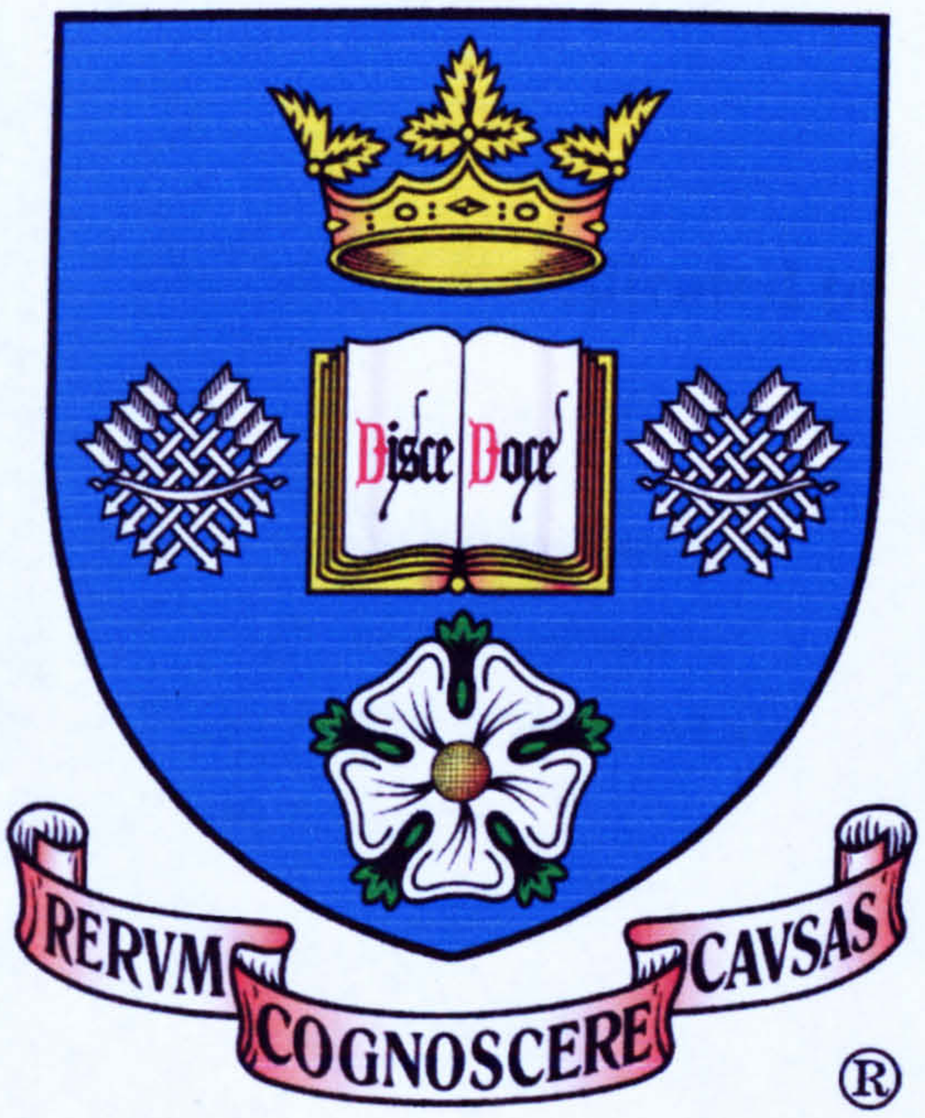
AN INVESTIGATION OF AUTOANTIBODIES TO THE MELANIN-CONCENTRATING HORMONE RECEPTOR 1 IN VITILIGO



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Thesis submitted for the degree of Doctor of Philosophy

June 2005

This thesis is dedicated to

Lord Venkateswara

My beloved parents

My Brother and Sister-in-Law

Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a higher degree. The work reported in this thesis has been carried out by myself and all sources of information have been specifically acknowledged by means of references.

Raju VSRK Gottumukkala

June, 2005

Summary

Vitiligo is an acquired depigmenting disorder characterised by the loss of functional melanocytes and of melanin from the cutaneous epidermis. A role for autoimmunity in vitiligo pathogenesis is supported by the presence of anti-melanocyte autoantibodies and autoreactive T lymphocytes in patients with the disorder. Recently, the melanin-concentrating hormone receptor 1 (MCHR1) has been identified as an autoantigen in vitiligo. The aim of this study was to characterise the properties of MCHR1 autoantibodies.

The epitopes on MCHR1 that are recognised by autoantibodies in vitiligo patients were identified using recombinant receptor in radio-binding assays. Multiple regions of MCHR1, including regions between amino acids 1-138 and 139-298, were identified as binding sites for MCHR1 autoantibodies. In addition, biopanning of a phage-display MCHR1 cDNA fragment library with vitiligo patient immunoglobulin G (IgG), identified more specifically the target sites of MCHR1 autoantibodies.

A stable Chinese hamster ovary cell line expressing MCHR1 was isolated. The cell line clearly showed expression of the receptor by flow cytometry. Stimulation of the cell line with melanin-concentrating hormone (MCH) reduced forskolin-stimulated cyclic adenosine monophosphate levels and increased the levels of intracellular calcium, both indicative of MCHR1 expression.

Functional blocking of MCHR1 by receptor autoantibodies was investigated by measuring changes in intracellular calcium levels in response to MCH-stimulation of MCHR1-expressing cells that had been pre-incubated with vitiligo patient IgG. The results revealed that 10/18 (56%) of the vitiligo patient IgGs tested were able to block receptor function. In contrast, IgG from healthy controls and from patients with other autoimmune disease had no effect upon receptor function. This suggested that MCHR1 function blocking autoantibodies are specific to patients with vitiligo and are not present in patients with other autoimmune disease.

The cell line expressing MCHR1 was also used to analyse MCHR1 autoantibodies for any immunological activities such as complement-fixation and antibody-dependent cell-mediated cytotoxicity (ADCC). None of the vitiligo patient sera tested appeared to have MCHR1 autoantibodies that were able either to fix

complement or mediate ADCC. This suggested that there are no such immunological activities against MCHR1.

In conclusion, key findings from this study were that the MCHR1 is an important autoantigen in vitiligo, with 56% of patients having function-blocking autoantibodies against the receptor, and that amino acids 1-138 and 139-298 are major epitopes for MCHR1 binding autoantibodies.

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Publications

Papers in peer-reviewed journals

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Abbreviations

A	Adenine
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophic hormone
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIRE	Autoimmune regulator
AIS	Autoimmune susceptibility
AMP	Adenosine monophosphate
APC	Antigen presenting cells
APS1	Autoimmune polyendocrine syndrome type 1
ASP	Agouti signal protein
BH_4	5,6,7,8-Tetrahydrobiopterin
bp	Base pairs
BSA	Bovine serum albumin
BSS	Balanced salt solution
C	Complement/ Cytosine/Carboxyl terminus
[Ca2+]i	Intracellular calcium
CAT	Catalase
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CHO-K1	Chinese hamster ovary cells-K1
CLA	Cutaneous lymphocyte-associated antigen
COMT	Catechol-*O*-methylthansferase
CTLA-4	Cytotoxic T lymphocyte antigen-4
dH_2O	Distilled water
DAG	Diacylglycerol
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
G	Guanine
g	Gravity
g	Gram/s
GAD	Glutamic acid decarboxylase
GPCR	G protein-coupled receptor
GTP-CH1	Guanosine triphosphate-cyclohydrolase 1
HBSS	Hanks balanced salt solution
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
IP	Immunoprecipitation
IP_3	Inositol 1,4,5-triphosphate
Kb	Kilobase
kDa	Kilodaltons
kHz	Kilohertz
L	Litre
LB	Luria-Bertani (medium and agar)
LC	Langerhans cells
LMP	Low molecular weight protein
M	Melanocyte
mA	Milli-amperes
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAP	Mitogen-activated protein
MART-1	Melanoma antigen recognised by T cells-1
MC-1R	Melanocortin-1 receptor

MC-4R	Melanocortin-4 receptor
MCH	Melanin-concentrating hormone
MCHR1	Melanin-concentrating hormone receptor 1
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
ml	Millilitre
mM	Millimolar
MOPS	3-[N-Morpholino] propanesulphonic acid
N	Amino terminus
NGF	Nerve growth factor
NK	Natural killer
OCA	Oculocutaneous albinism
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIA	Phenylisopropyl adenosine
PKC	Protein kinase C
PLC	Phospholipase C
PM	Plasma membrane
PTX	Pertussis toxin
PUVA	Psoralen and ultraviolet A
RIA	Radio-immunoassays
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SOC	Super optimal catabolite (medium)
SR	Sarcoplasmic reticulum
T	Thymine
TAP	Transporter involved in antigen-processing

TBAb	Thyroid blocking autoantibodies
Tc	T cytotoxic (lymphocytes)
TCA	Trichloroacetic acid
TE	Tris-EDTA (buffer)
TEMED	N, N, N, N-tetramethylethylenediamine
Th1/Th2	T helper (lymphocytes)
TM	Transmembrane
TNF-α	Tumour necrosis factor alpha
TRP-1	Tyrosinase-related protein-1
TRP-2	Tyrosinase-related protein-2
TSAb	Thyroid-stimulating autoantibodies
TSH	Thyroid-stimulating hormone
TSHR	Thyroid-stimulating hormone receptor
UV	Ultraviolet
V	Volts/vitiligo
VKH	Vogt-Koyanagi-Harada (syndrome)
XO	Xanthine oxidase
μg	Microgram
μl	Microlitre
μM	Micromolar
α-MSH	Alpha-melanocyte-stimulating hormone
°C	Degree centigrade

1 General Introduction

1.1 Structure and function of the skin

1.1.1 General skin functions

The functions of the skin allow man to adapt to a wide variation in environment. The skin is a complex organ, comprising approximately one-twelfth of total body mass (Millington and Wilkinson 1983), and forming the boundary between the body and its external environment. It performs many essential functions that include the maintenance of internal homeostasis, protection against chemical and physical assault (Millington and Wilkinson 1983) and the prevention of invasion by pathogenic micro- and macro-organisms (Reeves and Todd 1996).

1.1.2 Ultra-structure of the skin

Human skin is formed of two layers, the epidermis, a cellular, avascular epithelium that derives from the embryonic ectoderm, and the dermis, a dense vascularised connective tissue arising from the embryonic mesoderm. The ultra-structure of the skin is summarised in Figure 1.1. The cutaneous epidermis comprises a multi-layered epithelium, consisting mostly of keratinocytes. Five epithelial layers are described as follows: the basal cell layer (*stratum basale*), prickle cell layer (*stratum spinosum*), granular cell layer (*stratum granulosum*), transitional cell layer (*stratum lucidum*) and the dead squamous cell layer (*stratum corneum*) (Wood and Bladon 1985). The nomenclature is based on the different morphological appearances of the keratinocytes according to their stage in the process of keratinisation. The innermost basal layer consists of a single layer of cylindrically shaped cells attached to a basement membrane that forms the junction between epidermis and dermis. The basal keratinocytes continually proliferate by mitotic division and move outwards through the layers undergoing subsequent differentiation, to be shed eventually at the exterior. The prickle cells are keratinocytes, so called because their protruding desmosomes filled with keratin filaments, are visible as prickles on the cell surface under a light microscope. Prickle cells move upwards and, as new cells are formed

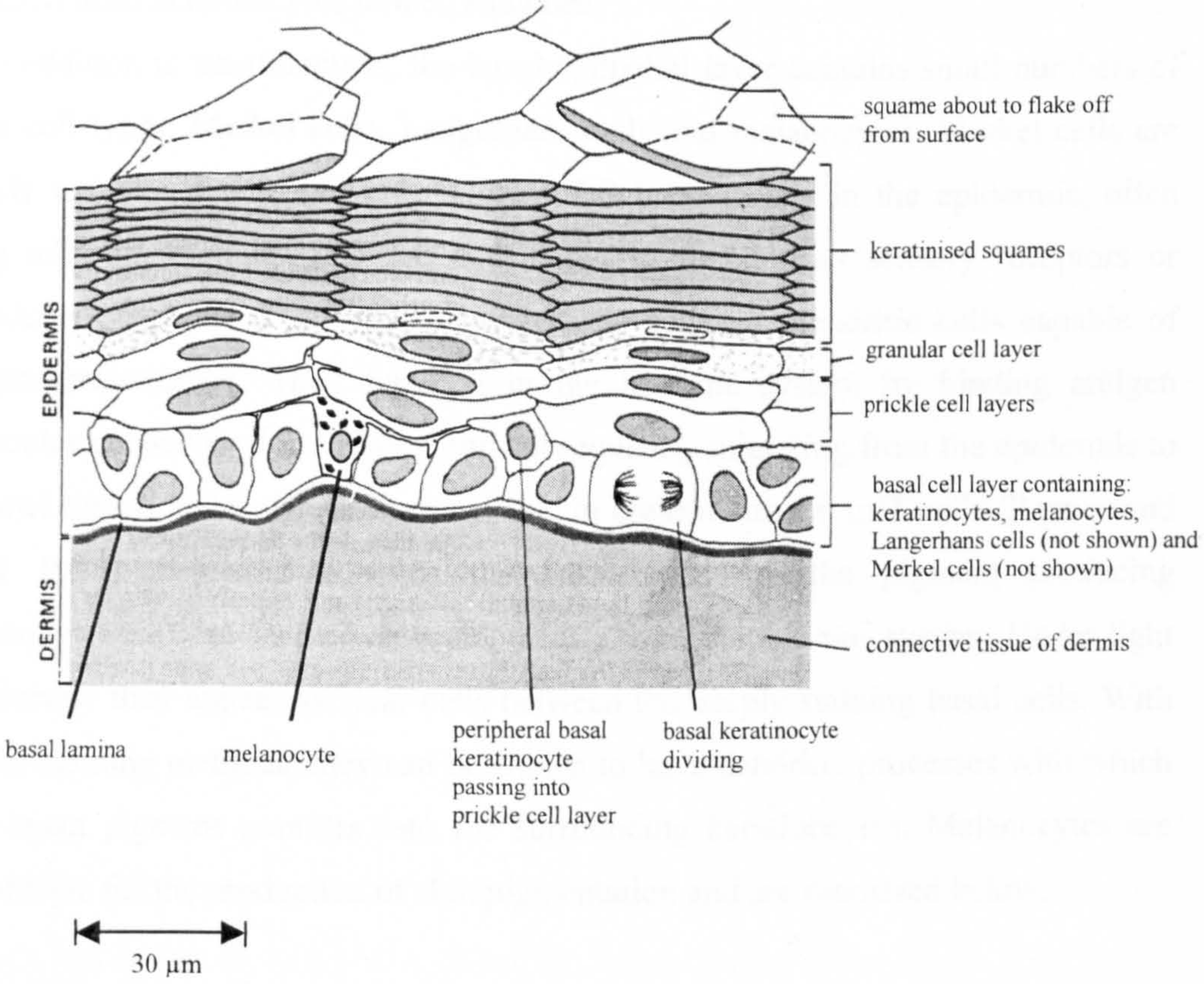


Figure 1.1 A schematic representation of the ultra-structure of the skin.

(Adapted from Alberts *et al.* 1989).

beneath them, they gradually change their polyhedral shape to a flatter configuration. As they approach the surface they take on their primary function, the production of the protein keratin. Above the prickle cell layer are keratinocytes in the penultimate stages of keratinisation. They appear granular because of the aggregation of keratohyaline. The transitional cells are terminally differentiated keratinocytes undergoing loss of their cellular contents. The outermost layer is made up of flattened dead keratinocytes termed squames.

In addition to keratinocytes, the basal epithelial layer contains small numbers of other cell types: Merkel cells, Langerhans cells and melanocytes. Merkel cells are closely associated with nerve terminals of Schwann cells in the epidermis, often lying adjacent to hairs, and are considered to function as sensory receptors or transducers (Lacour *et al.* 1991). Langerhans cells are dendritic cells capable of antigen presentation. They function in the immune system by binding antigen molecules encountered in the skin and subsequently migrating from the epidermis to regional lymph nodes, where they are able to present antigen to T cells (Reeves and Todd 1996). Scattered between the basal cells are the pigment producing melanocytes that are derived embryologically from the nervous system. Under light microscopy they appear as clear cells between the deeply staining basal cells. With special staining methods, they can be shown to have dendritic processes with which they inject pigment granules into the surrounding keratinocytes. Melanocytes are responsible for the production of skin pigmentation and are discussed below.

1.1.3 The melanocyte

Melanocytes comprise approximately 5% of the cellular component of the interfollicular epidermis. Melanocytes of the skin, uveal tract, inner ear and leptomeninges derive from melanoblasts that originate from the embryonic neural crest (Rawles 1947). In contrast, the ocular melanocytes of the retinal pigment epithelium arise from neural endoderm as it invaginates to form the outer wall of the optical cup (Feeny-Burns 1980). The melanoblasts migrate into the developing epidermis and eventually settle in the basal epithelial layer during seven weeks of early human embryonic gestation period (Holbrook *et al.* 1989). The control of migration and differentiation of embryonic melanoblasts is not fully understood, but

the involvement of several genes, including c-*kit* proto-oncogene, *pax* gene and stem cell factor gene, and transcription factors, especially microphthalmia-associated transcription factor (MITF) and SOX10, has been elucidated from mouse mutants (Boissy and Nordlund 1995a; Ortonne and Ballotti 2000).

Melanocytes reside in the basal epithelial layer of the interfollicular epidermis for the life of an individual at a density range of 1000–1500 per mm^2 (Quevedo *et al.* 1987). They have a roughly pyramidal morphology with numerous fine dendritic processes that can transfer melanin granules to surrounding keratinocytes. The appearance of melanocytes in culture is demonstrated in Figure 1.2. It was thought that melanocytes rarely proliferate or die under normal conditions. However, melanocytes can be induced to proliferate by stimuli such as ultraviolet (UV) light or during an inflammatory process. There is approximately one melanocyte per thirty-six keratinocytes in all races and ethnic groups in what is termed the 'epidermal melanin unit' (Fitzpatrick *et al.* 1967). Differences in the rate of melanin synthesis (Iwata *et al.* 1990), the type of melanin produced (Quevedo *et al.* 1974) and the way it is distributed within keratinocytes (Szabo *et al.* 1988), account for the different appearances of skin colour between ethnic groups.

The principal function of melanocytes is the synthesis of the pigment melanin that is primarily deposited in keratinocytes of the interfollicular epidermis. The process of melanin synthesis is discussed in Section 1.1.4. Melanin absorbs incident UV light and protects the genome of dividing basal keratinocytes and melanocytes (Boissy and Nordlund 1995a). Furthermore, melanin effectively absorbs oxygen free radicals (Korytowski *et al.* 1987) and, therefore, might serve to protect the metabolically active keratinocytes from oxidative stress (Boissy and Nordlund 1995a). In addition to their primary function of melanin synthesis, melanocytes can express major histocompatibility complex (MHC) class II molecules, cell adhesion molecules, and the Fc receptor. Moreover, they are also capable of producing several immunomodulatory cytokines (Zachariae *et al.* 1991) that assist in the maturation of antigen presenting cells, such as Langerhans cells, and are involved in the recruitment of immune infiltrates into the skin (Swope *et al.* 1994). Additionally, there is evidence to suggest that melanocytes can themselves act as antigen presenting cells (Le Poole *et al.* 1993a; Das *et al.* 2001).

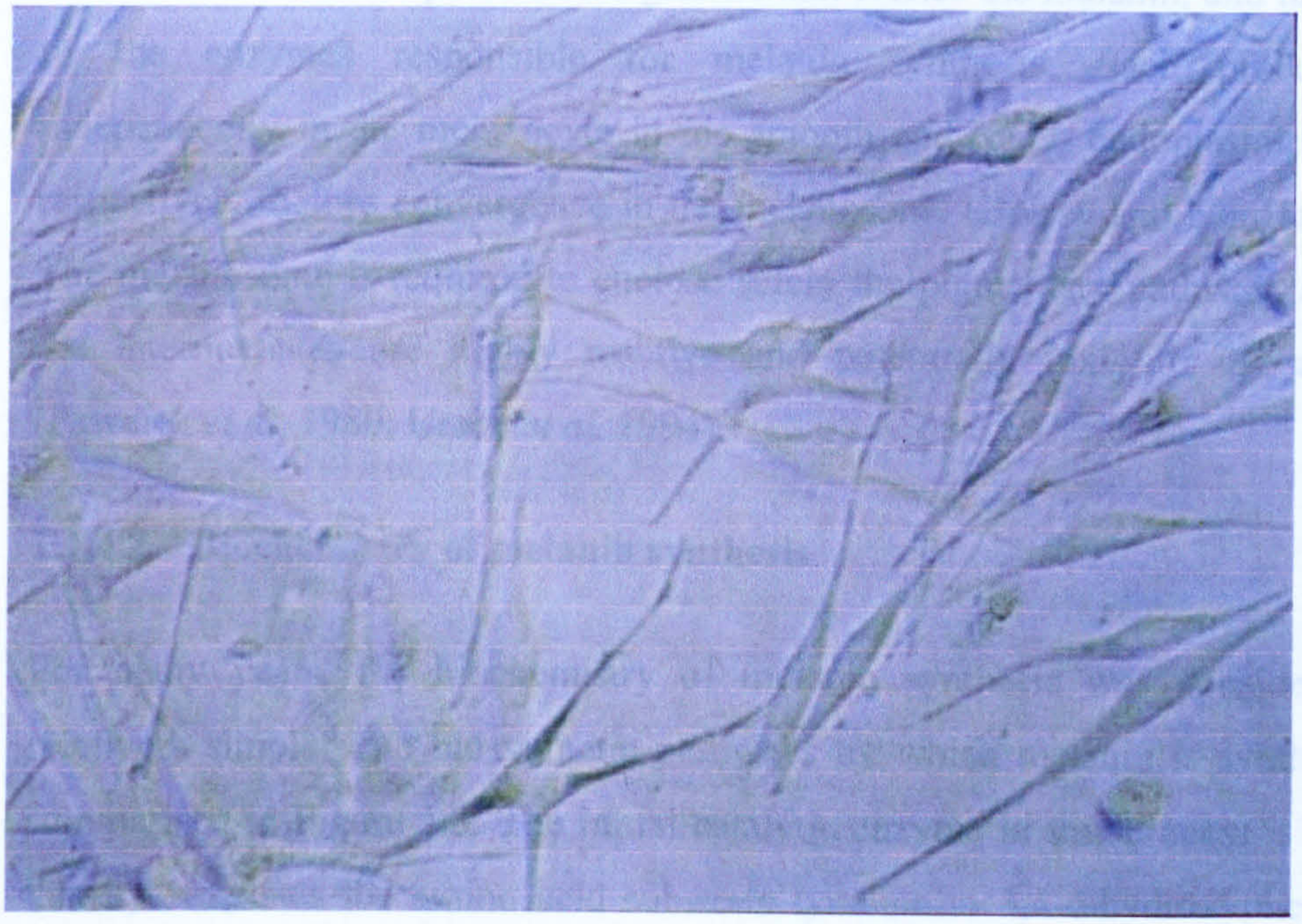


Figure 1.2 Bright field image of cultured melanocytes isolated from the human skin (X200 magnification).

(Provided by Miss C. Balafa and Professor S. MacNeil, Division of Clinical Sciences (North), University of Sheffield, UK).

1.1.4 Melanogenesis

1.1.4.1 The melanosome

In melanocytes, melanin synthesis or melanogenesis occurs in specialised organelles called melanosomes. The process of melanogenesis has two parts: the synthesis of the melanosome, the specialised organelle that carries the melanin, and the synthesis of the enzymes responsible for melanin synthesis. Each process occurs independently in the melanocyte in the smooth and rough endoplasmic reticulum, respectively, before convergence in the melanosome (Boissy and Nordlund 1995a). The melanosome is required to enclose safely the pigmentary pathway, as many of the intermediates are highly reactive and potentially toxic to the melanocyte (Pawelek *et al.* 1980; Urabe *et al.* 1994).

1.1.4.2 Biochemistry of melanin synthesis

For many years, the biochemistry of melanin synthesis was considered to be relatively simple. The biosynthetic pathways by which melanin is synthesised are summarised in Figure 1.3. The initial catalytic enzyme in this process is tyrosinase that hydroxylates the amino acid substrate tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and then converts DOPA to DOPAquinone. Subsequently, DOPAquinone appears to spontaneously progress to leucoDOPAchrome and then to DOPAchrome. At this stage a diversion can occur. In the presence of DOPAchrome tautomerase/tyrosinase-related protein (TRP)-2 and divalent metal cations, DOPAchrome is converted to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which, in turn, is enzymatically oxidised by DHICA oxidase/TRP-1 to indole-5,6-quinone-carboxylic acid. Without the catalytic contribution of TRP-2, DOPAchrome can spontaneously oxidise to 5,6-dihydroxyindole (DHI) which can then be converted to indole-5,6-quinone by the activity of tyrosinase. Again, Pmel17/gp100 is involved in the conversion of indole-5,6-quinone and indole-5,6-quinone-carboxylic acid to eumelanin which are black and brown polymers (Boissy and Nordlund 1995a).

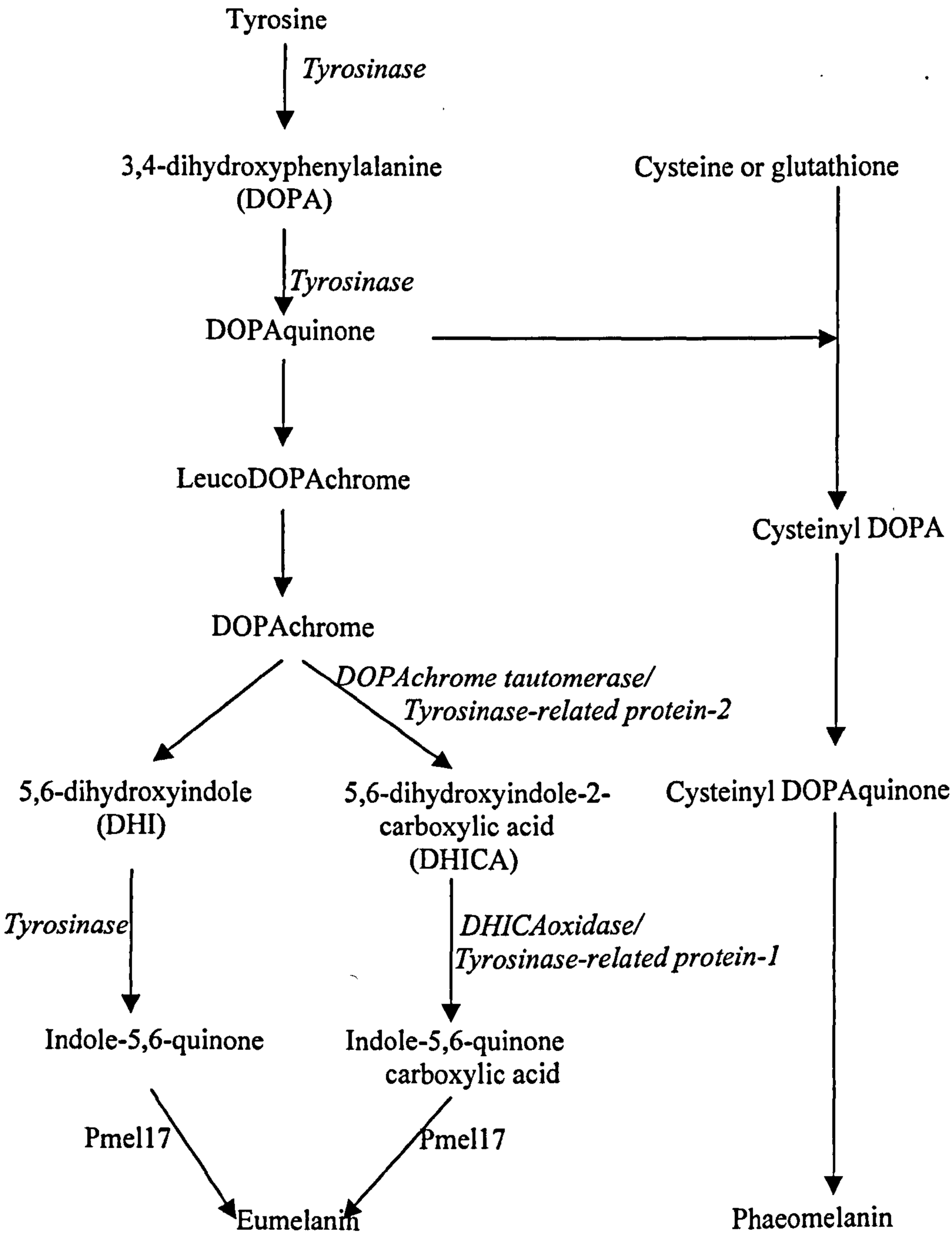


Figure 1.3 A diagrammatic overview of the melanin biosynthetic pathway.

