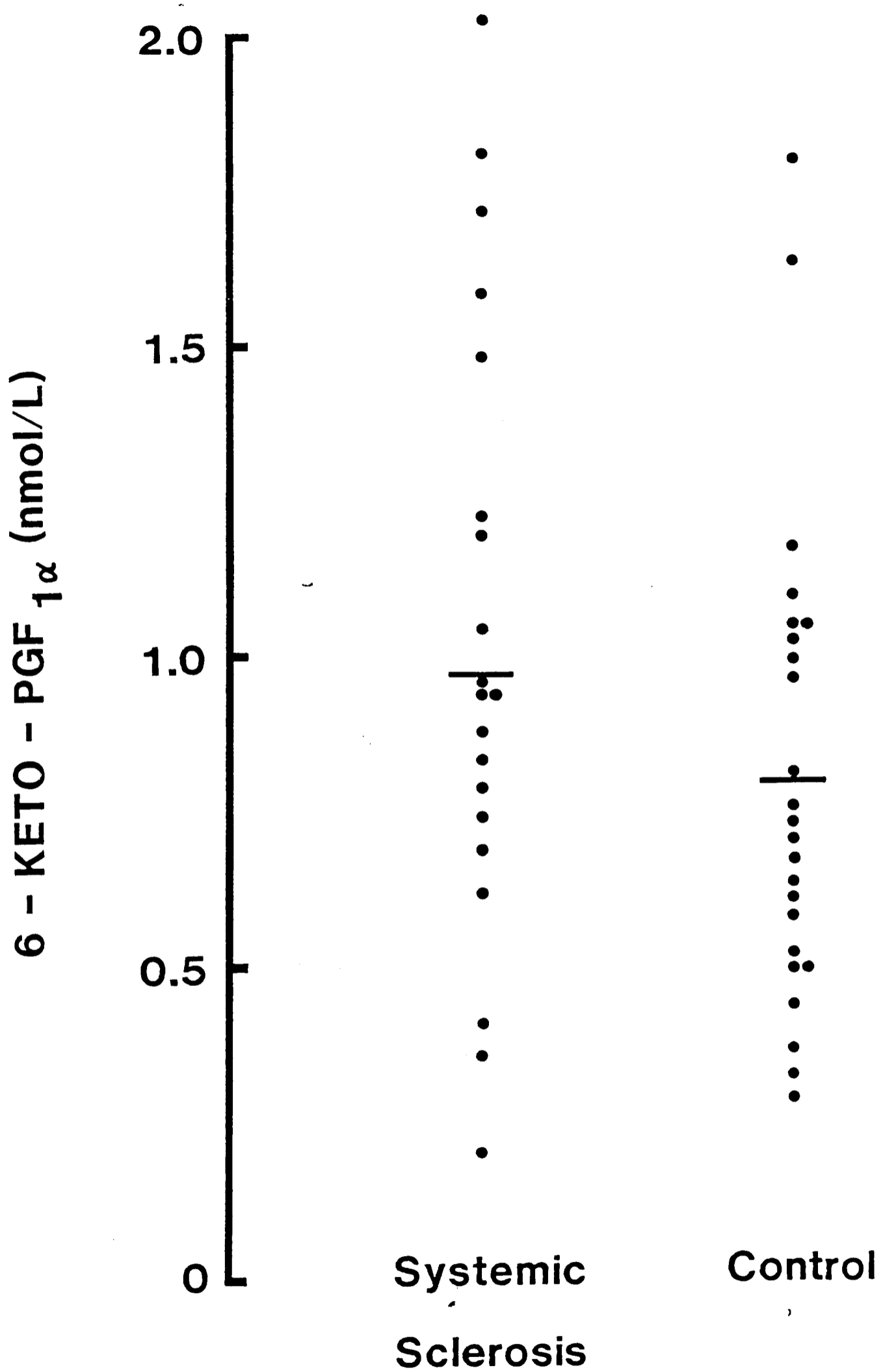


Figure 31
 The levels of 6-keto-PGF_{1α} present in serum from patients with systemic sclerosis and normal controls.

4.4.4 Discussion

4.4.4.1 Effects of sera from patients with systemic sclerosis on the release of prostacyclin from cultured human umbilical vein endothelial cells

The results obtained in these experiments clearly demonstrate that serum obtained from patients with systemic sclerosis does not alter the release of prostacyclin from cultured human umbilical vein endothelial cells, during both 15 minute and 72 hour exposure. There have been two previous reports on the effects of serum from patients with systemic sclerosis on prostacyclin production by vascular endothelial cells. A preliminary study by Evans et al (1984) also showed no difference between control (n=4) and systemic sclerosis (n=4) sera, in their effects on endothelial cell prostacyclin production of 6-keto-PGF_{1α}. However, a later study by Rustin et al (1987a) published during the course of the present study, showed a dose dependent inhibition of prostacyclin production from stimulated human umbilical vein endothelial cells produced by 13 sera from patients with systemic sclerosis. However, the differences between the findings of Rustin's study and this investigation may be more apparent than real, as the consequence of exposure to test serum may be impairment of a subsequent agonist response, even though no effect is produced during the period of exposure.

In addition, the variability in prostacyclin release from endothelial cells derived from different umbilical cords observed in this study, and has also been demonstrated by other authors, may be a factor accounting for the conflicting observations of Rustin et al (1987a) and those of the present study. The conversion of data to a stimulation index (as used in this study) could help to compensate for this variability.

A further factor that might also help to reduce the variability in prostacyclin release observed in different endothelial cell cultures would be the use of a stimulatory agent as used by Rustin et al (1987a). However, the calcium ionophore used by these authors is unphysiological and may even result in endothelial cell damage as demonstrated in some preliminary experiments carried out in this laboratory. Further studies might well consider the use of thrombin and perhaps bradykinin, both of which are more physiological stimulators of prostacyclin release.

Such future studies must also consider whether serum is the most appropriate test medium to use in investigations of patient effects on prostacyclin release by cultured endothelium. As mentioned previously, serum contains platelet derived factors that may stimulate prostacyclin release (Coughlin et al 1980) and in addition, platelet endoperoxides released during the clotting process may be used by endothelial cells in the synthesis of prostacyclin (Bunting et al 1976; Moncada et al 1977; Marcus et al 1980; Chesterman et al 1986). Thus, any inhibitory factors acting on the release of prostacyclin occurring in vivo would be masked by the stimulation produced by such factors present in serum. Platelet poor plasma is more relevant to in vivo situations and therefore should be used in future studies on the effect of prostacyclin release from cultured endothelial cells.

Thus, although the results obtained in this study did not demonstrate a serum effect from patients with systemic sclerosis on endothelial cell prostacyclin production, further studies employing platelet poor plasma and possibly a stimulated prostacyclin response, are required in order to clarify the discrepant findings of the present study and those obtained by Rustin et al (1987a).

4.4.4.2 Serum levels of 6-keto-PGF_{1α} in patients and controls

This study has also demonstrated that the serum levels of 6-keto-PGF_{1α} in patients with systemic sclerosis are not significantly different to the levels in normal control sera. These results are in agreement with the findings of Evans et al (1984) who also measured the levels of 6-keto-PGF_{1α} by radio-immunoassay, in 18 patients with systemic sclerosis and 12 normal controls, and found no significant difference between the two groups.

However, in contrast to these observations, Belch et al (1984) reported elevated plasma levels of 6-keto-PGF_{1α} in patients with systemic sclerosis. The determinations of Belch and co-workers were derived from plasma samples, in contrast to the serum samples used by both Evans et al (1984) and in this study. These differences may be partly responsible for the discrepancies but also methodological problems in the determination of plasma 6-keto-PGF_{1α} levels have been described (Morris et al 1981; Greaves and Preston 1982). In addition, differences in the extent and severity of the systemic sclerosis in the various subgroups of patients studied, could be a further factor responsible for the conflicting results obtained to date.

