THE 52 AND 60 kD Ro/SS-A ANTIGENS WHERE ARE THEY? DO anti-Ro/SS-A AUTOANTIBODIES CAUSE CUTANEOUS DISEASE?

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My interest in cutaneous lupus erythematosus was kindled more than 6 years ago when I became involved in the care of the cutaneous lupus erythematosus patients in Oxford.

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ABSTRACT

Systemic lupus erythematosus, considered a multifactorial autoimmune disease, is a disease affecting many systems, with associated immunological abnormalities. It has a striking diversity of clinical patterns, pathologies and prognoses. Genetic factors determine the inherited baseline, on which environmental, hormonal and infectious triggers act to produce autoantibodies.

Ro antibodies have been considered pathogenic in subacute cutaneous and neonatal lupus erythematosus. I affinity-purified antibodies to the 52 kD Ro from immunised rabbits (whole 52 kD protein) and human sera (using two immunodominant regions of the protein). I affinity-purified antibodies to the 60 kD Ro from immunised rabbits (whole 60 kD protein) and human sera (using two immunodominant regions of the protein, as well as the total "native" protein).

Using these purified antibodies, with immunofluorescence on normal neonatal human keratinocytes, I showed that the 52 kD Ro is mainly cytoplasmic and the 60 kD Ro is mostly nuclear, with some fine cytoplasmic staining.

I looked at the capacity of these purified antibodies to penetrate living keratinocytes under various conditions (hormones, drugs and vitamins). No antibody penetration was found, although one whole serum gave low levels of intracellular fluorescence.

I studied the putative membrane translocation of 52 kD and 60 kD Ro under conditions of stress (UVA or UVB with or without hormones, drugs, vitamins and heat shock). I could not identify translocation of the 52 or 60 kD antigens with purified antibodies, although some whole sera showed fluorescence.

I can find no evidence that antibodies directed against the 52 and 60 kD Ro antigens cause cutaneous disease.

ABBREVIATIONS

aa	:	Amino acids
ACLE	:	Acute cutaneous lupus erythematosus
ACR	:	American College of Rheumatology
ANA	:	Antinuclear antibody
BMZ	:	Basement membrane zone
C	:	Complement
CCLE	:	Chronic cutaneous lupus erythematosus
CIE	:	Counter- immunoelectrophoresisis
CR	:	Calreticulin
dd	:	double distilled
DEAE	:	Diethylaminoethyl
DIF	:	Direct immunofluorescence
DLE	:	Discoid lupus erythematosus
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme linked immunosorbant assay
ER	:	Endoplasmic reticulum
FCS	:	Fetal calf serum
FD	:	Fluorescein diacetate
FSH	:	Follicle stimulating hormone
GRP	:	Glucose regulated protein
GST	:	Glutathione S-transferase
HBSS	:	Hanks buffered salt solution
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCZ	:	Hydrochlorothiazide
HLA	:	Human leucocyte antigen
IF	:	Immunofluorescence
IIF	:	Indirect immunofluorescence
IL1RN	:	Interleukin-1 receptor antagonist gene

kD	:	Kilodalton
LB	:	liquid broth
LE	:	Lupus erythematosus
LH	:	Luteinising hormone
mA	:	milliamps
MCT	:	Microcentrifuge tube
MED	:	Minimal erythema dose
NLE	:	Neonatal lupus erythematosus
PCR	:	Polymerase chain reaction
PVA	:	Polyvinyl Alcohol
RA	:	Rheumatoid Arthritis
RFLP	:	Restriction fragment length polymorphism
rfp	:	Human ret finger protein
Ro52	:	52 kD Ro
Ro60	:	60 kD Ro
RNP	:	Ribonucleoprotein
rpt-1	:	Regulatory protein, T-lymphocyte, 1 gene
SCLE	:	Subacute cutaneous lupus erythematosus
SDB	:	Serum dilution buffer
SDS-PAGE:		Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	:	Systemic lupus erythematosus
Sm	:	Smith antigen
snRNP:		Small nuclear ribonucleoprotein
SPD	:	Serum pre-diffusion
SS	:	Sjögren's syndrome
SSCP	:	Single stranded conformation polymorphisms
TNF-∝:		Tumour necrosis factor alpha
µg	:	Microgram
µl	:	Microlitre
UVA	:	Ultraviolet A
UVB	:	Ultraviolet B
UVR	:	Ultraviolet radiation

1. CUTANEOUS LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a multisystem disease with associated immunological abnormalities. It has a striking diversity of clinical patterns, pathologies and prognoses. Skin manifestations are some of the most common symptoms and signs in SLE. They are important in diagnosing SLE: cutaneous lesions account for 4 of the 11 revised American College of Rheumatology (ACR) criteria for the classification of SLE (Tan *et al* 1982).

1.1 Epidemiology

The incidence of SLE in the UK is 24.6 per 100 000 population per year, 3.7/100 000/year for men and 45.4/100 000/year for women (Hopkinson *et al* 1993). The highest incidence for both men and women is in the 50-59 year age range. SLE is more common among Afro-Caribeans than other groups.

Skin involvement in SLE is seen in 72-85% of patients (Dubois & Tuffanelli, 1964; Harvey *et al* 1954). Cutaneous involvement is the first manifestation of lupus in 23-28% (Dubois & Tuffanelli, 1964; Harvey *et al* 1954) of patients.

9.9% to 12% of SLE patients have been reported to have an affected first degree relative (Grigor *et al* 1978), 7% with a first degree relative with photosensitivity (McCauliffe *et al* 1992). It is estimated that first-degree relatives of patients with SLE have an 11 fold greater chance of acquiring SLE than the general population (Lawrence *et al* 1987).

1.2 Pathogenesis

SLE is considered a multifactorial autoimmune disease. Genetic factors determine the inherited baseline, on which environmental, hormonal and infectious triggers act to produce autoantibodies. Autoantibodies, along with other mediators of tissue injury, including complement and inflammatory cells, are likely to cause disease.

1.2.1 Genetic factors

Genetic factors are important in the pathogenesis of LE. The major histocompatibility (MHC) class II antigen HLA-DR3 is associated with the presence of anti-Ro/SSA antibodies in subacute cutaneous lupus erythematosus (SCLE) (Lee *et al* 1983), neonatal lupus erythematosus (NLE) (Lee *et al* 1983) and Sjögren's syndrome (Harley *et al* 1986). The presence of both anti Ro and anti-La/SS-B in SLE is associated with HLA-DR3 and is seen in older patients (>50 years age at onset) (Hochberg *et al* 1985). A large SLE study group have subsequently shown that a strong association with HLA-DR3 was found for antibodies against Ro52 and La, rather than anti-Ro60 in the absence of anti-52 or anti-La (Ehrfeld *et al* 1992). This suggests that the DR association in SLE is an epitope-specific immune response. The SLE patients with anti-Ro alone, had an association with HLA-DR2 and tended to be younger (<22 years of age at onset). No HLA association has been found in infants with NLE or congenital heart block (Watson *et al* 1984).

Associations between an allele of the interleukin-1 receptor antagonist gene (IL1RN) and several inflammatory diseases have been described. There is an increase in both frequency and carriage rate of IL1RN*2 in SLE. This association is strengthened with extensive disease and particularly with the presence of photosensitivity and discoid skin lesions. Carriage of this allele seems to influence severity rather than susceptibility to SLE (Blakemore *et al* 1994).

Overexpression of heat shock protein is associated with the anti-phospholipid syndrome. This may be due to genetic factors, as this group of 94 SLE patients had an absence of the HLA allo-or haplotypes, commonly found in such a cohort (Dhillon *et al* 1994).

A study of 121 SLE patients, 119 of whom were complement typed, showed that both black and white patients with SLE were far more likely to have a C4a null allotype than were racially matched controls. A detailed evaluation of the relationship to different types of antibodies was performed. Anti-ds-DNA, anti-Sm, anti-Ro and anticardiolipin antibodies were less common in patients with homozygous C4a deficiency. C4a gene deletion was found in 23.4% of patients and was associated with SCLE and SS in SLE patients. The presence of one or two C4a null allotypes and the presence of a C4a gene deletion identify subgroups of SLE patients whose characteristics differ in many ways from those of other SLE patients (Petri *et al* 1993).

However, most people with lupus-associated genes, such as DR3, do not develop LE. Other triggering factors must operate. Viral infection may result in production of antibodies to a portion of the virus that is similar to a portion of an autoantigen, so that antibodies to the virus cross-react with the autoantigen. In a study of 80 SLE patients, 80 healthy controls, 47 rheumatic disease controls and a small number of patients infected with the vesicular stomatitis virus, there was significantly different binding to the viral proteins in the SLE patients compared with other groups. The reactivity of the SLE sera included binding to the internal viral matrix and the nucleocapsid proteins (Hardgrave *et al* 1993).

In a genetically susceptible individual at high rates of generation of novel fragments, such as in irradiation, heat shock, bacterial or viral infection, or xenobiotic exposure, the appropriate MHC class II molecules may capture and present self-peptides that were previously cryptic (Lipham *et al* 1991). As in other systems, the immune response to this self-peptide may subsequently diversify to other areas on the self-molecule to which the organism was previously tolerant (Mamula, 1993). Subsequent

re-exposure of the primed immune system to low levels of "apoptotic" autoantigen, such as in sun exposure, may lead to clinical flares (Casciola-Rosen *et al* 1994)

1.2.2 Environmental factors

Sunlight is a well established factor in the induction and exacerbation of LE. The relationship of ultraviolet irradiation (UVR) to the development of SCLE and NLE is well established. UVR appears to up-regulate the binding of anti-Ro/SSA antibodies to keratinocytes, both *in vitro* and *in vivo* (Furukawa *et al* 1990).

Clinically, it has been shown that lupus patients have a lowered minimal erythema dose (MED) to UVB compared with normal controls. A lowered MED was found most frequently in SLE (64%) and least frequently in DLE (32%). Prolonged persistence of erythema induced by 1-2 MED was a constant finding in SCLE. (Wolska *et al* 1989) Photoreproduction of lesions after single UVB exposure was observed most frequently in SCLE (62.5%) (Wolska *et al* 1989).

In addition, skin lesions in LE have been induced by both UVA and UVB irradiation. Lehmann et al showed that skin lesions clinically and histologically compatible with LE were induced in 64% of patients with SCLE, 42% of patients with DLE and 25% of patients with SLE. The action spectrum of induced lesions was within the UVB range in 33%, in the UVA range in 14% and in both the UVB and UVA range in 53% (Lehmann *et al* 1990).

UVA is the predominant UV component in solar radiation. UVA and UVB irradiation in both human and SLE derived cells *in vitro* shows increased sensitivity with a very low threshold for unscheduled DNA repair synthesis, coexisting with limited cellular DNA repair capacities (Golan & Boerel, 1984). In addition, UVA irradiation of murine SLE-derived cells (both morbid and pre-morbid) is accompanied by elevated oxygen consumption (which reflects increased accumulation of oxygen reactive radicals) in comparison to normal cells (Golan *et al* 1994). Based on this data, it has

been suggested that SLE-derived cells are defective in the enzymatic pathways of removing oxygen radical species and this may have a bearing on the lower threshold for the UV-induced DNA damage observed in such cells. In support of this, is a paper showing that peripheral blood SLE lymphocytes are hypersensitive to the toxic effects of hydrogen peroxide. The DNA of these cells exhibit raised levels of 8-oxo-7-hydrodeoxyguanosine (a DNA lesion induced by reactive oxygen species) (Bashir *et al* 1993).

The prevalence of SLE among uranium miners heavily exposed to silica, is 93 per 100 000 (Conrad *et al* 1996). The only differences to non-exposed SLE patients were a decreased frequency of arthritis and photosensitivity and an absence of anti-Sm and anti-U1-RNP antibodies. ANA were found in all definite SLE patients, with anti-dsDNA in 44.4%, anti-Ro in 55.6% and anti-La in 22.2% (Conrad *et al* 1996).

Reed et al in 1985 (Reed *et al* 1985) reported five patients with the clinical and histological features of SCLE and anti-Ro antibodies, while taking hydrochlorothiazide. All resolved after drug discontinuation. Anti-Ro positive SCLE has also been reported following hydralazine, penicillamine and glyburide (Sontheimer, 1989).

1.2.3 Hormonal factors

Sex hormones are an additional influence in LE. Concordant experimental studies have clearly demonstrated harmful effects of oestrogens in NZB/NZW F_1 mice, which develop an autoimmune disease similar to human SLE (Roubinian *et al* 1979; Steinberg *et al* 1979). The importance of oestrogen is suggested by the demography of LE. LE occurs more commonly in females. The female: male ratio in SLE can be as high as 10:1 in menstruating women. This ratio falls to 3:1 in non-menstruating girls or post-menopausal women (Kornreich, 1976). Although more males develop SCLE or DLE than SLE, even in these groups, women outnumber men, 70 to 80% of SCLE patients being female (Sontheimer *et al* 1979). In NLE, 75% with skin disease

23-26 misc

are female, whereas the number of girls with congenital heart block only slightly exceeds that of boys (McCune *et al* 1987)

Oestrogens can upregulate the binding of anti-Ro/SSA antibodies to cultured keratinocytes (Furukawa *et al* 1988). Clinical improvement in SLE has been reported following removal of the female sex organs (Yocum *et al* 1975). SLE may exacerbate with oestrogen containing oral contraceptives (Bole *et al* 1969).

Chronic oestrogenic stimulation due to elevated oestradiol levels may account for the association of SLE and Klinefelter's syndrome (Stern *et al* 1977). Both male and female SLE patients have elevated conversion of oestradiol to 16-hydroxylated metabolites, which behave as active oestrogens by binding to oestrogen receptors (Talal, 1982). Prolactin is an immunostimulatory hormone in experimental animals and high serum prolactin levels are found in SLE (Lavalle *et al* 1987). Serum prolactin levels have been found to be higher in pregnant SLE patients than in pregnant patients with rheumatoid arthritis or normal pregnant controls (Jara-Quezada *et al* 1991). Oestrogens stimulate the release of prolactin by the pituitary. In turn, prolactin inhibits the responsiveness of the pituitary to luteinising hormone release hormone, as well as inhibiting the action of luteinising hormone (LH) and follicle stimulating hormone (FSH) on the gonads.

It is possible that the low levels of oestradiol, testosterone and dihydrotestosterone in LE patients could be explained by the raised levels of prolactin. In LE, sex steroid hormones may be acting physiologically on a disordered immune system (Cohn, 1979). Normal immune responses, both cellular and humoral, are greater in females than males (Cohn, 1979). There are steroid-sensitive receptors in the hypothalamus, implying that neuroendocrine pathways also interact with the immune system (Besedovsky & sorkin, 1977). Oestrogens are reported to have the dual role of immunosuppresants and immunostimulants (Lahita, 1984). Differences in female immunity as compared to male immunity, include high levels of IgM, higher antigen specific antibodies and greater susceptibility to infection, more autoantibodies, higher T helper cell levels and decreased ability to accept skin grafts (Lahita, 1996).

In addition, a number of studies using liver, breast cancer, oviduct, uterus, anterior pituitary and mammary gland cells have all demonstrated that oestrogens can stimulate target cell DNA replication and cell division (Aprison *et al* 1982; Chalbos *et al* 1982; Oka & Schimke, 1969). Oestrogens are known to increase the mitotic rate of the epidermis of rodents and man (Bullough, 1955), to enhance the growth rate of cultured keratinocytes (Peehl & Ham, 1980), to stimulate the synthesis, maturation and turnover of collagen (McCarty & McCarty, 1977). In addition, they depress cell mediated immunity, attenuate natural killer cell function, decrease cancer cell immune surveillance and inhibit the release of thymic hormones, depending on the dose, with a constant relative lymphopaenia (Lahita, 1996). These oestrogen effects are thought to occur as a result of hormone interactions with nuclear receptors which result in an alteration of gene expression (Clark & Peck, 1976).

Although initially controversial (Cox, 1965; Tozman *et al* 1980), more recent work suggests that SLE does not change with pregnancy (Out *et al* 1989; Tincani *et al* 1991). SLE and pregnancy has been extensively studied but the studies have concentrated on renal involvement. Worsening of SLE is uncommon in pregnancy. Inactive disease is not associated with disease recurrence (Tozman *et al* 1980). In a prospective study of 80 pregnant SLE patients, disease worsened in less than 13%. A recent study showed that skin disease did not flare during pregnancy. 66.6% experienced no change in their disease, with 26.6% improving. 20% flared post-partum (Yell & Burge, 1993).

In a 1956 study of 80 patients, 13 with DLE were identified. In these, there were no clear cut changes which could be related to pregnancy (Frieman & Rutherford, 1956). Subsequently, in a 1961 study of 134 LE patients during 191 pregnancies, 29 DLE patients with 38 pregnancies were identified. DLE was essentially stable during pregnancy: 21 experienced no change in their disease; 7 patients improved and one worsened. In most patients, the disease remained stable or improved post partum (Bruce Donaldson & de Alvarez, 1962). Rowell and Goodfield in an unreferenced statement describe pregnancy as a precipitant of DLE in 1% of cases (Rowell &

Goodfield, 1992). In a recent series of DLE patients, 21% worsened, 43% did not change and 20% improved during pregnancy. 13% presented in pregnancy (Yell & Burge, 1993).

Sex hormonal levels fluctuate with the menstrual cycle. Oestradiol levels rise prior to ovulation, with a second peak in the luteal phase prior to menstruation. Rowell describes 13% of DLE patients deteriorating premenstrually (Rowell & Goodfield, 1992). A premenstrual cutaneous flare has been described in 25% of SLE patients and in 16% of DLE patients (Yell & Burge, 1993).

In the perimenopausal period, the decrease in the length of the follicular phase, results in a shorter interval between menses. In addition, mean levels of plasma FSH and LH increase. At the menopause, cessation of follicular development results in a drop in the production of oestradiol and other hormones, which in turn, leads to a loss of negative feedback on the hypothalamic-pituitary centres. The levels of plasma gonadotrophins increase, with FSH levels rising earlier and to a greater extent than those of LH. Plasma oestrogen and androgen levels are significantly reduced. In one series, 15% of SLE patients presented postmenopausally (Rowell & Goodfield, 1992). A perimenopausal cutaneous flare has been reported in 8% of SLE patients and in 35% of DLE patients. Presentation in the menopause occurred in 3% of SLE and 6% of DLE patients (Yell & Burge, 1993).

Exacerbation of SLE after oral contraceptive use was first reported in 1966 (Pimstone, 1966). Two further case reports suggest that a lupus diathesis may be unmasked by oestrogen containing oral contraceptives (Travers & Hughes, 1978; Garovich *et al* 1980). A prospective controlled study of normal women receiving oral contraceptives compared with a group using non hormonal contraception, revealed no altered serology or rheumatic complaints (Tarzyy *et al* 1972). However, in a study of 26 SLE patients receiving oral contraceptives, 43% experienced disease exacerbation during the 3 months after initiation of therapy (Jungers *et al* 1982a). However the trend to use low dose oestrogen pills may account for the apparent decrease in lupus like side effects recently. This is supported by a study which showed that oral contraceptives

were not a factor associated with the appearance of SLE in patients diagnosed between 1985 and 1987 (Strom *et al* 1994). The progesterone components of oral contraceptives are not related to SLE (Jungers *et al* 1982b).

1.2.4 Disturbance of immune regulation

The enormous array of non-organ-specific autoantibodies found in SLE has been termed an autoimmune thunderstorm. These antibodies are, however, directed at relatively few macromolecules of cell components. These are often highly conserved polypeptides that subserve very basic cellular functions, carried out in the nucleus, nucleolus and ribosomes. The reasons why only 30 or so basic cellular proteins become the targets of an autoimmune response in patients with LE, at the exclusion of the other 10 000 cellular macromolecules remain unknown. Molecular mimicry may be one answer. Highly conserved immunogenic molecules, expressed by infectious pathogens can trigger an immune response in a genetically predisposed host, that cross-reacts with cellular antigens (Sontheimer *et al* 1992).

The presence of ANA and antibodies to ds-DNA remain the mainstay of the diagnosis of SLE, although 5% of patients remain persistently ANA negative. Besides having diagnostic value, some of these antibodies are believed to be pathogenic, although antibody titers do not correlate with disease activity. Clinical interest has centered around defining subsets of SLE by antibody typing. However, the conversion of "serological subset" into "clinical subset" may rely more heavily on statistics than clinical observations. Despite these reservations, certain antibodies do appear to be associated with certain clinical features.

Anti-Ro antibodies are associated with the development of SCLE and NLE. Anti-Ro antibodies are found in 0.1% of hospitalised patients (Maddison *et al* 1979), 17.5% of normal people at low level (Gaither *et al* 1987), as well as some relatives of patients with autoimmune disease (Lehman *et al* 1984). Sera from 5000 female blood donors revealed a frequency of anti-Ro antibodies of 0.44% (Fritzler *et al* 1985). The

presence of antibodies in normal individuals, suggests that there are autoreactive B cell clones in the repertoire of normal people. It is possible that these autoreactive cells are un-stimulated precursors or that they are controlled by suppression rather than active deletion.

There are several animal models of SLE of which the murine ones are the most studied. These include the (NZB X NZW)F_1 and the MRL/1. The MRL/1 strain demonstrates the most aggressive form of the disease which is related to a single autosomal recessive gene. Anti-DNA antibodies are detected early in the NZB/NZW mouse and precede the first histologic signs of glomerulonephritis. If these animals are rendered unable to produce anti-DNA antibodies, they have few signs of autoimmune disease (Yoshida *et al* 1990). The evolution of the autoantibody response to nucleosomes in lupus has been studied during clinical flares, as well as in animal models of the disease (Mohan *et al* 1993; Burlingame *et al* 1993). These studies indicate that the antibody response in lupus is antigen-driven and T cell dependent. Autoantibodies undergo isotype switching, somatic mutation and affinity maturation, characteristic of rechallenge of a primed immune system by antigen. The presence of immune complexes containing dsDNA during acute flares, suggests that endogenous nucleosomes may be one antigen driving the immune response in lupus (Sano & Morimoto, 1981).

A striking feature of the different autoantigens in lupus is their lack of restriction to any one subcellular location. They may be exclusively nuclear, cytosolic, membrane associated, or distributed between the nucleus and cytoplasm under different conditions. It has been suggested that the total repertoire of autoantigens might be clustered on a limited number of distinct subcellular particles (Tan, 1991). It has been shown that autoantigens are clustered in two distinct populations of blebs at the surface of apoptotic cells (Casciola-Rosen *et al* 1994). The smaller blebs contain fragmented endoplasmic reticulum (ER), ribosomes and Ro. The larger blebs (apoptotic bodies) contain nucleosomal DNA, Ro, La and small nuclear ribonucleoproteins. These autoantigen clusters have in common, their proximity to

the ER and nuclear membranes, sites of increased generation of reactive oxygen species in apoptotic cells. Oxidative modification at these sites may be a mechanism that unites this diverse group of molecules together as autoantigens (Casciola-Rosen *et al* 1994). Apoptosis is however, a normal developmental process and alone may not be critical in the development of autoimmunity.

It has been proposed that some autoreactive T cells escape tolerance induction because they are directed against minor (cryptic) determinants on self-antigens that are not efficiently generated during antigen processing (Gammon & Sercarz, 1989). These autoreactive cells may become pathogenic if that cryptic determinant is subsequently revealed (Lipham *et al* 1991). A number of free radical-induced modifications of proteins have been described that may reveal potentially cryptic determinants. These include fragmentation, amino acid modifications and novel sensitivity to protease attack (Wolff & Dean, 1986). The strong association of particular autoantibody responses with specific MHC class II molecules in patients with SLE, may reflect the ability of these class II molecules to capture and present self-peptides to T cells (Reveille *et al* 1991).

Other pathogenic factors could result from the fact that immune complexes are abnormally processed. One group studied the clearance of large soluble immune complexes (HBsAg-anti-HBsAg) radiolabelled with ^{123}I in 12 healthy individuals and 10 SLE patients. Initially, clearance was more rapid in the SLE patients, with up to 12% of the complexes released from the liver after 30 to 50 minutes. Spleen uptake was reduced, as was the ability to retain the immune complex in this organ. Plasma complement levels and red cell complement receptor type 1 numbers were reduced in the SLE patients (Davies *et al* 1992).

Persistent B cell activation occurs in SLE. This may persist even with inactive disease. Lymphocyte activation at a cellular level in 13 nonactive SLE versus 15 controls was assessed. The expression of activation markers on B and T cells was higher in SLE patients. The percentage of activated B cells was related to levels of

total IgG and IgM but not to antibodies to ds-DNA (Spronk *et al* 1993). These findings suggest a persistently activated immune system.

1.2.5 Clinical Subsets

Cutaneous lupus erythematosus (LE) has been classified by Gilliam and Sontheimer (Gilliam & Sontheimer, 1981) into chronic cutaneous LE, subacute cutaneous LE and acute cutaneous LE. See table 1:

Table 1 The Gilliam classification of skin lesions associated with LE

1 LE-specific skin disease (Cutaneous LE [CLE])

- 1.1 Acute cutaneous LE (ACLE)
  - 1.1.1 Localized ACLE (malar rash, butterfly rash)
  - 1.1.2 Generalized ACLE (lupus rash, maculopapular lupus rash, photosensitive lupus dermatitis)
- 1.2 Subacute Cutaneous LE (SCLE)
  - 1.2.1 Annular SCLE (lupus marginatus, symmetric erythema centrifugum, autoimmune annular erythema, lupus erythematosus gyratus repens)
  - 1.2.2 Papulosquamous SCLE (disseminated DLE, subacute disseminated LE, superficial disseminated LE, psoriasiform LE, pityriasiform LE and maculopapular photosensitive LE)

1.3 Chronic Cutaneous LE (CCLE)

1.3.1 Classical discoid LE (DLE)

1.3.2 Localized DLE

1.4 Generalized DLE

1.5 Hypertrophic DLE/verrucous DLE

1.6 Lupus panniculitis/lupus profundus (see figure 14, pg. 42)

1.7 Mucosal DLE

1.7.1 Oral DLE

1.7.2 Conjunctival DLE

1.8 Lupus tumidus (urticarial plaque of LE)

1.9 Chilblain LE (chilblain lupus)

1.10 Lichenoid DLE (LE/lichen planus overlap, lupus planus)

2 LE-nonspecific skin disease

2.1 Cutaneous vascular disease

2.2 Vasculitis

2.2.1 Leukocytoclastic

2.2.1.1 Palpable purpura

2.2.1.2 Urticarial vasculitis

2.2.2 Periarteritis nodosa-like cutaneous lesions

2.2.3 Vasculopathy

2.2.3.1 Degos' disease-like lesions

2.2.3.2 Secondary atrophie blanche (livedoid vasculitis, livedo vasculitis)

2.3 Periungual telangiectasis

2.4 Livedo reticularis

2.5 Thrombophlebitis

2.6 Raynaud's phenomenon

1.2.5.1 Systemic Lupus erythematosus

Acute cutaneous LE, said to occur in 30-50% of SLE patients, includes localized, indurated erythematous lesions, widespread indurated erythema and bullous LE.

59% of patients (in a study of 73 patients, 33 had a butterfly eruption with an additional 10 having a transient flush) have lesions in the butterfly area of the face. The malar region may be preferentially involved due to its anatomical site - exposed to the environment, especially sunlight and susceptible to trauma. The malar dermal vessels are numerous, prone to flushing and superficial, accentuating telangiectasia (Yell *et al* 1996a). The high prevalence of malar rash is confirmed in children at 67% (Bosi Ferraz *et al* 1994).

Annular erythema, particularly of the cheeks, where thermography has shown that this is the coolest part of the face, has been described in Sjögren's syndrome patients positive for Ro. This syndrome is different histologically from SCLE (Katayama *et al* 1991).

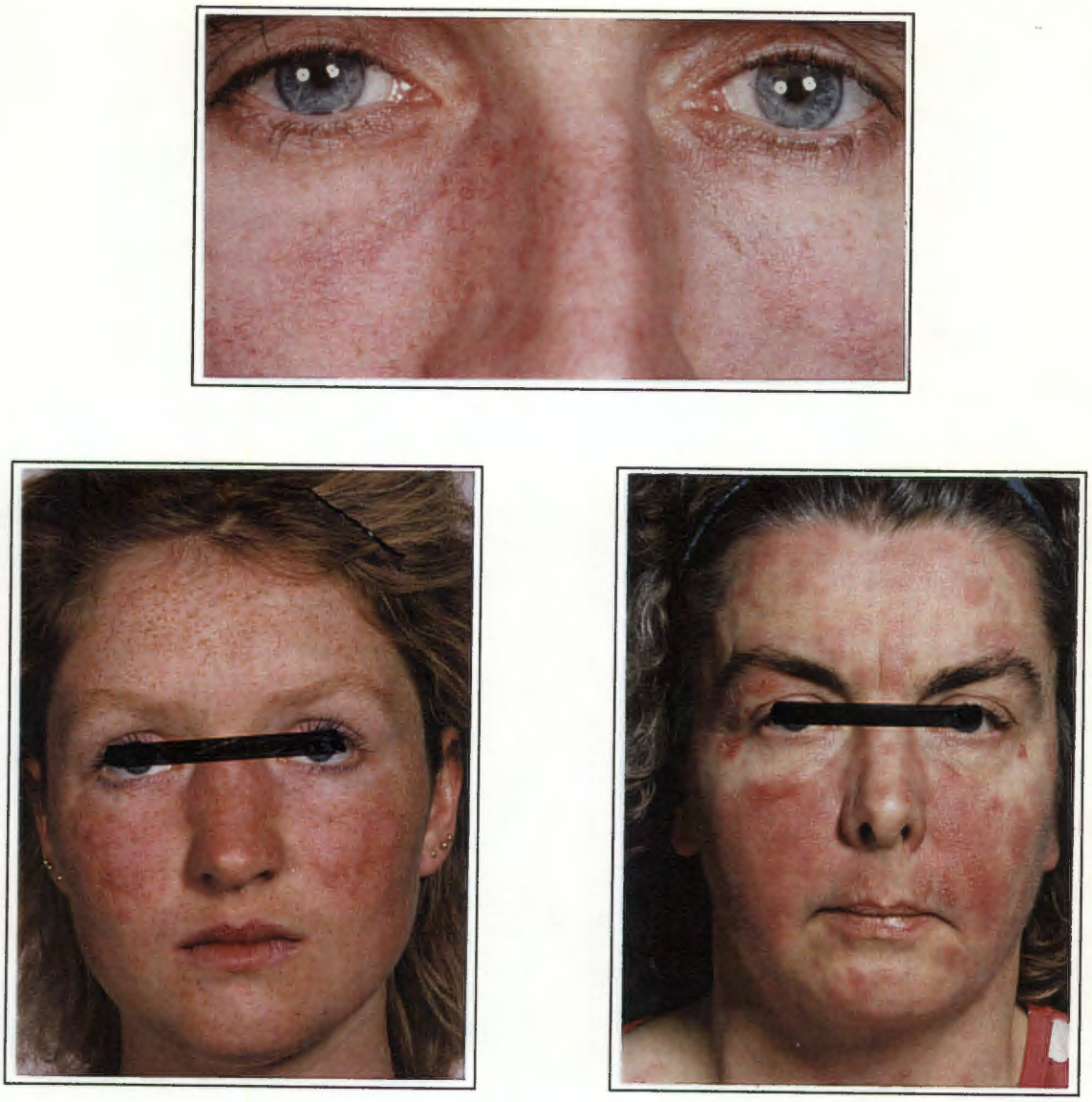


figure 1: The facial rash of SLE may vary from telangiectasia (top), through malar erythema (left), through more indurated erythema (right).

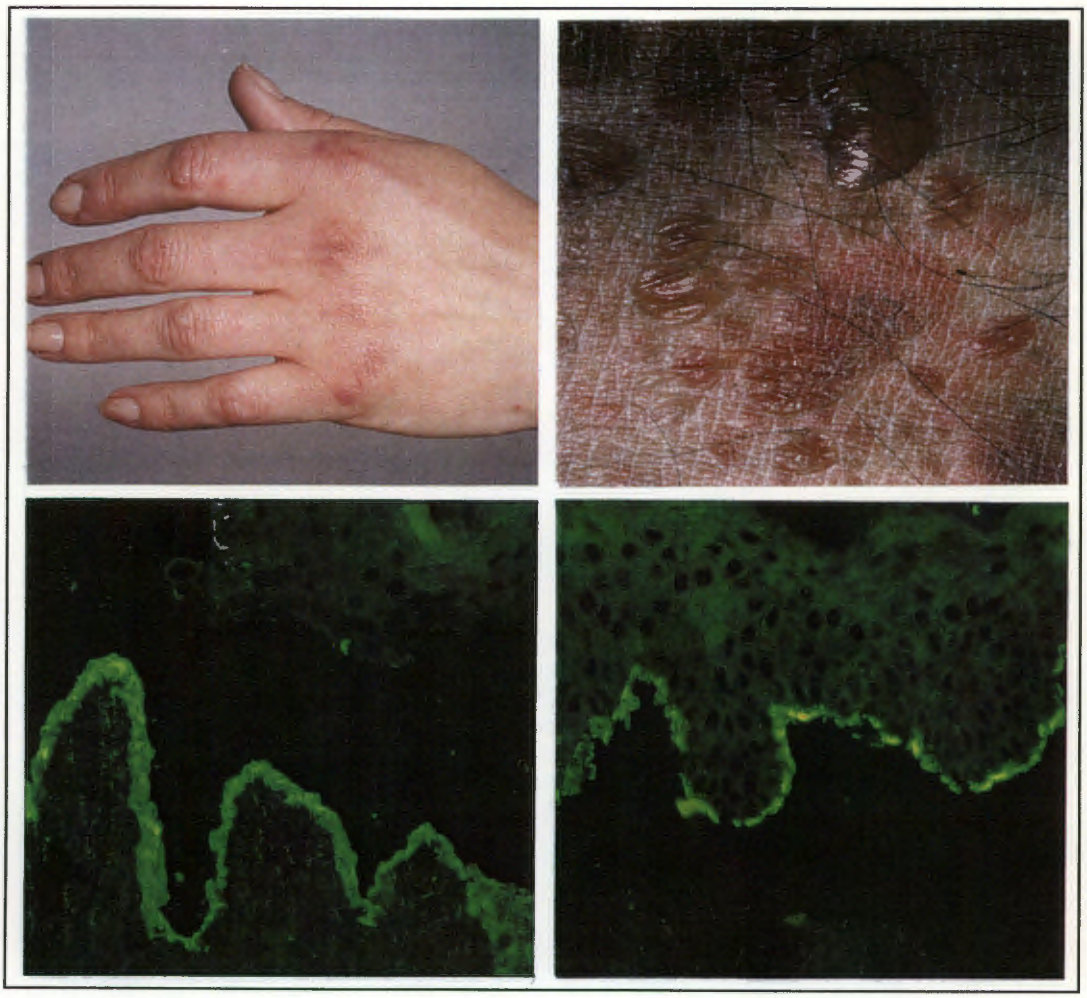
Other patterns of erythema are seen in 12%. They present with persistent erythematous patches, often worsened by sun, in various sites, including the "V" of the neck, ears, forehead and palms (Yell *et al* 1996a). A "non-specific rash" has previously been reported in 59% of SLE patients (Wysenbeek *et al* 1992).

Blistering is uncommon in SLE, occurring in < 5% of all patients. It may be due to a primary blistering disease in association with SLE, vasculitis, blistering of SLE lesions themselves, or "bullous SLE".

A number of criteria have been suggested in an effort to delineate "bullous SLE": SLE fulfilling the criteria of the American College of Rheumatology, a chronic widespread blistering eruption, a subepidermal blister with acute neutrophil-predominant inflammation in the upper dermis, immunoglobulin (Ig) and complement deposition at the BMZ on direct immunofluorescence, immune deposits ultrastructurally localised on or beneath the lamina densa and a clinical response to dapsone (Gammon & Briggaman, 1990), see **figure 2**, next page.

A number of patients fulfilling these criteria have antibodies to type VII collagen (Barton *et al* 1986; Gammon *et al* 1985). It has more recently been suggested that bullous SLE should include those patients with SLE and blistering with immune reactants at the basement membrane zone.

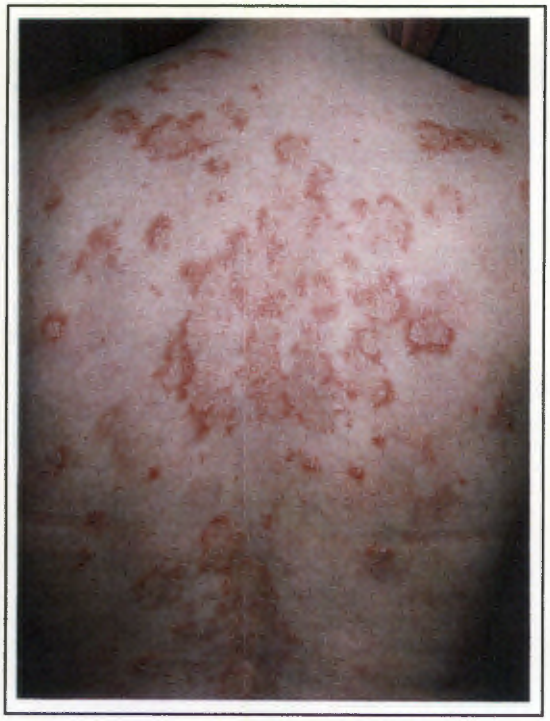
figure 2: Typical bullous SLE. Blistering not confined to typical lesions of SLE (top right and left). IF on salt split skin, either dermal, common (bottom left) or epidermal, uncommon (bottom right).



Subacute cutaneous LE (SCLE), said to occur in 10-15% of SLE patients, includes papulosquamous and annular-polycyclic lesions. Chronic cutaneous LE is estimated to occur in 15-20% of SLE patients. It includes classic discoid LE (DLE), hypertrophic DLE, lupus panniculitis, mucosal LE and chilblain LE.

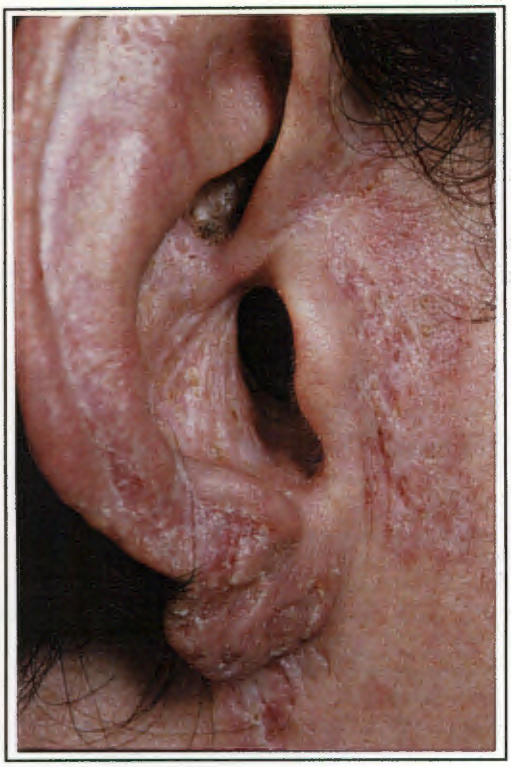
1.2.5.2 Subacute cutaneous lupus erythematosus(SCLE)

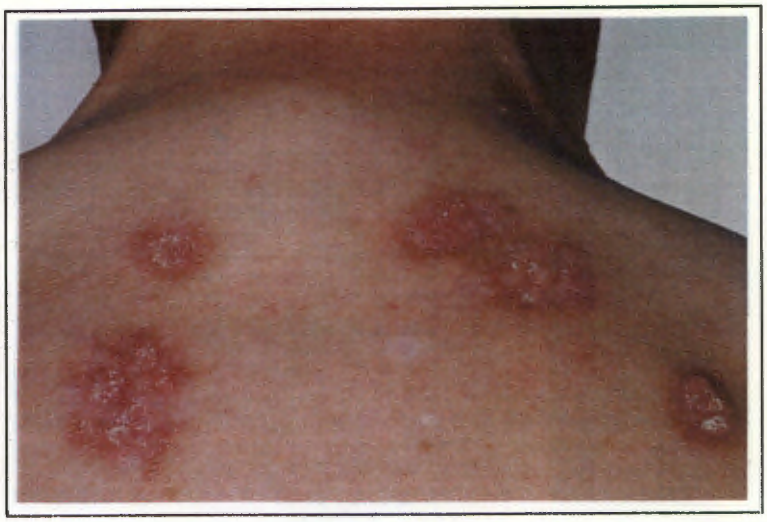
1.2.5.2.1 Subacute Cutaneous Lesions



SCLE was first described as a distinct subset of LE in 1979 (Sontheimer *et al* 1979). They characterised a group of 27 patients who had in common, a recurring, superficial, nonscarring type of cutaneous LE in a characteristic distribution. These patients had a mild systemic illness, marked by musculoskeletal complaints. Half fulfilled the ARA criteria for SLE. None had serious CNS or renal disease.

figure 3, left. These lesions are usually widespread, symmetrical and non-scarring. They occur in a characteristic, photoexposed distribution, with a LE-specific histopathology (Bangert *et al* 1984). A few patients may have a combination of both psoriasiform and annular lesions, although most have predominantly either one or the other subtype (Sontheimer, 1989). In Asians, annular erythema is the equivalent of the psoriasiform rash (Watanabe *et al* 1997). Patients falling into this subset of LE, frequently have anti-Ro antibodies (Sontheimer *et al* 1982; Miyagawa *et al* 1996) and are HLA B8 DR3/2 positive (Johansson-Stephansson *et al* 1989). SCLE may be associated with other rheumatic diseases (Provost & Watson, 1993). Photoactive medications may induce lesions of SCLE (Reed *et al* 1985), as may heat (Sontheimer, 1985). Systemic manifestations may be as varied in SCLE as in SLE and severe disease is not unusual. In particular, men with papulosquamous SCLE may be at higher risk for severe extracutaneous disease (Cohen & Crosby, 1994).





