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Induction of the Cellular Expression of Human Ro Autoantigens

by

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Abstract

Ro autoantigens are intracellular ribonucleoproteins of unknown function. Autoantibodies to these antigens are detected frequently in patients with systemic lupus erythematosus (SLE) and involved in the pathogenesis of lupus skin lesions. Although the mechanisms responsible for the induction of these autoantibodies and immunologic tissue damage are unclear, one possiblity is that Ro autoantigens are expressed on the cell surface and induce an immune response. Cell surface expression of Ro antigens has been reported previously following ultraviolet B (UVB) irradiation or estrogen stimulation of human keratinocytes. In this thesis, the effect of human cytomegalovirus (CMV) infection on the surface expression of Ro antigens and calreticulin on human fibroblasts and keratinocytes was investigated using a fixed cell enzyme-linked immunoassay (ELISA), immunofluorescence, flow cytometry (FACS) analysis and immunoblotting. CMV infection of cultured human fibroblast cells was found to increase the cell surface expression of calreticulin, but not 60kD/Ro antigen. However, CMV infection, in combination with UVB irradiation, synergistically induced the expression of 52kD/Ro antigen, but not 60kD/Ro or calreticulin, on the surface of these cells. This enhanced expression of 52kD/Ro autoantigen on CMV and UVB treated cells was significant and specific, compared with untreated cells, cells infected with CMV or irradiated with UVB only, and cells subjected to other treatments including low pH. These studies were then extended to human keratinocytes, which are relevant to the skin disease associated with the presence of anti-Ro antibodies in SLE. Human CMV was demonstrated to be capable of infecting keratinocytes in vitro and induced the surface expression of 60kD/Ro antigen, but not 52kD/Ro and calreticulin, on human keratinocytes. As there was no increase in total cellular expression of 60kD/Ro antigen after viral infection, 60kD/Ro antigen appears to be redistributed from the cytoplasm to the cell surface. In contrast to the effects of CMV infection on keratinocytes, UVB irradiation induced the expression of 60kD/Ro, 52kD/Ro and calreticulin on the surface of keratinocytes. These observations support the hypothesis that viral infection may induce the cell surface expression of Ro autoantigens, and thus make these cells targets for an autoimmune response. Demonstration of Ro autoantigen expression on the CMV-infected keratinocyte surface suggests that virus infection may play a role in the development of skin lesions in SLE.

Résumé

Les autoantigènes Ro sont des ribonucléoprotéines intracellulaires sans fonction connue. Les autoanticorps dirigés contre ces antigènes sont détectés fréquemment chez les patients atteints de lupus érythémateux disséminé (LED). Ils sont impliqués dans la pathogénie des lésions dermatologiques du lupus. Le mécanisme responsable de l'induction de ces autoanticorps ou celui causant immunologiquement des dommages tissulaires cutanés ne sont pas élucidés. Il est connu que le traitement de kératinocytes humains aux rayons ultraviolets B (UVB) ou qu'une stimulation de ces cellules avec des estrogènes provoque l'expression d'autoantigènes Ro à leur surface. Dans cette thèse, nous avons étudié les effets d'une infection à cytomégalovirus (CMV) sur l'expression des autoantigènes Ro à la surface des fibroblastes et des kératinocytes humains en utilisant différentes techniques soit, par titrage immunoenzymatique (ELISA) utilisant des cellules indicatrices fixées, par immunofluorescence, par analyse en cytofluorométrie et par immunobuvardage (Western blot). Les fibroblastes humains infectés au CMV in vitro ont un expression accrue de calréticuline à leur surface sans changement de l'expression de l'antigène 60kD/Ro. Par contre, lorsque cette infection à CMV est combinée à une exposition des fibroblastes aux rayons UVB, on observe une expression de l'antigène 52kD/Ro sans expression de l'antigène 60kD/Ro, ni de la calréticuline. L'augmentation de l'expression de l'autoantigène 52kD/Ro à la surface des fibroblastes infectés au CMV et traités aux rayons UVB est significative et spécifique lorsque comparée à celle observée chez des cellules témoins non traitées, ou chez des cellules soumises à un seul traitement i.e. soit l'infection à CMV, soit les rayons UVB ou encore chez des cellules traitées non-spécifiquement comme une exposition à un pH acide. Nous avons répété ce même type d'expérience en utilisant une culture de kératinocytes humains puisque ces derniers représentent le type de cellules affectées dans la pathologie cutanée associée à la présence d'anticorps anti-Ro chez les patients atteints de LED. Nous avons démontré que le CMV peut infecter les kératinocytes humains in vitro et provoquer ainsi l'expression de l'antigène 60kD/Ro sans l'expression de l'antigène 52kD/Ro ou de la calréticuline à leur surface. Cette expression accrue de l'antigène 60kD/Ro n'est pas accompagnée d'une augmentation de l'expression cellulaire totale de l'antigène, ce qui

suggère que l'accroissement à la surface cellulaire est une conséquence de la redistribution de l'antigène 60kD/Ro du cytoplasme à la surface des cellules. Contrairement à l'infection à CMV, l'exposition des kératinocytes aux rayons UVB induit l'expression des antigènes 60kD/Ro, 52kD/Ro, et de la calréticuline à leur surface. Ces résultats appuient donc l'hypothèse selon laquelle les infections virales pourraient provoquer l'expression des autoantigènes Ro à la surface des cellules, les rendant ainsi un cible lors d'une réaction autoimmune. L'expression de l'autoantigène Ro à la surface des kératinocytes infectés au CMV suggère qu'une infection virale pourrait jouer un rôle dans le développement de lésions cutanées chez les patients atteints de LED.

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cells
BSA	bovine serum albumin
C-terminus	carboxyl-terminus
CCHB	complete congenital heart block
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunoassay
ER	endoplasmic reticulum
$F(ab')_2$	$F(ab')_2$ fragment of IgG
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HICS	heat-inactivated calf serum
HLA	human histocompatibility locus antigen
hYRNA	human cytoplasmic ribonucleic acid
IE protein	immediate early protein
IFNs	interferons
Ig	immunoglobulin
IgG	immunoglobulin gamma
kD	kilodalton
KLH	keyhole limpet haemocyanin
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
M.W.	molecular weight

N protein	nucleocapsid protein
NLE	neonatal lupus erythematosus
O.D.	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque-forming units
RA	rheumatoid arthritis
RNA	ribonucleic acid
RNP	ribonucleoprotein
SCLE	subacute cutaneous lupus erythematosus
SDS	sodium dodecyl sulfate
SDS PAGE	SDS polyacrylamide electrophoresis
SE	standard error
SLE	systemic lupus erythematosus
SR	sarcoplasmic reticulum
SS	Sjögren's syndrome
SV40	simian virus 40
TCR	T cell receptor
UV	ultraviolet
UVA	ultraviolet A
UVB	ultraviolet B
UVC	ultraviolet C
VSV	vesicular stomatitis virus

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Preface

The Guidelines for Thesis Preparation of the Faculty of Graduate Studies and Research at McGill University read as follows:

> "Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored paper. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis."**

This thesis is organized into nine chapters. Chapter I is the "General Introduction" and is divided into four major sections. Chapter II is the "Rationale and Objectives". The subsequent three chapters constitute the experimental results and contain one published paper and two papers in press. Each of these three chapters

possesses its own Summary (Abstract), Introduction, Materials and Methods, Results, Discussion and References. Connecting texts for each of these chapters are located at the beginning of each chapter.

The paper presented in Chapter III, entitled "Viral Induction of the Human Autoantigen Calreticulin" by J. Zhu and M. Newkirk, has been published in Clinical and Investigative Medicine. The research described in this paper was done under the supervision of Dr. Marianna Newkirk. The paper presented in Chapter IV, entitled "Ultraviolet B Irradiation and Cytomegalovirus Infection Synergize to Induce the Cell Surface expression of 52kD/Ro Antigen" by J. Zhu, will be published in Clinical and Experimental Immunology. The paper presented in Chapter V, entitled "Cytomegalovirus Infection Induces Expression of 60kD/Ro Antigen on Human Keratinocytes" by J. Zhu, will be published in Lupus. I am the first author on all three papers and I performed all of the experiments in these papers. The research and writing of the papers in Chapters IV and V were performed under the guidance of my Ph.D. Thesis Committee.

Chapter VI is the "General Discussion", and Chapter VII is the "Appendix", which includes some experimental methods and data related to this study, but not in the published or submitted papers. Chapter VIII is the "Bibliography", which consists of the references cited in Chapters I, II and VI and listed in alphabetical order. Chapter IX is the "Statement of Originality" for the thesis.

CHAPTER I

GENERAL INTRODUCTION

SECTION 1

AUTOIMMUNITY AND AUTOIMMUNE DISEASES

The fundamental basis of the immune system is tolerance to self tissues and lack of tolerance to foreign antigens, marked by an appropriate immune response. However, in certain situations, the body can mount an response to itself and autoimmunity develops. The latter can be regarded as a failure of the organism to maintain tolerance to itself (breaking of self-tolerance), possibly due to unusual expression of the major histocompatibility complex (MHC) class II molecules on cells or dysregulation of the idiotype anti-idiotype network. Autoimmunity can occur in genetically susceptible individuals (presence of an MHC susceptibility allele), may be triggered by environmental agents (microbial or toxin) or can be associated with some physiologic or pathologic conditions (aging or cancer). It can exist without being expressed as a clinically overt autoimmune condition and, in some situations, such as the response to cancer cells, autoimmunity may benefit the individual. Autoantibodies can also be produced in apparently healthy individuals and do not necessarily lead to the development of a clinical condition (Cook et al., 1983, Tomer & Shoenfeld, 1988). Their presence may be required to rid the body of effete molecules and cells (Grabar, 1983).

When autoimmune reactions cause pathological tissue damage, autoimmune diseases occur. Based on the number of organs afflicted, human autoimmune diseases can be classified into two families: systemic and organ-specific. In systemic autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS), autoimmune responses to widely disseminated antigens (for example, autologous DNA in SLE) and the formation of circulating immune complexes typically produce multiple organ damage. On the other hand, in organ-specific autoimmune diseases, autoimmune responses against antigens with restricted tissue

distributions lead to organ-specific or tissue-specific injury, such as insulitis in diabetes mellitus. It is also likely that systemic autoimmune diseases characterized by multiple autoimmune phenomena are due to aberrant regulation or polyclonal activation of numerous clones of lymphocytes. In contrast, organ-specific autoimmune diseases may be due to failure of self-tolerance in lymphocytes specific for one or a few tissue antigens, or abnormal activation of lymphocyte clones reactive with a limited number of antigens.

Autoimmune diseases affect 5-7% of the population, often with severe disability, and are a major cause of chronic illness in humans (Sinha et al., 1990). Although there is much information available concerning the immunopathology of autoimmune diseases, it is still not known what causes them. However, a large body of evidence suggests that systemic autoimmune diseases are multifactorial in origin, with both genetic and environmental factors playing a role (Petranyi, 1992; Venables & Brookes, 1992; Behar & Porcelli, 1995; Atkinson, 1995). Their occurrence may result from events such as: (i) misdirection of the immune response due to molecular mimicry, (ii) emergence of previously cryptic antigens, (iii) reversal of the anergic state of autoreactive T cells, or (iv) inadequacy of immunosuppressor functions.

SECTION 2

SYSTEMIC LUPUS ERYTHEMATOSUS

2.1 CLINICAL ASPECTS

Systemic lupus erythematosus (SLE) is a multisystemic, rheumatic autoimmune disease characterized by the presence of multiple autoantibodies, which target cells and organs, such as the skin, joints, kidneys, brain, lungs, heart and the nervous system, resulting in inflammatory tissue damage. Patients with SLE can present with arthritis, skin rashes, glomerulonephritis, hematological disorder, neuropsychiatric manifestations, and immunological abnormalities (Tan et al, 1982). For many of them, SLE is a mild disease affecting only a few body organs, but for others, it can cause serious and even life-threatening problems.

SLE affects women nine times more frequently than men (Ahmed & Talal, 1989). Individuals of all ages are affected, but SLE most commonly strikes women of child bearing years. Although SLE affects people of all races, it is more common in Blacks, Asians and Native Americans than in Caucasians (Rothfield, 1985; Fessel, 1988). There are approximately 500,000 people in the United States who suffer from SLE and about 16,000 new cases are diagnosed each year.

2.2 ETIOLOGY

The etiology of SLE remains unknown, but evidence implicating some host and environmental factors as etiopathogenic agents in SLE will be discussed.

2.2.1 Host Factors

Sex Hormones. Hormonal effects are thought to be the basis for the female preponderance characteristic of SLE (Inman, 1987). In male patients with SLE, abnormal sex hormone metabolism, including elevated 16-α-hydroxyestrone and prolactin levels, has been demonstrated (Lahita et al., 1983; 1985). The most likely role of sex

hormones in predisposing to SLE relates to their immunostimulatory effect on humoral immune function. Estrogen binds directly to receptors on T-suppressor/cytotoxic cells, inhibiting their activity and resulting in increased antibody production (Ahmed et al., 1985). Prolactin also binds to specific receptors on T cells and may affect T helper or suppressor cell function (Russell et al., 1985). Experimental animal studies have also found that female NZB/NZW F_1 mice, a classical model for human SLE, have a more severe disease course, with earlier onset of lupus nephritis and death than their male littermates (Kelly et al., 1980). A study in BXSB male mice, without known hormonal abnormalities, demonstrated that estrogenic hormones did not induce SLE in this model (Eisenberg et al., 1980). These results suggest that hormonal effects likely facilitate, but do not cause, SLE.

Genetic Factors. Genetic factors clearly influence susceptibility to SLE. Based on family and twin studies, the incidence of this disease among first-degree relatives of lupus patients is approximately 100 times higher than that found in the general population (Arnett & Shulman, 1976; Reville et al., 1983), and the concordance rate of lupus in monozygotic twins varies between 24% and 69%, as opposed to 2% to 5% in dizygotic twins (Block et al., 1975, 1976; Deapen et al., 1992).

HLA serological studies have demonstrated a role for the MHC class II in this disease, in that approximately 75% of SLE patients carry HLA-DR3, HLA-DR2 or both, compared with about 26% of normal controls (Provost et al., 1988; Arnett, 1991; Reveille et al., 1991). Although the role of class II MHC molecules in the induction of SLE is not known, two potential mechanisms are of particular interest: (1) the differential capacities of the polymorphic regions of class II molecules to bind foreign or self-antigenic peptides; and (2) the effect of class II molecules on the selection of the T-cell repertoire during the maturation of the immune system in the thymus during early development (Sinha et al., 1990). Additionally, the class III region of the MHC contains the C4A and C2 genes of the complement system. The complement system components, C4 and C2, play a critical role in eliminating immune complexes. Heterozygous or homozygous deletion of the C4A gene, resulting in C4 deficiency, occurs in SLE patients

(approximately 11% of Caucasian SLE patients, compared with an incidence of 1% in the normal population) (Kemp et al., 1987), and C2 deficiency is associated with the development of SLE (approximately 6% of SLE patients) (Glass et al., 1976; Rynes et al., 1978; Agnello, 1986). Complement system dysfunction is considered to be associated with these deficiencies and the deficiency contributes to impaired clearance of immune complexes.

Genetic influences are important in susceptibility to lupus, but a proportion of lupus patients do not have the HLA-DR2 or DR3 haplotype and, conversely, many individuals with this haplotype do not develop SLE. Thus, other factors appear to be necessary for triggering the full expression of clinical disease.

2.2.2 Environmental Factors

A number of environmental factors have been reported to be important in the development of SLE. The following discussion will focus on two factors: viral infection and ultraviolet (UV) irradiation.

Viral Infection. Although no particular virus has been implicated in the etiology of SLE, the possibility of human viral infection as an important component in the pathogenesis of disease has been suspected (Phillips, 1975a, 1975b). Identification of virus-associated microtubular structures in SLE tissues (Norton, 1969; Gyorkey et al., 1969; Pincus et al., 1970) and elevated antibody titers to a number of viruses [herpes simplex, cytomegalovirus (CMV), Epstein-Barr virus (EBV), measles, rubella and parainfluenza] in SLE sera (Hollinger et al., 1971; Hurd et al., 1972; Phillips & Christian, 1973; Pincus et al., 1978) have been reported. The acceleration of murine SLE by lymphocytic choriomeningitis virus or polyomavirus has also been described (Tonietti et al., 1970; Crocker et al., 1974).

If viruses do precipitate SLE, it is likely that these infectious agents are present in the general population at a sufficiently high frequency to infect those individuals, carrying the particular combination of susceptible HLA and non-HLA genes required for SLE to develop, and target organs that are relevant to SLE. The most likely candidates,

to date, are human herpesviruses and retroviruses. Human CMV, one of the seven human herpesviruses, is attracting interest as a prime candidate in the induction of SLE owing to the fact that it is a common infectious agent worldwide (Gold & Nankervis, 1982; Alford & Britt, 1990), and elevated antibodies to CMV (Pincus et al., 1978; Zhang et al., 1991) and CMV-induced flares have been reported in SLE patients (Bulpitt & Brahn, 1989; Vasquez et al., 1992).

CMV is a DNA virus. Its infection is widely prevalent through the world. Fifty to 100% of the population, depending on the country in question, are infected (Gold & Nankervis, 1982), and all organ systems of the host can be targeted by the virus (Alford & Britt, 1990). In most cases, primary infection of children or adults occurs without overt disease. However, following primary infection, the virus persists, presumably as a latent infection, and can be reactivated under conditions of immunosuppression (Lamberson & Dock, 1992). There are a number of excellent reviews discussing the structure, the biology and the virus-host immune system interactions of CMV (Sissons et al., 1986; Griffiths & Grundy, 1987; Apperley & Goldman, 1988; Landini & Michelson, 1988; Stinski, 1990). In brief, CMV is a large virus of approximately 180 nm in diameter, composed of a nuclear core containing its genome (double-stranded DNA), a protein capsid (nucleocapsid) that surrounds the core, a poorly defined area (known as the tegument) that surrounds the nucleocapsid and a lipid bilayer that encloses the complete virion. The entire viral genome has recently been sequenced (Chee et al., 1990), and has the capacity to code for about 200 different proteins. Some of the viral proteins, such as CMV immediate-early (IE) proteins, have been demonstrated to play important roles in regulation of CMV gene expression and virus-host cell interactions (Yuo et al., 1992). Since the virus can persist in the host for life and has a complex biological interaction with the cells that it infects, the immune response to CMV is quite complex. Although some of the immune mechanisms, such as those involved in the lysis of CMV-infected cells or the production of virus-specific antibodies, have been elucidated (Sissons et al., 1986; Griffiths & Grundy, 1987; Landini & La Placa, 1991), the mechanisms responsible for the viral induction of SLE or other autoimmune disease are not clear.

Virus infections are known to give rise to autoimmune manifestations. CMV, EBV, hepatitis B and Coxsackie virus, for example, can all produce various immunologic abnormalities found in SLE (Christian, 1982). These include producing anti-nuclear autoantibodies; the presence of circulating immune complexes; enhanced interferon production; impaired lymphocyte responses to phytomitogens, antigens, and in mixed lymphocyte reactions; and changes in T-cell subpopulations. It has been suggested that viral-mediated pathogenesis in SLE likely occurs through direct injury to the cells by the virus, or the development of humoral and cellular immune reactions specific for virus-encoded proteins, virus-induced host molecules, or virus-altered host molecules. In addition, the ability of some viruses to transform and selectively alter the function of cells, including endothelial cells, macrophages, and lymphocytes, could also be the basis for the biological phenomena characteristic of SLE. More recently, superantigens have been proposed to play a role in autoimmune disease (Friedman et al., 1991). Superantigens are a group of unprocessed bacterial and viral proteins that activate T cells in vivo and in vitro by directly binding to the lateral exposed surface of MHC class II molecules on antigen presenting cells (APC) and to the variable region of the T cell receptor (TCR) ß chain on the responding T cells. Bacterial or viral superantigens can interact with a large fraction of T cells, resulting in cellular activation, proliferation, anergy, or deletion of specific T cell subset (Phillips et al., 1995). Studies have suggested that superantigens could stimulate autoantibody production by activating normal T cells and providing a molecular bridge that facilitates their interaction with autoantibody-producing B cells (Friedman et al., 1991; Tumang et al., 1990). Also, other studies have suggested that superantigens may trigger autoimmunity by directly facilitating the activation of previously silent (i.e. anergic or ignorant) autoreactive T cell clones. Superantigens may do this by promoting cell division, which may drive an anergic T cell out of its nonresponsive state, or by lowering the threshold level of a normally cryptic self antigen that is required for autoreactive T cell stimulation (Behar & Porcelli, 1995). Although there are as yet no compelling data showing that superantigens are responsible for causing human autoimmune diseases such as SLE, or

for inducing flares of disease activity, it is possible that certain autoimmune diseases associated with viral infection may be mediated by superantigen production.

Ultraviolet Irradiation. Ultraviolet (UV) irradiation influences the clinical and immunopathologic expression of skin disease in SLE (about 40-50% of SLE patients suffer from photosensitivity), and a small percentage of patients can experience systemic exacerbations after sun exposure. The role of UV irradiation in SLE has been reviewed (Sauder et al., 1993; Norris, 1993). The mechanisms mediating UV induction of the disease are not known, although autoimmunity involving photodenatured DNA has been demonstrated experimentally (Tan & Stoughton, 1969; Davis & Percy, 1978). Other potential mechanisms include: (1) increased release of immune mediators, such as interleukin-1, tumour necrosis factor-α, prostaglandin E, proteases and histamine in the skin; (2) direct impact on immunoregulatory cells, such as cutaneous T-cells, which normally help suppress cutaneous inflammation; (3) activation of latent virus infection; and (4) exposure of self-antigens, such as heat shock proteins or ribonucleoproteins, which could lead to changes (either locally or systemically) in immunoregulatory processes (Zappi and Sontheimer, 1993; Mckenzie and Sauder, 1994).

SECTION 3

ANTI-Ro AUTOANTIBODIES

An apparently large number of different autoantibodies occur in SLE, and their production is considered to be the primary abnormality of this disease (Smith & Steinberg, 1983). Autoantibodies, which are directed against DNA, histones, RNA, phospholipid and ribonucleoproteins (e.g. Ro, La, Sm and (U1)RNP), have been shown to correlate with clinical features of SLE, and some of them are directly involved in pathogenesis. Anti-DNA antibodies are associated with the development of lupus glomerulonephritis (Koffler et al., 1971) and have been isolated from nephritic kidneys of patients with that disease (Koffler et al., 1974). Anti-Ro autoantibodies are found in SLE patients who are more likely to develop skin lesions, cutaneous vasculitis, myositis, neurologic disease and pulmonary disease, and to have babies with neonatal lupus (Provost et al., 1988).

This section will focus on anti-Ro autoantibodies and discuss their clinical significance and possible origins.

3.1 CLINICAL ASSOCIATIONS

Anti-Ro autoantibody was first described in lupus patients more than 20 years ago (Clark et al., 1969), and was subsequently found to be identical to anti-Sjögren's syndrome antigen A (SS-A) autoantibody in the sera of patients with Sjögren's syndrome (Alspaugh & Tan, 1975, Alspaugh & Maddison, 1979). In fact, 90% of patients with Sjögren's syndrome have anti-Ro antibodies (Ben-Chetrit, 1993). Later, its clinical importance in SLE was demonstrated in subacute cutaneous lupus erythematosus and neonatal lupus erythematosus (Sontheimer et al., 1979; Bangert et al., 1984; France et al., 1981; Waston et al., 1984).

3.1.1 Subacute Cutaneous Lupus Erythematosus

Subacute cutaneous lupus erythematosus (SCLE) is a distinct subset of lupus erythematosus (Sontheimer et al., 1979; Bangert et al., 1984). Clinically, it consists of non-scarring papulosquamous or annular skin lesions, or both, which have an LE specific histopathology and occur on the sun-exposed areas of the body. Patients with SCLE have a low incidence of systemic disease with vital organ involvement, such as central nervous system or renal disease, compared with others. Serologic studies have demonstrated a unique association of the SCLE subset with the presence of anti-Ro antibodies (Provost & Waston, 1993). Depending on the method of antibody detection, approximately 70-90% of SCLE patients were found to have circulating anti-Ro antibodies (Ben-Chetrit, 1993). In contrast, these antibodies were detected only in 25-40% of unselected SLE patients.