CHAPTER 5

MEASUREMENT OF VON WILLEBRAND FACTOR ANTIGEN,
ANTICARDIOLIPIN ANTIBODIES AND IMMUNE COMPLEXES
IN PATIENTS WITH SYSTEMIC SCLEROSIS

5.1 Measurement of Factor VIII complex in patients with systemic sclerosis

5.1.1 Introduction

There is widespread evidence for the presence of vascular and endothelial cell abnormalities in patients with systemic sclerosis. Von Willebrand factor antigen and its functional activity, which is measured as ristocetin co-factor, are manufactured by the endothelium and are known to be elevated in various diseases characterised by endothelial cell injury, for example, diabetes mellitus with proliferative retinopathy; atherosclerosis and renal failure (Coller et al 1978; Green et al 1978; Warrel et al 1979).

In this study, therefore, patients with systemic sclerosis were investigated for evidence of endothelial damage by measurement of von Willebrand factor antigen and ristocetin co-factor. However, as von Willebrand factor antigen has been described as participating in acute phase responses, parallel measurements of both factor VIII coagulant and C reactive protein were also carried out.

5.1.2 Materials and methods

Patients

For this part of the investigation, 28 patients with systemic sclerosis were studied (24 women, 4 men; mean (\pm SD) age 52.8 ± 14.7 years).

The extent (and severity) of the visceral involvement produced by the disease was defined by a standard investigative protocol which enabled points to be awarded for cutaneous and visceral involvement and so produce a "disease score" for each patient (see table 2.) Patients were thus divided into categories of "severe" (disease score ≥ 6) and "mild" disease (disease score < 5)

Von Willebrand factor antigen

Von Willebrand factor antigen was determined by an enzyme-linked immunosorbent assay as described by Short et al (1982) (reference range 0.5-1.5 u/ml)

Factor VIII coagulant

Factor VIII coagulant was assayed by a 2-stage technique based upon the thromboplastin generation test (Biggs et al 1955) [reference range 0.5-1.5 u/ml].

Ristocetin co-factor

The ristocetin co-factor was measured using fixed washed platelets and a platelet counting technique (Evans & Austen 1977) [reference range 0.5-1.5 u/ml].

C-reactive protein

C-reactive protein was measured by single radial immunodiffusion (Milford-Ward 1986). Monospecific antiserum to human C-reactive protein was obtained from PRU Central Antiserum Purchasing Unit, Sheffield. Standardization of the assay was in relation to the WHO International Standard for C-reactive protein 1986 (NIBSC 85/506) [reference range 0-10 mg/l].

All reference ranges were based on determinations made in 40 normal, healthy laboratory staff.

Statistical methods

Comparison of factor VIII complex levels in "severe" and "mild" patient subgroups was by Student's t test while the relationship between disease score and factor VIII complex levels was examined by regression analysis.

5.1.3 Results

Von Willebrand factor antigen

Elevated levels of von Willebrand factor antigen were found in 12 patients with systemic sclerosis, 10 of whom showed severe extensive visceral disease. There was a positive correlation ($r=0.60$ $p<0.001$) between the extent of visceral involvement (ie severity of disease) and plasma levels of von Willebrand factor antigen (fig 32). 16 patients with severe disease had von Willebrand factor antigen levels of 1.73 ± 0.57 u/ml, whereas those with mild disease had levels of 1.03 ± 0.38 u/ml ($p<0.001$).

Factor VIII coagulant

By contrast, plasma levels of factor VIII coagulant were elevated in only 3 patients with raised von Willebrand factor antigen and in only 5 patients overall. The general failure of factor VIII coagulant to parallel increases in von Willebrand factor antigen was illustrated by a lack of correlation between factor VIII coagulant levels and disease score ($r = 0.30$; NS).

Ristocetin co-factor

Again, levels of ristocetin co-factor also failed to parallel the increases in von Willebrand factor antigen and so also failed to show any correlation with disease score ($r = 0.14$; NS).

C-reactive protein

Elevated levels of C-reactive protein were found in only 3 patients, 2 of whom were in the "severe disease" group.

5.1.4 Discussion

This study has shown that patients with systemic sclerosis have elevated levels of circulating von Willebrand factor antigen. This observation is in agreement with the smaller studies of Kahaleh et al (1982) and Lee et al (1985), who also showed increased levels of von

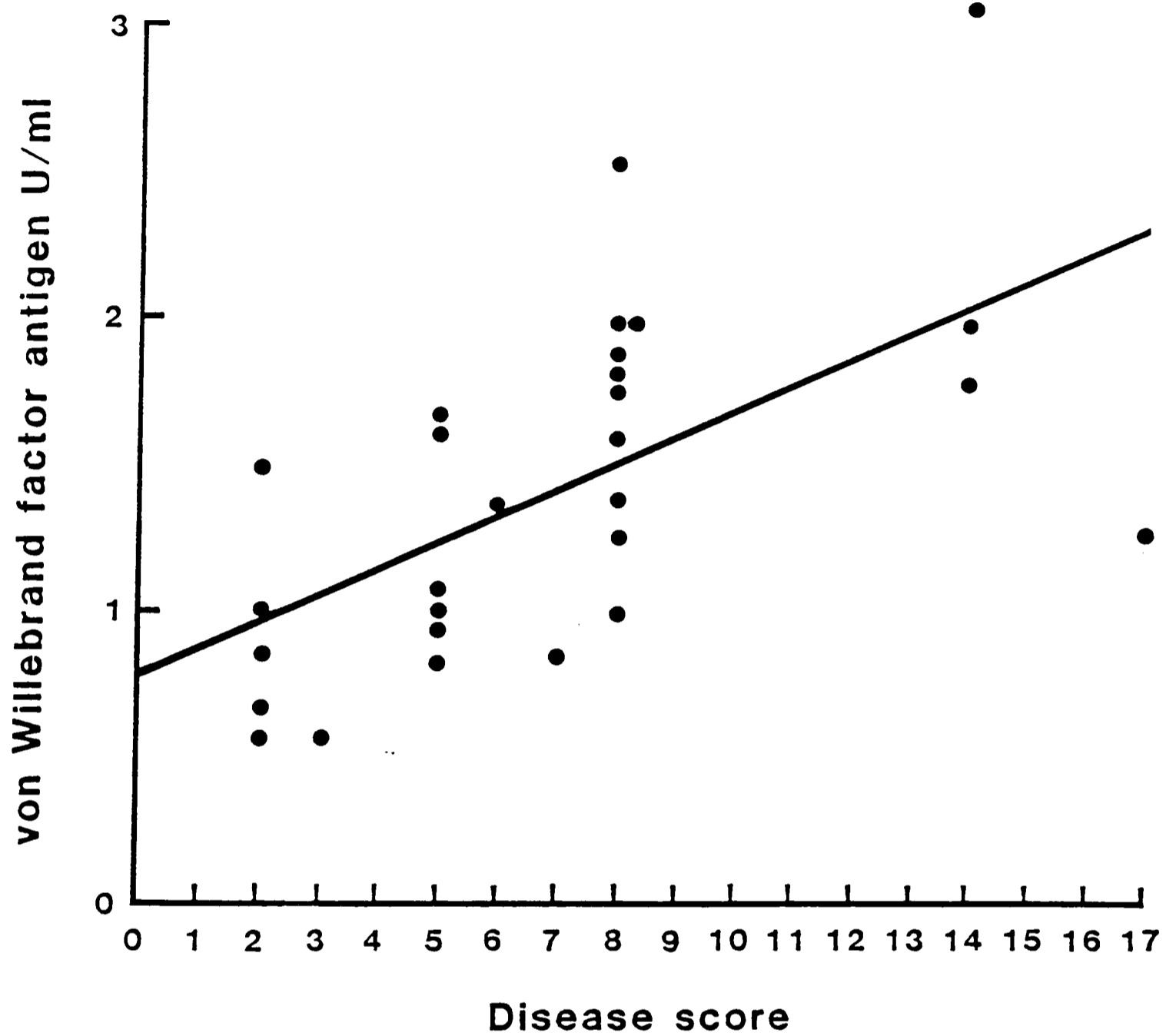


Figure 32

The correlation between the extent of visceral disease (disease score) and von Willebrand factor antigen in 28 patients with systemic sclerosis. $r = 0.60$; $p < 0.001$.