1.2.5.3 Chronic cutaneous lupus erythematosus

Chronic discoid lesions are typically inflammatory plaques with scaling, follicular plugging, atrophic scarring, central hypopigmentation and peripheral hyperpigmentation, see **figure 4 and figure 5 (above)**.

Discoid lesions occur in discoid LE but may also be seen in 23% of SLE patients (Yell *et al* 1996a). Chronic discoid skin lesions may be the initial manifestation of SLE, as found by Tuffanelli and Dubois in 10.8% (56/520) (Dubois & Tuffanelli, 1964). Discoid lesions occurred during the course of disease in 28.7% (149/520) of their patients. In 12% (9/73) of SLE patients, DLE was diagnosed prior to the development of systemic symptoms (Yell *et al* 1996a). It has been suggested that SLE patients with discoid lesions may have a more benign clinical course with less severe renal disease, than unselected SLE patients (Callen, 1985). A recent study of 136 patients with DLE, described the development of SLE in 11. Most developed SLE 5 years after the onset of the cutaneous lesions. 4 cases out of these 11, had a poor prognosis: renal and/or neurologic involvement (Le Bozec *et al* 1994).

1.2.5.4 Non specific cutaneous signs

Hair loss is a common and characteristic finding in SLE, occurring in about 50%. The variability in recording alopecia, resulted in its exclusion from the ARA criteria in 1982. Alopecia may be scarring, if preceded by DLE, see **figure 6 (below, left)**, or non-scarring. Various mechanisms have been cited, including telogen effluvium, see **figure 7 (below right)** with a systemic disease exacerbation, anagen arrest associated with the use of immunosuppressives or anticoagulants and growth retardation leading to "lupus hair"(Armaz-Cruz *et al* 1958). "Lupus hair" describes short irregular frontal hair, producing a disheveled appearance, a result of growth retardation, rather than hair breakage (Alarcon-Segovia & Cetina, 1974).

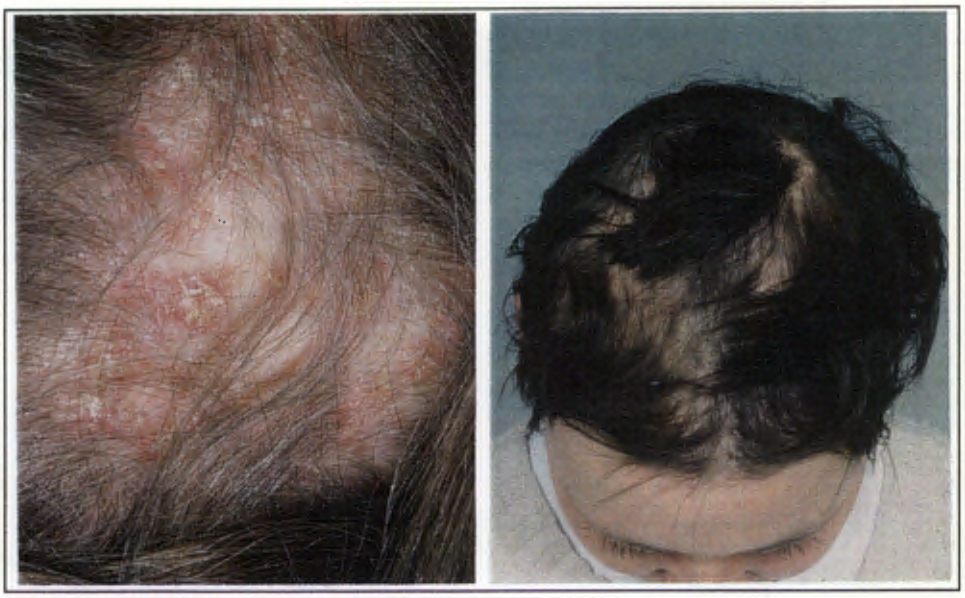


fig. 6 fig. 7

Chilblain lupus describes acral lesions induced by exposure to cold, with histologic evidence of LE (Su *et al* 1994). These become obvious lesions of DLE. They occur in 20% (15/73) of patients with SLE (Yell *et al* 1996a).

1.2.5.5 Mucosal Lesions

Mucosal lesions were considered uncommon in SLE. However, on careful inspection, see **Table 2,** more than 50% of SLE may have mucosal lesions (Jonsson *et al* 1984; Wysenbeek *et al* 1992; Robson *et al* 1992), see **figure 8 (top left, typical mouth ulcers), figure 9 (top right, thicker buccal plaques), figure 10 (below left, palatal ulceration), figure 11 (below right, DLE cheilitis) and figure 12 (bottom, episcleritis).**

fig. 8

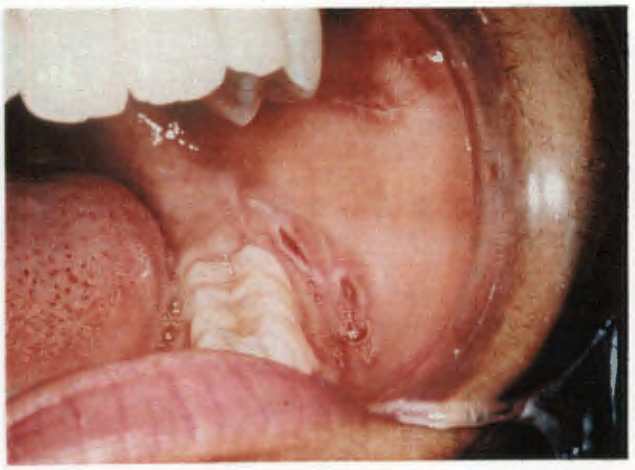


fig. 9

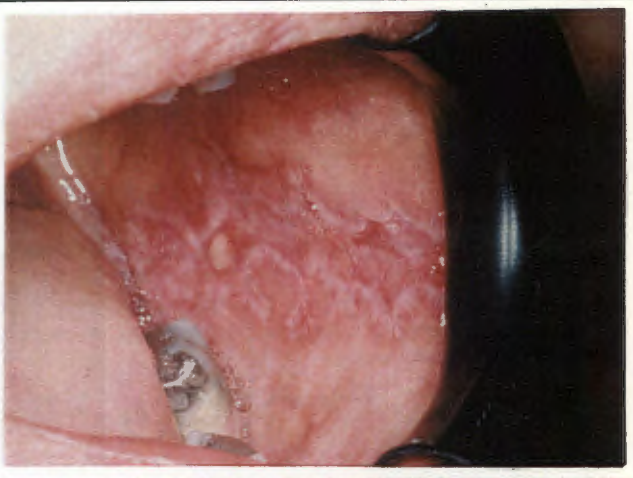


fig. 10

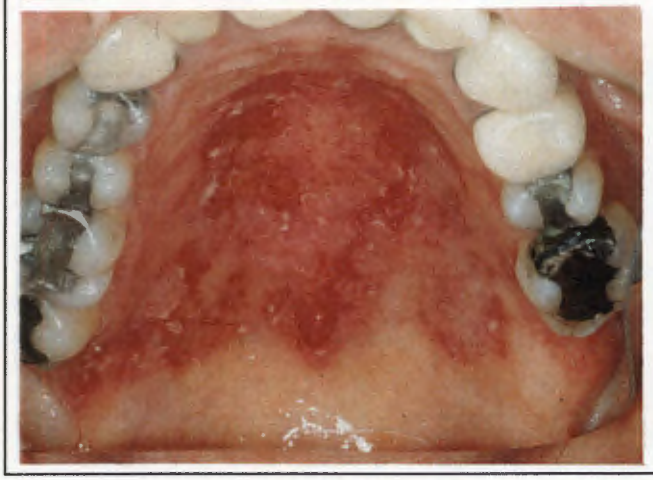
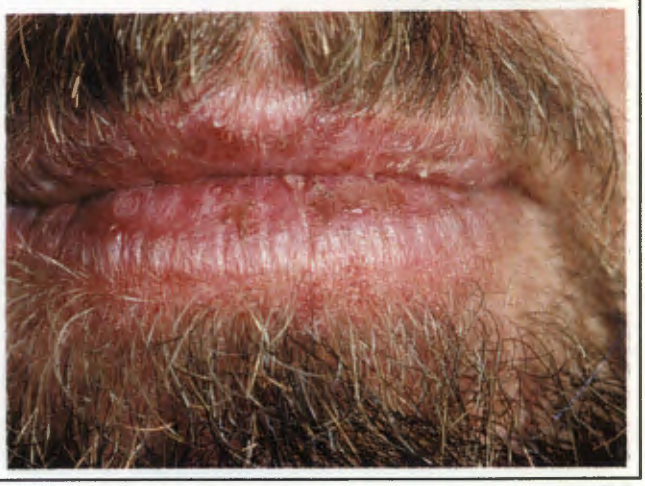


fig. 11



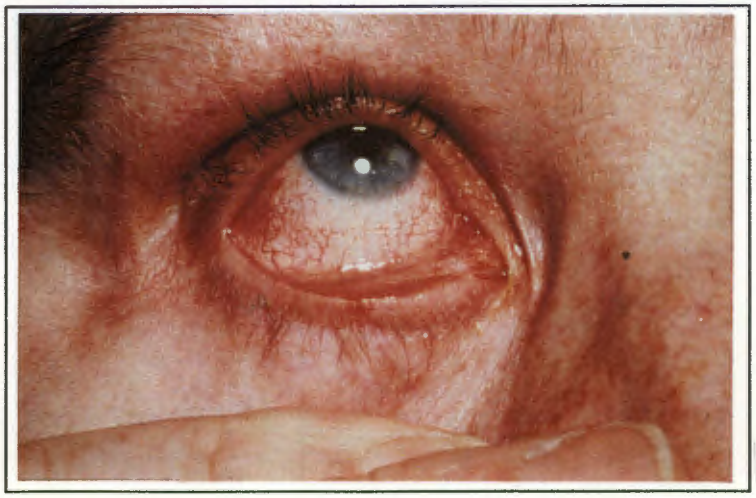


fig. 12

Photosensitivity occurs in: 32.7% (Dubois & Tuffanelli, 1964), 50% (Lee *et al* 1977), 28% (Grigor *et al* 1978), 63% (Wysenbeek *et al* 1992) and 71% (Rothfield, 1979), see **figure 13 (below)** and Table 2. It has been reported in 58% of children (Bosi Ferraz *et al* 1994). Photosensitivity, one of the major diagnostic criteria for SLE, is thought to be the result of 3 interrelated factors: susceptibility to ultraviolet radiation (UVR) -induced release of epidermal and dermal cytokines; susceptibility to UVR-induced release or translocation of sequestered antigens in the epidermis or dermis; and different specific immunologic effector mechanisms activated by cytokines and directed against discrete dermal or epidermal targets (Norris, 1993)·

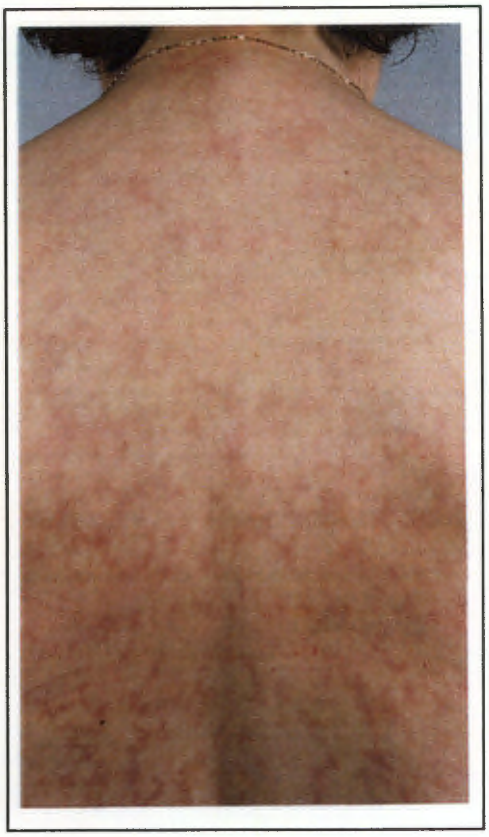


fig. 13

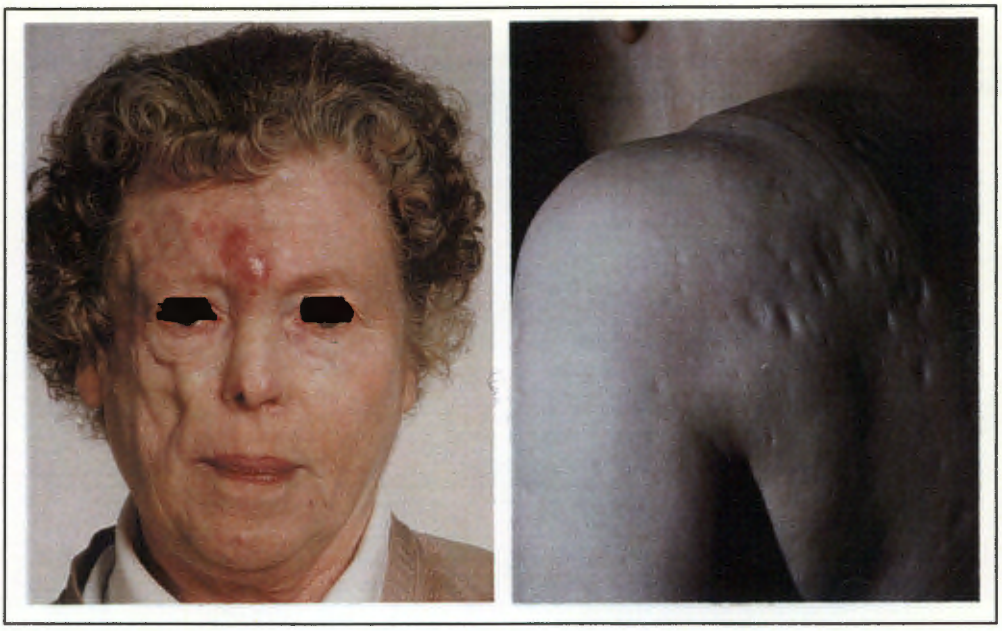
Photosensitivity precedes the clinical onset of internal manifestations of SLE in about one third of patients. This may begin as sunburn that persists for days or weeks despite no further sun exposure. The macular erythema may evolve into discrete papules that may resemble a drug eruption. The papular malar erythema and butterfly eruption which are characteristic of SLE may be due to photosensitivity. Some patients may not notice erythema after prolonged ultraviolet exposure, but hours or days later may note increased arthralgia, malaise and fever. Dubois and Tuffanelli noticed this in 32.7% of patients (Dubois & Tuffanelli, 1964). No seasonal variation has been found in the incidence of photosensitivity. It is possible that subjects whose disease is precipitated by sun exposure, may have a higher threshold for the disease, than those who develop the rash without sun exposure.

Urticaria and angioedema are common cutaneous vascular reaction patterns. Two North American studies have described prevalences of urticaria of 7% from a "neighbourhood" population) (Provost *et al* 1980) and 22% (from a national referral center)(O'Loughlin *et al* 1978), suggesting that it is more common in patients with severe disease. British studies have found urticaria in 50% of SLE patients (Scott & Rees, 1959; Yell *et al* 1996a). It suggests, therefore, that urticaria is commoner in the British population than the American population. There is no easy explanation for the American figures. It has recently been suggested that chronic idiopathic urticaria may well be autoimmune (Hide *et al* 1994) and as such it is not surprising that the rate in SLE is high.

The incidence of Raynaud's phenomenon in connective tissue diseases varies from 3% in rheumatoid arthritis to 95% in scleroderma. Raynaud's phenomenon may be the first manifestation of SLE (Lee *et al* 1977). When it is persistent and severe, patients develop other vascular complications. Raynaud's phenomenon is found in 18 - 60% of SLE patients, see Table 2.

Dermal vasculitis has been reported in 11 to 70% (Yell *et al* 1996a; Alarcon-Segovia & Osmundson, 1965) of patients with SLE. In severe cases, vasculitis may affect any part of the body. Livedo reticularis, an important marker of systemic disease, has been reported as an initial presentation of SLE (Weinstein *et al* 1987), occurring in 4% (Yell *et al* 1996a). Livedo reticularis may be associated with the antiphospholipid syndrome Weinstein *et al* 1987), or may be a cutaneous marker of CNS lupus (Yakshi, 1986).

A number of other skin conditions have been reported in SLE, see Table 1 The Gilliam classification of skin lesions associated with LE, including poikiloderma, erythromelalgia (Alarcon-Sergovia *et al* 1963), acanthosis nigricans (Brown & Winkelmann, 1968) and anetoderma, see figure 15.



Lupus profundus and anetoderma are rare cutaneous manifestations of lupus erythematosus.

Figure 14 (left) lupus profundus, figure 15 (right) anetoderma associated with SLE.

Table 2: Cutaneous manifestations in large SLE series

	Yell	Dubois	Estes	Lee	Grigor	Weinstein	Worral	Pisitiner
NO. OF CASES	73	520	150	110	375	84	100	464
YEAR OF STUDY	1995	1963	1976	1977	1982	1987	1990	1991
Skin lesion all types %	100	72	81			82	90	55
Raynaud's	60	18.4	21	46	32			25
Photosensitivity	63	32.7		50	28		48	37
Mucous membrane lesions	55	9.1	7		34	34.	36	19
Butterfly eruption	45	20.9				21		34
Urticaria	44	6.9	13	5				4
Alopecia non-scarring	40	21.3	37	38	64			
Chronic discoid lesions	23	28.6	14	28	22	11		23
Chilblains	20.5							
Alopecia scarring	14	3.6						
Butterfly blush	14	36.7	39	36	68			
Vasculitis	11		21		70	18		
Bullae	8	0.4	2					
Psoriasiform rash	7		2					
Facial oedema	5	4.6						
Diffuse hyperpigmentation	5	8.4						
Livedo reticularis	4							

2. THE Ro ANTIGENS

2.1 Historical Background

Anderson et al first used gel double diffusion, to detect precipitating antibodies in Sjögren's syndrome (SS) (Anderson *et al* 1961).

2.1.1 1969: A novel soluble cytoplasmic antigen is described

At the time when a variety of immunological reactions with tissue components in the sera of patients with SLE and related disorders was being described, a novel reaction was identified using a specific patient serum (Clark *et al* 1969). This patient (Ro), had a syndrome closely resembling SLE, with a systemic disease characterised by inflammatory lupus-like lesions over the malar eminence, arthritis and pleuropericardial disease. This patient's serum showed none of the typical reactions with nuclear components. However, this serum did show complement fixation reactions in very high titres with saline homogenates of various human tissues. The Ro antigen was subsequently isolated, partially purified and characterised. It was derived primarily from the soluble cytoplasmic fraction of a human tissue homogenate. It was found in many human organs as well as the tissue of the dog. This antigen was acidic, pH sensitive and heat labile, suggesting it was a protein. It was resistant to most proteolytic enzymes, including trypsin, pepsin and chymotrypsin. Reversible inactivation by parahydroxymercuribenzoate indicated that sulfhydryl groups may be essential for antigenicity.

This antigen was present in tissues in very small amounts. This group failed to demonstrate cellular localisation of the antigen with the indirect fluorescent antibody technique, using human, monkey and mouse tissue. They considered that this failure may have been due to the inaccessibility of the antigen, its low concentration, or that it

may be so soluble that it was removed during the washing procedures in slide preparation.

They did find very high titres in complement fixation reactions with some sera, suggesting that antibody directed toward the Ro antigen may be present in large amounts and that a significant part of a patient's hypergammaglobulinaemia may be due to this antibody. They thought it extremely interesting that a tissue component present in extremely small amounts, could elicit the production of large amounts of antibody. They found antibodies to this antigen in 40% of unselected LE sera.

2.1.2 1975: Sjögren's syndrome antibodies were described.

In attempts to find antibodies specific for Sjögren's syndrome (SS), 3 different antibodies were described in 18 SS sera. These were designated A, B and C. A Wil-2 cell line was used as a source for tissue extraction. An Ouchterlony double-diffusion method was used to demonstrate precipitating antibodies in human sera from SS patients with (SS-C) or without Rheumatoid Arthritis (RA) (SS-A and SS-B). There was complete non identity between SS-A, B and SS-C. The SS precipitating systems were not related to precipitating antibodies to native or denatured deoxyribonucleic acid (DNA), Smith (Sm), ribonucleoprotein (RNP) or small nucleoprotein (snRNP) (Alspaugh & Tan, 1975). This group suggested that at least one or more of the precipitins may be antibodies against cytoplasmic or non-nuclear components.

2.1.3 1979: Collaboration concludes that Ro and SS-A are similar

Double diffusion in agar gel and preparation of calf thymus extract, human spleen extract and Wil-2 cell extract were performed, exchanging both extracts and prototype sera between the two laboratories. This confirmed immunologic identity between sera containing anti-Ro and anti-SS-A, anti-La and anti-SS-B and confirmed immunologic

distinction between the Ro(SS-A) and La(SS-B) systems. In addition, they showed agreement between Ro and SS-A and La and SS-B with respect to their physicochemical properties, including acidic nature, behaviour in diethylaminoethyl (DEAE) - cellulose chromatography and an approximate molecular weight using gel filtration (Alspaugh & Maddison, 1979).

However, they did point out interesting discrepancies, including a difference in findings with regard to cellular localisation. Ro was considered cytoplasmic, based on demonstration of these systems only in cytoplasmic extract of fresh calf thymus and production of cytoplasmic fluorescence of calf thymocytes. On the other hand, nuclear localisation of SS-A and SS-B was provided by similar fluorescence studies using Wil-2 cells as tissue substrate.

2.2 Evidence for Pathogenicity

2.2.1 Epidemiology

Although the Ro particle is a relatively minor ribonucleoprotein, about 1-5 X 10^5 copies per cell (Wolin & Steitz, 1984), it is a potent antigen. The concentration of anti-Ro in an individual serum may reach 30 mg/dl, constituting a significant proportion of the total antibody concentration (Harley *et al* 1986). Anti-Ro antibodies are associated with the development of SCLE and NLE. Ro / SS-A antibodies are produced by most patients with subacute cutaneous and neonatal lupus erythematosus. It has been considered likely that these antibodies and the antigens against which they are directed, are pathogenic in SCLE and NLE (Buyon *et al* 1989; Sontheimer, 1989). In addition, anti-Ro antibodies may play a pathophysiologic role in the development of congenital heart block in neonatal lupus (Alexander *et al* 1992). More than 96% of SS patients have anti-Ro antibodies (Harley *et al* 1986). The levels of antibodies are much higher in patients with purpura, leucopaenia, lymphopaenia and increased polyclonal gammaglobulins than in those without these conditions (Harley *et al* 1986). About 10 % of normal controls had anti-Ro antibodies, in low titre (Harley *et al*

1986). Using immunoblotting, the positivity rates of Ro and/or La auto-antibodies were higher in sera from Japanese patients with SLE compared with titres reported for Caucasians but not in sera from healthy volunteers (Inagaki *et al* 1989). This may be due to a racial difference in HLA distribution and may explain some differences in the common signs, symptoms and laboratory anomalies in SLE between races (Inagaki *et al* 1989). Japanese SLE patients have a lower incidence of photosensitivity, sicca syndrome and NLE (Inagaki *et al* 1989).

2.2.2 Clinical

Anti-Ro antibodies are related to several clinical autoimmune disorders (McCauliffe, 1997b):

63% -82% of patients with SCLE (Sontheimer *et al* 1982; Deng *et al* 1984; Lopez-Longo *et al* 1997).
Virtually all patients with NLE (Laxer *et al* 1990; Brucato *et al* 1995).
75% of patients with homozygous complement (C) C2 and C4 deficiency with SLE-like disease (Provost *et al* 1983).
80% of patients with primary Sjögren's syndrome (Hochberg *et al* 1985).
62% of ANA-negative SLE Maddison *et al* 1981).
SLE with interstitial pneumonitis (Hedgepeth & Bouluare, 1988).
100% of patients with hypergammaglobulinaemic purpura with SLE (Senecal *et al* 1995).
A few patients with primary biliary cirrhosis and secondary SS (Dorner *et al* 1996a).

A 10-year follow up of one hundred anti-Ro antibody positive patients indicates that these patients have a diverse clinical presentation and that this anti-Ro response may persist for years. Some patients appear to have a static disease process for years. On the other hand, 65% had a progressive course.

At least 25% demonstrated a dynamic change in clinical presentation with the development of SS and/or a progressive 'rheumatoid-like' arthritis. Interstitial pulmonary disease and vasculitic insults occurred frequently in these patients. Renal disease occurred in 19 and in 47% of these, no anti-dsDNA antibodies were detected. Cutaneous manifestations were prominent, with photosensitivity and a malar rash being most common. 20% had discoid lesions, 20% had SCLE lesions (Simmons-O'Brien *et al* 1995).

Unlike anti-Ro antibody positive SCLE patients, anti-Ro antibody positive lupus patients with SS appear to have a more guarded prognosis. These SS/LE patients have an increased frequency of pulmonary, neurological, and renal disease compared to SCLE patients (Provost *et al* 1997).

Photosensitivity is one of the major criteria for the classification of systemic lupus erythematosus (Tan *et al* 1982). A comparative survey of 36 blacks and 56 whites with SLE in Johannesburg, showed photosensitivity in only 19% of blacks, compared to 71% of whites (Sutej *et al* 1989). A strong negative association with anti-Ro antibodies was noted as only 4% of the Ro-positive patients were photosensitive, compared with 55% of Ro-negative patients. The authors postulated a 'blocking' factor in blacks with SLE which blocked the photosensitivity caused by the interaction of Ro with UVR.

Anti-Ro antibodies can cross the placenta, by the 12th week of gestation (Deng *et al* 1987) where they are implicated in causing NLE. Isolated congenital heart block occurs in 1 in 15 000 to 22 000 live births. Skin lesions in affected infants are clinically similar to those of SCLE. In several months, as the maternally acquired antibodies are cleared from the infant's circulation, the skin lesions resolve. Anti-Ro maternal antibodies may also be associated with hepatitis and thrombocytopaenia as part of NLE (Laxer *et al* 1990; Watson *et al* 1988). Although controversial, despite having anti Ro autoantibodies, most of the mothers of offspring with congenital heart block remain healthy, with largely minor symptoms (arthralgia, dry eyes and photosensitivity) many years after delivery (Press *et al* 1996; Brucato *et al* 1995).

2.2.3 Histology

A direct immunofluorescence pattern of in vivo epidermal IgG binding that is associated with anti-Ro (Lee *et al* 1989; Velthuis *et al* 1989) has been suggested as displaying the same type of pattern as that obtained in vitro at the keratinocyte cell surface after UVB exposure (Furukawa *et al* 1990).

A different group has described basal fluorescence in human skin using a monoclonal Ro-antibody. This antibody reacts with a 60kD polypeptide in extracts from human spleen and a 60 and 48kD protein in extracts of human skin (Mayet *et al* 1988). This group suggested that, particularly in SCLE, cross-reaction of the Ro-antibody with one of the lower molecular weight keratins happens in that area of the human epidermis which histologically shows cellular destruction.

This is supported by the demonstration of fine granular IgG deposits in the epidermal basal layer with both nuclear and cytoplasmic localisation in 16/51 biopsies from spontaneously evolved lesions of SCLE and in 7/18 from UVR irradiated skin (Nieboer & Velthuis, 1991). In UVR irradiated skin, this was exclusively in the areas of extreme basal cell degeneration.

Using monocyte effectors, anti-Ro sera causes profound antibody dependent cellular cytotoxicity (Norris *et al* 1984). This may be related to the mononuclear cell - associated tissue change seen in cutaneous lupus lesions.

In an in vitro electrophysiological and immunocytochemical experimental model contrasting neonatal and rabbit cardiac tissue, it was found that sera and IgG-enriched fractions from anti-Ro antibody-positive mothers of infants with NLE and congenital heart block, bind to neonatal rather than adult rabbit cardiac tissue. In addition, they alter the transmembrane action potential (Alexander *et al* 1992). The additional presence of anti-La antibodies was not additive nor synergistic. Sera containing other antibody specificity's, anti-native DNA, cardiolipin, Sm and nuclear RNP, failed to stain the neonatal cardiac tissue or produce alterations in membrane polarization.

A further study using IgG fractions of sera from SLE patients with anti-Ro or La, with preparations of adult rabbit hearts and ventricular myocytes from young rabbit hearts, showed that anti-Ro or anti- La but not normal sera nor anti-RNP, induced cardiac conduction abnormalities similar to those observed in NLE (Garcia *et al* 1994).

2.2.4 Immunopathology

A number of papers from the same group, have shown that UVR induces Ro/SSA antigen expression on keratinocyte surfaces *in vitro* (LeFeber *et al* 1984), that targets coated with this antigen were lysed by mononuclear cells in the presence of antibodies (Norris *et al* 1984) and, *in vitro*, that human skin grafted onto nude mice bound Ro/SSA antibodies. This was increased by UV-irradiation (Lee *et al* 1989).

The effects of UVR on Ro expression of skin fibroblasts *in vitro* has shown an IgM-positive immunofluorescence in the nucleus, using 'monospecific' serum (Wollina *et al* 1991). Similar studies were performed on normal, uninvolved psoriatic and involved psoriatic skin (Wollina *et al* 1991). Psoriatic skin showed binding of anti-Ro after UVA. Normal and noninvolved psoriatic skin gave nuclear staining only after psoralens and UVA. Cells preincubated with anti-Ro serum, and subsequently UVA or PUVA treated, showed strong cytoplasmic fluorescence. Unfixed frozen normal skin and lesional psoriatic skin, showed no florescence with high dose UVB (10^3 to 2 X 10^5 J/m^2) but fluoresced after high dose UVA or PUVA (Wollina *et al* 1991).

2.2.4.1 animal experiments

To more directly determine if anti-Ro antibodies induced NLE skin disease, Lee *et al* immunoaffinity purified human Ro autoanibodies using purified Ro antigen from bovine spleen. These were given intravenously to immunodeficient mice engrafted with human skin. The human Ro autoantibodies, bound to the human basal keratinocytes. This binding was markedly augmented by UVR (Lee *et al* 1989). Antibody bound to the human skin graft, in a pattern of distribution indistinguishable

from that seen in lesions of anti-Ro SCLE donors (Lee *et al* 1989). In most areas, the epidermal staining appeared mainly cytoplasmic, with some nuclear staining. In occasional areas, there was accentuation of IgG deposition at the basement membrane.

Davis et al demonstrated that UVB irradiation of guinea pigs which had been passively sensitised by intradermal injections of anti-Ro positive or negative sera, produced elevated cutaneous blood flow, only in animals that had received anti Ro positive sera (Davis *et al* 1989).

2.2.4.2 translocation experiments

It is proposed that UVR may augment the release of epidermal and dermal inflammatory mediators and may disorder local and systemic immunity. In addition, it has been postulated that it may induce new antigens within the epidermis or at the basement membrane zone, or may induce the redistribution of normally sequestered antigen, from within the epidermal cell to the cell surface (Norris, 1993). This mechanism of translocation has been suggested as causal in the cutaneous lesions of SCLE and NLE (Jones, 1992b).

The binding of anti-Ro antibodies to the cell surface after ultraviolet irradiation (UVR) and oestradiol treatment, was described by the same group (LeFeber *et al* 1984; Furukawa *et al* 1990; Jones, 1992a; Furukawa *et al* 1988). They proposed that UVR or oestradiol, induced translocation of these antigens to the keratinocyte plasma membrane, exposing them to possible binding by extracellular antibodies. Further work on Ro translocation (Jones, 1992a) assessed whether injurious, metabolic, inflammatory, immunological or hormonal stimuli would induce Ro expression, or modulate that found in response to UVB.

2.3 There are a number of different Ro antigens

In 1981, Lerner *et al* showed that human Ro autoimmune sera precipitated a new class of small RNAs, the human cytoplasmic RNA (hYRNA) (Lerner *et al* 1981). RoRNPs in all mammalian cells examined, comprise one of several different but closely related RNA molecules.

In most nucleated cells there are at least 5 major RNAs: hY1, hY2 (a degradation product of hY1), hY3, hY4 and hY5 (Boire & Craft, 1989), which range in length from 84 to 112 nucleotides. hY5 RNA is significantly over-represented on Ro RNPs relative to hY1 and hY3, although the mechanism responsible for this pattern of expression is unknown. hY5 RNA occupies a large fraction of the 60 kD Ro protein in human Ro RNPs, has an atypical sequence motif and biochemical properties, not found on other Y RNAs and constitutes an RNA antigen in certain autoimmune patients. The four human Y RNA genes are arranged on chromosome 7q36 (Maraia *et al* 1996).

Indirect evidence indicates that each RoRNP probably contains a single hY RNA molecule (Wolin & Steitz, 1984). Physicochemical studies on native RoRNPs indicate that the particles segregate into three distinct subpopulations, one containing hY5, another containing hY4 and a third containing hY1, hY3 and hY4 (Boire & Craft, 1989).

Initially, it appeared that the 60kD Ro polypeptide was the sole stable protein component in the complex and that the La protein associated with hY RNAs only in a transient manner, just as it associates with all RNA polymerase III transcripts (Rinke & Steitz, 1982). However, it was then shown that hY RNAs in their mature forms were about 30% precipitable by anti-La sera (Wolin & Steitz, 1984). A further group were able to distinguish biochemically certain RoRNPs in which La was a stable component (Boire & Craft, 1990).

Both the 60 and 52kD Ro are associated with four (hY1, hY3, hY4, hY5) RNA species (Peek *et al* 1993; Ben-Chetrit *et al* 1988). Ro found in foetal red blood cells apparently associates with only 2 RNA species (Rader *et al* 1989), while in platelets, only hY3 and hY4 could be detected (Itoh & Reichlin, 1991a). Additionally, there are species differences in the structure of the Ro complex, with only 2 RNA species, mY1 and mY2, identified in the mouse (Wolin & Steitz, 1983).

Earlier data suggested that Ro autoimmune sera immunoprecipitated hYRNA. Protein was needed for this; this was shown to be a 60kD protein (Wolin & Steitz, 1984). Later, Ro antibodies were identified that specifically immunoprecipitated hY5RNA and a 60kD protein but not other types of hYRNA (Boire & Craft, 1989). They concluded that some Ro autoimmune sera contain antibodies directed at a conformational epitope that is expressed only on Ro-hY5RNA particles but absent on hY1, 2, 3 and 4 containing particles.

Additionally, anti-Ro antibodies from SLE and SS sera eluted from Western blots of human cell extracts, indicated that a 52-kD protein, in addition to a 60-kD Ro, is associated with hY RNA in nucleated cells (Ben-Chetrit *et al* 1988). While 60kD Ro has been shown to be directly associated with the hY RNAs in complexes, 52kD Ro has not been shown to directly contact any of the hY RNAs. However, 52-kD protein-specific antibodies affinity purified from SLE and SS patient sera, were able to immunoprecipitate RoRNPs along with the corresponding hY RNAs 1-5 (Ben-Chetrit *et al* 1988).

It has been suggested that, in fact, the 52kD Ro is not a detectable component of the RoRNP complex under some conditions, even though it reacts with Ro autoimmune sera (Kelekar *et al* 1994). Kelekar et al analysed HeLa cell extracts by glycerol density gradient fractionation, to determine the distribution of the individual protein and RNA components of RoRNPs. They found that 52kD Ro was not detectable in an RNP complex with the 60kD protein under a variety of conditions. Pretreatment of cell extracts with ribonuclease affected gradient migration of the 60kD Ro but not the 52kD protein, suggesting that the latter is not complexed to RNA. The migration of

the hY RNAs in these gradients closely followed that of 60kD and not 52kD Ro (Kelekar *et al* 1994). They suggested that other workers had shown coimmunoprecipitation of the 60kD when using affinity purified 52kD antibodies from patient antisera (Ben-Chetrit *et al* 1988) because certain autoimmune patient sera may not react against a protein immobilized on a membrane, while they would recognise it in solution. In other words, patient Ro antisera determined to be monospecific for 52kD Ro by Western blotting may contain antibodies to discontinuous epitopes on 60kD Ro that escape detection with this technique.

Further weight to this suggestion was added by a study of 12 patients sera, selected as a cross-section of anti-Ro associated disease states (Saitta *et al* 1994). They combined solution phase immunoprecipitation assays with solid phase ELISA and immunoblot experiments with recombinant Ags, to dissect the heterogeneity within the anti-60kD response and its relationship to the anti52kD response. Characterisation of the different serum reactivities along the length of the protein, allowed the identification of seven autoepitopes. These included both continuous and discontinuous determinants. The pattern of epitope recognition correlated with both the anti-60kD Ro antibody titer and the concomitant presence of antibodies to the 52kD Ro protein. It did not correlate with clinical diagnosis or HLA-DR/DQ genotypes in this small group of patients.

The 52kD Ro component was first described by Ben-Chetrit *et al* (Ben-Chetrit *et al* 1988). They showed a 52-kD protein in addition to the 60-kD protein, present in the sera of 80% of patients with detectable Ro precipitins. In addition, this study showed that these antigens contained different and unique epitopes which did not generate cross-reactive antibodies. However, this group suggested that the 52 and 60-kD antigens in their native state, may exist as a complex or as associated particles.

Besides the proteins mentioned above, Ro RNPs may contain additional components, as the presence of single copies of the Ro proteins, together with the La protein and one RNA molecule, cannot account for the molecular masses of 230 to 350kD of Ro RNPs, observed in gel filtration (Boire & Craft, 1990).

It has since been found that Ro autoantibodies react with at least four distinct proteins, of 3 different molecular weights, 52-, 54- and 60kD (Rader *et al* 1989). The 60 and 52kD antigens have been cloned (Chan *et al* 1991b; Chan *et al* 1991a). These 52 and 60 kD proteins are structurally dissimilar (Itoh *et al* 1991). The 54 and another 60 kD protein are thought to be variants of these 52 and 60 kD Ro proteins respectively. The 54kD Ro has been detected only in red blood cells and their nucleated precursors (Itoh *et al* 1991). The 60kD Ro in lymphocytes and red blood cells are distinct but related. Most patient autoantibodies which bind to the 60kD Ro lymphocyte polypeptide, cross react in most but not all cases with the 60kD red blood cell Ro. Similarly, antibodies binding to the lymphocyte 52kD Ro cross react in most cases with the red blood cell 54kD Ro. The 54 and 60kD red blood cell proteins are antigenically distinct from the previously described 52kD and 60kD Ro's respectively. Thus, the four isoforms of Ro fall into two non-cross-reacting families (Itoh *et al* 1990). More recently, a novel 52kD Ro protein was detected in human platelets, which further increase the heterogeneity found in human Ro RNP complexes (Itoh & Reichlin, 1991a).

The intricacy of the Ro polypeptide-RNA complex is further complicated by its association with the 48-kD La protein forming a larger assembly (Peek *et al* 1993; Deng *et al* 1985). The La protein does not share antigenic determinants with the Ro peptides and the genes encoding them show no homology (Chambers & Keene, 1985). The La component is complexed to a spectrum of RNAs that includes precursor forms of 5S rRNA and tRNA, certain small viral RNAs and the Ro associated RNAs (Rinke & Steitz, 1982).