3.1.2 Neonatal Lupus Erythematosus

Neonatal lupus erythematosus (NLE) is an uncommon clinical syndrome associated with the presence of maternal IgG autoantibodies, predominantly anti-Ro antibodies, which pass through the placenta into the fetus (France et al., 1981; Waston et al., 1984). The neonatal infants with NLE typically have nonscarring skin lesions in sun-exposed areas that are very similar to SCLE, and/or isolated complete congenital heart block (CCHB) (Scott et al., 1983). CCHB is characterized histologically by a lack of connection between atrial conduction tissue and the atrioventricular node, absence of the atrioventricular node, or disruption of the atrioventricular bundle. Maternal anti-Ro antibodies can be detected in almost 100% of the affected infants in their first few months of age, and then disappear from these infants' sera at about the time that cutaneous lesions resolve (Lee, 1984; Lee & Weston, 1988). Maternal anti-Ro autoantibodies are thought to be important in causing NLE.

3.2 PATHOGENETIC IMPORTANCE OF ANTI-Ro ANTIBODIES

Limited organ involvement has been shown in the anti-Ro-associated diseases, SCLE and NLE. Although it is not proven that anti-Ro antibodies are directly

responsible for the organ tissue damage, the following studies provide interesting support for such a possibility.

3.2.1 Skin Lesions

Photosensitive skin lesions are one of the dominant clinical features in SCLE and NLE (Tan et al., 1984). The observation that the duration of the skin lesions in NLE patients roughly parallels the duration of the maternally derived IgG anti-Ro antibodies in their serum supports a pathogenic role for anti-Ro antibodies in the production of the cutaneous disease (Lee, 1984).

When considering the possible pathogenetic role of anti-Ro antibodies in disease, one has to first establish that these antibodies bind to related target tissues, such as skin. The first evidence for anti-Ro antibody binding to skin was found in cultured neonatal keratinocytes (LeFeber et al., 1984). Anti-Ro antibody-containing sera demonstrated a particulate (speckled) pattern on cultured keratinocyte cell nuclei and cytoplasm. After UV irradiation of the cells, augmentation of antibody binding was seen on the keratinocyte surface (LeFeber et al., 1984). When monospecific anti-Ro sera were infused into nude mice grafted with human skin, an animal model of cutaneous lupus, IgG anti-Ro antibodies, but not control antibodies, preferentially bound to the grafted human skin (Lee et al., 1986; Jones et al., 1988). The adjacent mouse skin, which did not contain Ro antigens, showed no antibody binding. This IgG binding occurred in epidermal keratinocytes and was augmented by UV light exposure. Subsequent studies demonstrated that the pattern of this immunoglobulin deposition was identical to that found in biopsies from NLE and SCLE skin lesions (Lee et al., 1989). Other studies also found that anti-Ro antibodies are active in antibody-dependent cellular cytotoxicity (ADCC) (Norris et al., 1984) and their injection into the guinea pig skin, following UV irradiation, significantly enhanced the microvascular skin damage (Davis et al., 1989).

3.2.2 Cardiac Lesions

The characteristic cardiac lesion in NLE is isolated complete congenital heart block (CCHB), which occurs in approximately half of the cases of NLE (Lee et al.,

1988). The recognition that anti-Ro antibodies are important in the pathogenesis of CCHB was based on the observations that all mothers of affected infants had circulating anti-Ro antibodies. High titers of maternal IgG anti-Ro antibodies have been associated with an increased risk for this disease (Derksen & Meilot, 1992; Lee et al., 1994), and the greatest association between anti-Ro antibodies and CCHB was found with autoantibodies to 52kD/Ro rather than to the 60kD/Ro antigen (Buyon et al., 1989). More recently, high concentrations of anti-Ro antibodies were found in eluates from the heart of a child who died with CCHB (Reichlin et al., 1994).

Studies in human fetal heart tissues, using immunofluorescence analysis, have demonstrated that monospecific sera containing anti-Ro antibodies gave speckled nuclear staining in the myocardial cells as well as conduction system cells (Deng et al., 1987). The binding of anti-Ro antibodies to the surface of myocardial fibres in fetal cardiac tissue with CCHB has also been reported (Horsfall et al., 1991). These studies provided evidence that the Ro antigen is present in the human fetal cardiac tissue and expressed on the cell surface, where it is accessible for antibody binding. However, an intriguing question is how the intracellular Ro antigens are exposed on the cell surface.

The development of CCHB in affected infants is likely related to the ontogeny (development) of the neonatal heart. Studies of several hundred adult anti-Ro positive lupus patients demonstrated the rarity of conduction defects in adult SLE (Bilazzarian et al., 1989). When an in vitro rabbit heart model was developed to explore the pathologic role of anti-Ro antibodies in CCHB (Alexander et al., 1992), it was demonstrated that neonatal, but not adult, rabbit cardiac muscle tissue perfused with anti-Ro antibodies produced prolongation of the repolarization phase of the cardiac action potential. Similarly, another in vitro study indicated that 40% of rabbit hearts perfused with human anti-Ro positive sera developed electrocardiographic evidence of complete heart block (Garcia et al., 1992).

3.3 PRODUCTION OF ANTI-Ro ANTIBODIES

3.3.1 Genetic Control

Work from several laboratories has demonstrated that the anti-Ro antibody immune response in SLE patients is strongly associated with the HLA-DR2 and/or the HLA-DR3 phenotype (Hochberg et al., 1985; Batchelor et al., 1987). A gene interaction effect, found at the HLA-DQ locus, is associated with enhanced production of anti-Ro antibodies (Harley et al., 1986; Fujisaku et al., 1990; Reveille et al., 1991). Patients who were DQ1/DQ2 heterozygotes had higher titers of anti-Ro antibody responses than did patients with any other combination of HLA-DQ. Nucleotide sequence analysis of the alleles of HLA-DQ associated with enhanced antibody production showed that 100% of patients with anti-Ro antibodies had a glutamine residue at position 34 of the outermost domain of the DQA1 chain and/or leucine at position 26 of the outermost domain of the DQB1 chain. These amino acids map to the region that forms the "floor" of the major histocompatibility peptide binding cleft, and are located in the second hypervariable regions of the HLA DQ molecules (Figure 1). However, HLA-DQA1 and DQB1 alleles not associated with anti-Ro antibodies did not possess the same amino acids in these positions.

Associations have also been demonstrated between anti-Ro antibodies and TCR ß genes, in that a restriction fragment length polymorphism of the TCR ß chain was found in a high frequency of anti-Ro positive SLE patients (84%), compared with anti-Ro negative SLE patients (41%) or healthy controls (52%) (Frank et al., 1990).

3.3.2 Antigen Selection

One mechanism for the generation of autoantibodies is "antigen selection", which means that some antigens themselves select and drive the autoantibody response. There is evidence supporting antigen-selected anti-Ro antibody production. Firstly, of the 100,000 proteins in the cell, fewer than 100 are targets of the autoantibody response in rheumatic disease and they include the Ro ribonucleoprotein antigens (Venables et al., 1991). This argues against a random generation of autoantibodies and suggests a selective mechanism. Secondly, the autoantibody response in a given serum sample is

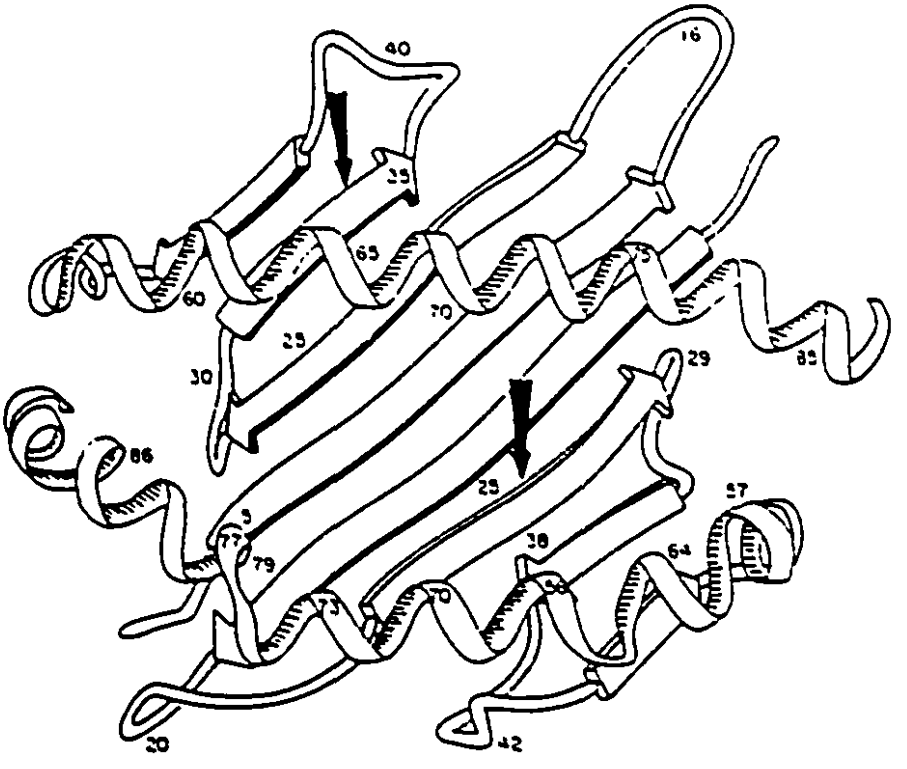

Figure 1. Model for the MHC class II peptide-binding groove in patients with anti-Ro autoantibodies. Arrows demonstrate glutamine substitution at position 34 of DQA1 chain and leucine at position 26 of the DQB1 chain. These amino acids map to the region that forms the "floor" of the MHC peptide binding cleft (adapted from Provost and Watson, 1993).

often directed against different polypeptides in the same molecular complex (Buchanan et al., 1983; Venables et al., 1983), or different epitopes on the same polypeptide (Williams, 1990). This suggests that the autoantibody response is driven by the molecule itself. Previous studies have shown that there are multiple epitopes on the Ro antigen, and that anti-Ro antibodies react preferentially with human Ro (Reichlin, 1991; Bozic et al., 1993; Blange et al., 1994; Ricchiuti et al., 1994). Lastly, idiotypes on autoantibodies to ribonucleoproteins are restricted or "private", suggesting antigen-selected affinity maturation of the antibody response (Horsfall et al., 1986).

3.3.3 Molecular Mimicry

Another theoretical mechanism for the induction of autoantibodies is "molecular mimicry" , which proposes that autoantibodies are induced by cross-reaction between foreign and self epitopes. Although there is limited data in support of molecular mimicry for anti-Ro antibody production, recent studies have found that a sequence in one of the major epitopes of the 60kD/Ro protein shares 7 of 8 amino acids with the nucleocapsid (N) protein from vesicular stomatitis virus (VSV) (Scofield et al., 1991; Scofield & Harley, 1991). Subsequent studies showed that anti-VSV N protein antibodies were detected more frequently in anti-60kD/Ro positive SLE sera (41%) than in anti-60kD/Ro negative SLE sera (20%) (Hardgrave et al., 1993). These data suggest that there may be a relationship between reactivity to the viral N protein and 60kD/Ro autoantigen (Routsias et al., 1994), but no direct causal effect of the virus has been established in this case.

3.3.4 Exposure of Self-antigen Ro

A further possibility for the production of autoantibodies is the exposure of self-antigens to the immune system. This raises the question of how Ro antigens, normally hidden within the cell, become accessible for the immune system to induce an autoantibody response. A traditional view is that cell damage, either by the inciting virus or by chronic inflammation, leads to the release of the cellular Ro antigens. However, a major difficulty with this theory is that there is no explanation as to why only a

minority of the 100,000 intracellular antigens released under these circumstances become targets of autoimmunity. The simplest explanation for immune recognition of intracellular Ro antigens is that they are presented on the cell surface under certain conditions. This challenging topic will be discussed in the following section, 4.3, and is a major theme of Chapters III, IV and V of this thesis.

SECTION 4

Ro AUTOANTIGENS

Ro antigen was first considered as a target of lupus specific autoantibodies (Clark et al., 1969) in 1969 and was later identified as SS-A antigen in Sjögren's syndrome (Alspaugh & Tan, 1975). In 1979, it was demonstrated that anti-Ro antibodies were immunological identical to anti-SS-A antibodies, suggesting that the Ro and the SS-A antigens were identical proteins (Alspaugh & Maddison, 1979).

4.1 CHARACTERIZATION OF Ro ANTIGENS

In human cells, Ro antigen is present as a ribonucleoprotein (RNP) complex (Lerner et al., 1981; Wolin & Steitz, 1984). The RNA moiety of the Ro RNP complex is comprised of a group of small, uridine rich RNA, called hYRNA (h-human; Y-cytoplasmic). Four unique types of hYRNA have been identified: hY1, hY3, hY4 and hY5 (hY2 is a cleavage product of hY1). These RNAs range from 80 to 112 nucleotides and are present in low abundance (only 10^5 copies per cell) (Hendrick et al., 1981; Wolin & Steitz, 1983). Each hYRNA is contained in a different RNP complex and the binding is via a highly conserved double-stranded stem formed by the base-pairing of the 5' and 3' ends of the RNA molecules. Four proteins associated with Ro hYRNAs have been described (Rader et al., 1989; Slobbe et al., 1991). Two of these, with molecular weights of 60kD and 52kD, were derived from lymphocytes, while the other two proteins (60kD and 54kD) were found in red blood cells. They are immunologically distinct proteins, as shown by the reactivity of different patient sera with each one of them (Rader et al., 1989). More recently, the genes encoding two of the four Ro proteins (60kD and 52kD) have been sequenced and no sequence homologies were found between them.

4.1.1 60kD/Ro

The 60kD Ro polypeptide was the first protein to be cloned and sequenced (Deutscher et al., 1988; Ben-Chetrit et al., 1989). Its amino acid sequence was found to contain a RNP consensus motif and a zinc finger motif. In addition, the 60kD/Ro protein has been demonstrated to associate directly with hYRNAs in reconstitution studies (Deutscher et al., 1988). It. hYRNA binding activity may be mediated by the RNP consensus motif, while the zinc finger is likely involved in nucleic acid or protein binding (Frankerl & Pabo, 1988). Homology between a highly antigenic region in the carboxyl-terminus (C-terminus) of the 60kD/Ro peptide and a nucleocapsid protein of vesicular stomatitis virus (VSV) has been reported (Scofield & Harley, 1991), raising the possibility of anti-Ro autoantibody development secondary to viral exposure.

4.1.2 52kD/Ro

A full length cDNA of the 52kD/Ro protein has been obtained (Chan et al., 1991; Itoh et al., 1991). The amino acid sequence predicted by this cDNA contains zinc finger sequences, a leucine zipper motif and a C-terminal "rfp-like" domain, but does not contain the RNA co..sensus sequence seen in the 60kD/Ro. The 52kD/Ro protein may associate with the hYRNA through protein-protein interactions with the 60kD/Ro (Slobbe et al., 1992). This is supported by the findings that: (1) autoimmune sera, specific for either a 52kD/Ro or a 60kD/Ro, can immunoprecipitate hYRNAs from cellular extracts and (2) the Ro 52kD protein associates with the hYRNAs via protein-protein interactions with the Ro 60kD protein in reconstitution experiments (Slobbe et al., 1992). The 52kD/Ro amino acid sequence has significant homology to the human ret transforming protein and the murine T-cell regulatory protein rpt-1 (Chan et al., 1991; Itoh et al., 1991), which are thought to play a role in gene regulation.

4.1.3 Calreticulin

cDNA cloning provided the complete amino acid sequence for the 46kD protein (McCauliffe et al., 1990a), which migrates aberrantly at 60kD in sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE) because of a charge-related gel migration

artifact and was initially thought to be a Ro component. Its amino acid sequence includes a hydrophobic leader, a KDEL C-terminal endoplasmic reticulum (ER) retention signal sequence, and a calcium binding domain. However, there are no RNP consensus sequences, zinc finger or leucine zipper motifs. UV light cross-linking studies demonstrated that the 46kD protein directly binds to hYRNA (McCauliffe et al., 1990a). Analysis of the protein sequence showed that this protein does not share homology with either the 52kD/Ro or the 60kD/Ro, but it is highly homologous to murine and rabbit calreticulin (McCauliffe et al., 1990a, 1990b), an ER and sarcoplasmic reticulum (SR) calcium binding protein (Smith & Koch, 1989). Recently, the 46kD protein has been redefined as a new human autoantigen, called calreticulin, since subsequent studies found that its recombinant form does not react with anti-Ro autoantibodies (Rokeach et al., 1991).

Studies on the relationships between 60kD/Ro, 52kD/Ro and calreticulin have indicated that these three proteins are heterogeneous, are associated with hYRNAs (directly or indirectly), and may physically interact with each other. Their proposed relationship is shown in Figure 2.

4.2 CELLULAR LOCALIZATION AND FUNCTION

4.2.1 Cellular Localization of Ro Antigens

Ro antigens appear to be virtually ubiquitous in human tissue, and they have been demonstrated in lymphocytes, erythrocytes, fibroblasts, keratinocytes and epithelioid cells. Although much is known about the constituents of Ro antigens, their cellular location is still controversial. Previously, some investigators have observed Ro antigens in the cytoplasm, while others found them in the nucleus, or both the nucleus and cytoplasm. At the time that these studies were performed, the antibody heterogeneity in anti-Ro sera was still unknown.

Recently, immunofluorescence studies, using highly monospecific antibodies, have allowed more precise cellular localization of Ro antigens. Using monospecific anti-60kD/Ro antibody, it was shown that 60kD/Ro localized primarily in the cytoplasm (Horsfall et al., 1991), similar to other hYRNA binding proteins. Also, 60kD/Ro

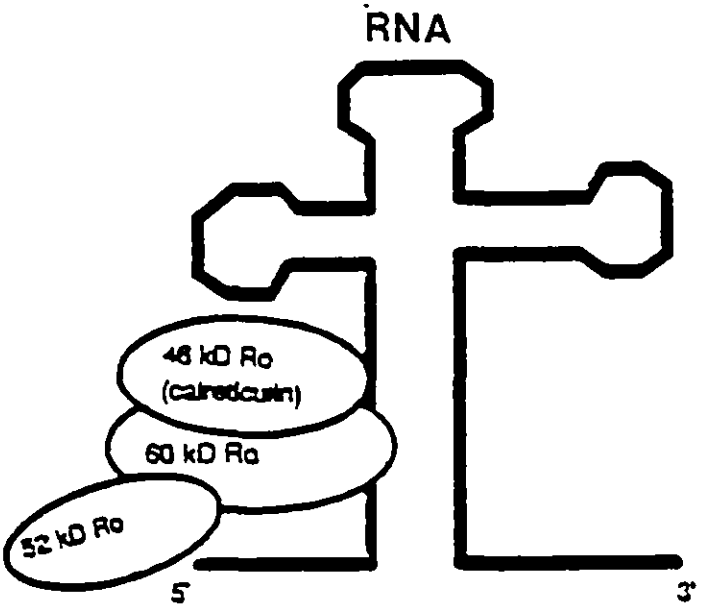

Figure 2. Model for Ro autoantigen structure. Ro antigens are heterogeneous and may exist as multimeric units consisting of hYRNA. The 60kD/Ro protein, which contains an RNA binding motif, is believed to be directly associated with hYRNA. The 52kD/Ro protein may associate with the hYRNA through protein-protein interactions with the 60kD/Ro. UV light cross-linking studies demonstrated that the 46kD/Ro protein (calreticulin) binds directly to hYRNA, and there is some evidence that the 60kD/Ro and 46kD/Ro proteins might be physically associated (adapted from Zappi and Sontheimer, 1993).

antigen was found in human erythrocytes (Rader et al., 1989; Byers et al., 1990), which do not contain a nucleus. In contrast, 52kD/Ro was detected predominantly in the nucleus by anti-52kD/Ro antibodies (Ben-Chetrit et al., 1988; Slobbe et al., 1991). Theoretically, 52kD/Ro could reside in the nucleus, since it has a C-terminal "rfp-like" domain that is usually shared by nuclear proteins (Chan et al., 1991). Antibodies specific for calreticulin, the 46kD protein, have revealed perinuclear cytoplasmic staining by immunofluorescence (Sontheimer et al., 1990; Milner et al., 1991). Based on the amino acid sequence anaiysis, the 46kD protein contains a C-terminal KDEL sequence. This sequence has been shown to be crucial for the retention of proteins in the ER/cytoplasm (McCauliffe et al., 1990a).

However, there is some evidence that 60kD/Ro and 52kD/Ro antigens colocalize in the nucleus as well as in the cytoplasm (Slobbe et al., 1991). There may be a cross-reactive epitope on these proteins recognized by antibodies specific for 60kD/Ro or 52kD/Ro antigens (Iton & Reichlin, 1991; Ben-Chetrit, 1993), or the presence of Ro RNP, such as 52kD/Ro, in the nucleus may represent a stage of RNP assembly in transit to the cytoplasm, where its action is required.

4.2.2 Cellular Function of Ro Antigens

Although much effort has been invested in studying Ro proteins, the cellular functions of Ro antigens remain obscure. Possible roles that have been proposed include: direct involvement in protein translation (Sontheimer et al., 1993); storage of processed mRNA (Wolin & Steitz, 1983, 1984); and a role as a transcription factor (Chan et al., 1991; Iton et al., 1991). However, different Ro proteins may exert distinct functions, which may be reflected in their different biochemical composition, behaviour, and cellular localization (Table 1).

4.3 CELL SURFACE EXPRESSION OF Ro ANTIGENS

Ro antigens are normally located within the cells, but certain cellular events can result in these intracellular proteins being expressed on the cell surface. UVB irradiation of cultured human keratinocytes has been demonstrated to provoke the appearance of Ro

Table 1. Characteristics of Ro autoantigens

M.W. (kD)	Localization	Function	Homology
60	Predominantly cytoplasm ± nucleus	RNA binding	VSV nucleocapsid protein[a]
52	Predominantly nucleus ± cytoplasm	Gene regulation	rpt-1[b], ret[c]
46	cytoplasm	Ca^{++} binding	Calreticulin[d]

[a]The C-terminal amino acid residues of the 60kD protein share seven of eight amino acids with the nucleocapsid protein from the Indiana serotype of VSV (Scofield et al., 1991).
[b]The amino-terminal halves of the 52kD protein show homology with the murine T-cell regulatory protein rpt-1 (39% identity) (Chan et al., 1991).
[c]The entire 52kD protein sequence shows a high degree of homology with human ret transforming protein (Chan et al., 1991).
[d]The amino acid sequence of the 46kD protein is homologous to murine and rabbit calreticulin (94% and 92%, respectively) (McCauliffe et al., 1990).

antigen on the cell surface (Lefeber et al., 1984). This finding was initially demonstrated by indirect immunofluorescence techniques (Lefeber et al., 1984) and, subsequently, it was shown by FACS analysis that UVB irradiation resulted in increased binding of anti-Ro antibodies to the surface of cultured keratinocytes (Furukawa et al., 1990). This was later confirmed by other studies (Jones, 1992; Golan et al., 1992). Another example of the cell surface expression of Ro antigens occurs with estrogen treatment of human keratinocytes (Furukawa et al., 1988). In the latter study, high concentrations of estradiol increased binding of IgG from sera specific for Ro antigens, and this augmentation was partially inhibited by nafoxidine, an anti-estrogen receptor agent. However, these studies did not demonstrate which component of the Ro antigens was expressed on the cell surface.

Studies have also demonstrated the cell surface expression of Ro antigens in human tissues. In fetal cardiac tissue, for example, Ro antigens were detected in the nuclei of normal myocardial cells by indirect immunofluorescence (Deng et al., 1987), while the strong myocardial surface fluorescence compatible with membrane expression of Ro was found in the CCHB fetal heart (Horsfall et al., 1991). These data suggest that cell surface expression of Ro antigens occurs in the organ targeted by the inflammatory response.

Two implications arise from such surface expression of Ro antigens: firstly, it provides a potential mechanism for the induction of specific antibodies by these autoantigens, and, secondly, it could contribute to pathogenesis by providing a target for immune recognition and destruction. Both of these are attractive hypotheses and, occurring together, they could explain many of the immunopathogenic features of SLE.