Willebrand factor antigen in patients with systemic sclerosis. These authors, however, failed to fully exclude an acute phase response by carrying out parallel determinations of all components of the factor VIII complex as well as C-reactive protein.

The results obtained in this study indicate that the rise in von Willebrand factor antigen is in fact due to in vivo endothelial cell damage rather than an acute phase response, since if this were the case, parallel rises would be shown by factor VIII coagulant and C-reactive protein.

The present study has also shown that ristocetin co-factor fails to parallel the rise in von Willebrand factor antigen, in patients with systemic sclerosis. The failure of the functional component of the factor VIII complex to parallel von Willebrand factor antigen levels provides further confirmation that the elevated levels of von Willebrand factor antigen are due to endothelial cell injury with the release of functionally abnormal or incomplete molecules (Carvalho et al 1982).

This study has reported a marked association between elevated levels of von Willebrand factor antigen and extensive visceral disease, confirming the previous smaller study of Lee et al (1985). This relationship is probably a reflection of the more widespread and extensive vascular pathology present in patients with visceral disease. Pulmonary involvement was present in all patients with severe and extensive visceral disease, and damage to the rich vascular bed present in the lungs, is likely to account for the elevated levels of von Willebrand factor antigen.

5.2 Measurement of anticardiolipin antibodies in patients with systemic sclerosis

5.2.1 Introduction

Various immunological abnormalities, such as autoantibodies and circulating immune complexes, which occur in SLE have also been described in patients with systemic sclerosis (Bernstein et al 1984; Catoggio et al 1983; Pisko et al 1979; Hughes et al 1983; and Seibold et al 1982). The presence of anticardiolipin antibodies in patients with SLE is particularly interesting in view of the association of these autoantibodies with a thrombotic tendency, through their possible interaction with endothelial cells and platelets (Harris et al 1983). It was felt that it would be potentially relevant, therefore, to look for these autoantibodies in patients with systemic sclerosis, especially in view of the evidence for endothelial cell damage and the vascular pathology occurring in the disease (Norton & Nardo 1970).

5.2.2. Materials and methods

The same 28 patients who were studied in relation to altered levels of the factor VIII complex were also investigated for the presence of anticardiolipin antibodies.

Plasma levels of anticardiolipin antibodies were determined by ELISA. 30 μ l of cardiolipin in ethanol (50 μ g of cardiolipin/ml) was added to each well of a rigid 96 well microtitre plate. The cardiolipin was evaporated overnight at 4 $^{\circ}$ C and the plate was washed 3 times with PBS. 75 μ l/well of 10% adult bovine serum was added to each well and incubated at room temperature for 1 hour. The adult bovine serum was discarded and the plate washed once with PBS. 50 μ l of either a standard or test plasma diluted in 10% adult bovine serum were added to each well and incubated at room temperature for 3 hours.

The wells were washed 3 times with PBS after which 50ul of a 1/1000 dilution of alkaline phosphatase conjugated goat antiserum was added to each well and the plates left for 1.5 hours. The plates were washed 3 times in PBS and 50ul of well mixed freshly prepared p-nitrophenyl phosphate solution (1µg/ml in diethanolamine buffer pH 9.8) was added to each well. After incubating for 30 ± 15 minutes in the dark at 37°C in a "wet box" the absorbance was measured at 405nm using a Titertek Multiscan. Results were expressed as anticardiolipin units calculated against IgG and IgM anticardiolipin standards kindly donated by Dr. N. Harris, the Rayne Institute, London, to give reference ranges of 0-5.2 u and 0-4.4 u for IgG and IgM antibodies, respectively, based on determinations (mean ± 2 SD) in 40 normal laboratory staff and blood donors. The specificity for cardiolipin was confirmed by the absence of any consistent reactivity of the assay with sera containing either high titre rheumatoid factors or myeloma paraproteins.

5.2.3 Results

The titres of anticardiolipin antibodies in the total group of patients with systemic sclerosis and in the mildly and severely affected subgroups are shown in Table 12. While the mean value in all the patient categories fell within the limits (mean ± 2 SD) of the normal reference range, 7 patients had elevated levels of IgG and/or IgM anticardiolipin antibodies (Table 13). 5 of these 7 cases were in severely affected patients, including all 3 cases with the highest titre of anticardiolipin antibody. By contrast, only 2 of the mildly affected subgroup had elevated levels of anticardiolipin antibody (Table 13)

Table 12

Anticardiolipin antibodies in patients with systemic sclerosis (SS).

	Patient groups		
	<hr/>		
Anticardiolipin antibodies (mean \pm SD)	Total SS (n = 28)	Mild SS (n = 12)	Severe SS (n = 16)
IgG (Ref. range 0-5.2 u)	3.55 \pm 2.97	2.70 \pm 1.75	4.21 \pm 3.58
IgM (Ref. range 0-4.4 u)	2.10 \pm 2.04	1.85 \pm 1.49	2.31 \pm 2.42

Table 13

Titres of anticardiolipin antibodies (SD above mean value of reference range) in relation to the severity of disease in 28 patients with systemic sclerosis (SS)

	Patient groups		
	Total SS	Severe SS	Mild SS
Anticardiolipin antibodies (IgG/IgM) (n = 28)	(n = 16)	(n = 12)	
> 2 SD	7	5	2
> 3 SD	4	3	1
> 5 SD	3	3	0

5.2.4 Discussion

This investigation has demonstrated the occurrence of raised levels of anticardiolipin antibodies in 20-25% of patients with systemic sclerosis, and is the most comprehensive study to date, enlarging on preliminary reports of occasional and generally low titre anticardiolipin antibodies in these patients (McHugh et al 1987; Baguley et al 1987b).

In the severely affected subgroup of patients, 4 of 5 patients with anticardiolipin antibodies also showed elevated levels of von Willebrand factor antigen. These associations suggest that anti-cardiolipin antibodies may somehow be involved in the pathogenesis of systemic sclerosis. Although none of the patients had a history of major thrombosis, the possibility that anticardiolipin antibodies may be capable of causing vascular damage by other means than the thrombotic tendency associated with their presence in SLE, has to be considered. While experiments suggesting that the anticardiolipin antibodies found in patients with SLE may inhibit prostacyclin production by vascular endothelium (Carreras and Vermylen 1982; Schorer and Watson 1987) have not been uniformly reproducible (Rustin et al 1987b; Petraiuolo et al 1987) there is evidence that these autoantibodies can react with phospholipid antigens in platelets (Cortelazzo et al 1987; Khamashta et al 1987). Such a reaction could lead to the release of platelet products, such as β thromboglobulin, transforming growth factor β and serotonin, which can either have an inhibitory effect on vascular endothelium (Heimark et al 1986) and/or stimulatory effects on fibroblasts with the resulting development of both vascular lesions and perivascular collagen deposition which are prominent features of systemic sclerosis.