A number of papers have emerged attempting to unravel the link between the 52 kD Ro, 60 kD Ro and La antibody development. A case report described a patient with typical Sjögren's syndrome and high titer anti-Ro antibodies, who subsequently developed anti-La antibodies, suggesting that anti-La antibodies may follow anti-Ro antibodies (Satoh *et al* 1996).

Immunization of mice with recombinant mouse La resulted in a specific, isotype-switched autoantibody response, which was initially directed toward the La C sub-fragment (aa 111-242) but rapidly spread to involve the La A (aa 1-107) and La F (aa 243-345) regions of the La antigen. Intramolecular spreading of the anti-La antibody response was further demonstrated by the appearance of autoantibodies to multiple, non-overlapping antigenic regions of La, after immunization of mice with the 107-aa La A sub-fragment. Moreover, immunization of mice with recombinant mouse or human La also elicited specific anti-60-kDa Ro IgG antibodies in all strains tested. Mice immunized with 60-kDa Ro produced a high titer anti-Ro antibody response, which was also associated with intermolecular spreading, resulting in the specific appearance of anti-La autoantibodies. These findings show that the development of autoantibodies to multiple components of the La/Ro ribonucleoprotein complex may follow initiation of immunity to a single component. In addition, the data reveal the incomplete nature of immune tolerance to La and Ro despite their endogenous expression in all nucleated cells. These observations are likely to account for the coexistence of anti-La/Ro antibodies in autoimmune disease and suggest a general explanation for the appearance of mixed autoantibody patterns in systemic autoimmune disorders (Topfer *et al* 1995).

The physical association of Ro52 with the Ro/La RNP has remained controversial, implying that antibodies to the 52 kD Ro are not a consequence of intermolecular spreading and may be triggered independently of the anti-60 kD Ro response.

To examine this relationship of the immune response to the 52 kD Ro and 60 kD Ro, mice were immunized with recombinant 52 kD Ro, 60 kD Ro, or La, and examined for autoantibody production. Immunization with 52 kD Ro resulted in rapid, high titre Ab production to the 52 kD Ro, followed 7 to 14 days later by lower titer autoantibody production to 60 kD Ro. Immunization with 60 kD Ro led to anti-60 kD Ro, which was also followed 7 to 14 days later by a lower titer anti-52 kD Ro response. Cross-reactivity of affinity-purified Abs from immune mouse sera was not observed (Keech *et al* 1996b).

To determine whether immunization of healthy non-autoimmune mice with 52-kD Ro induces a secondary antibody response to other components of the 48-kD La-60-kD /Ro RNP complex and vice versa, female BALB/c mice were immunized with recombinant human 48-kD La, 52-kD Ro, or 60-kD Ro proteins, or the polypeptide control, each purified by Ni2+ affinity chromatography (Tseng *et al* 1997). Mice subsequently received booster injections with the same antigen every 10-21 days. Immune responses were measured by ELISA, immunoblotting of recombinant antigens, and immunoprecipitation of 35S-methionine-labeled in vitro translation products.

Immunization with 48-kD SS-B/La resulted in anti-48-kD SS-B/La antibodies within 45 days, followed 10 days later by a secondary response to 52-kD SS-A/Ro, as measured by ELISA: Antibody spreading to 60-kD Ro was not detected. Immunization with 52-kD Ro resulted in rapid high-titer anti-52-kD Ro responses within 27 days. Spreading to 48-kD SS-B/La occurred in only 1 mouse and 60-kD Ro was detected in a minority of the mice after prolonged antigen exposure. Immunization with 60-kD Ro led to anti-60-kD Ro responses within 37 days, followed 3 months later by low-titer anti-48-kD SS-B/La and anti-52-kD Ro antibodies. All primary immune responses were confirmed by immunoblotting and immunoprecipitation. While immunoblotting of the recombinant proteins revealed reciprocal intermolecular spreading in the majority of mice, immunoprecipitation showed that predominant spreading was generated after immunization with 48-kD SS-B/La, which consistently resulted in antibodies to 52-kD Ro (Tseng *et al* 1997).

2.3.1 60 kD Ro

In 1988, the cloning of a gene encoding a 60kD Ro antigen was reported (Deutscher *et al* 1988). The next year, a cDNA sequence was reported that appeared to be a homologous gene, or a differentially spliced version of the same gene (Ben-Chetrit *et al* 1989). Both these sequences encode 60kD proteins that are virtually identical except for the carboxy terminal amino acids. More recently, these were confirmed as different versions of the 60kD protein, both of which exist in cells (Chan *et al* 1991b). They are thought to arise from differential splicing of the same gene transcript. The 60kD sequence published by Deutscher et al may be the most abundant type of 60kD Ro protein in cells.

A region of the 60kD Ro amino acid sequence showed a resemblance to the RNP consensus motif found in most RNA-binding proteins (Deutscher *et al* 1988). In addition, 60kD Ro contained a potential zinc binding finger motif, distinct from the RNP consensus region, which may participate in the interaction with human Y RNAs or other proteins. This zinc finger is not typical of other zinc finger proteins and is not conserved in other species (Wang *et al* 1996; O'Brien *et al* 1993). The 60kD Ro may bind hY RNA through the RNP consensus motif and interact with other proteins, such as the 52kD Ro protein through the Zinc finger (Deutscher *et al* 1988).

There is no extensive sequence homology between the 60kD protein and other published protein sequences (McCauliffe & Sontheimer, 1993b). However, six segments of this protein have limited homology to different portions of a vesicular stomatitis virus (VSV) nucleocapsid protein. Five of these homologous regions have significant reactivity to human Ro autoimmune sera by ELISA. In a study of 80 SLE patients, 80 healthy controls, 47 rheumatic disease controls and a small number of patients infected with the vesicular stomatitis virus, there was significantly different binding to the viral proteins in the SLE patients compared with other groups. The reactivity of the SLE sera included binding to the internal viral matrix and the nucleocapsid proteins (Hardgrave *et al* 1993). This suggests that an immune response

initially directed at the VSV, or a similar viral protein, might cross-react with the 60kD Ro protein by way of its cross-reacting epitopes (Scofield & Harley, 1991). Many features of VSV are consistent with it playing a role in causing anti-Ro associated disease, see (Scofield & Harley, 1991) discussion for a review of the literature supporting this.

The shared antigenic sequences between the 60kD Ro and the nucleocapsid protein of the VSV, plus the possibility that viral infection can alter the normal cellular distribution of Ro antigens, (Tesar & Armstrong, 1986) suggests that virus infection might have a dual impact on the Ro autoimmune response

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Anti-60kD Ro peptide Fab fragments from infiltrating salivary gland lymphocytes in a SS patient, have been cloned (Suzuki *et al* 1997). This group generated three human antibody Fab fragments by using a phage-display technique. Sequence analysis demonstrated that two of the three Fab clones (E-42 and E-60) used homologous heavy chains derived from the germline VH gene DP73 in combination with different light chains which were derived from germline V kappa gene L6 and V lambda gene DPL23. The third Fab clone (E-56) used another heavy chain derived from the germline VH gene DP31 in combination with the identical light chain as that of E-42. All three Fab clones revealed a high number of somatic mutations that probably occurred in antigen selection. This suggests that this patient's salivary gland lymphocytes have restricted usage of VH and VL genes of anti- Ro antibodies.

2.3.2 52 kD Ro

A 52-kD protein in addition to the 60-kD protein, present in the sera of 80% of patients with detectable Ro precipitins was found (Ben-Chetrit *et al* 1988). On SDS-immunoblot, the 52-kD protein appears to be the predominant SS-A/Ro component recognised by sera from patients with SS and, in particular, mothers of children with congenital heart block (Buyon *et al* 1994; Buyon *et al* 1989; Ben-Chetrit *et al* 1990).

Neonatal lupus is strongly associated with antibodies reactive with Ro and/or La proteins, independent of maternal disease activity or classification. The subclass distribution of anti-48 kDa La and anti-52 and 60 kDa Ro antibodies in the maternal and neonatal circulation, in pregnancies affected and unaffected by the development of congenital heart block was compared. Sera were obtained from 32 mothers (during 34 pregnancies 23 complicated by CHB and 11 healthy) demonstrated to have anti-Ro and/or La. Maternal and neonatal autoantibodies were evaluated for subclass distribution by ELISA. All 4 subclasses of anti-Ro and La antibodies crossed the placenta and were detectable in sera obtained from the umbilical cord. IgG1 and IgG3 were the major subclasses represented in the 48 kDa La and 52 kDa Ro responses. All subclasses, including IgG2 and IgG4, were observed in about one-third of the anti-52 kD Ro and 48 kD La responses. In contrast, anti-60 kD antibodies were, with rare exception, confined to IgG1. Except for anti-48 kD La IgG3 antibodies, no significant differences were observed between affected and unaffected pregnancies in the ratio of maternal to neonatal levels of any of the antibody subclasses. Overall, there were no significant differences in the subclass profiles between mothers whose children had heart block and those who did not. The IgG subclasses of anti-48 kDa La(SSB) and anti-52 and 60kD Ro do not account for the susceptibility of one fetus versus another for the development of CHB. Anti-60 kD Ro antibodies were more restricted in subclass distribution than anti-52 kD Ro(SSA) or 48 kDa La(SSB) responses (Tseng *et al* 1996a).

One study sought to determine whether the fine specificity of antibody profiles remains stable or evolves over time and whether these findings relate to clinical status. Sera from 23 mothers whose children had neonatal lupus (22 heart block, one skin) were evaluated by SDS-immunoblot. For each mother two samples were available at least 13 months apart; the mean duration of time between testing was 45 months +/- 27 S.D. (range 13-108 months). Twenty-two of the 23 initial profiles were identical to the results obtained in a later sample. The health status of seven (30%) of 23 mothers changed after the birth of the affected infant but the immunoblot specificity of the antibodies remained unchanged. SLE was the initial and final diagnosis in the only mother whose profiles differed, with development of weak reactivity to 48 kD SSB/La in addition to the 52kD Ro after 14 months. The fine specificity of anti-Ro/ La antibodies as assessed by immunoblot was highly stable for years. Progression of clinical status was not associated with a concomitant change in antibody profile (Tseng *et al* 1996b).

Reactivity to the denatured 52kD Ro component seems characteristic of these mothers (Brucato *et al* 1995). These mothers present with an increased prevalence of DR3, B44/DR5, DR3/DQ2 and A1/Cw7/B8/DR3/DQ2.

A recent study has shown that IgG-enriched fractions and anti-52-kD Ro antibodies affinity-purified from sera of mothers whose children have CHB induce complete atrioventricular (AV) block in the human fetal heart perfused by the Langendorff technique and inhibit L-type Ca2+ currents at the whole-cell and single-channel level. Immunization of female BALB/c mice with recombinant 52-kD SSA/Ro protein generated high-titer antibodies that crossed the placenta during pregnancy and were associated with varying degrees of AV conduction abnormalities, including complete AV block, in the pups (Boutjdir *et al* 1997).

Congenital heart block, associated with antibodies to Ro and La, is most often detected between 18 and 24 weeks of gestation, yet the maternal heart is unaffected. An alternatively spliced 52-kD Ro messenger RNA (mRNA) derived from the skipping of exon 4 which encodes a smaller protein, 52beta (MW 45 kD) is

recognized by CHB maternal antisera. Reverse transcriptase-polymerase chain reaction was performed using primers flanking exon 4 and mRNA from 22 human foetal hearts (age 11-25 weeks) and 3 adult hearts. The brain, kidney, liver, lung, and spleen were similarly evaluated in a 15-week, an 18-week, and a 24-week foetus. Expression of 52beta was greatest and 52alpha lowest between 14 and 16 weeks of gestation. In foetal hearts ages 22-25 weeks and adult heart, the 52beta transcript was markedly diminished and 52alpha clearly dominated. The 52beta mRNA was observed in a 15-week brain, kidney, lung, and spleen; however, its expression relative to 52alpha was greatest in the heart. Since expression of the alternative product 52beta is maximal at the time of cardiac ontogeny when maternal antibodies gain access to the foetal circulation, just prior to the clinical detection of bradyarrhythmia, a role for 52beta in the development of CHB is implicated. Although other foetal tissues express 52beta, there may be differences in accessibility of antigen or regenerative capacities (Buyon *et al* 1997).

It is not clear which of the Ro antigens are most important in pathogenesis. Certainly, antibodies directed against both the 52 and 60-kD proteins are present in patient sera. In one study, antibody to the 52-kD antigen without concomitant antibody to the 60-kD antigen was seen only in patients with primary SS, whereas antibody to the 60-kD antigen without concomitant antibody to the 52-kD antigen was seen only in SLE patients. Although this group found that antibodies to Ro are detected in both SS and SLE, they showed that there is a dissociation of immune responses to the 2 antigens, suggesting that this may be due to differing events initiating the autoimmune process in these diseases (Ben-Chetrit *et al* 1990). This dissociation of immune responses has not been observed in many subsequent reports.

In a study comparing 17 SCLE patients with 15 DLE patients, high titre, precipitating antibodies to Ro were found to be typical of SCLE, both anti-52 and anti-60 and unusual in DLE. Low titre, nonprecipitating antibodies to Ro were common in DLE and could be an indication of pathogenic factors shared with SCLE. However, low

titres of anti-Ro do not confer a significant risk for SCLE skin lesions (Lee *et al* 1994b).

In a study of maternal antibody response to the components of the Ro antigen in NLE, using immunoblotting, it was found that 80% of 20 sera had antibodies to Ro (Buyon *et al* 1989). Most had antibodies to the 52kD component. In contrast, antibodies to the 60kD Ro were least represented and not significantly increased in frequency among mothers of children with NLE, compared to a group of 31 mothers with autoimmune diseases, such as SLE, with healthy offspring. The Ro52 and La/SS-B were abundant in cardiac tissues from fetuses aged 18 to 24 weeks.

On the other hand, an abstract assessing the auto-antibody specificities in 20 maternal NLE sera found 20/20 had antibodies to 60kD Ro, 18/20 had antibodies to 52kD Ro , 9 had antibodies to La and 1 had antibodies to U1RNP. Titers of anti-60kD Ro were significantly lower in NLE skin disease sera than in the NLE heart block sera. They speculated that the low titers of anti-60kD Ro may be the result of a high affinity of these antibodies for the skin, leading to a consequent lowering of circulating titers. Alternatively, it may reflect a lower threshold for the development of skin disease compared with heart block (Lee *et al* 1994a).

A study comparing Japanese annular erythema of Sjörgren's syndrome and American SCLE, by ELISA, showed that these two syndromes share several types of anti-52 and anti-60 antibodies. However, these antibodies were found significantly more often and in significantly higher titers in sera from patients with Sjörgren's syndrome who did not manifest these skin diseases. The failure to identify disease-specific Ro autoantibodies in SCLE and annular erythema of SS sera, suggests that additional factors influence the development of these skin diseases. However, similarities in the Ro autoantibody responses and in the clinical features suggest that these two skin diseases might arise from a similar pathogenic process (McCauliffe *et al* 1996)

A molecular definition for the 52-kD component has been provided by the isolation of cDNA clones encoding full length 52-kD Ro independently by two groups (Chan *et al*

1991a; Itoh *et al* 1991). The complete 52-kD protein consists of 475 amino acid residues with a deduced molecular weight of 54 000. The deduced protein structure contains three distinct domains: an N-terminal region rich in Cys/His amino acid residues that may form DNA/RNA-binding motifs known as zinc fingers, a central region containing a leucine zipper motif (coiled coils) with potential for intermolecular dimerisation and a C-terminal "rfp-like" domain that is shared by several other proteins (Chan *et al* 1991a). The zinc finger DNA binding motif consists of regularly spaced cysteine and / or histidine residues that bind divalent cations (typically zinc). This gives rise to "finger-like" structures that enable the protein to bind DNA. There is some speculation that such metal binding domains facilitate protein folding that allows interaction with other macromolecules. There are innumerable examples of zinc finger containing proteins interacting with double-stranded DNA in a sequence specific fashion. Some evidence suggests that this domain may also participate in binding single stranded DNA, RNA or perhaps protein (Berg, 1990).

Antigenic regions on the 52-kD Ro were evaluated by immunoprecipitation of [^{35}S]-radiolabelled *in vitro* translation products. This was the first study to describe at least two antigenic determinants on the 52kD Ro protein: one immunodominant and the other recognised by a more "restricted" subset of anti-52kD Ro Abs. 95% of 99 sera containing anti-52kD antibodies by ELISA, reacted with a large fragment spanning amino acids (aa) 1-291 (Buyon *et al* 1994). Two antigenic regions were identified: aa 169-291 containing the leucine zipper, that was recognised by 83% of the anti-52kD sera tested and aa 1-78 containing the zinc finger domains, that was recognised by only half the sera. No sera immunoprecipitated the N-terminal fragment exclusively. Recognition of both or one region was not unique to any clinical subset of patients. Reactivity with both epitopes was demonstrated significantly more often in sera with high titers of Abs to the 60kD Ro by ELISA in association with the anti-52kD response, compared with anti-52kD responses associated with low titers of anti-60kD antibodies. 81% of 16 sera that recognised the N-terminal epitope were from patients

with the combination of HLA-DRb1*0301, DQA1*0501 and DQB1*0201 alleles, compared with 30% of 10 that recognised only the central epitope (Buyon *et al* 1994).

A further study determining the reactivity of recombinant 52 and 60 kD Ro proteins with sera from NLE, SCLE and SS patients, using CIE and ELISA, showed that 75% of NLE, 56% of SCLE and 83% of SS sera reacted with the 52kD protein (McCauliffe *et al* 1994). 75% of NLE, 63% of SCLE and 83% of SS sera reacted with the 60kD fusion protein. 17% of NLE, 25% of SCLE and 8% of SS sera were non reactive to both full length fusion proteins. Similar results were obtained for Chinese patients (Tsay *et al* 1996). ELISA studies with recombinant 52 and 60kD Ro protein fragments revealed at least 2 major epitopes on each Ro protein. A fragment of the 52kD Ro protein, containing the putative leucine zipper motif, reacted with 100% of ID defined Ro positive SS sera (McCauliffe *et al* 1994). It is interesting that this leucine zipper containing fragment is more reactive to the ID defined Ro positive NLE and SS sera than the full length 52kD fusion protein. An epitope is probably partially masked by the folding of the full length protein but more accessible to antibody binding in the partial length fragment.

Confirmation of the fact that anti-52 kD Ro autoantibodies in different autoimmune diseases preferentially recognize epitopes on the central region of the antigen, has appeared more recently. Sera were obtained from 20 patients with SLE, 10 with primary SS, 15 children with CHB, 6 healthy anti-52 kD Ro positive infants born to mothers with SLE and 7 anti-52 kD Ro positive patients with primary biliary cirrhosis / secondary SS. Epitope mapping was performed using different fusion proteins in ELISA and immunoblot. All sera reacted with whole recombinant antigen as well as with the protein carrying the amino acid sequence 1-245. The proportion of positive sera against the 52kD Ro fusion proteins tested was found in descending order in patients with CHB, down to primary SS, the healthy infants group, patients with SLE and finally primary biliary cirrhosis / secondary SS. In general, CHB and primary SS sera exhibited the broadest reactivity against the recombinant protein compared to the limited and lower reactivity of sera from patients with primary biliary cirrhosis. Sera

from infants with CHB had significantly higher antibody levels to aa 1-245 compared to SLE sera and to sera from healthy infants born to SLE mothers, as well as to serum samples from patients with primary biliary cirrhosis/SS. The strongest antigenicities recognized by anti-52 kD Ro autoantibodies were located within the aa 197-245 region. Further antigenic sites preferentially recognized by SS, CHB, and healthy infant sera were located within AA 153-196. The central region aa 153-245, was a major immunogenic region, containing a strong antigenic epitope between aa 197-245. The antibody response was directed at this major antigenic region regardless of the underlying autoimmune disease (Dorner *et al* 1996b).

The 52kD Ro has homology with the human ret finger protein(rfp), the regulatory protein, T-lymphocyte, 1 gene (rpt-1) and bovine butyrophilin. The rfp gene was originally described as part of the ret transforming gene (Takahashi & Cooper, 1987). The ret gene encodes a protein that consists of the amino terminal zinc finger portion of rfp, fused to a truncated tyrosine kinase. Later, when the gene that encodes this zinc finger containing portion of ret was isolated, it was called the rfp gene for ret finger protein (Takahashi *et al* 1988). Rfp is expressed at high levels in a variety of tumour cell lines, in mouse testis and in embryos. It is low or undetectable in other tissues, including brain, thymus, lymph node, muscle, kidney, liver, lung and ovary. Rfp may play a functional role in cells that are rapidly dividing, mitotically or meiotically.

Rpt-1 was first described as a protein selectively expressed by resting but not activated CD4+ inducer T cells. Rpt-1 is a 41kD nuclear protein that down regulates gene expression of IL-2 receptor α-chain gene and human immunodeficiency virus type 1 genes (Chan *et al* 1991a).

The carboxy terminal portion of the 52kD Ro shares +- 50% aa sequence identity with butyrophilin, thought to be involved in membrane stabilisation of milk-fat formation in mammary glands (Jack & Mather, 1990). The 52kD Ro may therefore be membrane associated. Ro has been shown to be membrane associated in apoptotic

keratinocytes (Casciola-Rosen *et al* 1994). Whether it was Ro52 and / or Ro60 was not clearly delineated.

It has been suggested that anti-52-kD autoantibodies appear to preferentially recognize the denatured rather than the native 52-kD Ro (Itoh & Reichlin, 1992). Another group, using a series of truncated 52-kD autoantigens, produced as beta-galactosidase fusion proteins in Escherichia coli, the B cell epitope distribution was probed with 18 anti-Ro-positive sera by immunoblotting and by ELISA (Kato *et al* 1995). Nearly all the antigenicity of the molecule was found to be linked to its leucine zipper region. In a further study using 9 of the 18 sera, the antigenicity of the molecule was found to be mainly formed by multiple conformational epitopes, and one of these epitopes appeared to be universally recognized by all the sera tested. The recognition of multiple epitopes indicates that the Ro 52-kD antigen itself drives the autoimmunity to this molecule. Further, it was suggested that the concentration of the antigenicity at the leucine zipper region may explain why anti-52-kD antibodies preferentially recognised the denatured protein rather than its native form. However, a different group (Buyon *et al* 1994), evaluated antigenic regions on the 52 kD Ro by immunoprecipitation of radiolabeled in vitro translation products. Two antigenic regions were identified: an immunodominant aa 169-291 region, containing the leucine zipper and the aa 1-78 region, containing the zinc finger domain, recognised by a more restricted subset of antibodies.

2.3.3 calreticulin

McCauliffe at al isolated a cDNA from an Epstein-Barr virus transformed cell line that was thought to encode a protein reactive with human Ro autoimmune sera (McCauliffe *et al* 1990). The encoded 46 kD protein migrated aberrantly at 60 kD and was shown to be calreticulin (CR) (McCauliffe *et al* 1990). Additional studies have failed to validate calreticulin's reactivity with Ro autoimmune sera, although it is reactive with some LE patient sera (Rokeach *et al* 1991; Routsias *et al* 1993). This criticism has been based predominantly on the lability of native calreticulin autoantigenic activity to denaturing conditions such as SDS-PAGE and the failure of prokaryotic forms of recombinant calreticulin to react preferentially with anti-Ro patients sera in Western blot and ELISA, as well as bind with hY RNA (Rokeach *et al* 1991). McCauliffe could later not confirm that CR was reactive with Ro autoantibodies nor that it was a component of the Ro ribonucleoprotein complex (McCauliffe & Gardner, 1992).

CR is a highly conserved calcium-binding protein. It resides mainly in the endoplasmic reticulum, where it may be involved in protein assembly (McCauliffe & Sontheimer, 1993b). Analysis of the CR promoter region shows a number of potential regulatory sites also found in the human GRP78, GRP94 and protein disulfide isomerase promoters (McCauliffe *et al* 1992). This suggests that these have similar transcriptional regulation and that their gene products, while structurally distinct, may have similar functions or co-functions (McCauliffe *et al* 1992). These observations are interesting in that all four of these genes encode acidic proteins that localise to the endoplasmic reticulum.

The sequence of calreticulin has significant homology to lambda Ral-1, a recombinant cDNA clone corresponding to a major antigen of the nematode, Onchocera volvulus, the infectious agent in onchocerciasis. Onchocerciasis patients produce antibodies that cross-react with the 46-kD CR autoantigen (Lux *et al* 1992).

It has been suggested that some form of post-translational modification such as phosphorylation with or without RNA binding, is necessary to produce the configuration of calreticulin that is recognised by anti-Ro antibodies (Sontheimer *et al* 1993). This is based on the observation that calreticulin binds to stem loop structures on the rubella virus genomic DRNA, only after its protein has been phosphorylated through a mechanism of autophosphorylation. A segment of the amino terminus of calreticulin has considerable homology to an RNA-binding motif employed by a member of the SR family of splicing factors. In addition, anti-Ro antibodies may precipitate a rubella virus RNA that is bound at the 5' end, to a pair of 60 and 52 kD proteins of unknown identity (Pogue *et al* 1993).

Further work suggests that approximately 10% of cellular calreticulin molecules are associated with hY RNA. This subpopulation of calreticulin has been suggested as binding Ro antibodies (Lieu *et al* 1994). Further work showed that unphosphorylated human rCR bound to in vitro transcribed forms of hYRNA, the RNA backbone of the Ro RNP particle. This interaction appeared to be mediated by binding through the N- and C-terminal domains of CR, but not by the central proline-rich domain. In addition, it was suggested that CR can facilitate the binding of the 60-kDa polypeptide component of the Ro RNP to hYRNA. In addition, CR and the 52-kDa Ro polypeptide appeared to be capable of interacting through direct protein-protein binding. Through these molecular interactions and its known functional role as a chaperone, it was suggested that CR plays a supportive role in the formation of the Ro RNP complex (Cheng *et al* 1996).

It was however, considered that a considerable proportion of cellular calreticulin molecules, perhaps including secreted forms of calreticulin, do not appear to have the conformational structure resulting from post-translational modification with or without hY RNA binding that would allow them to interact fully with Ro autoantibodies (Kawashima *et al* 1994).

In addition, complete congenital heart block is associated with anti-calreticulin autoantibodies. Anti-calreticulin autoantibody responses in serum samples from 18

infants with CCHB, their mothers and in a control group of 11 anti-Ro or anti-La positive infants without heart block and their mothers were analysed. Specific ELISA's were performed. Nine out of 18 sera with CCHB contained IgG anti-calreticulin antibodies. Four sera of those with IgG antibodies also had IgM antibodies. One serum contained anti-calreticulin IgM antibodies only. In the non-CCHB group two sera were positive for IgG and one serum was positive for IgM anti-calreticulin antibodies. Sera of healthy infants were negative both for anti-IgG and anti-IgM calreticulin antibodies. Calreticulin is involved in calcium storage and therefore anti-calreticulin antibodies might influence the development of CCHB. The finding of IgM autoantibodies and the observed differences in antibody response in infants and mothers support the hypothesis of a fetally mediated and passively acquired autoimmune disease (Orth *et al* 1996).

More recently, a subpopulation of human calreticulin that is reactive with human Ro autoimmune sera was identified in a nucleic acid-enriched Wil-2 cell fraction derived by anion exchange column chromatography. Further resolution of this fraction by gel filtration size separation demonstrated that the appearance of CR (true mol. weight 46kD) coincided with the emergence of Ro ribonucleoprotein (mol. weight > 250 kD) antigenic activity and increasing 260 nm ultraviolet absorbance. This high nucleic acid fraction could be further partitioned into four small RNA-containing Ro antigenic sub-fractions by a second passage over the anion exchange column. CR was enriched in one sub-fraction and present in the other three sub-fractions as well. No CR was found in the RNA-free fraction of the repartition eluate. These results represent the first direct demonstration that CR, a high-affinity calcium binding protein, exists in a form that is directly associated with all four varieties of native, human Ro ribonucleoprotein particles (hY1-5) (Lieu & Sontheimer, 1997).

2.3.4 54 kD Ro

Since the description of the 52kD component, (Rader *et al* 1989) described two human erythrocyte-specific Ro components of 60 and 54 kD. These were

immunologically related to the previously described 60 and 52 kD species respectively. This 54kD isoform was found in nuclei free platelets and red blood cells. It is unclear why mature red cells carry varying forms of Ro (Rader *et al* 1989). This has not been confirmed: a further group showed that the main reactivity of Ro sera was to a 52 kD, rather than a 54 kD protein in red cells (Manoussakis *et al* 1993), although a small percentage of sera detected a 54 kD red cell protein. An autoantigen defined by a band on western immunoblotting is less defined than one which has been cloned and characterised.

2.3.5 Cross-reacting epitopes

Controversy exists as to whether the 52kD protein is associated with the 60kD protein and hY RNA. It has been demonstrated that antibodies from Ro autoimmune sera specific for either a 52kD or 60kD protein on Western blot, immunoprecipitate hY RNA from cell extracts. However, antibodies specific for the 52kD protein, precipitated a small amount of 60kD protein, along with hY RNA and the 52kD protein. A number of groups would agree that the 52 kD Ro protein is a component of the Ro RNP complex (Itoh *et al* 1991; Itoh & Reichlin, 1991b; Ben-Chetrit *et al* 1988).

Some investigators have suggested that a major denaturation resistant epitope on the 52 kD Ro protein is cross reactive with the 60kD Ro protein (Itoh *et al* 1992). They suggest that it is not really a target of the Ro autoimmune response but simply is detected because of its shared epitope with the major immunogen.

2.4 Methods for detection of Ro antibodies

There are a range of assays to detect ANA. The first method of Ro detection was gel double diffusion. Newer assays are increasingly sensitive. When highly purified or recombinant autoantigens are used in versatile assays such as ELISA,

2.4.1 Indirect Immunofluorescence

The most widely used procedure for ANA screening, is the indirect immunofluorescence test (Tan, 1967). The standardisation of IF-ANA has been difficult and several recommendations have been made to improve testing procedures, including a quality assurance program to detect Ro antibodies. The inability to detect Ro antibodies may account for a significant percentage of so called ANA-negative SLE cases (Lopez-Robles *et al* 1986; Maddison *et al* 1981; Deng *et al* 1984). Most clinical laboratories are using ID, CIE or a purified native 60kD Ro ELISA to detect Ro autoantibodies in patients sera (McCauliffe *et al* 1997b).

The detection of 60kD Ro is hindered by its low cellular abundance. Two studies have used a transfected and overexpressed human 60 kD Ro autoantigen in HEp-2 cells for IF. In the first, the Ro60 (60kD) Ro gene was transfected and over-expressed into HEp-2 cells (Keech *et al* 1994). They used a mixture of Ro60 transfectants and untransfected HEp-2 cells (HEp-Ro60) as a substrate for IF-antinuclear antibody testing in a hospital laboratory. Screening of 240 routine serum specimens identified 14 Ro transfectant-positive sera which were confirmed by counterimmunoelectrophoresis. 3 of these sera were ANA-negative on untransfected cells and regular HEp-2. A comparison of HEp-Ro60 and regular HEp-2 showed strong concordance of the different ANA patterns between the 2 substrates. No increase in background staining was observed on the Ro transfectants when reacted with normal human sera. A comparison between HEp-Ro60 and CIE for 53 sera from patients with primary Sjögren's syndrome showed that HEp-Ro60 were a sensitive and specific substrate for detection of anti-Ro antibodies. Masking of positive Ro transfectants was observed rarely in sera containing multiple ANA specificities, but the Ro60 staining on these transfectants were unmasked at higher serum dilutions.

Dr. Tom Gordon holds a US patent for this transfected cell assay and has asked a company to market the product. Dr. Fritzler used the commercial product to determine the sensitivity and specificity of this assay (Fritzler & Miller, 1995).

Seventy-three sera with Ro autoantibodies as determined by double immunodiffusion and immunoblotting were tested by IF on a HEp-2 cell substrate that had been transfected with a full-length cDNA encoding a human 60kD Ro autoantigen. Controls included 30 normal human sera and 50 sera with a variety of other antinuclear antibodies. Prototype human and rabbit sera directed against the 60 kD Ro antigen produced intense speckled nuclear and nucleolar staining of transfected cells. Sixty-nine of 73 (95%) SS-A/Ro positive sera also produced this characteristic staining pattern. The endpoint autoantibody titers on transfected cells was fivefold greater than on un-transfected cells. The 30 normal human sera and the 50 sera with other antinuclear antibodies did not produce this characteristic staining. Six of 32 (19%) unselected sera that were sent for autoantibody testing had reactivity with transfectants by IF. Four of the six sera were confirmed to have anti-Ro antibodies by ID and 5/6 IB. By contrast, only three of these sera were scored as having a staining pattern compatible with SS-A/Ro antibodies by IIF on standard HEp-2 substrates.

2.4.1.1 Fixation

In addition, methods of fixation are important. Conventional cell fixation for ANA testing does not allow full visualisation of all proteins, such as CR, that reside in the ER. ER proteins can be seen better with IF with detergent permeabilisation of fixed cells or unfixed cells exposed to 9% sucrose (McCauliffe & Sontheimer, 1993b). Acetone fix for 1 - 10 minutes on KB or HEp-2 cells, with SS-A containing sera, produced discrete nuclear speckles and no cytoplasmic staining. However, when the same cell lines were used with either ethanol or methanol, the intensity of staining with anti SS-A sera dropped from 2+ to trace, as the fixation time increased from 1 to 10 minutes (Bylund & Nakamura, 1991). Periodate-lysine-paraformaldehyde (PLP) or 2% glutaraldehyde caused a different, non-specific immunofluorescent pattern of the entire cell, with anti SS-A sera (Harmon *et al* 1984). Using a variety of unfixed tissues, Lopez-Robles *et al* found mainly nuclear, with occasional cytoplasmic, fluorescence and suggested that Ro could be a mobile complex, whose location depends on the cell cycle (Lopez-Robles *et al* 1986).

2.4.1.2 Substrates

The Ro antigen is found in very low concentrations in mouse, rat, rabbit, hamster and chicken cells (Harmon *et al* 1984) and tissue culture cells are more sensitive substrates than tissue sections (Deutscher *et al* 1988). In addition, tissue culture cell nuclei contain certain nuclear materials that are not present in the nuclei of resting-phase cells. The presence of an ANA in certain patients with SCLE that binds to human HEp-2 cells but not to mouse kidney sections, suggests that this particular type of ANA reacts with a nuclear antigen present in HEp-2 cells but in low or absent concentrations in mouse kidney cells (Sontheimer *et al* 1992).

2.4.2 Immunodiffusion

Immunodiffusion, gel double diffusion (Ouchterlony) (ID), is the classical, simple method of detection of anti-Ro antibodies. Agar is used as a support medium. Wells are cut into the agar. Into the center well is placed the antigen, an extract of human spleen (HSE). The outer wells contain the test and reference sera. If the test serum contains an antibody reactive against an antigen in HSE, a precipitin line will form between the test and the antigen wells. If the test and reference sera have identical antibody systems, the precipitin line formed between the reference serum and the antigen wells will merge into a continuous line, with the precipitin line formed between the reference serum and the antigen wells (line of immunologic identity). Antibodies are detected by using double diffusion in 0.5% agarose. Precipitin lines form after interaction with Ro and dilutions of sera (Ben-Chetrit *et al* 1988). These precipitin lines are stained with Coomasie blue and compared against reference sera. This is a native assay (Boire *et al* 1991). This technique does not differentiate between antibodies to the 52 and 60-kD antigens (Ben-Chetrit *et al* 1988). With this technique, anti-Ro antibodies can be detected in 25% (Sontheimer *et al* 1992) to 57% (Ben-Chetrit *et al* 1990) of patients with SLE. Virtually no normal individuals have anti-Ro antibodies with immunodiffusion.

Counter-immunoelectrophoresis (CIE) is a more sensitive Ro autoantibody detection method (McCauliffe *et al* 1997b). In one study, CIE was found to be reliable, with a specificity of 100% and a sensitivity of 89% (Manoussakis *et al* 1993). It has not yet been determined which of the molecules reactive with Ro autoimmune sera are responsible for the immunoprecipitation, seen in immunodiffusion assays. It has been observed that non-reducing SDS-PAGE of eluted precipitins from CIE showed the precipitins to be composed of a 60kD protein (Rader *et al* 1989).

An improved method for the detection and identification of antibodies against extractable nuclear and cytoplasmic antigens has been described: counterimmunoelectrophoresis with serum pre-diffusion (SPD) (Walravens *et al* 1997). In this technique, serum is allowed to diffuse freely into the gel before pouring the antigenic extract in its trough (or wells) and starting the electrophoresis. Both the immunoprecipitations and the interaction with reference sera are strongly intensified by serum pre-diffusion, leading to higher sensitivity and specificity for the detection of anti- Ro, anti- La, anti-U1RNP, anti-Sm, anti-Jo1 and even anti-Scl-70 antibodies. The optimal serum pre-diffusion time was 2 h. To evaluate the relevance of SPD for the clinical laboratory, 92 antinuclear antibody (ANA) positive sera were tested on CIE without serum pre-diffusion and with 2 h serum pre-diffusion in identification tests with Ro, La, Sm, U1RNP and Jo1 reference sera. The precipitation lines and their interactions were evaluated by three independent observers. It was observed that serum pre-diffusion considerably improved the efficiency of CIE for antibody identification.

2.4.3 Immunoblotting

Electrophoretic blotting is performed using the method of Towbin (Towbin *et al* 1979). Proteins are separated by SDS-PAGE (Laemmli, 1970) and electrophoresed to a nitrocellulose sheet. These are then blocked to inhibit non specific binding. The nitrocellulose sheets are then incubated with dilutions of sera. This assay generally

uses denatured polypeptide versus native protein, although some proteins may be able to refold depending on experimental conditions.

Western blot analysis has high specificity (95% for the 52 kD Ro [Ro52], 97% for Ro60) for Ro autoantibody detection but low sensitivity (36% for Ro52 , 17% for Ro60) (Manoussakis *et al* 1993). One study showed 51/90 SLE sera (57%) Ro positive by ID. In Western blotting, of the same population, 24 (47%) possessed antibodies against both the 60 and 52-kD antigens, while 9 (18%) reacted only with the 60-kD protein. 18 (35%) were non-reactive by Western blot, although positive by ID (Ben-Chetrit *et al* 1990). In addition this biases for high affinity antibodies (Buyon *et al* 1989). Immunoblotting may fail to detect anti-60 kD antibodies in some sera because of the denaturing of the protein that takes place prior to electrophoresis and the importance of conformational epitopes in some sera. Thus, immunoblotting should not be relied on as the sole method for evaluating the presence or absence of anti-60kD Ro.

However this is to some degree controversial. One group (Zimmermann *et al* 1996) has found that IB is the most sensitive method for detection of anti-La and may help to characterize clinically distinct subgroups of anti-Ro positive patients with SLE. They suggest that determination of anti-Ro by IB may increase the prognostic value of this autoantibody.

2.4.4 Immunoprecipitation

In this technique, radiolabelled cell extracts are used as a source of antigen, against which patients' sera adsorbed to protein A-Sepharose beads, are immunoprecipitated (Lerner *et al* 1981; Francoeur & Mathews, 1982). A RNA immunoprecipitation assay detects Ro autoantibodies with greater sensitivity than CIE but is impractical for routine diagnostic work (Manoussakis *et al* 1993; Meilof *et al* 1990). This assay likewise detects native antigen.

In a study comparing 5 methods of Ro antibody detection, RNA precipitation was the most specific and sensitive of the assays (Manoussakis *et al* 1993). This is based on the sensitive detection of radiolabelled small hY-RNA particles precipitated by anti-Ro antibodies. Whole cell extracts are prepared from radiolabelled HeLa cell cultures. RNAs are extracted from the pellets with phenol and analysed on 10% polyacrylamide-7M urea gels. Bands are visualised by x-ray autoradiography in the presence of intensifying screens.

Owing to its complexity and the use of large amounts of radiolabelled reagents, this assay has little value for routine laboratory serological work (Manoussakis *et al* 1993), although protocols that do not need radioactivity exist (Forman *et al* 1985).