CHAPTER II

RATIONALE AND OBJECTIVES

Viruses have been suggested as triggers for the induction of SLE (Phillips, 1988) because of their elusive nature, their role in the induction of autoimmunity and evidence of viral infection in human SLE patients and murine lupus models, as discussed in Section 2 of Chapter I. In this study, human CMV was proposed as a potential candidate in the induction of Ro autoantigen expression based on a number of findings. First, CMV is a common infectious agent worldwide, which infects 50-100% of the population, targets all organ systems of the host, and persists in the host for life (Gold & Nankervis, 1982; Alford & Britt, 1990). Second, elevated anti-CMV antibodies (Pincus et al.,1978; Zhang et al., 1991), as well as CMV-induced flares (Bulpitt & Brahn 1989; Vasquez et al., 1992), have been reported in SLE patients. Third, CMV RNA has been immunoprecipitated from CMV-infected fibroblasts by SLE sera containing anti-Ro antibodies (Lord et al., 1989). These data suggest that patients with SLE may have a different immune response to CMV infection than normal individuals or that, following CMV infection, viral RNA may interact with cellular Ro proteins, resulting in "altered self" proteins. One possibility is that CMV infection induces the expression of intracellular Ro antigens on the cell surface and, thus, makes these cells targets of the immune response (Figure 1). Therefore, the objective of this study was to investigate the effect of virus infection on the induction of Ro autoantigen expression in human cells and to determine: (1) whether human CMV, alone or in combination with other stimuli (in particular, UVB irradiation), can induce the surface expression of Ro autoantigens on cultured human cells, and (2) which Ro autoantigens are perturbed by clinically relevant stimuli, such as CMV infection and UVB irradiation.

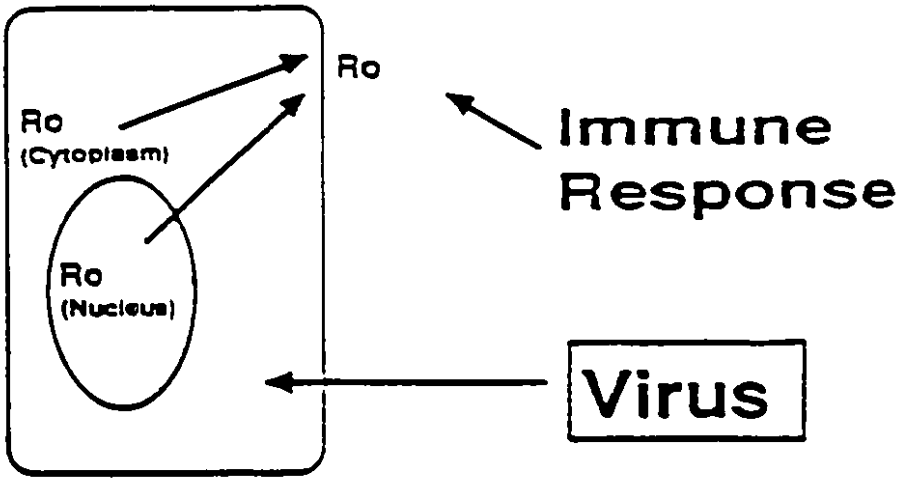

Figure 1. Model for viral induction of Ro antigen expression. A virus infection may result in Ro antigens being expressed on the cell surface and make these cells targets of the immune response. The immune response could then recognize these surface antigens and mediate cellular damage through mechanisms such as complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity (based on McCauliffe and Sontheimer, 1993).

CHAPTER III

Ro antigens are targets of specific IgG autoantibodies found in the circulation of many patients with SLE. The redistribution of Ro antigens from their normal intracellular location to the cell surface, which is potentially important in disease pathogenesis, may be affected by environmental factors. The paper presented in this chapter is a study on viral induction of the cellular expression of the Ro antigen complex. Total cellular and cell surface expression of 60kD/Ro antigen and calreticulin were examined in CMV-infected human fibroblast cells.

VIRAL INDUCTION OF THE HUMAN AUTOANTIGEN CALRETICULIN

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Abstract

Specific, but ubiquitous cytoplasmic proteins are the targets of autoantibodies such as anti-Ro/SS-A, anti-La/SS-B and anti-calreticulin. These antibodies may be pathogenic in systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). Tissue localization of the pathogenic process could be best explained by an abnormal expression of these cytoplasmic proteins. Several factors could likely displace the host proteins to the cell surface. This study was designed to use cytomegalovirus (CMV) infected human fibroblasts (MRC-5) as a model, to test whether a viral infection would induce the expression of the human autoantigen(s). Expression of Ro/SS-A, calreticulin and MHC class I antigens, both in the cytoplasm and on the cell surface, was examined by a fixed cell ELISA, immunofluorescence and immunoblotting. Infection of fibroblasts with CMV was found to increase the cell surface expression of calreticulin ($P=0.0314$), but not the 60kD Ro/SS-A. Cytoplasmic expression of both the autoantigens tested increased following CMV infection. Enhanced expression of class I MHC was detected on the cell surface in response to the virus infection. The expression of the autoantigens and MHC class I polypeptides as well as the virally induced elevated mitotic rate, diminished after 24 hrs of infection. Viral infection was found to alter the distribution of host cell proteins including autoantigens. Cell surface expression of calreticulin could provide a target for circulating autoantibody and contribute to the autoimmune process.

Résumé

Des protéines cytoplasmiques, mais très répandues, sont la cible d'autoanticorps spécifiques pour les Ro/SS-A, les La/SS-B, et les calréticuline. Ces anticorps peuvent être pathogéniques du lupus érythémateux systémique (SLE) et du syndrome de Sjögren (SS). La localisation des processus pathogéniques tissulaires est expliquée par une expression anormale de ces protéines cytoplasmiques. Plusieurs facteurs pourraient déplacer ces protéines intracellulaires vers la surface de la cellule. Notre étude a été effectuée pour vérifier si une infection virale pouvait induire l'expression de ces autoantigènes chez l'homme. Nous avons utilisé des fibroblastes humains MRC-5 infectés avec un cytomégalovirus (CMV) comme modèle. L'expression de Ro/SS-A, la

calréticuline, et les antigènes d'histocompatibilité de classe I ont été examinés sur la surface et dans le cytoplasme cellulaire au moyen d'ELISA, d'immunofluorescence, et d'immunobuvardage. L'infection des fibroblastes avec le CMV a augmenté l'expression de surface de la calréticuline (p = 0.0314), mais pas du Ro/SS-A de 60KD. L'expression cytoplasmique des autoantigènes testés a augmenté à la suite de l'infection. Une expression plus marquée des antigènes d'histocompatibilité de classe I a été retrouvée sur la surface cellulaire en réponse à l'infection virale. L'expression des autoantigènes et des polypeptides d'histocompatibilité, de même que l'élévation du taux de mitoses induites par l'infection, ont diminué après 24 h. L'infection virale a altéré la distribution des protéines cellulaires, incluant les autoantigènes. L'expression sur la surface cellulaire de la calréticuline peut devenir le site d'attaque d'autoanticorps circulants et contribuer ainsi au processus autoimmun.

Introduction

The Ro/SS-A complex includes intracellular ribonucleoprotein (RNP) particles consisting of several proteins complexed to one of 4 small RNAs [1]. Three Ro/SS-A proteins (60kD, 52kD and 54kD), calreticulin and La/SS-B, all apparently components of this complex, have been cloned and sequenced [2-6]. The function of this RNP complex has not been determined. These autoantigens appear to induce high levels of antibodies in patients with systemic lupus erythematosus (SLE) and/or Sjögren's syndrome (SS) [7,8]. The autoantibodies likely play an important role in the pathogenesis of these autoimmune diseases, as has been demonstrated by observations in infants with neonatal lupus [9-12]. The mechanisms leading to the production of autoantibodies are rather complex and both genetics and environmental factors are likely involved. There is much interest in determining how these proteins become autoantigens from normal components of human cells.

Viruses have been suggested as an etiological factor for autoimmune diseases. One attractive hypothesis is that a virus infection or the reactivation of a latent infection may cause certain cellular proteins to be expressed on the cell surface. Such altered expression could either induce a host immune response (in genetically susceptible

individuals) or become the target of circulating autoantibodies. The persistent autoantibodies could lead in turn to an increased risk in these subjects for the development of SLE or SS.

Epidemiology studies have shown a high infectivity rate of cytomegalovirus (CMV) in all populations [13]. Infection with CMV, a DNA virus of the herpes family, is associated with significant morbidity and mortality in a variety of clinical syndromes involving immunosuppression. Autoimmune diseases such as rheumatoid arthritis, SLE or SS [14-18] have been shown to follow CMV infection. The virus has been isolated from synovial cells [19] and viral antigens have been detected in phagocytosed serum complexes from the patients with rheumatoid arthritis [20]. In addition, viral RNA from transcribed CMV has been shown to complex with cellular RNP [21,22]. In this article we describe the first experimental *in vitro* model to study the impact of CMV infection on the induction of the human autoantigen calreticulin in human fibroblasts, and demonstrate that CMV infection enhances the expression of host proteins on the cell surface. This study may provide a mechanism to explain how the autoantigens are induced on the cell surface. This in turn could make them the targets of the circulating autoantibodies.

Materials and Methods

Cells and viruses

The human embryo lung fibroblast cell line MRC-5 obtained from American Type Culture Collection (ATCC) was cultured at 37°C in an atmosphere of 5% CO_2, in DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated calf serum (HICS) (GIBCO) and antibiotics. Stock cultures of human CMV (HCMV, Davis) were prepared by propagating the virus in MRC-5 cells. The titre of infectious virus was determined by plaque assays [23]. Cells were infected with 10^3 plaque-forming units (PFU) of CMV. After a 30 minutes adsorption period, the infected cells were maintained in DMEM containing 2% HICS and antibiotics.

Antibodies

Peptides specific for the amino terminal region (Cal 7-23) of calreticulin (60KD) protein (KEQFLDGDGWTSRWIES) [4], the carboxyl terminal region (489-499) of classical Ro/SS-A (60KD) protein (RKKMDIPAKLIVC) [3] and a region (amino acids 129-143) of the 52KD/Ro protein (EEAAQEYQEKLQVAL) [5] respectively were synthesized (Multiple Peptide, San Diego, CA). All of the peptides are hydrophilic based on the computer analysis (GCG, genetics computer group Sequence Analysis Software Package) of the nucleic acid sequence and its encoded amino acid sequence [24]. Anti-peptide hyperimmune sera were prepared by immunizing female rabbits (New Zealand White) with individual peptides coupled to keyhole limpet haemocyanin (KLH). Peptide specific antibodies were purified by affinity chromatography using Protein A-affigel (Bio-Rad, Richmond, Ont.) followed by peptide-affigel column chromatography. $F(ab')_2$ fragments were prepared by digesting with pepsin (Boehringer Mannheim, Bae d'Urfe, Que.) in 0.2 M sodium acetate IgG:pepsin = 100:2 (w/w). The reaction mixture was incubated at 37°C for 1.5 hours, after which the pepsin was inactivated by adding 3 M Tris to bring the pH to 7. Complete digestion of the IgG was monitored by SDS-PAGE.

The specificity of the antipeptide antibodies was measured in an ELISA. In brief, 96-well Immobulon I plates (Fisher, St. Laurent, Que.) were coated with peptide (conjugated or not-conjugated) overnight at 4°C in carbonate/bicarbonate buffer. After washing the plates with PBS/Tween 20 (1.5 mM monobasic potassium phosphate, 8 mM dibasic sodium phosphate, 0.15 M NaCl, 2.6 mM KCl, 0.05% Tween 20, pH 7.4) three times, 100 μl of the affinity purified fragments were added. After an incubation of 30 minutes at 4°C, the plates were washed and a biotinylated goat-anti-rabbit IgG (1/500 dilution) (Vector, Mississauga, Ont.) was added. After a further incubation of 30 minutes at 4°C, the plates were washed three times. Avidin D conjugated alkaline phosphatase (Vector) was added at the recommended dilution, and the plates were incubated for 30 minutes at 4°C. After washing, the substrate, p-nitrophenyl phosphate disodium (Sigma, St. Louis, MO) was added. Plates were incubated at 37°C for 20-30 minutes until sufficient colour developed. Results were expressed in OD405 nm units as the mean of duplicates and three identical experiments were done. Human class I

MHC glycoproteins were detected using mouse monoclonal anti-HLA-ABC IgG2a (1/800 dilution) (Chemicon, Temecula, CA). CMV immedate early (IE) antigens were detected using a mouse monoclonal anti-CMV IE antibody (1/10[illegible] dilution) (kindly provided by Dr. B. Brodeur, Ottawa). La/SS-B was detected using $F(ab')_2$ fragments of IgG isolated from a patient with SLE who was specific for anti-La and anti-52kD Ro by Immunoblot procedures.

Cell extracts

MRC-5 cells were growth in 10% HICS, DMEM medium as previously described. Cells were scraped and collected by centrifugation. Cells were then washed in PBS and suspended in Net-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Nonidet P-40, 50 mM TLCK, 0.1 mM PMSF, 0.1% pepstatin A) at 4°C for 30 minutes, followed by sonication (Model G112SP1T, 600 Volts, 80 KC, 15 AMPS) for 10 minutes. The supernatants were collected after centrifugation and samples were prepared for electrophoresis using a total protein content of 2×10^5 solubilized cell equivalents per lane.

Immunoblotting

Proteins of the human fibroblast cell (MRC-5) extract were separated by SDS-PAGE using 12% polyacrylamide gel electrophoresis (under reduced condition) and the ratio of acrylamide: bis-acrylamide was 30:0.8 in the resolving gel [25]. Proteins were transferred from the gel onto nitrocellulose using a constant voltage of 60V for 2 hours at 4°C [26]. The nitrocellulose sheet was incubated in PBS with 0.05% tween 20 at 4°C for 16 hours to block nonspecific binding sites. The nitrocellulose strips were incubated with a 1:25 dilution of sera at room temperature for 16 hours and then washed in PBS/Tween 20. For inhibition studies, the anti-peptide sera were pre-incubated with the specific peptide (1 mg/ml) at room temperature for 1 hour. Peroxidase-conjugated goat anti-rabbit IgG reagents (1:1000) (CAPPEL, Organon Teknika Corp, West Chester, PA.) was used to detect bound rabbit anti-peptide antibodies.

Detection of CMV and Ro/SS-A antigens by immunofluorescence

To visualize the infectivity of the fibroblast cells, MRC-5 cells were grown on tissue culture chambers (Nunc, Inc. Naperville, IL) in DMEM medium for three days and then infected with CMV as above. The cells were fixed to the chambers by adding 100 μl of 0.005% digitonin (Anachemia, Lachine, Quebec, Canada) in 2% paraformaldehyde at 37°C for 5 minutes for cytoplasmic staining. This step was critical, especially for the CMV infected cells, which tend to round up and lose their adherence to plastic. Double immunofluorescence cytoplasmic staining was conducted by adding 20 μl of the respective antibodies for peptide calreticulin or peptide Ro/SS-A and the immediate early (IE) antigen of CMV followed by an incubation of 30 minutes at 37°C. Control experiments (pre-immunized rabbit IgG antibody and PBS) were performed under similar conditions. After the cells were washed in PBS with 2% normal goat serum (GIBCO), cytoplasmic bound anti-calreticulin or anti-Ro/SS-A peptide antibodies were detected by incubation of the cells with FITC-conjugated anti-rabbit Ig (TAGO, Inc. Burlingame, CA, USA) and the mouse anti-CMV IE antibodies were detected by Rhodamine-conjugated anti-mouse Ig (CAPPEL) for 30 minutes at 37°C. Stained cells were analyzed by fluorescence microscopy (Zeiss, German).

[^{3}H]TdR incorporation

MRC-5 cells were cultured at a density of 10^5/ml cells, 100 μl per well in the 96-well flat-bottom plates (FALCON, Becton Dickinson, New Jersey) for 3 days. The cells were inoculated with CMV or with medium alone and then cultured for an additional time period of up to 3 days. The cells were pulsed for the final 23 hours with 1 μCi ^{3}H-thymidine (ICN, Irvine, California) per well. Cells were harvested by using a multichannel cell harvester (Skatron, VA., USA.). Filters were dried, immersed in liquid scintillation cocktail (Fisher) and then counted in a liquid scintillation counter (Beckman, Palo Alto, CA).

Fixed-cell ELISA

To minimize the manipulations of the adherent, fibroblast cells (MRC-5), 2 x 10^5/ml, 100 μl per well were cultured on 96-well flat-bottom tissue culture plates and incubated with CMV or with media alone, as above. At the termination of the tissue culture, the cells were fixed to the plates by adding 100 μl of 0.07% glutaraldehyde per well at room temperature for 5 minutes for surface staining. The cells were fixed with 0.07% glutaraldehyde and then 99% ethanol at -20°C for 30 minutes for cytoplasmic staining. The cells were then incubated with the respective anti-peptide antibodies, control pre-immunized rabbit IgG and PBS for 30 minutes at 4°C. The plates were then rinsed. In all instances a biotin-conjugated antibody was used that was appropriate for the primary antibody (i.e., anti-rabbit for Ro/SS-A and calreticulin, anti-mouse for class I MHC and CMV IE). Alkaline-phosphatase-conjugated Avidin D was used to detect the bound antibodies. Final incubation with the alkaline phosphatase substrate was at 37°C for 1 hour.

Cell quantification

To correct for differences in cell numbers between individual wells and between different experiments, the cell number was determined by staining with crystal violet [27]. Plates at the termination of the ELISA, were stained with 0.2% crystal violet at room temperature for 1 minute. After washing, PBS was added and the OD at 550 nm read on the ELISA reader. A standard curve was established with known dilutions of cells. Total antigen expression was then corrected for the number of cells in the well as determined from the standard curve. The amount of antigen was calculated as the value of OD405 (alkaline phosphatase) per cell and represents the mean of three experiments.

Statistical analysis

Data were analyzed by the T test using the Instant program (GraphPAD Software, San Diego, California). P values less than 0.05 were considered significant.

Results

Specificity of rabbit anti-peptide antisera

A computer analysis of the amino terminal region (Cal 7-23) of calreticulin (60KD) protein (KEQFLDGDGWTSRWIES) and the carboxyl terminal region (487-499) of classical Ro/SS-A (60KD) protein (RKKMDIPAKLIVC) predicted that these regions might be antigenic sites. Anti-sera were obtained from rabbits immunized with calreticulin and Ro/SS-A peptide. The anti-peptide antibodies reacted specifically with the corresponding peptide in ELISA. However, anti-peptide antibody raised to this Ro/SS-A peptide had a weak cross reactivity to calreticulin peptide (0.772 at OD405 to Ro/SS-A peptide, vs 0.134 to calreticulin (Cal 7-23)), but did not react with the controls.

Figure 1 is representative of an immunoblot analysis of MRC-5 cell extracts using the rabbit anti-peptide serum. As can be seen in lane 2, anti-Cal 7-23 antibody bound to a protein with a molecular mass of 60KD. Similarly a 60KD protein was specifically recognized by the anti-Ro/SS-A peptide antibody (lane 4). The remaining bands are due to nonspecific antibody binding. When anti-peptide sera were pre-incubated with the specific peptides in question, only the antibody reactivity to the respective protein (calreticulin or Ro/SS-A) was inhibited. Similarly a 52KD protein could be seen in lane 6, recognized by anti-peptide antibodies raised to the 52KD/Ro peptide. This binding was inhibited by the specific 52KD/Ro peptide (lane 7). The serologic analysis of 31 patients with SLE also confirmed that the anti-peptide sera that were used in this study are specific and recognize the same target proteins that the human autoantibodies bind (data not shown). Both human anti-Ro serum and rabbit anti-peptide/Ro or anti-peptide/calreticulin serum recognize 60KD protein extracted from human fibroblast cells.

Immunofluorescence for CMV IE, Ro/SS-A and calreticulin antigens

Cytoplasmic immunofluorescence staining patterns in MRC-5 cells obtained with anti-calreticulin, anti-Ro/SS-A and anti-CMV IE antibodies and those for pre-immunized rabbit and/or mouse IgG antibodies were done. MRC-5 cells are readily infected by CMV (standard laboratory line used to propagate CMV). Not only is the CMV IE antigen apparent in the nucleus (speckled staining pattern) (Figure 2, A) but also

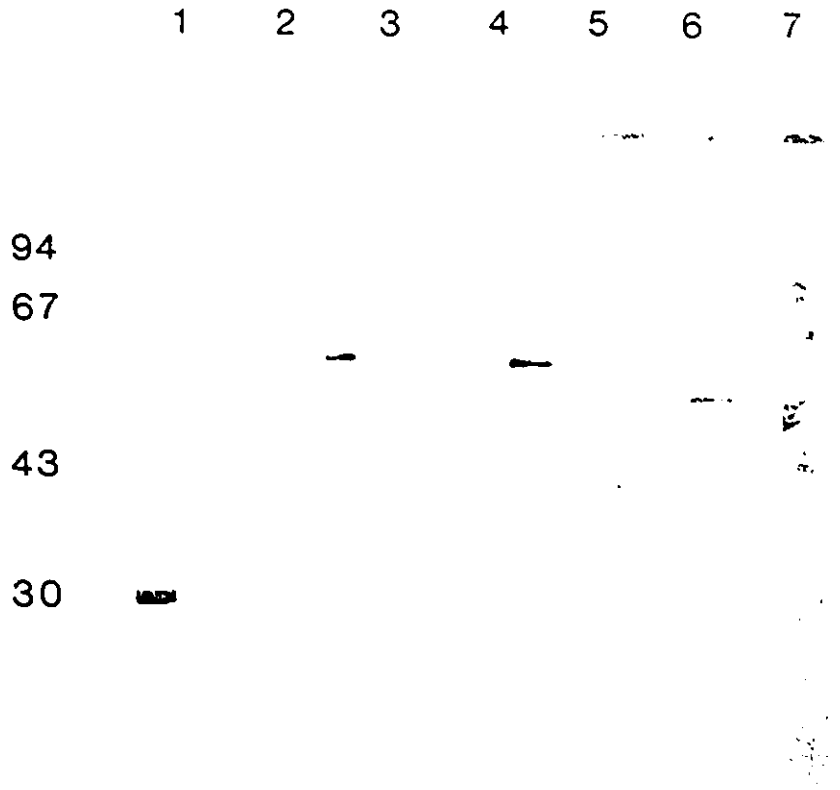

FIGURE 1. Detection of the 60KD Ro/SS-A, calreticulin and 52KD Ro/SS-A in MRC-5 cell extracts by immunoblotting. Nitrocellulose strips were incubated with, in Lane 1: rabbit pre-immune control serum; Lane 2: rabbit anti-calreticulin peptide antiserum; Lane 3: rabbit anti-calreticulin peptide antiserum pre-incubated with calreticulin peptide; Lane 4: rabbit anti-Ro/SS-A peptide antiserum; Lane 5: rabbit anti-Ro/SS-A peptide antiserum pre-incubated with Ro/SS-A peptide; Lane 6: rabbit anti-52kD/Ro peptide antiserum; Lane 7: rabbit anti-52kD/Ro peptide antiserum pre-incubated with 52kD/Ro peptide. Bound rabbit antibodies were detected by HRP-goat anti-rabbit antibodies. Molecular weight markers are indicated on the left (x 10^3 kD).