5.3 Measurement of immune complexes in patients with systemic sclerosis

5.3.1 Introduction

The presence of elevated levels of immune complexes in patients with systemic sclerosis is well described with, however, a highly variable incidence (Pisko et al 1979; Swierczynska et al 1984; Cunningham et al 1980; Hughes et al 1983). In this study, immune complexes were determined by the method of C1q binding, and the results correlated with severity of disease in patients with systemic sclerosis.

5.3.2 Materials and methods

Patients

The previous 28 patients with systemic sclerosis were also investigated for the presence of immune complexes.

C1q binding assay

Immune complexes were detected using the C1q binding assay, based on the method of Zubler & Lambert (1976). C1q was prepared from fresh normal human serum and iodinated by lactoperoxidase. The method was modified by the use of 4% polyethylene glycol to precipitate macromolecular-bound C1q, used heat aggregated (63°C for 30 minutes) Cohn fraction II to construct standard curves (0.1 mg/ml to 3.0 mg/ml) and gave a reference range of 0.8-13.8%, based on determinations made in 40 normal healthy laboratory staff.

5.2.3 Results

The results for C1q binding activity in the total group of patients with systemic sclerosis and in the mildly and severely affected subgroups are summarized in table 14. Increased C1q binding activity was found in 7 patients with systemic sclerosis, the

Table 14

C1q binding activity in patients with systemic sclerosis (SS)

	Patient groups		
	Total SS (n = 28)	Mild SS (n = 12)	Severe SS (n = 16)
C1q-binding (mean \pm SD) (Ref range 0.8-13.8%)	12.27 \pm 14.18	6.25 \pm 2.73	17.44 \pm 17.84

increases being confined to patients severely affected by the disease who had mean levels of $17.44 \pm 17.84\%$ in contrast to the $6.25 \pm 2.73\%$ of the mildly affected subgroup ($p < 0.05$, student's t test)

5.2.4 Discussion

This study has reported the occurrence of elevated levels of immune complexes in 7 of 28 patients with systemic sclerosis and further emphasizes earlier reports suggesting that systemic sclerosis is a disease characterised by immune complexes (Pisko et al 1979; Hughes et al 1983). In addition, immune complexes were found to be associated with extensive visceral disease as previously reported by these authors.

In patients with extensive visceral involvement, 4 of the 5 patients with anticardiolipin antibodies also had elevated levels of von Willebrand factor antigen, including the 3 patients with the highest titre of anticardiolipin antibodies. In addition, elevated C₁q binding activity and/or anticardiolipin antibodies occurred in 7 of the 10 patients with elevated levels of von Willebrand factor antigen.

Such a correlation between immune complexes, anticardiolipin antibodies and elevated levels of von Willebrand factor antigen, occurring in severely affected patients, emphasizes the multifactorial pathogenesis of systemic sclerosis, which is further discussed in the following chapter.

CHAPTER 6

FINAL DISCUSSION

6.1 Summary and conclusions

Although the pathogenesis of systemic sclerosis remained uncertain for many years, the last fifteen years has seen a steady accumulation of evidence in support of the concept of vascular involvement (Campbell and LeRoy 1975). Marked vascular lesions are a prominent feature of systemic sclerosis with Raynaud's phenomenon, digital ischaemia and telangiectasia occurring as characteristic features of the disease, while pathologically, a specific arterial lesion is a distinctive feature of visceral lesions (Norton & Nardo 1970).

In addition, it has over the same period been realized that marked immunological abnormalities are found in patients with systemic sclerosis, including a very high incidence of autoantibodies and immune complexes (Bernstein et al 1982; Hughes et al 1983). These findings begin to suggest that systemic sclerosis, like the related connective tissue disease of SLE, could also have an autoimmune aetiology. In SLE, pathological processes produced by the circulating immune complexes and various autoantibodies have been much more clearly defined than in systemic sclerosis. However, the clinical association and the occurrence in both, of autoantibodies, immune complexes and abnormalities of the cellular immune system, suggest very strongly that systemic sclerosis may also be an autoimmune disease (Tuffanelli & Winkelmann 1962).

The findings of this study provide further support for the occurrence of vascular injury in systemic sclerosis and more interestingly, suggest that such damage may be immunologically mediated. In addition to the in vitro demonstration of ADCC of vascular endothelial cells, produced by sera from approximately 20% of

patients with systemic sclerosis, in vivo evidence of endothelial injury has been provided by the detection of elevated plasma levels of von Willebrand factor antigen. The presence of immune complexes and anti-cardiolipin antibodies in patients with systemic sclerosis are additional important findings of this study. Such immunological abnormalities have the potential to cause vascular damage by mechanisms additional to ADCC and therefore it is probable that systemic sclerosis has a multifactorial pathogenesis similar to SLE.

No fully satisfactory in vivo model of systemic sclerosis is available to study the development of the vascular lesion. The models which do exist include experimentally induced graft versus host disease in the rat as described by Stastny et al (1963). However, although chronic graft versus host disease displays many of the pathological features of systemic sclerosis, several features not seen in human systemic sclerosis are also observed, including haemolytic anaemia, thrombocytopenia and polyarthrititis (Stastny et al 1963). In addition, the vascular lesions, when present in the graft versus host disease model, differ from those occurring in patients with systemic sclerosis (Jimenez 1987). Thus, this system does not provide an ideal model to investigate the vascular lesions which occur in systemic sclerosis.

The avian model of systemic sclerosis described by Gershwin et al (1981) is probably the best in vivo model so far described. However, the vascular lesions once more differ from those seen in human systemic sclerosis (Jimenez 1987). Another model, the tight skin mouse, although demonstrating the accumulation of collagen seen in systemic sclerosis, fails to show the inflammatory and immunological features of the disease (Green et al 1976).

Hence, in the present study, an in vitro system was adopted to investigate the vascular injury occurring in systemic sclerosis. ADCC of the vascular endothelium is a pathogenic mechanism which may well account for some of the vascular lesions which are seen in patients with this disease. The endothelium is an obvious primary target cell since it is in direct contact with circulating autoantibodies and peripheral blood effector cells which are thus ideally placed to interact and produce vascular damage by the mechanism of ADCC. However, in spite of this, ADCC has not been widely investigated as a pathogenic mechanism in systemic sclerosis, the only previous study being that of Penning et al (1983; 1984b). The advantages of the in vitro assay used in the present study is that it provides a totally homologous human system, in contrast to previously investigated assays where various transformed cell lines and chicken red blood cells have been used as target cells. Thus, heterologous systems have previously been employed despite the requirement for an entirely human system in which the mechanism of ADCC can be investigated.

The present study has demonstrated the occurrence of ADCC against cultured venous endothelial cells by sera from approximately 20% of patients with systemic sclerosis, following co-culture with normal human peripheral blood mononuclear cells. These results confirm the earlier study by Penning et al (1984b) who reported a similar incidence of ADCC caused by sera from these patients. In addition, this study has demonstrated, as a new finding, the occurrence of ADCC against human arterial endothelial target cells. This finding strengthens the view that ADCC is a pathogenic mechanism in systemic sclerosis, in view of the prevalence of arterial lesions in systemic sclerosis (Campbell and LeRoy 1975).