2.4.5 ELISA

Using an ELISA based on affinity purified Ro antigen, 50% of patients with SLE are found to have elevated levels of this autoantibody specificity (Sontheimer *et al* 1992). Some have found this test to be less specific than immunodiffusion (Manoussakis *et al* 1993; Meilof *et al* 1990), whereas others have found ELISA with purified native 60kD Ro protein to be more specific than ID (McCauliffe *et al* 1997b). Recombinant 60kD Ro protein ELISAs appear to be less sensitive in detecting Ro autoantibodies than are native 60kD Ro ELISAs, ID and CIE. One benefit of this new technology is the ability to measure antibody binding levels to individual epitopes. These are limited portions of an autoantigen's amino acid sequence that represent single antibody binding sites. Certain patterns of clinical disease may be linked to autoantibody production against individual autoepitopes rather than whole autoantigenic molecules (Sontheimer *et al* 1992). Certainly, it has been shown that commonly employed Ro serologic assays detect anti-60kD Ro autoantibodies but fail to detect anti-52 kD Ro autoantibodies, that can be detected using a recombinant 52kD Ro ELISA (McCauliffe *et al* 1997b). ELISA for anti-60 is preferably done using native 60 rather than denatured 60. In addition, human, rather than bovine 60 is required in some cases, as reactivity with 60-kD Ro from other species may be weak

or absent (Lee *et al* 1994b). On the other hand, the autoantibody response to Ro52 appears to be due to largely epitopes present on the denatured or recombinant molecule (Lee *et al* 1994b), although a study evaluating Ro52 antigenic regions by immunoprecipitation in a relatively non-denaturing system, confirmed similar results to those obtained with ELISA (Buyon *et al* 1994).

SS sera are more likely to contain anti-52 and anti-60kD Ro autoantibodies, or anti-52kD Ro autoantibodies alone, than anti-60kD Ro autoantibodies alone (McCauliffe *et al* 1997b; Ben-Chetrit *et al* 1990; Ricchiuti *et al* 1994; St.Clair *et al* 1994; Slobbe *et al* 1991). The 52kD Ro ELISA assay is a better assay for detecting Ro autoantibodies in SS sera, than commonly employed serologic techniques (McCauliffe *et al* 1997b).

A study looking at the reactivity of recombinant 52 and 60kD Ro proteins with sera from SCLE, NLE and SS patients, found that the ID assay and the recombinant Ro ELISA together are more sensitive in detecting Ro antibodies than either assay alone (McCauliffe *et al* 1994). Eight (57%) of 14 ID defined Ro negative NLE, SCLE and SS sera were reactive with both the 52 and 60kD Ro fusion proteins by ELISA.

3. ANTIBODY PURIFICATION

3.1 INTRODUCTION

The early controversy about the location of Ro, could be explained by the later recognition of different Ro proteins. These initial studies used "generic Ro" sera, that may have contained both anti-52 and anti-60 kD Ro antibodies, or only anti-52, or only anti-60 kD Ro antibodies. Later studies attempting to differentiate the position of the 60 kD Ro from the 52 kD Ro were flawed in their purification of anti 52kD Ro antibodies.

Slobbe *et al* in their immunofluorescence study (Slobbe *et al* 1991), immunoaffinity purified antibodies monospecific for either the Ro60 or La antigen, using recombinant antigen, coupled to a CNBr-activated Sepharose 4B, yielding monospecific anti-La and anti-Ro60 antibodies from patients and monospecific rabbit anti-Ro60 antibodies. Anti-Ro52 antibodies were not purified. Rather, a serum was used, which did not contain detectable levels of anti-Ro60 or anti-La antibodies on immunoblot. They then passed this serum repeatedly over a Ro60-column, in an effort to remove anti-Ro60 antibodies. The western blot showing the specificity of the Ro52 antibodies however, revealed a band running just above 60 kD.

The subsequent paper by Kelekar *et al*, used 60 or 52-kD rabbit antiserum produced against partially purified recombinant forms of the corresponding proteins overexpressed in E coli (Kelekar *et al* 1994). No further characterisation details were given. Ben-Chetrit *et al*, in their paper describing the 52 kD antigen, used anti-52kD antibodies which precipitated both a 52 kD and also a 60 kD component (Ben-Chetrit *et al* 1988). Perhaps this explains the similar bright nuclear staining they found with both anti-52 and anti-60 kD antibodies. It is possible that the results one obtains, may be dependent in part, on the sera from which one purifies the antibody. It has been

shown that a number of patients with anti-Ro antibodies also have rheumatoid factor (Scofield *et al* 1991; James *et al* 1990).

Where there are two different Ro antigens, it is necessary in efforts to unravel physiological and disease processes, to have purified antibodies to work with. In addition these antibodies need to target the relevant epitopes, whether conformational or not. For this reason it was important to purify antibodies to the major regions of Ro52 and Ro60. For double immunofluorescence, antibodies from two different species were needed.

3.2 METHODS

3.2.1 Recombinant Gst Ro Fusion Protein

3.2.1.1 Isolation of 52 and 60 kD Ro encoding clones

cDNA clones encoding the entire 52 and 60 kD Ro proteins were isolated by the polymerase chain reaction (PCR) using Wil-2 B-cell cDNA (ATCC CRL 8885) and synthetic oligonucleotides corresponding to published sequences (Deutscher *et al* 1988; Chan *et al* 1991a; Itoh *et al* 1991). Ro cDNA fragments that encode different portions of the antigens were also produced by PCR using the isolated cDNA clones as templates. These cDNA were subsequently used to make protein fragments of the 52 and 60 kD Ro antigens. This work was performed by Dr. Daniel P McCauliffe.

3.2.1.2 Expression vector construction and sequencing

The 52 and 60kD Ro encoding cDNA were ligated into the pGEX-2T vector (Pharmacia, Piscataway, NJ) so that full length and partial length glutathione S-transferase (GST)-Ro fusion proteins could be generated. dsDNA sequence analysis of each construct was performed by the Sanger dideoxy method with modified T7 DNA polymerase (Sequenase) according to the manufacturer's recommendations (United States Biochemical Corp., Cleveland, Ohio, USA) and compared to published

sequences (Deutscher *et al* 1988; Chan *et al* 1991a; Itoh *et al* 1991; Ben-Chetrit *et al* 1989). This work was performed by Dr. Daniel P McCauliffe.

3.2.1.3 Recombinant protein production and purification

Recombinant GST-Ro fusion proteins were expressed in JM 109 cells (ATCC 53323) and purified using glutathione linked agarose (Sigma) as described (Smith & Corcoran, 1990). The 52 and 60 kDa GST fusion proteins could be cleaved from GST with thrombin as described (Smith & Corcoran, 1990). However, previous work has shown that the GST portion of the fusion protein does not interfere with Ro antibody binding to the recombinant Ro proteins (McCauliffe *et al* 1994). Large scale recombinant protein production was performed using the following protocol:

Protocol 1: Large scale recombinant protein production

On the first day, 150 ml of liquid broth (LB)/ ampicillin (ampicillin: add 150µl of stock to final conc. of 50µg/ml) was seeded with the bacteria containing plasmid of interest and grown overnight in a 37°C shaker. During this process, 3l of LB was prepared in three 2l flasks and autoclaved. In addition, the glutathione agarose was prepared by adding 249 mg of glutathione agarose to 20 ml of PBS, letting it sit at room temperature for about one hour and then spinning it for 10 min. in a Dorvall RT 6000 centrifuge at 2 500 RPM. The wash was repeated twice. After the third wash, the agarose pellet was resuspended in 3ml of PBS, to bring up the total volume to 6 ml.

On the second day, 1 ml of 50 mg/ml ampicillin stock was added to each one litre of broth. Each flask was seeded with 50 ml of overnight culture and grown for an optimal 4 hours at 37°C. Then 1 ml of 100mM IPTG was added to each flask and grown for one hour. During this hour, the following were prepared on ice: 6 centrifuge bottles, rinsed with ddH_2O, six 50 ml tubes, four scintillation tubes and the PBS/protease inhibitor solution (30 ml 1 X PBS + 900µl 500 mM EDTA + 450 µl 100 mM PMSF [added only to one bottle as viable for only 1/2 hr, during 2nd

centrifugation] + 300µl DTT + 300µl 1M DTT + 450µl Triton X-100). Then the cells/media were poured into six 250 ml Nalgene centrifuge bottles, balanced and centrifuged for 10 minutes in a Sorvall GSA rotor at ~5 500 RPM (5 000Gs). One flask was put into the -20°C freezer. The supernatant was decanted and the centrifugation was repeated with the rest of the cells/media in the same bottles. Each cell pellet was then resuspended on ice in 4 - 5 ml of ice cold PBS/protease inhibitor solution and poured into four 20 ml scintillation tubes. This solution was sonicated on ice with a 13 mm probe at a setting high enough to clarify the cells in about 30 sec but low enough to avoid frothing. [setting 5, 20 sec. thrice]. The sonicate was spun for 10 min. in a Sorvall SS-34 rotor at 6500 rpm (~5000 G). The supernatants were transferred to a clean 50 ml tube and the 6 ml solution of glutathione agarose was added. This was mixed gently on a rocker for about 6 min. at room temperature and centrifuged for 10 min. in a Dorvall RT 6000 centrifuge at 2 500 RPM. The supernatant was aspirated with a vacuum. 30 ml PBS/protease inhibitor solution was added to the pellet and rocked for 6 min. at 4°C. The centrifugation was repeated, followed by two washes with 1 X PBS. The supernatant was decanted, aspirated and the agarose pellets were washed once with 20 ml ice cold 1% Triton-X/PBS solution. The pellets were washed once with 20 ml. ice cold 1X PBS, transferred to a 15 ml tube, spun and the excessive supernatant removed , to ~ 5 ml. The 2 tubes were combined to ~10 ml, spun and aspirated to 6 ml. Using a 1 ml pipette, the 50 ml bottles were washed to remove remaining agarose and this was transferred to 6 ml. A sample was then taken for western blotting It was aliquoted and stored at -70°C.

*For thrombin cleavage, after the addition of the ice cold 1% Triton-X/PBS solution as above, the agarose pellets were washed once with 30ml ice cold thrombin wash buffer, followed by a wash with 30ml ice cold thrombin cleavage buffer without calcium. This was then spun and the excessive supernatant was removed, to a final volume of 6ml.

3.2.2 Rabbit Immunisation

New Zealand white rabbits were immunized with recombinant 52 and 60 kD fusion proteins, linked to glutathione - agarose, according to established protocols (Oettinger *et al* 1992). This work was performed, in part, by Mr. L Wang. In total 6 rabbits were immunised. The sera from three rabbits were used to purify antibodies used in these experiments.

In more detail, the following methodology was applied:

Protocol 2: Rabbit Immunisation

RABBIT #1 52-1-b

The rabbit was immunised subcutaneously with 1mg of recombinant 52-1-b protein (0.5 ml agarose, 0.5 ml Normal saline and 0.5 ml complete Freund's medium). 22 days later, a further 1mg of recombinant 52-1-b protein (1 ml [agarose + supernatant], 0.5 ml incomplete Freund's medium) was injected. 17 days later, 4 ml of blood was drawn. 15 days later, the rabbit was immunised with >1mg of recombinant 52-1-b protein (1 ml [agar + supernatant, 1 ml incomplete Freund's medium). 13 days later, the rabbit was bled. 16 days later the rabbit was re-immunised with ~800 μg of recombinant 52-1-b protein (0.5 ml agar and 0.5 ml incomplete Freund's medium). 13 days later, 15 ml blood was drawn. 34 days later, 15 ml blood was drawn. 19 days later the rabbit was re-immunised with ~500 μg of recombinant 52-1-b protein (0.5 ml agar and 0.5 ml incomplete Freund's medium). 17 days later, 35 ml of blood was drawn. The next day, 100 ml blood was drawn and the rabbit was sacrificed.

RABBIT #2 whole 52

The rabbit was immunised subcutaneously with ~200 μg of recombinant whole 52 protein (0.5 ml agar, 0.5 ml Normal saline and 0.5 ml complete Freund's medium). 22 days later, a further ~150 μg of recombinant whole 52 protein(1 ml [agar + supernatant, 0.5 ml incomplete Freund's medium) was injected. 17 days later, 4 ml of

blood was drawn. 15 days later, the rabbit was immunised with ~500 μg of recombinant whole 52 protein (1 ml [agar + supernatant, 1 ml incomplete Freund's medium). 13 days later the rabbit was bled. 16 days later the rabbit was re-immunised with ~500 μg of recombinant whole 52 protein (0.5 ml agar and 0.5 ml incomplete Freund's medium). 13 days later, 15 ml blood was drawn. 34 days later, 15 ml blood was drawn. 19 days later the rabbit was re-immunised with ~400 μg of recombinant whole 52 protein (1 ml agar and 1 ml incomplete Freund's medium). 27 days later, 20 ml of blood was drawn. 36 days later the rabbit was re-immunised with ~100 μg of recombinant protein (50 μg whole 52 and 50 μg 52-2, 1 ml agar and 1 ml incomplete Freund's medium). 16 days later, 10 ml of blood was drawn. 81 days later the rabbit was re-immunised with ~150 μg of recombinant protein (50 μg whole 52, 50 μg 52-1 and 50 μg 52-2, 1 ml agar and 1 ml incomplete Freund's medium). 21 days later, 12 ml of blood was drawn. 7 days later, 25 ml of blood was drawn. 18 days later the rabbit was re-immunised with ~150 μg of recombinant protein (50 μg whole 52, 50 μg 52-1 and 50 μg 52-2, 1 ml agar and 1 ml incomplete Freund's medium). 22 days later, 30 ml of blood was drawn. 6 days later the rabbit was re-immunised with ~150 μg of recombinant protein (50 μg whole 52, 50 μg 52-1 and 50 μg 52-2, 1 ml agar and 1 ml incomplete Freund's medium). 14 days later, 100 ml of blood was drawn and the rabbit was sacrificed.

RABBIT #3 whole 60

The rabbit was immunised subcutaneously with ~100 μg of recombinant whole 60 protein (0.5 ml agar and 0.5 ml complete Freund's medium). 23 days later, a further ~150 μg of recombinant whole 60 protein (0.5 ml agar and 0.5 ml incomplete Freund's medium) was injected. 13 days later, 15 ml of blood was drawn. 34 days later, 12 ml of blood was drawn. 42 days later, the rabbit was immunised with ~300 μg of recombinant whole 60 protein (1 ml [agar + supernatant, 1 ml incomplete Freund's medium). 27 days later, 20 ml of blood was drawn. 36 days later the rabbit was re-immunised with ~500 μg of recombinant whole 60 protein (0.5 ml agar and 0.5 ml incomplete Freund's medium). 16 days later, 20 ml blood was drawn. 32 days

later the rabbit was re-immunised with ~200 μg of recombinant protein (50 μg whole 60, 50 μg 60-1, 50 μg 60-4 and 50 μg 60-2, 1 ml agar and 1 ml incomplete Freund's medium). 27 days later, 20 ml of blood was drawn. 17 days later, 25 ml of blood was drawn. 32 days later the rabbit was re-immunised with ~200 μg of recombinant protein (50 μg whole 60, 50 μg 60-1, 50 μg 60-4 and 50 μg 60-2, 1 ml agar and 1 ml incomplete Freund's medium). 22 days later, 5 ml of blood was drawn. 16 days later the rabbit was re-immunised with ~200 μg of recombinant protein (50 μg whole 60, 50 μg 60-1, 50 μg 60-4 and 50 μg 60-2, 1 ml agar and 1 ml incomplete Freund's medium). 14 days later, 100 ml of blood was drawn and the rabbit was sacrificed.

3.2.3 Characterisation Of Human Sera

A number of whole sera from well documented patients were used, in addition to the specific antibodies. Autoimmune patient sera were collected from the University of North Carolina sera bank in the division of rheumatology. Patients notes were reviewed to verify patient diagnosis. A diagnosis of NLE was based on the presence of congenital heart block and / or characteristic skin lesions in an infant born to a mother with Ro antibodies. A diagnosis of SCLE required the presence of characteristic skin lesions by clinical and histopathological examination. A diagnosis of primary SS was based on the presence of the sicca complex (dry eyes and dry mouth) in the absence of another connective tissue disease. ID defined Ro negative SCLE sera were provided by Dr. Richard Sontheimer, Southwestern Medical Center, Dallas, Tx, U.S.A.

In addition, a number of sera were chosen after assessing their antibody yield on both immunoblotting and ELISA:

2429 SS. Immunodiffusion (ID) positive for SSA. ELISA positive for native 60kD Ro, recombinant 52kD Ro, recombinant 60kD Ro and La.

93-1 Glyburide induced SCLE. ID positive for SSA. + native 60

2109 Subacute cutaneous LE. ID Ro negative

123 Neonatal LE. ID positive for SSA. ELISA positive for native SSA, negative for recombinant 52kD Ro, recombinant 60kD Ro and La.

92-1, C2 Subacute cutaneous LE. Paired sera from same patient, one month apart. C2 antinuclear factor (ANF) negative, 92-1 ANF positive.

3.2.4 Antibody Purification

3.2.4.1 Anti-52 kD Ro

3.2.4.1.1 SDS-PAGE and Western Blotting

Ro fusion proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidine difluoride paper (Immobilon-P, Millipore Corp., Bedford, MA.), electrophoretically with a semi-dry transfer unit (Semi-phor TE70, Hoefer Scientific Instruments, San Francisco, CA.) (Harlowe & Lane, 1988) according to the following protocol:

Protocol 3: SDS-PAGE

Prior to pouring the gel, the glassware and spacers were cleaned with 70 - 95% ETOH and Kimwipes. The plates were then fixed together using the alignment card and fitted into the casting stand. The resolving gel was made with ddH_2O 3.35 ml + Tris(8.8) 2.5 ml + 1% SDS 100µl + 30% acrylamide 4 ml, then degassed, followed by the addition of 50µl 1% APS and 5µl Temed. The resolving gel was added carefully from the top. The plates were filled and immediately overlaid with water using a

needle and syringe. When the gel had polymerised (about one hour) the water was removed from the surface and the stacking gel poured with the comb in place. Once polymerised (about 30min), the comb was removed and the wells rinsed with distilled water.

The samples to be run on the gels were prepared by adding 2 ml of sample buffer (for 2 double gels) to the recombinant protein and mixing on a rocker at room temperature. The molecular weight markers, 10µl / single and the samples were loaded onto the relevant lanes of the gel. The gels were then fitted into the upper chamber of an electrophoresis tank (Biorad) and the top and bottom chambers filled with cooled running water. The gels were then run at 200mA for 30 -45 mins.

Western Blotting

Just prior to the completion of gel electrophoresis, the PDF (polyvinylidine difluoride) paper and 4 sheets of filter paper, cut to the size of the gels, were soaked for a few seconds in Methanol, then ultrapure water, then transfer buffer. Once electrophoresis was complete, the gel was removed from the tank. The glass plates were separated. The plate holding the gel was then immersed in buffer and overlaid with a sheet of nitrocellulose. On top of this was added one sheet of filter paper, followed by a piece of cellophane, then a sheet of filter paper, then the second gel, then two sheets of filter paper.

The resulting sandwich (held between two plastic plates) was then placed in the transfer unit (Semi-phor TE70, Hoefer Scientific Instruments, San Francisco, CA.). The proteins were then transferred to the poly (vinylidine difluoride) paper by blotting at 150 mA/gel for about 50 mins. Coomassie blue dye was then added to the glass chamber. Two ends were cut off the PDF paper and stained. The rest of the PDF paper was then blocked with 4 g milk powder in 80 ml ELISA coating buffer for 1.5 to 2 hrs. It was then removed, and strips from Coomassie blue were added to find the protein. The protein strips were then cut out and stored in SDB and Ampicillin.

3.2.4.1.2 **Reduction**

From preliminary experiments it was realised that to keep the antigenicity of 52-1b, a reducing agent was necessary. The best reducing agent was DTT, which prevents denaturation by stopping the formation of disulphide bonds. The 52-1b strips needed to be incubated overnight in DTT, before being washed repeatedly and using for antibody purification.

3.2.4.2 **Stripping**

The PDF strips were incubated with human Ro autoimmune sera, or rabbit anti-sera, overnight at 4°C and then washed three times with a large volume of 1X PBS at room temperature. The PDF strips were stripped of the bound antibodies with 0.2 M glycine (pH 2.8), for 10 minutes, as described below. A number of preliminary experiments were performed to determine the best stripping agent for each antibody, as assessed by specific activity in ELISA and western blot. The following agents were used for 2, 5, 10, 15 and 30 minutes: 0.1M Glycine (pH 1.8), 0.2M Glycine (pH 2.8), Glycine pH 2.5 and Trimethylamine.

Protocol 4: Summary of antibody stripping and storage of strips

Strips were stored in 50 ml tubes with 20 ml SDB, to cover the strips completely, with 25 µl Ampicillin at 4°C. The 52-1b strips were stored in 20 ml SDB + 200 µl DDT overnight, then washed 4 times with 1 X PBS the next morning, before storing as for other strips. SDB was made with 0.5 ml Tween 20, 2.5 g bovine serum albumin (BSA 0.25%), 1.0 g goat gamma globulin (0.1%), 1.0 g sodium azide (0.1%), 1 X PBS to 1 liter (dilute 10X PBS 1:10 in H_2O), filter sterilised.

Antibody purification The strip was taken out of the storage tube and washed twice with 1 X PBS. The storage tube was kept. 12-20 ml fresh SDB was added to the strips then ~40-50 µl serum. The tube was rotated overnight at 4°C. The liquid phase (unbound fraction) was saved in a labeled tube for ELISA. The strips were

washed in 25 ml wash buffer (0.5 ml Tween 20 (0.05%) in 1 liter sterile 1 X PBS). After the final wash, all the wash buffer was poured out.

Antibody stripping ~7 ml 0.2 M Glycine (pH 2.8) was added and rotated at room temperature for 5 min. After decanting and saving the first glycine wash, ~5 ml additional glycine was added to the strips and rotated at room temperature for ~ 5 min. The liquid phase was saved and combined with the other ~7 ml glycine wash. The strip was briefly spun to collect any remaining liquid. Immediately ~0.9 ml of 1 M Tris (hydroxymethyl)-aminomethane (pH 9.5) was added. This was mixed well and checked with pH paper to ensure that the pH = 8, if not, more Tris was added. After the final glycine wash had been removed, the strips were washed twice with 1 X PBS, then stored as described above.

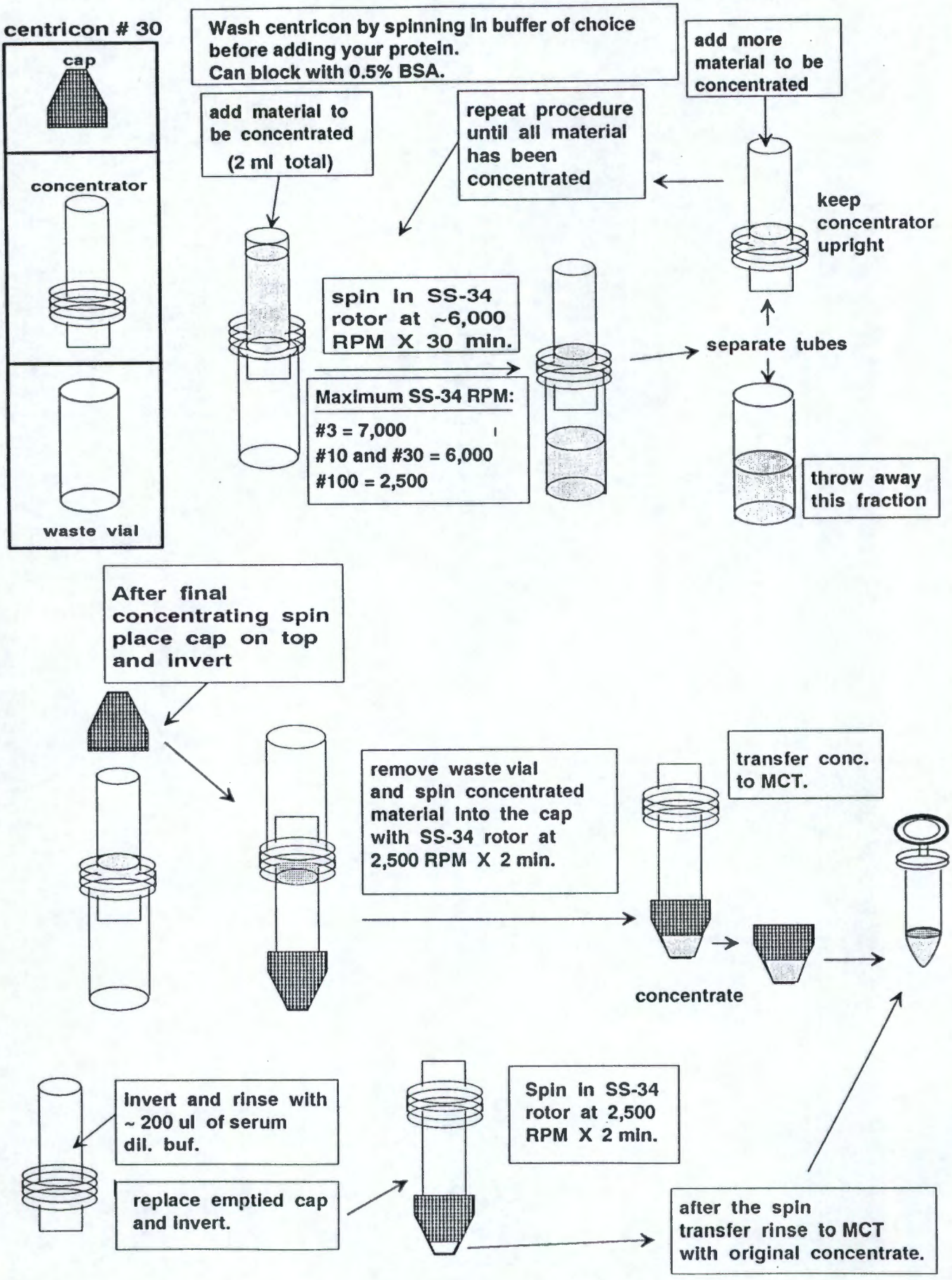
3.2.4.3 Human Anti-native 60kD Roantibodies

3.2.4.3.1 Novel ELISA method

Protocol 5: ELISA method for purification of anti-native 60kD Ro

24 wells of a 96-well microtiter plate were coated with bovine Ro (Immunovision, Springdale, AK) diluted to 8 units/ml in coating buffer. These were incubated overnight at 4°C, blocked for one hour (0.25% bovine serum albumin and 0.1% goat gamma globulin in 1 X PBS) and incubated with a high titer anti native 60 kD Ro containing autoimmune serum, diluted 1:5000, for one hour. After two washes, the bound antibodies were stripped from each well with glycine 0.2M, pH 2.8 for five minutes. The pH was immediately equilibrated to pH 8.0, with 1M Tris, pH 9.5. The antibodies from each well, were pooled and concentrated (Spin-X UF concentrator, Costar, Cambridge, MA), according to the following protocol:

Protocol 6: Antibody concentration If the antibodies were to be kept for later use on fixed cells, a preservative, Sodium Azide was added. If the antibodies were to be used on viable cells, for the penetration experiments, no preservative was added.



3.2.5 Antibody Characterisation

3.2.5.1 Verification of antibody specificity

3.2.5.1.1 Immunoblotting

3.2.5.1.1.1 Skin extract preparation

Protocol 7: Cell extract preparation

A human skin extract was prepared from a freshly isolated plastic surgery specimen. The dermis was separated from the epidermis with 1M NaCl, as previously described (Woodley *et al* 1984). The epidermis was then snap frozen in liquid nitrogen and pulverised. The pulverised epidermis was suspended in a lysis buffer (50mM NaCl, 0.3% Nonidet P-40, 10mM dithiothreitol, 15mM ethylenediamine tetraacetic acid) containing protease inhibitors [1.4mM phenylmethanesulfonyl fluoride, pepstatin (2.5 μg/ml), leupeptin (0.5μg/ml) and aprotinin (0.4 μg/ml)]). The supernatant fraction was collected by centrifugation. Serial dilutions of this extract were subjected to SDS-PAGE and Coomassie blue staining to determine the relative amount and integrity of protein in the extract. Extracts with the least protein degradation were used for western blot analysis. Wil-2 cell (ATCC CRL 8885) and cultured human keratinocyte extracts were similarly made by suspending $X10^{6-7}$ cells in 1ml of lysis buffer followed by sonication with three 20 sec bursts.

3.2.5.1.1.2 Western Blot analysis

Western blot analysis was performed on the human skin extract (Martinez-Lavin M *et al* 1979), a cultured keratinocyte and a human Wil-2 cell extract (ATCC CRL 8885), a human lymphoblastoid cell line, known to express both the 52 and 60 kD Ro proteins (McCauliffe *et al* 1994) and GST (negative control protein). They were subjected to SDS-PAGE and then transferred to PVD paper, in a miniblot apparatus (Miniblotter 28, Immunetics, Cambridge, MA) as described in Protocol 3. The blot was then developed with bromochloroindoyl phosphate and nitro blue tetrazolium (Sigma) as described (Harlowe & Lane, 1988).

3.2.5.1.2 ELISA

Protocol 8: ELISA for confirming purified antibody specificity

Recombinant proteins and native bovine Ro antigen were coated on 96-well microtiter plates at 2-5 μg/ml concentration as previously described (Winston *et al* 1990), see protocol in Appendix. Antibodies were incubated. Antibody binding was detected using goat antihuman IgG antibodies conjugated with alkaline phosphatase (sigma Chemical Co, St. Louis, MO, USA). P-nitrophenyl phosphate (Sigma) substrate was added and colour development measured at 405 nm by a microplate reader (Vmax, Molecular Devices, Corp., Menlo Park, CA, USA). Antibodies were judged reactive if their optical density values were at least 2 standard deviations above the mean value of 15 ID defined Ro negative autoimmune sera. The autoimmune control sera were obtained from patients with SLE [9 (4 anti-Sm, 1 anti-U1 RNP, 4 anti-Sm and anti-U1 RNP)], rheumatoid arthritis (3), dermatomyositis (2) and mixed connective tissue disease (1 anti-U1 RNP).

3.3 RESULTS

3.3.1 Multiple types of anti-52 and anti-60 kD Ro antibodies were purified

Rabbit antibodies were purified against full length recombinant 52 and 60 kD Ro proteins. Human autoantibodies were affinity purified against two different recombinant 52 kD Ro fragments [52-1b (amino acids 1-98) and 52-2 (amino acids 138-340)], see **figure 16, pg. 94** and two different recombinant 60 kD Ro fragments [60-2 (amino acids 139-326) and 60-4 (amino acids 410-538)], see **figure 17, pg. 95**. Each of the four fragments had previously been shown to contain at least one major Ro epitope [52-2, 60-2, 60-4: (McCauliffe *et al* 1994); 52-1b (Yell *et al* 1996b)]. Human antibodies were also purified against the native 60-kD Ro protein, which contains one or more epitopes not expressed by the recombinant Ro proteins (McCauliffe *et al* 1994).

3.3.2 Cultured keratinocytes and human epidermis express the 52- and 60-kD Ro proteins

The specificities of the purified antibodies were demonstrated by immunoblot, see **figure 18, pg. 96** and ELISA, see **figure 19, pg. 97, figure 20, pg. 98**.

None of the purified Ro autoantibodies reacted with both 52- and 60-kD Ro proteins.

There was no convincing evidence of cross-reactivity between the anti-52 and anti-60 kD Ro antibodies by immunoblot. Furthermore, the ELISA results of the entire panel of Ro antibodies failed to validate the existence of the previously described cross-reactive epitope shared by the 52 and 60 kD Ro proteins (Itoh *et al* 1992).

figure 16: Schematic view of the 52 kD Ro protein, showing the areas against which various antibodies were purified. Human autoantibodies were affinity purified against two different recombinant 52 kD Ro fragments [52-1b (amino acids 1-98) and 52-2 (amino acids 138-340)].

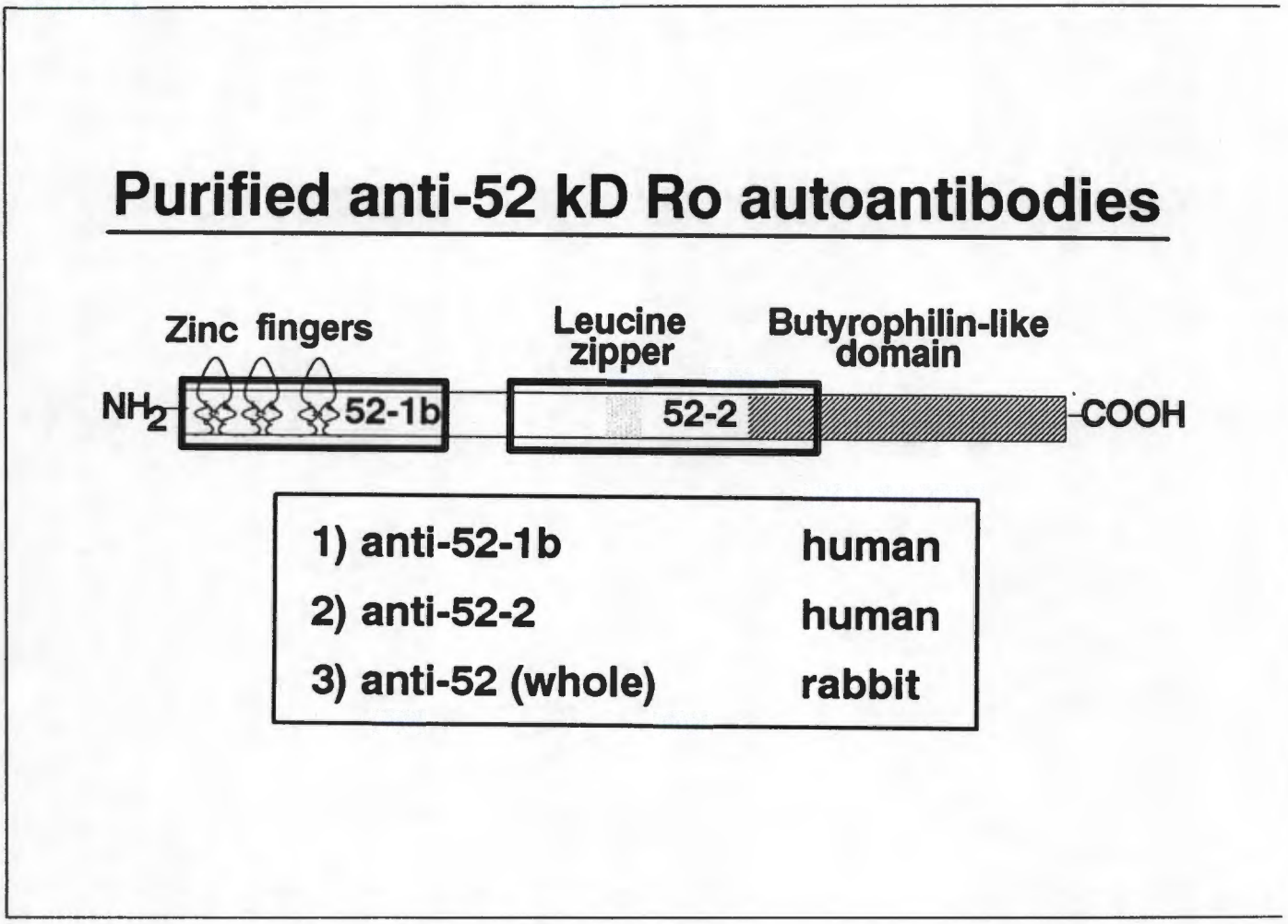


figure 17: Schematic view of the 60 kD Ro protein, showing the areas against which various antibodies were purified. Human autoantibodies were affinity purified against two different recombinant 60 kD Ro fragments [60-2 (amino acids 139-326) and 60-4 (amino acids 410-538)]. Human antibodies were also purified against the native 60-kD Ro protein, which contains one or more epitopes not expressed by the recombinant Ro proteins (McCauliffe *et al* 1994).

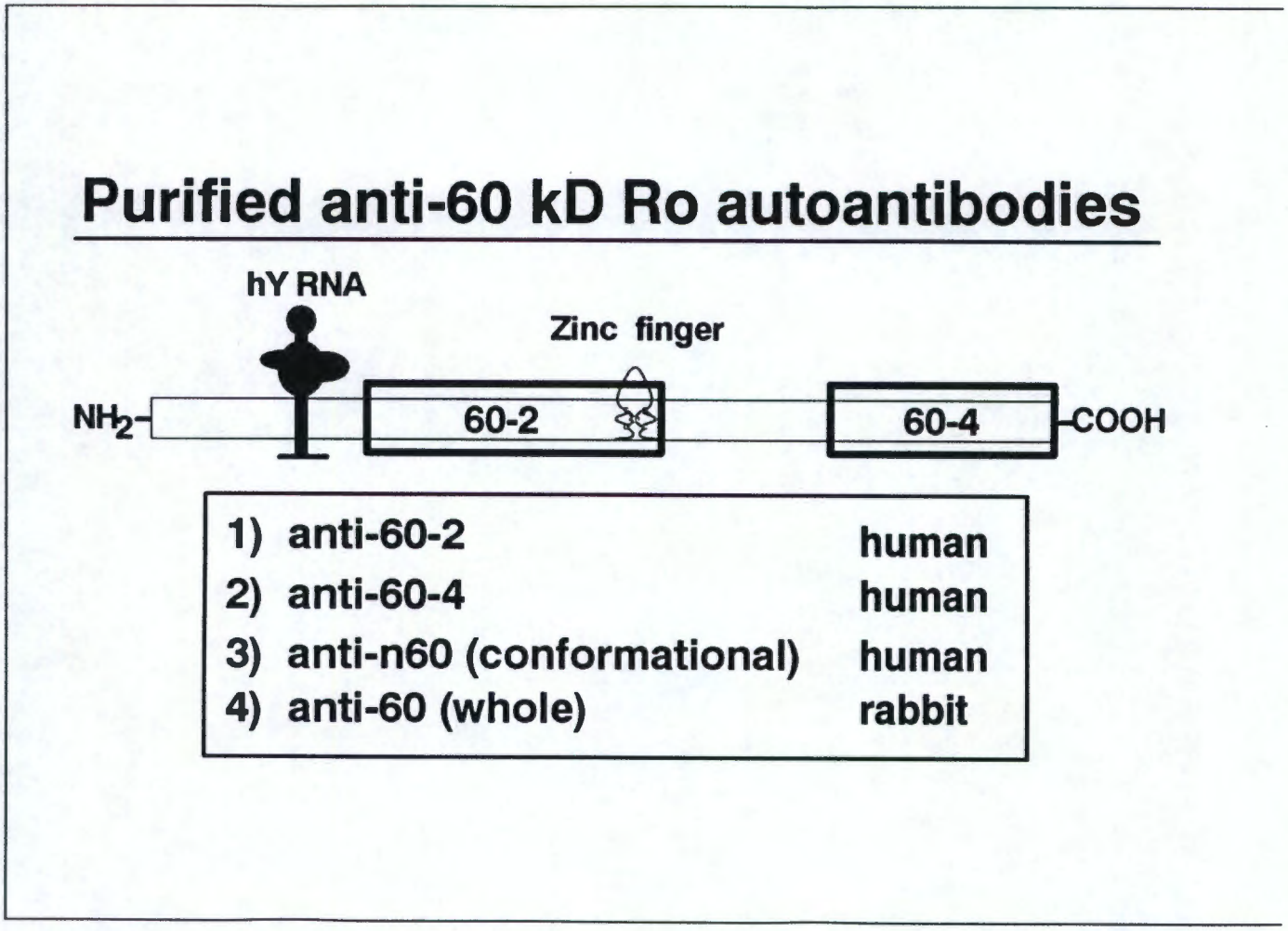


figure 18: Western immunoblot detects the 52 and 60 kD Ro proteins in human skin extract (SE), cultured normal human keratinocyte extract (KE) and Wil-2 cell extract (WE). Autoimmune sera (AS) was used as a negative control. Each extract was subjected to SDS-PAGE, transferred to PVDF paper and then incubated with a 1:25 dilution of Ro-negative autoimmune control sera (AS), purified human anti-60-2 antibodies (60) and human anti-52-2 antibodies purified from two different autoimmune sera (52 and 52').