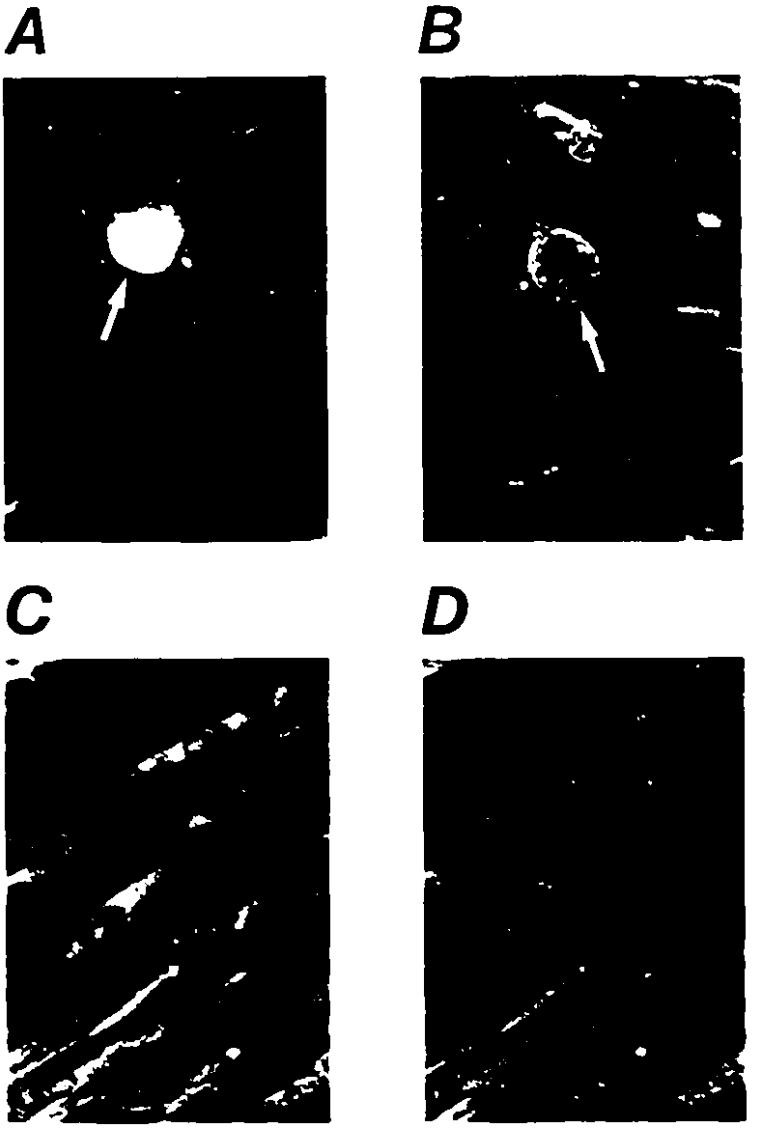

FIGURE 2. Detection of CMV IE and Ro related antigens by immunofluorescence. Human fibroblasts (MRC-5) were grown on multitest chambers either infected with CMV strain Davis or uninfected for 1 day. (A) CMV infected MRC-5 cells stained with monoclonal anti-CMV-IE antibodies. The arrow denotes one of the cells where fluorescence is restricted to the nucleus. (B) CMV infected MRC-5 cells stained with purified rabbit anti-calreticulin antibodies. The arrow indicates a cell where fluorescence is restricted to the cytoplasm. (C) Non-infected MRC-5 cells stained with monoclonal anti-CMV-IE antibodies. (D) Non-infected MRC-5 cells stained with purified rabbit anti-calreticulin antibodies. There is weak background fluorescent material in both C and D.

recognizable cytopathic changes in the cells (cells tend to round up) are evident when compared to the uninfected cells (Figure 2, C). Anti-calreticulin antibody bound proteins in the cytoplasm with a speckled staining pattern in the infected cells (Figure 2, B). This was not seen in the uninfected cells (Figure 2, D). No binding of the anti-peptide Ro/SS-A to cytoplasmic proteins was demonstrated by immunofluorescence. Pre-immunized rabbit and/or mouse IgG did not stain any of the CMV infected and uninfected cells. In addition, no MHC class I antigens cytoplasmic fluorescence staining was seen in cells post CMV infection (data not shown).

[^{3}H]-Thymidine incorporation in DNA

DNA synthesis in MRC-5 cells was measured with and without CMV infection in order to monitor the impact of CMV infection on the host cells (Figure 3). As well, determination of the time of maximal synthesis could possibly coincide with the point where viral RNA might complex to the RNP proteins. Peak ^{3}H-thymidine incorporation in DNA occurred in the first 24 hours after viral infection. By comparison with the uninfected cells, net DNA synthesis (that likely represents both cellular and viral DNA replication) increased significantly, especially for the CMV infected MRC cells in the first 24 hours ($P=0.0119$). At 48 hours, there still was a higher incorporation in infected cells than that in uninfected cells ($P=0.0193$). By 72 hours, however, DNA replication was not found to be significantly different between infected and uninfected cells. With time in culture, the amount of ^{3}H-thymidine incorporation declined, in both infected and uninfected cells.

Host cell antigen expression

A fixed-cell ELISA assay was established to characterize the antigens expression on and/or in the cells. The expression of MHC class I glycoproteins was studied by incubating CMV infected or uninfected MRC-5 cells with the antibodies. MHC class I was more readily detected on the cell surface than in the cytoplasm (Table 1). The peak expression was detected at 24 hours after CMV infection and with time, the surface MHC class I expression diminished. When a comparison of the expression of MHC class

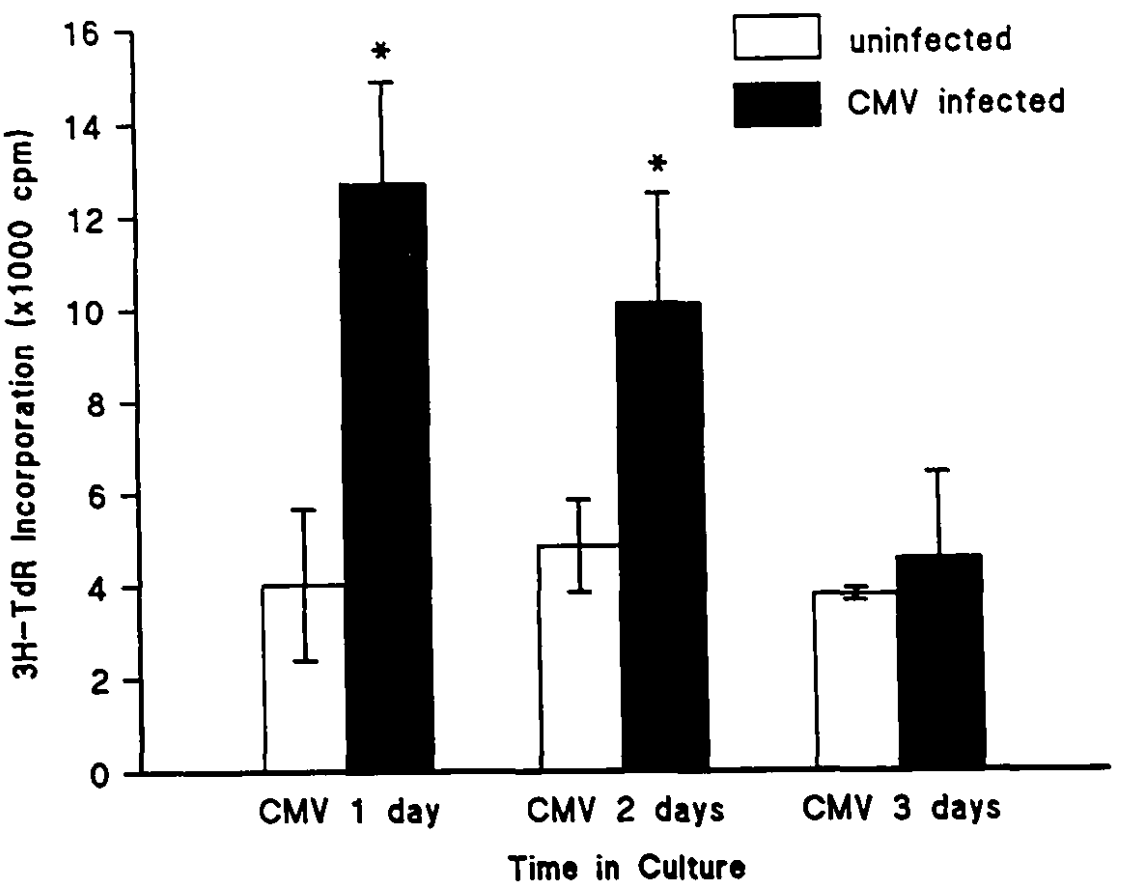

FIGURE 3. ^{3}H-Thymidine incorporation of MRC-5 cells following CMV infection with time, in culture. Significant differences between groups are indicated by asterisks.

Table 1. Detection of selected human proteins on the surface (A) or in the cytoplasm (B) of fibroblasts (MRC-5) by fixed-cell ELISA, in the presence and absence of CMV.

Protein	CMV	Protein expression* Days after infection 1	2	3
A. Surface expression				
MHC class I (ABC)	−	1.25±0.08	1.15±0.03	0.71±0.06
	+	1.67±0.06[a]	1.36±0.06	0.95±0.08
Calreticulin	−	1.00±0.04	0.51±0.02	0.30±0.01
	+	1.24±0.01[b]	0.70±0.04	0.36±0.02
Ro/SS-A	−	0.22±0.15	0.38±0.02	0.20±0.04
	+	0.31±0.19	0.44±0.05	0.26±0.03
La/SS-B	−	0.26±0.09	0.23±0.02	0.08±0.02
	+	0.39±0.11	0.24±0.03	0.13±0.01
B. Cytoplasmic expression				
MHC class I (ABC)	−	0.28±0.03	0.27±0.01	0.15±0.01
	+	0.37±0.05	0.27±0.01	0.21±0.02
Calreticulin	−	0.52±0.06	0.38±0.04	0.36±0.11
	+	0.79±0.05[c]	0.59±0.06	0.44±0.11
Ro/SS-A	−	0.32±0.10	0.44±0.08	0.33±0.10
	+	0.39±0.15	0.66±0.13	0.43±0.10
La/SS-B	−	0.38±0.03	0.32±0.04	0.19±0.06
	+	0.55±0.03	0.42±0.06	0.24±0.04

*Mean ± SE of antigen equivalents per cell of three experiments as described in Materials and Methods. a, $p = 0.0364$; b, $p = 0.0314$; c, $p = 0.0023$.

I is made between the infected and uninfected cells, there was a similar trend with peak expression occurring at 24 hours, followed by the loss of expression in the cells. However, only the results of surface MHC class I expression for the first 24 hours of infection were significantly elevated (P=0.0364) when compared with the noninfected cells. By 48 and 72 hours, there was no significant difference in the surface expression of MHC class I by the cells both infected and uninfected by CMV.

Autoantigen expression

Using the fixed cell ELISA, all of the autoantigens (Ro/SS-A, calreticulin and La/SS-B) studied could be detected in the cytoplasm of the cells. This assay was clearly more sensitive than the immunofluorescence studies. CMV infection appeared to greatly affect the expression of calreticulin in the cytoplasm, which increased by 52% compared with uninfected cells (Table 1). Calreticulin increased most noticeably in the first day post inoculation (P=0.0023) compared with classical Ro/SS-A. Interestingly, after viral infection, only calreticulin was detected on the cell surface (an increase of 24% compared to uninfected cells). The surface expression of the calreticulin antigen significantly increased after viral infection, primarily at the first 24 hours (P=0.0314), but not at 48 and 72 hours post infection (Table 1). With time in culture the expression of the calreticulin autoantigen per cell diminished regardless of whether or not the cells were infected with CMV. The expression of 60kD Ro/SS-A or La/SS-B antigens did not change with viral infection either in the cytoplasm or on the cell surface.

Discussion

Human fibroblasts (MRC-5), as is well known, are readily infected with CMV as demonstrated by both the CMV induced cytopathic change and the expression of the CMV IE antigen by immunofluorescence in our study. In permissively infected cells, the expression of CMV IE antigen first occurs after infection, which controls the activation of CMV early (E) genes and activates cellular gene expression. E antigens are required for viral DNA synthesis, which is followed by the synthesis of structural proteins during the late phase of infection [28]. Following CMV infection, it appears

that fibroblasts are initially activated with a net increase both the cellular DNA and viral DNA replication as has previously been shown [29]. This suggests that CMV activates the host cells at the onset of infection and the enhanced replication of DNA in infected cells is stimulated by CMV IE and E antigens [30-32].

Alterations in the level of expression of MHC class I molecules was found to occur during the initial phase of the virus infection. This surface glycoprotein is not normally a target of pathogenic antibodies in patients with SLE. In our study, MHC class I molecules served as the control for the detection of the cell surface expressed proteins as the majority of the class I MHC molecules are detected on the cell surface rather than in the cytoplasm. As well, interferons (IFNs) produced by virally infected fibroblasts can increase the expression of the MHC class I molecules [33]. Interestingly CMV itself encodes a glycoprotein homologous to the MHC class I heavy chain antigen [34,35] and we cannot totally role out a cross reactivity of the antibody specific for MHC class I and the CMV mimicked heavy chain protein which could account for the increase observed with infection. The regulation of cell surface MHC class I induced by CMV is dose-dependent [36]. Our studies, using a low virus dose, have shown that CMV infection of human fibroblasts results in an enhanced expression of MHC class I molecules on cell surface, most likely, on the uninfected bystander cells.

The target autoantigens recognized by circulating antibodies in patients with SLE and SS include selected cytoplasmic proteins. Whereas La/SS-B antigen has been identified as a termination of transcription factor and is clearly important in transcription [37], the functions of Ro/SS-A antigens have not been determined. Calreticulin has recently been described as a rheumatic disease-associated autoantigen [38]. It is located in the endoplasmic reticulum (ER) where it may bind calcium [39]. It may play a role in protein assembly [40]. During the time calreticulin is localized within the ER and trans-Golgi complex, structural modifications may take place leading to transport to the cell surface [41]. The relocated and modified proteins could become immunogenic in the appropriate environment. The displacement of the various cytoplasmic proteins to the cell surface may be a result of "stress" by a number of mechanisms. For example, our results revealed that the fibroblast cells were found to have an increased cell surface and

cytoplasmic expression of calreticulin antigen after CMV infection. The expression of this antigen coincides with the onset of cellular DNA synthesis, as was shown by studies of the incorporation of ^{3}H-thymidine and time course studies. The increased expression of calreticulin on cell surface may reflect a more mobile calreticulin and/or virus utilization of the protein. Our study suggests that virus infection may have an effect on cells and that host proteins could be increased, or the expression in subcompartments of the cells could change after infection.

The growth characteristics of the fibroblast cells *in vitro*, which reach confluence with time, leading to a cessation of cell growth, could account for the loss of DNA replication and antigen expression with time in culture. This decline in DNA replication is not likely to be due to the depletion of nutrients, as the same trend was seen when the experiment was conducted in the presence of 10% HICS (data not shown). Alternatively the virus could severally damage the cell in the late stage of infection, however, this is less likely to be the case since the Ro/SS-A and La/SS-B antigens appeared not to be increased on the cell surface following CMV infection. This suggests that the alteration of cell surface expressed cytoplasmic proteins is not a general consequence of CMV infection or cell death.

In this *in vitro* assay system, we have demonstrated that CMV induces the expression of the autoantigen calreticulin, during permissive infection of human diploid fibroblasts. Although very little is known about the regulation of the autoantigen in response to viral infection, our experiments have made it possible to investigate the role of viral infection on autoantigen expression.

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Key words: calreticulin, Ro/SS-A, systemic lupus erythematosus (SLE), cytomegalovirus (CMV), immediate early (IE) antigens, fibroblasts

CHAPTER IV

The study described in the previous chapter (Chapter III) demonstrated enhanced expression of calreticulin, but not 60kD/Ro antigen, on the surface of CMV-infected human fibroblasts. The present chapter addresses the question of whether other stimuli (UVB irradiation and low pH) in combination with CMV infection, can modulate the cellular expression of Ro autoantigens. The effects of these stimuli on the expression of different Ro antigens (60kD/Ro and 52kD/Ro) and calreticulin in human fibroblasts are described.

ULTRAVIOLET B IRRADIATION AND CYTOMEGALOVIRUS INFECTION SYNERGIZE TO INDUCE THE CELL SURFACE EXPRESSION OF 52kD/Ro ANTIGEN

This work is in press in

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SUMMARY

Cultured human fibroblasts (MRC-5) have been previously demonstrated to express calreticulin, but not Ro autoantigen, on their surface after human cytomegalovirus (CMV) infection. The present study addresses the question of whether other stimuli, alone or in combination with CMV, can induce the surface expression of Ro autoantigens on human fibroblasts. Using a fixed-cell ELISA to detect autoantigen expression, a synergistic effect between ultraviolet B (UVB) exposure and CMV infection on the surface expression of 52kD/Ro antigen, but not 60kD/Ro or calreticulin, was observed. The enhanced expression of 52kD/Ro antigen was significant and specific, compared with untreated cells, cells infected with CMV alone or irradiated with UVB only, and cells subjected to other treatments, such as low pH. Immunofluorescence studies confirmed these findings and indicated that cells expressed 52kD/Ro protein on their surface at 24 hours after a combined UVB and CMV treatment. These studies provide evidence that synergy between UVB irradiation and CMV infection may play a role in the induction of cell surface expression of the human autoantigen, 52kD/Ro.

INTRODUCTION

Ro (60kD and 52kD) and calreticulin (46kD) autoantigens are small intracellular ribonucleoproteins, which have been isolated and cloned [1-4], but their biological functions remain unclear. Antibodies directed against Ro and calreticulin antigens are of clinical interest, as they are found in approximately 50% of patients with systemic lupus erythematosus (SLE) and associated with lupus skin lesions [5-7]. However, the mechanisms by which autoantibodies to these intracellular proteins are induced are not fully understood.

In previous studies, ultraviolet B (UVB) irradiation has been demonstrated to induce the expression of Ro antigens on the surface of cultured human keratinocytes [8-11]. This expression appeared to be an active process rather than a passive leakage of antigens to the cell surface. Estradiol has also been reported to augment the binding of IgG from anti-Ro antibody-containing sera to the surface of cultured keratinocytes [12]. This augmentation was not induced by other hormones, such as dihydrotestosterone,

testosterone and progesterone, and was partially inhibited by the anti-estrogen, nafoxidine [12].

In addition to UVB and estradiol, viruses have been proposed as important factors in disturbing normal cellular levels of autoantigens [13,14]. Human cytomegalovirus (CMV) is a potential candidate in the induction of Ro antigen expression. CMV is a common viral agent worldwide, which infects 50-100% of the population, targets all organ systems of the host, and persists in the host for life [15,16]. Elevated anti-CMV antibodies [17,18], as well as CMV-induced flares [19,20], have been reported in SLE patients, suggesting that patients with SLE may have a different immune response to CMV infection than normal individuals. In addition, CMV RNA, including the abundant 2.8 kb RNA as well as the much less abundant 2.5, 1.9, 1.45 and 1.3 kb RNAs, has been immunoprecipitated from infected fibroblasts by SLE sera containing anti-Ro antibodies [21], suggesting that viral RNA may interact with cellular Ro proteins, resulting in "altered self" proteins.

In a previous study [22], we reported the enhanced expression of calreticulin, but not Ro antigen, on the surface of cultured human fibroblasts infected with CMV. The purpose of the present study was to investigate whether other stimuli (in particular, UVB irradiation) in combination with CMV infection can modulate the expression of Ro antigens in cultured human fibroblasts, which are the only cells that are permissive for a full cycle of human CMV replication in vitro [23,24]. The demonstration of Ro antigen expression on the surface of human fibroblasts, after combined UVB and CMV treatment but not either treatment alone, suggests that multiple factors may synergize in the induction of cell surface expression of the human autoantigen, Ro.

MATERIALS AND METHODS

Cell culture and CMV infection

The human embryo lung fibroblast cell line, MRC-5, and human CMV (Davis strain) were both obtained from American Type Culture Collection (ATCC, Rockville, MD). MRC-5 cells were expanded and passaged in Dulbecco's minimum essential medium (DMEM) (GIBCO Laboratories, Grand Island, NY), supplemented with 10% heat-

inactivated calf serum (HICS) (GIBCO) and 0.01% (w/v) gentamycin sulfate (USB, Cleveland, OHIO), and grown at 37°C with 5% CO_2. When MRC-5 monolayers were grown to approximately 90% confluency, cells were infected with 10^3 plaque-forming units (PFU) of CMV, as previously described [22]. Cell viability following CMV infection was assessed by trypan blue dye exclusion. The mean viability (± SE) of the infected cells was 96.7% (± 0.5) at 24 h, 95.5% (± 0.7) at 48 h, and 90.0% (± 2.3) at 72 h of CMV infection, compared with uninfected controls (95.8% ± 1.2 at 24 h, 96.0% ± 0.4 at 48 h, and 94.8% ± 0.9 at 72 h, respectively).

Effects of UVB irradiation and low pH treatment on cells

MRC-5 cells (2 x 10^5/ml) were grown for three days in 56.7 cm^2 plastic Nunclon dishes (GIBCO) or in 96-well flat-bottom plates (FALCON, Becton Dickinson, Lincoln Park, NJ). For UVB exposure, cells were irradiated for 5 seconds with UVB (2.0 mJ/cm^2, 302 nm) emitted from a transilluminator (Spectroline, Fisher, St Laurent, Quebec). This source of irradiation does not contain any UVA or UVC component. The dose of 2.0 mJ/cm^2 was chosen, since this amount of UVB irradiation achieves cell injury, without killing the cells [8]. For low pH treatment, cells were incubated with DMEM medium at pH 5.0 for 5 seconds. Five-second exposures were used to be consistent with the UVB experiments. Cell viability was assessed after each treatment by trypan blue dye exclusion (mean cell viability ± SE was 95.2% ± 1.3 after UVB irradiation and 94.4% ± 0.6 after low pH treatment, compared with 96.2% ± 2.1 for untreated control cells). After treatment, the medium was replaced with fresh DMEM medium at 37°C. Cells were returned to the 37°C incubator, and grown in the presence and absence of CMV for up to 3 days.

Antibodies

Rabbit anti-peptide antibodies, specific for the amino terminal region (amino acids 7-23) of calreticulin (KEQFLDGDGWTSRWIES), the carboxyl terminal region (amino acids 489-499) of 60kD/Ro (RKKMDIPAKLIVC), and the amino acid region (amino acids 129-143) of 52kD/Ro (EEAAQEYQEKLQVAL) were kindly provided by Dr. Marianna

Newkirk (The Montreal General Hospital Research Institute, Montreal, Quebec) and have been described previously [22]. These anti-peptide antibodies are capable of binding to the native antigens and such binding can be inhibited by the respective peptides or by anti-Ro positive SLE serum. IgG F(ab')$_2$ fragments from the anti-peptide antibodies were prepared as previously described [22]. The monoclonal antibody, W6/32 (ATCC), directed against a nonpolymorphic determinant on HLA-ABC, was used to study the surface expression of MHC class I antigens.

Fixed-cell ELISA

Fibroblast cells (2 x 10^5/ml , 100 μl per well) were cultured in 96-well flat-bottom plates and treated with CMV and/or UVB, as described above. For the detection of surface antigens, cells were washed with PBS and fixed to the plates by adding 100 μl of 0.07% glutaraldehyde per well and incubating for 5 min at 25°C [22]. For the detection of cytoplasmic and nuclear antigens, cells were washed with PBS, and permeabilized and fixed with 0.005% digitonin in 2% paraformaldehyde (100 μl/well) for 5 min at 37°C, followed by 0.07% glutaraldehyde (100 μl/well) for 5 min at 25°C. Under these conditions, cytoplasmic, nuclear and surface antigens could be detected (referred to as "total cellular" antigen expression). After 3 washes with PBS, the fixed cells were incubated with anti-peptide IgG F(ab')$_2$ fragments (20 μg/ml in PBS), control pre-immunized rabbit IgG F(ab')$_2$ fragments, or PBS for 30 min at 4°C. Following 3 washes with PBS, the cells were incubated with biotin-conjugated anti-rabbit IgG (Vector, Mississauga, ON). Alkaline phosphatase-conjugated Avidin D (Vector) was used to detect the bound antibodies. To correct for differences in cell numbers between individual wells, the cell number in each well was determined by staining with crystal violet [22]. At the termination of the ELISA, plates were stained with 0.2% crystal violet for 1 min at 25°C. After PBS washing, 100 μl per well of PBS was added and the OD at 550 nm read on an ELISA reader. A standard curve was established by plating known concentrations of cells and staining with crystal violet. Total antigen expression was corrected for the number of cells in the same wells, as detected by crystal violet staining, and was expressed as OD_{405} units per cell, as described previously [22]. In

each experiment, wells coated with positive control peptides (2.5 μg/ml) and incubated with the appropriate anti-peptide antibody served as positive controls. Each sample was run in duplicate in three separate experiments. The results were analyzed for variance (SE) between the three experiments for control and test samples.