This study has also shed further light on the nature of the serum factor capable of causing cytotoxicity of vascular endothelium. Preincubation of target cells with serum has revealed that the responsible factor is able to bind to and presensitize vascular endothelial cells. This finding suggests that an antiendothelial cell antibody may be the ADCC inducing factor, and it was further supported by evidence obtained following column chromatography of serum. IgG prepared by both ion exchange and affinity chromatography, revealed that IgG was in fact the responsible cytotoxic factor in the majority of cases. In addition, gel filtration chromatography of sera with ULTROGEL provided further evidence that ADCC inducing activity of sera was restricted to monomeric 7S IgG fractions. These observations suggest that antiendothelial cell antibodies may be responsible for mediating ADCC against vascular endothelium and have been further supported by recent studies which have used an ELISA technique to demonstrate the presence of antiendothelial cell antibodies in patients with systemic sclerosis. It is also interesting to note that the incidence of these antibodies, as detected by ELISA, was similar to the incidence of ADCC described in this study (Hashemi et al 1987; Baguley et al 1987a; Byron et al 1987).

A further observation made in this study was that, in the majority of cases, sera from patients with systemic sclerosis and SLE maintained the ability to mediate ADCC of vascular endothelial cells, when investigated serially over a period of two years. Thus, it is likely that damage to the vascular endothelium by ADCC is an important pathological mechanism occurring throughout the duration of these diseases, and may be one of the initial events contributing to the fibrosis which is such a characteristic feature of systemic sclerosis.

Antiendothelial cell antibodies, which may be responsible for the vascular damage observed in systemic sclerosis, have previously been described in patients undergoing renal allograft rejection, where they may be important in mediating endothelial cell damage (Cerilli et al 1977). In addition, antiendothelial cell antibodies have also been described in patients with SLE (Cines et al 1984; Shingu and Hurd 1981 and Le Roux et al 1986). The occurrence of ADCC of vascular endothelium by sera from 7 of 49 patients with SLE was also observed in this study, and further analysis of these positive sera produced findings similar to those obtained with scleroderma sera. Thus, antiendothelial cell antibodies appear to mediate ADCC in patients with both systemic sclerosis and SLE, although it should be stressed, with a much lower incidence in the latter disease.

In contrast to the ability of sera to presensitize target cells, the ability to presensitize effector cells only occurred with serum from one patient. Thus, in the majority of sera capable of mediating ADCC, "arming" of effector cells as described by Greenberg & Shen (1973) did not occur. However, sera from one patient which consistently demonstrated ADCC following preincubation of effector cells, may be capable of this effector cell "arming", an effect which could well be due to the presence of small sized immune complexes. Alternatively, a cytokine could cause the activation of effector cells to induce endothelial cell cytotoxicity such as occurs with lymphokine activated killer cells (Miltenburg et al 1987). It has actually been suggested that various cytokines may be involved in the pathogenesis of systemic sclerosis. For example, interleukin 1 is capable of enhancing fibroblast proliferation (Schmidt et al 1982). Interleukin 2 may cause the activation of killer cells which could then be an additional factor in inducing endothelial cell damage. However

induced, endothelial damage would lead to exposure of the underlying collagen, with resulting platelet activation and the release of various platelet products, such as Transforming growth factor β , serotonin, platelet derived growth factor and β thromboglobulin, which would then exert their effects on fibroblasts, as described later (Miltenberg et al 1987).

The occurrence of ADCC against vascular endothelial cells demonstrated in this study tended to occur in patients with mild rather than extensive visceral involvement. This indicates that ADCC could be important in the initial stages of systemic sclerosis. It is likely that vascular injury occurs as a primary event (Campbell and LeRoy 1975; Norton and Nardo 1970) with fibrosis occurring after the initial vascular lesion. Thus ADCC is potentially an important pathogenic mechanism in the development of systemic sclerosis.

In addition to the direct demonstration of vascular damage in vitro produced by ADCC, further evidence of endothelial cell injury in vivo, has also been provided by the occurrence of elevated plasma levels of von Willebrand factor antigen in patients with systemic sclerosis. Von Willebrand factor antigen has previously been reported as elevated in patients with various diseases characterised by vascular damage, for example diabetes, atherosclerosis and renal failure (Coller et al 1978; Green et al 1978; Warrel et al 1979). The findings described in this study confirm and extend the previous work of Kahaleh et al (1981); Pagono et al (1986); and Lee et al (1985) who also demonstrated elevated levels of von Willebrand factor antigen in patients with systemic sclerosis. However, these earlier studies failed to measure all three components of the factor VIII complex (von Willebrand factor antigen, factor VIII coagulant and ristocetin co-factor) simultaneously. The findings of the present study imply

that the elevated levels of von Willebrand factor antigen result from endothelial cell damage rather than an acute phase response, since factor VIII coagulant levels failed to show a parallel increase. In addition, the absence of C reactive protein response in most of the patients adds further support that the increase in von Willebrand factor antigen is not an acute phase response. Finally, this study has shown that ristocetin co-factor activity also fails to parallel von Willebrand factor antigen elevation, a finding which provides additional support for the view that in vivo endothelial damage occurs in patients with systemic sclerosis.

4 of 10 patients who demonstrated "excess % specific cytotoxicity" of >10% also had elevated plasma levels of von Willebrand factor antigen. 3 of these patients had the highest disease scores in the group of patients capable of mediating ADCC against vascular endothelium, and were in the "severe disease" subset of patients. Thus the occurrence of both ADCC of vascular endothelial cells and the in vivo finding of elevated plasma levels of von Willebrand factor antigen appears to define patients characterized by extensive visceral involvement. In patients where a correlation was not observed between elevated levels of von Willebrand factor antigen and ADCC of vascular endothelium, the extent and severity of the vascular involvement in these patients may not be sufficient to cause increased levels of von Willebrand factor antigen, thus reflecting the sensitivity of ADCC as an indicator of vascular damage.

In addition to the autoimmune mechanism of ADCC which has been demonstrated by this study, in 20% of patients with systemic sclerosis, various other immunological abnormalities have also been detected in these patients.

The demonstration of anticardiolipin antibodies in 20-25% of patients with systemic sclerosis confirms and extends the results of earlier and smaller studies (McHugh et al 1987; Baguley et al 1987b). Anticardiolipin antibodies have previously been described in patients with SLE, where they distinguish a subset of patients characterised by a thrombotic tendency (Harris et al 1983). Although thrombosis is not a common feature of systemic sclerosis, it is possible that these antiphospholipid antibodies could play a pathogenic role in systemic sclerosis and may be responsible, at least in part, for the vascular lesions present in patients with this disease, via a mechanism different to that occurring in SLE. Such a view is supported by the interesting association between the presence of these antibodies, severe vascular damage and elevated plasma levels of von Willebrand factor antigen, as revealed by this investigation. Another interesting finding was the occurrence of anticardiolipin antibodies in 4 of 5 patients with SLE who also demonstrated ADCC of vascular endothelial cells. This association strongly suggests the importance of these antibodies in mediating ADCC of vascular endothelial cells and is supported by the recent observation of Vismara and co-workers who showed that affinity purified anticardiolipin antibodies were able to react with intact human endothelial cells (Vismara et al 1988).

Other mechanisms by which anticardiolipin antibodies could mediate vascular injury in systemic sclerosis include damage via complement activation (Norberg et al 1987). Even more important, anticardiolipin antibodies are capable of reacting with phospholipid antigens in platelets, the resulting platelet activation being followed by the release of platelet derived products (Cortelazzo et al 1987; Khamashta et al 1987). These platelet products would then be available to exert their effects on both the endothelium and underlying

vascular and connective tissue. For example, transforming growth factor β has been shown to inhibit endothelial cell proliferation and angiogenesis in vitro (Heimark et al 1986; Takehara et al 1987; Muller et al 1987; Frater-Schroder et al 1986). β thromboglobulin may also be important, in that it causes an inhibition of prostacyclin release (Hope 1979) and has been shown to be elevated in systemic sclerosis (Kahaleh et al 1982). Transforming growth factor β also exerts effects on fibroblasts and may alter the phenotypic expression of these cells to produce more collagen (LeRoy 1987). The recent study by Falanga et al (1987) which demonstrated a selective increase in glycosaminoglycan synthesis by cultures of fibroblasts obtained from patients with systemic sclerosis, is thus potentially relevant.