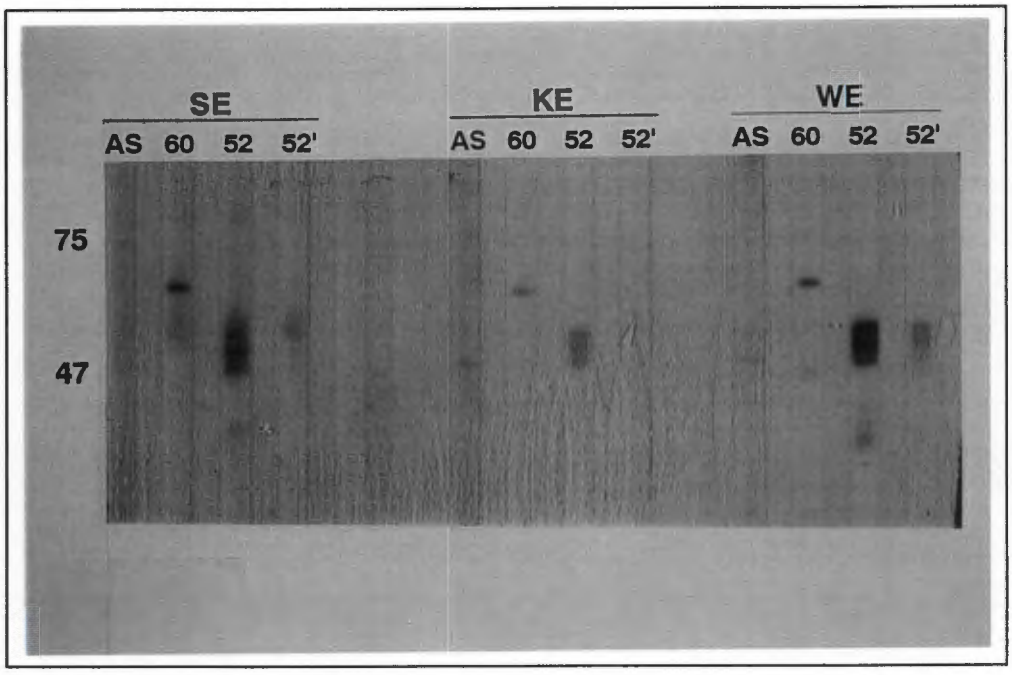


figure 19: Purified Ro antibodies were specific for either the 52- or the 60-kD Ro protein as demonstrated by ELISA. (a) Purified human anti-52-1b, anti-52-2, rabbit antisera raised against whole recombinant 52 kD Ro (rab anti-52) and a 1:100 dilution of normal sera (normal) were reacted with a panel of antigens. Purified antigens: n60, native 60 kD Ro;whole 60, full length recombinant 60 kD Ro;60-2, recombinant 60-2 fragment (aa139-326);60-4, recombinant 60-4 fragment (aa 410-538); whole 52, full length recombinant 52 kD Ro; 52-1, recombinant 52-1 fragment (aa 1-178); 52-2, recombinant 52-2 fragment (aa 138-340); La, native La/SS-B autoantigen; and GST, recombinant glutathione S-transferase, which was a component of each recombinant fusion protein in this antigen panel.

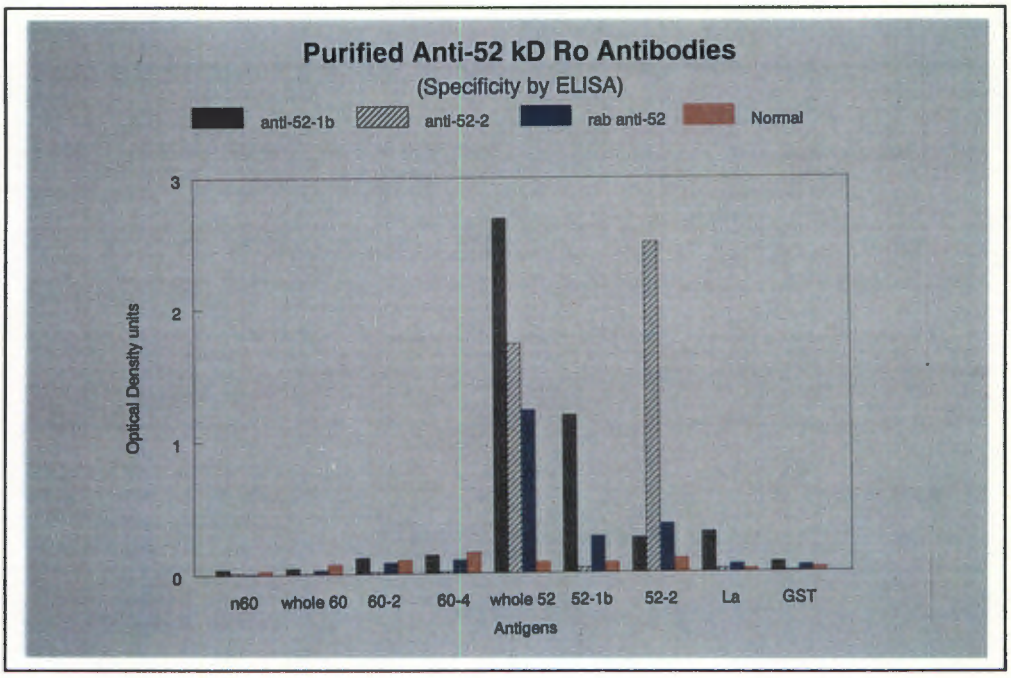
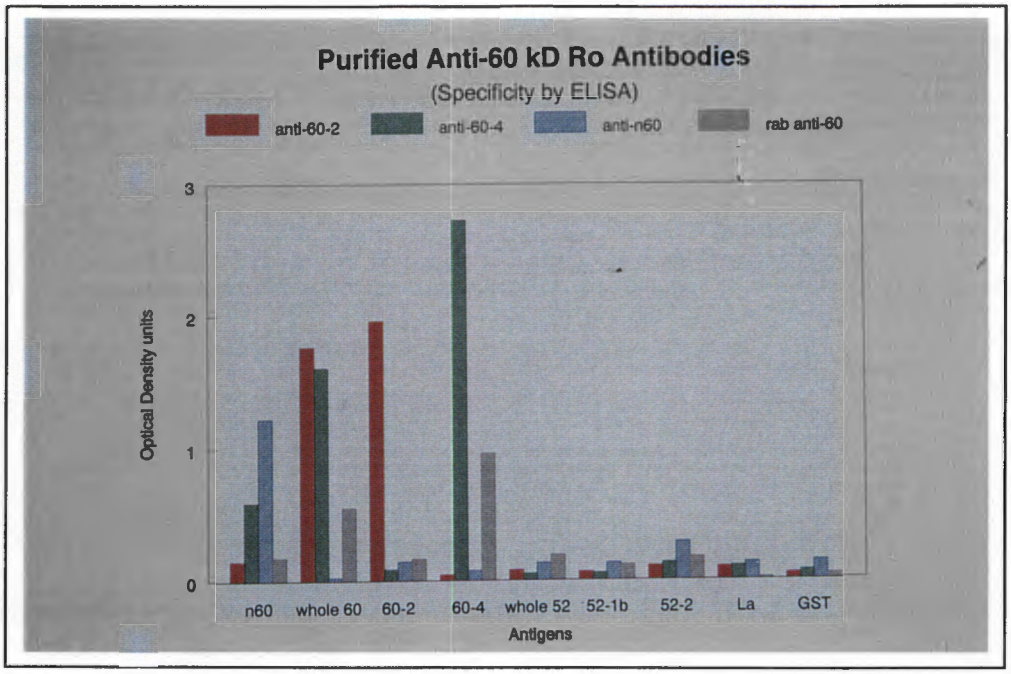


figure 20: Purified Ro antibodies were specific for either the 52- or the 60-kD Ro protein as demonstrated by ELISA. (b) Purified human anti-60-2, anti-60-4, anti-native 60 kD Ro (anti-n60) and rabbit antisera raised against the full-length recombinant 60 kD Ro (rab anti-60), were similarly tested. Purified antigens: n60, native 60 kD Ro;whole 60, full length recombinant 60 kD Ro;60-2, recombinant 60-2 fragment (aa139-326);60-4, recombinant 60-4 fragment (aa 410-538); whole 52, full length recombinant 52 kD Ro; 52-1, recombinant 52-1 fragment (aa 1-178); 52-2, recombinant 52-2 fragment (aa 138-340); La, native La/SS-B autoantigen; and GST, recombinant glutathione S-transferase, which was a component of each recombinant fusion protein in this antigen panel.



3.4 DISCUSSION

3.4.1 Multiple types of anti-52 and anti-60 kD Ro antibodies were purified

A 52-kD Ro protein was first found in addition to the 60-kD protein, in the sera of 80% of patients with detectable Ro precipitins (Ben-Chetrit *et al* 1988). In addition, this study showed that these antigens contained different and unique epitopes which did not generate cross-reactive antibodies. However, this group suggested that the 52 and 60-kD antigens in their native state, may exist as a complex or as associated particles. Ro autoantibodies have been shown to react with at least four distinct proteins, of 3 different molecular weights, 52-, 54- and 60kD (Rader *et al* 1989). The 60 and 52kD antigens have been cloned (Chan *et al* 1991b; Chan *et al* 1991a) and are structurally dissimilar (Itoh *et al* 1991).

Considerable evidence has implicated passive immunisation with anti-Ro antibodies in the pathogenesis of NLE. However, the low frequency of the disease in infants born to mothers seropositive for anti-Ro, suggests that significant qualitative differences exist among humoral anti-Ro responses. Further definition of these qualitative differences has been possible with epitope mapping. This has been applied to a number of autoimmune responses. Theoretically, a dominant autoepitope may represent an important stimulus for initiation or maintenance of an autoantibody response.

Methods of identifying B cell epitopes with short synthetic peptides have been criticised (Horsfall *et al* 1991). Based on molecular modeling of antibody-antigen interactions, greater than 90% of epitopes are thought to be conformational and dependent on discontinuous sequences and therefore unlikely to be identified with short synthetic peptides (Blundell *et al* 1987; Laver *et al* 1990). Recombinant fusion proteins have been used to identify conformational epitopes that are missed with

synthetic peptides. ELISA studies with recombinant 52 and 60kD Ro protein fragments revealed at least 2 major epitopes on each Ro protein (Tsay *et al* 1996).

The first study to describe at least two antigenic determinants on the 52kD Ro protein, showed one immunodominant and the other recognised by a more "restricted" subset of anti-52kD Ro antibodies. 95% of 99 sera containing anti-52kD antibodies by ELISA, reacted with a large fragment spanning amino acids (aa) 1-291 (Buyon *et al* 1994). Two antigenic regions were identified: aa 169-291 containing the leucine zipper, that was recognised by 83% of the anti-52kD sera tested and aa 1-78 containing the zinc finger domains, that was recognised by half the sera. Confirmation of the fact that anti-52 kD Ro autoantibodies in different autoimmune diseases preferentially recognize epitopes on the central region of the antigen, has appeared more recently. The strongest antigenicities recognized by anti-52 kD Ro autoantibodies were located within the aa 197-245 region. Further antigenic sites preferentially recognized by SS, CHB, and healthy infant sera were located within aa 153-196. The central region aa 153-245, was a major immunogenic region, containing a strong antigenic epitope between aa 197-245. The antibody response was directed at this major antigenic region regardless of the underlying autoimmune disease (Dorner *et al* 1996b).

The immune response to the 60kD Ro antigen is heterogeneous. Some autoimmune sera specifically recognise the native Ro antigen but fail to bind the corresponding denatured polypeptides. A number of groups have described about 15% of anti-Ro positive sera which do not recognise either the 52 nor 60-kD polypeptides in immunoblots (Elkon & Culhane, 1984; Ben-Chetrit *et al* 1988; Boire *et al* 1991). In one study (Boire *et al* 1991), after immunodepletion using the denatured 60-kD Ro polypeptide, all anti-Ro positive sera still contained high titers of antibodies recognising conformational determinants on the Ro antigen. The frequent immunodominance of anti-Ro antibodies targeted to conformational determinants suggests that native autoantigens may directly drive the autoimmune response (Boire *et al* 1991). Heterogeneity in recognition of autoepitopes of the 60-kD Ro protein has

been detected using both synthetic peptides by ELISA and denatured fusion proteins by immunoblot. Discontinuous epitopes exist (Saitta *et al* 1994).

Several investigators have tried to identify 60kD Ro epitopes based on the reactivity of autoimmune sera with synthetic peptides. Scofield et al identified a major epitope on the 60kD Ro protein that has been localised to the sequence EYRKKMDI contained within my 60-4 fragment (Scofield *et al* 1991). They showed 28/45 (67%) ID defined Ro positive SS sera reacted by Western blot with a 3kD fragment of the 60kD Ro that contains the EYRKKMDI sequence. McCauliffe et al have found that 42% of ID defined Ro positive SS sera react by ELISA to a recombinant 60 fragment containing this sequence (60-4) (McCauliffe *et al* 1994). They found the 60-2 fragment more reactive, with 9/12 (75%) of ID defined Ro positive sera reactive. NLE, SCLE and SS patient sera all reacted with these two 60kD Ro epitopes.

A different group has shown that a synthetic peptide corresponding to amino acid residues 21-41 of the 60kD Ro protein had significant reactivity to SS but not LE patient sera (Barakat *et al* 1992). My recombinant 60kD Ro protein starts with residue 24. The 60-1 fragment that contains all but three of the amino acids used to make the 21-41 synthetic peptide, has little reactivity with SS patient sera (McCauliffe *et al* 1994). Another group (Scofield & Harley, 1991), synthesised 531 overlapping octapeptides for the entire 60kD Ro sequence.

A further group used 22-mer, synthetic peptides overlapping by eight residues, covering the entire sequence of the Ro60 kD autoantigen, to evaluate three groups of sera according to their autoantibody specificites. The first group consisted of monospecific anti Ro60 kD sera from four patients with SLE and one with SS, the second one was composed of anti-Ro60 kD+anti-La(SSB)-positive sera from four patients with SS and the third group included three normal sera and one anti Ro52 kD serum. It was found that sera from SLE patients interact with a common antigenic site spanning the sequence TKYKQRNGWSHKDLLRSHLKP (169-190) of the Ro60 kD protein. On the other hand, sera from SS patients recognise the ELYKEKALSVETEKLLKYLEAV (211-232) region. Determination of the minimal

required peptide length for optimal antibody recognition showed that the defined epitopes can be shortened to the NGWSHKDLLR(175-184) and KALSVETEKLLKYLEAV (216-232) sequences respectively. Inhibition experiments using the Ro60 kD antigen and soluble peptides corresponding to the 175-184 and 216-232 segments further confirmed the specific antibody binding (Routsias *et al* 1996).

I have purified multiple types of anti-52 and anti-60-kD Ro antibodies. Rabbit antibodies were purified against full length recombinant 52-and 60-kD Ro proteins. Human autoantibodies were affinity purified against two different recombinant 52-kD Ro fragments [52-1b (amino acids 1-98) and 52-2 (amino acids 138-340)] and two different recombinant 60-kD Ro fragments [60-2 (aa 139 -326) and 60-4 (aa 410-538)]. Each of the four fragments had previously been shown to contain at least one major Ro epitope(McCauliffe *et al* 1994). Human antibodies were also purified against the native 60-kD Ro protein, which contains one or more epitopes not expressed by the recombinant Ro proteins (McCauliffe *et al* 1994). I have previously used the stripping method to reuse ELISA plates for antibody testing, without significant loss of coated protein. This stripping technique has been previously published and demonstrated that the stripping procedure does not significantly diminish antigenic capacity (of denaturation resistant epitopes) (Baunoch et al 1992).

3.4.2 Cultured keratinocytes and human epidermis express the 52- and 60-kD Ro proteins

The specificities of the purified antibodies were demonstrated by immunoblot and ELISA. These antibodies identified appropriately sized proteins in keratinocyte extracts prepared from surgical and cultured specimens, indicating that both Ro proteins are expressed in normal human adult skin and in cultured human keratinocytes respectively, see **figure 18**. Multiple protein fragments were labeled with the anti-52-kD Ro antibodies. Whether these fragments represent protein degradation, different forms of the 52-kD Ro protein, or cross-reactive proteins is

uncertain. The smear -like nature of the fragments with a greater proportion of smaller fragments in the skin extract preparation, which takes longer to prepare than the cultured keratinocyte and Wil-2 cell extracts, would suggest that these fragments arise from protein degradation.

3.4.3 No evidence that Ro autoantibodies cross-react with the 52- and 60-kD Ro proteins

Itoh *et al* previously reported that a major denaturation-resistant epitope on the 52 kD Ro protein is cross-reactive with the Ro autoantibodies directed against an epitope expressed on the native 60 kD Ro protein (Itoh *et al* 1992). There was no convincing evidence of cross-reactivity between the anti-52 and anti-60 kD Ro antibodies by immunoblot, see **figure 18**. Furthermore, the ELISA results of the entire panel of purified Ro antibodies failed to validate the existence of the previously described cross-reactive epitope shared by the 52- and 60-kD Ro proteins (Itoh *et al* 1992), see **figure 19**. 52-2 contains the previously described denaturation resistant epitope. Whether the discrepancy between my data and that of Itoh et al resulted from differences in patient sera reactivities, source of Ro antigen, or methodology is uncertain. Other studies have also failed to reveal cross-reactivity of anti-52 and anti-60 kD Ro autoantibodies (Keech *et al* 1994; St.Clair *et al* 1994; Ben-Chetrit *et al* 1988; Peek *et al* 1993).

4. LOCATION OF Ro ANTIGENS IN CELLS

4.1 Introduction

Anti-52 and anti-60 kD Ro autoantibodies are produced by the vast majority of patients with SCLE and NLE (McCauliffe *et al* 1994). Although there is substantial evidence that these autoantibodies are pathogenic in these two diseases (McCauliffe, 1997a; Lee *et al* 1989), the cellular function and location of their target antigens have remained controversial.

Multiple studies have characterised the cellular location of Ro antigens by immunofluorescence techniques but with discrepant results. These discrepancies have arisen in part from differences in fixation techniques (Harmon *et al* 1984; Peek *et al* 1993), the use of non-human substrates (Harmon *et al* 1984) and the utilisation of human Ro autoimmune sera of varying specificities (Hendrick *et al* 1981; Maddison *et al* 1981; Harmon *et al* 1984; Lopez-Robles *et al* 1986; Miyagawa *et al* 1988).

Studies using anti-Ro antibodies in IF reported both nuclear and cytoplasmic staining (Ben-Chetrit *et al* 1988; Slobbe *et al* 1991; Lopez-Robles *et al* 1986; Mayet *et al* 1988), while others showed only nuclear staining (Lopez-Robles *et al* 1986; Harmon *et al* 1984), or a predominantly cytoplasmic location (Alspaugh & Maddison, 1979; Bachmann *et al* 1986; Hendrick *et al* 1981). It is interesting that purification of the Ro antibody system by a quantitative precipitin technique, followed by IF, has shown conclusively that anti-Ro antibodies showed only cytoplasmic staining when calf thymus, KB cells, human normal lymphocytes and cells from chronic lymphocytic leukaemia patients, were used as substrates. However, many whole sera when employed in similar experiments using KB and Wil-2 cells, produced a speckled nuclear fluorescence as well as cytoplasmic staining. This was especially obvious when La antibodies were additionally present. When, however, mouse liver, the most commonly used substrate at the time, was employed, with purified anti-Ro antibodies, neither nuclear nor cytoplasmic staining was found (Sontheimer *et al* 1982).

In 1986, a group used eight sera directed against the Ro antigen in an indirect immunofluorescence technique, on several mammalian tissues. They checked the sera for mono specificity by Ouchterlony and immunoblotting techniques, using partially purified Ro antigen from human spleen. They found a mainly speckled nuclear pattern in the nucleus of dog liver and HEp-2 cells. Cytoplasmic fluorescence appeared in one serum (Lopez-Robles *et al* 1986).

A further group has fractionated human Jurkat and mouse 3T3 cells into a cytoplasmic and nuclear fraction and analysed these fragments separately. They found that the Ro and La protein were both present in the cytoplasm and nucleus, while the Y RNAs are exclusively cytoplasmic. In addition, they showed that the majority of the human Y stably associated with Ro60 and Ro52, as well as with the La protein. In the nucleus, Ro60 and Ro52 were not associated with Y RNA (Peek *et al* 1993)

Antiserum specific for CR has revealed predominantly perinuclear cytoplasmic staining with lesser amounts of nuclear staining (McCauliffe & Sontheimer, 1993b). Most hY5RNA has been reported in the cytoplasmic fraction of HeLa cells (Kato *et al* 1982). A different study subsequently found that more than 90% of the Ro particles were recovered from the cytoplasmic fraction of HeLa cells (Bachmann *et al* 1986). The Ro antigen was originally described as a saline-soluble, cytoplasmic antigen (Clark *et al* 1969).

A further study using six monoclonal anti-Ro antibodies, showed either a cytoplasmic binding or a nuclear binding to HEp-2 cellular antigens (Rosario *et al* 1988). They found that the first mouse serum and 3 of the 6 monoclonal antibodies bound cytoplasmic antigens. One monoclonal antibody bound a nuclear antigen in HEp-2 cells in a homogenous pattern. This binding represented that of the monoclonal antibodies, rather than other immunoglobulins, as it was reproduced when supernatants, ascites fluid, purified IgG, or hybridoma-bearing SCID mouse sera were tested.

Recent studies, with standard fixation methods, human substrate and more specific Ro antibodies, however, have also produced discrepant results (Bachmann *et al* 1986; Ben-Chetrit *et al* 1988; Slobbe *et al* 1991; Peek *et al* 1993; Casciola-Rosen *et al* 1994; Kelekar *et al* 1994; Peek *et al* 1994). Kelekar et al used immunofluorescence analysis of two different cell lines with monospecific antibodies against 52 and 60kD proteins to suggest that these two proteins are not present on overlapping sets of structures in vivo (Kelekar *et al* 1994).

In a study using monospecific antibodies on simian virus 40-transformed keratinocytes, Ro antigen was detected in both the nucleus and cytoplasm. Primary cultured adult keratinocytes showed localised immunofluorescence within the nucleus. When the calcium concentration of the medium was switched to 0.05 mM, these cells expressed cytoplasmic Ro antigens within 48 hours. Surface expression of this antigen could not be detected in either primary or transformed cells (Miyagawa *et al* 1988).

The interpretations of these data are even less certain because it has been reported that some Ro autoantibodies cross-react with the 52 and 60kD Ro proteins (Itoh *et al* 1992). Slobbe et al used indirect IF to look at the intracellular location of 52 and Ro60, using a monospecific anti-Ro52 serum and a monospecific rabbit anti-Ro60 serum on human HEp-2 cells. The monospecific human anti-Ro52 serum was determined by negativity to Ro60 and La on immunoblotting. However a cross reactive epitope has been found with the conformational 60 and 52 on immunoblot. This may well have accounted for their findings of identical localisation of both the 52 and 60kD Ro in a diffuse cytoplasmic staining next to a strong punctuated nuclear staining, with nucleoli (Slobbe *et al* 1991).

My aims were to determine the localisation of 52 and 60 kD Ro antigens in human keratinocytes. I also hoped to clarify the discrepancies reported in previous localisation studies by using a panel of anti-52 and anti-60 kD Ro specific antibodies and to reassess whether previous inconsistencies may have resulted from cross-reacting antibodies.

4.2 METHODS

4.2.1 Keratinocyte culture

Keratinocyte culture has been comprehensively reviewed (Karasek, 1983; Fuchs *et al* 1988). Before 1983, most keratinocyte culture media needed serum supplementation (Briggaman *et al* 1967). Improved keratinocyte growth was shown on lethally irradiated mouse 3T3 feeder layers in media supplemented with epidermal growth factor, cholera toxin and hydrocortisone (Rheinwald & Green, 1975). Ham's group pioneered the use of serum free media for many cell types, including MCDB-153 for keratinocytes (Boyce & Ham, 1983). This medium was then enriched with increased concentrations of a number of essential amino acids.

A number of preliminary experiments were performed (not described here) to assess the best way of establishing primary human keratinocyte culture, see **Protocol 14, pg. 110**. It was decided to use serum free media *ab initio* to decrease confounding factors.

Protocol 9: Definitive keratinocyte culture

Neonatal foreskins were stored in Dulbecco's modified Eagle's medium-high glucose (DMEM-high glucose), with 1 mM sodium pyruvate, 2% fetal calf serum (FCS), 5 ug/ml of ketoconazole, 100 ug/ml of chloramphenicol, 100 U/ml of penicillin, 100 ug/ml of streptomycin, 250 ng/ml amphotericin B and 20 mM HEPES (pH 7.3), for between one to 24 hours at 4°C. They were washed 2 - 3 times with HBSS. The foreskin was flattened, epidermis up and cut into pieces 3 mm^2. They were then trypsinised overnight, in 0.25% trypsin (make fresh: weigh the trypsin, dissolve in the buffer, then filter) at 4°C. The next morning, the epidermal layers were coming off the dermal chunks. The epidermal layers were peeled off and shaken vigorously into Medium 154 (Cascade Biologics Inc., Portland, OR). This shaking dislodged the viable basal keratinocytes. The basal keratinocytes from the epidermis were resuspended in Medium 154 (Cascade Biologics Inc., Portland, OR) supplemented with human keratinocyte growth supplement. These primary cultures were incubated

Medium 154 (Cascade Biologics Inc., Portland, OR). This shaking dislodged the viable basal keratinocytes. The basal keratinocytes from the epidermis were resuspended in Medium 154 (Cascade Biologics Inc., Portland, OR) supplemented with human keratinocyte growth supplement. These primary cultures were incubated at 37°C 5% CO_2. The medium was changed as needed. After about 5 days, the keratinocytes were about 50% confluent, at which stage they were subcultured.

The following protocol was followed for subculturing:

Protocol 10: Subculturing of keratinocytes

The media was aspirated and the parent dish rinsed twice with HBSS cmf. 5 ml of 0.1% trypsin solution containing 0.02% EDTA was added. The dish was put into the incubator and checked about 4 mins later. The cell solution was aspirated. The dish was rapped vigorously against the palm to remove the cells. (Total < 10 min.). 5 ml trypsin neutralising solution was added. This was transferred to a 15 ml tube and centrifuged at 1000 rpm for 3-5 min.. The supernatant was removed. The pellet was loosened and resuspended in 4 ml Med 154. An aliquot was added to the daughter flask containing Med 154. The next day, each flask was rinsed twice with HBSS cmf and fed with Med 154. This important rinse removed dead cells and debris.

When the cells were 60-80% confluent, they were frozen. The following freezing protocol was used:

Protocol 11: Freezing keratinocytes

Freezing medium was made up (MED 154 with DMSO to 10%, sterile filter, store at 4°C). The NUNC cryotubes were labeled with cell type, passage number, number of cells per tube and date. The keratinocytes were trypsinised as for routine subculturing and resuspended in HBSS cmf 0.1% FCS. The cell suspension was spun for 5 minutes at 1000 rpm in GLC-3(200 X g). The supernatant was removed, the pellet was loosened and resuspended in the cold freezing medium. The cryotubes were placed on ice for 20 minutes to thoroughly cool them, then transferred to the -20°C

freezer for 2 hours, followed by the -70°C freezer overnight, then into the liquid nitrogen.

These keratinocytes were thawed and seeded onto 8-chamber Lab Tek slides (Nunc Inc., Naperville, IL). The following thawing protocol was used:

Protocol 12: Thawing keratinocytes frozen in Liquid Nitrogen

The cryotube was removed from the liquid nitrogen and thawed in a 37°C water bath as rapidly as possible. In the hood, the cryotube was drenched with 70% ethanol to sterilise it. It was wiped dry with a sterile wipe. Thawed keratinocytes were diluted with appropriate volumes of Med 154 and the chambers were seeded.

In an effort to increase the yield of basal keratinocytes from adult cells, Dispase, rather than Trypsin was used according to the following protocol:

Protocol 13: Dispase for epidermal separation

The skin was placed into foreskin medium and washed three times HBSS without Ca or Mg. With the epidermis up, it was cut into 3 mm^2 pieces. It was then incubated in Dispase 2.4u/ml at 37°C for 3 - 5 hours. When ready, the epidermis slid off the dermis. The epidermis was transferred to a 15 ml centrifuge tube, containing 2 ml Gibco trypsin. The dermis was scraped with a scalpel to remove basal keratinocytes and these were added to the tube. This was incubated at 37°C in a water bath for 20 - 30 min., shaking the tube from time to time. 10 ml Trypsin Neutraliser was then added and pipetted up and down to help dissociation. This was centrifuged for 8 min. at 1800 and resuspended in plating medium. Basal keratinocytes were seeded at 2 - 3 X 10^6 cells/75 c^{m2} flask with 10 - 15 ml medium. They were washed at 24 - 48 hours.

Protocol 14: Comparing various described keratinocyte culture methods

DAY	WITH 3T3 CELLS	IN SERUM FREE MEDIA
1	Incubate foreskins for 24 hrs in foreskin media	Incubate foreskins for 24 hrs in foreskin media
2	a)In petri dish, wash foreskin X 3 with HBSS b)Epidermis up, cut into 2X2mm squares c)Fill dish with 15 ml 0.25% trypsin in trypsin/EDTA buffer (without EDTA) d) incubate overnight at 4C	a)In petri dish, wash foreskin X 3 with HBSS b)Epidermis up, cut into 2X2mm squares c)Fill dish with 15 ml 0.25% trypsin in trypsin/EDTA buffer (without EDTA) d) incubate overnight at 4C
3	treat 3T3 cells with Mitomycin a)T/F epidermal sheets to clean dish with 10ml plating media b) shake vigorously c)pipette and expel cells X5-10 d)aliquot suspension into new tissue culture dishes e)Add $2X10^6$ mitomycin treated 3T3 cells/dish f) add plating medium up to 15ml g)incubate at 37C for 3-4 days	a)remove and pool epidermal sheets b)vortex epidermal sheets in HBSS c)centrifuge cell suspension for 5min at 250 g d)resuspend in Medium 154 supplemented with KGM and antibiotics e)seed each 25 cm^2 flask with 10^5 cells
4		After 24 hours, wash cells with warm HBSS to remove dead cells before adding further warmed Medium 154.
5		
6	change medium every 3-4 days to growth medium	change medium every 2-3 days until cells are confluent, typically, 7-10 days.
7	after one week, or when keratinocytes cover 50% of dish, remove 3T3 cells	
10	subculture keratinocytes before they become confluent a)aspirate medium and rinse X2 with HBSS b)add 5ml 0.1% trypsin/0.02% EDTA c)put dish in incubator and check 4 mins later d) aspirate cell suspension e) add 5ml trypsin neutralising soln plus 1-2ml FCS (mixed beforehand) f)T/F to a 15ml tube and spin at 1000rpmX 3-5min g)remove supernatant h)resuspend pellet in 4ml KGM i)add aliquot to daughter flask	once confluence reached, a)remove medium b)add 0.05% trypsin/0.01% EDTA for 1 min to remove fibroblasts c)discard supernatant d)add more trypsin to detach keratinocytes (+-4-5 minutes) e) stop trypsinisation with HBSS with 10% FCS f)centrifuge resulting suspension for 5min at 250g g)resuspend pellet in warmed medium 154 h)1XT25 flask to 1T75 flask 1XT75 flask to 2xT150 flasks
11	next day, rinse each flask with HBSS and feed with KGM	

4.2.2 Immunofluorescence

A number of preliminary experiments (not shown here) were performed to determine: the best fixative (3% paraformaldehyde, versus 4% paraformaldehyde, versus neat Acetone, versus Ethanol, versus 2% glutaraldehyde) the best permeabilising agent: (0.1% Triton X in PBS, versus neat Acetone for 30 seconds, versus 0.1% Saponin, versus Tween 20, diluting buffer) and the best dilutions of both primary and secondary antibodies. All the primary antibodies used were checked at concentrated through to dilute dilutions. The secondary antibodies were diluted according to manufacturer's recommendations with one dilution either side in addition.

Protocol 15: Immunofluorescence of fixed keratinocytes

The keratinocytes were fixed in 3% paraformaldehyde at 4°C for 15 minutes, followed by 2 brief washes with 1X phosphate buffered saline (PBS). They were permeabilised for 5 minutes with 0.1% Triton X in PBS, washed twice with 1X PBS and then incubated for one hour at 4°C with the primary purified Ro antibodies. Following another 2 PBS washes, secondary goat anti-human IgG conjugated to Texas Red (Jackson Immunoresearch Laboratories, West Grove, PA) and / or (depending on whether the cells were labeled singly or doubly) goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO), was added for 30 minutes at 4°C. The slides were washed twice, the chambers were removed and the slides were coverslipped with polyvinyl alcohol mounting media (PVA).

To ensure that the negative nuclear staining observed with the anti-52kD antibodies was not due to inability of the secondary antibody to detect the primary antibody, as it was bound to DNA, the experiments were repeated with pre-treatment with both DNAse and RNAse. The following modified IF protocol was used:

Protocol 16: Immunofluorescence on pre-treated DNAse and RNAse keratinocytes

The cells were washed twice with cold dulbecco PBS (X 5 min. max.) and then fixed in paraformaldehyde (3% in PBS) for 15 min. at room temperature. Following a further two washes with cold dulbecco PBS (X 5 min. max.), the keratinocytes were permeabilised with Triton X-100 (0.2% in PBS) for 15 mins. The cell chambers were then incubated with DNAse I (Pharmacia) at a concentration of 10 000u/ml stock, diluted 1:20 for 45 min. or incubated with DTT 200 μl in 20 ml SDB overnight. Negative controls were incubated with RNAse/DNAse dilution buffer only. Thereafter, the keratinocytes were treated as for previous immunofluorescence protocols.

4.2.2.1 Microscopy

4.2.2.1.1 Epifluorescence microscopy

The slides were viewed with a conventional epifluorescence microscope, followed by confocal microscopy.

4.2.2.1.2 Confocal Microscopy

Two confocal microscopy systems were used. Images were collected with a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad Ltd., Hemel Hempstead, Herts, UK) mounted onto a Nikon Diaphot inverted microscope, with a 100X objective and a pinhole setting of 3 to maximise optical sectioning, at 0.8 - 0.9μm

krypton laser to the sample. Excitation light was attenuated with a 1% neutral density filter to minimise photobleaching.

The other confocal system used, was a Zeiss invert LSM 410, with software version 3.50 (Gottingen, Germany) with a pinhole of 20 and a similar neutral density filter. The contrast and brightness adjustments were maintained at constant levels for all experiments and controls. Each digital image was obtained with a laser exposure time of 8 seconds, averaged over four, to reduce noise. Confocal images were processed using Adobe Photoshop 3.0 software and printed on an Epson colour printer (Epson America, Inc., Torrance, CA.)

4.3 RESULTS

The specific antibodies, described in the previous chapter, were used to determine the cellular locations of the 52 and 60 kD Ro proteins, by conventional epifluorescence and scanning confocal microscopy, including Z-series.

4.3.1 Anti 52 kD Ro autoantibodies

The human anti-52-1b and 52-2 autoantibodies stained the cytoplasm in a fine granular pattern, see **figure 21, pg. 115**. There was similar nuclear staining that was most apparent in cells that had not yet reached confluence. Cells that had reached confluence often demonstrated minimal nuclear staining, see **figure 22, pg. 116**. To ensure that the poor nuclear staining observed with the anti-52kD antibodies was not due to inability of the secondary antibody to detect the primary antibody, as it was bound to DNA, the experiments were repeated with pre-treatment with both DNAse and RNAse. These results were the same as those obtained with the standard IF method.

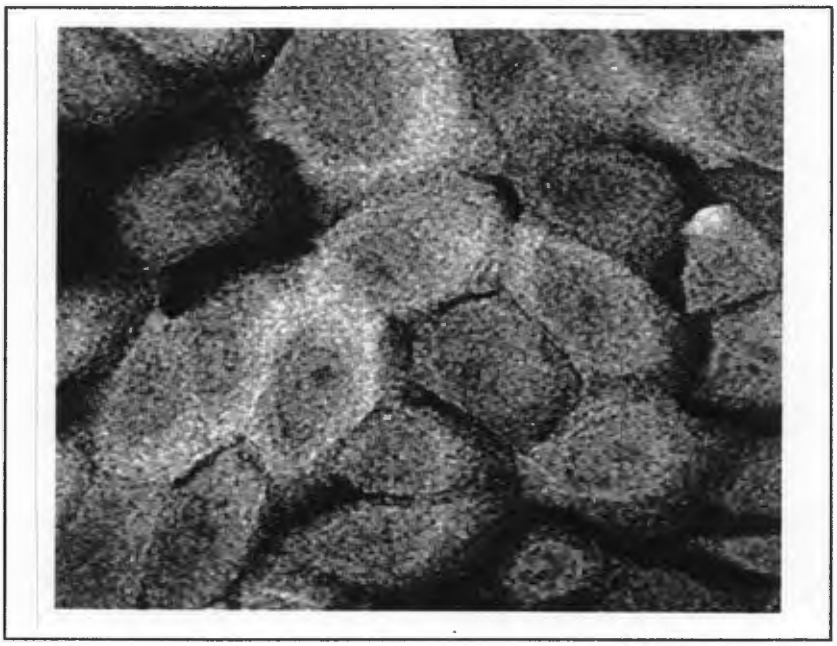
4.3.2 Anti 60 kD Ro autoantibodies

Both the human anti-60-2 and 60-4 autoantibodies strongly stained the nucleus in a coarse granular pattern, with no staining of nucleoli. In addition there was some fine cytoplasmic staining, **see figure 23 pg.117, figure 24 pg. 117, figure 25 pg. 118, figure 26 pg. 118.**

Differences in 52 and 60 kD Ro localisation were confirmed by double immunofluorescence with both human and rabbit purified anti-52 and anti-60 kD Ro antibodies, see **figure 27 pg. 119, figure 28 pg. 120.**

figure 21: The 52 kD Ro antigens are immunolocalised to the cytoplasm in normal cultured keratinocytes. Normal neonatal cultured keratinocytes were fixed and stained as described. Purified human antibodies to the recombinant 52-2 kD fragment (a) and the 52-1-2 kD fragment (b), both stained the cytoplasm, as viewed by confocal microscopy.

52-2 kD fragment (a)



52-1-2 kD fragment (b)

figure 22: Confocal laser microphotographs reveal both nuclear and cytoplasmic localisation of 52 kD Ro, depending on cell confluence. The anti-52 kD antibodies, showed more cytoplasmic staining (fine granular) than the 60 kD antibodies. Occasionally, nuclear staining, although much weaker than that obtained with the 60 kD antibody, was present. This was most apparent in cells that had not yet reached confluence. Cells that had reached confluence often demonstrated minimal nuclear staining. Immunofluorescence of cultured human keratinocytes with purified human anti-52-1b antibodies (two panels on the left) and anti-52-2 antibodies (two panels on the right). The two uppermost panels depict nonconfluent cells and the two lowermost panels show cells that have reached confluence. Scale bar, 25 μm.

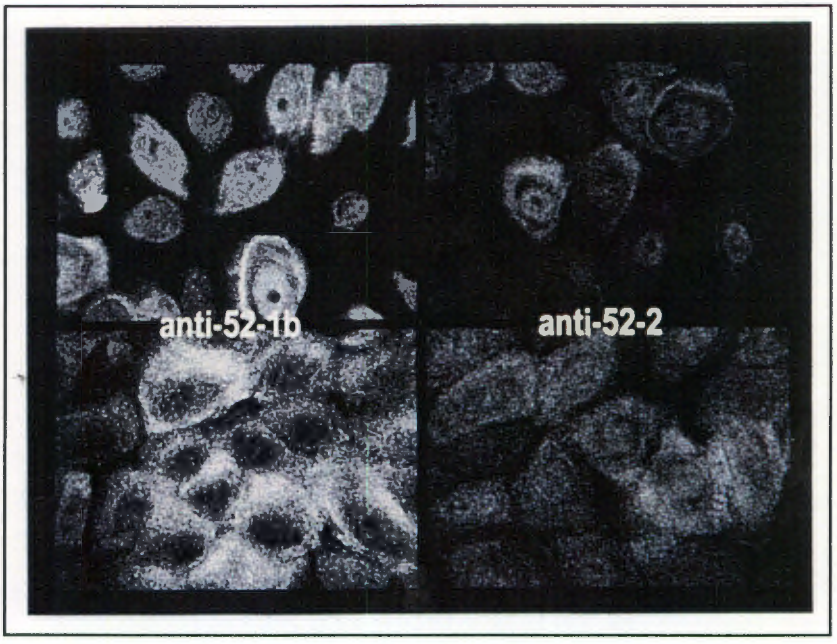


figure 23: The 60 kD Ro antigens are immunolocalised to the nucleus in normal cultured keratinocytes. Purified human antibodies to the recombinant 60-2 kD fragment stained the nucleus brightly, as viewed by confocal microscopy. *Bar,* 25 μm.