(Adapted from Boissy and Nordlund 1995a; Ortonne and Ballotti 2000)

A second group of pigments called phaeomelanins are yellow and red polymers and predominantly appear in hair. The synthesis of phaeomelanins is initiated by the same mechanism as eumelanins up to the production of DOPAquinone. At this point, a high intracellular or intramelanosomal concentration of cysteine or glutathione, provided by glutamyl transpeptidase/glutathione reductase, will convert DOPAquinone to cysteinyl DOPA and then to phaeomelanin (Mojamdar *et al.* 1982; Jimenez *et al.* 1986; Jara *et al.* 1988). This alternative pathway proceeds by largely unknown mechanisms to the phaeomelanins that, unlike eumelanins, are considerably photolabile and may produce highly cytotoxic and mitogenic free radicals on photo-excitation.

Following melanin synthesis, the melanosome is transported to surrounding keratinocytes via the dendritic processes of the melanocyte. The keratinocytes take up the melanosome by endocytosis of the dendritic ends (Wolff 1973).

1.1.4.3 Melanogenic enzymes

Table 1.1 summarises some key melanosomal proteins and their known functions in melanogenesis. Tyrosinase is the rate-limiting enzyme in the melanogenesis pathway through its action of the hydroxylation of tyrosine to DOPAquinone (Oetting 2000). The presence of tyrosinase activity was first described in mammalian skin by Durham (Durham 1904) who also noted an absence of tyrosinase activity in the skin of albino animals. The tyrosinase protein is a membrane glycoprotein containing 529 amino acids, including a signal peptide at the amino end, two copper binding sites and a transmembrane region at the carboxy-end (Oetting 2000). The requirement for two copper atoms in the catalytic site of tyrosinase is demonstrated by the expression of hypopigmentation in the phenotype of Menke's disease, a disorder in which copper transport is deficient (Ortonne and Ballotti 2000). The control of tyrosinase gene expression, in response to stimuli such as UV light and α-melanocyte-stimulating hormone (α-MSH) production, has yet to be fully elucidated, but an important regulatory element contained within the tyrosinase promoter has been identified and termed the 'M' (melanocyte)-Box (Oetting 2000). The 'M'-Box is known to bind the family of transcription factors that include MITF (Oetting 2000).

Table 1.1 Melanosomal proteins.

Protein	Size (kDa)	Function	Gene location	Phenotype of gene mutations	References
Tyrosinase	70	Catalsyes the principle regulatory step in melanogenesis, the conversion of L-tyrosine→DOPAquinone.	Chromosome 11	Oculocutaneous albinism type (OCA)-1.	Oetting 2000 King *et al.* 1995
TRP-1 (gp75)	60	Catalyses steps in the eumelanin pathway. May also stabilise tyrosinase and other melanosomal enzymes. Precise role remains unclear.	Chromosome 9	OCA-3.	Kobayashi *et al.* 1994a Hearing 2000
TRP-2	59	Catalyses DOPAchrome→5,6-DHICA.	Chromosome 13	OCA-2.	Aroca *et al.* 1990 Hearing 2000
Pmel17 (gp100)	73	Controls the deposition of DHI and DHICA into eumelanin.	Chromosome 12	Mutations in the mouse Pmel17 gene result in loss of melanocytes and a subsequent greying of the coat.	Boissy and Nordlund 1995a Johnson and Jackson 1992
MelanA/MART-1 (Melanoma Antigen Recognised by T cells- 1)	13	Unknown	Chromosome 9	None described	
P-protein	90	Regulation of melanosomal pH.	Chromosome 15	OCA-2.	Puri *et al.* 2000 Rinchik *et al.* 1993
Lysosome-associated membrane protein-1	90-120	Purported to protect the melanosomal membrane.	Chromosome 13	None described	Das *et al.* 2001

Oculocutaneous albinism type (OCA)-1, characterised by a complete lack of melanin synthesis in the hair, eyes and skin, is an autosomal recessive disorder caused by mutations of the tyrosinase gene (King *et al.* 1995), of which more than ninety patients have been described (Oetting 2000).

The tyrosinase gene family (Kwon 1993) includes two other structurally homologous glycoproteins: TRP-1 and TRP-2. The primary amino acid sequences of TRP-1 and TRP-2 are overall approximately 45% identical to that of tyrosinase and contain a similar signal sequence and transmembrane domain (Kwon 1993). However, both proteins differ significantly from tyrosinase at their carboxyl-end (Orlow *et al.* 1993) and have distinct catalytic activities. Tyrosinase-related protein-2 acts as a DOPAchrome tautomerase, catalysing the conversion of DOPAchrome to DHICA (Aroca *et al.* 1990). The function of TRP-1 has not been determined in human melanogenesis, despite its role being characterised as DHICA oxidase in murine melanosomes (Hearing 2000). It is thought to stabilise tyrosinase activity (Kobayashi *et al.* 1994a) and mutations of TRP-1, which affect its ability to do this, may lead to OCA-3 (Hearing 2000).

Pmel17 is a melanosomal matrix glycoprotein (Kobayashi *et al.* 1994b; Zhou *et al.* 1994) with a limited structural homology to the tyrosinase gene family proteins (Kwon 1993). Indeed, Pmel17 complementary DNA (cDNA) was originally isolated from a human melanocyte cDNA library using anti-tyrosinase antibodies (Kwon *et al.* 1991). However, the homologous regions are confined to five short regions of amino acids only and it is classified within its own gene family (Kwon 1993). Mutations at the mouse silver locus, which encodes Pmel17, result in a loss of melanocytes in the coat hair and subsequent silvering (Johnson and Jackson 1992). Similar mutations in the human Pmel17 gene, D12S53E, have been speculated to cause greying of human hair (Ortonne and Ballotti 2000). The Pmel17 protein is thought to function as a stablin, more specifically an indole-blocking factor, that regulates the deposition of DHI and DHICA into eumelanin (Boissy and Nordlund 1995a).

1.1.4.4 Control of melanogenesis

In addition to UV light exposure, several cytokines and growth factors, including tumour necrosis factor-α (TNF-α), endothelin, stem cell factor and basic fibroblast

growth factor, are known to mediate melanogenesis (Nordlund 1998). Of particular importance in the control of melanogenesis are the melanotrophic peptides α-MSH and adrenocorticotrophic hormone (ACTH), which are synthesised in both the pituitary gland and locally in the human skin and which competitively bind to the melanocortin-1 receptor (MC-1R) on melanocytes (Thody and Graham 1998). Cell-signalling pathways, including the cyclic adenosine monophosphate (cyclic AMP)-dependent protein kinase pathway and the mitogen-activated protein (MAP) kinase pathway, are activated in response to α-MSH binding to MC-1R and ultimately bring about an up-regulation of melanogenesis (Ortonne and Ballotti 2000).

In humans, the mechanism that controls the type of melanin synthesized by melanocytes (eumelanin vs phaeomelanin) has not been elucidated. The switch to produce eumelanin or phaeomelanin in mouse follicular melanocytes is controlled by the extension and the agouti loci (Barsh 1996). The former encodes MC-1R and the latter encodes the agouti signal protein (ASP), which acts as a competitive antagonist of α-MSH on MC-1R. Activation of MC-1R by α-MSH induces a significant increase in the intracellular cyclic AMP content of melanocytes resulting in increased eumelanogenesis. Agouti signal protein binds to MC-1R, thereby antagonising the effects of α-MSH, including the α-MSH-induced activation of adenylate cyclase (Ortonne and Ballotti 2000). It is well established that α-MSH increases the synthesis of eumelanin in human melanocytes. A human homologue for the mouse agouti gene has been cloned and its product functions in a similar manner to the mouse ASP both *in vivo* and *in vitro*. However, its physiological function in humans remains to be elucidated.

1.2 Vitiligo

1.2.1 Definition

Vitiligo is an acquired depigmentary disorder characterised by the loss of functional melanocytes and of melanin from the cutaneous epidermis. Additionally, the disorder may affect melanocytes in the hair bulb, mucous membranes, inner ear and eye (Biswas *et al.* 2003). Clinically, the disorder is typified by pigment loss from the

skin in circumscribed maculae that have a tendency to enlarge peripherally with disease progression.

1.2.2 Prevalence

In approximately 50% of cases vitiligo presents before the age of 20 years (Majumder *et al.* 1993). The prevalence of the disease in the world ranges from 0.38% (Howitz *et al.* 1977) to 1.13% (Mehta *et al.* 1973) with a consensus prevalence of around 1% (Lerner 1971). The incidence of the disease in both sexes is equal. There is no preponderance of vitiligo in a particular racial skin type, although the disease is more visually apparent in darker skin.

1.2.3 Inheritance and genetics

A genetic involvement in vitiligo is supported by observations on familial aggregation of the disorder in over 20% of vitiligo patients (Ortonne *et al.* 1983; Majumder *et al.* 1993). In addition, concordance in monozygotic twins has been reported (Mohr 1951; Siemens 1953; Mayenburg *et al.* 1976; Alkhateeb *et al.* 2002) but the exact frequency of concordance has not been determined and the reporting of isolated cases is subject to ascertainment bias. Genetic studies of familial cases of vitiligo conclude that the disorder is most likely due to the action of genes at multiple loci (Majumder *et al.* 1993; Kim *et al.* 1998; Acros *et al.* 1999) and is not transmitted by a simple Mendelian mechanism. However, some authors propose that an autosomal dominant gene with variable expression and incomplete penetrance may be responsible (Mosher *et al.* 1979; Lorincz 1985). In the Smyth line chicken (Smyth 1989), an animal model of vitiligo, the disorder results from a genetically inherited defect that is expressed in the melanocytes and which appears to be transmitted to offspring as an autosomal recessive trait. Similarly, depigmentation in the mouse model C57BL/6J Ler-*vit/vit* results from a genetic mutation that is inherited as an autosomal recessive trait (Lerner *et al.* 1986). The mutation in the *vit/vit* mouse occurs at the microphthalmia locus that encodes MITF, and, therefore, interest arose in the human homologue as a susceptibility gene for vitiligo. However, linkage studies have proven negative (Tripathi *et al.* 1999) with respect to this locus.

1.2.3.1 Immune response related genes

Since vitiligo may have an immune involvement (Sections 1.3.6 and 1.5), candidate genes include those that have a regulating role in the immune system. In particular, the genes of the MHC have been associated with the presence of autoimmune disorders (Larsen and Alper 2004). The study of MHC genes has revealed associations of vitiligo with certain human leukocyte antigen (HLA) specificities, but there is no consistent association with MHC class I or class II alleles (Table 1.2) and the reported relative risks have been modest. Several autoimmune disorders are associated with homozygous or heterozygous deficiencies of the MHC class III molecules complement (C) components C4 and C2 (Kahl and Atkinson 1988) and an increased frequency of heterozygous C4 and C2 deficiency has been found in patients with vitiligo (Venneker *et al.* 1992).

The cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene encodes a T cell receptor that is involved in the regulation of T cell activation (Walunas *et al.* 1994). Several polymorphisms of the *CTLA-4* gene are associated with autoimmune disorders such as Graves' disease (Kotsa *et al.* 1997), autoimmune hypothyroidism (Kotsa *et al.* 1997), Addison's disease (Kemp *et al.* 1998a) and type 1 diabetes mellitus (Ide *et al.* 2004). With respect to vitiligo, the 106 base pair allele of the *CTLA-4* microsatellite polymorphism has been associated with vitiligo in patients who have at least one autoimmune disease in addition to their cutaneous depigmentation (Kemp *et al.* 1999b).

The autoimmune regulator (*AIRE*) gene encodes a T cell-specific protein that contains motifs indicative of a transcription factor (Heino *et al.* 2001; Myhre *et al.* 2001) and mutations in *AIRE* result in autoimmune polyendocrine syndrome type 1 (APS1), a disorder with which vitiligo can be associated (The Finnish-German APECED consortium 1997; Nagamine *et al.* 1997). However, it is unlikely that *AIRE* gene is related to the onset of vitiligo that develops independently of APS1. Analysis of 552 vitiligo patients and their families, with respect to polymorphic markers in a panel of genes encoding proteins involved in the immune response, has also been undertaken (McCormack *et al.* 2001). The genes analysed included: low molecular weight protein (LMP)-2 and LMP-7; transporters involved in antigen-processing (TAP)-1 and TAP-2; the proteosome multi-catalytic endopeptidase

Table 1.2 Major histocompatibility complex antigen associations with vitiligo.

Origin of population	Number of patients in study	Associated specificity	Reference
Slovakia	67	A2, Dw7	Buc *et al.* 1996
Italy	87	A30, B27, Cw6, DQw3	Finco *et al.* 1991
Holland	42	DR4, C4BQ0	Venneker *et al.* 1992
Holland	50	DRB4, DQB1	Zamani *et al.* 2001
Holland	Not given	Cw7, DR06	Venneker *et al.* 1993
Kuwait	40	B21, Cw6, DR53	Al-Fouzan *et al.* 1995
Oman	50	Bw6, DR7	Venkataram *et al.* 1995
Hungary	88	DR1, DR3	Poloy *et al.* 1991
USA (African)	24	DR4, DQw3	Dunston and Halder 1990
USA (Caucasian)	48	DR4	Foley *et al.* 1983
USA	6	HLA-DR	Le Poole *et al.* 2003
Italy (Northern)	86	A3	Lorini *et al.* 1992
Italy (Northern)	93	A30, Cw6, DQw3	Orecchia *et al.* 1992
Morocco (Jewish)	18	B13	Metzker *et al.* 1980
Yemen	16	Bw35	Metzker *et al.* 1980
Germany (Northern)	102	A2, B13, Bw60	Schallreuter *et al.* 1993
Germany	34	DRB1, DQB1	Buc *et al.* 1998
Colombia	56 families	D6S265, D6S276, D6S273	Arcos-Burgos *et al.* 2002
Turkey	41	DRB1*03, DRB1*04, DRB1*07	Tastan *et al.* 2004
China (Hans)	187	HLA-A*2501, HLA-A*30, HLA-B*13, HLA-B*27 HLA-Cw*0602	Zhang *et al.* 2004

complex-like 1; CD4; CD59; membrane cofactor protein; interleukin (IL)-1β; IL-1 receptor-α; TNF-α; intercellular adhesion molecule-1 (ICAM-1); co-stimulatory lymphocyte activation ligands CD28 and *CTLA-4*. Only polymorphic markers for LMP/TAP and CD28/*CTLA-4* appeared to be positively associated with vitiligo susceptibility.

A recent genome wide linkage scan of 102 white multiplex families with vitiligo from the USA and the UK mapped a new autoimmune susceptibility 1 (*AIS1*) gene, a locus on chromosome 1 that apparently confers susceptibility to generalized vitiligo (Alkhateeb *et al.* 2002; Fain *et al.* 2003; Spritz *et al.* 2004). In addition, Spritz *et al.* reported two more loci *AIS2* and *AIS3* on chromosomes 7 and 8, respectively, that confer susceptibility to vitiligo (Spritz *et al.* 2004). A European-American pedigree study detected a susceptibility gene *SLEV1* on chromosome 17 in families with vitiligo-related systemic lupus erythematosus (Nath *et al.* 2001). Subsequently, Spritz *et al.* confirmed the presence of *SLEV1* on chromosome 17 in the genome scan of 102 vitiligo families from the UK and the USA (Spritz *et al.* 2004).

1.2.3.2 Genes related to metabolism

An association of vitiligo with mutations in the gene encoding guanosine triphosphate-cyclohydrolase 1 (*GTP-CH 1*) has been reported (de la Fuente-Fernandez 1997). Sequencing of the *GTP-CH 1* gene showed missense mutations in vitiligo families. This enzyme catalyses the initial step in 5,6,7,8- tetrahydrobiopterin synthesis (BH_4) from guanosine triphosphate. The BH_4 is an essential cofactor for phenylalanine and tyrosine synthesis that are involved in the production of melanin (Schallreuter *et al.* 1994a). Patients with active vitiligo have abnormalities in BH_4 and *GTP-CH 1* activity compared to controls (Schallreuter *et al.* 1994c).

Both case-control and family-based genetic studies of a T/C single nucleotide polymorphism in exon 9 of the catalase (*CAT*) gene demonstrated that T/C heterozygotes of the *CAT* gene are more frequent among vitiligo patients than controls and that the C allele is transmitted more frequently to patients than controls (Casp *et al.* 2002). The *CAT* gene encodes catalase that is responsible for the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). A reduction of this enzyme and the concomitant accumulation of excess H_2O_2 have

been observed in the entire epidermis of vitiligo patients (Schallreuter *et al.* 1991; Schallreuter *et al.* 1999). An accumulation of H_2O_2 can directly oxidise BH_4 to 6-biopterin that is toxic to melanocytes (Schallreuter *et al.* 1999).

It has been demonstrated that the G/A polymorphism at codon 158 of the catechol-*O*-methyltransferase (*COMT*) gene leads to low activity of *COMT* and results in the development of acrofacial vitiligo (Tursen *et al.* 2002). Catechol-*O*-methyltransferase catalyses the *O*-methylation of biologically active or toxic catechols, that are produced at the nerve endings, and plays a major role in the metabolism of drugs and neurotransmitters. It has been suggested that the low activity of *COMT* might lead to an increase in *O*-quinones that are highly toxic to melanocytes (Pavel *et al.* 1983; Tursen *et al.* 2002).

Recently, it has been found that I/D polymorphism at intron 16 of angiotensin converting enzyme (*ACE*) gene has a strong association with the development of vitiligo in Korean patients (Jin *et al.* 2004). The *ACE* gene encodes angiotensin converting enzyme which is capable of degrading neuropeptides released from sensory nerves in response to chemical stimuli and mechanical injury (Scholzen *et al.* 2003). In addition, it also plays an important role in the physiology of the vasculature, blood pressure and inflammation. The I/D polymorphism of *ACE* gene is also associated with other autoimmune diseases (Papadopoulos *et al.* 2000).

1.2.3.3 Other genes

By comparing the different gene expression levels of non-lesional melanocytes from vitiligo patients and control melanocytes, a novel candidate gene '*VIT1*' has been found which is associated with vitiligo (Le Poole *et al.* 2001). The *VIT1*-encoded protein contains an amino acid sequence that is identical to a putative zinc-finger motif found in *N*-recognin, which is involved in the degradation of cellular proteins (Kwon *et al.* 1998). The authors, therefore, suggest that the *VIT1*-encoded protein might be involved in protein degradation and reduced *VIT1* expression, as found in vitiligo patient melanocytes, might help to explain protein accumulation leading to dilated endoplasmic reticulum profiles, a hallmark of vitiligo pigment cells (Le Poole *et al.* 2001).

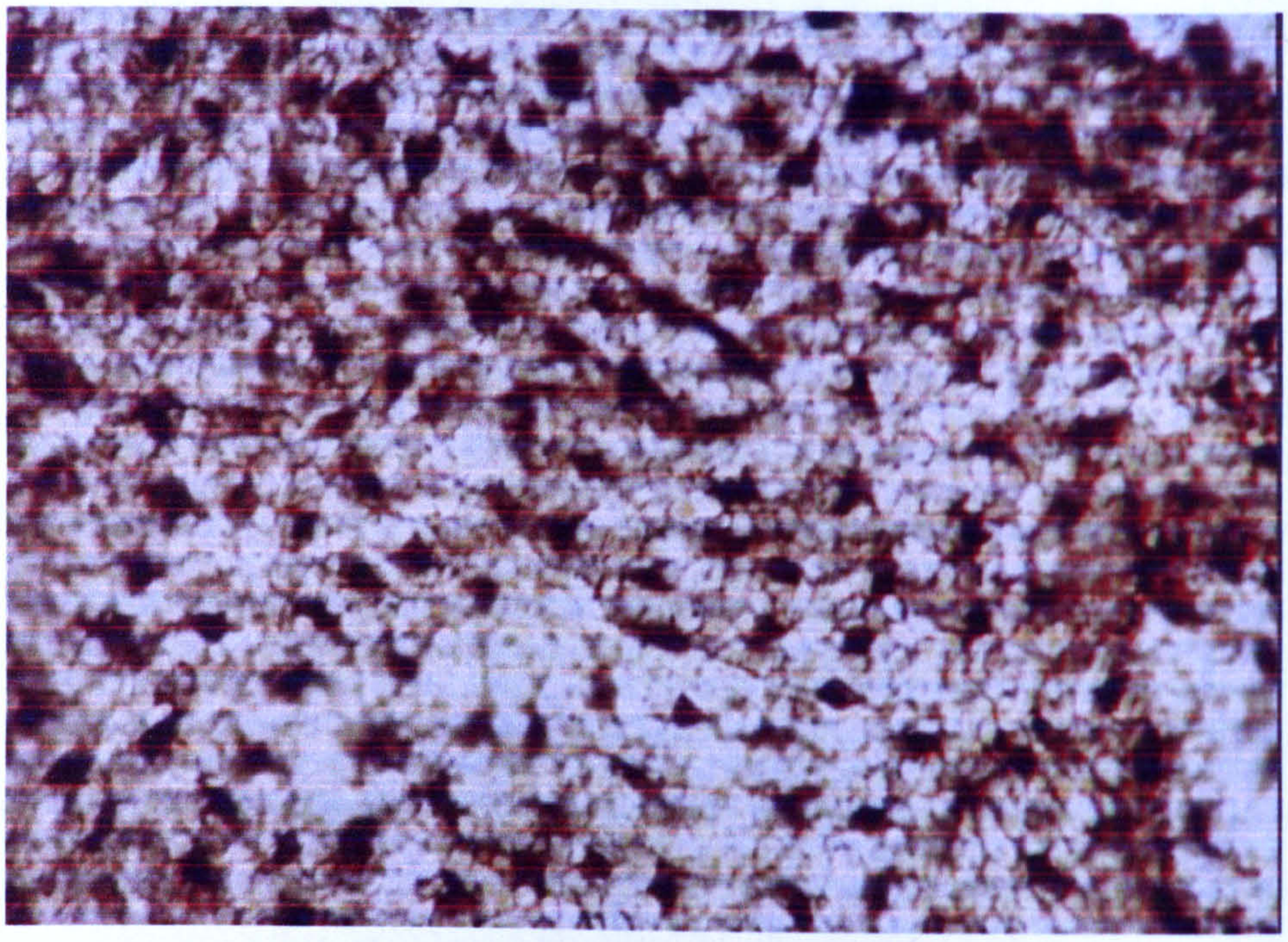
A high frequency of the A allele of G/A polymorphism in codon 274 of *MC-1R* gene and the G allele of G/A polymorphism in codon 8818 of *ASP* gene has been found in vitiligo patients compared to controls but the study could not able to find any statistically significant association of these polymorphisms with vitiligo (Na *et al.* 2003).

A study involving 552 vitiligo patients and their families, examined polymorphic markers in a panel of twenty-four candidate genes that might confer susceptibility to vitiligo (McCormack *et al.* 2001). The genes analysed in this study included those encoding tyrosinase, TRP-1, TRP-2, tyrosine hydroxylase, phenylalanine hydroxylase, GTP-CH 1, the transcription factor nuclear factor 1, and two genes involved in melanocyte embryogenesis, *kit* and *pax*3. No association was found between any of these genes involved in melanocyte function and an increased risk of vitiligo.

1.2.4 Pathology

Affected skin shows a loss of melanin and a decrease in the number of melanocytes in the epidermis when compared to normal skin (Figures 1.4 a and b). Electron microscopic analysis and immunohistochemical studies of vitiligo lesions using a variety of monoclonal and polyclonal antibodies confirm the degeneration of melanocytes and also the presence of abnormal keratinocytes (Moellmann *et al.* 1982; Le Poole *et al.* 1993b). In peri-lesional areas, abnormal pigment cells are seen that are fragmented, grossly enlarged or show the appearance of vacuoles (Le Poole *et al.* 1993b). In addition, in the peri-lesional regions of inflammatory vitiligo, a lymphocytic infiltrate can be identified in the basal layer of the epidermis in proximity to the melanocytes (Bleehan, 1979). It has also been reported that the epidermis of vitiligo lesions has reduced thioredoxin reductase activity (Schallreuter *et al.* 1987), defective calcium uptake (Schallreuter and Pittelkow 1988), low catalase activity (Schallreuter *et al.* 1991) and high expression of β-adrenoreceptors (Schallreuter *et al.* 1992).