Immunofluorescence

MRC-5 cells, grown on coverslips in DMEM medium, were stained as either fixed or living cells. Staining of fixed cells was performed as described previously [22]. Living cells were washed with cold (4°C) PBS and then incubated with antibodies for 1 h at 4°C. Three $F(ab')_2$ affinity-purified rabbit anti-peptide antibodies, anti-calreticulin, anti-52kD/Ro and anti-60kD/Ro, were used to detect antigen expression (20 μg/ml in PBS). A monoclonal antibody to HLA-ABC was used to detect cell surface expression of HLA-ABC antigens under the different conditions. Cells were washed with cold PBS and immediately fixed by incubation with 0.005% digitonin in 2% paraformaldehyde for 5 min at 37°C. Surface binding of rabbit IgG $F(ab')_2$ fragments was detected with FITC-conjugated anti-rabbit IgG (1:50 dilution) for 30 min at 37°C, while binding of the murine monoclonal anti-HLA antibody was detected with a rhodamine-conjugated anti-mouse IgG [22]. After three washes in PBS, the slides were mounted in a 90% glycerol/PBS solution containing paraphenylenediamine (0.1 mg/ml), and viewed through a Zeiss fluorescent microscope (Zeiss, Germany), using oil immersion and a 40x objective. In each experiment, negative controls included untreated cells that were stained with the same anti-peptide antibody and treated cells that were incubated with pre-immunized rabbit IgG $F(ab')_2$ fragments or PBS, and the second antibody. Each sample was run in duplicate and experiments were repeated three times.

Immunoblotting

This method has been described previously [22] and is briefly summarized as follows. The proteins in MRC-5 cell extracts were separated using standard 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membrane (0.45 micron) (Bio-Rad, Richmond, CA). After blocking with PBS containing

0.4% BSA for 16 h at 4°C, blots were incubated with antiserum 1:50 diluted in PBS for 2 h at 37°C. The nitrocellulose filters were then washed three times with PBS containing 0.05% Tween 20 (PBST) and incubated with biotinylated anti-rabbit IgG (1:2000 dilution) for 1 h at 37°C. After three washes with PBST, the bound antibody was detected by incubation with horseradish peroxidase (HRP)-labelled Avidin D (Vector) (1:3000 dilution) and 3,3'-diaminobenzidine tetrahydrochloride substrate (Sigma, St. Louis, MO). The positions of the bands were compared to standard molecular weight markers.

Statistical analysis

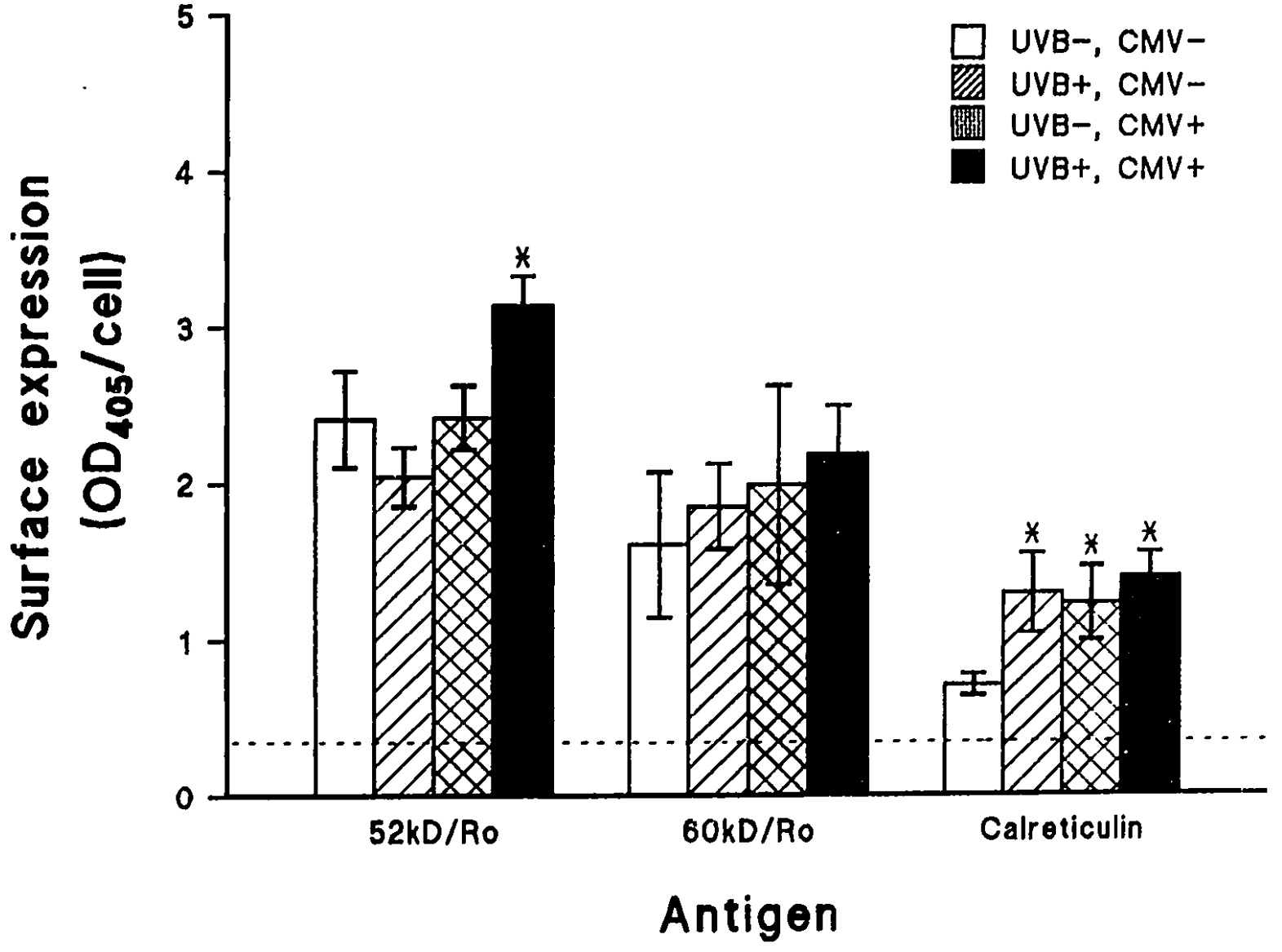
Data were analyzed by the paired t-test using the Instat program (GraphPAD Software, San Diego, CA). P values (one-tailed) less than 0.05 were considered significant.

RESULTS

Effects of UVB and CMV stimuli on cell surface expression of antigens

The analysis of Ro and calreticulin autoantigen expression was performed using a fixed-cell ELISA and the results are shown in Figures 1 and 2. As shown in Fig. 1, CMV infection did not significantly increase 52kD/Ro or 60kD/Ro antigen expression on the fibroblast surface at 24 h of infection, while cell surface expression of calreticulin was enhanced, as in our previous report [22]. When cells were exposed to UVB alone, there was also an increase in the cell surface expression of calreticulin, but not 52kD/Ro or 60kD/Ro antigen. This surface expression was similar to that observed with CMV infection alone. However, when fibroblast cells were treated with a combination of UVB irradiation and CMV infection, 52kD/Ro antigen expression on the cell surface was significantly increased at 24 h of treatment, compared to untreated cells, CMV-infected cells and UVB-irradiated cells ($p<0.05$). A specific increase of 52kD/Ro antigen expression for UVB plus CMV-treated cells, compared to cells treated with UVB or CMV alone, was also observed when the results were expressed as paired comparisons of the test versus control cells, where the control cells were normalized to 1.0. The mean value $\pm$ SE of the ratio of this antigen expression for UVB plus CMV-infected

Figure 1. Cell surface expression of 52kD/Ro, 60kD/Ro and calreticulin antigens. The binding of anti-Ro or anti-calreticulin peptide antibodies to human fibroblasts untreated (control) or treated with UVB, CMV or UVB plus CMV was assessed by fixed-cell ELISA. Results shown are the mean binding (OD_{405}/cell $\pm$ SE) of three independent experiments. The dashed line represents the mean binding (OD_{405}/cell) of pre-immunized rabbit serum for all experiments. Asterisks denote significant differences in paired comparisons to untreated control cells ($p<0.05$).


Surface expression
(OD405/cell)
5
4
3
2
1
0
UVB−, CMV−
UVB+, CMV−
UVB−, CMV+
UVB+, CMV+
*
*
*
*
52kD/Ro
60kD/Ro
Calreticulin
Antigen

cells to untreated control cells was 1.40 ± 0.23, compared to 0.86 ± 0.14 for UVB-treated cells and 1.00 ± 0.05 for CMV-infected cells.

In contrast to the observation that 52kD/Ro expression was increased only after combined UVB and CMV treatment, cell surface expression of 60kD/Ro antigen was not significantly induced by this treatment. Although cell surface expression of calreticulin was increased by either UVB irradiation or CMV infection, no further increase was caused by these two treatments together. In addition, the cell surface expression of MHC class I was increased by CMV infection, with or without UVB irradiation (data not shown).

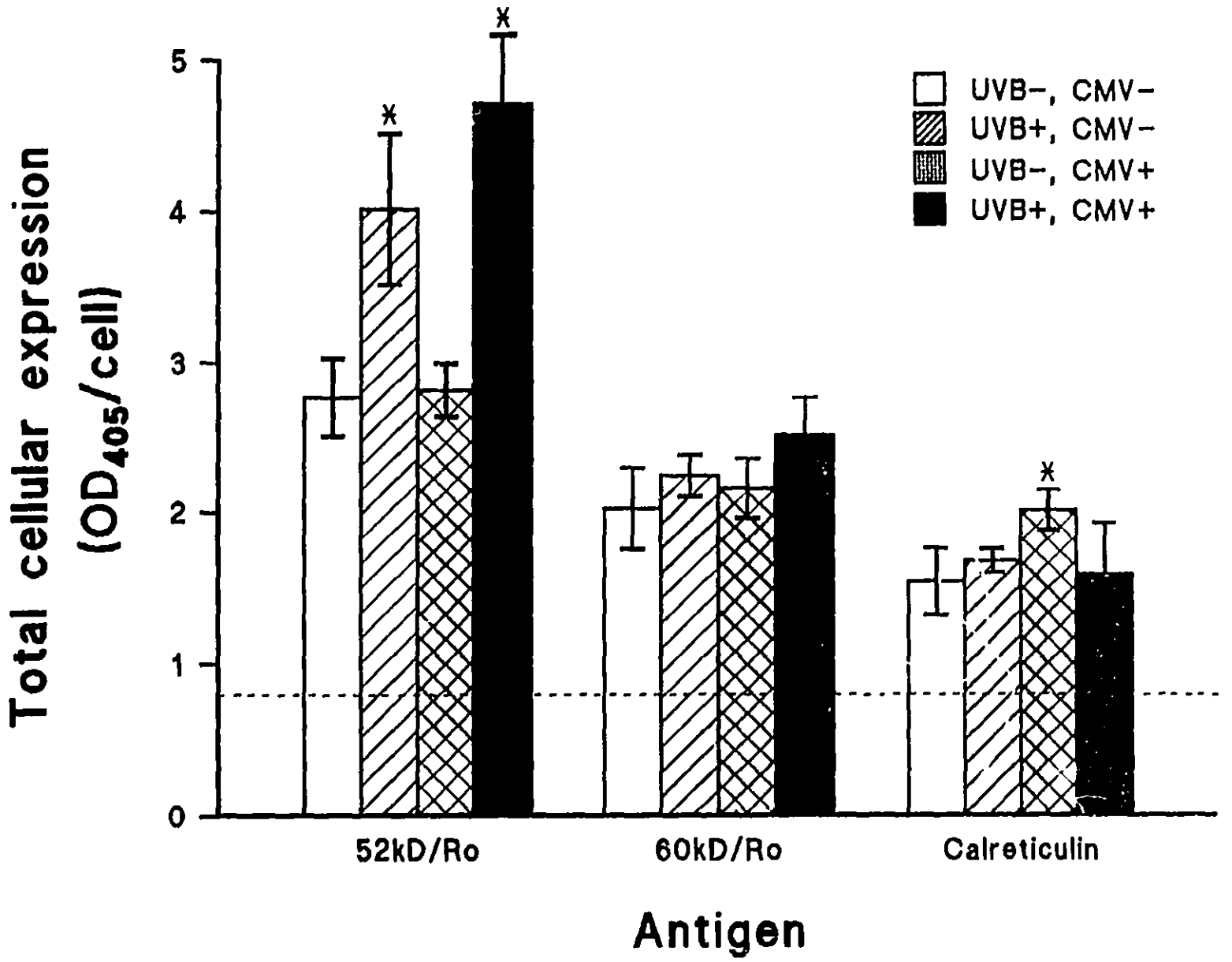
Effects of UVB and CMV stimuli on total cellular expression of antigens

The total cellular (surface plus nuclear and cytoplasmic) antigen expression, obtained by fixed-cell ELISA, is shown in Fig. 2. CMV infection did not cause an increase of 52kD/Ro antigen expression in cells, while UVB exposure alone or UVB plus CMV treatment showed an augmentation in total cellular 52kD/Ro antigen ($p < 0.05$), compared with untreated cells. The cellular expression of 60kD/Ro antigen did not change significantly with any stimulation, including CMV infection, UVB irradiation and UVB plus CMV treatment. Although cellular calreticulin expression was increased in CMV-infected cells ($p < 0.05$), there was no marked increase in calreticulin in cells after UVB irradiation or UVB plus CMV treatment, compared with untreated cells.

Effects of low pH treatment on surface and total cellular expression of antigens

Experiments using other stimuli, such as low pH treatment, were performed to determine whether the synergistic effect between UVB exposure and CMV infection on the cell surface expression of 52kD/Ro antigen was specific. Cells were pre-incubated at low pH (pH 5.0) prior to CMV infection, and compared with incubation at pH 7.2. For surface expression of cells incubated at low pH, the OD_{405}/cell values (mean ± SE) of uninfected and CMV-infected cells, respectively, were 2.36 ± 0.35 and 2.46 ± 0.29 (compared to 2.41 ± 0.07 and 2.42 ± 0.50 at pH 7.2) for 52kD/Ro; 1.6.' ± 0.47 and 2.10 ± 0.45 (compared to 1.61 ± 0.46 and 2.11 ± 0.63) for 60kD/Ro; and 1.04 ± 0.10 and 1.06

Figure 2. Total cellular (surface, nuclear and cytoplasm) expression of 52kD/Ro, 60kD/Ro, and calreticulin antigens. The binding of anti-Ro or anti-calreticulin peptide antibodies to human fibroblasts untreated (control) or treated with CMV, UVB or UVB plus CMV was assessed by fixed-cell ELISA. Results shown are the mean binding (OD_{405}/cell $\pm$ SE) of three independent experiments. The dashed line represents the mean binding (OD_{405}/cell) of pre-immunized rabbit serum for all experiments. Asterisks denote significant differences in paired comparisons to untreated control cells ($p < 0.05$).


Total cellular expression
(OD405/cell)
5
4
3
2
1
0
*
*
*
UVB−, CMV−
UVB+, CMV−
UVB−, CMV+
UVB+, CMV+
52kD/Ro
60kD/Ro
Calreticulin
Antigen

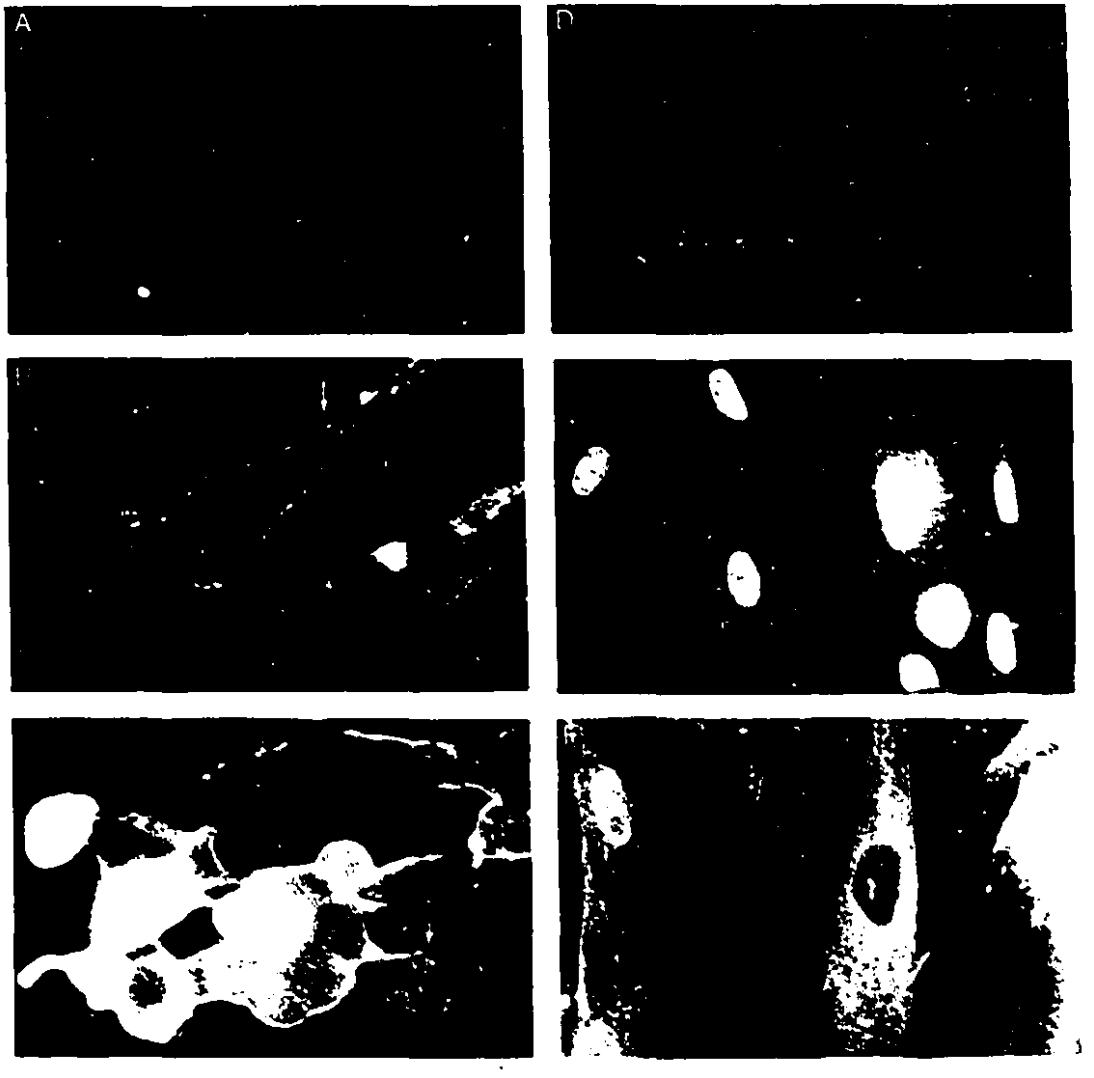
± 0.17 (compared to 0.59 ± 0.09 and 1.18 ± 0.28) for calreticulin. For total cellular expression of cells incubated at low pH, the mean OD_{405}/cell values for uninfected cells and CMV-infected cells, respectively, were 2.83 ± 0.27 and 2.52 ± 0.11 (compared to 2.60 ± 0.28 and 2.82 ± 0.17 at pH 7.2) for 52kD/Ro; 2.20 ± 0.22 and 2.17 ± 0.35 (compared to 2.02 ± 0.27 and 2.16 ± 0.20) for 60kD/Ro; and 1.89 ± 0.49 and 1.75 ± 0.34 (compared to 1.54 ± 0.22 and 1.83 ± 0.30) for calreticulin. Treatment with low pH did not induce a consistent increase in either the cell surface or total cellular expression of the 52kD/Ro antigen or 60kD/Ro antigen. In contrast, calreticulin surface expression, but not total cellular expression, was induced by low pH and was similar to that observed following treatment with CMV, UVB or UVB plus CMV.

Immunofluorescence localization of 52kD/Ro antigen in UVB and CMV stimulated cells

The synergy between UVB irradiation and CMV infection in the induction of surface expression of the 52kD/Ro antigen on fibroblast cells suggested by the ELISA results was confirmed by immunoflorescence, which is shown in Fig. 3. Untreated cells showed no cell surface staining with anti-52kD/Ro antibody (Fig. 3A). However, dense granular surface staining with anti-52kD/Ro antibody was seen on UVB-irradiated CMV-infected cells at 24 h (Fig. 3B), and at 72 h of treatment (Fig. 3C). When cells were infected with CMV only, or irradiated with UVB alone, cell surface staining for 52kD/Ro antigen was also evident, but much weaker than that observed with UVB plus CMV treatments (data not shown). In contrast, there was no evidence of surface fluorescence staining on CMV and/or UVB treated cells incubated with anti-60kD/Ro antibody, pre-immunized rabbit IgG or PBS, compared with untreated cells (data not shown). Anti-calreticulin antibody showed weak surface staining on CMV and/or UVB treated cells (data not shown).

When cells were fixed before reaction with the primary antibody, typical nuclear or cytoplasmic staining was clearly distinguishable from the cell surface staining (Fig. 3D-F). After 24 h of treatment, nuclear 52kD/Ro staining was increased in CMV-infected cells, UVB-irradiated cells and UVB plus CMV treated cells (Fig. 3E), compared with untreated cells (Fig. 3D). This staining appeared to shift from the

Figure 3. Indirect immunofluorescent staining of human fibroblasts with anti-52kD/Ro antibody. (A,D) untreated cells; (B,E) cells at 24 h after UVB plus CMV treatment; (C,F) cells at 72 h after UVB plus CMV treatment. In panels A to C, cells were fixed after incubation with the primary antibody (surface staining). In panels D to F, cells were fixed before incubation with the primary antibody (cytoplasmic staining). Arrows indicate membrane fluorescence. Results shown are representative of three independent experiments.


A
D

nucleus to the cytoplasm after 72 h of treatment (Fig. 3F). However, no differences were observed between the fluorescence patterns of untreated cells and treated cells incubated with anti-60kD/Ro antibody (data not shown). Although anti-calreticulin antibody showed increased nuclear fluorescence staining in CMV-infected cells, no increased nuclear or cytoplasmic staining was found in UVB-irradiated cells or untreated cells (data not shown).

Analysis of total cellular 52kD/Ro antigen in fibroblast cell extracts

To examine whether the changes in the cellular distribution of 52kD/Ro antigen observed by the fixed-cell ELISA or immunofluorescence were associated with changes in the total cellular content of Ro, cellular extracts were made from MRC-5 cells that had undergone different treatments. By Western blotting, there was a slight increase in the 52kD/Ro protein in the extracts of treated cells, compared with untreated cells (data not shown), but there was no obvious quantitative difference between the extracts of cells treated with either CMV, UVB or CMV plus UVB (data not shown).

DISCUSSION

Our results demonstrate a significant synergy between UVB exposure and CMV infection on the surface expression of 52kD/Ro antigen in human fibroblasts (MRC-5). In a fixed-cell ELISA, this phenomenon was significant and specific, compared with untreated cells and cells treated with CMV or UVB alone, or subjected to other treatments, such as low pH. Immunofluorescence confirmed these findings and showed that cells expressed 52kD/Ro protein on their surface after combined UVB and CMV treatment. These results extend our previous observations, which showed that MRC-5 cells treated with CMV alone demonstrated an increased expression of calreticulin on the surface, whereas no increase in Ro antigen surface expression was observed [22].