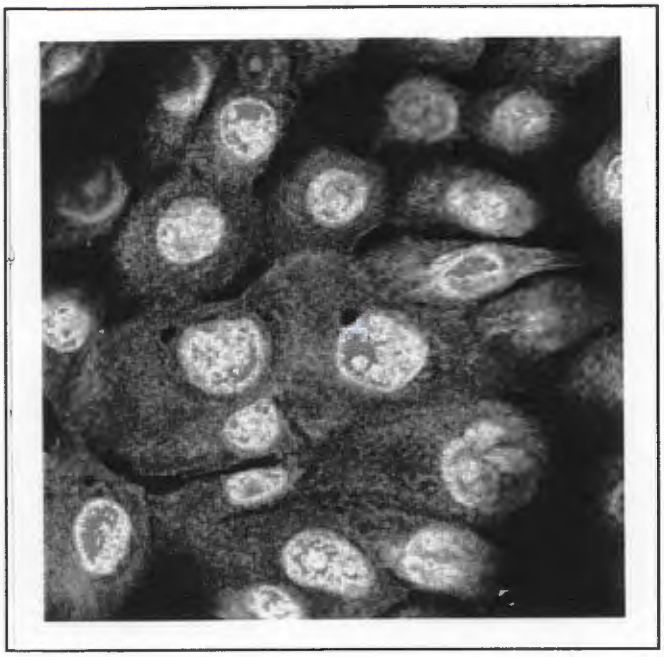


figure 24: The 60 kD Ro antigens are immunolocalised to the nucleus in normal cultured keratinocytes. Purified human antibodies to the recombinant 60-4 kD fragment stained the nucleus brightly, as viewed by confocal microscopy. *Bar,* 25 μm.

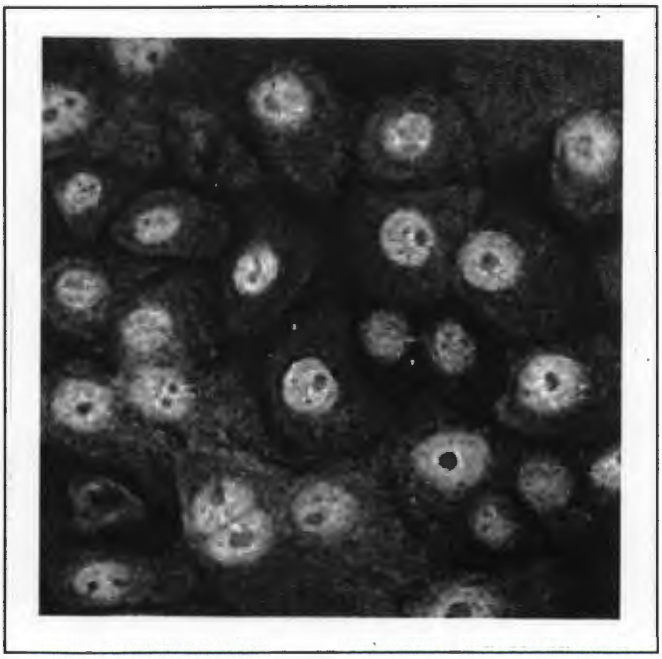


figure 25: The 60 kD Ro antigens are immunolocalised to the nucleus in normal cultured keratinocytes. Purified human antibodies to the native 60 (c), stained the nucleus brightly, as viewed by confocal microscopy. *Bar,* 25 μm.

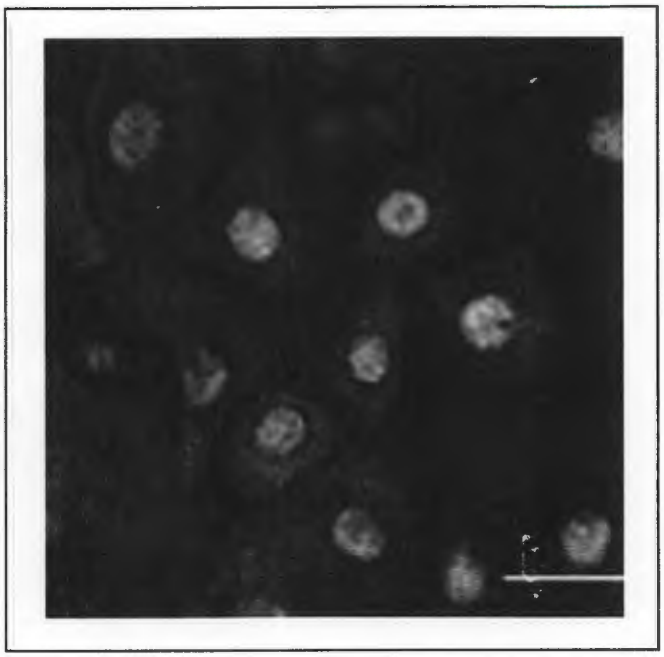


figure 26: Confocal laser microphotographs reveal predominantly nuclear localisation of 60 kD Ro. Immunofluorescence of cultured human keratinocytes with purified human anti-60-2, anti-60-4, anti-native bovine 60 kD Ro and a 1:100 dilution of normal serum, as depicted. *Bar,* 25 μm.

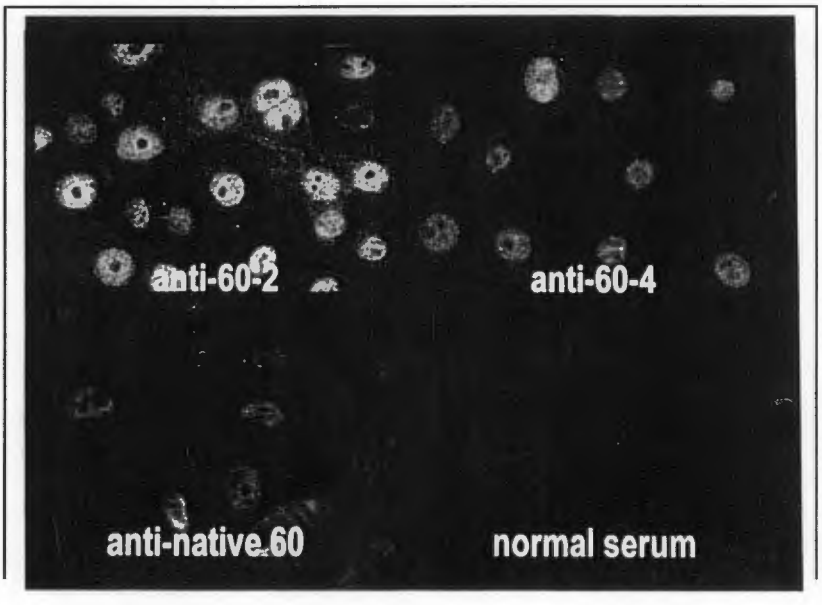


figure 27: Confocal laser microphotographs of the double-immunofluorescent localisations of the 52 and 60 kD Ro confirm disparate locations of the 52 and 60 kD Ro proteins in confluent keratinocytes. Double immunofluorescence of cultured human keratinocytes with human antibodies to the recombinant 52-2 kD fragment, (labeled with Texas Red-conjugated goat anti-human antibodies) seen as *orange* fluorescence and purified rabbit anti-60 kD (labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies) seen as *green* fluorescence. *Bar,* 25 μm.

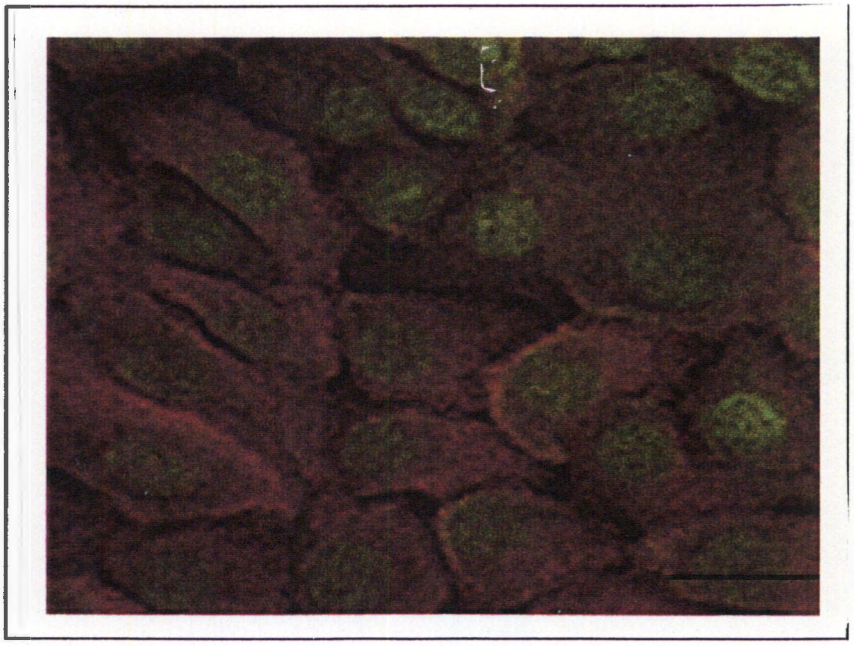
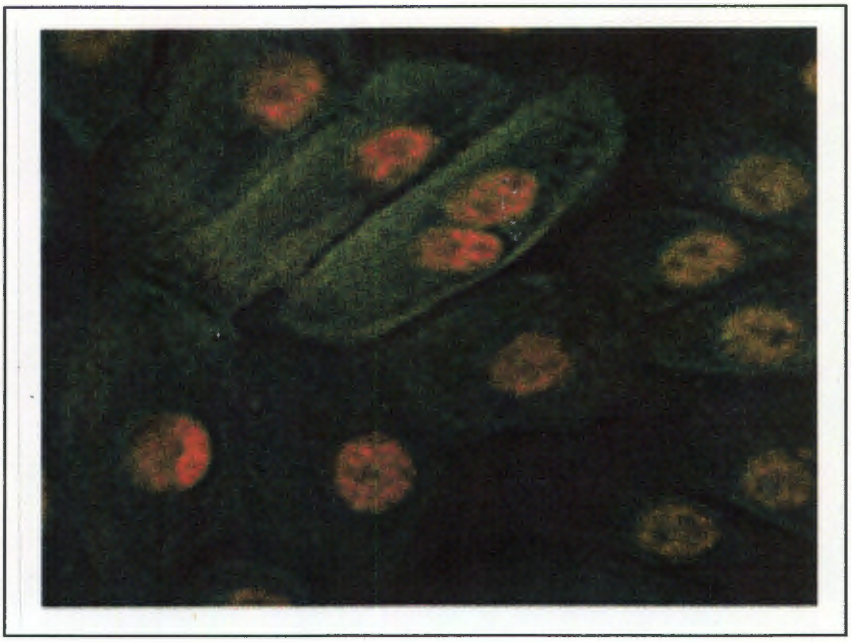


figure 28: Confocal laser microphotographs of the double-immunofluorescent localisations of the 52 and 60 kD Ro confirm disparate locations of the 52 and 60 kD Ro proteins in confluent keratinocytes. Double immunofluorescence of cultured human keratinocytes with human anti-60-4 antibodies (labeled with Texas Red-conjugated goat anti-human antibodies) seen as *orange* fluorescence and purified rabbit anti-52 kD Ro antibodies (labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies) seen as *green* fluorescence. *Bar,* 25 μm.



4.4 DISCUSSION

The position of Ro in cells has been variable in numerous different experiments over many years. Recent localisation studies have found predominantly nuclear and minor cytoplasmic localisation of both the 52- and 60-kD Ro antigens in HEp-2 and HeLa cells (Peek *et al* 1994; Ben-Chetrit *et al* 1988; Slobbe *et al* 1991; Casciola-Rosen *et al* 1994). Another study, however, found predominantly cytoplasmic localisation of 60-kD Ro and mostly nuclear localisation of 52-kD Ro in HeLa and HEp-2 cells (Kelekar *et al* 1994). Unfortunately, most of these studies relied on a single monospecific autoimmune sera and / or antisera with anti-52 or anti-60-kD Ro activity. The specificity of these reagents was often not well defined. Monospecific anti-52-kD Ro sera were defined by immunoblot analysis and may have contained anti-60kD Ro antibodies as this method does not detect all anti-60-kD Ro antibodies (Boire *et al* 1991). My immunofluorescence data are more convincing than any yet published because I used a panel of non-cross-reactive anti-52 and anti-60 kD Ro antibodies, as shown in chapter 3, all of which produced consistent yet disparate locations for the 52 and 60 kD Ro antigens.

4.4.1 Anti 60 kD Ro autoantibodies

My predominantly nuclear localisation of the 60 kD Ro antigen is consistent with most of the earlier studies and a recent study that used monoclonal anti-60 kD Ro antibodies (Veldhoven *et al* 1995). A German group had earlier developed a monoclonal 60 kD Ro antibody. This and a 60kD Ro purified from a particular SCLE patient serum, showed (in contrast to all other Ro antisera) nuclear speckles, diffuse cytoplasmic staining and, in addition, staining of the intermediate filament network (Mayet *et al* 1988). In addition, in contrast to their other Ro sera which they could not identify in whole epidermis, these antibodies, stained frozen sections of epidermis. Only a pale fluorescence was seen on non-sun exposed skin. Sun-exposed skin gave a bright staining of basal epidermis. The highest concentration of antigen was detected in the basal keratinocytes of a cutaneous lesion from the patient from whom this anti-

60kD antibody was purified. They assumed that this specific binding observed only with their monoclonal and the antibody purified from one particular patient's serum, cross-reacted with a cytoskeletal protein or was directly interacting with an antigen directly related to the intermediate filament. This study had no positive control.

In the previous chapter, I showed by immunoblot analysis that normal human epidermis expresses both the 52- and 60-kD Ro antigens. I have been unsuccessful, however, in localising the 52 and 60-kD Ro antigens in human skin biopsies by indirect IF, despite using different techniques on sections obtained from different donors (data not shown). I speculate that my inability to localise antigens may be related to technical matters but it is possible that my skin biopsies came from individuals that produce low amounts of Ro antigen. An up to 2 000-fold difference in 60-kD Ro skin expression has been reported among individuals, recently (Niimi *et al* 1995). I have not noticed such a difference in the level of 52- and 60-kD Ro antigen expression in cultured keratinocytes harvested from several donors.

Overexpression of the 60 kD Ro-encoding gene in HEp-2 and LTA-5 cells showed bright finely speckled nuclear staining. Those cells with the highest intensity staining also showed weak cytoplasmic staining. No surface expression was detected (Keech *et al* 1994). A further study has used a transfected and overexpressed human 60 kD Ro autoantigen in HEp-2 cells for IF. Prototype human and rabbit sera directed against the 60 kD Ro antigen produced intense speckled nuclear and nucleolar staining of transfected cells (Fritzler & Miller, 1995).

4.4.2 Anti 52 kD Ro autoantibodies

I have found greater cytoplasmic localisation of the 52 kD Ro than previous work. Most of the recent localisation studies used "monospecific" 52 kD Ro autoimmune sera (Ben-Chetrit *et al* 1988; Slobbe *et al* 1991; Casciola-Rosen *et al* 1994), or partially purified rabbit antisera (Kelekar *et al* 1994) that may have contained anti-native 60 kD Ro or other autoantibodies that could produce nuclear fluorescence that

overshadowed the cytoplasmic fluorescence generated by the anti-52 kD Ro antibodies. The Casciola-Rosen study that found no difference in the location of Ro52 and Ro60 in unprovoked cultured keratinocytes, used an anti-52 kD Ro monospecific serum defined by immunoblot results, which may have contained anti-60 kD Ro antibodies, as immunoblot analysis does not detect all anti-60 kD Ro antibodies (Boire *et al* 1991).

Isolation of antibodies specific for each Ro antigenic polypeptide has allowed more precise cellular localisation of the Ro polypeptides. These results have helped explain the discrepancies encountered previously with immunofluorescence staining used whole Ro patient sera. Part of this difficulty has been due to the difficulty in obtaining reactive monoclonal Ro52. Anti-nuclear autoantibodies found in human autoimmune diseases frequently cross-react with homologous autoantigens in distant species, supporting the notion that autoantibodies target conserved functional domains. However, the 52-kD Ro protein is an exception, in that human autoantibodies are not known to recognize any equivalent antigen in the cells of rodents and other non-primate species. To understand this lack of cross-reactivity, one group isolated cDNAs encoding the mouse 52-kD Ro molecule. The cDNA encoding mouse 52-kD Ro revealed an open reading frame of 470 amino acids, with 70% sequence identity to the human 52-kD Ro antigen. The putative leucine-zipper and zinc-finger motifs present in human Ro52 were conserved in the mouse protein. Recombinant mouse 52-kD Ro protein reacted with human autoantibodies by ELISA and immunoblot, but with approximately 10-fold lower reactivity than recombinant human 52-kD Ro protein under the same conditions. Detection of both human and mouse 52-kD Ro by immunoblot was dependent on antigen concentration which was limiting in the cell equivalents generally used in immunoblot assays. Differential chaotropic disruption of antibody binding suggested a lower avidity of human autoantibody binding to the mouse 52-kD Ro protein compared with the human antigen. Structural differences between the human and mouse 52-kD Ro autoantigens are associated with poorly conserved autoantibody activity across species (Keech *et al* 1996a). Our group (work not shown here) had similar difficulties.

My results are supported by the work of other laboratories. In a paper focusing on methodology for detecting Ro and La antibodies in Japanese patients with autoimmune disease, Ro antigens were prepared from cultured human cell lines (KB and KBE). They isolated a 58kD (probably equivalent to the now well recognised 52kD protein), in high concentration from the cytoplasmic fraction of KB cells (Inagaki *et al* 1989).

Overexpression of the 52 kD Ro-encoding gene in HEp-2 and LTA-5 cells has demonstrated a predominantly cytoplasmic localization of the 52kD protein (Keech *et al* 1995).

My immunofluorescence findings are unique in that I have shown the location of the 52 and 60-Ro in normal human cultured keratinocytes. Human antibodies directed against two different recombinant 52 protein fragments showed similar immunofluorescence. This was true too, of the two different anti-recombinant 60 antibodies, as well as the antibody directed against the native 60 epitope(s). The differences between the anti-52 kD and anti-60 kD antibodies were further confirmed by double labeling with human and rabbit antibodies. I would argue that the 60 kD Ro protein resides largely in the nucleus and the 52 kD Ro is located mainly in the cytoplasm.

5. DO THE Ro ANTIBODIES CAUSE CUTANEOUS DISEASE?

5.1 CAN Ro ANTIBODIES PENETRATE LIVING CELLS?

5.1.1 Introduction

The view has long been held that antinuclear antibodies cannot penetrate living cells. However there is evidence to suggest that this may be possible. Patients have been described in whom epidermal nuclear staining is present, without circulating antinuclear antibodies (Burrows *et al* 1993, Velthuis *et al* 1990; Parodi *et al* 1985; Herrera-Esparza & Avalos-Diaz, 1982).

One group has made the interesting observation that deposits of Ig (as shown by DIF) in the cytoplasm of human skin keratinocytes, are associated with high levels of serum antibodies to keratinocytes in the superficial epidermal cell layers (upper cytoplasmic antigens) and with LE (found in 22/98, 22.5%) (Ioannides & Bystryn, 1993). They speculate that that this may be due to antibody penetration of cells in vivo.

Furukawa *et al* during their work on oestradiol's influence on keratinocyte surface binding of sera with Ro and La reactivity, made the observation that in some cells, incubated with antibody probes before fixation, antibody internalization and nuclear staining could be seen. They suggested that this antibody internalization could be minimized by shortening incubation times and performing incubations in the cold (Furukawa *et al* 1988).

Another group have identified IgG antibodies capable of penetrating an epithelial cell line, COLO 16 in 5/36 (14%) ANA+ SLE patient sera (Golan *et al* 1993). Thirty

minutes following incubation of cells with dilutions of either whole sera, globulin fractions, or $F(ab')_2$ fragments of IgG, ~80-90% of cells showed intranuclear IgG by indirect IF. The percentage of DIF-positive staining cells was directly related to the length of time that the sera were incubated with the cells. The binding process was rapid (30% of nuclei were positive after a 10-min. incubation with serum) and 95% of cells were positive after a 30-min. incubation. In addition, the process was temperature dependent. 50% of cells showed faint nuclear fluorescence after incubation with serum for 60 min. at 2°C, whereas 100% of cells showed intense nuclear staining when incubated at 37°C for the same length of time. Viability of the cells prior to assay was >98% as determined by Trypan blue staining. Penetration of IgG into the nuclei did not affect viability or DNA synthesis of cells in short-term culture.

Other antibodies have been shown to penetrate cells, including antibodies to proteins 3, ribosomal protein P, lymphocytes, synaptosomes and neurons (Zack *et al* 1996).

Assuming that the observations of antibody penetration of cells is a true *in vivo* phenomenon, it is possible that a number of mechanisms may be involved in allowing immunoglobulin molecules to penetrate the cytoplasm or nucleus of living cells. The expression of keratin antigens on the surface of viable keratinocytes might account for the internalisation of such antibodies into cells (Sontheimer, 1993).

For anti-DNA antibodies, cellular penetration and translocation to the cell nucleus were thought to require the presence of DNA (Zack *et al* 1996). Since some antibodies have been shown to bind membrane proteins cross-reactive with DNA, these proteins may be instrumental in cellular penetration. In other cases, DNA binding proteins usually thought of as intracellular, have been described in association with the membrane of some cells (Zack *et al* 1996).

5.1.2 Methods

5.1.2.1 Cell Culture

See Protocol 9: Definitive keratinocyte culture pg. 107.

Neonatal foreskins were washed and trypsinised overnight at 4°C. The basal keratinocytes from the epidermis were resuspended in Medium 154 (Cascade Biologics Inc., Portland, OR) supplemented with human keratinocyte growth supplement. These keratinocytes were seeded onto 8-chamber Lab Tek slides (Nunc Inc., Naperville, IL). They were grown to 50% confluence.

5.1.2.2 Determination Of Cell Viability

Protocol 17: Assessment of viability

This was assessed by the propidium iodide (Yeh *et al* 1981) or trypan blue (Golan *et al* 1993) methods.

Propidium iodide (Molecular probes, Eugene, OR) stock solution was made by dissolving 5 mg of propidium iodide in 100 ml of 1% Trisodium citrate and stored in a brown bottle at 4°C. To make a working solution, this was diluted 1:3 with PBS. Two methods were then employed: 20 µl PI was added to cells following 3rd wash after addition of conjugate, mixed thoroughly, mounted and read immediately, or 100 µl PI was added to cells following the first wash after addition of conjugate, mixed, incubated at 4°C for 5 min., washed twice, mounted in glycerol and examined.

The trypan blue dye exclusion test was performed by adding trypan blue 0.1.% in PBS at 4°C to the keratinocytes after the primary antibody incubation for 45 sec at room temperature, before being fixed in 3% paraformaldehyde. The mean percentage of viability was determined from a total count of 500 cells in three different chambers.

5.1.2.3 Viable cells

Protocol 18: Immunofluorescence on viable keratinocytes

Old media was removed. Primary antibody diluted to 75 µl in Media 154 was added and incubated for 30 min, or for varying durations as described below. Incubation took place at 4°C or 37°C, in separate experiments. The keratinocytes were then washed with PBS for 5 min, followed by fixation in paraformaldehyde (3% in PBS) for 15 min. at room temperature. Following two washes with cold dulbecco PBS (x5 min. max.). The cells were immersed in Triton X-100 (0.2% in PBS) for 5 min. at room temperature. Next followed two washes with cold dulbecco PBS, followed by incubation with 100 µl of secondary antibody at room temperature for one hour. The plastic chamber was removed, the slides were dipped for 10 sec in propidium iodide (100µl in 10 ml PBS) to counterstain the nucleus and mounted in PVA. The primary antibody was incubated on viable cells for varying time periods: 5 min., 15 min., 1 hr, 3 hrs, 7 hrs, 24 hrs and 48 hrs. The keratinocytes were irradiated with UVB, 1.5 and 4.5 mJ/cm^2 at 5 mins, 30 mins and one hour post irradiation. The positive control was the same antibody or whole serum 2429, without UVB. The negative control was secondary antibody alone. The following antibodies were used: 60-2 (1:100), 60-4 (1:50), 52-1b (1:25), 52-2 (1:50). These were the dilutions previously found to yield the best results on immunofluorescence of fixed normal keratinocytes.

In the next stage of the experiments, all the above variables remained constant, with the addition of a supplement to the medium. Hydrochlorothiazide was supplemented at 100 ng/ml, 200 ng/ml and 400 ng/ml for 24 - 48 hours. Media in 2 chambers of each slide were supplemented with Oestradiol at 10^{-7} M for 24 - 48 hours. Using colonies of 10 - 50 cells in Lab Tek chambers, Vit C was added to give a concentration of 27 µg /10^8 cells, Vit E was added to give a concentration of 2.7 µg /10^8 cells and HCZ 100 ng/ml plus oestradiol were used together.

The above experiments were similarly performed on keratinocytes seeded into wells on an ELISA grid, as annotated later in **Protocol 19: ELISA to assess surface antigen expression on unfixed keratinocytes.**

Protocol 19: ELISA to assess surface antigen expression on unfixed keratinocytes

2 X 10^4 third passage keratinocytes were seeded onto each well of a 96-well microtiter, strip culture plate (Costar). They were seeded at a density of 2 X 10^4 cells in 200 µl complete medium. 3 separate cell lines were used in triplicate, on at least 3 separate occasions. They were incubated in medium 154 (Cascade Biologics Inc., Portland, OR) at 37°C, supplemented with human keratinocyte growth supplement, until the keratinocytes in all wells were confluent. For the last 3 days of growth, any experimental supplements were added, in the same concentration as used for immunofluorescence. Viability of two columns of wells were assessed using trypan blue. The wells were then washed twice with 1 X PBS. The final wash was gently aspirated carefully and completely, using a Pasteur pipette whose tip had been pulled in a flame, to a fine tip. 200 µl primary antibody was incubated in each well overnight at 4°C. The wells were then washed X 3 with PBS, incubated with goat anti-human IgG for 2 - 4 hours and developed at 37°C for 2 - 3 hours.

Protocol 20: ELISA to assess surface antigen expression on fixed keratinocytes

2 X 10^4 third passage keratinocytes were seeded onto each well of a 96-well strip, microtiter culture plate (Costar) and treated exactly as described in **Protocol 19: ELISA to assess surface antigen expression on unfixed keratinocytes**. Once the final wash had been aspirated, the keratinocytes were fixed in 200µl 0.25% glutaraldehyde, diluted fresh in PBS. They were incubated for 5 minutes at room temperature. The glutaraldehyde was aspirated. The wells were then washed gently twice with 1X PBS. The plate was flicked to remove the PBS. For storage, 200µl/well PBS and 0.02% sodium azide were added. The plate was then stored in

the dark in a tight-sealed container. 200 µl primary antibody was incubated in each well overnight at 4°C. The wells were then washed three times with PBS, incubated with goat anti-human IgG for 2 - 4 hours and developed at 37°C for 2 - 3 hours.

Protocol 21: Surface ELISA Permutations

The permutations performed on keratinocytes for immunofluorescence, were mirrored in the keratinocytes assessed for ELISA, with the addition of irradiation with 160 mJ UVB/cm^2. All keratinocytes were used one hour post irradiation. The wells containing the 52-1b were incubated overnight with 10 mM DTT. Fixed versus un-fixed cells were compared.

5.1.3 Microscopy

See 4.2.2.1 Microscopy pg: 112, 4.2.2.1.1 Epifluorescence microscopy pg: 112, 4.2.2.1.2 Confocal Microscopy pg: 112

5.1.4 RESULTS

All studies were performed in triplicate, each experiment with a different cell line, for each of the variables studied.

5.1.4.1 Immunofluorescence

For every permutation on viable cells, no purified human antibodies displayed any intracellular fluorescence. All the anti-52 kD Ro (see **figure 29, pg. 131**), the anti-60 kD Ro (see **figure 30, pg. 132**) autoantibodies (labeled with Texas Red-conjugated goat anti-human antibodies and/or fluorescein isothiocyanate-conjugated goat anti-human antibodies) did not fluoresce. Normal serum, used as a negative control, likewise showed no cellular penetration (see **figure 31, pg. 133**). However, a single whole autoimmune serum, 2429, showed fluorescence under all conditions but it was of great interest to observe, that this occurred in only those cells staining positive for

trypan blue (see **figure 32, pg. 134**). Serum 2429 was obtained from a patient with Sjögren's syndrome. This serum was immunodiffusion positive for Ro, ELISA positive for native 60kD Ro, recombinant 52kD Ro, recombinant 60kD Ro and La. The positive control was whole autoimmune serum, 2429 in fixed, rather than viable keratinocytes (see **figure 33, pg. 135**). To clarify these negative results, the experiments, with the addition of UVB or UVA were repeated on at least two separate occasions some months apart. The negative results were reproducible.

figure 29: No intracellular fluorescence was obtained for or any anti-52kD antibody. These results were negative for all perturbations.

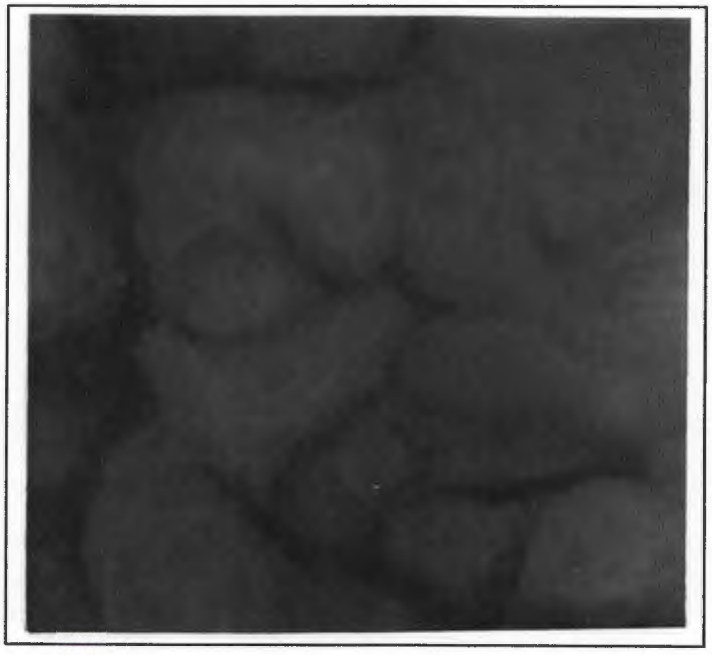


figure 30: No intracellular fluorescence was obtained for any anti-60kD antibody . These results were negative for all perturbations.

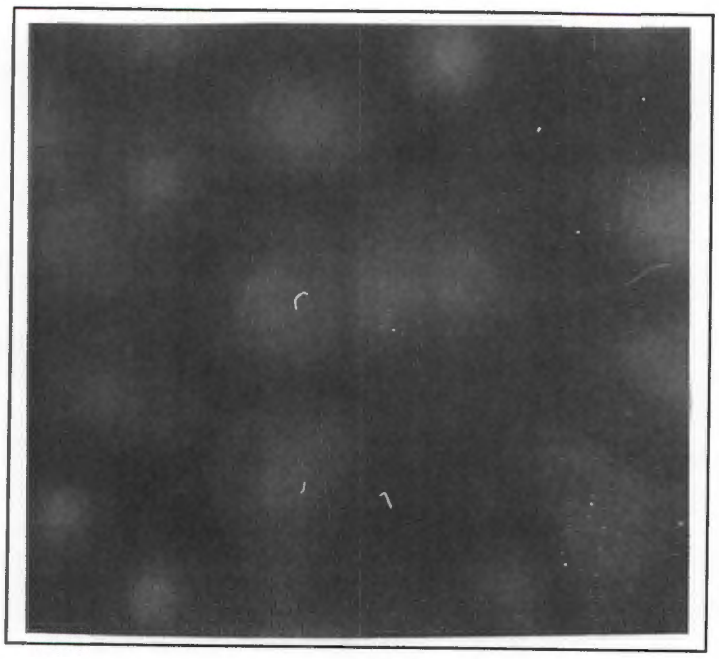


figure 31: No intracellular fluorescence was obtained for normal serum, used as a negative control. These results were negative for all antibodies, normal sera and for all perturbations

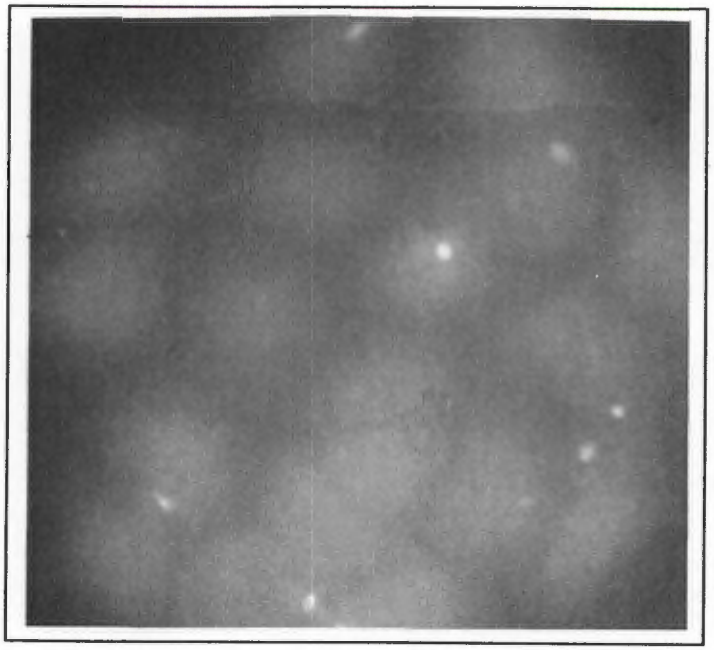


figure 32: Serum 2429 showed fluorescence when incubated with viable normal keratinocytes for 5 min. Positive control was serum 2429 on fixed keratinocytes (see figure 33, pg. 135). This occurred in both perturbed and un-perturbed cells

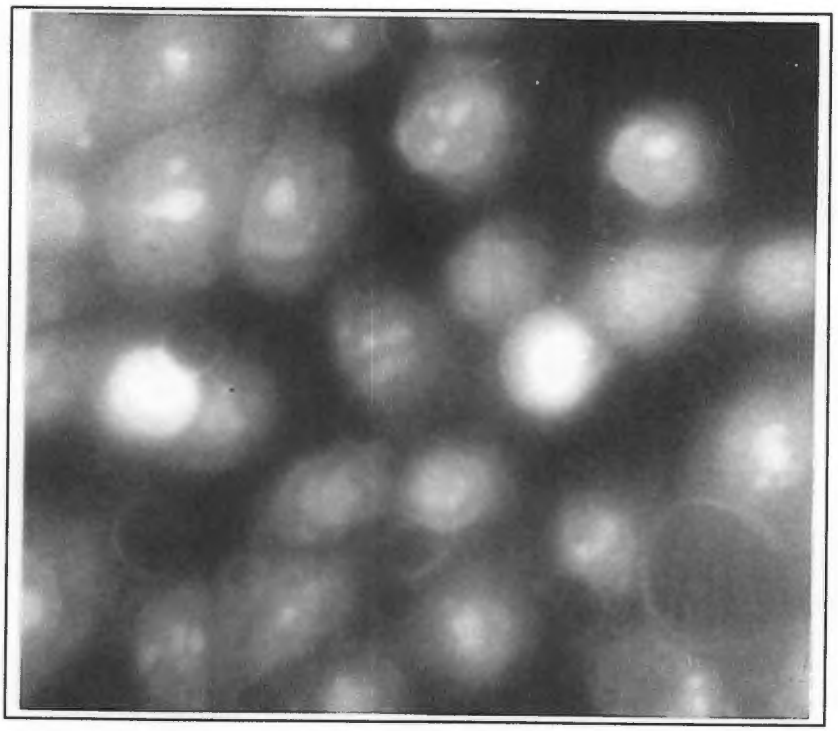
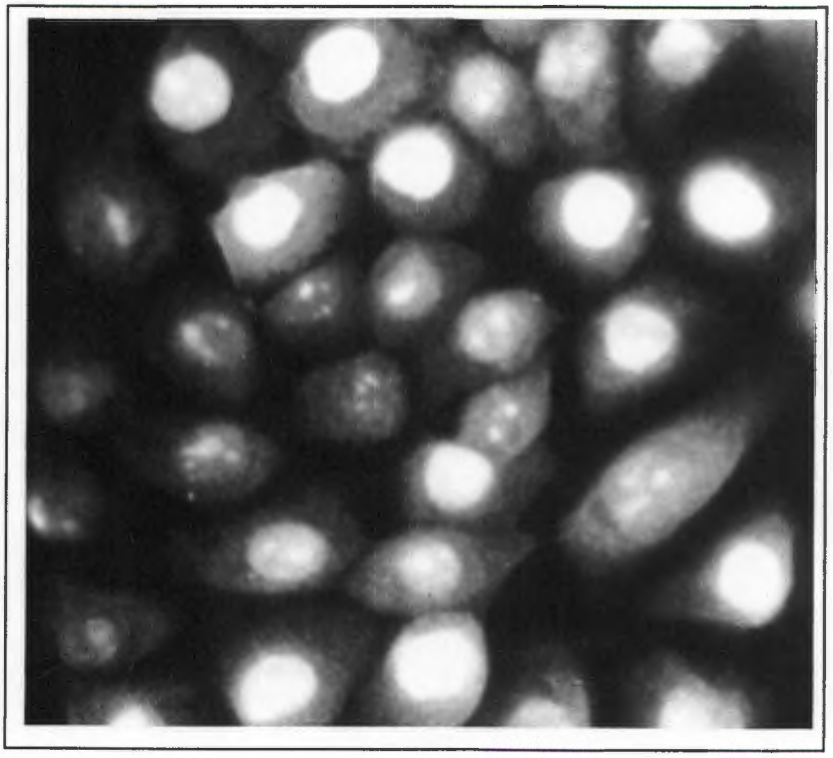


figure 33: Serum 2429 showed fluorescence incubated with viable normal keratinocytes for 5 min. Positive control was serum 2429 on fixed keratinocytes, shown here. This occurred in both perturbed and un-perturbed cells



5.1.5 DISCUSSION

Patients have been described in whom epidermal nuclear staining is present, without circulating antinuclear antibodies. In a study of 22 patients exhibiting epidermal nuclear staining, 7 did not have a positive serum ANA at the time of biopsy for DIF. In addition, in 11 of 16 cases tested, antibodies to RNP were absent (Burrows *et al* 1993). Other groups have reported cases of epidermal nuclear staining occurring in the absence of serum ANA (Velthuis *et al* 1990; Parodi *et al* 1985; Herrera-Esparza & Avalos-Diaz, 1982).

I have no evidence that purified monospecific anti-52 and anti-60 Ro antibodies are capable of penetrating normal human neonatal keratinocytes. These studies were performed under a multitude of experimental stresses, including all those factors known to precipitate or aggravate cutaneous disease. It is possible that the previously reported epidermal nuclear staining occurred as a result of contamination by diffusable ANA in the dermis gaining access to the epidermal nuclei during tissue processing (Gilliam, 1975).

Other workers agree, showing that it is associated with high titers of anti-RNP antibodies. Other experiments that would address this issue further, would include disaggregation of the blister tops into a single cell suspension, followed by extensive washing, cytospin fixation and staining for intracellular immunoglobulin. Another approach would be to formalin fix suction blister tops or intact biopsy specimens prior to sectioning, followed by the application of a staining protocol capable of detecting human immunoglobulin in formalin-fixed tissue.

Ioannides & Bystryn in their work showing that deposits of Ig in the cytoplasm of human skin keratinocytes, are associated with high levels of serum antibodies to keratinocytes in the superficial epidermal cell and LE, speculate that that this may be due to antibody penetration of cells in vivo. This may possibly due to antibody binding to an antigen like structure on the cell surface, or to potential functional or cell-cycle changes in the epidermal cell membrane in SLE (Ioannides & Bystryn,

1993). These deposits were very uncommon in skin specimens from patients with bullous dermatoses, or non lupus skin disease (Ioannides & Bystryn, 1993). More details of the actual experiments performed including the conditions and durations of washing the epidermal suction blister tops prior to freezing and sectioning, would have allowed for critical analysis as to whether their findings may have occurred because of contamination by small amounts of extracellular immunoglobulin.