In addition, anticardiolipin antibody containing sera have been shown to inhibit the release of prostacyclin from cultured endothelial cells (Carreras and Vermylen 1982; Schorer and Watson 1987) although other studies have failed to confirm these observations (Rustin et al 1987b; Pet raillo et al 1987). Thus further work is required in this area to clarify these discrepant observations.

This study has also confirmed previous reports demonstrating the occurrence of immune complexes in the sera of patients with systemic sclerosis and has reemphasized the association of these immune complexes with extensive visceral disease. The formation of immune complexes is likely to result from the interaction of autoantibodies found in patients with systemic sclerosis, with cellular antigens released following cell breakdown. Although there is no direct proof that these immune complexes are pathogenic in systemic sclerosis, immune complexes occurring in other diseases are known to be capable of causing lesions. For example, in SLE, immune complexes are frequently found deposited in vessel walls and in the kidney where

they give rise to vasculitis and lupus nephritis. Immune complexes have also been described deposited in the kidneys of patients with systemic sclerosis (McGiven et al 1971) where they may give rise to vascular injury by various mechanisms. For instance, by interaction with components of the complement system, a chemotactic stimulus for neutrophils can be produced with resulting tissue and endothelial cell damage (Weiss et al 1981). In addition, immune complexes could interact with Fc receptors on lymphocytes and produce the effector cell arming described by Greenberg and Shen (1973) resulting in further vascular damage by the mechanism of ADCC (Penning et al 1984c). However, in this study, only two patients with systemic sclerosis capable of causing ADCC of vascular endothelial cells showed elevated levels of immune complexes by the method of C1q binding. Although serum from one patient observed in this study was capable of such arming of effector cells, with subsequent vascular endothelial cell damage, and small sized immune complexes could well be responsible for this observation. Finally, immune complexes are also capable of reacting with platelet Fc receptors (Penttinen 1977) resulting in platelet activation and subsequent release of platelet derived products as previously described. In addition, a disturbance of prostacyclin release may occur following platelet activation. Thus various mechanisms may be initiated by immune complexes, all of which could be important in the pathogenesis of systemic sclerosis.

One of the aims of this study was to investigate patients with systemic sclerosis, for the presence of serum factors capable of either damaging the vascular endothelium directly, or else capable of altering the interaction between platelets and endothelial cells. Hence, the production of prostacyclin by endothelial cells during incubation with control and scleroderma serum was investigated. A

recent study by Rustin et al (1987a) reported diminished agonist stimulated prostacyclin release by cultured endothelial cells following incubation with sera from patients with systemic sclerosis. However, in contrast to these findings, the experiments performed in this study failed to demonstrate such an inhibition, an observation which was also made in the preliminary study of Evans et al (1984).

However, prostacyclin is likely to play a key role in the pathogenesis of systemic sclerosis. Endothelial cell injury, occurring in systemic sclerosis, whether it is mediated immunologically via antiendothelial cell antibodies, anticardiolipin antibodies and immune complexes, may well result in altered prostacyclin release either directly, or via platelet effects occurring as a result of endothelial cell injury. Altered prostacyclin release may exert further effects on the aggregation of platelets resulting in the release of various platelet products.

It is likely that the failure to detect any alterations in prostacyclin release observed in this study, may be due to the large variability in endothelial cell cultures derived from different umbilical cords. In addition, serum may produce a masking effect by supplying exogenous arachidonic acid for the synthesis of prostacyclin. The apparently differing results of the present study and those of Rustin et al (1987a) may not in fact be conflicting since in the study of Rustin et al (1987a) the release of prostacyclin was not measured during incubation with sera, but in a subsequent agonist stimulated release. It is possible that the prolonged stimulation of prostacyclin release, or even an independent cytotoxic effect, occurring in the presence of test sera, may cause a decrease in the

subsequent ability of these cells to respond to an agonist. Thus further studies are required in order to characterize any prostacyclin imbalance which may occur in systemic sclerosis.

Finally, perhaps the single most important conclusion produced in this study is that multiple pathogenic mechanisms are likely to be involved in the pathogenesis of systemic sclerosis. Thus, of all the patients investigated, 75% exhibited abnormalities such as ADCC of endothelium, the presence of circulating immune complexes and elevated levels of von Willebrand factor antigen and the occurrence of anticardiolipin antibodies which could well be capable of causing vascular damage. All these immunological abnormalities are also present to varying degrees in the related connective tissue disease of SLE, in which it is known that multiple autoimmune mechanisms are responsible for the differing disease manifestations.

This study has produced clear evidence in support of a vascular pathogenesis for systemic sclerosis, which arises as a result of autoimmune mechanisms. These observations carry a therapeutic implication as well as stimulating further investigations of the multiple mechanisms responsible for this highly complex disease.