(a)



(b)

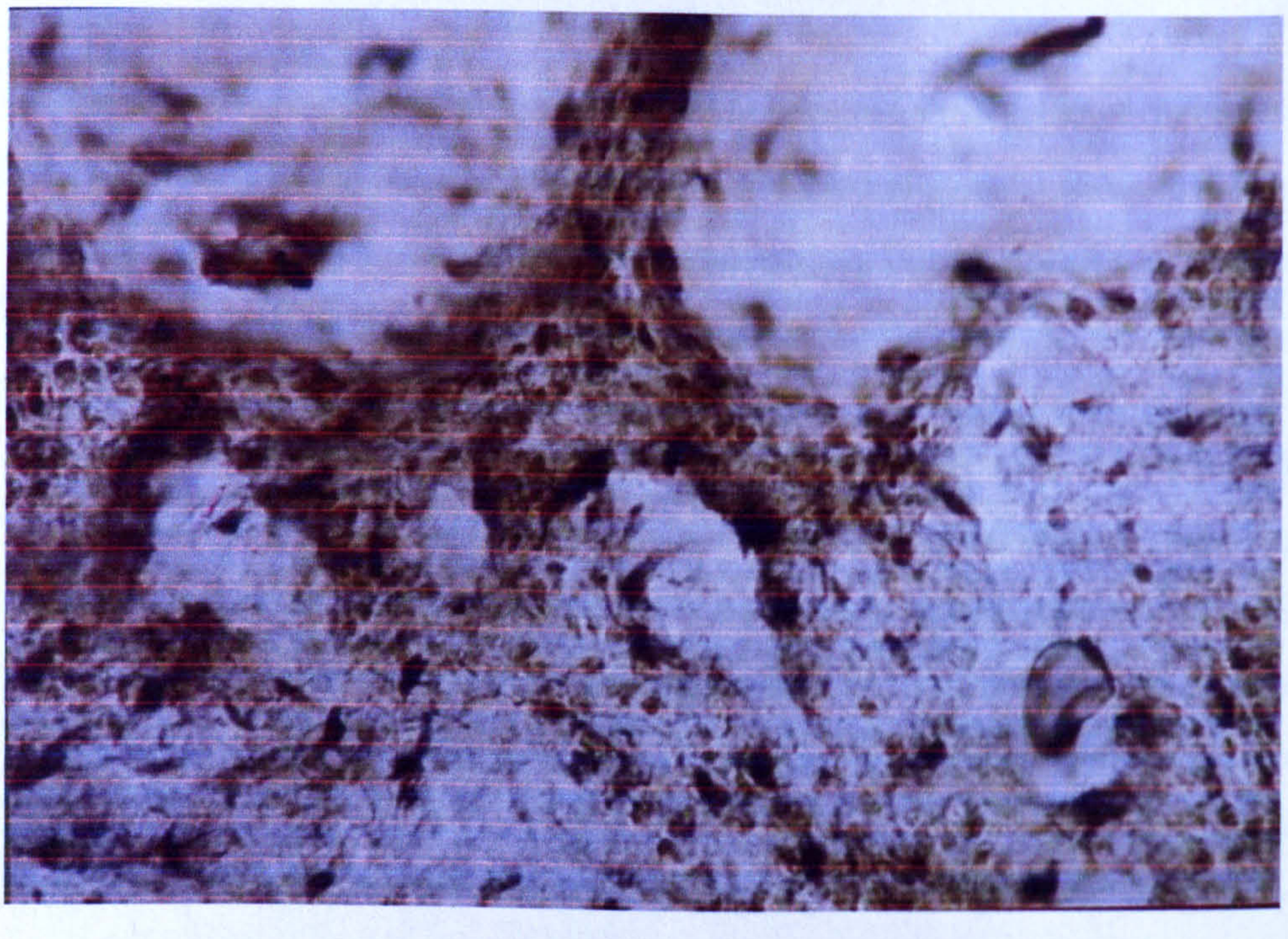


Figure 1.4 (a) Melanocytes in normal cutaneous epidermis. Photomicrograph showing a whole mount of a sheet of epidermis from normal skin, spread upon a slide and viewed from the underside. The epidermis was separated from the dermis by the generation of a suction blister. The epidermal sheet was then incubated with DOPA that selectively stains melanocytes brown (X300).

(b) Melanocytes in the cutaneous epidermis of a vitiligo patient. Photomicrograph of a whole mount of a sheet of epidermis from an area of vitiligo skin, spread upon a slide and viewed from the underside. The epidermis was separated from the dermis by the generation of a suction blister. The epidermal sheet was then incubated with DOPA that selectively stains melanocytes brown (X300). Melanocytes are observed at a much lower density in the vitiligo skin than in the normal skin.

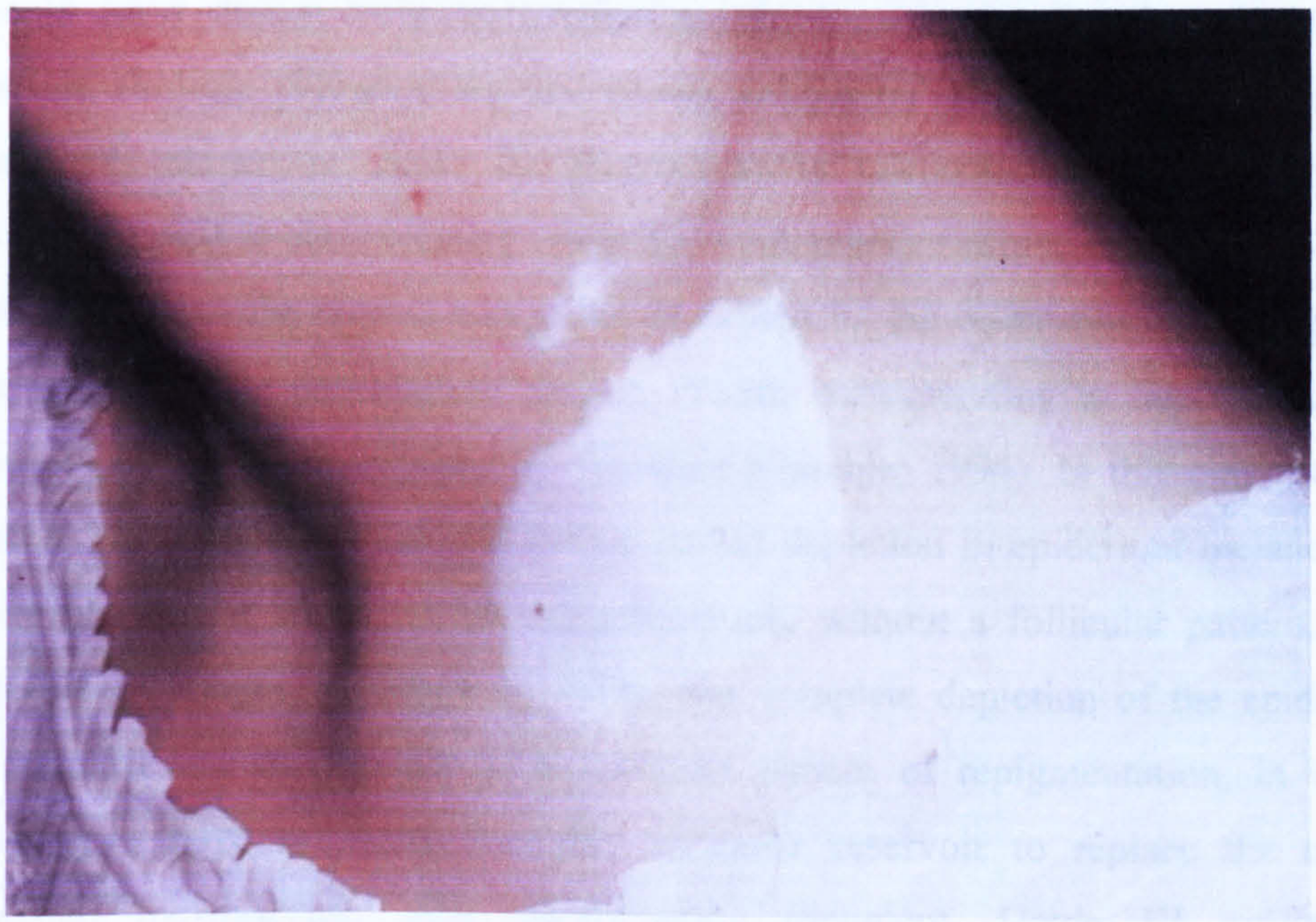
(Provided by Professor D.J. Gawkrodger, Royal Hallamshire Hospital, Sheffield, UK).

1.2.5 Clinical features

Vitiligo can be classified by the extent and distribution of cutaneous depigmentation. There are five main disease categories: generalised or symmetrical, segmental, focal, acrofacial and universal (Kovacs 1998). Generalised or symmetrical type vitiligo is the most common presentation with a bilateral, symmetrical depigmentation of the face (especially peri-orificial areas), neck, torso and the extensor surfaces or bony prominences of the hands, wrist and legs. Segmental vitiligo has a dermatomal, asymmetrical distribution of depigmentation, usually with one or more macules in one area of the body. This type of vitiligo often begins in childhood, is rarely associated with autoimmune disorders and has stable results after autologous grafting. In contrast, symmetrical vitiligo has a later age of onset, is frequently associated with autoimmunity and has unstable results with autologous grafting. These contrasts have lead to the speculation that symmetrical and segmental vitiligo represent two quite separate disease manifestations (Kovacs 1998; Taieb 2000). Focal type vitiligo is localised and non-dermatomal in distribution, whereas acrofacial vitiligo is characterised by depigmentation of the distal fingers and facial orifices only, the latter being in a circumferential pattern. Finally, patients with universal vitiligo exhibit a loss of pigment over the entire body surface, a type of vitiligo that occurs rarely. The two most common clinical presentations are segmental and symmetrical vitiligo and these are shown in Figures 1.5 a and b, respectively.

Trichrome, inflammatory, and occupational vitiligo represent unusual forms of the disease. Trichrome vitiligo involves macules in which there are areas of both complete and partial loss of pigment, forming a pattern of varying hues between the affected and non-affected skin (Fitzpatrick 1964; Gawkrodger 1998). Uniquely, areas of normally pigmented skin adjacent to an area of vitiligo may become hyperpigmented (Gawkrodger 1998). Inflammatory vitiligo is characterised by actively spreading lesions that are inflamed, presenting a raised erythematosus peri-lesional border (Grab and Wise 1948; Le Poole *et al.* 1996). Chemically induced occupational vitiligo can occur in workers exposed to compounds such as catechols, phenols, hydroquinone and monobenzene. The depigmentation is usually confined to the parts of the body exposed to the chemical, e.g. the hands and forearms, but can

(a)



(b)

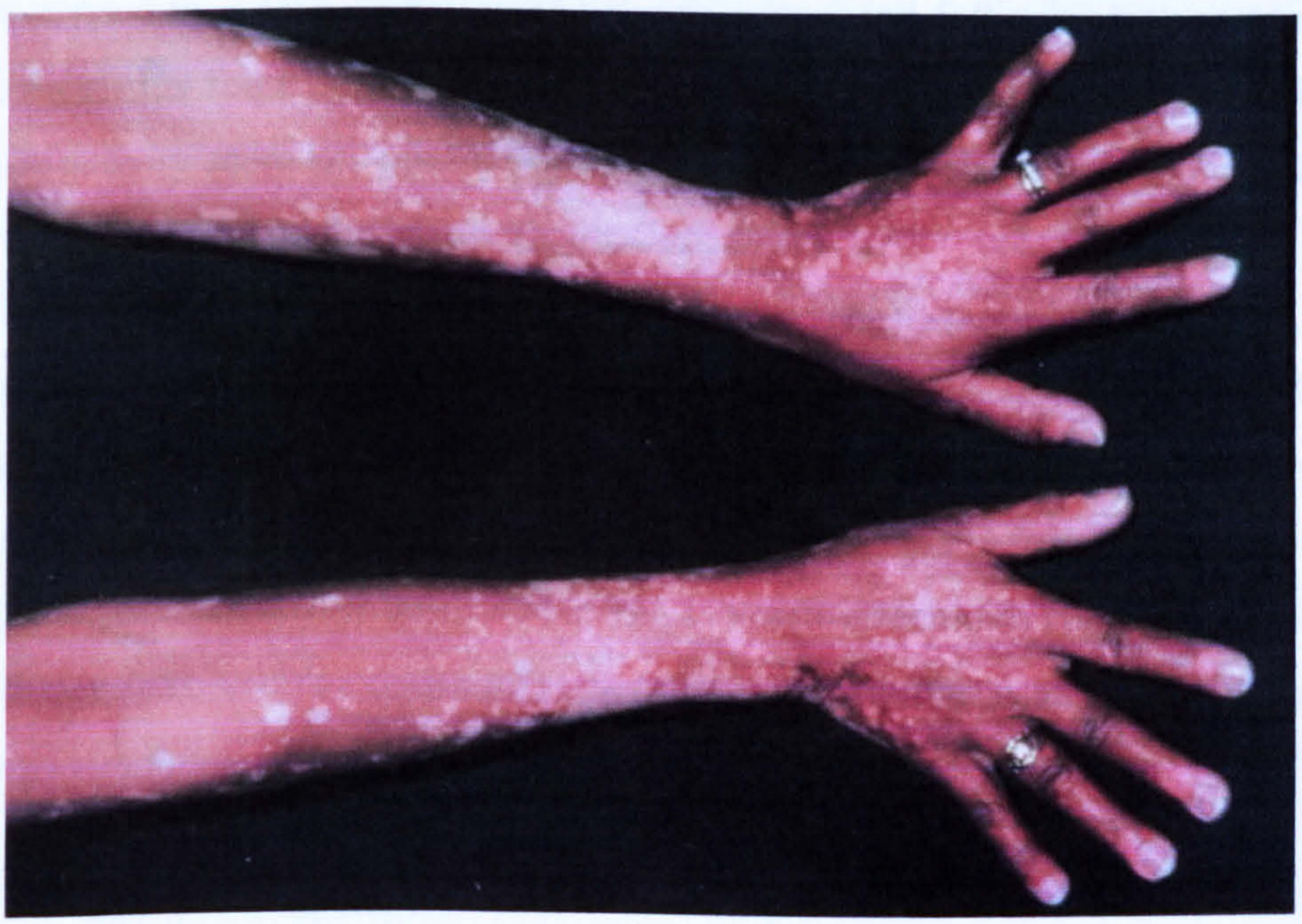


Figure 1.5 (a) Segmental vitiligo affecting one side of the neck.

(Provided by Professor A.P. Weetman, Division of Clinical Sciences (North), University of Sheffield, UK).

(b) Symmetrical vitiligo on the dorsal aspects of the hands.

(Provided by Professor D.J. Gawkrodger, Royal Hallamshire Hospital, Sheffield, UK).

arise in areas distant from the original point of contact, suggestive of systemic exposure through percutaneous adsorption, inhalation or ingestion. Some of the compounds shown to cause depigmentation in occupational vitiligo are now specifically used in depigmenting therapies in idiopathic vitiligo.

In addition to the disease categories described by the distribution of vitiliginous skin, a three-grade pathological staging system corresponding to depletion of the melanocytes has been proposed by Gauthier (Gauthier 1994). In this classification scheme, grade I vitiligo exhibits only a partial depletion in epidermal melanocytes and results in the possibility of repigmentation, without a follicular pattern, after phototherapy treatment. Grade II vitiligo has complete depletion of the epidermal melanocytes and corresponds to a follicular pattern of repigmentation, in which melanocytes have migrated from the follicular reservoir to replace the absent epidermal melanocytes, after phototherapy treatment. Grade III vitiligo is characterised by a total depletion of both epidermal and follicular melanocytes and patients at this stage do not respond to medical therapy. More recently, a vitiligo disease activity score has been proposed which measures disease activity in relation to time, as assessed by the patient (Njoo *et al.* 1999).

Although vitiligo may be considered a relatively minor disorder, the psychological effects can be considerable, particularly in those with racially darkly pigmented skin in whom the appearance of the lesions is more marked. Detrimental feelings of stress, embarrassment or self-consciousness when in contact with strangers, perception of discrimination and low self-esteem are common, especially in patients with visible lesions (Porter *et al.* 1986; Porter *et al.* 1990; Kent and Al'Abadie 1996).

1.2.6 Associated disorders

Vitiligo is frequently associated with other autoimmune disorders including autoimmune thyroid disease, type 1 diabetes mellitus, alopecia areata, pernicious anaemia, Addison's disease, autoimmune polyendocrine syndromes and other disease conditions and rare syndromes (Table 1.3). Patients with vitiligo are more likely to suffer from an autoimmune condition than those in the general population. Autoimmune thyroiditis, for example, has a prevalence of up to 30% in vitiligo

Table 1.3 Association of vitiligo with autoimmune and other disorders.

Disorder	Reference
Thyroid disease	Grimes *et al.* 1983; Shong and Kim 1991; Schallreuter *et al.* 1994b; Hegedus *et al.* 1994; Barnadas *et al.* 2000; Dervis *et al.* 2004; Kurtev and Dourmishev 2004
Type 1 diabetes mellitus	Gould *et al.* 1985
Addison's disease	Mulligan and Sowers 1985
Systemic lupus erythematosus	Mihailova *et al.* 1999
Alopecia areata	Grimes *et al.* 1983; Sharma *et al.* 1996
Myasthenia gravis	Tan 1974
Pernicious anaemia	Howitz and Schwartz 1971; Abraham *et al.* 1993; Barnadas *et al.* 2000
Rheumatoid arthritis	Abraham *et al.* 1993
Sarcoidosis	Barnadas *et al.* 2000; Terunuma *et al.* 2000
Chronic active hepatitis	Sacher *et al.* 1990
Vogt-Koyanagi-Harada syndrome	Okada *et al.* 1996; Tsuruta *et al.* 2001
Autoimmune polyendocrine syndromes	Ahonen *et al.* 1990
Psoriasis	Dhar and Malakar 1998; Hwang *et al.* 1998; Sandhu *et al.* 2004
Lichen planus	Anstey and Marks 1993; Rubisz-Brzezinska *et al.* 1996

(Adapted from Ongenae *et al.* 2003)

patients (Cunliffe *et al.* 1968), a figure that is significantly higher than that of 1% as reported in the general population (Turnbridge *et al.* 1977). Autoimmune gastritis, pernicious anaemia, alopecia areata and type 1 diabetes mellitus are also found more commonly in vitiligo patients compared with normal subjects with frequencies of 15%, 5.3%, 16% and 7.2%, respectively (Cunliffe *et al.* 1968; Turnbridge *et al.* 1977; Zauli *et al.* 1986). In addition, circulating organ-specific autoantibodies, particularly to the thyroid, adrenal glands and gastric parietal cells, are commonly detected in the sera of vitiligo patients (Brostoff *et al.* 1969; Betterle *et al.* 1976; Mandry *et al.* 1996).

Halo naevi, areas of depigmentation seen surrounding a melanocytic naevus or 'mole', may be associated with vitiligo and sometimes pre-date the onset of the disorder (Lerner 1971). Premature greying of the hair (canities) may also be found in patients with vitiligo (Nordlund *et al.* 1981). Vitiligo can affect all active melanocytes, including those of the retinal pigment epithelium and uveal tract of the eye and the scala vestibuli of the inner ear, which may lead to ocular abnormalities such as iris, conjunctival and chorioretinal depigmentation and uveitis (Albert *et al.* 1979; Cowan *et al.* 1986) or auditory problems including hearing impairment in the 2-8 kHz range (Thurmon *et al.* 1976; Tosti *et al.* 1986; Tosti *et al.* 1987). The combination of vitiligo with uveitis, auditory anomalies, neurological involvement and canities is known as the Vogt-Koyanagi-Harada (VKH) syndrome (Barnes 1988). It has been proposed that VKH syndrome may represent a systemic manifestation of vitiligo, since the neurological involvement may also result from melanocyte destruction in the leptomeninges of the brain (Kovacs 1998).

1.2.7 Treatment

Since the aetiology of vitiligo is not yet understood, there are no effective treatments available to correct the basic defect/s that lead to the destruction of melanocytes. Current therapies can be categorised as medical or surgical and these are summarised in Table 1.4. Medical therapies include topical steroids and phototherapy and surgical treatments include various forms of melanocyte grafts and tattooing. On-going psychological support is also important, particularly for patients who have suffered serious psychological affects or social stigma as a result of the disease.

Table 1.4 Therapies for vitiligo.

Treatment	Reference
I. MEDICAL TREATMENTS	
Cosmetic modalities	Kovacs 1998
Corticosteroids (topical or systemic)	Boissy and Nordlund 1995b; Gawkrodger 1998; Kovacs 1998; Parsad *et al.* 2004
Calcineurin antagonists	Hartmann *et al.* 2004
Tacrolimus (topical immunomodulator)	Grimes *et al.* 2002; Tanghetti 2003; Lepe *et al.* 2003; Castanedo-Cazares *et al.* 2003; Grimes *et al.* 2004
Psoralen (topical or systemic) and ultraviolet A (PUVA)	Boissy and Nordlund 1995b; Gawkrodger 1998; Kovacs 1998
Khellin (topical or systemic) with ultraviolet A or natural sunlight	Gawkrodger 1998
Phenylalanine (topical or systemic) with ultraviolet A	Kovacs 1998
Topical pseudocatalase with ultraviolet B	Gawkrodger 1998
Oral antioxidants (e.g. vitamin E acetate; selenio-methionine)	Gawkrodger 1998
5-Fluorouracil (topical)	Kovacs 1998
Depigmentation with monobenzyl ether of hydroquinone	Kovacs 1998
II. SURGICAL TREATMENTS	
Epidermal grafting	Boissy and Nordlund 1995b; Kovacs 1998
Punch grafting	Agarwal 2004
Autologous minigrafts	Boissy and Nordlund 1995b; Kovacs 1998
Transplantation of cultured epidermis	Boissy and Nordlund 1995b; Kovacs 1998
Transplantation of non-cultured melanocytes	Boissy and Nordlund 1995b; Kovacs 1998
Tattooing	Boissy and Nordlund 1995b

Steroids, both systemic and topical, have been used extensively to treat vitiligo by inducing repigmentation in affected areas of skin. However, since treatment might need to be continued for many months, systemic steroids are not usually appropriate because of their numerous side effects. Topically applied steroids avoid systemic toxicity (Boissy and Nordlund 1995b) and can be useful in children under the age of 10 years or for those patients with limited depigmentation.

Psoralen in combination with exposure to UV-A light (PUVA) in the 320-400 nm spectra is the most common treatment for vitiligo (Boissy and Nordlund 1995b). The molecular mechanism involved in PUVA-induced repigmentation has confirmed that the re-colonising melanocytes are derived from pigment cells reservoirs in hair follicles (Cui *et al.* 1991). In addition, it has been proposed that PUVA treatment may stimulate the production of melanocyte growth factors and may deplete vitiligo-associated antigen expression on melanocytes thus blocking the binding of specific autoantibodies (Kao and Yu 1992). Furthermore, it has been shown that PUVA therapy depletes epidermal Langerhans cells *in vivo* which may reduce immunological responses against pigment cells in vitiligo skin (Kao and Yu 1992).

The observation that the cutaneous epidermis in vitiligo lesions has low levels of catalase activity and defective catecholamine metabolism, led to the development of pseudocatalase (Schallreuter *et al.* 1995). Vitiligo patients treated with a pseudocatalase cream, that also received UV-B and a topical calcium preparation, were reported to show complete repigmentation of the face and dorsa of the hands in 90% of cases (Schallreuter *et al.* 1995).

If topical steroids or PUVA treatments fail to induce repigmention, surgical alternatives exist. However, because of the time consuming nature of surgical therapies, these procedures are most often limited to segmental or localised vitiligo, although they can be successful in generalised vitiligo (Kovacs 1998). Surgical modalities are only considered in the case of inactive and non-progressive disease.

1.3 Theories of vitiligo aetiology

The aetiology of vitiligo is unknown and it is possible that there may be more than one mechanism involved in the cause of the disorder. Various theories have been put forward to explain the destruction of melanocytes in vitiligo and the main hypotheses are:-

i. Autoimmune effects that destroy melanocytes
ii. Neurochemical-mediated effects on melanocytes
iii. Self-destruction of melanocytes due to an intrinsic melanocyte defect

In addition to the above separate theories, a convergence theory states that psychological stress, the accumulation of toxic compounds, infection, autoimmunity, an inherent genetic predisposition (Section 1.2.3), mechanical stress injury and impaired melanocyte migration and/or proliferation can all contribute to vitiligo pathogenesis in varying proportions (Le Poole *et al.* 1993c; Taieb 2000).

1.3.1 Psychological stress

Many vitiligo patients report that their vitiligo lesions first appeared or expanded under conditions of extreme stress (Lerner 1959; Papadopoulos *et al.* 1999). Stress induces an increase in the production of catecholamines, the possible influences of which on the destruction of melanocytes are described in Table 1.5.

1.3.2 Physical stress (Koebner's phenomenon)

Vitiligo maculae may appear at the site of mechanical stress or friction injury to the skin (Taieb 2000). Gauthier demonstrated that the distribution of vitiligo lesions commonly correlates to areas of the body subjected to the most mechanical stresses (Gauthier 1996). It has been reported that there is some correlation between the activity of non-segmental vitiligo and the presence of experimentally-induced Koebner's phenomenon (Njoo *et al.* 1999).

Table 1.5 Influence of chemical agents and enzymes implicated in vitiligo aetiology.

Chemical agents or enzymes	Normal function	Experimental observations in relation to vitiligo	Proposed contribution to disease aetiology	References
Tetrahydrobiopterin	Regulatory role in melanogenesis since it is a rate-limiting cofactor in tyrosine→phenylalanine and tyrosine→L-DOPA conversions.	Phenylalanine accumulation seen in epidermis of vitiligo patients. 6-biopterin, metabolite of tetrahydrobiopterin, is highly toxic to cultured melanocytes.	Defective melanogenesis leads to toxic metabolites which cause the melanocytes to self-destruct.	Schallreuter *et al.* 1994c Schallreuter *et al.* 1998
Thioredoxin reductase	A free-radical scavenging enzyme located on melanocyte membrane. Also involved in conversion tyrosine→phenylalanine. Action inhibited by calcium.	Vitiligo melanocytes and keratinocytes have decreased capacity for calcium uptake. Extracellular calcium is, therefore, higher in vitiligo skin.	Inhibition of thioredoxin reductase, by ↑ extracellular calcium, leads to a build-up in free-radicals which may lead to melanocyte damage. Inhibition may also affect melanogenesis leading to the build-up of toxic metabolites.	Schallreuter *et al.* 1996 Schallreuter and Pittelkow 1988 Ortonne and Bose 1993
Catalase	H_2O_2 scavenging enzyme ($H_2O_2 \rightarrow H_2O+O_2$). ↓oxidative stress in epidermis after UVB irradiation.	Decreased levels of catalase recorded in vitiligo patient blood and in cultured vitiligo melanocytes.	Toxic levels of H_2O_2 may accumulate destroying the melanocyte.	Schallreuter *et al.* 1991
Glutathione reductase	Anti-oxidant enzyme involved in maintaining intracellular redox status.	Decreased levels of glutathione reductase in vitiligo epidermis.	Imbalance of intracellular redox status renders melanocytes susceptible to damage by toxic free-radicals.	Schallreuter *et al.* 1987
Catecholamines (Adrenaline, Noradrenaline, melatonin, etc.)	Neurotransmitters. Synthesised by adrenal glands but adrenaline and nor-adrenaline also recently shown to be synthesised by epidermal keratinocytes and melanocytes.	Increased catecholamines in vitiligo skin and vitiligo patient urine. Increased synthesis by epidermal keratinocytes in vitiligo patients.	Directly cytotoxic in high levels. Indirectly cytotoxic by causing a build-up of oxidative stress as follows; abnormal release of catecholamines may cause increased vasoconstriction→ hypoxia-ischaemia→ reoxygenation→ increased production of oxidative species.	Schallreuter *et al.* 1992 Iyengar and Misra 1987 Morrone *et al.* 1992

Chemical agents or enzymes	Normal function	Experimental observations in relation to vitiligo	Proposed contribution to disease aetiology	References
Melatonin	Control of circadian rhythms.	Met-enkephalin and β-endorphin oscillations no longer circadian in vitiligo patients.		Mozzanica *et al.* 1992
		Melanocytes at the margins of vitiligo lesions are receptive to melatonin, and, therefore, may have melatonin receptors. These melanocytes can become less dendritic in response to melatonin, thereby possibly modulating depigmentation.	Hyperactivation of melatonin receptors on vitiligo melanocytes initiates a dysregulation of melanogenesis by ↑ activity of some of the enzymes involved in melanin synthesis.	Iyengar 1994 Slominski *et al.* 1989
Catechol-*O*-methyl transferase (COMT)	Enzyme which inactivates catecholamines by methylation.	Increased COMT in lesional vitiligo skin, indicative of high levels of catecholamines.	As catecholamines.	Le Poole *et al.* 1994
Melanocyte growth factor/s	Stimulate proliferation of melanocytes.	Cultured vitiligo melanocytes demonstrate growth defects.	Decreased concentrations of melanocyte growth factor/s → melanocytes unable to recover or replenish after damage.	Puri *et al.* 1987 Ramaiah *et al.* 1989
Malondialdehyde (MDA)	An end product of lipid peroxidation of polyunsaturated fatty acids induced by reactive oxygen species.	Increased levels of MDA in vitiligo patients serum.	Lipid peroxidation of cellular membrane of melanocytes by free radicals may have a significant role in the pathogenesis of vitiligo.	Yildirim *et al.* 2003 Koca *et al.* 2004
Xanthine oxidase (XO)	An enzyme that catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid.	Increased levels of XO in vitiligo patient serum.	Increased XO levels could contribute to the oxidative stress in the aetiology of vitiligo.	Koca *et al.* 2004

1.3.3 Infections

It is possible that infections can result in melanocyte destruction by triggering an immune response leading to vitiligo in some patients. The melanocyte may be destroyed as a result of harbouring an infectious agent or because it exposes antigens similar to a micro-organism to immune surveillance (Le Poole *et al.* 1993c). Vitiligo-like hypopigmentation has been reported in some infectious diseases such as leprosy (Shegan 1971), candidiasis (Howanitz *et al.* 1981) and acquired immunodeficiency syndrome (Ivker *et al.* 1994). Cytomegalovirus DNA was found in skin biopsies in 11 out of 29 patients with vitiligo, with particular prominence in biopsy specimens of active lesions (Grimes *et al.* 1996), and has, therefore, been suggested as a trigger for vitiligo.