Ioannides and Bystryn showed that the upper epidermal cytoplasmic staining pattern is sometimes extremely focal and spotty (Ioannides & Bystryn, 1993). If free keratin reactive autoantibodies from the dermis were washing onto intracellular keratinocyte antigens exposed by processing, one may expect this staining to be even and diffuse, rather than either in localised keratinocyte clumps or in scattered single keratinocytes. It is unlikely that specific keratin antigens are differently expressed in these targeted areas.

Assuming that previous observations of antibody / cell penetration are true (Ioannides & Bystryn, 1993), it is possible that some keratinocytes in LE patients suffer subtle injury, not morphologically apparent. Such stressed keratinocytes may be unable to prevent circulating antibodies from leaking in. There is some precedent for anti-keratin antibodies gaining access to stressed mammary epithelial cells in the absence of morphologic evidence of cell injury (Dairkee *et al,* 1991).

However, (Ioannides & Bystryn, 1993) did not observe epidermal cytoplasmic staining in basal keratinocytes, the focus of injury in SLE. In addition, it is likely that penetration of keratinocytes by antibody would lead to some type of functional consequence but epidermal cytoplasmic staining was frequently observed in normal-appearing skin.

In the Denver experiments on 'translocation', they found that IgG from Ro and La positive serum, bound to UV irradiated keratinocytes in culture. They produced the same pattern of binding in intact human skin harvested by suction blister 24 hours after irradiation with UVB and then incubated as intact epidermis with serum In these

experiments they found that antibody bound to cell surfaces, was internalised with time and rapidly appeared in the nucleus if these cells were observed early enough (Furukawa *et al* 1990). I observed my cells within minutes of addition of the anti-Ro antibodies, without detecting intracellular penetration. It is interesting that I showed a very low penetration rate in those cells incubated with whole serum (in this respect, duplicating the Denver work, as they failed to use purified antibodies). One must therefore presume that either a completely different antibody present in the "Denver" sera penetrated cells, or more likely, as shown in the work of Casciola-Rosen *et al*, their findings may be changes associated with apoptosis, rather than penetration of normal cells.

As discussed in the introduction to this chapter, the group that identified IgG antibodies capable of rapidly penetrating an epithelial cell line, COLO 16 in 5/36 (14%) ANA+ SLE patient sera (Golan *et al* 1993), showed that the percentage of DIF-positive staining cells was dependent on the time of sera incubation and temperature. Interestingly, intracellular IgG could not be detected following exposure of the cells to high titer reference autoantibodies of known specificities, against Ro, La, Sm, RNP, ribosomes or DNA. This would support my work showing no cell penetration with a whole range of anti-Ro antibodies, directed at the major Ro epitopes.

Antibody uptake was relatively selective for transformed epithelial cell lines, as intranuclear IgG was not detected in cell lines of normal human keratinocytes, or those of lymphoid origin, exposed to the sera. Penetration may be limited to either the state of differentiation or activation of the cell. These findings suggest that a subset of SLE patients have IgG capable of penetrating a transformed cell line of epithelial origin. It supports my work that anti-Ro antibodies are incapable of penetrating normal human keratinocytes.

For molecules to penetrate into the nucleus, they would need to do so through a very selective gatekeeper sorting mechanism, recognising specific amino acid sequences, not possessed by immunoglobulins. For these to penetrate into the nucleus, they would need to do so via a rather unconventional mechanism. One group has

suggested that they may be imported into the nucleus in association with a carrier protein such as a cell surface receptor. They, in addition suggest that, the association of the carrier protein with the immunoglobulin, may prevent the antigen-binding site of the antibody from reacting with its antigen in the nucleus accounting for the absence of visible cellular pathology (Golan *et al* 1993). The requirement for free DNA and the role of Fc binding for cellular penetration appears to be different in the various antibodies. The antibodies studied have distinct specificities for binding antigen and they target different cell types. Therefore, multiple mechanisms may be operative in cellular penetration and nuclear localisation.

Some have argued that Fc receptor-mediated endocytosis is the mechanism by which U1RNP antibodies penetrate human lymphoid cells (Alarcon-Segovia *et al* 1978). Keratinocytes have been shown to express Fc and C3 receptors as a result of herpes virus infection (Kubota *et al* 1987). However, normal epidermal keratinocytes have not been shown to express such receptors (Galoppin & Saurat 1981, Stingl *et al* 1977).

There are a number of functional implications of immunoglobulins directed against specific vital intracellular components, gaining access to cells. Overall, there is little precedent within the conventional view of the immune system for immunoglobulins finding their way into healthy living cells. In favour of this is the fact that infectious organisms that hide in cells are dealt with by cellular rather than humoral responses.

5.2 DO THE Ro ANTIGENS TRANSLOCATE TO THE CELL SURFACE?

5.2.1 Introduction

5.2.1.1 Ultraviolet Radiation

In 1984, the first report appeared of ultraviolet-irradiated, cultured keratinocytes which bound IgG antibodies from the sera of LE patients with either monospecific anti-SSA/Ro, anti-RNP, or anti-Sm activity (LeFeber *et al* 1984). A study of the viability and permeability of irradiated keratinocytes showed that this apparent cell membrane expression of extractable nuclear antigens was seen on injured keratinocytes whose cell membranes were intact but not on dead cells. Neonatal human keratinocyte monolayers of 10-50 cells were irradiated with 0, 0.2, or 2.0 mJ/cm^2 UVR (predominantly UVB). They chose these doses, suggesting that since 10% of UVB light incident on Caucasian skin penetrates to the basal layer, an in vitro dose of 2 mJ/cm^2 would be equivalent to 20 mJ/cm^2 in vivo in human skin. One MED in Caucasians, ranges from 15 to 75 mJ/cm^2 for UVB. At 8 and 24 hours after irradiation the cultures were examined for viability or antibody binding. Human IgG binding to cell surfaces was assessed by first blocking irradiated cultures with normal rabbit serum and then incubating with a 1:10 dilution of monospecific antiserum followed by acetone fixation, second antibody incubation and sequential propidium iodide dip. Slides were mounted in a paraphenylenediamine-containing mounting medium.

The degree of positive staining was determined qualitatively and quantitatively by visual counting of at least 200 cells in each specimen using a Zeiss epi-illuminated microscope. Cell viability in irradiated cultures was measured by ethidium bromide with acridine orange, propidium iodide exclusion, or fluorescein diacetate uptake. They found strong granular staining of unfixed irradiated keratinocytes with all six monospecific sera used. The percentage of positive cells ranged from 10 - 20%.

Irradiated cells which showed granular staining with anti-Ro or anti-RNP, also stained with fluorescein diacetate but less intensely than did cells without granular staining. This suggested that these cells were injured. 45-55% of there cells were dead. They interpreted these findings to suggest that UV irradiation of cultured keratinocytes causes cell death and irreversible membrane damage to many cells, producing a population of injured cells with expression of certain nuclear antigens on the cell membranes (LeFeber *et al* 1984).

Furukawa et al from the same laboratory set out to confirm Le Feber's findings. Using three different protocols, they concluded that Ro autoantibodies (and anti-La and anti-RNP) bound to UVB (not UVA) irradiated keratinocytes (Furukawa *et al* 1990). They used short-term 2% paraformaldehyde fixation of suspensions of cultured human keratinocytes previously incubated with monospecific antiserum probes for Ro. UVB (280-320 nm) induced the binding of monospecific antibody probes for Ro and La on keratinocytes in a dose dependent fashion. Maximal induction was observed at the dose of 200 mJ/cm^2. UVA (320-400 nm) had no effect. Identical results were obtained with standard immunofluorescence techniques, incubating viable cells with antibody at 4°C for one hour before fixation and labeling.

Jones, previously from the same group and continuing his work in the UK, used the same sera that the Denver group had used for all their work (Jones, 1992a). For injurious stimuli he used hydrogen peroxide. Metabolic stimuli were assessed because of their effects on various aspects of cellular metabolism, such as protein synthesis. He used cholera toxin, as it has been shown to increase DNA and subsequently protein synthesis in human melanocytes and keratinocytes. In addition, he used Phorbol-12-myristate-13-acetate, tumour promoters, with a number of effects on cellular metabolism and cell membrane function. A number of immunological stimuli were assessed, including gamma interferon (reportedly stimulates the expression of class II HLA-DR on the surface of cultured human keratinocytes and induces the synthesis of new proteins by cells), interleukin 1 (a cytokine produced by keratinocytes, monocytes and Langerhans cells, which, in the presence of antigen,

activates T-cells to produce IL-2 and IL-2 receptors and enhances the protein synthesis and cell proliferation of keratinocytes) and interleukin 2 (thought to be responsible for the proliferation of T cells). In addition, he used the inflammatory stimuli, leukotriene B4, one of the most active of the inflammatory products of the lipoxygenase and cyclo-oxygenase pathways of arachidonic acid metabolism. The hormonal stimuli used were 17-β oestradiol, progesterone and dihydrotestosterone.

5.2.1.2 Drugs

Several drugs have been shown to induce SCLE lesions, associated with Ro autoantibodies (Furner, 1990; Fine, 1989). The mechanism, here, is unknown. Most cases of drug induced SCLE have been linked with Hydrochlorothiazide. The effects of drugs on Ro translocation have not been studied.

Vitamin E (Vit) has been proposed as a treatment for lupus skin disease (Ayres Jr & Mihan, 1971). α tocopherol (5,7,8 - trimethyltocopherol) comprises about 90% of tocopherols in animal tissue. Discovered in 1922, it quickly became known as the "fertility" vitamin and was christened tocopherol from tocos (childbirth) and phero (to confer), based on the ability of Vit E to restore fertility to experimental animals on vit E-deficient diets. Tocopherols are the body's chief antioxidants, protecting the labile unsaturated fats, e.g.: cell membranes, from lipid peroxidation. They also enhance the intestinal absorption of Vitamin A. Vit E deficient mice have been shown to develop a defect in the formation of inter and intramolecular collagen cross-linkages and premature aging (Ayres Jr & Mihan, 1971). The daily recommended allowance is 30 I.U., approximately 30g (Ayres Jr & Mihan, 1971).

In addition, no work has been done on the effect of Vitamin C (Vit C) on Ro translocation. Ascorbic acid acts as a cofactor by transferring electrons to enzymes that provide reducing equivalents. As such they are important in collagen synthesis, carnitine synthesis, the conversion of folic to folinic acid and dopamine to noradrenaline, microsomal drug metabolism, they increase intestinal iron absorption and play a role in adrenal steroid genesis. Vit C can also increase the effectiveness of

Vit E through antioxidant synergism. As for Vit E, the concentration in tissues is probably more real than plasma concentrations.

5.2.1.3 Oestrogens

The hormonal milieu may also play an important role in Ro associated disease. Approximately 75% of SCLE patients and infants with NLE skin disease are female. Oestrogen treatment of cultured keratinocytes has been reported to increase the expression of Ro antigens on the cell surface (Furukawa *et al* 1988). It is not known which of the Ro antigens are so displaced.

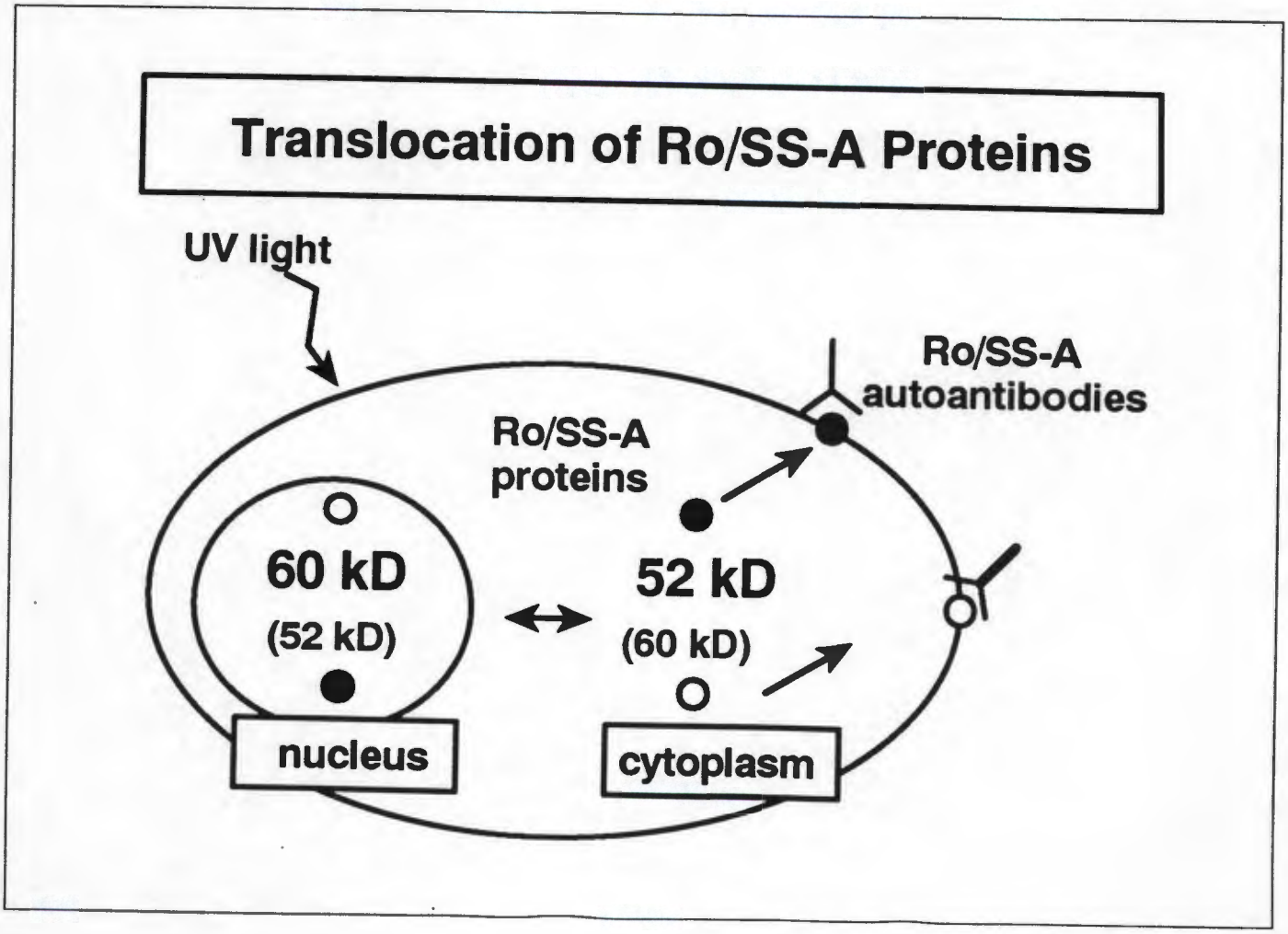
5.2.1.4 Heat

Some SCLE patients have found that heat can exacerbate their disease (McCauliffe & Sontheimer, 1993a). Heat shock-like proteins may be translocated to the cell surface, where they may participate in antigen presentation or be a target for gamma/delta T cell directed cytotoxicity. The induction of heat shock proteins has been reported to be a primary cellular response to tissue injury and their induction may enhance post-traumatic tissue recovery. Interleukin-2-treated T-lymphocytes and cyclopentanone prostaglandin-treated HeLa cells, induce 68kD and 80kD heat-shock proteins. These may function in the regulation of the cell cycle or form part of a defensive reaction to cytokine or mediator synthesis. Immunofluorescence has demonstrated a rapid and reversible accumulation of the 72-kD heat shock protein within the nucleolus of heat-stressed keratinocytes (Edwards *et al* 1991). There has been great interest in the role of hsp as molecular chaperones, binding and escorting molecules to the nucleus, cytoplasmic vacuoles, the ER and the plasma membrane during stress. In addition, hsp themselves may be targets of autoimmune reactivity. In human keratinocytes, hsp can be induced by UVR as well as heat (Edwards *et al* 1991; Maytin *et al* 1993). A number of investigators have shown that hsp induction is greater in peripheral blood mononuclear cells from lupus patients compared to normal subjects or in lymphoid cells from infiltrates in lupus nephritis. Lupus patients also make antibodies to hsp (Norris, 1993).

5.2.1.5 State Of Keratinocyte Differentiation And Proliferation

Human keratinocytes grown in low calcium containing media, have greater amounts of cytoplasmic Ro antigen as detected by IF than those grown in high calcium containing media. At lower calcium concentrations, cultured keratinocytes are in a less differentiated, more rapidly dividing state, more like the basal keratinocytes. This may explain why the more rapidly proliferating basal keratinocytes are preferentially targeted in NLE and SCLE cutaneous disease (McCauliffe & Sontheimer, 1993b). I reproduced the previous experiments, on normal human keratinocytes under physiological doses of UVA and UVB and other physiological stresses, including drugs, vitamins and hormones, using the purified anti-52 and anti-60 kD Ro autoantibodies previously discussed in chapter 3 to unravel which of the Ro antigens were translocated to the cell surface, see **figure 34, below.**

figure 34: proposed model for Ro translocation.



5.2.2 Methods

5.2.2.1 Cell Culture

Keratinocytes were cultured as previously described, see Protocol 9: Definitive keratinocyte culture pg:107. Second passage keratinocytes were frozen in liquid nitrogen for later use. These keratinocytes were thawed and seeded onto 8-chamber Lab Tek slides (Nunc Inc., Naperville, IL). They were grown to 50% confluence.

5.2.2.2 Determination Of Cell Viability

See Protocol 17: Assessment of viability

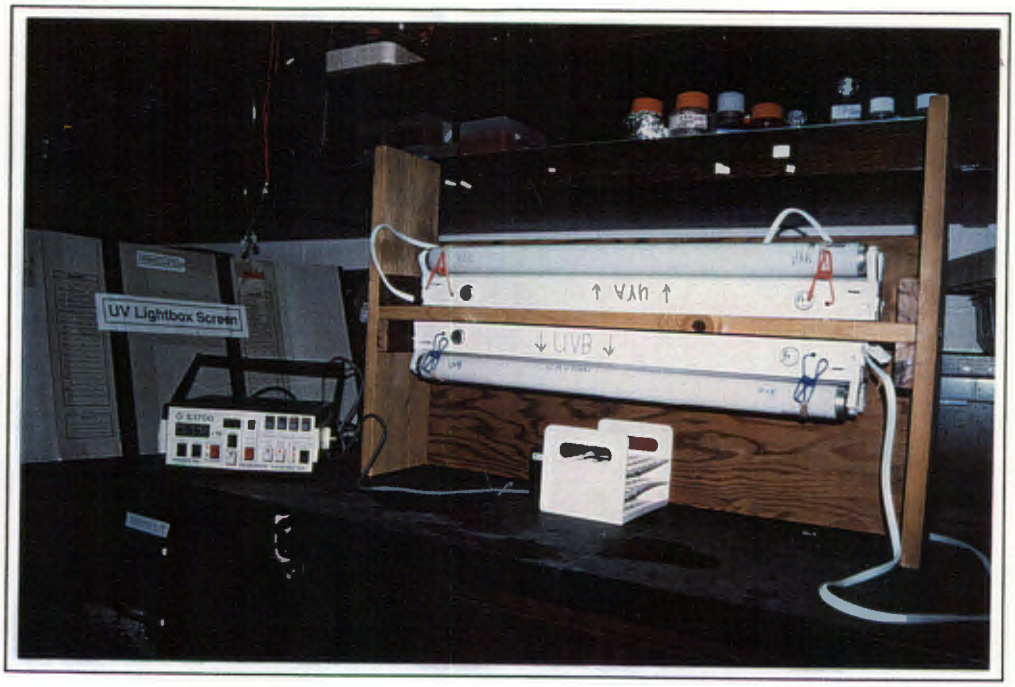
This was assessed by the propidium iodide (Yeh *et al* 1981) or trypan blue (Golan *et al* 1993) pg.: 127

5.2.2.3 Ultraviolet Irradiation

The ultraviolet lamps used were two Phillips black light lamps, mainly UVA, peaking at 370 nm and two FS40 lamps, mainly UVB, peaking at 305 nm (Strato-Ray Co. Minneapolis, MN). These were mounted on opposing sides of a wooden gantry and shielded appropriately from the operator. See **figure 35, pg. 146**. Irradiance was measured with a IL 1700 Radiometer (International light, Newburyport, Mass). Keratinocytes were irradiated with ultraviolet A (UVA) at 4.795J/cm^2. They were then returned to the 37°C incubator. They were incubated with the primary antibody at one and 20 hours after UVA exposure. Keratinocytes were irradiated with ultraviolet B (UVB) at the following doses: 0 mJ/cm^2, 1.5 mJ/cm^2, 4.5 mJ/cm^2, 160 mJ/cm^2, 200 mJ/cm^2 and then incubated with primary antibody at one hour and at 20 hours post irradiation.

Viability was assessed at 20 hours post irradiation by counting the number of cells out of 500, that were positive for trypan blue.

figure 35: The ultraviolet lamps were mounted on opposing sides of a wooden gantry and shielded appropriately from the operator. Irradiance was measured with a IL 1700 Radiometer (International light, Newburyport, Mass).



5.2.2.4 Addition Of Oestradiol, Hydrochlorothiazide, Vit E and C

Keratinocytes were incubated in a 10^{-7} M solution of 17 β oestradiol. d α tocopherol acetate was added to the chambers or ELISA wells, to give a concentration of 2.7μg/10^8 cells, L Ascorbic acid was added to give a concentration of 27μg/10^8 cells. Hydrochlorothiazide was added to give a dose of 400 ng/ml, 200 ng/ml and 100 ng/ml.

5.2.2.5 Heat Shock

Keratinocytes at 50% confluence on chamber slides, were exposed to 42°C for 90 minutes and then either used immediately, or allowed to recover at 37°C for 4 hours. Control slides were maintained at 37°C. These slides were stained with 2 different monospecific anti-52 and anti-60 kD antibodies and C2 whole sera.

5.2.2.6 Immunofluorescence

5.2.2.6.1 Fixed cells

Keratinocytes were fixed as previously described, see Protocol 15: Immunofluorescence of fixed keratinocytes pg. 111, followed by manipulations, see 5.2.1.1 Ultraviolet Radiation pg. 140, 5.2.1.2 Drugs pg.142, 5.2.1.3 Oestrogens pg. 143, 5.2.1.4 Heat pg. 143.

5.2.2.7 Viable cells

See Protocol 18: Immunofluorescence on viable keratinocytes pg: 128

At varying time intervals after manipulations, see 5.2.1.1 Ultraviolet Radiation pg. 140, 5.2.1.2 Drugs pg.142, 5.2.1.3 Oestrogens pg. 143, 5.2.1.4 Heat pg. 143, the keratinocytes were washed in 1X PBS, stained with trypan blue to assess viability and then incubated at 37°C, for varying times (5 min., 60 min., 3 hr, 4 hr, 5 hr and 7 hr) with the primary antibodies or whole sera. This was followed by fixation in 3% paraformaldehyde at 4°C for 15 minutes, followed by 2 brief washes with 1X PBS. They were permeabilised for 5 minutes with 0.1% Triton X in PBS, washed twice with 1X PBS and then incubated for 30 minutes at 4°C with secondary goat anti-human IgG conjugated to Texas Red. The slides were either dipped in propidium iodide to counterstain the nucleus, or the nucleus was left unstained. They were then washed twice and coverslipped with polyvinyl alcohol mounting media.

5.2.2.8 Microscopy

The slides were viewed with a conventional epifluorescence microscope, followed by confocal microscopy, as previously described in chapter 4. Confocal images were processed using Adobe Photoshop 3.0 software and printed on an Epson Stylus colour printer

5.2.2.9 ELISA to assess surface antigen expression

5.2.2.9.1 Unfixed

2 X 10^4 third passage keratinocytes were seeded onto each well of a 96-well microtiter culture plate (Costar), as previously described, see Protocol 19: ELISA to assess surface antigen expression on unfixed keratinocytes pg: 129. They were subjected to manipulations before fixation, as already described, see 5.2.1.1 Ultraviolet Radiation pg. 140, 5.2.1.2 Drugs pg.142, 5.2.1.3 Oestrogens pg. 143, 5.2.1.4 Heat pg. 143

5.2.2.9.2 Fixed

2 X 10^4 third passage keratinocytes were seeded onto each well of a 96-well microtiter culture plate (Costar), as previously described, see Protocol 20: ELISA to assess surface antigen expression on fixed keratinocytes. They were subjected to various manipulations described earlier, see 5.2.1.1 Ultraviolet Radiation pg. 140, 5.2.1.2 Drugs pg.142, 5.2.1.3 Oestrogens pg. 143, 5.2.1.4 Heat pg. 143, mirroring those permutations performed on keratinocytes for immunofluorescence. The wells containing the 52-1 were incubated overnight with 10 mM DTT.

5.2.2.9.3 Autoimmune sera

A number of whole sera from well documented patients were used, in addition to the specific antibodies:

2429	Sjörgen's syndrome. Immunodiffusion (ID) positive for SSA. ELISA positive for native 60kD Ro, recombinant 52kD Ro, recombinant 60kD Ro and La.
93-1	Glyburide induced SCLE. ID positive for SSA.
2109	Subacute cutaneous LE.
123	Neonatal LE. Immunodiffusion positive for SSA. ELISA positive for native SSA, negative for recombinant 52kD Ro, recombinant 60kD Ro and La.
92-1, C2	Subacute cutaneous LE. Paired sera from same patient, one month apart. 92-1: antinuclear factor (ANF) positive. C2: (ANF) negative, Ro +, +whole 52, +-52-1, 52-2, -whole 60, +-60-2, +60-4

5.2.3 Results

5.2.3.1 Ultraviolet irradiation

5.2.3.1.1 Viability

Viability was excellent among the keratinocytes subjected to UVR, hormonal, drug or vitamin manipulations, see tables 3 and 4. On average, 99% of cells were viable 20 hours after irradiation with UVB 1.5 mJ/cm^2, with or without supplementation with varying doses of Hydrochlorothiazide, oestradiol, vit E or vit C. Viability was poor (9%) at the end of the 90 min. 42°C incubation, as measured by the ability of cells to exclude propidium iodide. Fewer cells were propidium iodide positive (33% able to exclude propidium iodide) at the end of a two hour 37°C recovery phase. I found that cells, although trypan blue negative, may be positive for propidium iodide. This propidium iodide positivity is much less after the recovery phase in the heat shock experiments, suggesting that these changes may be related to apoptosis.

Table 3: Viability of normal neonatal keratinocytes with varying doses of UVB at one or twenty hours post irradiation, as determined by the trypan Blue exclusion method. Expressed as number of dead cells per 500 cells counted.

	One hour	Twenty hours
no UVB	2	5
1.5 mJ/cm^2	14	28
4.5 mJ/cm^2	6	12
160 mJ/cm^2	80	102
200 mJ/cm^2	93	120

Table 4: Viability of cells after supplementation +/- UVB, at 20 hrs post irradiation

Viability assessed by number of trypan blue staining cells. 100 cells counted in each corner of the chamber and 100 counted in the middle of the slide. This table shows the number of trypan blue positive cells per 500 cells. Donors refer to different normal neonatal keratinocyte cell lines.

	DONOR 9	DONOR 11	DONOR 6
oestradiol	2	0	0
oestradiol /UVB	3	1	4
HCZ 100 ng/ml	6	1	2
HCZ 100 ng/ml /UVB	3	2	4
HCZ 400 ng/ml	9	3	3
HCZ 400 ng/ml /UVB	2	9	6
vit c	2	0	2
vit c /UVB	6	10	4
HCZ 200 ng/ml	2	0	0
HCZ 200 ng/ml /UVB	0	4	4
vit e	12	3	3
vit e/ UVB	4	3	4

5.2.3.1.2 Translocation / surface fluorescence

The negative controls for translocation were established in chapter 4, from the work on the normal location of the 52 and 60 kD Ro autoantibodies in un-perturbed fixed keratinocytes, see figure 36. In addition, as previous work had used sera, a range of sera were additionally used as negative controls, see 5.2.2.9.3 Autoimmune sera pg.149, see figures 36 and 37.

5.2.3.1.2.1 Manipulations

Following UVA or UVB irradiation, with or without supplements, as described in methods, there was no surface staining for any of the comprehensive range of monospecific antibodies, suggesting no translocation of the 52 or 60 kD antigens to the keratinocyte surface. Some surface staining was found when whole sera was used, see figure 38, figure 39, figure 40.

5.2.3.1.2.2 Heat shock

No translocation of the 52 or 60 kD antigens occurred, following the heating or recovery phase of heat shock. The C2 whole sera did not show positive results either.

figure 36: Translocation / surface fluorescence negative controls. The negative controls for translocation were established in chapter 4, from the work on the normal location of the 52 and 60 kD Ro autoantibodies in un-perturbed fixed keratinocytes. In addition, as previous work had used sera, a range of sera were additionally used as negative controls, see 5.2.2.9.3 Autoimmune sera pg.149. Immunofluorescence of cultured un-perturbed human keratinocytes, fixed before incubation with serum C2, purified human anti-52 and anti-60 kD Ro and a 1:100 dilution of normal serum

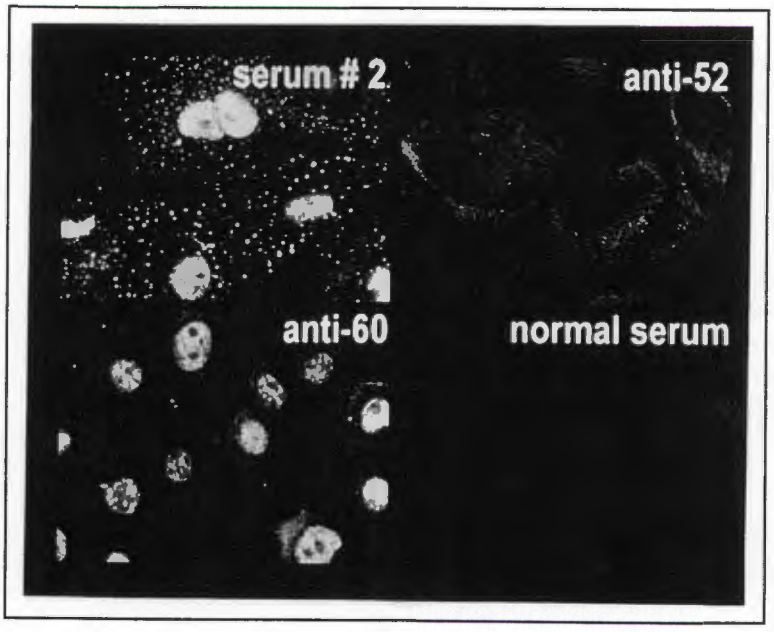


figure 37: Translocation / surface fluorescence negative controls. The negative controls for translocation were established in chapter 4, from the work on the normal location of the 52 and 60 kD Ro autoantibodies in un-perturbed fixed keratinocytes. In addition, as previous work had used sera, a range of sera were additionally used as negative controls, see 5.2.2.9.3 Autoimmune sera pg.149. Immunofluorescence of cultured un-perturbed human keratinocytes, fixed before incubation with serum 2429.

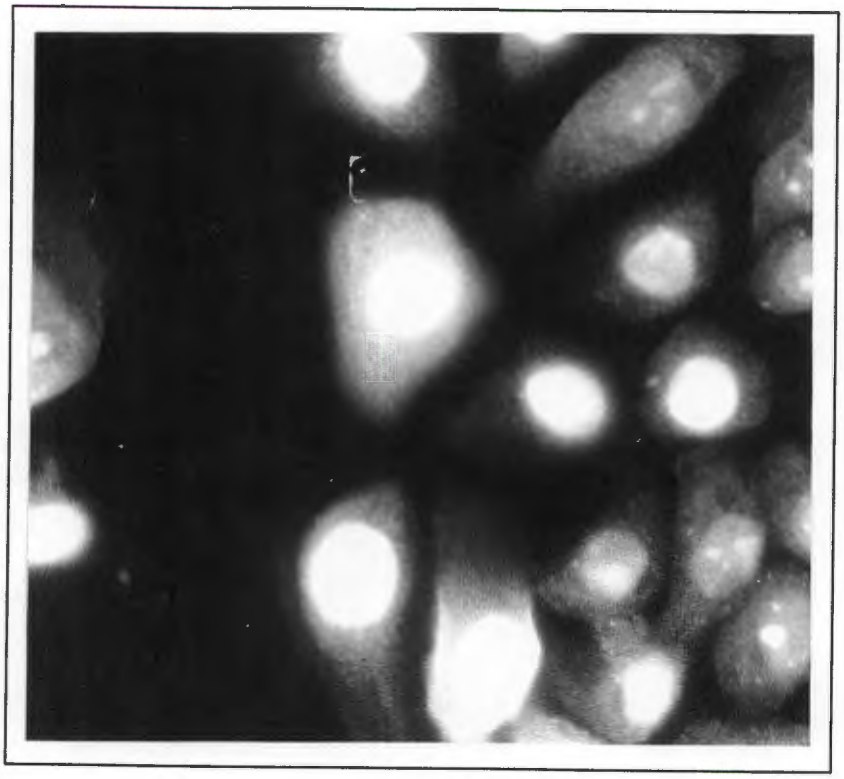


figure 38: Translocation / surface fluorescence No supplements, with or without UVR, caused translocation of the 52 or 60 kD Ro antigens. Some speckling of viable keratinocytes was found when incubated with serum 2429 after irradiation with UVB 1. 5 mJ/cm^2 and prior incubation with a 10^{-7} M solution of 17 β Oestradiol

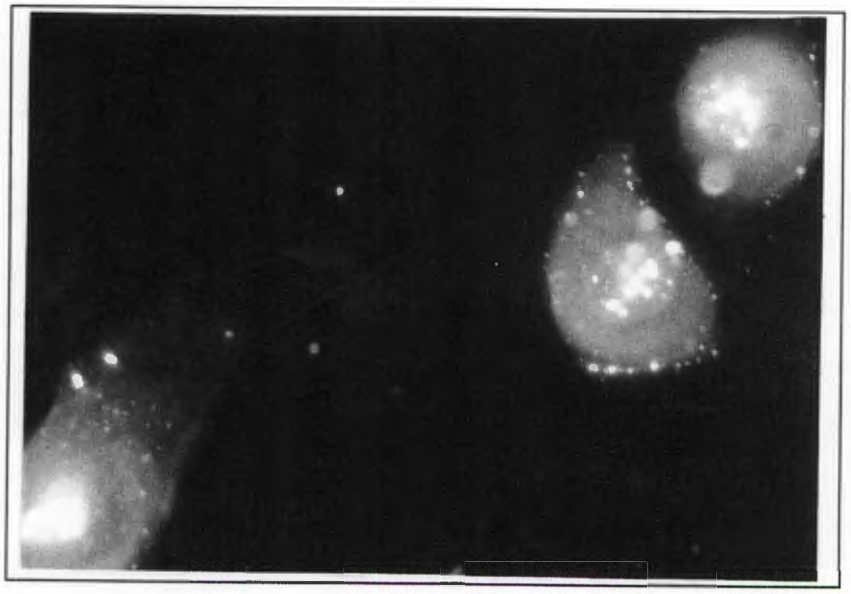


figure 39: Translocation / surface fluorescence No supplements, with or without UVR, caused translocation of the 52 or 60 kD antigens. Some speckling of viable keratinocytes was found when incubated with serum 2429 after prior incubation with Hydrochlorothiazide 100 ng/ml, as described in Methods.

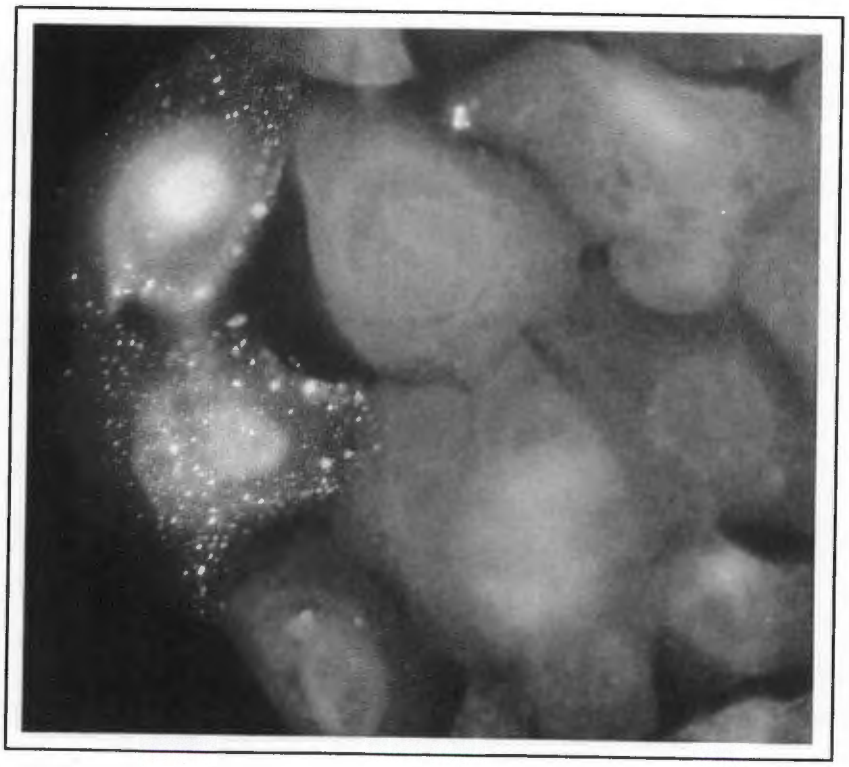


figure 40: Translocation / surface fluorescence No supplements, with or without UVR, caused translocation of the 52 or 60 kD Ro antigens. Some speckling of viable keratinocytes was found when incubated with serum 123, 1 hour post UVB 1.5 mJ/m^2, as described in Methods.