The specific induction of the surface expression of 52kD/Ro antigen, but not 60kD/Ro or calreticulin, by UVB plus CMV treatment is intriguing. Although this may be due to specific structural or functional features of the 52kD/Ro protein, the limited data available makes it difficult to correlate the antigenic properties with known

characteristicsof the protein. Surface expression of the 52kD/Ro antigen occurred only in UVB-irradiated cells that had subsequently undergone CMV infection, while UVB irradiation caused an augmentation in total cellular 52kD/Ro antigen. The 52kD/Ro protein, which contains zinc finger motifs, may play a role in the DNA repair process after UVB irradiation of cells [3,25-27], as proteins sharing this motif have been found to be involved in DNA repair, regulation of gene expression, or protein transformation [27,28]. In addition, a recent study has shown that apoptotic skin keratinocytes contain surface blebs expressing autoantigens, such as Ro [29]. Apoptosis may occur in response to many different stimuli, including DNA damage [30,31], heat shock [32] and viral infection [33,34]. Although the mechanism responsible for the synergistic effect of UVB and CMV in the induction of 52kD/Ro antigen surface expression is not clear, it is possible that apoptosis, induced by combined UVB and CMV treatment, results in the surface expression of 52kD/Ro and other nuclear autoantigens.

In this study, calreticulin was the only one of the three antigens to show increased cell surface expression with all of the various stimuli, including CMV, UVB and low pH. This phenomenon may be explained by its cellular functions. Previous studies have suggested that calreticulin is a stress protein [35,36]. Homology between the promoter region of calreticulin and other related stress proteins has been reported [37], and a link between heat shock/stress proteins and autoimmunity has been described [38-40]. Recently, Erkeller-Yuksel et al. [41] demonstrated that a group of patients with SLE expressed heat shock protein 90 (hsp 90) on the surface of their blood mononuclear cells and this increased level correlated with high disease activity. As there was no difference in cytoplasmic expression of hsp 90 in blood mononuclear cells from SLE patients and controls, these authors suggest that heat shock protein may chaperone integral proteins and/or damaged intracellular proteins to the cell surface, or that heat shock proteins, released from stressed cells, may bind to the surface of viable cells [41].

Anti-52kD/Ro antibody was found to bind to the surface of UVB plus CMV treated cells by both the fixed-cell ELISA assay and immunofluorescence analysis. However, binding of the same anti-52kD/Ro antibody to the surface of CMV-infected or UVB-irradiated cells was observed only by immunofluorescence analysis. These

observations are consistent with the findings of Peek et al. [42], who reported that surface expression of the La autoantigen could be detected on adenovirus-infected cells by immunofluorescence, but not by other techniques. As there was no difference between the cellular content of 52kD/Ro antigen in cells subjected to the different treatments, it is likely not the total cellular content of Ro protein that changes during stimulation, but the cellular distribution of the protein or its ability to be recognized by specific anti-Ro antibodies. However, it is also possible that Western blot analysis is a less sensitive quantitative measure of cellular protein expression than the ELISA [43].

The Ro antigen/antibody system is highly associated with cutaneous lupus syndromes [44-47]. The results described here demonstrate that synergy between UVB irradiation and CMV infection can induce cell surface expression of the 52kD/Ro antigen in cultured human fibroblasts, and may help to explain why environmental stimuli can promote the development of lupus skin lesions. Future studies are required to better understand the role of multifactorial induction of Ro in the skin. Human keratinocytes in culture should provide a useful model to study these effects in vitro.

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CHAPTER V

Chapters III and IV have described the effects of CMV infection and UVB irradiation on the surface expression of Ro antigens in human fibroblasts. The present chapter extends these studies to human keratinocytes, which are relevant to SLE skin disease associated with the presence of anti-Ro antibodies. The first experiments in the study were performed to determine whether human CMV is able to infect human keratinocytes in vitro. Second, the effects of CMV infection and UVB irradiation on the induction of Ro antigens on the keratinocyte surface are compared.

CYTOMEGALOVIRUS INFECTION INDUCES EXPRESSION OF 60kD/Ro ANTIGEN ON HUMAN KERATINOCYTES

This work is in press in *Lupus*

Summary

To investigate the effect of human cytomegalovirus (CMV) infection on the expression of Ro autoantigen in human keratinocytes, the binding of anti-Ro peptide antibodies (anti-60kD/Ro, anti-52kD/Ro and anti-calreticulin) to cultured human keratinocytes was detected by fixed cell enzyme-linked immunoassay (ELISA), immunofluorescence, flow cytometry (FACS) analysis and immun blotting. There was a significant increase in the binding of anti-60kD/Ro antibody, but not anti-52kD/Ro or anti-calreticulin antibody, to the surface of cultured keratinocytes at 24 h after CMV infection, compared with uninfected cells, by ELISA and immunofluorescence. Surface binding of anti-60kD/Ro was found in 71.2% (±5.5) of CMV-infected cells, compared with 26.2% (±4.1) of untreated cells ($p<0.05$) by FACS analysis. Similar observations were made with a human serum, which contained anti-60kD/Ro antibodies. Immunoblotting was used to analyze total cellular 60kD/Ro antigen expression in keratinocytes infected with CMV or without infection. No increase in the intensity of the 60kD band was found in extracts of the CMV-infected cells, suggesting that the 60kD/Ro antigen is redistributed from the cytoplasm to the cell surface after viral infection. The effects of CMV infection on cell cultures were compared with those of ultraviolet B (UVB) irradiation. 60kD/Ro, 52kD/Ro and calreticulin were all induced on the UVB-irradiated cell surface, but no significant synergistic effect of UVB and CMV was found. This study provides evidence that CMV infection induces 60kD/Ro antigen expression on the surface of human keratinocytes, suggesting that CMV may play a role in development of skin lesions in systemic lupus erythematosus (SLE).

Introduction

Patients with connective tissue diseases frequently have circulating autoantibodies directed against cytoplasmic or nuclear proteins. One such protein is Ro, an intracellular ribonucleoprotein, which is composed of at least two immunologically distinct polypeptides, 60kD and 52kD, non-covalently bound to a family of small RNAs[1-3]. Antibodies to this protein are most commonly found in patients with systemic lupus erythematosus (SLE) and have been detected in 95% patients with subacute cutaneous

lupus erythematosus (SCLE)[4] and up to 100% patients with neonatal lupus erythematosus (NLE)[5]. Although it is not known how antibodies to these sequestered intracellular proteins arise, the strong association of anti-Ro antibodies with SLE suggests that these antibodies may participate in the development of clinical disease, especially cutaneous lupus[6-9].

Human keratinocytes are the most abundant cells in the epidermis. The expression of Ro antigens on the keratinocyte surface is of considerable interest, because the skin is a prominent target organ of immunological damage in patients with SCLE and NLE, and lupus cutaneous lesions occur in the epidermal region and are associated with the binding of anti-Ro antibodies to keratinocytes[10-12]. Clinical and experimental observations have demonstrated that exposure to ultraviolet B light (UVB) induces Ro antigen expression on the surface of human keratinocytes[13,14], allowing the binding of circulating antibody, complement activation, and thus potential cell damage[15-17]. Hormones, such as estradiol, were reported to enhance binding of IgG from anti-Ro antibody-containing sera to the surface of cultured keratinocyte cells in vitro[18]. In addition to UVB and hormones, viral infection has been shown to affect the cellular distribution of Ro antigens. In a previous study[19], we have demonstrated that human cytomegalovirus (CMV) infection increased the surface expression of calreticulin in human fibroblast cells. Calreticulin, a 46kD protein that migrates aberrantly at 60kD on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), was initially reported to be a Ro component, but was subsequently redefined as a new human autoantigen because of its high degree of amino acid sequence homology to murine and rabbit calreticulin and the failure of anti-Ro sera to react with its recombinant form[20,21]. In a subsequent study, we found that UVB irradiation and CMV infection acted synergistically to induce the surface expression of 52kD/Ro antigen on human fibroblasts[22]. Also, there has been a report showing that adenovirus infection displaced the Ro antigen from the nucleus to the epithelial cell surface[23]. Although simian virus 40 (SV40) infection has been shown to modulate the cytoplasmic expression of Ro in human keratinocytes[24], there was no evidence that the virus increased the Ro antigen

expression on the cell surface. No reports have established a particular virus as etiologically relevant to the development of lupus skin lesions.

There has been considerable evidence showing an association between CMV and SLE: (1) human CMV infects and establishes latency in a majority of the population and targets all organ systems of the host, including the skin[25,26]; (2) elevated titers of antibodies to CMV have been found in SLE sera[27,28]; and (3) CMV induced flare of SLE has been reported, including a case-report that CMV caused cutaneous lesions by damage of the vascular endothelium in SLE[29,30]. One possible explanation could be that patients with SLE respond differently to CMV infection than normal individuals. After CMV infection, the virus may act as a trigger and induce the expression of intracellular Ro antigens on the cell surface and make these cells targets for the immune response. Therefore, the present study was undertaken to investigate the effect of CMV infection on the expression of Ro antigen in cultured human keratinocytes. Demonstration of Ro antigen expression on the CMV-infected keratinocyte surface suggests that CMV infection may play an etiological role in the initiation or development of lupus skin lesions in vivo.

Materials and Methods

Cell culture

Normal human epidermal keratinocytes (NHEK 894 1'), purchased from Clonetics (San Diego, CA), were expanded and passaged in keratinocyte growth medium (KGM) (Clonetics), and grown at 37°C in an atmosphere containing 5% CO_2. For all experiments, fifth-passage cells were used when they reached to 70% to 80% confluency.

Viral preparation and infection of keratinocytes

Human CMV (Davis strain) (ATCC, Rockville, MD) was harvested from the culture medium of infected human embryonic lung fibroblasts (MRC-5) (ATCC) grown in the presence of 2% fetal calf serum (GIBCO, Laboratories, Grand Island, NY). The virus was clarified by centrifugation at 1500 g for 20 min and stored in liquid nitrogen until required. The titre of infectious virus was determined by plaque assay using MRC-5

cells[41]. 10^3 plaque-forming units (PFU) of CMV were used to infect keratinocyte cells. After adsorption of the virus for 30 min at 37°C, the infected cells were maintained in KGM medium.

UVB irradiation

Keratinocytes were grown in 56.7 cm^2 plastic Nunclon dishes (GIBCO) or in 96-well flat-bottom plates (FALCON, Becton Dickinson, Lincoln Park, NJ). Cells were irradiated for 5 seconds with UVB (302 nm, 2.0 mJ/cm^2) emitted from a transilluminator (Spectroline, Fisher, St Laurent, QC). This source of irradiation does not contain any UVA or UVC components. The dose of 2.0 mJ/cm^2 was chosen, since this amount of UVB irradiation achieves cell injury, without killing the cells[13]. After irradiation, the medium was replaced with fresh KGM medium. Cells were returned to the 37°C incubator, and grown in the presence or absence of CMV for 24 h.

Antibodies

Anti-peptide antibodies, specific for the amino terminal region (amino acids 7-23) of calreticulin (KEQFLDGDGWTSRWIES), the carboxyl terminal region (amino acids 489-499) of 60kD/Ro (RKKMDIPAKLIVC), and the amino acid region 129-143 of 52KD/Ro (EEAAQEYQEKLQVAL) were kindly provided by Dr. Marianna Newkirk (The Montreal General Hospital Research Institute, Montreal, QC) and have been described previously[19]. These anti-peptide antibodies are capable of binding to native antigens and such binding can be inhibited by the respective peptide or by anti-Ro/SS-A positive SLE serum. IgG $F(ab')_2$ fragments from the anti-peptide antibodies were prepared as described previously[19]. The monoclonal antibody, W6/32 (ATCC), directed against a non-polymorphic determinant on HLA-ABC, was used to study the surface expression of MHC class I antigens. Monoclonal antibodies CIE and CL-1 (kindly provided by Dr. Bernard Brodeur, National Laboratory for Immunology, Laboratory Center for Disease Control, Ottawa, ON) were used to detect CMV immediate early (IE) antigen and CMV late antigen, respectively. Human anti-Ro autoantibody-containing serum, which was specific for 60kD/Ro by ELISA and immunoblotting analysis, was kindly provided by

Dr. John Esdaile (Division of Rheumatology, The Montreal General Hospital, Montreal, QC). As controls, pre-immune rabbit IgG F(ab')$_2$ fragments, normal human serum, and mouse IgG1 isotype control antibodies were used.

Fixed-cell ELISA

The fixed-cell ELISA was performed as previously described[19]. Briefly, for the detection of surface antigens, keratinocytes were fixed to 96-well flat-bottom plates (FALCON) by adding 100 μl of 0.07% glutaraldehyde per well and incubating for 5 min at 25°C. For the detection of cytoplasmic antigens, cells were permeabilized and fixed with 0.005% digitonin in 2% paraformaldehyde (100 μl/well) for 5 min at 37°C, followed by 0.07% glutaraldehyde (100 μl/well) for 5 min at 25°C. Under the latter conditions, both cytoplasmic and surface antigens were detected (referred to as "total cellular" antigen expression). After 3 washes with 0.02 M sodium phosphate buffered saline, pH7.4 (PBS), the fixed cells were then incubated with the anti-peptide IgG F(ab')$_2$ fragments or control pre-immunized rabbit IgG F(ab')$_2$ fragments for 30 min at 4°C. Following 3 washes with PBS, cells were incubated with biotin-conjugated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA). Alkaline phosphatase-conjugated Avidin D (Vector) was used to detect the bound antibodies. Total antigen expression was corrected for the number of cells in the same wells and was expressed as OD_{405} units per cell, as described previously[19]. In each experiment, wells coated with positive control peptides (2.5 μg/ml) and incubated with the appropriate anti-peptide antibody served as positive controls (mean OD_{405} was 2.5 for 60kD/Ro, 2.4 for 52kD/Ro and 1.2 for calreticulin, compared with 0.2 for normal rabbit IgG F(ab')$_2$ control). Each sample was tested in duplicate and in three separate experiments.

Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described[19] with slight modifications. Keratinocytes were grown on glass coverslips, washed with cold (4°C) PBS containing 0.4% bovine serum albumin (BSA), and then stained as either living or fixed cells. For live cell staining, cells were incubated with primary antibodies (affinity-

purified rabbit anti-peptide IgG $F(ab')_2$ fragments or murine monoclonal antibodies) for 1 h at 4°C. Cells were then washed with cold PBS and immediately fixed in 0.005% digitonin in 2% paraformaldehyde for 5 min at 37°C. For fixed cell staining, cells were fixed before incubation with primary antibodies. The binding of rabbit IgG $F(ab')_2$ fragments was detected by incubation with a FITC-conjugated anti-rabbit IgG (1:50 dilution) (TAGO, Inc., Burlingame, CA) for 30 min at 37°C, while the binding of the murine monoclonal antibodies was detected with a rhodamine-conjugated anti-mouse IgG (TAGO)[19]. After three washes with PBS, the slides were mounted in a 90% glycerol/PBS solution containing paraphenylenediamine (0.1 mg/ml). The cells were examined and photographed with a Zeiss fluorescent microscope, using oil immersion, 40x objective and TMAX film (EASTMAN KODAK COMPANY, Rochester, NY). Positive and negative controls that were run in all experiments included: 1) a monoclonal antibody to HLA-ABC, as a positive control for cell surface staining; and 2) pre-immune rabbit IgG $F(ab')_2$ fragments and PBS, in place of the primary antibody. Each sample was run in duplicate and experiments were repeated three times.

Flow cytometry analysis (FACS)

Cultured keratinocytes were collected by treatment with trypsin and EDTA solution (Clonetics) and washed twice with PBS containing 0.5% BSA. 10^6 cells were resuspended in 3 ml KGM and incubated for 3 h at 37°C. The cells were then centrifugated at 220 g for 5 min at 25°C and resuspended in cold PBS containing 1% BSA for 1 h at 4°C. The cells were then incubated with the diluted anti-peptide antibodies or human serum for 1 h at 4°C. After 2 washes with PBS, cells were fixed with 2% paraformaldehyde solution for 30 s at 4°C. Next, cells were incubated with FITC-conjugated goat IgG $F(ab')_2$ anti-rabbit IgG (1:50 dilution) (TAGO) or FITC-conjugated goat IgG $F(ab')_2$ anti-human IgG (1:100 dilution) (TAGO) for 4 h at 4°C. After 3 washes with PBS, these cells were analyzed immediately using a flow cytometer (FACScan, Becton-Dickinson & Co., Mountain View, CA). As controls for each sample, the following specimens from the same lot of cultured keratinocytes were analyzed: 1) untreated cultured keratinocytes that were incubated with the same primary

and second antibodies; and 2) treated cultured keratinocytes that were incubated with pre-immunized rabbit IgG F(ab')$_2$ fragments, normal human serum, or PBS, and the appropriate second antibody.

Immunoblotting

Cultured keratinocytes were used to prepare total cellular extracts according to methods described previously[19]. Cell were washed with cold PBS, scraped and collected by centrifugation. The cells were then suspended in Net-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Nonidet P-40, pH7.4), containing protease inhibitors (50 mM TLCK, 0.1 mM PMSF, 0.1% pepstain A) and incubated for 30 min at 4°C, followed by sonication (Model G112SP1T, 600 Volts, 80 KC, 15 Amps) for 10 min at 25°C. The supernatants were collected after centrifugation. Protein concentration was measured by BCA Protein Assay (Pierce, Rockford, IL) and samples were stored at -70°C until required. The proteins in the keratinocyte extracts were separated using standard 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membrane (0.45 micron) (Bio-Rad, Richmond, CA). After blocking with PBS containing 0.4% BSA for 16 h at 4°C, blots were incubated with serum diluted in PBS for 2 h at 37°C. The nitrocellulose filters were washed three times with PBS containing 0.05% Tween 20 (PBST) and incubated with biotinylated anti-rabbit IgG (1:2000 dilution) for 1 h at 37°C. After 3 washes with PBST, the bound antibody was detected by incubation with horseradish peroxidase (HRP)-labelled Avidin D (Vector) (1:3000 dilution) and 3,3'-diaminobenzidine tetrahydrochloride substrate (Sigma, St. Louis, MO). The positions of bands were compared to those of standard molecular weight markers run in the same gel.

Statistical Analysis

Data were analyzed by the paired t-test using the Instat program (GraphPAD Software, San Diego, CA). P values less than 0.05 were considered significant.

Results

CMV infection of cultured human keratinocytes

The initial experiments were designed to determine whether CMV was able to infect human keratinocytes in vitro. Immunofluorescence data for the cytoplasmic staining of keratinocytes is shown in Figure 1. CMV infected cells after 24 h of infection (Figure 1A), but not uninfected cells (Figure 1B), exhibited bright, sharp nuclear fluorescence with monoclonal antibody CIE, specific for the CMV IE protein. The same typical nuclear fluorescence pattern for IE protein was observed in CMV-infected human fibroblasts, which served as a positive control for CMV infection (data not shown). In contrast, the monoclonal antibody CL-1, specific for the viral late protein, showed Golgi-like fluorescence staining in the cytoplasm of approximately 5% keratinocytes at day 4 of CMV infection (Figure 1C), but not at 24 h of infection (data not shown). No Golgi-like fluorescence staining was found in uninfected cells (Figure 1D).

To determine whether these viral proteins can be expressed on the surface of infected keratinocytes, monoclonal antibody CIE or CL-1 was incubated with whole unfixed cells with or without CMV infection at 24 h or 48 h. Neither CMV IE nor CMV late proteins were detectable on the keratinocyte surface, while both uninfected and CMV-infected cells, treated under the same conditions, revealed intense surface staining with an anti-MHC class I monoclonal antibody (Figure 2). No cell surface, cytoplasmic or nuclear staining was seen with the mouse IgG control antibody or PBS (data not shown). Finally, keratinocytes were found to produce infectious virus, as demonstrated by the infection of human fibroblasts with supernatant derived from CMV-infected keratinocytes (data not shown).

Effect of CMV on the induction of Ro antigens in keratinocytes

The effect of CMV infection on the induction of Ro antigens in keratinocytes was evaluated in parallel with the effect of UVB irradiation, a known stimulus of Ro antigen expression in human keratinocytes. Figure 3 shows the binding of antibody specific for 60kD/Ro, 52kD/Ro or calreticulin to the surface of keratinocytes left untreated or 24 h after treatment with CMV and/or UVB, using a fixed-cell ELISA. With CMV infection

FIGURE 1. Indirect immunofluorescent cytoplasmic staining of CMV-infected (A, C) and uninfected (B, D) human keratinocytes with anti-CMV monoclonal antibodies. Cells were incubated with either anti-CMV IE monoclonal antibody at 24 h (A, B) or anti-CMV late protein monoclonal antibody at 4 days (C, D) after infection or uninfection.

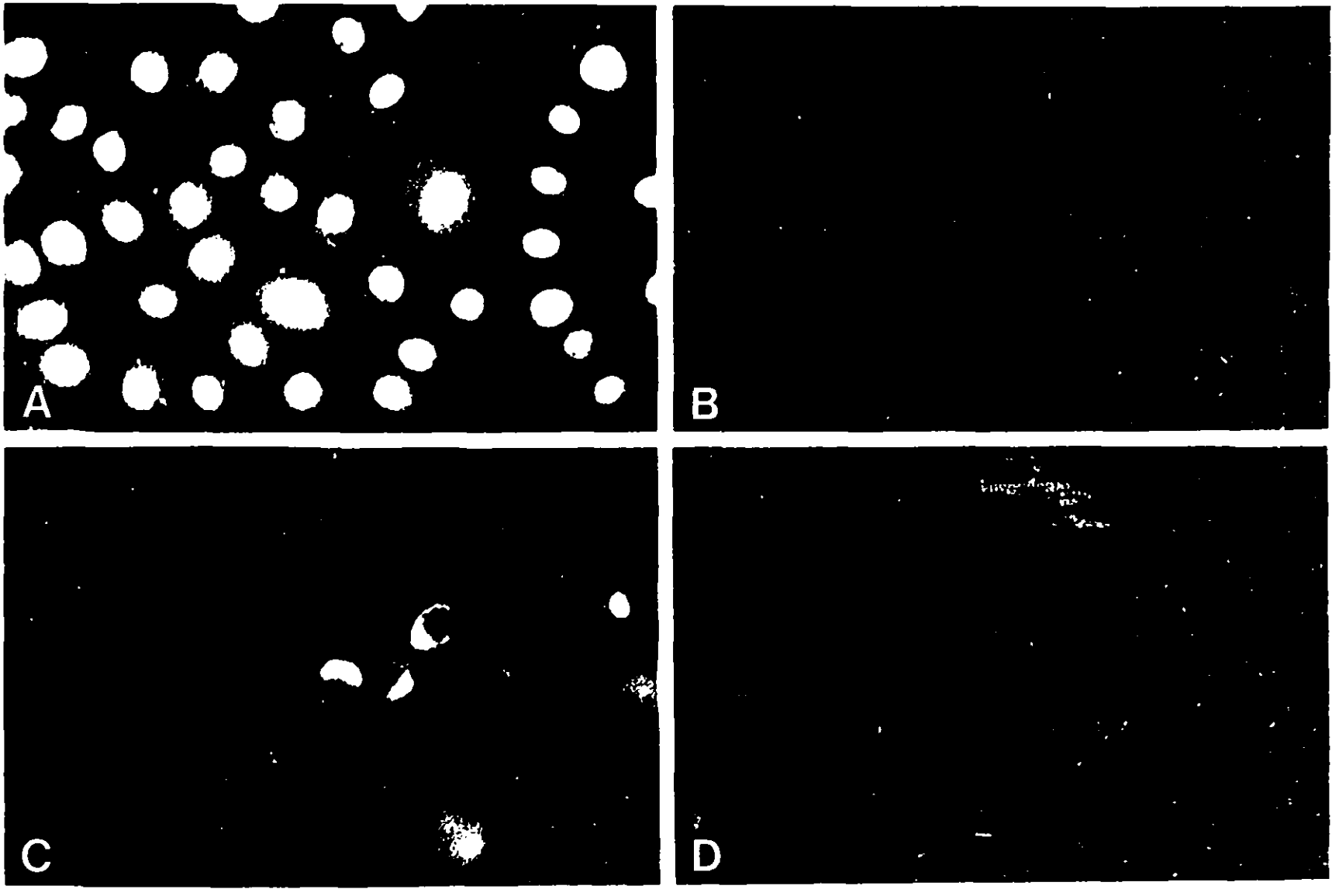
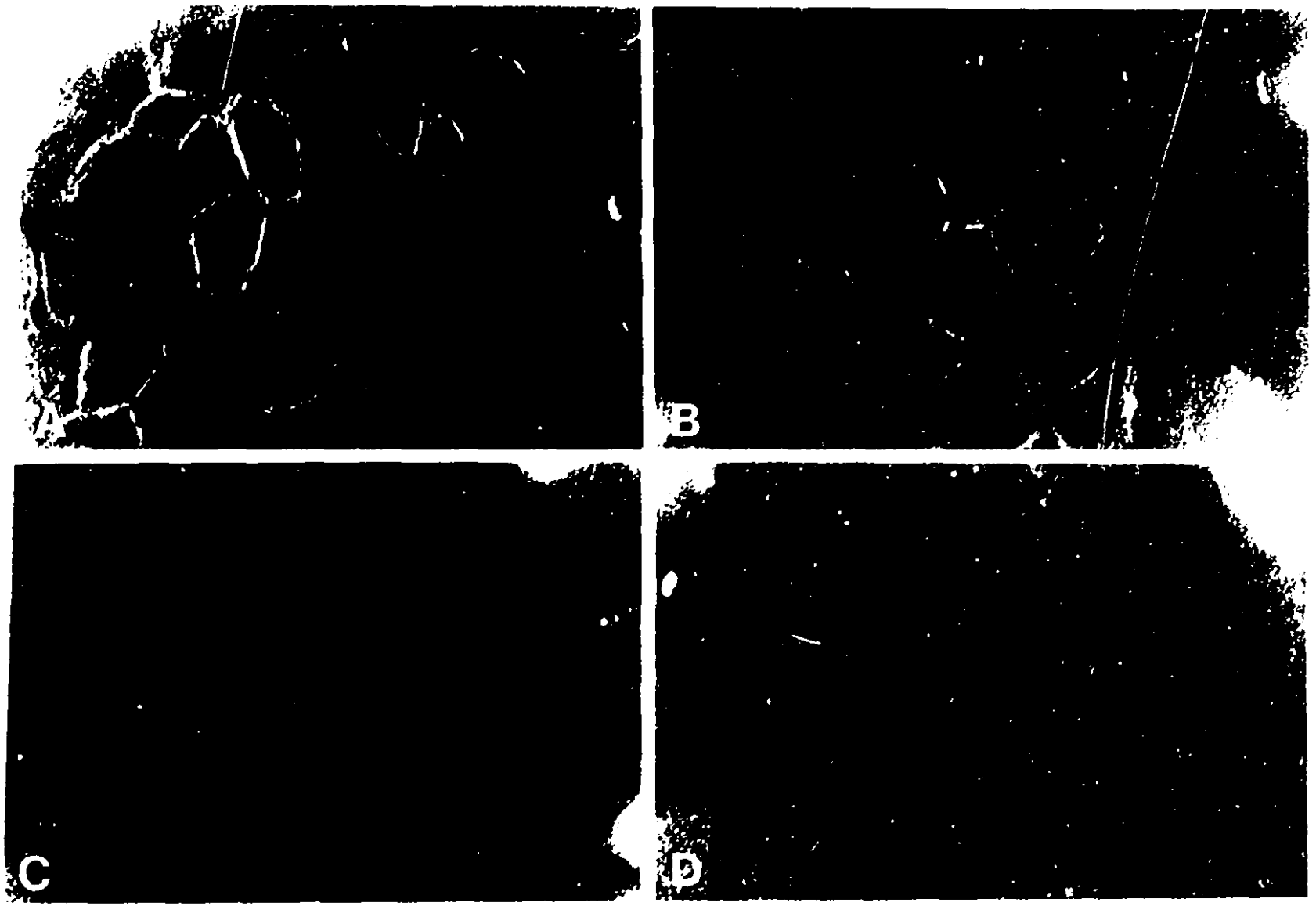