1.3.4 Neuronal hypothesis

Neurochemical-mediated effects on melanocytes would explain the often symmetrical distribution of vitiligo lesions or the dermatomal pattern of segmental-type vitiligo (Lerner 1959). Indeed, immunohistochemical studies of lesional and perilesional vitiligo skin have demonstrated abnormalities in the levels of skin neuropeptides. It has been reported that 5 of 10 patients with symmetrical type vitiligo had increased levels of neuropeptide Y in the margins of vitiligo lesions and those with active disease also had elevated neuropeptide Y within lesional skin (Al'Abadie *et al.* 1994). In addition, the number of nerve fibres immunoreactive to nerve growth factor (NGF) and calcitonin gene-related peptides has been shown to be increased in vitiligo lesions compared to the uninvolved and control skin (Liu *et al.* 1996). The secretion of both NGF and calcitonin gene-related peptide is thought to influence normal melanocyte function (Yaar *et al.* 1991; Hara *et al.* 1995) and could be implicated in vitiligo pathogenesis (Liu *et al.* 1996). Electron microscopy studies have also demonstrated regenerative and degenerative changes in dermal nerves in lesional and perilesional skin of vitiligo patients (Al'Abadie *et al.* 1995). Furthermore, melanocytes originate from the neural crest and, in many cases of vitiligo, perilesional melanocytes appear progressing more neural in their behaviour, both in an increasingly dendritic morphology and in ability to synthesise adrenalin

(Iyengar and Misra 1988; Iyengar 1989). A reduced activity of acetylcholinesterase has been reported in vitiliginous skin compared to normal skin (Iyengar 1989), and this is due to high concentrations of H_2O_2 in the skin of vitiligo patients (Schallreuter *et al.* 2004). Investigations into the levels of neurotransmitters in vitiligo patients are summarised in Table 1.5.

Several clinical observations provide further evidence for an association of the nervous system with vitiligo. It has been noted that vitiligo spares denervated skin, for example, below the level of neurological damage in certain patients with severe spinal cord injury (Lerner 1959). Spontaneous repigmentation of lesions occurs occasionally in vitiligo patients whose nervous system has been compromised by diabetic neuropathy. In contrast, vitiligo-like hypopigmented macules are sometimes produced in inflammatory diseases affecting the peripheral nervous system such as leprosy, and in neurodysplasias such as neurofibromatosis and tuberous sclerosis. Additional indirect evidence is provided by the requirement for innervation for skin transplants to repigment an area. While vitiligo patients do not complain of sensory abnormalities in lesional skin, autonomic dysfunction has been reported, including: increased skin surface temperature and an increased sweating response in vitiliginous areas, when compared to normal skin, and an abnormal sympathetic skin response quantified by electrical measurement of sympathetic nerve activity (Chanco-Turner and Lerner 1965; Dutta and Mandal 1982; Merello *et al.* 1993). However, this could be associated with the absence of Merkel cells from the basal layer of vitiligo lesions (Bose and Ortonne 1994) that are thought to interact with nerve endings (Lacour *et al.* 1991).

1.3.5 The melanocyte defect/self-destruction hypothesis

It has been hypothesised that intrinsic melanocyte defects such as the accumulation of toxic intermediate products of melanin synthesis (Pawelek *et al.* 1980; Moellmann *et al.* 1982), the breakdown of free radical defence (Nordlund and Lerner 1982), an increase of serum nitric oxide levels (Rocha and Guillo 2001; Yildirim *et al.* 2003) and the build-up of excess H_2O_2 (Schallreuter *et al.* 1991; Schallreuter *et al.* 1994c, Schallreuter *et al.* 2004) might result in the self-destruction of melanocytes in vitiligo. The theory is also supported by the clinical observation that certain chemical

compounds can produce a pattern of depigmentation indistinguishable from idiopathic vitiligo, e.g. mercaptoamines (Bleehan 1979) and phenolic agents (Le Poole *et al.* 1999; Boissy and Manga 2004). The suggestion that vitiligo melanocytes show an increased sensitivity to oxidative stress (Schallreuter 1999; Jimbow *et al.* 2001) has also led to the use of the antioxidant pseudocatalase in the treatment of vitiligo (Section 1.2.7). The proposed involvement of some of the biochemicals thought to contribute to vitiligo pathogenesis through melanocyte autocytotoxicity is summarised in Table 1.5.

1.3.6 The autoimmune theory

The concept of autoimmunity and the development of autoimmune disease are discussed in Section 1.4. Here, the evidence for autoimmunity as a pathogenic mechanism for the development of vitiligo is outlined. Initially, a role for autoimmunity was suggested due to the frequent clinical association of vitiligo with diseases of an autoimmune origin (Table 1.3) and by the presence of organ-specific autoantibodies in vitiligo patients (Table 1.6). Subsequently, autoantibodies that target pigment cell antigens and are capable of causing damage to the melanocyte have been identified in vitiligo patient sera (Naughton *et al.* 1983a; Naughton *et al.* 1983b; Norris *et al.* 1988b; Cui *et al.* 1992; Gilhar *et al.* 1995). Autoreactive T lymphocytes targeting melanocyte-specific proteins have also been identified both circulating in the blood and at the margins of advancing lesions of vitiligo patients (Ogg *et al.* 1998; van den Wijngaard *et al.* 2000; Lang *et al.* 2001). These aberrant cellular and humoral responses in vitiligo are described in further detail in Section 1.5.

The association of vitiligo with certain HLA specificities and other immune response genes (Section 1.2.3.1) adds credence to an autoimmune hypothesis. All autoimmune endocrinopathies are associated with particular alleles of the MHC class II HLA-DR (Dahlberg *et al.* 1981; Platz *et al.* 1981; Maclaren and Riley 1986). Although studies of association of MHC specificities with vitiligo have shown variability, several studies have reported a significant association of HLA-DR with

Table 1.6 The frequency of organ-specific autoantibodies in vitiligo patients.

Autoantibody reactivity	Number of patients in study	Percentage with antibody reactivity	Reference
Gastric parietal cells	65	17	Zauli *et al.* 1986
Gastric parietal cells	80	21	Brostoff 1969
Gastric parietal cells	96	13.7	Betterle *et al.* 1976
Gastric parietal cells	20	30	Mandry *et al.* 1996
Thyroid cytoplasm	80	28	Brostoff 1969
Thyroid microsomes/peroxidase	96	20	Betterle *et al.* 1976
Thyroid microsomes/peroxidase	20	50	Mandry *et al.* 1996
Thyroglobulin	80	9	Brostoff 1969
Thyroglobulin	20	40	Mandry *et al.* 1996
Adrenal cortex	80	4	Brostoff 1969
Pancreatic islet cells	96	7.2	Betterle *et al.* 1976

(Adapted from Kemp *et al.* 2001c)

vitiligo patients in different ethnic populations (Foley *et al.* 1983; Dunston and Halder 1990; Poloy *et al.* 1991; Venneker *et al.* 1992; Venkataram *et al.* 1995; Zamani *et al.* 2001; Tastan *et al.* 2004).

The therapeutic response of some vitiligo patients to PUVA (Boissy and Nordlund 1995b), topical corticosteroids (Kovacs 1998; Parsad *et al.* 2004) and topical cytotoxic drugs such as fluorouracil (Tsuji and Hamada 1983) is believed to result from immunosuppression of the local immune reactions responsible for damaging melanocytes. Approximately 50% of vitiligo patients will note a significant return of pigment in those lesions that retain melanocytes after the application of topical steroids (Boissy and Nordlund 1995b).

Despite the persuasive evidence detailed above for an autoimmune aetiology, exactly how aberrant immunological reactivities (Section 1.5) might contribute to vitiligo development remains open to speculation. It is currently unknown whether abnormal immune responses are the primary cause of the disease or if they arise as a secondary phenomenon resulting from damage to melanocytes via other mechanisms and, in turn, exacerbates the condition. It is also possible that different pathogenic mechanisms account for the different clinical subtypes of vitiligo (Section 1.2.5). For example, associated autoimmunity is rare in patients with segmental vitiligo and, therefore, immune mechanisms might play a lesser role than in symmetrical vitiligo that is often associated with autoimmune disorders. Several animal models of vitiligo also support the autoimmune theory of vitiligo development and these are discussed below.

1.3.6.1 Animal models

There are several spontaneous animal models of vitiligo (Boissy and Lamoreux 1988) but how the acquired depigmentation relates to that of the human disease remains to be established. The well-documented Smyth line chickens (Smyth 1989), express a genetically inherited form of vitiligo-like depigmentation resulting from the loss of melanocytes in feather and ocular tissues. In this avian model, vitiligo begins with an inherent melanocyte defect that is followed by an autoimmune response involving both humoral and cellular reactions that eliminate abnormal pigment cells (Lamont and Smyth 1981; Boyle *et al.* 1987; Pardue *et al.* 1987). Autoantibodies to chicken melanocytes have been detected in the sera of 100% of

Smyth line chickens but not in the sera of normally pigmented birds (Austin *et al.* 1992). These antibodies were found to be present both before and during the presentation of vitiligo (Searle *et al.* 1991). In addition, an increase of T cells is seen in the feather pulp and in the circulation of Smyth chicks prior to the onset and during development of vitiligo-like depigmentation (Erf *et al.* 1995; Erf and Smyth 1996). Recently, it has been demonstrated that enhanced apoptosis in the feathers of Smyth line chickens is at least one pathogenic mechanism involved in the death of melanocytes and this appears to be induced by infiltrating cytotoxic T cells (Wang and Erf 2004). Depigmentation is reduced by cyclosporine A, a selective inhibitor of inflammatory T cells, and by performing neonatal bursectomy that causes B cell deficiency (Lamont and Smyth 1981). The vitiligo-like disorder of Smyth chickens is accompanied by autoimmune hypothyroidism and an avian equivalent of alopecia areata (Smyth 1989).

Autoantibodies targeting pigment cell surface antigens have also been identified in several other animal models of vitiligo, including Arabian horses, Siamese cats and Tervuren dogs (Naughton *et al.* 1986a) as well as in the Sinclair pig, an animal model for regressive melanoma with vitiligo-like hypopigmentation (Cui *et al.* 1995a). These antibodies recognise a similar pattern of melanocyte antigens in immunoprecipitation experiments as antibodies in vitiligo patients (Naughton *et al.* 1986a), indicating that similar immunological responses may occur in both animals and humans.

1.3.7 The convergence theory

The convergence theory (Figure 1.6) proposes that all of the previously described mechanisms can contribute to the development of vitiligo (Le Poole *et al.* 1993c; Taieb 2000). Moreover, the different causal factors can act independently or synergistically to bring about the local destruction of melanocytes and are differentially involved in separate vitiligo patients (Le Poole *et al.* 1993c).

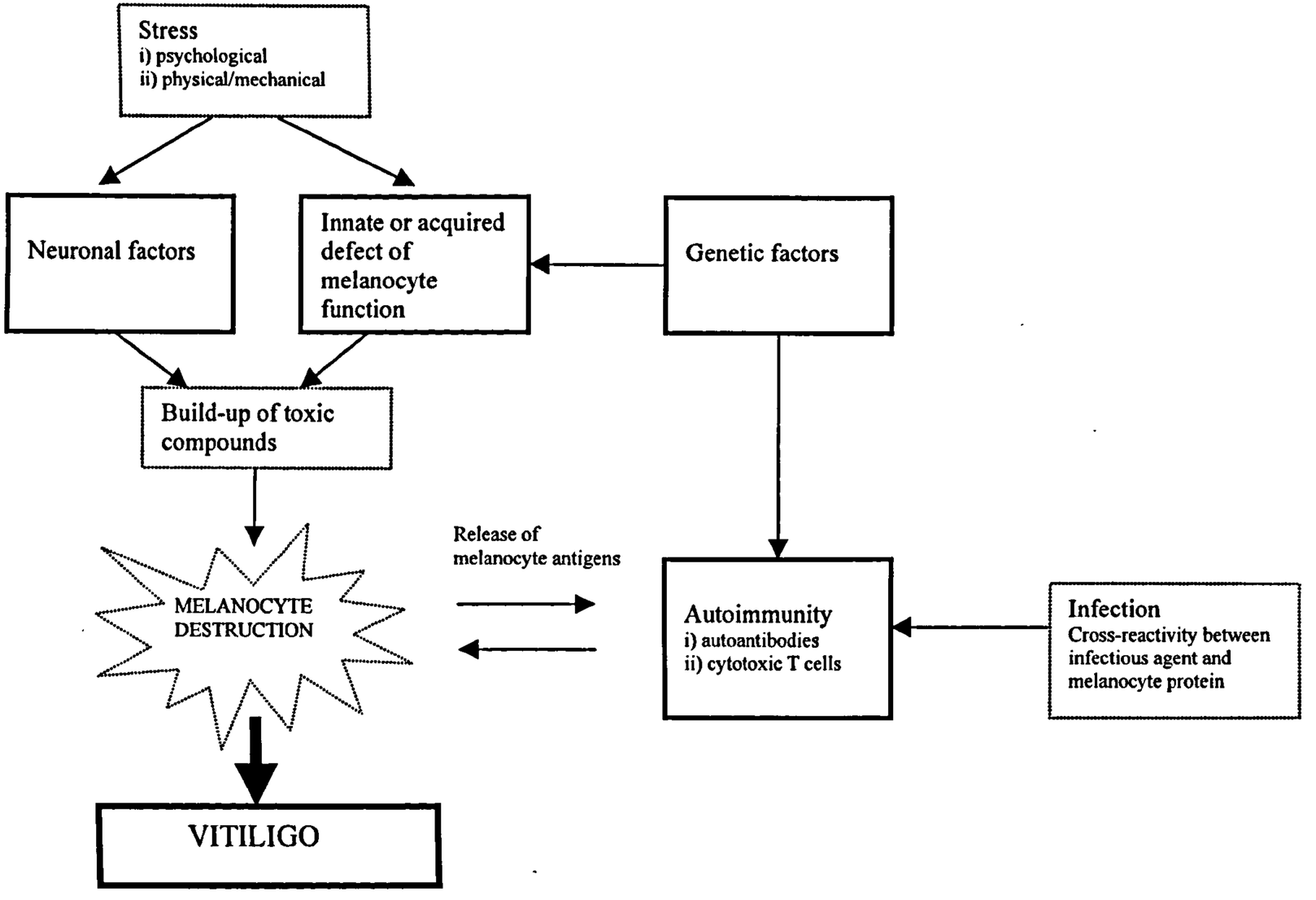


Figure 1.6 A schematic representation of the convergence theory for vitiligo aetiology.

(Adapted from Le Poole *et al.* 1993c).

1.4 Autoimmunity and autoimmune disease

Normally, individuals do not form potentially destructive autoantibodies and autoreactive T cells to their own tissues, but only to foreign antigens (Janeway 1992). This is because the body has developed a tolerance to self-antigens that is maintained by a complex network of T and B lymphocytes and their regulatory products. However, the intrinsic variability of the regulatory mechanisms governing the immune repertoire can allow for the development of autoreactive immune cells and any subsequent failure of the regulatory networks that maintain self-tolerance can result in the transition from autoreactivity to autoimmune disease (Avrameas 1991; Viglietta *et al.* 2004).

1.4.1 Self-tolerance

The processes leading to the discrimination between self and non-self by T cells include: central tolerance induced in the thymus, peripheral tolerance and active suppression. T cells are predominantly subject to clonal deletion in the thymus (Nossal 1989; Schwartz 1989; Kroemer and Martinez 1992) where the recognition of the complex of self-peptide with self-MHC antigen during development results in the elimination by apoptosis of the majority of potentially self-reactive T cells. This thymic negative selection of T cells occurs primarily by the interaction with MHC molecules/self-peptides on macrophages and dendritic cells (Speiser *et al.* 1989), although the thymic epithelium may also cause tolerance (Salaun *et al.* 1990). T cells can be subject to positive selection in the thymus. This involves the selection of T lymphocytes with moderate but not high affinity for self-MHC antigens (von Boehmer and Kisielow 1990) and this selection procedure ensures that circulating T cells have some affinity for self-MHC molecules and can thus recognise foreign antigens when appropriate. In addition to the aforementioned mechanisms of T cell regulation, T cells are also controlled in the thymus by clonal anergy. In this case, the interaction of a specific T cell with an antigen results in the production of a state of non-responsiveness or anergy in which T cells do not proliferate or produce cytokines and thus do not trigger an immune response (Rammensee *et al.* 1989). Clearly, the combination of mechanisms so far described cannot guarantee there will

be complete discrimination between self and non-self and prevent the development and release of potentially autoreactive T cells into the peripheral circulation. Two additional levels of extrathymic control are known to deal with autoreactive T lymphocytes that escape central tolerance, namely, peripheral tolerance and active suppression.

Peripheral tolerance has been largely explored through the use of transgenic mice and it has been shown that the expression of recombinant antigen in different cells and tissues can lead to anergy or deactivation in the corresponding specific T cells (Markmann *et al.* 1988). This effect appears to be due to the presentation of antigen by non-professional antigen presenting cells and the concomitant absence of secondary signalling molecules necessary for the generation of an immune response. Finally, studies have demonstrated a role for specific T cell subsets in the suppression of autoreactive T cells. Developments in the technology of cell cloning have led to the isolation of several T cell clones with suppressor function. Antigen-specific $CD8^+$ suppressor T cell clones have been established from patients with leprosy and these are able to inhibit T cell proliferative responses to mycobacterial antigen (Modlin *et al.* 1987). Recently, it has been shown that the depletion of T cell populations can give rise to autoimmune thyroid disease in human patients and this may be due to T helper lymphocyte type-1 (Th1)/ T helper lymphocyte type-2 (Th2) switching (Coles *et al.* 1999).

There is compelling evidence that immune responsiveness is also controlled by a sub-population of other regulatory T cells that are enriched in a naturally activated subset of $CD4^+$ T cells, which constitutively express CD25. The thymus is the primary source of this regulatory $CD4^+CD25^+$ T cell subset, which participates in the active suppression of potentially autoreactive T cells in the periphery (Sakaguchi *et al.* 1995). Regulatory $CD4^+CD25^+$ T cells are naturally anergic *in vitro* and they inhibit the proliferation of co-cultured conventional $CD4^+CD25^-$ T cells in a contact-dependent manner and have been shown in a variety of experimental systems to provide protection from T cell-mediated autoimmune disorders (Shevach 2002).

A decrease in the number of circulating $CD4^+CD25^+$ T cells has been reported in type-1 diabetes mellitus (Kukreja *et al.* 2002) and the loss of functional activity for circulating $CD4^+CD25^+$ T cells (Viglietta *et al.* 2004) has been shown in multiple sclerosis. In contrast, the $CD4^+CD25^+$ T subset was shown to be enriched with

functionally active regulatory T cells in the inflamed joints of patients with rheumatoid arthritis (Cao *et al.* 2003). In myasthenia gravis patients, although a normal number of $CD4^+CD25^+$ T cells was found, a severe functional defect in their regulatory activity together with a decreased expression of the transcription factor Foxp3, which is essential for T cell regulatory function (Balandina *et al.* 2005), was apparent.

Similar tolerising mechanisms of clonal deletion and anergy have been described for autoreactive B cells (Goodnow *et al.* 1989). However, in contrast to T cells, antigen concentration is critical for the induction of B cell tolerance as low amounts may fail to tolerise B cells. In some transgenic animal models, it has been shown that autoreactive B cells can escape the tolerisation process and this may occur in tissues where the corresponding antigen is not present or is sequestered (Murakami *et al.* 1992).

Despite the safety mechanisms, self-tolerance can breakdown and several triggers have been shown to cause this.

1.4.2 Mechanisms of breakdown in self-tolerance

A breakdown in self-tolerance can be triggered by defects in the immune network that strictly monitors the recognition of self-antigens by immune cells (Ring and Lakkis 1999). This can include: the failure to delete autoreactive lymphocytes or to render them anergic in the thymus, the failure of the mechanism of peripheral tolerance to again render autoreactive immune cells anergic and the failure of active suppression of autoreactive T cells in the peripheral circulation (Ring and Lakkis 1999). Self-reactive B lymphocytes may also be stimulated by polyclonal activators such as lipopolysaccharides with the subsequent formation of antibodies to self components (Tsubata 2000). Overall, the breakdown of the immune network can occur at several levels resulting in the presence of autoreactive lymphocytes.

Self-tolerance of non-exposed (cryptic) antigens means that potentially autoreactive T cells are not subjected to the normal deletion response. Self-tolerance to these antigens continues as far as they remain immunologically hidden, sequestered in a tissue compartment devoid of immune cells. Exposure of these antigens through tissue damage could trigger an autoimmune response. Examples of

this include the immune response against lens protein after eye trauma (Goldschmidt *et al.* 1982) and Dressler's syndrome after myocardial infarction, due to ischemic tissue damage (Bartels *et al.* 1994).

An attractive concept explaining the initiation of autoimmunity is the existence of cross-reactive antigens or molecular mimicry (Wucherpfenning 2001). If a pathogenic antigen resembles a host self-antigen, then tolerance of self may be broken. A classical example is rheumatic fever, where antibodies to a streptococcal protein cross-react with cardiac muscle myosin leading to carditis (Williams *et al.* 1985). However, there is no firm evidence that such a mechanism plays a role in the aetiology of organ-specific autoimmune diseases.

An event that could lead to a breakdown in self-tolerance is viral infection. Since viruses can cause the display of viral antigens on the surface of cell, viral antigenic expression could act to directly induce autoimmune reactivity (Lernmark 2001). Viruses can also elicit autoimmune diseases by the polyclonal activation of lymphocytes, the release of subcellular organelles after viral lysis of the cell, by antigenic mimicry and by the functional impairment of regulatory immune cells such as T-helper lymphocytes. Viral infections prior to disease have been associated with systemic lupus erythematosus (SLE), multiple sclerosis and type 1 diabetes mellitus (Lernmark 2001).

Once self-tolerance has broken down, the resulting production of autoreactive T cells and autoantibodies can cause an array of autoimmune diseases.

1.4.3 Autoimmune disease

Autoimmune diseases are generally classified into those affecting a single organ or tissue, the organ-specific, and those affecting more than one organ, the non-organ specific or systemic (Roitt and Delves 2001). In organ-specific diseases, the tissue destruction occurs is restricted to the specific antigens localised in that organ. Examples of this type of autoimmune diseases are Graves' disease, type 1 diabetes mellitus, multiple sclerosis, pernicious anaemia and myasthenia gravis (Roitt and Delves 2001). In contrast, systemic autoimmune diseases can affect many regions of the body and circulating immune complexes are often deposited in several different

organ regions. Examples of systemic autoimmune diseases are rheumatoid disease and SLE (Roitt and Delves 2001).

In autoimmune disease, damage to tissue results from autoreactive T cells and autoantibodies. With respect to cellular autoimmune responses, the recognition of self-antigens as immunostimulatory can lead to the initiation and expansion of T lymphocyte subsets (Lang *et al.* 2003). Populations of phagocytic cells and cytotoxic T cells are then drawn to the antigenic site and proceed to destroy cells displaying the specific self-antigen. An example of this type of immune mechanism is autoimmune thyroiditis where an accumulation of cytotoxic T cells can be found in thyroid lesions (Martin *et al.* 1990). As the present study concerns humoral immune reactivity in vitiligo, the role of autoantibodies in autoimmune disease is considered in more detail in the next section.

1.4.4 Autoantibodies

In autoimmune disease, autoantibodies may be the actual pathogenic agents of the disease or arise as a secondary consequence due to tissue damage. They can be directed against cell surface molecules such as hormone receptors, extracellular targets such as circulating soluble antigens and intracellular components such as DNA (Naparstek and Poltz 1993). With respect to intracellular antigens, there is evidence that autoantibodies can penetrate living cells and can interact with their target altering function or even causing apoptosis (Reichlin 1995). To establish that an autoantibody is pathogenic, it should fulfil certain criteria (Rose and Bona 1993):

i) The autoantibody should be capable of causing the lesions attributed to it in an experimental system or by transplacental transfer in man, and suitable immunisation that leads to the production of similar autoantibodies in animals should lead to a similar disease process.
ii) Autoantibody level and disease activity should, in general, correlate.
iii) Removal of autoantibody should ameliorate the disease process.
iv) The antibody has to be found in all patients with the relevant disease in a significantly higher frequency or titre than in normal.

In pemphigus, anti-epidermal antibodies correlate with disease activity and the removal of these antibodies by plasmapheresis leads to clinical improvement (Grando *et al.* 1990). In addition, the injection of pemphigus foliaceus autoantibodies into neonatal balb/c mice reproduced the human form of the disease in the animals (Rock *et al.* 1990). Similarly, in myasthenia gravis, autoantibodies to the acetylcholine receptor also fulfil the main criteria for the pathogenicity of disease (Eymard *et al.* 1991).

There are a variety of ways in which autoantibodies can cause tissue damage in autoimmune disease including: damage by complement fixation, detrimental effects on normal cellular function, the formation of immune complexes that initiate a destructive inflammatory response and antibody-dependent cell-mediated cytotoxicity (ADCC). These are discussed below with examples of the resulting autoimmune disorders.