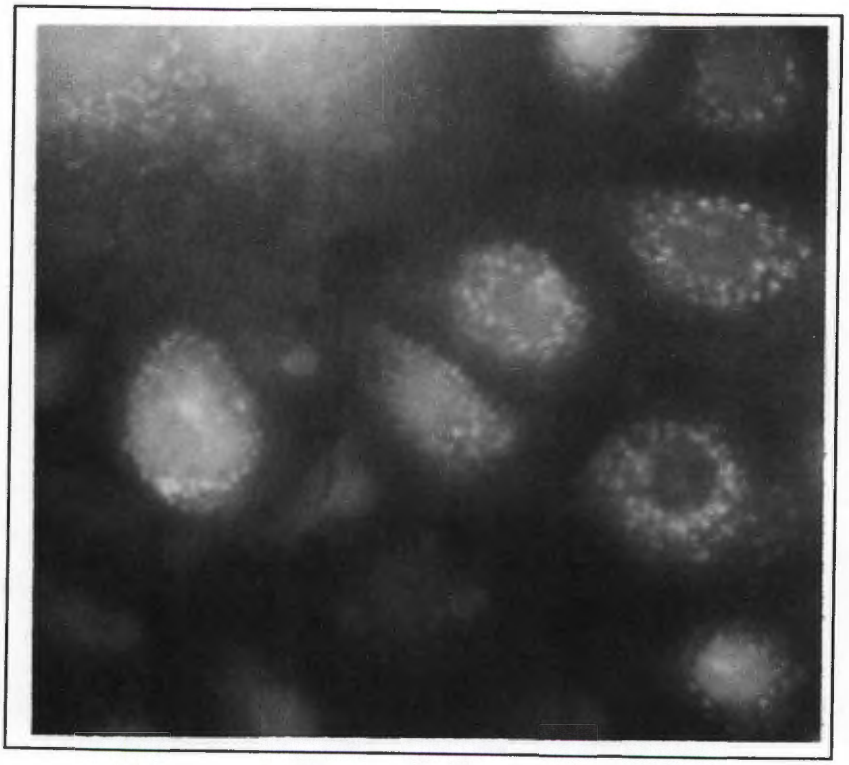
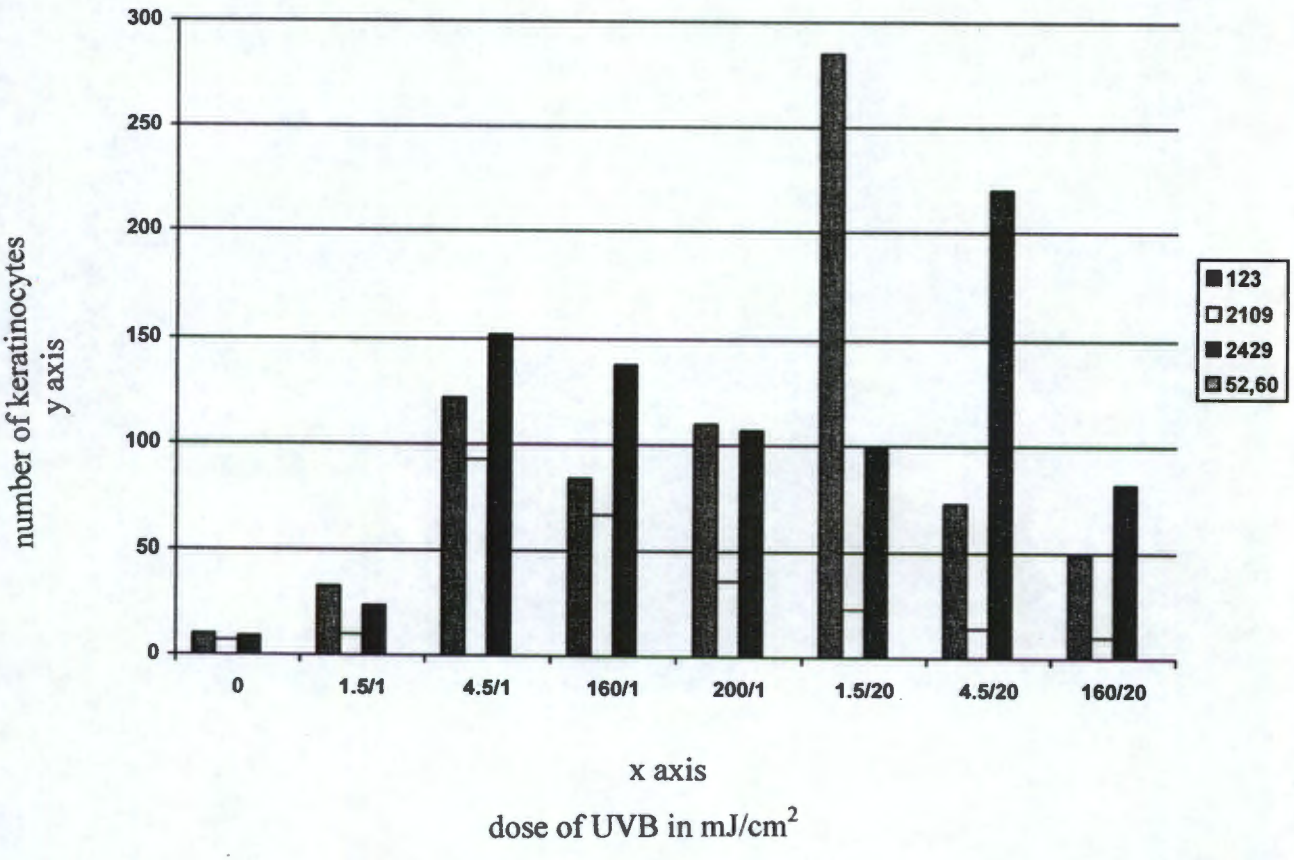


figure 41: Translocation / surface fluorescence No supplements, with or without UVR, caused translocation of the 52 or 60 kD Ro antigens. Some surface fluorescence of viable keratinocytes was found when incubated with whole autoimmune sera, as previously described. (*2429*: SS, ID + Ro, ELISA + n Ro60, + r Ro52, + r Ro 60kD, + La. *123*: NLE, ID + Ro, ELISA + n Ro60, - r Ro52, - r 60kD, - La. *2109*: SCLE, , ID + Ro, ELISA - n Ro60, - r Ro52, - r 60kD, + La.) Number of keratinocytes (y axis) showing surface fluorescence after varying doses of UVB in mJ/cm^2 (x axis), at one and twenty hours after irradiation, expressed as UVB dose in mJ/cm^2/duration post UVR after which cells were incubated with the secondary antibody or whole autoimmune serum.



5.2.3.1.2.3 ELISA to assess surface antigen expression

There was no detectable surface binding one hour after 0, 1.5, 4.5 or 20, 160 mJ/cm^2 UVB, using antibodies directed against two different recombinant 52 proteins or a recombinant 60 kD protein or whole sera 2429 and 123, with or without supplemental Oestradiol, HCZ, Vit C or Vit E.

5.2.4 Discussion

As previous work has described translocation of Ro to the keratinocyte surface, under the influence of a number of agents (LeFeber *et al* 1984; Furukawa *et al* 1990; Golan *et al* 1992; Lee *et al* 1989; Edwards *et al* 1991; Furukawa *et al* 1994) I thought this would be an ideal model, to further unravel the role of the different Ro antigens. As these studies used poorly characterised monospecific Ro sera, it remained to be determined whether the 52-kD Ro, 60-kD Ro or both proteins were so translocated.

With my panel of Ro autoantibodies, I have found that neither the 52 nor 60kD Ro antigens change position in normal human keratinocytes under the influence of physiological doses of UVB or UVA, with or without the additional influences of oestrogen, Vit E or C, Hydrochlorothiazide, a drug clinically causing SCLE, or heat. This is not all together surprising, given that most of the previous studies relied on a single monospecific autoimmune sera and / or anti-sera with anti-52 or anti-60 kD Ro activity; the specificity of these reagents was often not well defined.

Furukawa *et al*, used only four sera for immunofluorescence, one with La (43kD) reactivity, two with reaction to a 60 kD protein (Ro) and one with reactivity to both Ro and La (Furukawa *et al* 1988). However the 52kD Ro antigen was not addressed. They found augmentation of antibody binding on keratinocyte cellular surfaces 24 hours after the addition of oestradiol, maintained for 3 to 4 days. The highest

incidence (24%) was seen on day 2, with the both La and Ro-reactive serum. This was double that observed with just Ro reactive sera alone (13%). Using flow cytometry, these figures were confirmed: higher (21.4%) for both Ro and La reactive serum than for just Ro (13.1%) or just La (14.4%). However, by immunoblotting, comparing the extracts of cultures treated with oestradiol with control cultures, the amount of La was augmented but Ro levels were inadequate for detection (Furukawa *et al* 1988).

Jones showed that the percentage of keratinocytes expressing cell-surface Ro antigen after exposure to hydrogen peroxide showed no increase above control slides, despite the fact that the concentrations used did induce injury and at higher doses, the death of a proportion of the cells (Jones, 1992a). None of the other stimuli tested, induced any consistent change in cell-surface antigen expression, compared with controls. None of stimuli, when tested in conjunction with UVA, led to any increase in antigen expression over the background levels exhibited with UVA alone. When UVB was tested with and without the various stimuli in the culture medium, the results were similar. Most stimuli induced no significant up or down regulation of the antigen expression as produced by UVB alone. The one exception was the addition of 17-β oestradiol to the culture medium prior to UVB exposure. Alone, it induced no increase in cell-surface antigen expression. When used in conjunction with UVB, it produced a two-fold increase over that induced by UVB alone. This increased expression did not show any dose dependence, being induced equally by all concentrations used. There was, however, a correlation with the time of pre-incubation prior to UVB irradiation, expression being greater at the 24-and 48-time points than the 12-h time point (Jones, 1992a). This work suggested that cell injury alone is not enough to produce cell-surface antigen expression.

My findings contrast with those of Furukawa et al (Furukawa *et al* 1988) who found that oestradiol at concentrations of 10^{-5} - 10^{-7} M augmented cell-surface antigen expression using a similar test system. The reasons for these differences are uncertain and cannot be explained on the basis of differences of experimental technique.

Furukawa does add that the issue of cross-reactivity of anti-Ro or anti-La antibodies with other antigen cannot be ignored (Furukawa *et al* 1988). Especially in autoimmune sera, the presence of autoantibodies with widely cross-reactive specificities has been appreciated.

This is especially interesting, since clinical studies have shown that 70% of patients with Ro precipitating antibodies also have rheumatoid factor (Mamula *et al* 1986). In SS, the rheumatoid factor titre and the concentration of anti-Ro are closely related. In addition, rheumatoid factor and antinuclear antibody contribute to Ro precipitation by double diffusion. In other disorders, selected rheumatoid factors (some restricted to particular idiotypes) have been shown to bind to non-IgG cellular components including histones, DNA and bacterial peptidoglycan and nitrophenyl groups. In a study comparing the binding characteristics of heteroimmune anti-Ro generated in a rabbit host with human autoimmune anti-Ro, it was found that both autoimmune and heteroimmune anti-Ro not only bind the Ro protein but also react with IgG (Mamula *et al* 1986). Affinity purification of the IgG and Ro-binding fractions of the rabbit anti-Ro, demonstrated that both the anti-Ro and anti-IgG activities were concentrated in these fractions. Therefore, Ro and IgG share epitopes that are bound by anti-Ro antibody. Inhibition experiments suggest that this antibody is found in most human anti-Ro autoimmune sera and that the epitope (s) are found in the $F(ab')_2$ fragment of IgG (Mamula *et al* 1986). It was therefore postulated that the anti-Ro component of sera may contribute to the detection of positive rheumatoid factors.

The same group analysed the fine specificity of anti-Ro for the IgG molecule. They concluded that the Ig G cross-reactivity of anti-Ro does not resemble conventional rheumatoid factors, since Fc regions of IgG are not bound by anti-Ro (Mamula & Harley, 1992). The anti-Ro antibody has binding specificity for the $F(ab')_2$ heavy chain region of IgG. The 52kD Ro protein does not possess the cross-reactive epitope. These cross-reactive antibodies are most likely to appear as the anti-Ro responses mature and become high-titered in the patient (Mamula & Harley, 1992).

Additionally, the UVB dose described has been somewhat inconsistent. My doses of UVB and UVA are physiological, terrestrial doses. Likewise, those used by Le Feber at 2-20 J/m^2 (LeFeber *et al* 1984), Jones at 10 - 40 J/m^2(Jones, 1992b) and Kawashima at 50 - 150 J/m^2 (Kawashima *et al* 1994) were physiological.. However, Furukawa used 2000 J/m^2 (Furukawa *et al* 1990) and Golan used 200 - 2500 J/m^2 (Golan *et al* 1992), representing up to 50-fold mean erythema doses for Caucasian skin. It has been established that irradiation of neonatal keratinocytes with 1 650 J/m^2 generates apoptotic cells within 6 hours (Casciola-Rosen *et al* 1994). This group has shown that the Ro antigen is clustered in two discrete populations of blebs at the surface of apoptotic cells (Casciola-Rosen *et al* 1994). The previous papers may therefore have been describing changes with apoptosis, rather than cell surface translocation of Ro with physiological doses of UVR.

It is interesting that Furukawa *et al* make the observation that oestradiol doubled the incidence of small or cuboidal cells showing cell surface IgG binding. In contrast, the percentage of large or polygonal cells binding IgG decreased from 39% to 21% by oestradiol stimulation. The relative percentage of medium-sized cells binding IgG was not changed by oestradiol stimulation. Looking at their figures and with the benefit of hindsight and all the work done by Casciola-Rosen *et al* on apoptosis, these cells (small, polygonal) were probably apoptotic. They suggested that these differences may relate to differentiation but then went on to find that strontium, which produces uniform, small, cuboidal cell colonies, did not enhance cell surface antigen expression (Furukawa *et al* 1988).

It has been felt that the translocation occurred in viable cells, on the basis of Trypan blue exclusion. However I have found that the cells showing these changes with whole sera, although Trypan blue negative, are positive for Propidium iodide. This propidium iodide positivity is much less after the recovery phase in the heat shock experiments. This suggests that these changes may be related to apoptosis. This is supported by the work of Kawashima *et al* (Kawashima *et al* 1994) who analysed Ro/SSA surface binding using fluorescence activated cell sorter analysis (FACS), by

which dead cells are gated out by propidium iodide staining. They found there was no plasma membrane expression of the 52 and 60kD antigens in transformed keratinocytes. In addition, Le Feber (LeFeber *et al* 1984) found that their keratinocytes showing granular Ro/SSA staining, stained positively but less intensely with fluorescein diacetate (FD) than did cells without granular Ro/SSA. This confirms that these cells were viable but apparently injured. In fact, they found no Ro staining in the 25 - 35% of "live" cells, namely cells which avidly hydrolyzed and retained FD.

Casciola-Rosen et al have shown a conspicuous movement of cytoplasmic Ro to small surface blebs early in apoptosis (Casciola-Rosen *et al* 1994). Ro is also found in the apoptotic nucleus (Casciola-Rosen *et al* 1994). Much of this nuclear Ro is reorganised during apoptosis. Ro is excluded from the nucleoplasm as the chromatin becomes condensed. It becomes concentrated around the rim of apoptotic bodies after nuclear fragmentation. After forming within the cell, the apoptotic bodies move to the surface where they give rise to a population of larger surface structures containing fragmented DNA. These apoptotic bodies also contain a distinct cluster of autoantigens that are targeted in patients with lupus, including nucleosomes (Mohan *et al* 1993; Burlingame *et al* 1993), Ro, La and snRNPs.

Other work on the anti-phospholipid autoantibodies has shown that apoptotic, not viable thymocytes may be the natural immunogen and/or target for anti-phosphlipid autoantibodies. In addition, this group suggested that the interaction of β2-glycoprotein 1 with redistributed anionic phospholipid may itself generate a novel ligand by which apoptotic cells are recognised directly for phagocytic clearance (Price *et al* 1996).

Previous work has shown a great deal of inter individual keratinocyte donor variability. Percentages of translocation have been quoted as varying from 3 - 17% in one report (Jones, 1992b). The percentage of cell showing granular staining increased significantly with each increase in dose of UVB, although the expression did not vary significantly when assessed at the different time points after irradiation (Jones,

1992b). This study showed an increase in both injured and dead cells with higher doses of UVB. In addition, this work showed that blocking protein synthesis with cycloheximide, induced a dose dependent reduction in the percentage of cell-surface antigen expression as induced by UVB. Le Feber (LeFeber *et al* 1984) found 10 - 20%. Golan (Golan *et al* 1992) showed a maximum of 8.2% for the combined anti-Ro/anti-La probe. However this level was still extremely low, the cell line showing the highest positive number of fluorescent cells showed a doubling of the mean percentage of Ro positive normal keratinocytes from 4 to 8% after 1 600 - 2 500 J/m^2 UVB. UVA light at doses of 50 -200 kJ/m^2 had no effect on cell viability or autoantibody binding. In addition, in 10/12 SLE derived keratinocyte cell lines, this Ro/La surface expression was higher than normal controls. This was UVB dose dependent, although expression varied between cell lines, ranging from about 4% in 2, to 60% in one. This study in addition, was done using autoimmune patient sera, rather than purified antibodies. They used a serum with both Ro and La combined, rather than one specific for either. They admit that they could not exclude the possibility that their sera could also contain antibodies that could have bound to other antigens or to neoantigens created by UVR (Golan *et al* 1992). Golan (Golan *et al* 1992) used one single anti - SSA/SSB (Ro/La) antiserum from a patient with primary Sjögrens syndrome, which did not bind to other proteins on immunoblotting. Their results may be due to La translocation, as described by Bachmann (Bachmann *et al* 1990).

Bachmann *et al* (Bachmann *et al* 1990) have shown that the La antigen translocates from the nucleus to the cytoplasm of human keratinocytes. This shuttling includes a translocation to the cell surface. Their method differed somewhat from those of Le Feber *et al* (who incubated antisera for an unspecified period and examined cells 8 and 24 hours after irradiation) (LeFeber *et al* 1984), in incubating irradiated viable cells for 5 minutes with the antibody at 1 hr, 5 hrs and 24 hrs post UVB. They found that anti-La stained in a punctate manner on the cell surface at 1 hour after UVB but that this staining disappeared during further incubation, being absent at 5 hours. Golan (Golan *et al* 1992) incubated the LE keratinocytes for one hour with the

primary antibody at 20 hours post irradiation, having found this the best time point after assessing surface expression at 4, 8, 16, 20 and 24 hours after irradiation.

Furukawa *et al* in their work on UVR irradiated keratinocytes (Furukawa *et al* 1990), commented that since the degree of UVL-augmented antibody binding varied with different cell sources, the cell source was carefully selected and cells from the same source were used for all experiments. However in their results the lowest scoring cell lines are not recorded. Rather, results represent the mean of positive cells from four experiments, rather than different cell lines, which would more likely resemble the situation in the real world. *In vivo* Ro augmentation on the keratinocyte surface was examined in suction blister roofs, 24 hours post 100 and 200 mJ/cm^2. Anti-Ro serum bound to epidermal keratinocytes but was also seen to bind to cytoplasm and nucleus. This report states in the first line of discussion that their work verified that of Le Feber et al, that antibodies specific for Ro were induced to bind to the cell surface by UVB. However, it is interesting that no comment was made on the very differing doses of UVB used! In addition, by using "monospecific" whole sera rather than purified antibodies, it is possible that cross-reacting antibodies or other antibodies not detected by immunoblotting, CIE, or immunodiffusion might be responsible for the IgG binding to keratinocytes.

Furukawa in continuing his USA work in Japan, described lupus keratinocytes as showing a higher susceptibility to single-dose UVB compared to those of normal individuals. They however give no indication of the magnitude of this upregulation and in addition their figures do not convince (Edwards *et al* 1991).

A further study, using only two cell lines, one from a patient with NLE and a normal neonatal control, suggested that NLE keratinocytes were more likely to show surface expression than the normal, although they did not differentiate between Ro or La. A dose of 200 mJ/cm^2 UVB irradiation on NLE keratinocytes induced a 2.5-3-fold increase in Ro/SSA and La/SSB expression compared to non-irradiated cells. Sera derived from both the NLE patient and from his mother exhibited a cytotoxic effect on NLE keratinocytes, but not on control cells, in the presence of complement.

Furthermore, the cytotoxicity of the sera was enhanced on UVB-irradiated NLE keratinocytes, whereas it had no cytotoxic effects on UVB-irradiated control cells (Yu *et al* 1996).

It has been suggested that 52kD Ro and La may be membrane expressed by TNF alpha. Normal neonatal keratinocytes were treated with tumour necrosis factor alpha (TNF alpha) and incubated with antibodies to 52 kD Ro isolated and purified from patients with systemic lupus erythematosus or Sjögren's syndrome, with mouse monoclonal antibody to La, normal sera and a mouse monoclonal antibody to U1RNP 68 kDa. Membrane expression of 52 kD Ro and La was detected using cyto ELISA, laser scanning microscopy and indirect immunofluorescence. After incubation with TNF alpha, cyto ELISA revealed an increased membrane binding of 52 kD Ro antibodies at a maximum of two hours, followed by enhanced 52 kD Ro expression during the subsequent 24 hours. The La antigen was expressed rapidly after TNF alpha treatment (within one hour), with a fast decrease to the pre-incubation value within three hours. Indirect immunofluorescence with fixed normal human keratinocytes confirmed increased 52 kD Ro and La antigen expression after the incubation with TNF (Dorner *et al* 1995).

Some SCLE patients describe cutaneous exacerbations with heat (Sontheimer, 1985) Heat shock like proteins can be translocated to cell surfaces where they may participate in immune mediated cell injury. More recently, a group studied the quantitative and qualitative changes of Ro induced by stress, such as with heat shock and UV irradiation, and found that only Ro52 could be expressed on the cell surface of human peripheral lymphocytes by either heat shock or UV irradiation. Flow cytometric analysis showed that heat shock-treated and UV-treated lymphocytes could be stained with patient sera (Igarashi *et al* 1995). In my model, I could find no keratinocyte surface expression of the 52 or 60kD Ro under the influence of heat shock.

The importance of using monospecific antibodies is emphasised by a paper published concurrently with my work (Kawashima *et al* 1994). They studied the effect of UVB

irradiation on the expression of 60kD Ro, 52kD Ro and calreticulin in A431 cells, a transformed human epidermal keratinocyte cell line. Total cellular and cell surface expression of each antigenic polypeptide was examined by a whole cell ELISA and FACS using rabbit anti-synthetic peptide antisera as probes. They found that both total cellular and cell surface calreticulin but not the 60 and 52 kD Ro polypeptides was increased after $100J/m^2$ of UVB irradiation. The mean fluorescence intensity of cells that had been irradiated with UVB 24 hrs earlier and then stained with the anti-calreticulin antiserum, was approximately 50% greater than control levels. No such differences were noted with the 60 and 52kD Ro peptide antisera.

I have systematically examined the influence of physiological, rather than apoptosis - inducing doses of UVB and UVA in a number of normal neonatal human keratinocyte cell lines. I have additionally documented the effect of oestradiol, Vit E and C, Hydrochlorothiazide and heat shock on the expression of the 52 and 60 kD Ro antigens. I conducted my experiments on viable keratinocytes at a whole range of incubation durations and intervals post UVR to include all those previously described. I could find no translocation of these Ro antigens to the cell surface.

5.3 POSSIBLE ROLES PLAYED BY THE Ro ANTIGENS

The functions of the 52- and 60-kD Ro proteins remain unknown. There is convincing data that the 60kD Ro protein associates with cellular hY RNA as a ribonucleoprotein complex. It has also been argued that the 52-kD Ro protein is also a component of this complex (Peek *et al* 1993) but others dispute this claim (Slobbe *et al* 1992; Kelekar *et al* 1994). My findings indicate that the majority of 52- and 60-kD Ro proteins are not complexed together. I would have to add, that given some similarities in distribution, it is possible that some 52- and 60-kD Ro molecules are components of the same ribonucleoprotein particles. Cell fractionation methods and hY RNA and Ro protein injection studies have demonstrated that the Ro ribonucleoprotein complex is located primarily in the cytoplasm (Peek *et al* 1994; Peek *et al* 1993). My localisation of the 60-kD Ro protein predominantly to the nucleus, would therefore indicate that only a minority of the total cellular 60-kD Ro protein is complexed with cytoplasmic hY RNA. Peek et al have reported that the majority of 60-kD Ro is in fact located in the cytoplasm but antibodies cannot detect it because of epitope masking when the protein is part of the ribonucleoprotein particles (Peek *et al* 1993). It is possible that my IF methods of fixation and / or permeabilisation may have failed to preserve and / or unmask epitopes on the cytoplasmic form of 60-kD Ro. This is less likely, however, as I used antibodies reactive with at least 3 different 60-kD Ro epitopes. In addition, other investigators, using different IF techniques, have also found a predominantly nuclear distribution of the 60-kD Ro antigen (Lopez-Robles *et al* 1986; Ben-Chetrit *et al* 1988; Casciola-Rosen *et al* 1994; Veldhoven *et al* 1995).

It has been suggested that Ro may play a part in the control of the distribution of mRNAs into translationally active or inactive forms (Wolin & Steitz, 1983). It has also been reported that the Ro antigen may be a part of a cellular complex possessing endoribonuclease VII activity (Bachmann *et al* 1987). The fact that the 60kD Ro protein contains a zinc finger, potentially capable of binding DNA, in addition to the putative RNA-binding domains RNP1 and RNP2 may suggest that the 60kD Ro

resembles transcription factor IIIa. This is able to bind both DNA and RNA (Miller *et al* 1985) and may therefore play a role in transcriptional regulation.

The conspicuous movement of cytoplasmic Ro to small surface blebs early in apoptosis, implies that cytoplasmic Ro has an affinity for the other components in these blebs. It suggests that Ro has a function that is performed at the site of ER-ribosome interaction. Such functions might include a role in protein synthesis and control of translational protein translocation into the ER (Peek *et al* 1993; Slobbe *et al* 1991).

At present very little is known about the 52kD protein but it would be interesting to speculate that the 52kD Ro may be involved in regulating RoRNP function. This regulation may be manifest at the level of protein-DNA interaction. To test this hypothesis, the human gene which encodes this protein was cloned in a baculovirus and expressed in Spodoptera frugipoda cells. Extracts from these infected insect cells were used as a source of protein for this study. The protein is similar in size and antigenicity to that expressed in human cells. This protein binds to DNA at a physiological temperature and is eluted with high concentrations of sodium chloride. Striking similarities were found between the sequence in, and adjacent to, the nucleic acid-binding motifs of 52 kD Ro and a growing family of zinc finger proteins which have been shown to bind to DNA or regulate gene expression (Frank *et al* 1995).

6. PROPOSALS FOR FUTURE WORK

Skin manifestations are some of the most common symptoms and signs in the multifactorial autoimmune disease of SLE. Genetic factors determine the inherited baseline, on which environmental, hormonal and infectious triggers act to produce autoantibodies.

Ro / SS-A antibodies are considered pathogenic in SCLE and NLE. Ro autoantibodies react with at least two distinct proteins. The 52 and 60 kD Ro antigens have been cloned and are structurally dissimilar. Immunoaffinity purified anti-52 and 60kD Ro antibodies directed against all major epitopes, are expressed in keratinocytes, with no cross-reaction between the two. There are differences in the position of the 52 and 60 kD Ro proteins. The Ro52 is predominantly cytoplasmic, with the Ro60 mainly nuclear.

There is, as yet, little evidence to implicate a specific Ro protein or Ro epitope in a particular type of Ro autoantibody-associated autoimmune disorder. Anti-Ro antibodies are incapable of penetrating normal viable human keratinocytes. Ro antigens are not translocated to the surface of normal keratinocytes under physiological stress, although other work has shown a wide variation in cell surface expression related to presumed UVB induced apoptosis. Certainly, SLE patients show double the concentration of surface Ro than do keratinocytes from normal individuals (Golan *et al* 1992). Autologous sera from SLE patients sometimes increased the level and percentage of binding, supporting the possibility that autoantibodies or cytokines in patients' sera might prime cells for this UVR effect.

There is evidence of at least two forms of the 60kD Ro encoding gene (Rader *et al* 1989; Deutscher *et al* 1988; Ben-Chetrit *et al* 1989). Kutsch et al have reported variability in Ro displacement to the keratinocyte membrane after treatment with

calcium ionophore or tumour necrosis factor alpha, which they suggest may arise from genetic differences in the keratinocyte (Kutsch *et al* 1991). It is possible that certain forms of a Ro protein may be more immunogenic, expressed at higher levels, or more easily displaced to the cell surface where it could participate in immune mediated injury. I used anti-60 and 52-Ro antibodies, in addition to using at least 3 different donors.

In addition, other authors have shown a striking variation in the expression of the Ro antigen in normal human skin between different individuals, by more than 2 000-fold (Niimi *et al* 1995). This difference in expression does not appear to be related to age or sex, nor due to regional differences in expression, as occurs with bullous pemphigoid or pemphigus antigens. There was no difference in Ro and La antibody binding between sun-exposed and sun-protected skin. Whatever the cause, the heterogeneity in expression of Ro antigens is not a unique phenomenon, since individual variability in the expression of other keratinocyte components, such as that of La, cytokines, or UV light-induced intercellular adhesion molecule 1, has been described (Norris, 1993).

In the course of characterising the gene encoding the human 52kD Ro protein, a restriction fragment length polymorphism (RFLP) was shown. Homozygosity for this 1.5kb RFLP was strongly associated with lupus in African Americans (Frank *et al* 1993). This polymorphism, though present in White and Japanese populations, was not associated with SLE in these two populations. The structure of this gene has been shown to be six exons spanning over 5.5kb of the human genome (Tsugu *et al* 1994). This was considered to be in the promoter region. This would be an interesting polymorphism, as it is present in a gene encoding an autoantigen but the association is with the disease as a whole, rather than with the presence of anti-52kD Ro antibodies. In addition, it is restricted to African-American lupus patients. However, Tsugu *et al* did not report the 5' most exon, as reported earlier (Chan *et al* 1991a). Therefore the RFLP was not in the promoter region but in the first intron (Chan *et al* 1995).

Differences in the presence of particular anti-Ro antibodies between patients may be based on the ability of that patient's immune system to process and recognize epitopes which are unique to each form of the Ro polypeptide. Given the complexity of this antigenic system, a reevaluation of polymorphisms of the immune response genes of patients with anti-Ro antibodies is in order. It is possible that polymorphisms of the 52kD Ro gene, contribute to the SLE disease process. SLE associated polymorphisms of the 52kD Ro antigen may affect response to UV radiation and cell surface expression. This would explain the controversy surrounding the effect of UV irradiation on this antigen in normal keratinocytes. It would therefore be sensible to detect SLE associated 52kD Ro gene polymorphisms and to define their role in the autoimmune disease process. This could be achieved by detecting polymorphisms in both the 52kD Ro gene and its promotor by SSCP and characterising these polymorphisms by sequencing. PCR based methods could then be designed to assign polymorphism frequency in both normal controls and SLE patients. It would then be necessary to determine functional differences due to disease associated polymorphisms

APPENDIX

7. PROTOCOLS NOT PREVIOUSLY DESCRIBED

Protocol 22: POLYVINYL ALCOHOL (PVA) MOUNTING MEDIUM FOR IF

PVA (Sigma P 8136)	4.8g (crystalline type II)
Glycerin	12.0g
ddH_2O	12 ml

Tris Buffer (0.1M, pH 8.5)

1. 21g Tris base in 95 ml ddH_2O and 3 ml 1N HCl (Adjust pH as necessary)

Place 12g glycerol in a small beaker. Add 4.8g PVA and stir well without getting PVA into the sides of the tube. To stop the PVA from clumping, mix the glycerol and PVA thoroughly before adding the ddH_2O. Stir well and incubate for 4 hours at room temperature. Then add 24 ml Tris buffer and place tube in a water bath (50°C) for about 10 mins with occasional stirring to dissolve the PVA. Clear the reagent by centrifuging at 200 for 10 mins. Store in dropper bottles at 4°C.

Protocol 23: BUFFERS USED FOR WESTERN BLOTTING

The following Tris-Glycine stock solutions and buffer systems were used:

30% acrylamide: dissolve 30g acrylamide and 0.8g bis-acrylamide (30.8%T, 2.6% C) in ddH_2O to a final volume of 100 ml.

Electrophoresis buffer: dissolve 3.03g Tris base, 14.4 glycine and 1g SDS in 1l ddH_2O

10% Ammonium persulphate: dissolve 100 mg Ammonium persulphate in 1 ml ddH_2O. Store the solution at 4°C. Discard after 24hrs.

Sample buffer X 2: mix 1 ml glycerol, 0.5 ml 2-mercaptoethanol, 0.3g SDS, 0.25 ml 0.05%(w/v) bromophenol blue and 1.25 ml Upper Tris (4X) buffer. Add ddH_2O to a final volume of 5 ml

1.5M Tris-HCl pH 8.8: dissolve 36.34g Tris base and 0.8 SDS in 150 ml ddH_2O. Titrate to pH 8.8 with 6N HCl. Add ddH_2O to final volume of 200 ml

.5M Tris-HCl pH 6.8: dissolve 12.11g Tris base and 0.8g SDS in 150 ml ddH_2O. Titrate to pH 6.8 with 6N HCl. Add ddH_2O to final volume of 200 ml.

20% SDS: Avoid creating dust and wear a mask. Dissolve 200g electrophoresis grade SDS on 800 ml ddH_2O. Heat to 68°C with magnetic stir bar mixer. Adjust pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1 l. No need to sterilize.

Loading buffer: 0.5M Tris HCl (6.8) 2 ml + ddH2O 1.6 ml + 10% SDS 3.2 ml + 2-6-mercaptoethanol 0.8 ml

Protocol 24: BUFFERS USED FOR ELISA

ELISA

Materials:

1. antigens

a) recombinant Ro protein

b) control protein

2. antisera

a) human Ro/SSA autoimmune sera

b) goat anti-human immunoglobulin conjugated to alkaline phosphatase (SIGMA A3150 - gamma chain specific)

3. buffers/solutions

a) coating buffer

b) diluting buffer

c) PBS

d) wash buffer

e) alkaline phosphatase wash solution

f) substrate solution

a)Coating buffer

0.1M carbonate buffer, pH 9.6 =

stock A= 0.2M: 21.2 g Na_2CO_3 (anhydrous) per liter H_2O

stock B=0.2M: 16.8 g $NaHCO_3$ per liter H_2O

80 ml stock A+ 170 ml stock B + 250 ml H_2O

b)Diluting buffer 0.5 ml Tween 20 + 2.5 g bovine serum albumin (BSA 0.25%) + 1.0 g goat gamma globulin (0.1%) + 1.0 g sodium azide (0.1%) + 1 X PBS to 1 liter (dilute 10X PBS 1:10 in H_2O). Filter sterilize

c)10 X PBS phosphate buffered saline, pH~7.3 = 80 g NaCl + 2 g KCl + 11.5 g $Na_2HPO_4 \bullet 7\ H_2O$ + 2 g KH_2PO_4 + H_2O to 1 liter. pH to ~7.4 with a few drops of conc. HCl, then autoclave.

d)Wash buffer 0.5 ml Tween 20 0.05%) in 1 liter sterile 1 X PBS

e) Alkaline phosphatase wash solution

10 mM diethanolamine, pH 9.5, containing 0.5 mM $MgCl_2$

10X stock solution = 0.53 gm diethanolamine + 0.05 gm $MgCl_2/6\ H_2O$ in 50 cc ddH_2O, pH with 10M NaOH (~100-150 ul) and filter sterilise

For 1X working solution, add 5 cc to 45 cc sterile H_2O

f) Substrate solution

prepare just before use

10 mM ethanolamine HCl, pH 9.8, containing 0.5 mM $MgCl_2$

10X stock solution = 0.49 gm diethanolamine + 0.05 gm $MgCl_2/6\ H_2O$ in 50 cc ddH_2O, pH with 10M NaOH (~100-150 ul) and filter sterilise

For 1X working solution, add 5 cc to 45 cc sterile H_2O

add a 5 mg *p*-nitrophenyl phosphate tablet (sigma #N9389)to 5 cc of 1X working solution to get enough substrate for 96 wells

STRIPPING AN ELISA PLATE

Wash the plate 2-3 times with wash buffer

150µl stripping buffer at room temperature in the hood into each well . Leave for 2 hours at 37°C (molecular biology room)

Wash buffer 6-10 times, until no smell remains

Add DTT to T1 and T-1b wells, 100µl/well overnight

Wash 3-4 times to remove DTT

Use a new plate to titre accurately

ELISA PROTOCOL

1. Designate the protein and sera to be placed in each well on the blank grid form.
2. Dissolve each recombinant protein antigen and control protein in coating buffer. The final concentration of each protein in coating buffer should be ~5µg/ml for each batch of protein. Some proteins are already dissolved in coating buffer and stored in the -70°C freezer.
3. Coat wells with protein according to grid sheet:
   a) Add 50 µl of control antigen in coating buffer to as many wells as needed.
   b) Do the same for each recombinant protein antigen to be tested.
   c) Leave one row or column of wells empty as BLANKS.
4. Cover plate with saran wrap and incubate overnight at 4°C.
5. a) Shake plate over sink to remove coating buffer and pat semi-dry on a paper towel.

   b) Without delay, add 100 µl of diluting buffer to each well and let plate incubate X 1 hr. on shaker at room temperature. Use this time to make the serum dilutions (see 6 below).

   c) Empty plate, add 100 µl wash buffer and empty.

6. Dilute sera:

   a) Determine the amount of serum needed, as determined by the number of wells loaded (50µl each) and the serum dilution to be tested. Overestimate the amount of serum needed to ensure that you have a little extra to make up for possible pipetting errors.

   b) Add 50 µl of dilute sera to each designated well.

7. Cover plate with saran wrap and incubate on a shaker bed at room temperature for 1 - 2 hours. While plate is incubating, dilute 1 µl of goat anti-human immunoglobulin (IgG, A or M) in 999 µl of diluting buffer for every ~19 wells that have been coated).

8. Empty and wash wells at least twice with 100 µl of wash buffer.

9. Add 50 µl of goat anti-human immunoglobulin (diluted 1:1000 in dilution buffer) to each well, cover with saran wrap and incubate at room temperature for ~1 hour on shaker.

10. Wash wells:

    a) Shake plate out over the sink and wash each well X 3 with 100 - 200µl wash buffer.

    b) Then wash each well once with 100 µl of alkaline wash solution.

11. Add 50 µl of substrate solution to each well, cover with foil, place on shaker platform and watch closely for colour change (this usually takes between 15 - 30 minutes).

12. As the wells darken, check the ODs at 405 nM frequently until the darkest well has an OD ~1.5 to 1.9 OD units.

8. PUBLICATIONS

8.1.1 Letters

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Yell JA, Burge SM. Cyclosporin and Discoid Lupus Erythematosus. *British Journal of Dermatology* 1994 **131** (1) p85.

8.1.2 Papers

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Yell JA, Burge SM. Warts and Lupus Erythematosus *Lupus 2* 1993(1) p 21-23.

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J.A.Yell, L. Wang, H. Yin, D.P. McCauliffe Disparate locations of the 52- and 60-kDa Ro/SS-A Antigens in Cultured human keratinocytes *Journal of Investigative Dermatology* 1996 107 **4** 622-626.

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D.P.McCauliffe, L.Wang, H.Yin, J.A.Yell. 52 and 60 kD Ro/SS-A autoantigens have disparate intracellular locations in human keratinocytes. Arthritis and Rheumatism 1996 **39** 9 S 181 No 927

9. DECLARATION

I, Jennifer Anne Yell hereby declare that the work on which this thesis is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

.....Signed..... DATE: 20 March 1998

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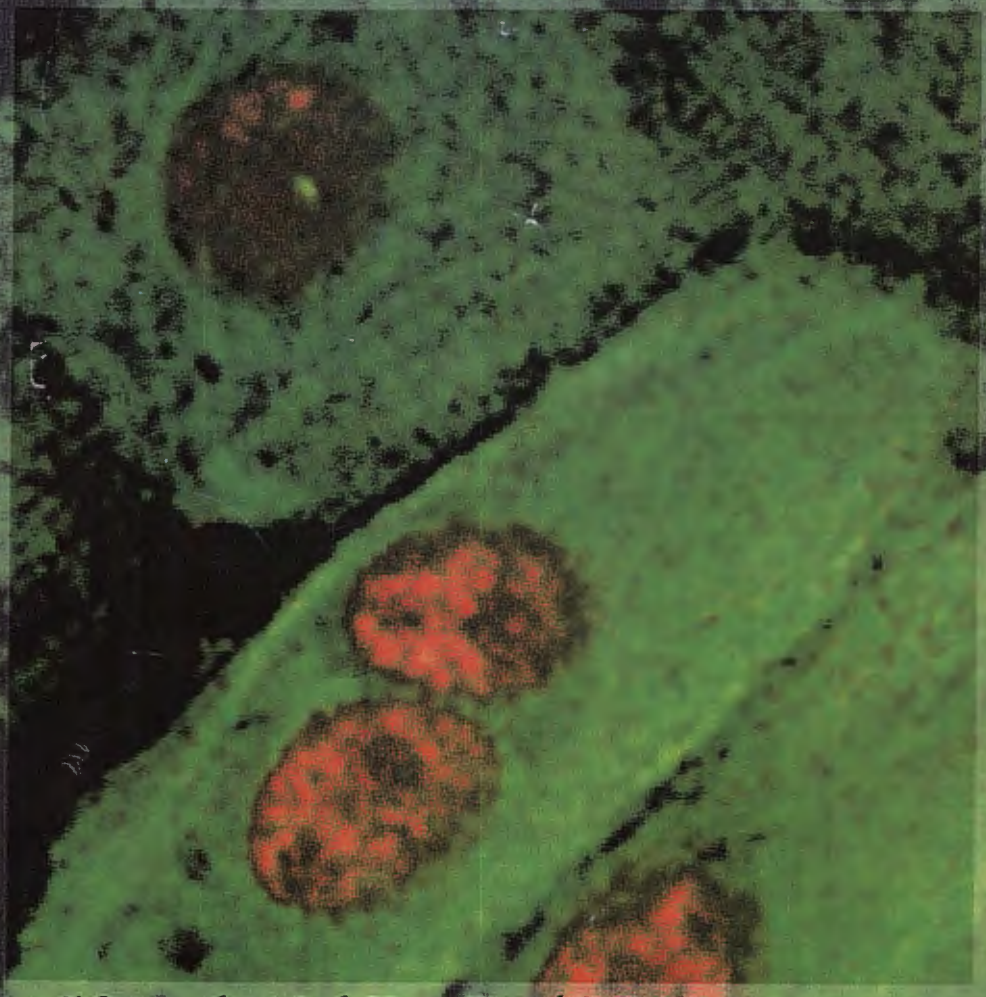

52 and 60 kDa Ro/SS-A antigens

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