A
B
C
D

FIGURE 2. Indirect immunofluorescent surface staining of human keratinocytes with anti-HLA-ABC (A, B), anti-CMV IE (C) or anti-CMV late protein (D) monoclonal antibodies. (A) uninfected cells; (B, C) cells infected with CMV for 24 h; (D) cells infected with CMV for 4 days. Cells were fixed after incubation with the primary antibody.


A
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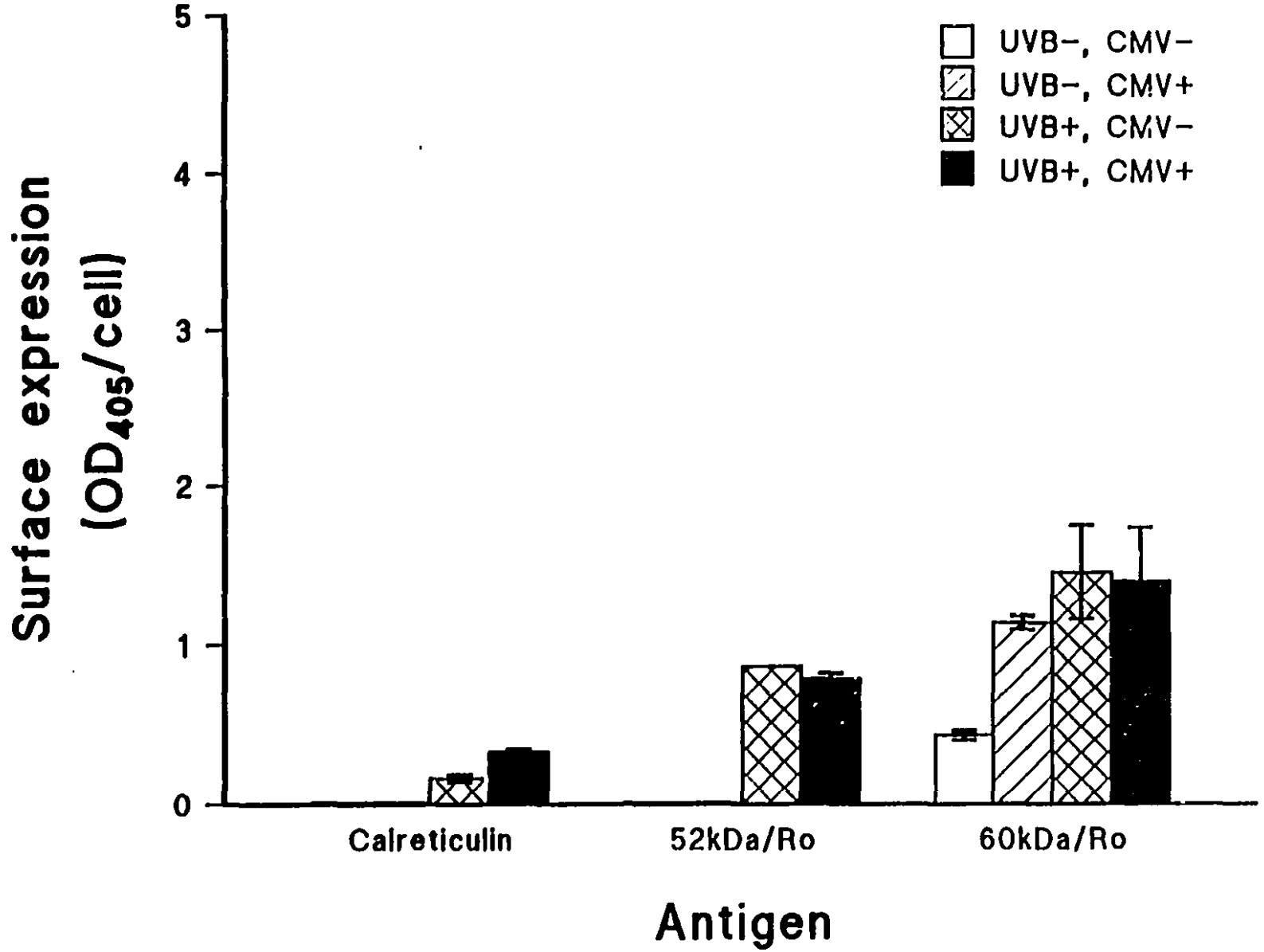
D

FIGURE 3. Ceil surface expression of calreticulin, 52kD/Ro and 60kD/Ro antigens. Mean binding (OD_{405}/cell $\pm$ SE) of anti-peptide antibodies to human keratinocytes left untreated or 24 h after treatment with CMV, UVB or UVB plus CMV was assessed by fixed-cell ELISA.


Surface expression
(OD405/cell)
5
4
3
2
1
0
UVB−, CMV−
UVB−, CMV+
UVB+, CMV−
UVB+, CMV+
Calreticulin
52kDa/Ro
60kDa/Ro
Antigen

alone, increased cell surface binding of anti-60kD/Ro occurred, but no surface binding of anti-52kD/Ro and anti-calreticulin antibodies was found. In contrast, with UVB irradiation alone, anti-60kD/Ro, anti-52kD/Ro and anti-calreticulin antibodies all bound to the cell surface. With a combination of UVB irradiation and CMV infection, results were similar to those obtained on UVB-irradiated cells, and there appeared to be no synergistic effect of UVB and CMV. There was no surface binding of anti-52kD/Ro or anti-calreticulin antibody to untreated keratinocytes, while anti-60kD/Ro antibody bound, but at a much lower level than to treated cells ($P < 0.05$).

The total cellular (surface and cytoplasmic) binding of these antibodies to keratinocytes left untreated or 24 h after CMV and/or UVB treatment is shown in Figure 4. Anti-60kD/Ro, anti-52kD/Ro and anti-calreticulin antibodies all bound to CMV-infected cells, UVB-irradiated cells, and cells treated with UVB and CMV. Although the levels of detectable antibody binding varied with the different antibodies or stimuli, only the anti-60kD/Ro antibody binding was markedly increased after treatment and was maximally induced by CMV infection alone. Untreated keratinocytes showed low detectable levels of total cellular binding of anti-52kD/Ro and anti-60kD/Ro antibody, but not anti-calreticulin antibody.

Distribution of Ro antigens after CMV infection of keratinocytes

Immunofluorescence analysis was used to study the effect of CMV infection on the cellular distribution of Ro antigens in keratinocytes (Figure 5). In live cells infected with CMV alone, the anti-60kD/Ro peptide antibody gave a continuous staining pattern on the cell surface at 24 h after infection (Figure 5B). The staining pattern of the anti-60kD/Ro antibody was similar to that observed with the anti-MHC class I monoclonal antibody (Figure 2). Surface binding of anti-60kD/Ro antibody was also observed on cells irradiated with UVB (Figure 5C), or cells treated with a combination of UVB and CMV (Figure 5D), but the fluorescence intensity appeared to be weaker than that of cells infected with CMV alone (Figure 5B) and less granular than staining previously observed with human anti-Ro sera[13,14]. The incubation of treated cells with pre-immune rabbit IgG or PBS did not show detectable cell surface staining (data not shown) and untreated cells

FIGURE 4. Total cellular (surface and cytoplasmic) expression of calreticulin, 52kD/Ro and 60kD/Ro antigens. Mean binding (OD_{405}/cell $\pm$ SE) of anti-peptide antibodies to human keratinocytes left untreated and 24 h after treatment with CMV, UVB or UVB plus CMV was assessed by fixed-cell ELISA.

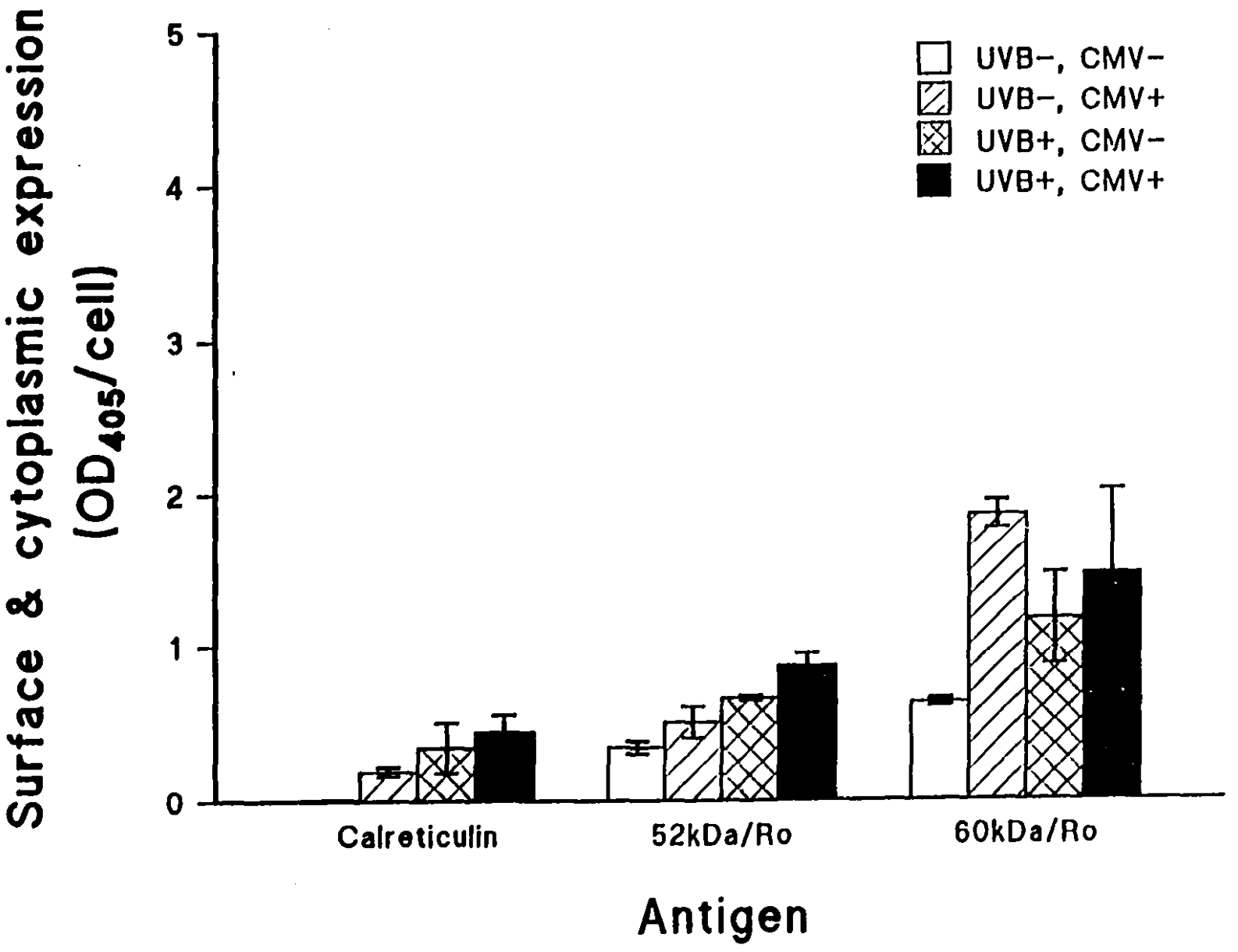
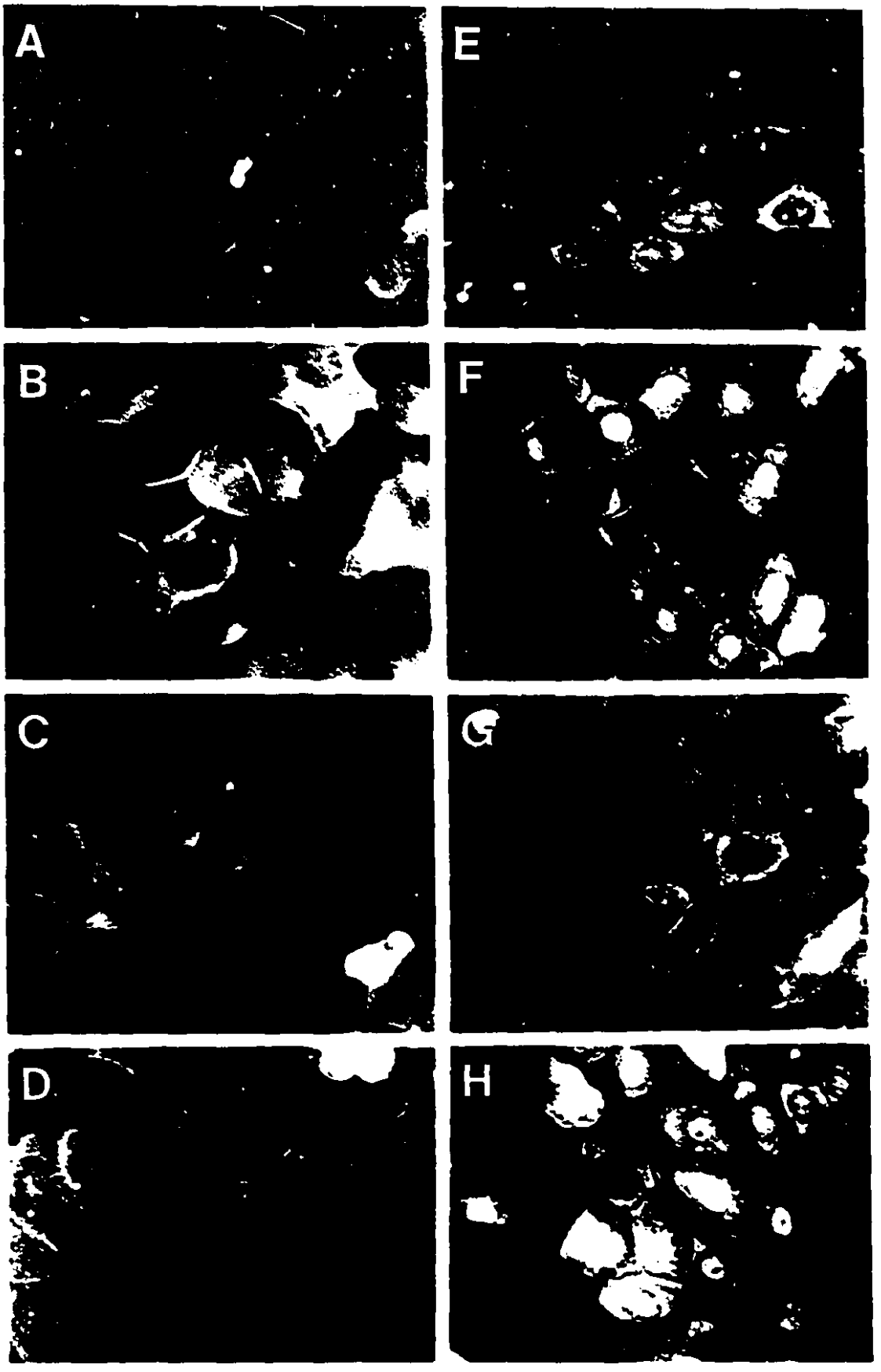

Surface & cytoplasmic expression
(OD405/cell)
5
4
3
2
1
0
UVB−, CMV−
UVB−, CMV+
UVB+, CMV−
UVB+, CMV+
Calreticulin
52kDa/Ro
60kDa/Ro
Antigen

FIGURE 5. Indirect immunofluorescent surface and cytoplasmic staining of human keratinocytes with anti-60kD/Ro peptide antibody. (A, E) untreated cells; (B, F) at 24 h after CMV infection; (C, G) at 24 h after UVB irradiation; (D, H) at 24 h after UVB plus CMV treatment. In panels A to D, cells were fixed after incubation with the primary antibody (surface staining). In panels D to H, cells were fixed before incubation with the primary antibody (cytoplasmic staining).


A
E
B
F
C
G
D
H

did not bind anti-60kD/Ro peptide antibody (Figure 5A). In contrast, anti-52kD/Ro peptide antibody stained only a few live CMV-infected cells and produced granular surface staining (data not shown) that was different from the staining with anti-60kD/Ro antibody. However, no visible cell surface staining with anti-52kD/Ro antibody was found in UVB-irradiated cells, UVB plus CMV treated cells, or untreated cells (data not shown). Anti-calreticulin peptide antibody did not bind to the surface of CMV-infected or uninfected cells, but showed weak surface staining of cells treated with UVB irradiation or a combination of UVB and CMV (data not shown).

The reaction of anti-60kD/Ro peptide antibody with fixed keratinocytes showed an increase in the intensity of cytoplasmic and/or nuclear staining in treated but not in untreated cells (Figures 5E-H). Interestingly, anti-52kD/Ro peptide antibody also caused dense granular cytoplasmic staining in the cytoplasm of CMV-infected cells, but not in UVB-treated cells with or without CMV infection or in uninfected cells (data not shown). Anti-calreticulin peptide antibody showed no difference in cytoplasmic staining between treated and untreated cells (data not shown).

Cell surface expression of Ro antigens during CMV infection

FACS analysis was performed as a more quantitative measure of the CMV-induced Ro and calreticulin antigen expression on the keratinocyte surface. Figure 6 shows that CMV infection or UVB irradiation induced the augmentation of cell surface binding of anti-60kD/Ro antibody. Treated cells, but not untreated cells, incubated with specific anti-60kD/Ro peptide antibody (Figure 6A-D) or human anti-60kD/Ro antibody (Figure 6E-H) showed positive staining.

Table I shows the results of the flow cytometric analysis based on three separate experiments. At 24 h after treatment of keratinocytes, surface binding of anti-60kD/Ro was found in 71.2% (±5.5) of CMV-infected cells, 79.2% (±4.9) of UVB-irradiated cells, and 80.6% (±3.4) of UVB and CMV-treated cells. In contrast, only 26.2% (±4.1) of untreated cells showed positive staining with the same antibody. Similar observations were made with a human serum that contained anti-60kD/Ro antibodies, in that 48.3% (±4.0) of cells were positive after CMV infection, 57.2% (±0.5) of cells

FIGURE 6. FACS analysis of binding of anti-60kD/Ro antibodies to human keratinocytes treated with CMV and/or UVB. (A, E) untreated cells; (B, F) at 24 h after CMV infection; (C, G) at 24 h after UVB irradiation; (D, H) at 24 h after UVB plus CMV treatment. Cells were incubated with either specific rabbit anti-60kD/Ro peptide antibody (A to D) or human serum containing anti-60kD/Ro antibodies (E to H). The shaded peaks represent treated or untreated cells that were incubated with either pre-immune rabbit IgG (A to D) or normal human serum (D to H), while the unshaded peaks represent cells incubated with anti-60kD/Ro antibodies. All cells were fixed after incubation with the primary antibody and stained with FITC-conjugated antibodies. In this figure, the percentages of positive cells are 25.7% in (A), 63.1% in (B), 73.2% in (C), 75.3% in (D), 27.5% in (E), 52.3% in (F), 57.7% in (G), and 56.3% in (H).