1.4.4.1 Effects on cellular functions

In autoimmune thyroid disease, antibodies against thyroid-stimulating hormone receptor (TSHR) play a role in causing the different manifestations of the disease (Weetman and McGregor 1994). Autoantibodies in Graves' disease are directed against the TSHR and mimic the action of thyroid-stimulating hormone (TSH). Since their production is not subject to the feedback control of the thyroid hormone whose synthesis they stimulate, the thyroid gland overproduces thyroid hormones and enlarges under the tropic stimulus delivered through the receptor. The antibodies associate strongly with the disease. In autoimmune hypothyroidism, certain TSHR autoantibodies may cause blockage of TSH function leading to hypothyroidism. Similarly, blocking autoantibodies in autoimmune hypoglycaemia are directed against the insulin receptor and mimic the actions of insulin (Taylor *et al.* 1989). In myasthenia gravis, autoantibodies are produced to acetylcholine receptors located at the neuromuscular junction. These antibodies interfere with proper neurotransmission and diminish the contractility of the muscle cells (Fabien *et al.* 2001).

1.4.4.2 Complement activation

The complement system (Figure 1.7) comprises a complex group of proteins whose activation leads to the production of biologically active molecules with several discrete functions (Whaley and Schwaeble 1997). Activation of complement may occur by two pathways, the classical and the alternative, both of which lead to splitting of the C3 and C5 components and the formation of a membrane attack complex (MAC) capable of producing cell lysis. The classical pathway is usually activated by immune complexes of antigen and antibody of the IgM, IgG1, IgG2, or IgG3 isotypes. Immunoglobulin bound to antigen as an immune complex undergoes conformational changes that expose a C1q binding region in the C_H2 domain of the Fc portion of the antibody and thus stimulates classical pathway activation. The initial components of the alternative pathway are factors B, D and P. These factors are activated by substances like endotoxin and IgA and feed into MAC formation through C3, as does the classical pathway.

Autoantibodies directed at self-antigens located on the cell surface may bind to the recognised antigen and activate the complement system resulting in cytolysis. This occurs in autoimmune haemolytic anaemia where IgG autoantibodies bind to certain blood group antigens on erythrocytes causing haemolysis (Silverstein 1989). In antiphospholipid syndrome, autoantibodies that react with lipid-binding proteins on the surface of endothelial cells trigger complement activation and subsequent thrombosis (Munakata *et al.* 2000).

1.4.4.3 Antibody-dependent cell-mediated cytotoxicity

Unlike cell killing by cytotoxic T lymphocytes or natural killer (NK) cells, ADCC depends on the presence of specific and high-affinity antibodies directed to the surface of target cells. The mechanism responsible for this type of killing is the capture of such antibodies on the surface of certain cell types including: NK cells, neutrophils, eosinophils and macrophages, through receptors that bind the Fc portion of the antibody thus leaving the antigen-binding site of the antibody molecule ready to interact with membrane antigens (Yokoyama 1999). Binding of such antibodies to

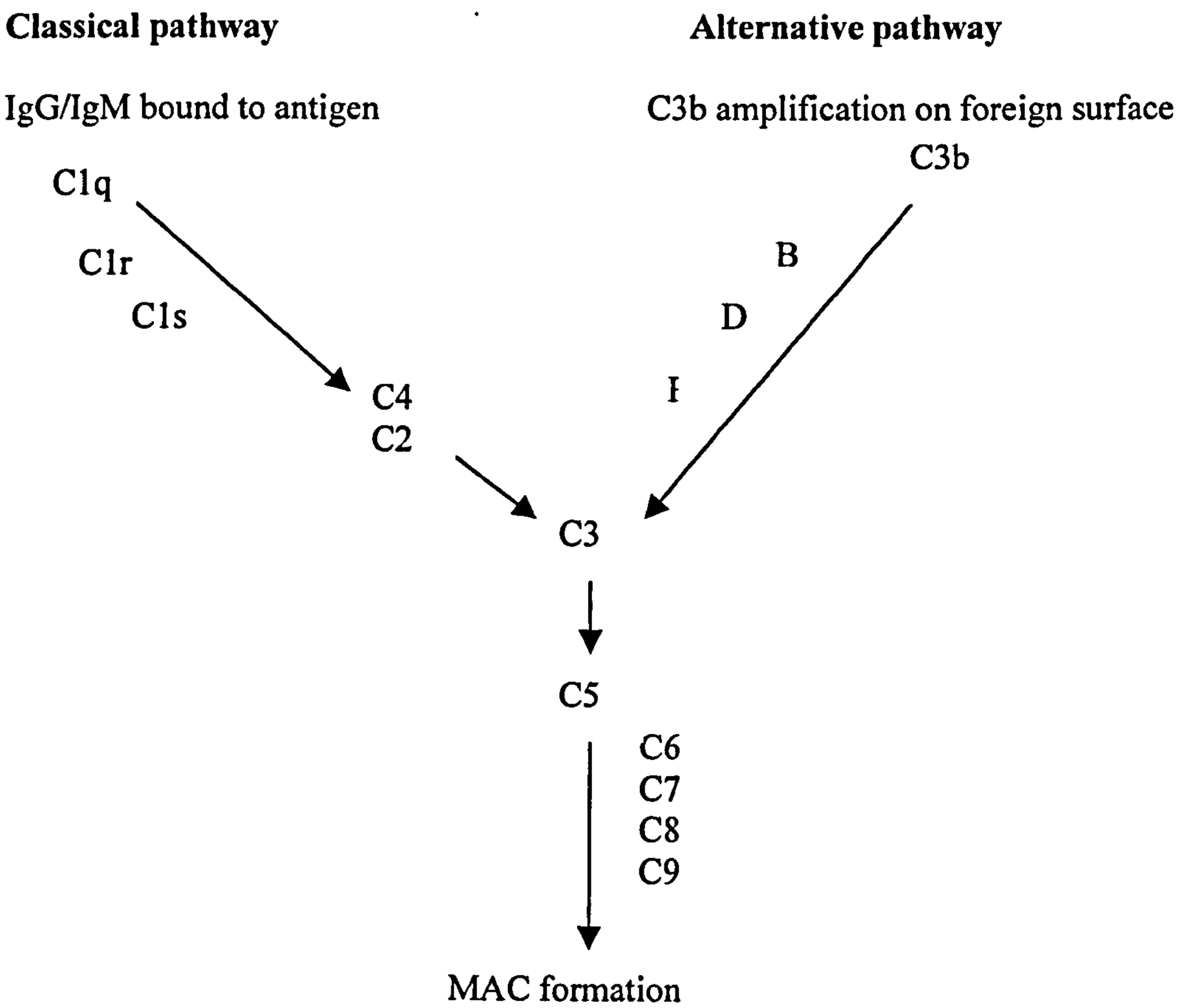


Figure 1.7 An overview of the complement pathway. Initiation of the classical pathway requires the presence of antibodies, either IgG or IgM, bound to cell surface antigen. The serum protein C1 binds to antibody that, in turn, activates C4 and C2 leading to the deposition of C3. Following the activation of C5, C6, C7, C8 and C9, the membrane attack complex (MAC) is formed. The alternative pathway is initiated by non-immune activators such as lipopolysaccharides and is characterised by a two-phase system in which six proteins participate. Initiation is the first phase with particle-bound C3b acting like the C1 recognition complex of the classical pathway. The second phase is called amplification that involves bound C3b, Factors B, D, P and that finally leads to the formation of MAC. At the end of both pathways MAC, mediates lysis of the target cell.
(Adapted from Prodinger *et al.* 1999)

the target cell signals to the cell bearing the Fc receptor to initiate cell killing. In the case of NK cells and eosinophils, this involves perforin but in the case of macrophages and neutrophils killing results entirely from secretion of granzymes and TNF-α (Yokoyama 1999).

Examples of autoimmune disorders in which ADCC can play a pathogenic role include autoimmune thyroid disease. Autoantibodies mediating ADCC have been found in patients with Hashimoto's thyroiditis but not in those with Graves' disease (Bogner *et al.* 1984). In the same study, a strong association between anti-thyroid peroxidase antibody titres and cell lysis was evident (Bogner *et al.* 1984). Antibodies mediating ADCC can also be associated with primary myxoedema (Bogner *et al.* 1995; Metcalfe *et al.* 1997).

1.4.4.4 Formation of immune complexes

Autoantibodies produced against soluble self-antigens and extracellular molecules can form immune complexes that lead to the activation of the complement cascade. The subsequent formation of the complement components C3 and C5 (Figure 1.7) results in the release of histamine and an increase in phagocytic activity, respectively, causing an inflammatory response that is destructive to tissues at the site of immune-complex formation. This is the usual series of events that occurs in SLE with anti-DNA/DNA complex formation. In autoimmune thyroid disease, autoantibodies to thyroid peroxidase are able to fix complement resulting in tissue damage. The deposition of the early components of complement has been identified in the thyroid of patients with Hashimoto's thyroiditis (Weetman and McGregor 1994) and the terminal complement complexes have been found around thyroid follicles and in elevated circulating levels in both Hashimoto's thyroiditis and Graves' disease patients (Weetman *et al.* 1989a). In epidermolysis bullosa acquisita, an autoimmune skin disease characterised by autoantibodies to type VII collagen, complement activation by autoantibody-antigen complexes occurs at the basement membrane zone of the skin resulting in inflammatory subepidermal blistering (Mooney *et al.* 1992).

1.5 The immune responses in vitiligo

The possible mechanisms by which both cellular and humoral immune reactivities in vitiligo may arise and contribute to its pathogenesis are summarised in Figure 1.8.

1.5.1 Cellular immunity

1.5.1.1 T lymphocytes

Autoimmune diseases are often associated with an expansion of $CD4^+$ T lymphocytes (Stites 1994). However, with respect to vitiligo, inconsistent data regarding abnormalities in circulating T cells have been reported. An increase in the number of $CD4^+$ T lymphocytes as well as an elevated $CD4^+/CD8^+$ ratio were detected in patients with stable vitiligo and in their first-degree relatives (Soubiran *et al.* 1985; D'Amelio *et al.* 1990; Al-Fouzan *et al.* 1995). In addition, a higher proportion of activated peripheral T cells, as determined by the expression of HLA-DR, has also been found in vitiligo patients compared to healthy individuals (Abdel-Naser *et al.* 1992). In contrast, a decrease in the $CD4^+$ T lymphocytes and reduced $CD4^+/CD8^+$ ratio has been observed in vitiligo patients (Grimes *et al.* 1986; Halder *et al.* 1986). These contradictory results may arise from differences in the techniques used to evaluate T-cell subpopulations, as well as from differences in the vitiligo patients studied e.g. clinical type, disease activity. The studies conclude that circulating T-cell subpopulations have rarely been found to be normal in vitiligo patients.

Of more functional relevance, a recent study has demonstrated the presence of circulating MelanA-specific cytotoxic T lymphocytes, expressing a skin-homing receptor cutaneous lymphocyte-associated antigen (CLA), in a significant number of patients with vitiligo (Ogg *et al.* 1998). These results were confirmed in a subsequent study, in which the presence of MelanA-specific cytotoxic T cells expressing CLA was also found to be significantly greater in the peripheral blood of vitiligo patients with active, progressive disease (Palermo *et al.* 2001; Lang *et al.* 2001). These studies have also demonstrated the presence of $CD8^+$ cytotoxic cells that react with

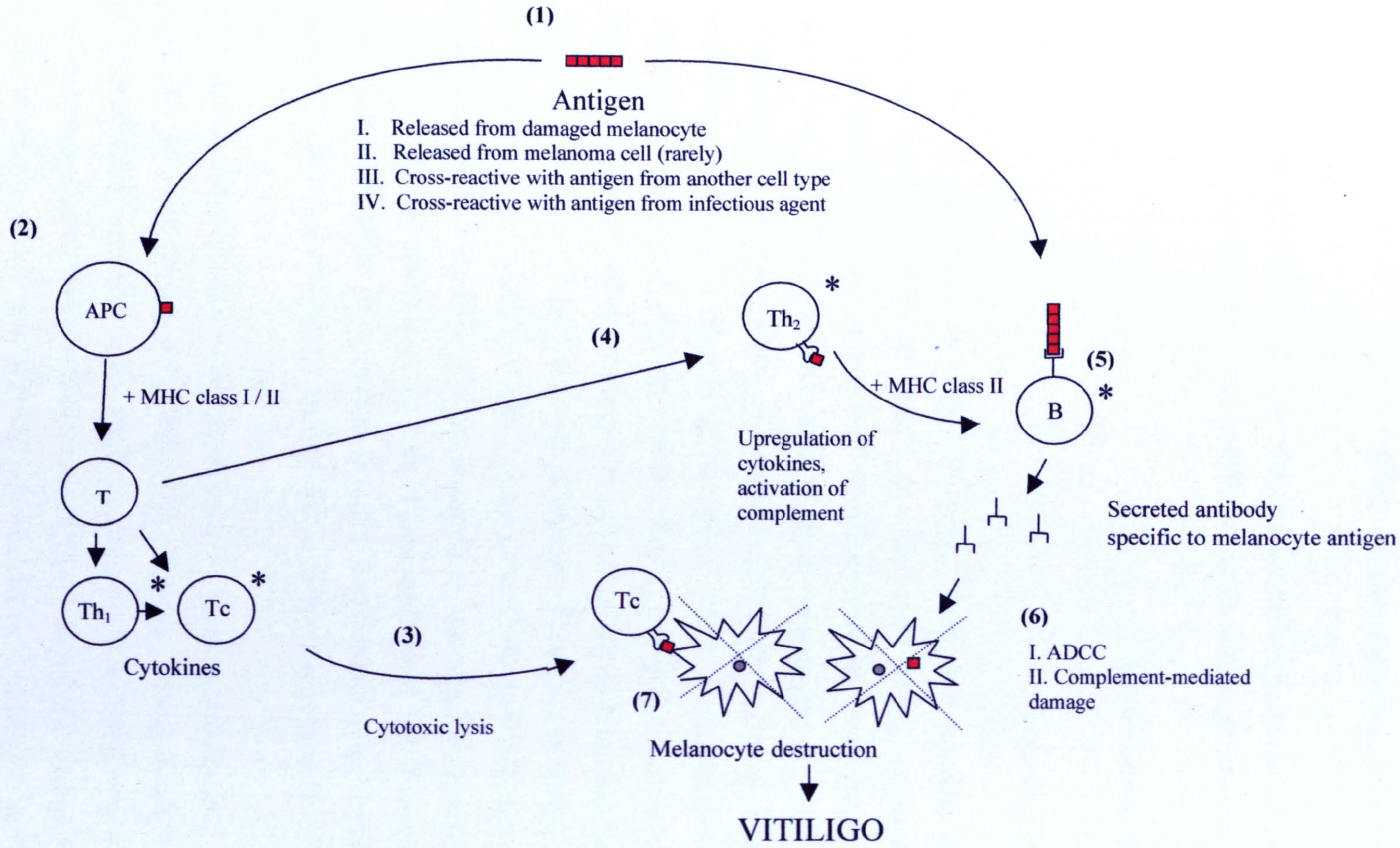


Figure 1.8 A summary of the mechanisms by which cell-mediated and humoral immune responses may arise and bring about vitiligo.

(1) Melanocyte antigens are produced by melanocytes, which include proteins released from damaged melanocytes, or from melanoma cells, which may provoke an immune response from specific immune effector cells directly, or by cross-reactivity with antigens from other cell types or infectious agents. (2) Antigen is processed by antigen-presenting cells (APC) and presented to T cells in association with the appropriate major histocompatibility complex (MHC) molecules. Different effector functions are subsequently carried out by different T cell subsets. $CD8^+$ cytotoxic T cells (Tc), which recognise antigen in the context of MHC class I molecules can proceed to destroy cells bearing the presented antigen by cytotoxic lysis (3). The cytotoxic T cell response is upregulated by cytokines produced by $CD4^+$ helper T cells of type-1 (Th1), which recognise antigen in the context of MHC class II. (4) A second type of helper T cell, type-2 (Th2) can activate a B cell response via the production of cytokines and the subsequent antibody production can also activate complement. (5) B cells may also recognise antigen directly through antigen-specific membrane immunoglobulins. (6) Anti-melanocyte antibodies can destroy melanocytes by either ADCC or antibody-dependent complement-mediated damage. (7) Destruction of the melanocytes by cellular (2,3,4)- and humoral (5,6)-mediated immune attack may result in vitiligo.

* A failure of immunoregulatory mechanisms to destroy/switch off self-reactive lymphocytes perpetuates the autoimmune response (may be due to mutations in immunoregulatory genes).

(Adapted from Kemp *et al.* 2001c)

peptides from tyrosinase (Ogg *et al.* 1998; Lang *et al.* 2001) and Pmel17 (Lang *et al.* 2001).

A dermal and epidermal inflammatory infiltrate consisting of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and macrophages, closely associated with areas of melanocyte depletion, has been observed in vitiligo patients (Al Badri *et al.* 1993b). The infiltrate is most prominent at the margins of vitiligo lesions and contains a significant number of activated T cells, as measured by expression of MHC class II antigen HLA-DR and CLA antigen (Al Badri *et al.* 1993b). There is also evidence of expression of the IL-2 receptor (IL-2R) and the interferon γ (IFN-γ) receptor by the lymphocytic infiltrate (Abdel-Naser *et al.* 1994). In rare cases of inflammatory vitiligo, similar observations with regard to perilesional, epidermis-infiltrating T cells have been made (Le Poole *et al.* 1996). A recent study isolated MelanA-specific $CD8^+$ T cells expressing CLA antigen from vitiligo skin biopsies, demonstrating the presence of a cytotoxic response to a melanocyte-specific antigen within vitiligo lesions (van den Wijngaard *et al.* 2000). However, the absolute numbers of infiltrating cells in lesional and perilesional skin are small when compared with other inflammatory skin disorders (van den Wijngaard *et al.* 2000) and it remains to be seen whether the infiltrate arises as a result of the disease process as opposed to being the cause of vitiligo.

1.5.1.2 Cytokines

Cytokines mediate many functions of cellular immunity and a number of studies have analysed the levels of various cytokines in patients with vitiligo (Table 1.7). Studies have demonstrated that the level of soluble IL-2R in patients with vitiligo is significantly increased compared with that of controls, indicating an activation of T lymphocytes may be involved in the disease pathogenesis (Caixia *et al.* 1999; Yeo *et al.* 1999). A recent study also demonstrated that serum IL-2R concentration in vitiligo patients depends on the activity and intensity of the disease process (Franczuk *et al.* 2004). The levels of production of IL-6 and IL-8 by peripheral mononuclear cells are also elevated in vitiligo patients, both of which can act to recruit lymphocytes to the site of an immune response (Yu *et al.* 1997). In contrast,

Table 1.7 The involvement of cytokines in vitiligo.

Cytokine	Source	Function	Level in vitiligo patients compared with healthy individuals	Method of detection	Number of patients in study	Reference
IL-2	Activated T cells	Induces growth, differentiation and proliferation of T and B cells and natural killer cells.	Levels of soluble IL-2 receptor are increased, indicating a rise in IL-2	ELISA (serum, skin tissue fluid)	41 79	Caixia *et al.* 1999 Yeo *et al.* 1999
IL-6	T cells, macrophages	Stimulates T and B cell differentiation and proliferation and upregulation of ICAM-1 expression on melanocytes, facilitating lymphocyte-melanocyte interaction.	Increased	ELISA (mononuclear cell supernatants obtained from peripheral blood)	12	Yu *et al.* 1997
IL-8	Monocytes, fibroblasts, keratinocytes	Chemotactic to lymphocytes.	Increased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997
GM-CSF	T cells, macrophages	Induces growth of macrophages and granulocytes.	Increased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997
TNF-α	T cells	Anti-tumour activity. General immunostimulant.	Decreased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997
IFN-γ	T cells	Anti-viral, modulates expression of MHC[7] class I antigens. May inhibit melanocyte growth.	Decreased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997

the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α by peripheral mononuclear cells in active vitiligo was found to be reduced compared with healthy controls (Yu *et al.* 1997). The expression of cytokines by lymphocytes from the margins of vitiligo lesions has yet to be fully characterised but initial data suggest that cytokines which promote inflammation and cytotoxicity, that is, those of the T helper lymphocyte type-1 subset, particularly IFN-γ and TNF-α, are predominantly expressed (Das *et al.* 2001).

1.5.1.3 Adhesion molecules

Adhesion molecules, which allow cell-cell attachments, are required for lymphocyte migration and infiltration through the epidermis and also for interaction with antigen presenting cells. Several studies have shown expression of ICAM-1 by keratinocytes and melanocytes in areas of vitiligo (Norris 1990; Al Badri *et al.* 1993a; van den Wijngaard *et al.* 2000). The expression of ICAM-1 by melanocytes at the edge of vitiligo lesions was found to be increased by six-fold, and was present in nineteen of twenty biopsies from perilesional skin, compared to six of seven biopsies from non-lesional skin and five of eight samples from control skin (Al Badri *et al.* 1993a). Immunohistochemistry of serial sections of vitiligo skin biopsies has shown that the epidermal expression of ICAM-1 coincides with the expression of HLA-DR (van den Wijngaard *et al.* 2000). Similar abnormal expression of HLA-DR and ICAM-1 are shown by the pancreatic islet cells in type 1 diabetes mellitus (Cambell and Harrison 1990) and by the thyroid follicular cells in autoimmune thyroid disease (Hanafusa *et al.* 1983; Weetman *et al.* 1989b; Zheng *et al.* 1990).

Vascular expression of E-selectin, the adhesion molecule responsible for capture of leucocytes from the circulation to initiate migration to the site of a local immune response, appears neither to be upregulated nor more widely dispersed in post-capillary venules in vitiligo, when compared to control skin (van den Wijngaard *et al.* 2000). However, immunohistochemical analysis demonstrated that where there is expression of E-selectin in perilesional skin, it is co-localised with T cell infiltration (van den Wijngaard *et al.* 2000). The expression of E-selectin is dependent on cytokines, particularly TNF-α (Pilewski *et al.* 1995).

1.5.1.4 Langerhans cells

The role of Langerhans cells (LC), which act as antigen-presenting cells to primed T lymphocytes, in vitiligo is unclear (Facchetti *et al.* 1984). The density of LC in vitiligo has been reported as normal (Claudy and Rouchouse 1984) or increased (Riley 1967), relative to that of the non-lesional skin and skin from control subjects. An increase in the numbers of LC might contribute to the immunological responses to melanocytes in vitiligo. Patients with repigmenting lesions in response to PUVA or Fluocinonide treatment show a decrease in epidermal LC further supporting a role for these cells in the disease (Kao and Yu 1990).

1.5.1.5 Natural killer cells

It has been shown that the number of NK cells present in affected vitiligo skin is no different from that present in the skin of control subjects (Abdel-Naser *et al.* 1992; Hann *et al.* 1993b). Although previous studies have implicated an involvement of these cells in the destruction of melanocytes in vitiligo (Halder *et al.* 1986; Mozzanica *et al.* 1989), a recent study found no significant differences between the level of activity of NK cells from vitiligo patients and control subjects, against both normal and malignant melanocytes (Durham-Pierre *et al.* 1995).

1.5.2 Humoral Immunity

1.5.2.1 Organ-specific autoantibodies

In addition to the frequent association of vitiligo with autoimmune disorders, previously described (Section 1.2.6 and Table 1.3), a variety of organ-specific autoantibodies are commonly detected in the sera of vitiligo patients (Table 1.6).

1.5.2.2 Anti-melanocyte antibodies

Significantly, antibodies to melanocytes are present in the circulation of most patients with the vitiligo (Naughton *et al.* 1983a). In one study, 12 out of 12 of vitiligo patients but none of control subjects were found to have anti-pigment cell antibodies in their sera (Naughton *et al.* 1983b). In addition, a correlation has been

described between the incidence and level of melanocyte antibodies and disease activity in vitiligo: 8/10 patients with active vitiligo, 0/14 with inactive disease and 0/19 controls were found to have circulating anti-pigment cell antibodies (Harning *et al.* 1991). Furthermore, the presence of these antibodies is related to the extent of disease: they were detected in 50% of patients with minimal vitiligo, less than 2% of skin area involved, compared to 93% of patients with greater depigmentation covering 5-10% of their skin (Naughton *et al.* 1986b). Furthermore, the level of melanocyte antibodies appears to decrease in vitiligo patients who respond to PUVA therapy or following systemic steroid treatment, suggesting that the disease activity is somehow related to the incidence of these antibodies (Park *et al.* 1996).

Generally, vitiligo antibodies belong to the IgG class (Harning *et al.* 1991) with subclasses IgG1, IgG2 and IgG3 represented (Xie *et al.* 1991). Deposits of IgG have been observed in the basal membrane zone and in the keratinocytes of vitiligo lesions (Yu *et al.* 1993; Uda *et al.* 1984) and fluorescence staining for IgG-binding to cultured normal melanocytes and keratinocytes is more prominent in IgG samples from patients with active and extensive vitiligo (Yu *et al.* 1993). However, other investigators have reported that IgA anti-pigment cell membrane antibody levels also correlate well with vitiligo activity (Aronson and Hashimoto 1987).

The pathogenic role of antimelanocyte antibodies in vitiligo still remains unclear. However, the capacity of anti-melanocyte antibodies to injure pigment cells has been demonstrated experimentally: vitiligo antibodies are able to destroy melanocytes *in vitro* by complement-mediated cytotoxicity and ADCC (Norris *et al.* 1988b) and *in vivo* following passive immunisation of nude mice grafted with human skin (Gilhar *et al.* 1995). Furthermore, it has been shown that the purified IgG fraction of vitiligo sera has a destructive effect on melanoma cells both *in vivo* and *in vitro* (Fishman 1993). More recently, a reduced expression of the membrane regulators of complement activation in both lesional and perilesional keratinocytes and melanocytes has been reported, possibly indicating an increased vulnerability of these cells to autologous complement attack (van den Wijngaard *et al.* 2002). The selective destruction of melanocytes in vitiligo may result from antibody reactivity directed to the antigens preferentially expressed on pigment cells (Cui *et al.* 1992). Alternatively, an antibody response against antigens expressed on a variety of cell types may selectively destroy melanocytes because they have been shown to be

intrinsically more sensitive to immune-mediated injury than, for example, keratinocytes or fibroblasts (Norris *et al.* 1988a).

Vitiligo antibodies were initially identified in immunoprecipitation experiments with melanocyte protein extracts and vitiligo patient sera. The antibodies were most commonly directed against pigment cell antigens with molecular weights of 35, 40-45, 75, 90 and 150 kDa, which are located on the surface of the cell (Cui *et al.* 1992; Cui *et al.* 1995b). Although the proteins have not been specifically identified, some (40-45, 75 and 150 kDa) are expressed both on pigment cells and in other tissues, while others (35 and 90 kDa) are preferentially expressed on pigment cells (Cui *et al.* 1992). Immunoblotting studies have revealed antibody reactivity in vitiligo patient sera to melanocyte proteins of 45, 65, 68, 90, 110 and 165 kDa (Park *et al.* 1996; Hann *et al.* 1996; Rocha *et al.* 2000). Again, however, the identity of these antigens has not been determined.