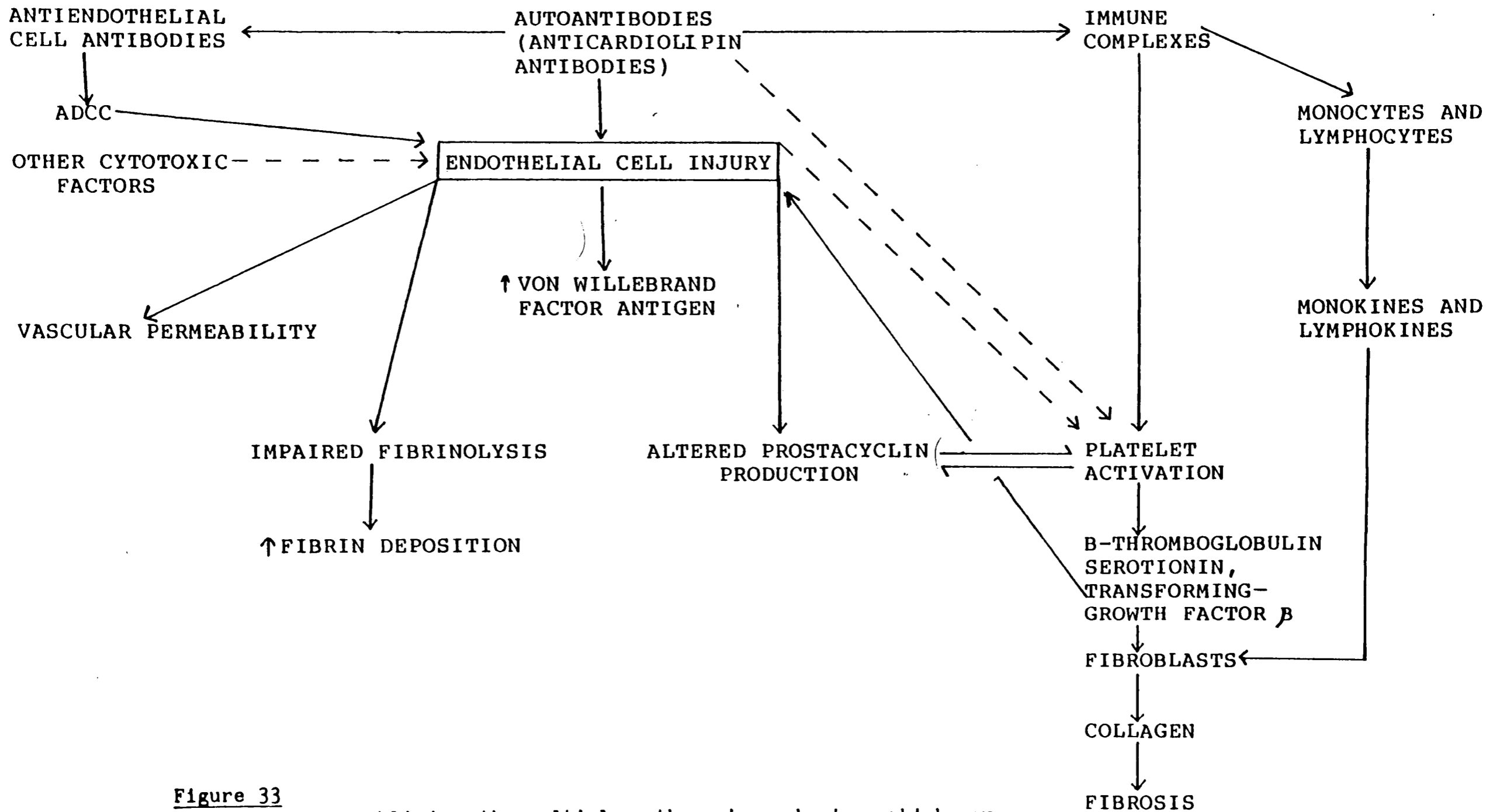


Figure 33

Diagram outlining the multiple pathogenic mechanisms which may occur in systemic sclerosis.

6.2 Further work

The work described in this thesis provides a basis for further investigation, which is discussed below.

Evidence has been presented that antiendothelial cell antibodies may be responsible for some of the vascular pathology seen in patients with systemic sclerosis. Further evidence to confirm the role of these antibodies could be provided by using the ELISA technique recently described (Hashemi et al 1987; Baguley et al 1987a; Byron et al 1987). Final proof could then be obtained by affinity chromatography. The ability of the purified antibody to produce ADCC of vascular endothelial cells could then be tested and fully confirmed. In addition, the antibody subclass of the involved antiendothelial antibodies should also be defined, using purification techniques.

The antigenic determinants present on endothelial cells which are recognised by specific antibodies also require elucidation. Immunoblotting techniques using solubilized cultured endothelial cells and antiendothelial cell antibody containing sera could provide information of this nature.

The mechanism of ADCC, in particular, the nature of the responsible effector cells and the Fc receptors involved in mediating the reaction, also require further investigation. Various monoclonal antibodies directed against the different Fc receptors exist, and these could be used to eliminate or block the action of corresponding effector cell populations. In addition to peripheral blood mononuclear cells, other effector cell populations, for example neutrophils, have been shown to mediate ADCC (Shen et al 1987). These cells could be isolated and used in ADCC reactions against endothelial cells. In addition, neutrophils may produce endothelial damage via

oxygen radicals (Weiss et al 1981; Varani et al 1985; Sacks et al 1978). This phenomenon should also be investigated as a possible pathogenic mechanism in systemic sclerosis.

Cytokines have been shown to enhance ADCC (Ralph 1984). Since altered levels of Interleukin 1 have been reported in patients with systemic sclerosis, the possible involvement of this factor in ADCC reactions would be an interesting study. In addition, various growth factors, including platelet release products such as transforming growth factor β and β thromboglobulin exert effects on the vascular endothelium (Heimark et al 1986; Takehara et al 1987; Hope et al 1979). These and other factors require further investigation to determine the precise mechanisms involved in their actions on endothelial cells.

Other diseases which feature a vascular pathology should be investigated for the occurrence of ADCC against endothelial cells. Although an earlier study failed to show any cytotoxicity of endothelium in patients with atherosclerosis (Penning 1984a), ADCC of vascular endothelium is also produced in SLE, although to a lesser extent than in systemic sclerosis, and may well be found in a variety of other autoimmune and connective tissue diseases, in particular rheumatoid arthritis. A recent report by Fattorossi et al (1988) demonstrated the occurrence of antiendothelial cell antibodies in patients with autoimmune hypoparathyroidism, therefore sera from patients with this disease should also be examined for the occurrence of ADCC of vascular endothelial cells.

In addition to the use of human umbilical vein and arterial endothelial cells, there is a need to investigate microvascular target cells in ADCC reactions, especially as the vascular lesion occurring in systemic sclerosis is prominent in the microcirculation (Kahaleh

and LeRoy 1988). Human capillaries have been isolated from various sites, including the skin and foreskin (Folkman et al 1979; Sherer et al 1980; Davison et al 1980, 1981; Marks 1985). However, at the present time, human capillary endothelial cell culture is in its infancy, and improved techniques for the isolation and growth of these cells will be necessary to obtain the numbers necessary for their use as target cells in ADCC assays. Nevertheless, large vessel endothelial cells from human adult tissue, for example the lung, could perhaps be used to provide a more relevant target cell compared with those derived from umbilical cords.

The effect, on endothelial cells, of the anticardiolipin antibodies detected in this investigation also requires further investigation. Anticardiolipin antibodies can be affinity purified by absorption to cardiolipin liposomes followed by elution with 1M sodium iodide (Alving and Richards 1977). Antibodies purified in this way could then be tested in ADCC and other cytotoxicity assays and perhaps also studied for their effects on prostacyclin production by endothelium. Such experiments on prostacyclin production could be combined with further studies on the effect of platelet poor plasma on endothelium in order to minimize the effect of platelet derived arachidonic acid, on the synthesis of prostacyclin. In addition, the effect on stimulated prostacyclin release could be investigated, as used in the study by Rustin et al (1987a) using, however, more physiologically relevant agonists, such as thrombin and bradykinin, rather than the calcium ionophore originally used by Rustin et al (1987a).

There is, therefore, a vast amount of further work to be carried out in order to unfold the complex mechanisms involved in the pathogenesis of systemic sclerosis.

APPENDICES

APPENDIX I

Double immunodiffusion (Ochterlony)

This technique was used for the qualitative determination of immunoglobulins

Principle

In this technique, antigen and antibody are allowed to migrate towards each other through a gel and a line of precipitation occurs where they meet. The precipitate is soluble in excess antigen, hence a sharp line is formed at equivalence, the position of which is determined by the concentration of antigen and antibody in the agar.

Procedure

Plates were coated with 1% agar in PBS and allowed to set at 4°C. Wells were punched into the agar using a 5µl borer attached to a suction pump. Antiserum was added to the centre well and samples placed in the outer wells. The plate was then left overnight at room temperature in a wet box. A single line of precipitation occurred at the sight of antigen and antibody interaction.

APPENDIX II

Radial immunodiffusion (Mancini)

This technique was used for the quantitative determination of immunoglobulins.

Principle

Single radial immunodiffusion is a diffusion in gel reaction in which antiserum is incorporated into a thin layer of agar on a plate. Antigen is then placed into wells cut into the plate. As the antigen diffuses radially, a precipitation ring forms around the well and moves outwards. At equivalence, the ring becomes stationary. The diameter of this ring is equivalent to the antibody concentration. A standard curve is constructed using known antibody concentrations and thus the unknown concentration can be determined by reference to this standard curve.

Procedure

Antisera was added to 6% PEG in PBS, which had been heated to 56°C. This was mixed with an equal volume of 2% agarose in PBS also at 56°C. The mixture was then poured onto a level plate which had previously been precoated with 0.5% agar. The coated plate was then allowed to cool at 4°C in a wet box. 16 wells were evenly punched into the gel using a 5 μ l borer.

IgG standards and dilutions of test samples were added to the wells, and the plate was then incubated in a covered wet box at room temperature for 3-4 days. Following incubation, the plate was soaked in 0.9% saline over night, then rinsed in water, and allowed to dry. Plates were then stained and the ring diameters were read using a vernier micrometer. Immunoglobulin concentrations were determined by plotting the diameter on a linear scale, against the log of the antigen concentration.