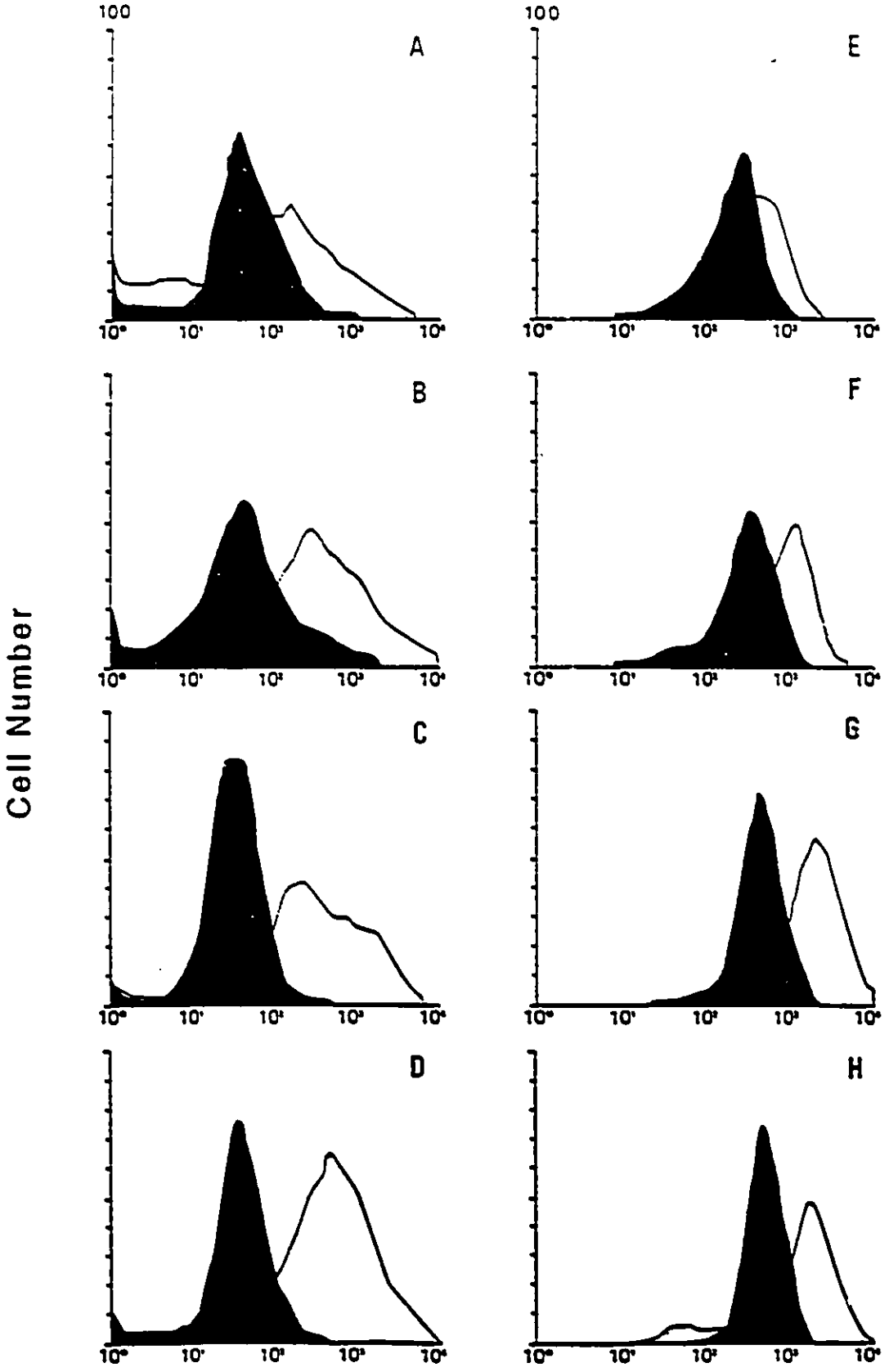

100
A
E
B
F
C
G
D
H
Cell Number
Fluorenscence Intensity

Table I. Binding of Anti-Peptide Antibody to Cultured Keratinocytes Treated with CMV and UVB.

	Specificity of Antibody Binding[a]		
Treatment	60kD/Ro	52kD/Ro	calreticulin
UVB−, CMV−	26.2 ± 4.1	17.1 ± 9.9	4.5 ± 0.8
UVB−, CMV+	71.2 ± 5.5[b]	14.8 ± 7.8	2.5 ± 0.3
UVB+, CMV−	79.2 ± 4.9[b]	64.2 ± 5.4[b]	23.2 ± 1.9[b]
UVB+, CMV+	80.6 ± 3.4[b]	61.3 ± 1.2[b]	17.0 ± 0.7[b]

[a]Binding was assayed by FACS analysis and represents the mean percentage ± standard error of positive cells from 3 separate experiments.

[b]$p < 0.01$ compared to untreated cells.

after UVB irradiation and 69.6% (±15.7) of cells after UVB plus CMV treatment, compared with 22.3% (±11.2) of untreated cells (data not shown). In contrast, the binding of anti-52kD/Ro antibody to the surface of CMV-infected cells was low (14.8% ±7.8) and not significant, compared with untreated cells (17.1% ±9.9). However, significant positive cell staining was observed after UVB irradiation (64.2% ±5.4) and UVB plus CMV treatment (61.3% ±1.2). Similarly, CMV infection did not increase the binding of anti-calreticulin antibody to cells (2.5% ±0.3 in CMV-infected cells, compared with 4.5% ±0.8 in untreated cells), while the antibody binding occurred following UVB irradiation (23.3% ±1.9) and UVB plus CMV treatment (17.0% ±0.7).

Total cellular levels of Ro proteins in CMV-infected keratinocytes

Immunoblotting was used to examine changes in the total cellular 60kD/Ro, 52kD/Ro and calreticulin in keratinocytes after CMV infection, UVB irradiation, or combined treatment with UVB and CMV. In these experiments, no significant increase in the intensity of the 60kD or 52kD band, recognized by anti-60kD/Ro or anti-52kD/Ro sera, respectively, was found in the extracts of treated cells, compared with untreated cells (data not shown). Similarly, anti-calreticulin sera showed a strong 60kD protein band in the extracts of untreated keratinocytes, but there was no increased intensity in the extracts of treated keratinocytes (data not shown).

Discussion

CMV is an ubiquitous, potentially pathogenic herpesvirus. Its infection in humans is common, affecting approximately 50-100% of adults worldwide[25] and involving virtually all organ systems, despite the relative rarity of clinical disease. In studies of cutaneous CMV infection, investigators indicated that CMV causes damage to the skin only if the host immune response is compromised, as in patients with AIDS[32-34]. Although CMV can cause cutaneous lesions by damage of the vascular endothelium[30], there is very little information on CMV in human keratinocytes, especially with regard to its possible role in autoimmune disease.

The present study demonstrates that human CMV can infect human keratinocytes in vitro and that 60kD/Ro antigen surface expression is induced by this infection. Infected keratinocytes expressed immediate early and late viral proteins, and cells continued to produce virus throughout culture. When three different methods, a fixed-cell ELISA, immunofluorescence and FACS analysis, were used to detect the binding of anti-Ro antibody to the cell surface as a measure of Ro antigen surface expression, the data show that anti-60kD/Ro antibody, but not anti-52kD/Ro or anti-calreticulin antibody, significantly bound to the CMV-infected keratinocyte surface. However, there was no detectable increase of total 60kD/Ro protein in extracts of the CMV-infected cells by Western blot analysis, suggesting that 60kD/Ro antigen appears to be redistributed from the cytoplasm to the cell surface as a consequence of CMV infection.

Previous studies have demonstrated that UVB irradiation has a profound effect on the cellular redistribution of Ro antigens[13,14]. In the present study, the effects of CMV infection on cell cultures were compared with those of UVB irradiation. The anti-60kD/Ro antibody staining pattern on the surface of CMV-infected keratinocytes was quite unlike the pattern of punctate fluorescence of Ro antigens that have been described by others[13,14] on the surface of keratinocytes following UVB exposure. This may be accounted for by differences in the treatment of the cells, donors of keratinocyte cells, or anti-Ro antibodies (rabbit anti-peptide antibodies versus human anti-Ro antibody-containing sera) used for the studies. In addition, the present studies show that 60kD/Ro, 52kD/Ro and calreticulin were all induced on the UVB-irradiated cell surface, while CMV induced cell surface expression of 60kD/Ro only. No significant synergistic effect of UVB and CMV was found for any of these antigens. Ro antigen expression appears to be more selected by CMV infection than UVB irradiation, which may arise from differences in the mechanism of the induction of Ro antigens to the cell surface. Furthermore, the findings in this study differ from those of Kawashima et al.[35], who recently reported that both total cellular and surface calreticulin, but not 60kD/Ro and 52kD/Ro, were increased in a transformed human epidermal keratinocyte cell line after UVB irradiation. A possible explanation for this discrepancy is that transformed keratinocytes were used in the latter study.

An intriguing question is how CMV functions in the redistribution of the 60kD/Ro protein. Although this has not been addressed by the present study, Ro antigen expression has been suggested by others to be associated with cell proliferation. Previous studies, using immunofluorescence, found that the amount of 60kD/Ro antigen in normal human adult skin was barely detectable, while this antigen was readily detected in fetal and newborn skin, as well as in rapidly proliferating adult skin explant outgrowth cells[36,37]. Increased surface and cytoplasmic Ro antigen expression was found in keratinocytes grown in a low calcium environment[13,18,24,38], in which cultured keratinocytes are in a less-differentiated, more rapidly proliferating state. Another observation in agreement with these findings is the increase in cytoplasmic Ro antigen in SV40-transformed human keratinocytes, but not in primary cultured keratinocytes derived from adult skin[24]. Following SV40 infection, keratinocytes acquire a high growth potential and lose their ability to differentiate in an orderly fashion in vitro[24]. Human CMV infection also can cause proliferation of a variety of cells. Recently, the ability of human CMV infection to induce cell proliferation has been explained by the demonstration that the viral protein, CMV IE, combines with and inactivates the p53 protein in cells, resulting in excessive cell growth[39]. In addition, a recent study has shown that apoptotic skin keratinocytes, induced by UVB irradiation, contain surface blebs expressing autoantigens, such as Ro[40]. As apoptosis may occur in response to viral infection[41-42], future studies on the relationship between CMV infection and apoptosis of target cells may shed light on how CMV infection affects the cellular redistribution of Ro antigens.

The increased expression of Ro antigen on the CMV-infected keratinocyte surface has important clinical implications. Increased expression of Ro antigen on the cell surface could provide an initial antigenic stimulus for the induction of specific autoantibodies. Expression of Ro antigen on the cell surface, in the presence of circulating autoantibodies, could result in in situ formation of antigen-autoantibody complexes within the skin. These complexes could then activate complement or other proinflamatory pathways within the skin and result in the relevant tissue damage. Although CMV infection and UVB irradiation appear to be clinically relevant disease triggers in the induction of Ro antigen expression on the keratinocyte surface, it is likely

that the clinical appearance of skin lesions is due to the interplay of a number of variables including viral infection, UVB exposure, circulating of autoantibodies, expression of autoantigens at different epidermal levels, and genetic predisposition.

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CHAPTER VI

GENERAL DISCUSSION

The studies presented in this thesis demonstrate that Ro antigens, normally detected in the nucleus and cytoplasm of human cells, can be induced to appear on the cell surface by CMV infection. This cell surface expression was selective for components of the Ro antigen complex, and the autoantigen expression varied with the treatment of the cells and the cell type. These observations are of particular relevance to those interested in the pathogenesis of SCLE and NLE, and support the hypothesis that viral infection can cause the cell surface expression of Ro antigens and, thus, make these cells targets of an autoimmune response.

In previous studies, LeFeber et al. demonstrated that UVB irradiation could induce the expression of Ro antigens on the cell surface of cultured human keratinocytes (LeFeber et al., 1984). Their experiments showed that this expression appeared to be an active process, rather than a passive leakage of antigens to the cell surface, because the cells had only undergone sublethal injury without altered membrane permeability and blocking protein synthesis in these cells prevented the surface antigen expression. Subsequently, Furukawa et al. reported that estradiol could augment the binding of IgG from antisera specific for Ro antigen to the surface of cultured human keratinocytes (Furukawa et al., 1988). This augmentation was not induced by other hormones, such as dihydrotestosterone, testosterone and progesterone, and was partially inhibited by the anti-estrogen, nafoxidine. These studies suggest that certain clinically relevant perturbations can affect the cellular distribution of Ro antigens, and prompted us to investigate the effect of viral infection on the expression of Ro antigens in human cells.

In this thesis, the initial studies were designed to use CMV-infected human fibroblasts as a model, to test whether a virus infection would induce the expression of the human Ro autoantigens. The observations were that CMV infection of fibroblasts resulted in the expression of calreticulin, but not the classical 60kD/Ro antigen, on the cell surface (Chapter III). The second study addressed the question of whether CMV infection, in combination with other stimuli, can induce the surface expression of Ro antigens in human fibroblasts. A synergy between UVB irradiation and CMV infection

on the fibroblast surface expression of 52kD/Ro antigen, but not 60kD/Ro and calreticulin, was found. This enhanced surface expression of 52kD/Ro was significant and specific, compared with untreated cells, cells infected with CMV or irradiated with UVB only, and cells subjected to other treatments, including low pH and increased temperature (Chapter IV). To better understand the potential role of viral induction of Ro antigens in target tissues relevant to SLE, such as skin, the third study was undertaken in human keratinocytes, the most numerous cells in the epidermis. Human CMV was demonstrated to be capable of infecting keratinocytes in vitro and induced the expression of 60kD/Ro antigen, but not 52kD/Ro and calreticulin, on the keratinocyte surface. As there was no increase in total cellular 60kD/Ro antigen expression after viral infection, 60kD/Ro antigen appears to be redistributed from the cytoplasm to the cell surface. In contrast to the effects of CMV infection on keratinocytes, UVB irradiation induced the expression of 60kD/Ro, 52kD/Ro and calreticulin on the keratinocyte surface (Chapter V).

It should be noted that there are four antigenically distinct Ro molecules, ranging in size from 52kD to 60kD. Although the physiological functions of the Ro complex remain unclear, it is important to examine the regulation of expression of each of the Ro antigens independently to better understand their potential pathogenic role in anti-Ro antibody-related diseases. The present studies have addressed this question with two Ro proteins (60kD and 52kD) and calreticulin, a protein previously thought to be a Ro antigen, and indicate that all components of the Ro complex are not equally perturbed by the same stimuli.

There were some discrepancies in the surface expression of Ro antigens between human fibroblasts and keratinocytes. One possible explanation for this is that the HLA status of the donors, from whom the fibroblasts or keratinocytes were derived, may influence the cell surface antigen expression, as HLA status is known to play a role in patient susceptibility to developing SLE. Another possible influence could relate to the fact that anti-Ro associated diseases, such as SCLE and NLE, are very limited in their

organ involvement. This could account for the present variation, because the fibroblasts and keratinocytes were obtained from different tissue sources. The content of Ro antigens may, in fact, differ between these two cell types. A further possibility could be that fibroblasts and keratinocytes have different susceptibilities to each of the stimuli tested.

The underlying mechanisms responsible for the enhanced and selective expression of Ro antigens on the cell surface induced by CMV infection was not directly addressed in this study. However, the induction of Ro antigen expression on the CMV-infected cell surface may be as relevant as UVB irradiation in the pathogenesis of the disease. Patients with SCLE and NLE almost always have antibodies to Ro antigens in their serum. Upon CMV infection (such as primary infection, persistent infection, reactivation of latent infection, reinfection, or congenital and perinatal infections), the virus may act directly, or might potentiate other factors, to induce Ro antigen expression on the keratinocyte surface. The exposed Ro antigens can then be recognized by circulating anti-Ro antibodies and lead to immunological cell lysis by complement activation or by antibody-dependent cellular cytotoxicity (ACDD). Thus, the demonstration of increased surface expression of Ro antigens on CMV-infected keratinocytes could account for the skin disease in SCLE and NLE. It is also possible that a similar mechanism operates in isolated complete congenital heart block (CCHB) in NLE, although it is not known whether the Ro antigen is induced by CMV infection on the surface of fetal cardiac tissue cells. However, Ro antigen expression on the surface of myocardial fibres in fetal cardiac tissue from NLE patients with CCHB (Horsfall et al., 1991) and in intra-uterine CMV infection affecting infants, including affecting one twin and not the other (Cooper, 1985; Taylor et al., 1986; Park et al.,1987), has been previously documented.

These studies present new evidence that human CMV may be involved in the development of skin lesions in SCLE and NLE. Further identification of CMV genomes and their products in diseased tissues will provide a better understanding of the biological function of CMV and the significance of CMV infection, and may help to explain at least

one of the mechanisms that result in cutaneous or cardiac tissue damage in SLE patients. In addition, a recent study has shown that apoptotic skin keratinocytes, induced by UVB irradiation, contain surface blebs expressing autoantigens, such as Ro (Casciola-Rosen et al., 1994). As apoptosis may occur in response to viral infection (Levine et al., 1993; Dawson et al., 1995), future studies should focus on the correlation between CMV infection and apoptosis of target cells and altered autoantigen location. This approach may shed light on how CMV infection affects the cellular redistribution of Ro antigens.

CMV infection and UVB irradiation appear to be clinically relevant disease triggers in the induction of Ro antigen expression on the keratinocyte surface. However, it is likely that the clinical appearance of skin lesions is due to the interplay of a number of variables including genetic predisposition, viral infection, UVB exposure, circulating autoantibodies, and expression of autoantigens at different epidermal levels.

CHAPTER VII

APPENDIX

EFFECTS OF CMV INFECTION AND UVB IRRADIATION ON Ro ANTIGEN EXPRESSION IN HUMAN EPITHELIAL-LIKE TUMOR CELLS

Objective: To determine the effects of CMV infection and UVB irradiation on the expression of Ro antigens and calreticulin in human epithelial-like tumor cells (HT-1080).

Methods: HT-1080 cells (ATCC, Rockville, MD) were infected with CMV and/or irradiated with UVB, as outlined previously (Chapter III). Viral protein expression (CMV IE and late antigens) was determined by indirect immunofluorescence. Ro and calreticulin antigen expression were examined by a fixed-cell ELISA, indirect immunofluorescence and immunoblotting.

Results: No viral protein expression could be detected in CMV-infected HT-1080 cells. No observed increase of the surface and total cellular expression of 60kD/Ro, 52kD/Ro and calreticulin were found in HT-1080 cells with CMV infection, UVB irradiation or a combined UVB with CMV treatment, compared with untreated cells (data not shown).

Conclusion: In contrast with human fibroblasts and keratinocytes (Table 1), human epithelial-like tumor cells (HT-1080) do not appear to be infected by CMV in vitro. No increased Ro or calreticulin autoantigen expression was found in these cells either before or after CMV infection or UVB irradiation. This provides further evidence that Ro expression is specifically induced by clinically-relevant perturbations, including CMV infection and UVB irradiation, in human fibroblasts and keratinocytes.

Table 1. Comparison of antigen expression in three human cell lines treated with CMV infection and UVB irradiation

	Antigen Expression*				
	CMV IE	CMV late	60kD/Ro	52kD/Ro	Calreticulin
Fibroblasts					
UVB−,CMV−	−	−	+C	+N	+C
UVB−,CMV+	+N	+C	+C	+N	+S
UVB+,CMV−	−	−	+C	+N	+S
UVB+,CMV+	+N	+C	+C	+S	+S
Keratinocytes					
UVB−,CMV−	−	−	+C	+N	+C
UVB−,CMV+	+N	+C	+S	+N	+C
UVB+,CMV−	−	−	+S	+S	+S
UVB+,CMV+	+N	+C	+S	+S	+S
HT-1080 cells					
UVB−,CMV−	−	−	+C	+N	+C
UVB−,CMV+	−	−	+C	+N	+C
UVB+,CMV−	−	−	+C	+N	+C
UVB+,CMV+	−	−	+C	+N	+C

* CMV IE and late antigen expression were detected by indirect immunofluorescence; 60kD/Ro, 52kD/Ro and calreticulin antigen expression were detected by a fixed-cell ELISA and confirmed by indirect immunofluorescence. +: positive; −: negative; S: surface; N: nuclear; C: cytoplasmic.

PREDICTION OF ANTIGENIC DETERMINANTS FROM AMINO ACID SEQUENCES OF Ro PROTEINS

Objective: To identify putative antigenic determinants from the amino acid sequences of Ro proteins in order to prepare synthetic peptides for this study.

Methods: The hydrophilicity analysis of Ro proteins, sequence comparisons and data base searches were performed (Hopp & Woods, 1981; Kyte & Doolittle, 1982) using the University of Montreal Genetics Computer Group (GCG) Sequence Analysis Software Package.

Results: A hydrophilicity analysis predicted several regions in the amino acid sequence of each Ro protein that might be antigenic. Then, regions with considerable sequence homology to rabbit proteins (to avoid immunological tolerance) and proteases (this could complicate the immunoassays) were eliminated. The antigenic regions from amino acid residues 487 to 499 in 60kD/Ro protein and 129 to 143 in 52kD/Ro protein (Figure 1) were chosen for synthesizing peptides chemically. Subsequent procedures, including raising antisera against such synthetic peptides and testing the specificities of these antisera, have been described in Chapter III.

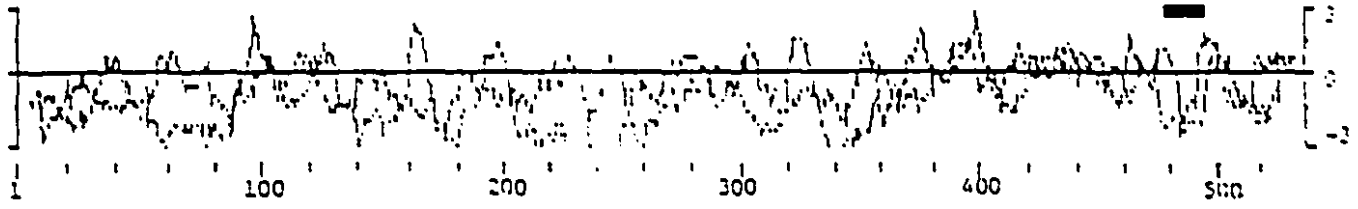
Conclusion: Based on the ability of these peptides to induce an antibody response in rabbits, the synthetic peptides corresponding to residues 487-499 of 60kD/Ro or 129-143 of 52kD/Ro sequence had antigenic activity (Chapter III). Identification of antigenic determinants from amino acid sequence data by hydrophilicity analysis is very useful in the prediction of immunogenic peptides.

Figure 1. Hydrophilicity analysis of 60kD/Ro (A) and 52kD/Ro (B) proteins. The x axis represents each amino acid in the sequence of the Ro protein. The y axis represents the range of hydrophilicity values. The solid bars indicate the location of the amino acid residues in the hydrophilic regions chosen for peptide synthesis in this study, which are shown above each figure in single letter code.

60KD/Ro amino acids 487-499

RKKMDIPAKLIVC

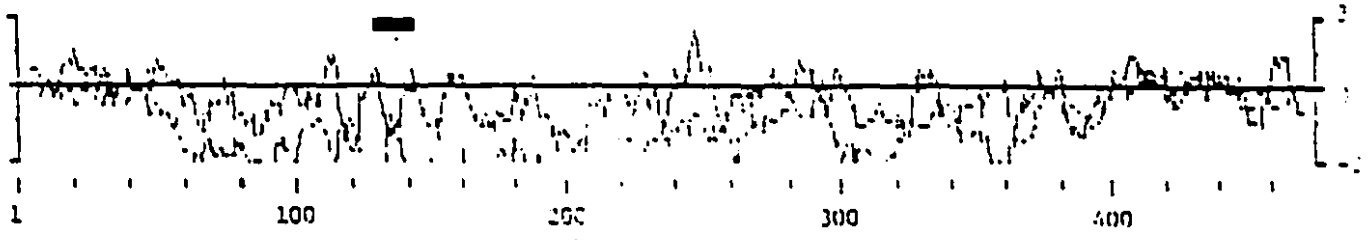
A



52KD/Ro amino acids 129-143

EEAAQEYQEKLQVAL

B



CHAPTER VIII

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CHAPTER IX

STATEMENT OF ORIGINALITY

1) Infection of cultured human fibroblasts with human CMV was found to increase the cell surface expression of calreticulin, but not 60kD/Ro autoantigen. This is the first demonstration of viral induction of the expression of the human autoantigen, calreticulin, on the cell surface.

2) Human CMV infection, in combination with UVB irradiation, synergistically induced the expression of 52kD/Ro, but not 60kD/Ro or calreticulin, on the surface of human fibroblasts. This enhanced expression of 52kD/Ro autoantigen on cells was significant and specific, compared with untreated cells, cells infected with CMV or irradiated with UVB only, and cells subjected to other treatments, including low pH. This is the first report showing that environmental stimuli synergize in the induction of the cell surface expression of the human autoantigen, 52kD/Ro.

3) Human CMV was demonstrated to be capable of infecting human keratinocytes in vitro. Infected keratinocytes expressed CMV immediate early and late proteins, and continued to produce virus throughout culture. This study provides an important model to study the pathogenic role of CMV in the development of human skin diseases.

4) CMV infection induced the expression of 60kD/Ro autoantigen, but not 52kD/Ro or calreticulin, on the human keratinocyte surface. As there was no increase in total cellular expression of 60kD/Ro antigen after viral infection, 60kD/Ro antigen appears to be redistributed from the cytoplasm to the cell surface. This is the first demonstration of the effect of CMV infection on the expression of Ro autoantigens in human keratinocytes.

5) UVB irradiation induced the expression of 60kD/Ro, 52kD/Ro and calreticulin on the keratinocyte surface, while CMV infection caused the keratinocyte surface expression of 60kD/Ro antigen only. Demonstration of 60kD/Ro antigen expression on the CMV-infected keratinocyte surface suggestes that virus infection may play a role in the initiation and/or development of skin lesions in SLE.