In addition, antibodies to the melanocyte-specific proteins tyrosinase (Song *et al.* 1994b; Baharav *et al.* 1996; Kemp *et al.* 1997a; Okamoto *et al.* 1998), TRP-1 (Kemp *et al.* 1998b), TRP-2 (Kemp *et al.* 1997b; Okamoto *et al.* 1998) and Pmel17 (Kemp *et al.* 1998c) have been detected in the sera of patients with vitiligo. In a recent study, antibodies to transcription factors SOX9 and SOX10 were identified in vitiligo patients with polyendocrine syndrome type 1 (Hedstrand *et al.* 2001). In addition, the melanin-concentrating hormone receptor 1 (MCHR1) has been identified as an autoantigen in 16.5% of vitiligo patients (Kemp *et al.* 2002) and this is discussed further in Section 1.6. Although all these proteins may be considered cytoplasmic autoantigens a *de novo* mechanism may exist whereby antibodies recognise autoantigens directly via cell surface or cytoplasmic penetration (Okamoto *et al.* 1998). For example, antibodies have been shown to be taken up by non-haemopoietic cells and react with nuclear antigens *in vivo* (Isenberg *et al.* 1997). Alternatively, antigens may be released if the melanocyte is damaged by another mechanism such as cytotoxic T cell lysis. A summary of the autoantigens implicated in vitiligo is given in Table 1.8.

The stimulus for the production of anti-melanocyte antibodies has not been identified and it is unknown whether vitiligo autoantibodies initiate the development of the disease. It is possible that cross-reacting antigens expressed either on other cell

Table 1.8 Identified target autoantigens for vitiligo antibodies.

Autoantigen	Detection method	Percentage reactivity in patients	Reference
Tyrosinase	Immunoblotting with recombinant human tyrosinase	61	Song *et al.* 1994b
Tyrosinase	ELISA with mushroom tyrosinase	38.8	Baharav *et al.* 1996
Tyrosinase	Immunoprecipitation dopa essay/ELISA with human tyrosinase	0	Xie *et al.* 1999
Tyrosinase	Radiobinding assay with recombinant human tyrosinase	10.9	Kemp *et al.* 1997a
TRP-2	ELISA with recombinant human TRP-2	67	Okamoto *et al.* 1998
TRP-2	Radiobinding assay with recombinant human TRP-2	5.9	Kemp *et al.* 1997b
TRP-1	Radiobinding assay with recombinant human TRP-1	5.7	Kemp *et al.* 1998b
Pmel17	Radiobinding assay with recombinant human Pmel17	5.9	Kemp *et al.* 1998c
MelanA/MART1	Radiobinding assay with recombinant MelanA/MART1	0	Waterman *et al.* 2002
MCHR1	Radiobinding assay with recombinant MCHR1	16.4	Kemp *et al.* 2002
SOX9	Immunoprecipitation of recombinant human SOX9	47	Hedstrand *et al.* 2001
SOX10	Immunoprecipitation of recombinant human SOX10	63	Hedstrand *et al.* 2001
40-45 kDa	Immunoprecipitation of human melanocytes	72	Cui *et al.* 1995b
75 kDa	Immunoprecipitation of human melanocytes	76	Cui *et al.* 1995b
90 kDa	Immunoprecipitation of human melanocytes	45	Cui *et al.* 1995b
65 kDa	Immunoprecipitation of human melanocytes	44	Park *et al.* 1996
35 kDa	Immunoprecipitation of human melanocytes	4	Cui *et al.* 1992
150 kDa	Immunoprecipitation of human melanocytes	4	Cui *et al.* 1992
110 kDa	Immunoblotting of melanocytes membrane extract	31	Hann *et al.* 1996
165 kDa	Immunoblotting of human melanoma cells	11	Rocha *et al.* 2000
90 kDa	Immunoblotting of human melanoma cells	26	Rocha *et al.* 2000
68 kDa	Immunoblotting of human melanoma cells	37	Rocha *et al.* 2000

(Adapted from Kemp *et al.* 2001c and Ongenae *et al.* 2003)

types or on infecting micro-organisms may elicit their production. For example, common antigens have been identified in mycobacteria and malignant melanocytes (Bystryn 1997). Alternatively, vitiligo antibodies might result from a secondary immune response to melanocyte antigens following damage to pigment cells by other mechanisms, and these antibodies might further exacerbate the condition. It is presently unknown if humoral immune responses actually damage melanocytes *in vivo* and it is still possible that they play no direct part in vitiligo aetiology. Nonetheless, these antibodies serve as relevant markers to identify potential autoantigens, even if the disease is T cell mediated.

1.5.2.3 Other antibody reactivities

Aside from organ-specific antibodies (Table 1.6), anti-keratinocyte intracellular antibodies that correlate with disease extent and activity have been detected in vitiligo patients (Yu *et al.* 1993) and specific IgA reactivity against human melanoma cells in patients with active vitiligo has been reported (Aronson and Hashimoto 1987). An increased incidence of anti-nuclear, anti-microsomal, and anti-smooth muscle cell antibodies has also been observed in the sera of vitiligo patients (Hann *et al.* 1993a). A significant number of vitiligo patients also have antibodies against compounds containing the benzene ring structure (Wojdani *et al.* 1992; Vojdani and Grimes 1996). These were investigated for immune reactivity because compounds containing the benzene ring, including catechols, phenols, hydroquinone and mono-benzene, can induce cutaneous depigmentation (Section 1.2.5). Although the nature of the action of the anti-benzene ring antibodies is not proven, it has been suggested that exposure to compounds containing this structure may induce aberrant immunological responses in some individuals with vitiligo (Wojdani *et al.* 1992; Vojdani and Grimes 1996). As with melanocyte antibodies, although to a greater degree, it is presently unclear how these antibodies relate temporally and pathogenically to the lesions in vitiligo.

1.6 Melanin-concentrating hormone and melanin-concentrating hormone receptor 1

1.6.1 Melanin-concentrating hormone

Melanin-concentrating hormone (MCH) is a cyclic polypeptide that was first isolated from the pituitary of salmon fish. In teleost fish, MCH circulates and lightens skin colour by stimulating melanosome aggregation within the melanophores (Kawauchi *et al.* 1983). In fish, MCH consists of seventeen amino acids with a cysteine-cysteine disulfide bond, whereas in mammals, it consists of nineteen amino acids and, although it has high homology to salmon MCH, the N-terminus is extended by two additional amino acids, Asp^{1} and Phe^{2} (Pissios and Maratos-Flier 2003). Among mammals, MCH is highly conserved in all species and, indeed, rat MCH is identical to human MCH. Melanin-concentrating hormone is produced from pro-MCH that encodes neuropeptide E-I and neuropeptide G-E, in addition to MCH (Nahon *et al.* 1989). The human MCH gene was assigned to chromosome 12 (Pedeutour *et al.* 1994).

In contrast to the salmon MCH gene that is intronless (Takayama *et al.* 1989), the mammalian gene has two introns and three exons (Nahon 1994). In lower invertebrates, MCH acts to regulate skin colour by antagonising the melanin-dispersing actions of α-MSH (Baker 1988; Baker 1993). In addition, it has been found that MCH evokes an increase in proliferation of the erythrophoroma cell line, GEM-81, a skin tumour cell line developed from teleost fish (Isoldi *et al.* 2004). The physiological roles of MCH in mammals have yet to be fully elucidated but since it is highly expressed in the regulatory centres of the brain, particularly the hypothalamus, and throughout the central and peripheral nervous systems, it is thought to act as a neuromodulator or neurotransmitter mediating a broad range of behavioural responses (Skofitsch *et al.* 1985; Bittencourt *et al.* 1992; Mouri *et al.* 1993; Knigge *et al.* 1996). Most functional studies of MCH have concentrated on its influence on feeding (Qu *et al.* 1996), a role which is highlighted by the lean phenotype created by knocking-out the function of MCH in mice (Segal-Lieberman *et al.* 2003) and by the obese phenotype of mice engineered to over-express MCH (Ludwig *et al.* 2001). Furthermore, a recent study reported the expression of prepro-

MCH by human immune cells such as peripheral blood mononuclear cells, thymocytes and splenocytes (Verlaet *et al.* 2002).

So far, very little is known about the actions of MCH on the regulation of pigmentation in higher vertebrates including humans. Recently, it has been demonstrated that human skin expresses MCH and the hormone inhibits α-MSH-induced melanogenesis in cultured human melanocytes (Hoogduijn *et al.* 2001; Hoogduijn *et al.* 2002). Some of the characterised physiological functions of MCH, with reference to the antagonistic effects of MCH on α-MSH, are shown in Table 1.9.

Several studies have identified a somatostatin-like receptor SLC-1 (Kolakowski *et al.* 1996) as the receptor for MCH, which has been renamed as the melanin-concentrating hormone receptor 1 (MCHR1) (Bachner *et al.* 1999; Chambers *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999) and this is discussed in the Section 1.6.2.

1.6.2 The melanin-concentrating hormone receptor 1

1.6.2.1 Expression of MCHR1

The gene encoding MCHR1 has been localized to chromosome 22 and contains two exons (Kolakowski *et al.* 1996). The receptor is widely and strongly expressed in the brain, major sites of expression being the cerebral cortex, hippocampus, hypothalamus, cerebellum and the olfactory organs (Kolakowski *et al.* 1996; Bachner *et al.* 1999; Chambers *et al.* 1999; Saito *et al.* 1999; Hervieu *et al.* 2000; Kokkotou *et al.* 2001). Moderate expression has also been noted in the eye, skeletal muscle and tongue of the rat, although the function of the receptor at these locations is unknown (Saito *et al.* 1999; Saito *et al.* 2000). In addition, MCHR1 is expressed in human skin, (Hoogduijn *et al.* 2002), melanocytes (Hoogduijn *et al.* 2001; Hoogduijn *et al.* 2002), keratinocytes (Burgaud *et al.* 1997), melanoma cell lines (Hoogduijn *et al.* 2001; Hoogduijn *et al.* 2002), mouse melanoma cell lines (Drozdz *et al.* 1995; Hoogduijn *et al.* 2001; Hoogduijn *et al.* 2002), human immune cells such as peripheral blood mononuclear cells, thymocytes and splenocytes (Verlaet *et al.* 2002).

Table 1.9 Physiological functions influenced by MCH and α-MSH.

Location	Ligand	Receptor	Function	Reference
Fish melanophore	Salmon α-MSH	Not characterised	Pigment dispersion.	Kawauchi *et al.* 1983.
	Salmon MCH	Not characterised	Pigment aggregation.	
Human melanocyte	Human α-MSH	MC-1R	Increases intracellular calcium, Melanogenesis.	Hoogduijn *et al.* 2001.
	Human MCH	MCHR1	Decreases α-MSH stimulated intracellular calcium. Partially inhibits α-MSH induced melanogenesis.	Hoogduijn *et al.* 2002.
Mouse melanoma cells	Rat α-MSH	MC-1R	Melanogenesis.	Ludwig *et al.* 1998.
	Rat MCH	?	No apparent effect on melanogenesis.	
Mammalian brain	Rat α-MSH	MC-4R[1]	Inhibits feeding.	Ludwig *et al.* 1998.
	Rat MCH	MCHR1	Increases feeding.	

The large brackets indicate a functionally antagonistic relationship between MCH and α-MSH.

[1] MC-4R, melanocortin 4 receptor.

1.6.2.2 Structure and function of MCHR1

The melanin-concentrating hormone receptor 1 is a G protein-coupled receptor (GPCR; Bachner *et al.* 1999; Chambers *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999) that specifically binds MCH. The GPCRs are classified in a superfamily of membrane proteins, with a distinguishing arrangement of seven transmembrane (TM) domains, encompassing hundreds of receptors for a diverse range of chemical messengers including hormones and neurotransmitters (Iismaa and Shine 1992; Eckard and Beck-Sickinger 2000). The GPCRs share a common core domain consisting of seven TM helices connected by three intracellular and three extracellular loops, with the N- and C-terminal domains protruding, respectively, on the extracellular and intracellular sides of the membrane. The MCHR1 is 402 amino acids long, with all the hallmark features of a G protein-coupled receptor, including seven TM helices, an Asp-Arg-Phe motif at the end of the third intracellular loop and three potential glycosylation sites at the N-terminus. The receptor shows the highest degree of homology (35%) to the somatostatin receptor family. Comparison of the human and the rodent receptors shows a high degree of conservation between species with 98% identity between rat and mouse and 91% identity between rat and human (Schlumberger *et al.* 2002).

Mutational analysis of several GPCRs has revealed that the extracellular regions and the TM domains contribute to the formation of the ligand-binding site, whereas the intracellular loops interact with G proteins, as well as with other regulatory proteins (Bockaert and Pin 1999; Gether 2000). Biochemical analyses have identified two amino acid residues important for rat MCH binding to the receptor: Asp^{123} in the transmembrane domain (Macdonald *et al.* 2000) and Asn^{23} in the extracellular amino-terminal region (Saito *et al.* 2003). Glycosylation is a common posttranslational feature of the GPCRs superfamily. It has been shown that glycosylation of the receptor may be involved in a variety of biological activities. These activities include maintenance of receptor stability, folding, trafficking of the receptor to the cell surface, ligand binding and signal transduction. In human MCHR1, *N*-linked glycosylation of Asn^{23} is necessary for expression of the receptor on the cell surface, signal transduction and ligand binding (Saito *et al.* 2003).

Binding of agonist to GPCRs results in the activation of one or more class of G proteins that regulate numerous signal transduction pathways (Hawes *et al.* 2000).

G proteins are heterotrimeric proteins composed of α-, β- and γ- subunits and are categorised by the signalling characteristics of the Gα-subunit. Over 20 distinct Gα-subunits are grouped into four classes, G_s, G_i/G_o, G_q and G_{12}. G_s proteins stimulate adenylate cyclase, activate cyclic guanosine monophosphate phosphodiesterase and regulate potassium and calcium channels. G_q proteins activate phospholipase C (PLC), leading to increased production of inositol 1,4,5- triphosphate (IP_3) and diacyl glycerol (DAG) and consequently the activation of protein kinase C (PKC) (Hawes *et al.* 2000). G_{12} proteins regulate sodium/potassium exchange (Hawes *et al.* 2000).

It has been demonstrated that binding of MCH to cells expressing MCHR1 inhibits forskolin-stimulated cyclic AMP production, increases intracellular calcium and IP_3, and activates MAP kinase (Figure 1.9; Chambers *et al.* 1999; Lembo *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999; Hawes *et al.* 2000; Verlaet *et al.* 2002). Studies have been carried out to determine with which G protein class MCHR1 interacts. Pre-treatment of Chinese hamster ovary cells (CHO-K1) expressing MCHR1 with pertussis toxin (PTX), which blocks G_i/G_o protein activity, reverses the MCH-provoked inhibition of cyclic AMP production and indicates that MCHR1 couples to G_i/G_o proteins to mediate the inhibition of adenylate cyclase (Figure 1.9; Hawes *et al.* 2000). Melanin-concentrating hormone also stimulates increased IP_3 production and increased levels of intracellular calcium that are attenuated, but not abolished, by PTX pre-treatment in CHO-MCHR1 cells. These results suggest that multiple G protein types (a PTX-sensitive G_i/G_o-type and a PTX-insensitive G_q-type) are responsible for coupling the MCH receptor to increases in IP_3 production and intracellular calcium (Hawes *et al.* 2000).

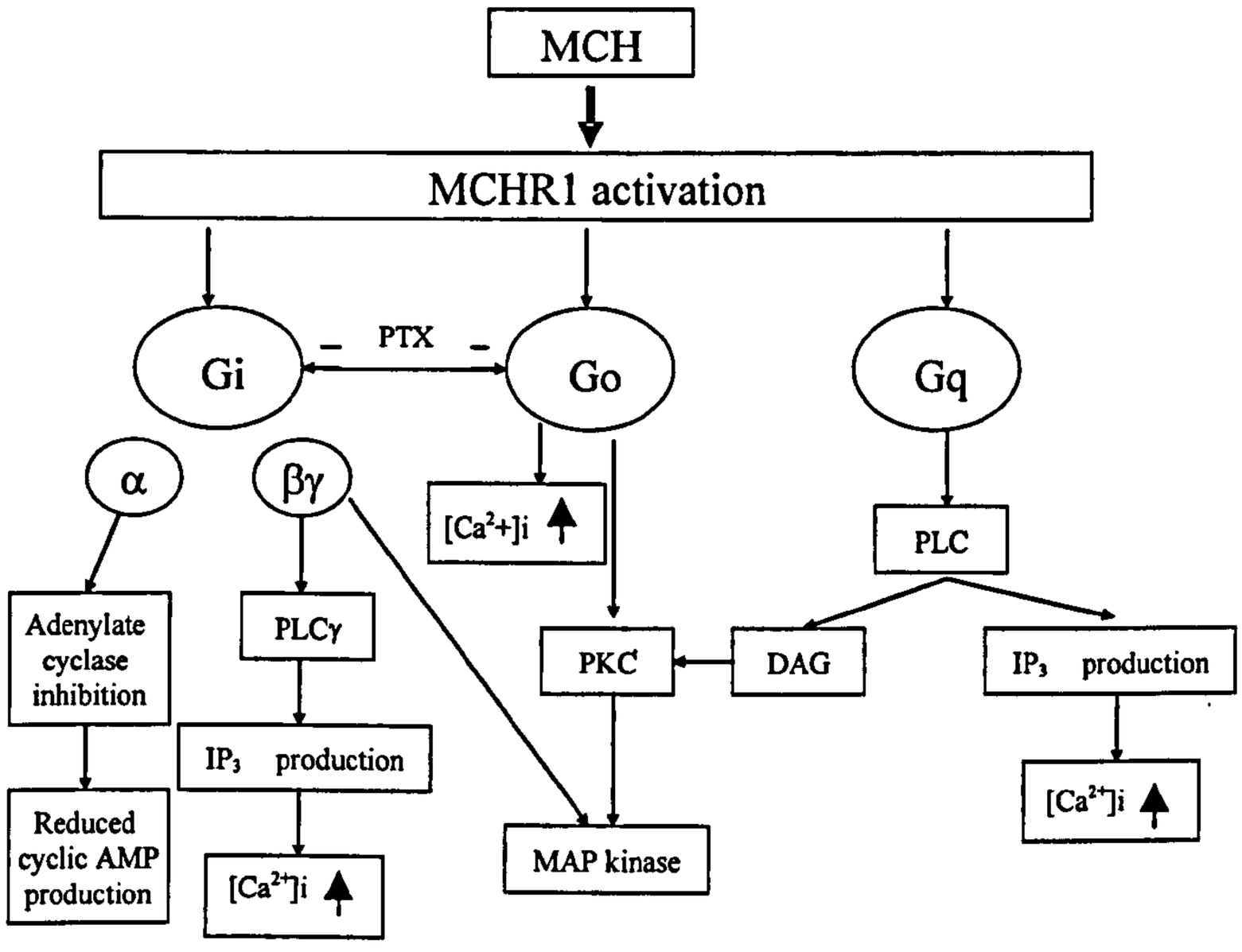


Figure 1.9 A model of the intracellular signalling pathways coupled to MCHR1. The MCHR1 couples to multiple G proteins, including G_i, G_o and G_q. G_i activation results in a decrease in adenylate cyclase activity and reduced cyclic AMP production. The βγ-subunit of G_i mediates a portion of MCH-stimulated MAP kinase activation. In addition, G_i is responsible for mediating a component of MCH-stimulated IP_3 production and increases intracellular calcium levels. G_o mediates a portion of MCH-stimulated MAP kinase activation and might also mediate increases intracellular calcium levels. G_q mediates one component of MCH-stimulated IP_3 production and increases intracellular calcium levels. Pertussis toxin inhibits G_i and G_o activity. $[Ca^{2+}]_i$, intracellular calcium.

(Adapted from Hawes *et al.* 2000)

1.6.2.3 The melanin-concentrating hormone receptor as an autoantigen in vitiligo

Using IgG from vitiligo patients in biopanning experiments with a melanocyte cDNA phage-display library, MCHR1 was identified as a novel autoantigen recognised by autoantibodies in vitiligo (Kemp *et al.* 2002). Antibodies against the receptor were demonstrated in 9/55 (16.4%) of the vitiligo patient sera tested in radiobinding assays and were at a significantly increased frequency compared with a control population (p=0.025). Among sera from healthy controls and from patients with other autoimmune disease, SLE, none exhibited immunoreactivity to MCHR1, indicating a high degree of disease specificity for antibodies against the receptor. Although the frequency of MCHR1 antibodies appears low, it is possible that some receptor antibodies were not detected in the radiobinding assay since recombinant *in vitro* translated MCHR1 was used as the radioactive ligand. Indeed, the incidence of MCHR1 antibodies may be increased if native receptor was employed in an assay to detect MCHR1 antibodies that recognise conformational epitopes.

As discussed (Section 1.6.1), the effect of MCH on pigmentation has not been fully elucidated in mammalian systems. Initial studies have shown that the stimulation of cultured human melanocytes with MCH reduces the α-MSH-induced increase in cyclic AMP production via binding to MCHR1 (Hoogduijn *et al.* 2002). Furthermore, the melanogenic actions of α-MSH were inhibited by the interaction of MCH with its receptor (Hoogduijn *et al.* 2002). In conclusion, the authors proposed that the MCH/MCHR1 signalling system might have a role, along with the melanocortins, in regulating the physiological reactivities of melanocytes, including melanin synthesis. As MCHR1 is expressed on the cell surface, it could be accessible to autoantibodies that might adversely affect the functioning of the receptor, leading to the disruption of normal melanocyte behaviour. Interestingly, IgG from MCHR1 antibody-positive vitiligo patients inhibited the binding of ^{125}I-MCH to membranes of CHO-K1 cells expressing the receptor (Kemp *et al.* 2002). However, the effects of MCHR1 antibodies on the functioning of the receptor and on normal melanocyte physiology as well as their possible relationship to the aetiology of vitiligo have yet to be established.

1.7 Aims of this study

Although both aberrant cellular and humoral immune responses have been reported in vitiligo patients (Section 1.5), the exact contribution of these to the pathogenesis of the disorder have yet to be fully elucidated. However, characterising the autoimmune response in vitiligo has several potential clinical implications and applications. It is possible that analysing the abnormal immune response in vitiligo might lead to the development of more effective treatments, as well as to a greater understanding of the action of current immunosuppressive treatments, such as PUVA, which are effective in some cases of vitiligo. If autoimmune responses could be identified routinely, more appropriate treatments could be applied to individual patients. It might also be possible to establish whether particular clinical subtypes of vitiligo do indeed have separate disease aetiologies.

Part of the elucidation of the autoimmune response in vitiligo is the identification and characterisation of melanocyte autoantigens that are recognised by autoantibodies and/or autoreactive T cells in vitiligo patients. In the long term, this might be of use in the diagnosis of the disorder and in the development of markers for disease activity and progression. With respect to this, the aim of this study was to characterise autoantibodies to MCHR1, an autoantigen in vitiligo. The specific aims were:

1) To identify B cell epitopes on the MCHR1 using recombinant protein and phage-display technology.

2) To isolate a stable Chinese hamster ovary (CHO-K1) cell line expressing MCHR1.

3) To examine the effects of MCHR1 autoantibodies on the functioning of the receptor by using fluorimetry to measure changes in intracellular calcium levels in response to MCH-stimulation in a CHO-K1 cell line expressing MCHR1.

4) To investigate complement fixation activity and ADCC with respect to MCHR1 autoantibodies.

2 General Materials and Methods

2.1 Patient sera

Sera from patients with vitiligo and other autoimmune diseases were collected from dermatology and endocrinology clinics at the Royal Hallamshire Hospital, Sheffield, UK, and the Northern General Hospital, Sheffield, UK, respectively, between 1990-2004. Patient details are given in Table 2.1. Control sera were also obtained from healthy laboratory volunteers with no personal or family history of vitiligo or of autoimmune disorders their details are given in the relevant chapters. The details of other patient groups are also summarised in the relevant chapters. Serum was separated from whole blood samples (10-20 ml) by centrifugation in a Sorval® RT6000-D centrifuge at 2800 revolutions per minute (rpm) for 20 minutes. Sera were kept frozen at –20°C until use. All work was approved by the Ethics Committee of the Northern General Hospital NHS Trust, Sheffield, UK and all subjects gave informed consent.

2.2 Chemicals

The majority of chemicals were purchased from either Sigma (Poole, UK) or BDH (Poole, UK), and were of either molecular biology grade or 'AnalR' grade, respectively. The source of some chemicals is indicated in the text where appropriate.

2.3 Media

The media used in this study are given below. All media were sterilised either by autoclaving or by filtration using Millipore GS disposable filters (Millipore Corp., Bedford, MA, USA).

Table 2.1 Details of the vitiligo patients used in this study

Patient	Sex[1]	Age[2] (years)	Vitiligo Type	Autoimmune Disease	Antibodies to MCHR1[3]	Assay Used in this Study[4]
V1	M	75	Symmetrical	None	Yes	1,3,4,5
V2	M	27	Symmetrical	None	Yes	1,2,3,4,5
V3	F	50	Symmetrical	None	Yes	1,2,3,4,5
V4	M	23	Symmetrical	None	Yes	1,3,4,5
V5	M	64	Peri-orificial and symmetrical	None	Yes	1,3,4,5
V6	M	50	Peri-orificial and segmental	Thyroid disease	Yes	1,3,4,5
V7	F	42	Symmetrical	None	Yes	1,2,3,4,5
V8	F	31	Peri-orificial	None	Yes	1,3,4,5
V9	F	61	Symmetrical	Thyroid disease	Yes	1,3,4,5
V10	M	27	Symmetrical	None	No	1,3,4,5
V11	F	77	Segmental	Thyroid disease	No	1,3,4,5
V12	F	45	Symmetrical	None	No	1,3,4,5
V13	M	31	Symmetrical	None	No	1,3,4
V14	F	53	Symmetrical	Thyroid disease	No	1,3,4
V15	F	28	Symmetrical	None	No	1,3,4
V16	M	29	Peri-orificial	None	No	1,3,4
V17	F	56	Symmetrical	None	No	1,3,4
V18	F	60	Symmetrical	None	No	1,3,4
V19	F	62	Symmetrical	None	No	1

[1]M, male; F, female.
[2]Age of patient at time that serum sample was taken for analysis.
[3]MCHR1 antibodies were reported in the study of Kemp *et al.* 2002.
[4]1, Radio-binding assay; 2, Phage-display assay; 3, Functional blocking assay; 4, Complement fixation assay; 5, Antibody-dependent cell-mediated cytotoxicity assay.

2.3.1 Luria Bertani medium and agar

Luria Bertani (LB) medium was prepared in double distilled water and contained: 1% (w/v) Bacto-tryptone (Sigma); 0.5% (w/v) yeast extract (Sigma); 0.5% (w/v) NaCl. Luria Bertani agar was made by adding 1.5% (w/v) Bacto-agar (Sigma) to LB medium. After sterilisation, the agar was left to cool to 40-45°C before the addition of appropriate antibiotics. The agar was then poured into 90-mm petri-dishes, allowed to set and the plates stored at 4°C until required.

2.4 Antibiotics

Antibiotics (Sigma) were prepared as 100x concentrated stocks in double-distilled water, sterilised by filtration with a Millex® Filter Unit (Millipore Corp.) and used in medium at either the final concentration shown below or as stated in the text. Tetracycline was dissolved in 50% (v/v) ethanol/H_2O. The stocks were stored at -20°C.