APPENDIX III

Lowry Method of Protein Determination

Principle

The assay used for the determination of protein content was adopted from the method of Lowry (1951). The method involves a two-step process in which protein is first reduced with an alkaline copper compound and then causes the reduction of a phosphomolybdic-phosphotungstic reagent. Protein is then quantified colorimetrically by measurement of changes in absorbance at 750nm.

Procedure

Human umbilical vein endothelial cells were hydrolysed overnight in situ with 1N sodium hydroxide. 100ul distilled water was added to 100ul test sample. A reference standard curve, ranging from 0-100 µg protein, was prepared with bovine serum albumin as the standard. Standard were diluted to 100ul with distilled water and the volume adjusted to 200ul with 1N sodium hydroxide. Each test and standard was reformed in duplicate. 1ml solution A was added to each test and standard and allowed to stand at room temperature for 10 minutes. 100ul solution B was then added, the samples mixed and left for a further 30 minutes. Optical density was read at 750nm in a Gilford spectrophotometer.

Solution A	100ml 2% sodium carbonate
	1ml 2% sodium potassium tartarate
	1ml 2% copper carbonate (CuSO ₄ .5H ₂ O)
Solution B	1:1 v:v Folin's reagent:distilled water

APPENDIX IV

Pattern of visceral involvement in patients with systemic sclerosis who were examined for cytotoxicity of vascular endothelium.

Age (years)	Sex	Visceral involvement	Disease score
Severe Disease			
61	M	Skin, oesophagus, lung, small bowel, heart, polymyositis	17
39	F	Skin, oesophagus, lung, heart, kidney	14
68	F	Skin, oesophagus, lung, heart, kidney	14
36	M	Skin, oesophagus, lung, heart, polymyositis	14
73	F	Skin, oesophagus, lung, heart	11
74	F	Skin, oesophagus, lung, arthritis	11
17	F	Skin, oesophagus, lung, small bowel	11
64	F	Skin, oesophagus, lung, small bowel	11
79	F	Skin, oesophagus, lung	8
30	F	Skin, oesophagus, lung	8
68	M	Skin, oesophagus, lung	8
	F	Skin, oesophagus, lung	8
78	F	Skin, oesophagus, lung	8
46	F	Skin, oesophagus, lung	8
53	F	Skin, oesophagus, lung	8
77	F	Skin, oesophagus, lung	8
69	M	Skin, oesophagus, lung	8
49	F	Skin, oesophagus, lung	8
59	F	Skin, oesophagus, kidney	8
66	F	Skin, lung, heart	8
60	M	Skin, oesophagus, lung	7
50	F	Skin, lung	6
41	F	Skin, oesophagus	6
Mild disease			
43	F	Skin, oesophagus	5
76	F	Skin, oesophagus	5
70	F	Skin, oesophagus	5
53	M	Skin, oesophagus	5
66	F	Skin, oesophagus	5
62	F	Skin, oesophagus	5
63	F	Skin, oesophagus	5
66	F	Skin, oesophagus	5
48	F	Skin, oesophagus	5
39	M	Skin, oesophagus	5
63	F	Skin, oesophagus	5
34	F	Skin	3
49	F	Skin	2
54	F	Skin	2
63	F	Skin	2
60	F	Skin	2
29	F	Skin	2
67	F	Skin	2
40	F	Skin	2
43	F	Skin	2
43	F	Skin	2
49	F	Skin	2

APPENDIX V

Pattern of system involvement in patients with SLE who were examined for cytotoxicity of vascular endothelium.

Age (years)	Sex	Visceral involvement
49	F	Joints, lung, heart, gut
26	F	Joints, cerebral, skin
45	F	Joints, cerebral, renal
38	F	Joints, renal
41	F	Joints, skin
75	M	Joints, renal, splenomegaly
47	M	Joints, lung
57	F	Joints, splenomegaly, polymyositis
31	F	Cerebral, renal, skin
37	F	Joints, lung, skin
52	F	Joints, lung, heart
44	F	Joints, lung, heart
23	F	Joints, lung
50	F	Lung, heart, cerebral
57	F	Renal, skin
24	F	Joints, lung
64	M	Heart, skin, haemolysis
63	F	Joints, splenomegaly, anaemia
32	F	Lung, neuropathy
46	F	Cerebral
34	F	Lung, cerebral, DVTs
34	M	Joints, lung, heart
42	M	Joints, lung
43	F	Joints, heart, skin
41	F	Joints, lung, heart, renal
56	F	Joints, cerebral, DVTs
21	F	Joints, heart, neuropathy
58	M	Skin, DVTs
61	F	Joints, lung, cerebral, thrombocytopenia
29	F	Abortion
41	F	Joints, lung, gut, skin
30	F	Lung, renal, skin
43	F	Joints, skin
45	F	Joints, skin, myopathy
65	F	Joints, cerebral
22	F	Renal, skin
57	F	Joints, skin
40	F	Splenomegaly, optic neuritis, arthritis
49	F	Cerebral, gut, arthritis

APPENDIX VI

List of suppliers

Tissue culture

Minimum essential medium (MEM)	Flow laboratories Rickmansworth Hertfordshire England
Medium 199	Flow laboratories
HEPES	Boehringer-Mannheim Lewes E Sussex England
Sodium bicarbonate	BDH Poole England
Penicillin	Glaxo laboratories Greenford England
Streptomycin sulphate	Glaxo laboratories
Fungizone	ER Squibb & sons Princeton USA
L-glutamine	Flow laboratories
Gelatin	Sigma chemical Co Poole England
Collagenase	Sigma chemical Co
Typsin	Sigma chemical Co
EDTA	Sigma chemical Co
Foetal calf serum	Gibco Paisley Scotland
Thyoglycolate	Oxoid Basingstoke Hampshire England
T25 & T75 flasks (Nunclon)	Gibco
96 well microtitre plates (Nunclon)	Gibco

24 well plates (Falcon) Beckton & Dickinson
Cowley
England

Staining

Coverslips (13mm round) Miles scientific
Naperville
USA

Peroxidase labelled anti-human
von Willebrand factor antigen Dakopatts
Glostrup
Denmark

Peroxidase labelled rabbit
anti-mouse immunoglobulin Dakopatts

DAB Sigma chemical Co

Hydrogen peroxide Sigma chemical Co

DPX mountant (microscopical reagent) BDH

Trypan blue BDH

Haemalin BDH

Giemsa BDH

6-keto-PGF_{1α} radio-immunoassay

Tritiated 6-keto-PGF_{1α} NEN
Dupont (UK) Ltd.
Stevenage
Hertfordshire
England

6-keto-PGF_{1α} antibody Dr M Greaves
Dept Haematology
Hallamshire Hospital
Sheffield
England

Tris hydrochloric acid Sigma chemical Co

Bovine serum albumin Sigma chemical Co

dextran T70 Pharmacia LKB
Milton Keynes
England

Norit A charcoal BDH

Optiphase safe scintillant Pharmacia LKB

Column chromatography

ULTROGEL	Pharmacia-LKB
DEAE-Sepharose	Whatman chromatography services Wirral Cheshire England
Staphylococcal Protein A-sepharose	Bio-processing Ltd Consett England

Cytotoxicity assays

Sodium chromate - ^{51}Cr	NEN & Amersham Internatinal Amersham England
Saponin (biochemicals)	BDH
Lymphoprep	Nycomed (UK) Ltd Sheldon Birmingham England
Nycodenz monocytes	Nycomed
Carbonyl iron powder	GAF (GB) Ltd Wythenshawe Manchester England
Formic acid	BDH
Heparin	CP Pharmaceuticals Ltd Wrexham England

Biochemicals

Biochemicals for buffers etc were obtained from BDH, and were ANALAR grade

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