	Final concentration in medium (μg/ml):
Ampicillin (Sodium salt)	100
Kanamycin (Monosulfate)	12
Tetracycline (Hydrochloride)	10

2.5 Bacterial strains

The bacterial strains used in this study were derivatives of *Escherichia coli* K-12 and were strains JM109 (Promega, Southampton, UK; Yanisch-Perron *et al.* 1985) and XL-1 Blue (Tetracycline resistant; Stratagene, La Jolla, CA, USA; Bullock *et al.* 1987). Derivatives of strains carrying various plasmids were constructed by transformation (Section 2.14) and are defined in the text.

2.6 Plasmids

The plasmids used in this study are listed in Table 2.2. They were kept at -20°C in sterile TE buffer (10 mM Tris-HCl; 1 mM ethylenediaminetetraacetic acid (EDTA); pH 8.0). Maps of the plasmid vectors used in DNA cloning experiments are given in the relevant chapters.

2.7 Growth and maintenance of bacterial strains

When first obtained, strains were checked for the presence of relevant antibiotic markers by streaking onto selective LB agar. All *E. coli* strains were routinely grown from single colonies on LB agar plates in LB medium, with the appropriate antibiotics, at 37°C in a rotary incubator shaking at 200 rpm.

For storage, bacterial strains were streaked onto LB agar plates, containing appropriate antibiotics, and incubated overnight at 37°C and then placed at 4°C for up to one month. Additionally, 10 ml bacterial cultures were grown overnight at 37°C and then mixed with an equal volume of 50% (v/v) sterile glycerol/H_2O for long-term storage at –20°C.

2.8 Small-scale plasmid preparations

The Wizard™ Minipreps DNA Purification System (Promega) was used to purify plasmid DNA from a 5-10 ml overnight culture of a required bacterial strain, according to the manufacturer's protocol. Briefly, single colonies of the desired bacterial strain were isolated by streaking out 20 µl of frozen bacterial stock onto LB agar containing the appropriate antibiotic. A single colony from a selective plate was then used to inoculate 10 ml of LB containing the relevant antibiotic. This culture was shaken in a rotary incubator at 200 rpm at 37°C overnight. A bacterial cell pellet was obtained by centrifugation at 10,000 *g* for 10 minutes. The pellet was resuspended in 300 µl of Wizard™ resuspension solution (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A), and the cells were lysed by addition of 300 µl of Wizard™ cell lysis solution (0.2 M NaOH; 1% (w/v) sodium dodecyl sulphate (SDS)) and then neutralised by addition of 300 µl of Wizard™ neutralisation buffer

Table 2.2 Plasmids used in this study.

Plasmid	Characteristics	Source	Reference
pcMCHR1	pcDNA3 with a 1.3 kb *Eco*RI-*Xba*I insert of MCHR1 cDNA. Contains promoters for T7 and SP6 polymerases flanking a diverse multiple cloning site. (Amp^R)[1].	Dr. E.H. Kemp, Division of Clinical Sciences (North), University of Sheffield, Sheffield, UK	Kemp *et al.* 2002
pcDNA3	Multicopy 5.4 kb expression vector. Contains promoters for T7 and SP6 polymerases flanking a diverse multiple cloning site. (Amp^R).	Invitrogen (Paisley, UK)	Akrigg *et al.* 1985 Boshart *et al.* 1985
pComb3	Multicopy 4.0 kb expression vector with *Eco*RV insert of MCHR1 cDNA fragments. (Amp^R).	Prof. C.F. Barbas, The Scripps Research Institute, La Jolla, CA, USA	Barbas *et al.* 1991

[1]Amp^R, ampicillin resistance.

(1.32 M potassium acetate). The resulting solution was mixed gently and centrifuged at 10,000 *g* for 10 minutes at room temperature. The clear lysate was then mixed with 1 ml of Wizard™ resin and loaded on to a Wizard™ minicolumn via a 2 ml syringe, followed by washing with 2 ml of Wizard™ column wash solution (80 mM potassium acetate; 8.3 mM Tris-HCl, pH 7.5; 40 μM EDTA; 55% (v/v) ethanol). The Wizard™ minicolumn was centrifuged at 10,000 *g* for 2 minutes to remove excess column wash solution prior to DNA elution. Fifty microlitres of TE buffer, heated to 65°C, was subsequently added to the column, left for 1 minute and then centrifuged at 10,000 *g* for 30 seconds. The concentration of the DNA was determined by spectrophotometry at 260 nm.

2.9 Large-scale plasmid preparations

A large-scale overnight culture of a bacterial strain carrying the desired plasmid was prepared by inoculation of 0.5-1 L of LB, containing relevant antibiotics, followed by incubation of the culture with shaking at 37°C. The culture was then centrifuged at 5000 *g* (Sorval® RC-3B) for 30 minutes and plasmid extracted from the cell pellet using a Qiagen Plasmid DNA Maxiprep Kit (Qiagen Ltd., Crawley, UK) as per the kit instructions. The bacterial cell pellet was first resuspended in 10 ml of buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 μg/ml RNase A). An equal volume of buffer P2 (0.2 M NaOH; 1% (w/v) SDS) was added to the resuspended cells and mixed by gentle inversion, followed by incubation at room temperature for 5 minutes. The tube was then mixed gently again after the addition of 10 ml of buffer P3 (1.32 M potassium acetate, pH 5.5) and incubated on ice for 20 minutes. The mixture was subsequently centrifuged at 20,000 *g* for 30 minutes at 4°C resulting in a clear supernatant. A Qiagen column was equilibrated by adding buffer QBT (750 mM NaCl; 50 mM 3-[N-morpholino] propanesulphonic acid (MOPS), pH 7.0; 15% (v/v) isopropanol; 0.15% (v/v) Triton X100) and was allowed to empty by gravity flow. Clear supernatant was then loaded onto the column and left to flow through, followed by washing of the column twice with 30 ml of QC buffer (1 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol). Plasmid DNA was subsequently eluted with 15 ml of buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% (v/v) isopropanol) and precipitated by the addition of 10.5 ml of isopropanol and centrifugation at 15,000 *g* for 30 minutes at 4°C. The resulting DNA

pellet was washed with 70% (v/v) ethanol, centrifuged at 15,000 *g* for 10 minutes at 4°C and finally resuspended in 300 µl of TE buffer that had been pre-heated to 65°C. The concentration of the DNA was ascertained by spectrophotometry at 260 nm.

2.10 Restriction enzyme digestion

All restriction endonucleases and restriction endonuclease reaction buffers were supplied by Promega. Restriction enzyme digests of plasmid and polymerase chain reaction (PCR) amplified products were carried out in a volume not normally exceeding 25 µl, containing 10 U of enzyme(s) and 0.1 volumes of an appropriate 10x concentration restriction buffer. Each reaction proceeded for 2 hours at 37°C. A typical reaction comprised:

1 µl 1 µg/µl DNA
16 µl sterile distilled H_2O
2 µl 10x reaction buffer
1 µl 10 U/µl restriction endonuclease

2.11 Agarose gel electrophoresis

Agarose gels, 0.8-1% (w/v), were prepared by boiling molecular biology grade agarose (Sigma) in TAE electrophoresis buffer (40 mM Tris-base; 0.1% (v/v) glacial acetic acid; 100 mM EDTA, pH 8.0) for 1-2 minutes in a microwave oven. One microlitre of ethidium bromide (10 mg/ml) was added for every 50 ml of the gel solution. The molten agarose was cooled and poured into the casting deck of a dedicated gel electrophoresis apparatus. After it had set, the combs were removed, and the electrophoresis tank was filled with TAE as a running buffer. A loading dye (6x concentration contained: 0.25% (w/v) bromophenol blue; 40% (w/v) sucrose) was added to the DNA, acquiring approximately $1/6^{th}$ of the volume to be loaded, and this was then pipetted into the gel slots. A 'marker' lane was always included which either contained *Hin*dIII-restricted bacteriophage λ DNA (Promega) or 100 base pair DNA ladder (Promega), with which to size the DNA products after they had migrated through the gel. The gels were run at 100 volts (V) and subsequently viewed using an UV transilluminator.

2.12 Preparation of DNA fragments from agarose gels

When electrophoresis was performed to purify a particular DNA fragment, either from a PCR or a restriction digest, the DNA fragment of interest was recovered from the gel using a Wizard™ PCR Prep DNA Purification Kit (Promega). Briefly, the area of the gel containing the relevant piece of DNA was excised using a clean scalpel and placed in a 1.5 ml eppendorf tube. One millilitre of purification resin was used to dissolve the gel slice and the resulting mixture was applied via a 2 ml syringe to a Wizard™ minicolumn followed by washing with 2 ml of 80% (v/v) isopropanol. The Wizard™ minicolumn was centrifuged at 10,000 *g* for 2 minutes, to remove excess isopropanol, prior to DNA elution with 30 µl of sterile TE buffer, which had been pre-heated to 65°C.

2.13 DNA ligations

Ligation of vector and DNA fragments was performed using T4 DNA ligase (Promega). An estimation of the concentration of each vector and insert was performed by agarose gel electrophoresis next to base pairs (bp) markers of a known concentration. Approximately 200 ng of vector DNA were mixed together with insert (the amount of which to be added was calculated using a molar ratio, vector: insert, of between 1 and 3), and sterile H_2O added to a final volume of 18 µl in a clean 0.5 ml eppendorf tube. The mixture was heated to 65°C and gradually cooled to 16°C in a thermal cycler block, to allow DNA to anneal slowly, before addition of 1 µl of T4 DNA ligase enzyme (Promega) and 2 µl of 10x ligase buffer (300 mM Tris-HCl, pH 7.8; 100 mM $MgCl_2$; 100 mM dithiothreitol (DTT); 10 mM ATP; Promega). The reaction was subsequently incubated at 16°C overnight.

2.14 Bacterial transformation

2.14.1 Transformation of chemically-competent bacterial cells

When required, a 100-µl aliquot of competent *E. coli* JM109 (Promega) cells was thawed. The appropriate DNA sample, usually 0.5-1 µg, was gently mixed with the cells and this was incubated on ice for 10 minutes. The cells were then heat shocked

at exactly 42°C for 45 seconds and returned to ice. After 2 minutes, the cells were transferred to a culture tube containing 990 µl of chilled SOC medium (Invitrogen, Paisley, UK) which comprises: 2% (w/v) tryptone; 0.5% (w/v) yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose. The culture tube was placed in a shaking incubator at 37°C and grown for 1 hour, to allow expression of the antibiotic resistance genes carried by the transforming plasmid DNA. A 100-µl aliquot of undiluted transformed cells, and of 1:10 and 1:100 dilutions, were then spread onto LB agar plates containing the appropriate antibiotic, and incubated at 37°C overnight. A proportion of the colony forming units were then picked with a sterile pipette tip and streaked on to fresh LB agar plates for growth overnight at 37°C.

2.14.2 Transformation of electrocompetent bacterial cells

A 50-µl aliquot of electrocompetent *E. coli* XL-1 Blue cells (Stratagene) were thawed on ice and then added to a pre-chilled 0.1 cm cuvette (Invitrogen). The appropriate DNA sample, usually 0.5-1 µg, was added, mixed by pipetting, and the cuvette placed into an *E. coli* Pulser electroporator (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and a charge of 1800 V applied. The cuvette was removed from the electroporator and the cells resupended in 500 µl of super optimal catabolite (SOC) medium and then incubated with shaking at 37°C for 1 hour. A 100-µl aliquot of undiluted transformed cells, and of 1:10 and 1:100 dilutions, were spread onto LB agar plates containing appropriate antibiotics, and incubated at 37°C overnight. A proportion of the colony forming units were then picked with a sterile pipette tip and streaked on to fresh LB agar plates for growth overnight at 37°C.

2.15 Polymerase chain reaction amplification

Preceding the PCR amplification, oligonucleotide primers were appropriately designed to amplify the relevant DNA fragment (details are given where appropriate in the text). Reactions were carried out in 50-µl volumes comprising, unless indicated, 50 ng of template DNA, 0.3 mM of each required (forward and reverse) primer, 1 U of Expand™ High Fidelity Taq polymerase (Roche Diagnostics Ltd., Lewes, UK), 0.1 mM deoxynucleotides (Promega), in buffer containing: 1.5 mM

$MgCl_2$; 10 mM Tris-HCl (pH 8.3); 0.01% gelatin; 50 mM KCl; 0.1% Tween 20; 0.1% Nonidet P-40 (Roche Diagnostics Ltd.). Each reaction was overlaid with a drop of mineral oil to prevent evaporation during heating and was subject to PCR amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT, USA) using the conditions stated in the text.

2.16 Direct purification of PCR products

Polymerase chain reaction amplification products were directly extracted from the PCR constituents using a Wizard™ PCR Prep DNA Purification Kit (Promega). Briefly, one or more PCR reactions assuming a volume of no more than 300 µl, were mixed with 100 µl of Wizard™ direct purification buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 0.1% (v/v) Triton X100). One millilitre of Wizard™ PCR Prep DNA Purification resin was added and the mixture vortexed briefly three times during a period of 1 minute. The mixture was then applied to a Wizard™ minicolumn, which was treated as previously described in Section 2.12.

2.17 DNA sequencing

2.17.1 Manual DNA sequencing

Sequencing of plasmid DNA was performed using a T7 Sequenase® Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech, Little Chalfont, UK) by the dideoxy-chain termination method (Sanger *et al.* 1977) using the appropriate oligonucleotide primer and [α-^{35}S]dATP (NEN Life Science Products, Hounslow, UK), as outlined below.

One-to-three micrograms of plasmid DNA in a volume of 10 µl of TE were denatured by incubation at 37°C with 1 µl of 2 mM EDTA (pH 8.0) and 1 µl of 2 M NaOH for 30 minutes. One microlitre of 3 M sodium acetate (pH 5.2) and 50 µl of 100% ethanol were added and the DNA was precipitated at –20°C for 1-2 hours. A pellet was collected by centrifugation at 12,000 *g* for 15 minutes and was air-dried, before being resuspended in 10 µl of diluted sequencing primer mixture containing 7 µl sterile distilled H_2O, 2 µl of T7 Sequenase® reaction buffer and 1-5 pmoles of the relevant primer (details are given where appropriate in text). The reaction was

then heated to 65°C for 2 minutes, to allow annealing of the primer with its target sequence, prior to slow cooling to 0°C.

Two-point-five microlitres of each of four termination mixes (ddGTP, ddATP, ddTTP, ddCTP) were aliquoted into separate tubes and heated to 37°C. Termination mixes contained: 80 μM of each dNTP; 8 μM of the relevant ddNTP. To the annealed DNA, 1 μl DTT, 2 μl of diluted labelling mix (5x concentration contained: 7.5 μM dGTP; 7.5 μM dTTP; 7.5 μM dCTP; diluted to 1x with sterile H_2O), 1 μl [α-^{35}S]dATP (1250 Ci/mmol; 12.5 mCi/ml; NEN Life Science Products) and 2 μl diluted T7 Sequenase® polymerase (diluted 1:8 with enzyme dilution buffer) were added. This was incubated at room temperature for 2 minutes before 3.5 μl was removed from the reaction into each of the four tubes containing dideoxynucleotides. These were then incubated at 37°C for five minutes after which 4 μl of stop solution were added. The tubes were subsequently heated at 85°C to denature the DNA and then applied to sequencing gels for electrophoresis as described in the Section 2.18.

2.17.2 Automated DNA sequencing

Automated DNA sequencing was carried out by the genomic facility of the Division of Genomic Medicine at the University of Sheffield, Sheffield, UK and also at the Department of Biological Sciences at University of Durham, Durham, UK. Sequencing reactions were performed using the ABI PRISM BigDyeTM Terminators Version 3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Generally, 1-2 μg of the DNA samples (plasmid, phagemid or PCR product) and 3.2 pmoles of the relevant primers were sent to the automated DNA sequencing facility. The facility performed cycle sequencing reactions using fluorescent dye terminators, ran the gel, acquired the data, and provided the sequence as chromatogram and text files. DNA sequence homology searches against the GenBank database were performed using the BLAST service of the National Centre for Biotechnology Information (Bethesda, MD, USA).

2.18 Sequence gels and autoradiography

The sequencing reaction products were resolved on 6% (w/v) polyacrylamide gels containing: 7 mM urea; 89 mM Tris-base; 2 mM EDTA (pH 8.0); 89 mM boric acid; 0.03% (w/v) ammonium persulphate; 0.07% (v/v) of N, N, N, N'-tetramethylethylenediamine (TEMED). A standard non-gradient sequencing gel apparatus, (Bio-Rad Laboratories Ltd.), was used in the set up. The gel running buffer contained: 89 mM Tris-base; 2 mM EDTA (pH 8.0); 89 mM boric acid. The gel and buffer were warmed to 50°C prior to electrophoresis by running at 2000 V for one hour. Gel slots were flushed with buffer to remove any urea and unpolymerised acrylamide. DNA sequence reactions were then loaded. Gels were run at a voltage of approximately 1800 V for 2 to 4 hours at 50°C depending on the length of the sequence to be studied. After the reactions had run to the desired extent, the current was turned off, apparatus dismantled and the gel fixed in 12% (v/v) methanol, 10% (v/v) glacial acetic acid. The gel was then transferred to a sheet of 3MM blotting paper (Whatman International Ltd., Maidstone, UK) and dried on a Bio-Rad Gel Dryer 583 (Bio-Rad Laboratories Ltd.) at 80°C for 2 hours.

The dried gel was subjected to autoradiography by exposure to Fuji RX X-ray film (Genetic Research Instrumentation Ltd., Dunmow, UK) in a Hypercassette™ (Amersham Pharmacia Biotech) at room temperature for 24 hours. The film was subsequently developed using Photosol CD18 X-ray developer (Photosol Ltd., Basildon, UK) for 3 minutes, rinsed in water and then fixed for 3 minutes in Photosol CF40 fixer (Photosol Ltd.).

2.19 SDS-PAGE and autoradiography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was performed in 12.5% (w/v), or 15% (w/v) SDS-polyacrylamide resolving gels. The exact constitution of gels and associated buffers is shown in Table 2.3. The percentage acrylamide of the gel varied according to the size of the protein products to be electrophoresed. The gels were created using a Bio-Rad Protean II apparatus (Bio-Rad Laboratories Ltd.).

Table 2.3 Constitution of SDS-PAGE gels.

Resolving gel:

Constituent	Final concentration in gel
Buffer A:	
Tris-base	0.4 M
SDS	0.1% (w/v)
Acrylamide: bisacrylamide (37.5: 1) (Bio-Rad Laboratories Ltd.)	12.5% (w/v) or 15 % (w/v)
Ammonium persulphate	0.04% (w/v)
TEMED	0.0004% (v/v)

Stacking gel:

Constituent	Final concentration in gel
Buffer B:	
Tris-base	0.125 M
SDS	0.1% (w/v)
Acrylamide: bisacrylamide (37.5: 1)	4% (w/v)
Ammonium persulphate	0.05% (w/v)
TEMED	0.075% (v/v)

Briefly, two glass plates (12 cm^2 and 12 x 16 cm) and 1 mm spacers were assembled using the dedicated equipment according to the manufacturer's instructions. The resolving gel solution was poured into the space between the plates, (to a level approximately 5 cm from the top of the smaller plate), and overlaid with 1 ml isopropyl alcohol. After the gel mixture had polymerised the isopropyl alcohol was poured off, and a comb was inserted before the solution for the stacking gel was poured in on top. Subsequently, the comb was removed and the full apparatus assembled. Laemmlli buffer (250 mM Tris-base; 0.1% (w/v) SDS, 0.2 M glycine) was poured into the tank until the bottom of the plates was covered and the top reservoir was full.

Prior to loading, samples to be analysed were mixed with 2x SDS sample buffer (4% (w/v) SDS; 20% (v/v) glycerol; 0.002% (w/v) bromophenol blue; 2% (v/v) 2-mercaptoethanol; 25% (v/v) buffer B as detailed in Table 2.3) and heated at 85°C for 5 minutes. Protein molecular weight standards (Bio-Rad Laboratories Ltd.) used were: bovine serum albumin, 77 kilodaltons (kDa); ovalbumin, 50 kDa; carbonic anhydrase, 35 kDa; soybean trypsin inhibitor, 29 kDa; lysozyme, 21 kDa. Gels were run at a current of 35 milli-amperes (mA) for 3-5 hours or until the visible dye-front had reached the bottom of the plates. The apparatus was subsequently dismantled and the gel transferred to a plastic tray and covered with Coomassie® Blue stain containing: 0.05% (w/v) Coomassie® Brilliant Blue (Bio-Rad Laboratories Ltd.); 10% (v/v) glacial acetic acid; 25% (v/v) isopropanol. This was placed on a rocking platform. After a minimum of 30 minutes staining, the gel was destained by repeated fresh additions of a solution containing: 10% (v/v) glacial acetic acid; 25% (v/v) isopropanol. The destain was discarded and the gel was soaked for a further 30 minutes in Amplify fluorographic reagent (Amersham Pharmacia Biotech), before being dried for 2 hours at 60°C onto 3MM Whatman paper. Gels were exposed to Fuji RX X-ray film at –70°C for the required time (usually overnight) and then developed as in Section 2.18.

2.20 Chinese hamster ovary (CHO-K1) cell culture

All cell culture media and reagents were purchased from Invitrogen, unless otherwise stated. Chinese hamster ovary (CHO-K1) cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). CHO-K1 cells were routinely grown in culture media containing Nutrient Mixture F-12 (Ham) supplemented with 10% foetal bovine serum (FBS; Bio West Ltd., Ringmer, UK), 2 mM L-glutamine, 100 U/ml pencillin G (sodium salt), 100 µg/ml streptomycin sulphate and 1.25 µg/ml fungizone (amphotericin) in T75 tissue culture flasks (Nalge Nunc International, Rochester, NY, USA) in a 5% CO_2 incubator at 37°C. Transfected CHO-K1 cell lines were cultured in media containing 1 mg/ml geneticin sulphate (G-418; Invitrogen).

Cells were passaged when 80-90% confluent, usually every 2-3 days. At each time of passage, the CHO-K1 cells were washed once with phosphate-buffered saline (PBS; 0.01 M phosphate buffer; 0.0027 M KCl; 0.137 M NaCl; pH 7.4; Oxoid, Basingstoke, UK) and then incubated in a solution containing 0.05% trypsin and 0.53 mM EDTA at 37°C in a 5% CO_2 incubator for 5 minutes. After incubation, the cells were resuspended in a culture media and centrifuged at 1000 *g* for 5 minutes. The cell pellet was resuspended in culture media and the cells plated at a 1:10 dilution in T75 tissue culture flasks.

For long-term maintenance of CHO-K1 cells, cells were frozen and stored in liquid nitrogen. Cells were grown to 80-90% confluence and then the cells washed once with PBS and incubated with trypsin/EDTA at 37°C for 5 minutes in a 5% CO_2 incubator. The cells were then centrifuged in culture media at 1000 *g* for 5 minutes and the pellet resuspended in 1 ml of freezing mixture containing FBS and dimethyl sulfoxide (Fisher Scientific UK Ltd., Loughborough, UK) at a ratio of 9:1. The cells were then transferred to cryogen vials (Nalge Nunc International) and stored at –80°C for 24 hours. The cells were then transferred to liquid nitrogen until required.

3 B Cell Epitope Mapping of the Melanin-Concentrating Hormone Receptor 1 Using Recombinant Protein

3.1 Introduction

3.1.1 Antibody-antigen interaction

In order for binding to take place between an antibody and its antigen, there must be complementarity between surface structures on the antigen and the combining site of the antibody. The precise region of the antibody's combining site which contacts with antigen is called the paratope and the part of the antigen recognised by the paratope is termed the epitope. Antigens are highly diverse in size and in primary sequence, as well as in their secondary structure. Consequently, antigenic epitopes are extremely varied and are usually classified as either continuous or discontinuous. An epitope that constitutes part of a linear amino acid sequence on a polypeptide chain is known as a continuous or linear epitope. An epitope that is formed by two stretches of the primary sequence that are distant from each other but are brought together in the folded molecule's secondary or tertiary structure is known as a discontinuous or conformational epitope (Westwood and Hay 2001). The size of a linear epitope can range in length from 5-6 amino acids (Roitt and Delves 2001) up to 15-22 amino acids (von Mikecz *et al.* 1995). Conformational epitopes can consist of approximately discontinuous 16 amino acid side-chains (Roitt and Delves 2001).

For binding of an antibody to an antigen to occur, the specific epitope must be exposed on the surface of the antigen (Roitt and Delves 2001). There are a number of criteria that might, therefore, determine which areas of an antigen can act as antibody binding sites including:

1. The mobility of the region of the protein. X-ray crystallography and nuclear magnetic resonance techniques may indicate surface amino acid residues with higher mobility that are more likely to act as an epitope than areas on the molecule where there are rigid structures such as α-helices and β-sheets (Westwood and Hay 2001).

2. The nature of the primary sequence, in the case of protein antigens. For example, where there is the potential to form a loop or a turn, e.g., in the presence of proline residues, amino acid residues can be exposed for antibody binding (Westwood and Hay 2001).

3. The hydrophilicity of the protein sequence. Generally, the hydrophobic stretches of sequence will reside in the interior of the molecule, with the hydrophilic stretches found on the surface. Therefore, highly hydrophilic sequences are likely to be on the surface of a molecular fold and thus be putative epitopes (Westwood and Hay 2001).

Epitope mapping is the identification and localisation of the specific regions of protein molecules that are recognised by the immune system (Roitt and Delves 2001). It is possible to predict areas of probable antigenicity on a particular protein (Parker *et al.* 1986). However, in order to determine the exact location of epitopes on an antigen, experimental epitope mapping techniques need to be applied.

3.1.2 Methods of B cell epitope mapping

Several epitope mapping strategies for the identification of autoepitopes have been used (Table 3.1). The most precise available method of defining the area of an antigen making contact with an antibody is X-ray crystallography of the antigen-antibody complex. This technique has the advantage of allowing the study of the three-dimensional structures and even can provide information about conformational epitopes. However, it is costly and requires sufficiently large quantities of a pure monoclonal antibody and its antigen to produce crystal. If the primary amino acid sequence of the antigen is known, there are two main approaches to B cell epitope mapping. The first method relies on molecular manipulation at the cDNA level using techniques such as polymerase chain reaction amplification, site-directed mutagenesis, and restriction with DNA exonucleases and endonucleases. Substitutions and/or deletions are created in the amino acid sequence followed by comparative analysis of the immunoreactivity of these recombinant proteins either by immunoblotting, immunoprecipitation or enzyme-linked immunosorbent assay

Table 3.1 A summary of techniques for mapping linear and conformational B cell epitopes.

Technique	Suitable for which type/s of epitope	Reference
X-RAY CRYSTALLOGRAPHY[1]	Conformational and linear	Amit *et al.* 1986
PHAGE-DISPLAY[2] i) Random-peptide library	Conformational and linear	Williams *et al.* 2001
ii) Single chain Fv[3] fragment (scFv) library		
SYNTHETIC PEPTIDES[4] i) Peptide synthesiser (Generates peptides >10 amino acid residues)	Mostly linear	Petterson 1992
ii) 'PEPSCAN'[5] (Peptides ≤ 10 amino acid residues)		Sumar 2001
PEPTIDE FRAGMENTS[6] Derived enzymatically from the whole protein	Mostly linear	Petterson 1992
RECOMBINANT PROTEIN[7] Subcloning and expression[8] of cDNA encoding peptide fragments generated by:	Mostly linear	Petterson 1992
i) Naturally occurring restriction sites		Petterson 1992
ii) Exonuclease digestion		Petterson 1992
iii) Polymerase chain reaction (PCR) amplification		Petterson 1992
iv) Site-directed mutagenesis		Perdue 2001

[1]Allows direct analysis of the 3-dimensional structure of an antigen-antibody complex.

[2]Epitope regions are determined by affinity selection screening with either (i) antibody, or (ii) antigen.

[3]Fv, variable region of the fragment with antigen binding (Fab) of an immunoglobulin.

[4]Can be used in a number of different assay systems including enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation using monoclonal antibody (mAb) or polyclonal sera.

[5]'PEPSCAN' involves the synthesis of short overlapping peptide sequences on to solid supports (polystyrene pins) which are then used in a modified ELISA.

[6]Can be used in a number of different assay systems including ELISA and immunoprecipitation using mAb or polyclonal sera.

[7]Can be used in a number of different assay systems including immunoblotting, ELISA, immunoprecipitation and radioimmunoassays (RIA) using mAb or polyclonal sera.

[8]A variety of expression vectors/systems may be used. Vectors may fuse a tag to the protein for purification, or allow the incorporation of a radiolabelled amino acid into the recombinant protein for RIA.

(ELISA) (Petterson 1992). Examples of studies in which B cell epitopes have been identified in several autoimmune diseases using recombinant proteins are given in Table 3.2. The second technique begins at the peptide level and involves the analysis of antibody reactivity to either synthetic peptides or enzymatically digested protein fragments (Petterson 1992). By using synthetic peptides the entire sequence of the antigen can be represented by sets of consecutive or overlapping peptides that are then assayed for antigenicity. However, both these methods can only accurately detect linear epitopes and short sequences that may constitute part of a conformational epitope. More recently, the use of phage-display technology has allowed the identification of conformational peptide epitopes (Williams *et al.* 2001), and this will be discussed further in Chapter Four.

3.1.3 The role of B cell epitope mapping in autoimmunity

Mapping of B cell epitopes on autoantigens can provide understanding of the association of an autoantigen with a particular autoimmune pathogenesis. For example, epitopes may reside in functional domains of autoantigenic proteins. Indeed, autoantibodies in myasthenia gravis, directed against the acetylcholine receptor, are known to bind to an epitope in the acetylcholine-binding site which can directly inhibit the receptor function (Hoedemaekers *et al.* 1997). Similarly, the autoantigenic epitopes in SLE have been found to reside in highly conserved regions of proteins and can thus inhibit functions of both structural proteins and enzymes (Casiano and Tan 1996). For example, antinuclear antibodies targeting tRNA synthetase and DNA polymerase delta auxiliary protein have been shown to functionally inhibit the aminoacylation of tRNA and DNA replication and repair, respectively, in *in vitro* studies (Tan *et al.* 1994).

Epitope mapping can also provide an insight into the initiation of the autoimmune process. Autoimmunity may be triggered by an initial infection with a pathogenic organism (Wucherpfenning 2001). One of the mechanisms by which this might induce immune disease is the phenomenon of molecular mimicry in which a microbial peptide has sufficient structural similarity with a self-peptide to evoke a cross-reactive autoimmune response. For example, an epitope on the bacterium

Table 3.2 Examples of previous studies employing recombinant proteins to map B cell epitopes in autoimmune disease.

Study	Antigen	Disease	Techniques
Burch *et al.* 1993	Thyroid stimulating hormone-receptor	Graves' disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria. Immunoprecipitation (IP) experiments with patient sera.
Peterson and Krohn 1994	Steroid 17α-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments and exonuclease digestion. Expressed in bacteria and immunoblotted with patient sera.
Wedlock *et al.* 1993	Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments. Expressed in bacteria and immunoblotted with patient sera.
Song *et al.* 1994a	Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblotted with patient sera.
Volpato *et al.* 1998	Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments. Site-directed mutagenesis. Expressed in bacteria. IP experiments with patient sera.
Nikoshkov *et al.* 1999	Steroid 21-hydroxylase	Autoimmune Addison's disease	Site-directed mutagenesis. Expressed in bacteria. IP experiments with patient sera. Also expressed in mammalian cells and immunoblotted with patient sera.
von Mikecz *et al.* 1995	Ribosomal protein L7	Systemic lupus erythematosus and mixed connective tissue disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblot-ted with patient sera. Also IP experiments with patient sera.
Syren *et al.* 1996	Glutamate decarboxylase	Type 1 diabetes mellitus	Subcloning of endonuclease restricted cDNA fragments and cDNA amplified by PCR. Expressed in bacteria. IP experiments with patient monoclonal antibodies (*m*Abs). Also expressed in mammalian cells and immunoblotted with mAbs.
Daw *et al.* 1996	Glutamate decarboxylase	Type 1 diabetes mellitus	Subcloning of endonuclease restricted cDNA fragments and cDNA amplified by PCR. Expressed in bacteria. IP experiments with patient sera.
Lin *et al.* 1999	'BP 180' (collagen XVII)	Bullous pemphigoid	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblotted with patient sera.
Kemp *et al.* 2001a	Sodium iodide symporter	Autoimmune thyroid disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria. IP experiments with patient sera.

Yersinia entericolitica has homology with the extracellular domain of human thyroid-stimulating hormone receptor and could therefore cross-react with autoantibodies in Graves' disease (Tomer and Davies 1993). There is also evidence that similarities between epitopes on the parasite *Trypanosoma cruzi* and the cardiac muscle protein myosin may cause the aberrant immune response in Chagas' disease (Roitt and Delves 2001). The inflammation that results from a viral or bacterial infection can lead to local activation of antigen-presenting cells resulting in enhanced processing and presentation of self-antigens present at that tissue.

As well as providing insights into the mechanisms of autoimmune pathogenesis, the molecular characterisation of B cell epitopes may also allow new and more specific assays for autoimmune diseases to be established (Lernmark 2001). For example, if the epitope of a pathogenic autoantibody can be precisely defined, recombinant proteins containing only the significant epitope region could be used to measure titres of pathogenic autoantibodies within the heterogeneous antibody population of a patient's serum (Ishii *et al.* 1997). A further application of epitope mapping lies in the development of specific-active immunotherapies: the ability to determine a single relevant antigenic site within a target protein is the basis for the development of synthetic vaccines for the prevention of and protection against disease (Atabani 2001). There is a need for vaccines to be epitope specific to eliminate the possibility of cross-reactivity with self-protein or related antigens and also to make the production of the synthetic peptides economical.

3.1.4 Aim

The aim of this part of the study was to identify B cell epitopes on the MCHR1, a protein previously reported as an autoantigen in some vitiligo patients (Kemp *et al.* 2002). Deletion derivatives of MCHR1 cDNA were constructed by PCR amplification and then translated *in vitro* with the concomitant incorporation of [^{35}S]-methionine into the protein products. The [^{35}S]-labelled MCHR1 derivatives were subsequently used in radio-binding assays to investigate the reactivity of sera from vitiligo patients to the receptor. This method of epitope mapping has been successfully used to identify antibody-binding sites on the vitiligo autoantigens tyrosinase (Kemp *et al.* 1999a) and Pmel17 (Kemp *et al.* 2001b).

3.2 Materials and Methods

3.2.1 Patients and controls

Sera from nine vitiligo patients (V1-V9; Table 2.1), that were previously shown to contain MCHR1 antibodies (Kemp *et al.* 2002), were analysed in these experiments. In addition, sera from ten vitiligo patients (V10-V19; Table 2.1), that were previously shown not to contain MCHR1 antibodies (Kemp *et al.* 2002), were analysed. Sera from twenty healthy individuals (9 male, 11 female; mean age: 32 years with range 24-48 years) with no history of either vitiligo or autoimmune disorders were used as controls.

3.2.2 Generation of MCHR1 cDNA deletion constructs by PCR amplification

Full-length MCHR1 cDNA in pcDNA3 (Figure 3.1; Invitrogen) and referred to as pcMCHR1 is as detailed previously (Kemp *et al.* 2002). Fragments of MCHR1 cDNA, including base pairs 1-414, 1-534, 1-654, 1-774, 1-894, 1-1014 and 1-1134, 415-894, 895-1206, where the A residue of the initiating ATG codon is assigned as base pair number 1 (Kolakowski *et al.* 1996), were generated from pcMCHR1 by PCR amplification using the oligonucleotide primers (Life Technologies, Paisley, UK) listed in Table 3.3. Restriction sites for *Eco*RI and *Xba*I were incorporated into the 5'and 3' primers, respectively, in order to allow subcloning of the PCR products into pcDNA3 and the subsequent expression of the MCHR1 cDNA fragments from the T7 promoter present in the vector.

Samples of plasmid DNA containing MCHR1 cDNA (50 ng) were subjected to 30 cycles of PCR amplification in a DNA Thermal Cycler using the following conditions: denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute; extension at 72°C for 2 minutes; 72°C for 10 minutes to terminate the reaction. The composition of each 50-µl PCR reaction was as previously described (Section 2.15).

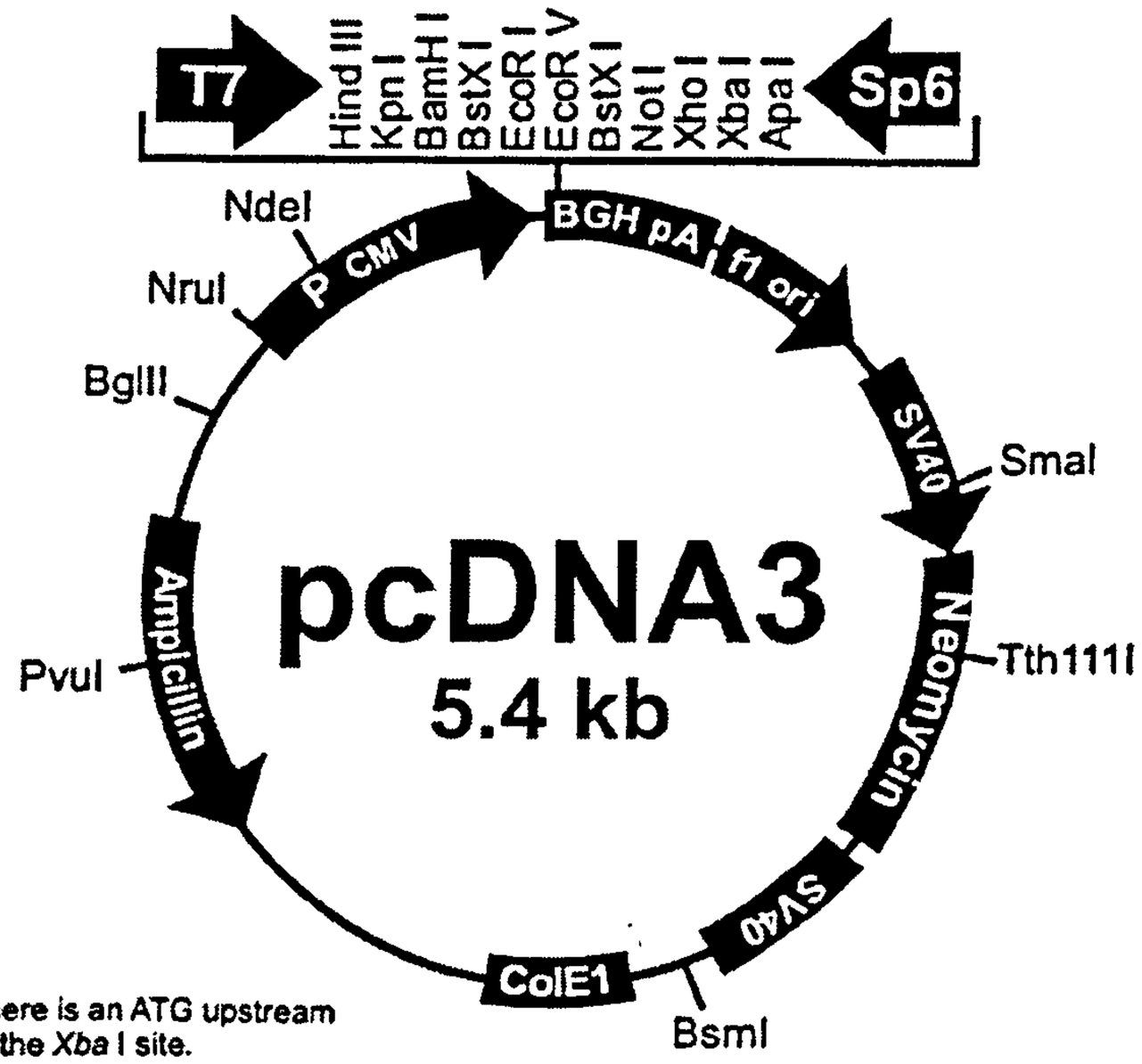


Figure 3.1 A map of the pcDNA3 vector detailing restriction sites. MCHR1 cDNA fragments generated by PCR amplification were cloned into the *Eco*RI-*Xba*I sites of pcDNA3 so that expression of the cDNA fragments is controlled by the T7 promoter.

Table 3.3 Oligonucleotide primers used to generate MCHR1 cDNA fragments by PCR amplification.

Base pairs[1]	Primer sequences[2]
1-414	Forward 5'-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCAATCTACTACCGAGAG-3'
1-534	Forward 5'-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCAGGTGAACTGACTATT-3'
1-654	Forward 5'-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCAGGCCCACAGGAGGCA-3'
1-774	Forward 5"-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCAGGTGAACCAGTAGAG-3'
1-894	Forward 5'-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCACAGCCGGATGCTGCG-3'
1-1014	Forward 5'-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCAAAAGGTGAGGGTCGG-3'
1-1134	Forward 5'-TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG-3' Reverse 5'-AATCTAGACGCCTATCATGCAGGCTTCACCGA-3'
415-894	Forward 5'-TTGAATTCGCCGCCATGCTCCTCTTTCTCCTG-3' Reverse 5'-AATCTAGACGCCTATCACAGCCGGATGCTGCG-3'
895-1206	Forward 5'-TTGAATTCGCCGCCATGCGGACAAAGAGGGTG-3' Reverse 5'-AATCTAGACGCCTATCAGGTG CCTTTGCTTC-3'

[1] The numbers correspond to base pairs of MCHR1 cDNA included in the construct with the A residue of the initiating ATG codon assigned as base pair number 1.

[2] The *Eco*RI and *Xba*I restriction sites are underlined. The translation initiation codon and the translation termination codons in the forward and reverse primers, respectively, are shown in bold-type face.

The PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega) from 0.8% (w/v) agarose gels as detailed in Section 2.12. Each PCR product was restricted with *Eco*RI and *Xba*I and then re-purified from a 0.8% (w/v) agarose gel. The PCR products were ligated into pcDNA3, previously restricted with *Eco*RI and *Xba*I, as described in Section 2.13. The ligation reactions were used to transform *E. coli* JM109 as in Section 2.14.1. Plasmid DNA was purified from individual transformants using a The Wizard™ Minipreps DNA Purification System (Section 2.8), digested with *Eco*RI and *Xba*I and analysed by gel electrophoresis in 0.8% (w/v) agarose. Appropriate recombinant plasmids were sequenced (Section 2.17.1) using T7 and SP6 primers (Promega) by the dideoxy termination methods according to a Sequence Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech) with [α-^{35}S] dideoxy adenosine triphosphate (NEN Life Science Products), to verify that no sequence errors had been introduced. The plasmids created are summarised in Table 3.4.

3.2.3 *In vitro* coupled transcription and translation

The plasmid constructs (Table 3.4) were used in a TnT® T7 Coupled Reticulocyte Lysate System (Promega) to produce and label MCHR1 and its deletion derivatives with [^{35}S]methionine *in vitro*. Each MCHR1 cDNA fragment was inserted into pcDNA3 in the correct orientation to allow expression from the T7 promoter, and each template contained appropriate start and stop codons to ensure accurate translation. The TnT® Coupled Reticulocyte Lysate System is 'cell-free' and contains purified reticulocyte lysate with ribosomes, tRNA, and all the initiation, elongation and termination factors necessary for protein synthesis. It contains little endogenous RNA that would produce unwanted background protein.

A standard reaction mixture of 50 µl contained: rabbit reticulocyte lysate, 25 µl; TnT® T7 RNA polymerase, 1 µl; TnT® reaction buffer, 2 µl; amino acids minus methionine, 1 µl; RNasin® RNase Inhibitor (Promega), 40 U; plasmid template, 2 µg; [^{35}S]methionine (1000 Ci/mmol; 10 mci/ml; Amersham Pharmacia Biotech), 4 µl. The reaction was incubated for 120 minutes at 30°C and then stored at –20°C until required.

Table 3.4 Deletion derivatives used in determining the antigenic domains on MCHR1 that are recognised by autoantibodies from patients with vitiligo.

MCHR1 construct	Amino acids encoded[1] (base pairs)[2]	Predicted molecular weight of expressed protein (kDa)[3]	Estimated molecular weight of expressed protein (kDa)[4]
pcMCHR1[5]	1-402 (1-1206)	44	45
pMCHR1-378	1-378 (1-1134)	42	39
pMCHR1-338	1-338 (1-1014)	37	34
pMCHR1-298	1-298 (1-894)	33	30
pMCHR1-258	1-258 (1-774)	28	27
pMCHR1-218	1-218 (1-654)	24	23
pMCHR1-178	1-178 (1-534)	20	21
pMCHR1-138	1-138 (1-414)	15	19
pMCHR1-139	139-298 (415-894)	18	20
pMCHR1-299	299-402 (895-1206)	11	10

[1] Numbers correspond to the amino acid residues of MCHR1 with the initiating methionine as residue number 1.

[2] Numbers correspond to base pair of MCHR1 cDNA with the A residue of the initiating ATG codon assigned as base pair number 1.

[3] Predicted from the amino acid sequence of the protein.

[4] Estimated from the mobility of the protein in SDS-polyacrylamide gels.

[5] Encodes full-length MCHR1.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of *in vitro* translated products was performed in either 12.5% (w/v) or 15% (w/v) polyacrylamide resolving gels as described in Section 2.19. An aliquot of each of the *in vitro* translation reaction mixtures was added to SDS sample buffer (Section 2.19) and boiled for 2 minutes before electrophoresis. Prior to drying under vacuum, gels were stained, destained and soaked in Amplify fluorographic reagent (Section 2.19). Autoradiography was carried out at –70°C.

3.2.4 Radio-binding assays

Vitiligo and control sera were tested for binding to [^{35}S]-MCHR1 and its labelled deletion derivatives in radio-binding assays as follows. For each assay, an aliquot of *in vitro* translation reaction mixture (equivalent to 50,000-100,000 counts per minute (cpm) of trichloroacetic acid (TCA)-precipitable material) was suspended in 50 µl of immunoprecipitation buffer containing: 20 mM Tris-HCl pH 8.0; 150 mM NaCl; 1% (v/v) Triton X-100; 10 µg/ml aprotinin (Bayer, Newbury, UK). Serum was then added to a final dilution of 1:50. After overnight incubation with shaking at 4°C, 50 µl of protein G Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech), prepared according to the manufacturer's protocol, were added and incubation continued for 1 hour at 4°C. The protein G Sepharose-antibody complexes were then collected by centrifugation and washed six times for 15 minutes in immunoprecipitation buffer at 4°C. Immunoprecipitated radioactivity was evaluated in a LKB 1217 Rackbeta liquid scintillation analyser (Wallac UK, Milton Keynes, UK). All samples were analysed in duplicate and the mean cpm immunoprecipitated were determined.

The binding reactivity of each patient and control sera to full-length MCHR1 and each deletion derivative was expressed as an antibody index calculated as: cpm immunoprecipitated by tested serum divided by mean cpm immunoprecipitated by 20 healthy control sera. Each serum was analysed in two experiments and the mean antibody index was calculated from these. The upper level of normal for each assay with a different ligand was calculated using the mean antibody index + 3 standard deviations (SD) of 20 control sera. Patient sera with an antibody index greater than the upper level of normal were regarded as positive for binding to the radio-labelled ligand used in the assay.

For analysis of immunoprecipitated proteins by SDS-PAGE and autoradiography, the protein G Sepharose-antibody complexes were resuspended in 50 µl of SDS sample buffer, boiled and the supernatant recovered for electrophoresis in SDS-polyacrylamide gels that were processed as described in Section 2.19.

3.2.5 Computer analysis

Computer analysis predictions of B cell epitopes were carried out as previously described (Parker *et al.* 1986). Since antigenic regions are those recognised by antibodies, it is most likely that these sites are accessible on the surface of a protein and are, therefore, probably hydrophilic in nature. Indeed, alogorithms for hydrophilicity and accessibility have been used to predict antigenicity (Parker *et al.* 1986). DNA sequence homology searches against the GenBank database were performed using the BLAST service of the National Centre for Biotechnology Information (Bethesda, MD, USA).

3.3 Results

3.3.1 *In vitro* translation of MCHR1 cDNA and its deletion derivatives

For identification of autoepitopes on MCHR1, PCR amplification was used to generate MCHR1 cDNA fragments of varying lengths that were restricted with *Eco*RI and *Xba*I and then cloned into pcDNA3 (Figures 3.2a and b). DNA sequencing of the fragments was carried out to verify that no sequence errors had been introduced. All the constructs used in the study are listed in Table 3.4. Products generated from *in vitro* translation of MCHR1 cDNA and its deletion derivatives were evaluated by SDS-PAGE in a 12.5% (w/v) SDS-polyacrylamide gel and subsequent autoradiography (Figure 3.3). Major bands representing the intact and modified [^{35}S]-labelled MCHR1 proteins were found in each case (Table 3.4). The deletion derivative encoded by pMCHR1-299 was detected on a 15% (w/v) SDS-polyacrylamide gel (data not shown).

3.3.2 Radio-binding assays of MCHR1 deletion derivatives with sera from vitiligo patients and controls

Sera from nine MCHR1 antibody-positive vitiligo patients, 10 MCHR1 antibody-negative vitiligo patients and 20 healthy controls were tested for their ability to immunoprecipitate [^{35}S]-labelled MCHR1 and its radio-labelled deletion derivatives. The results of the reactivity of the vitiligo sera with the MCHR1 deletion derivatives are summarised in Tables 3.5 and 3.6. The reactivity of each serum with each [^{35}S]-labelled ligand was expressed as an antibody index. For each assay, the mean antibody index + 3 SD of 20 control sera was used to calculate the upper level of normal. Any patient serum that had an antibody index greater than the upper level of normal was regarded as positive for binding to the labelled MCHR1 ligand used in the assay.

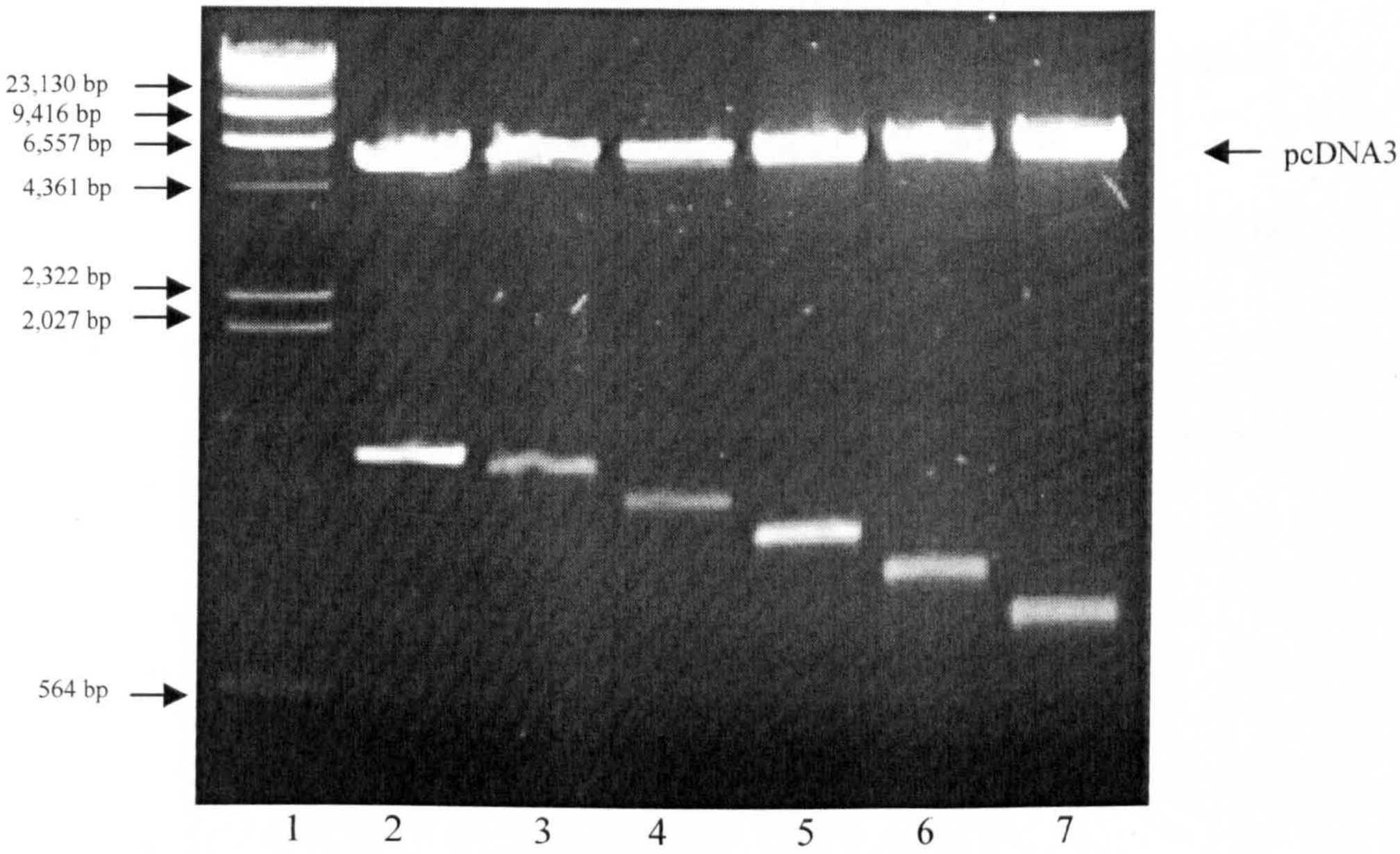


Figure 3.2a Agarose gel electrophoresis of MCHR1 cDNA deletion constructs. Plasmid DNA was restricted with *Xba*I and *Eco*RI and the DNA fragments generated were analysed by electrophoresis in a 0.8% (w/v) agarose gel as described in Section 2.11. The above gel shows *Hin*dIII-restricted bacteriophage λ DNA (*lane 1*); pcMCHR1 (*lane 2*); pMCHR1-378 (*lane 3*); pMCHR1-338 (*lane 4*); pMCHR1-298 (*lane 5*); pMCHR1-258 (*lane 6*); pMCHR1-218 (*lane 7*).

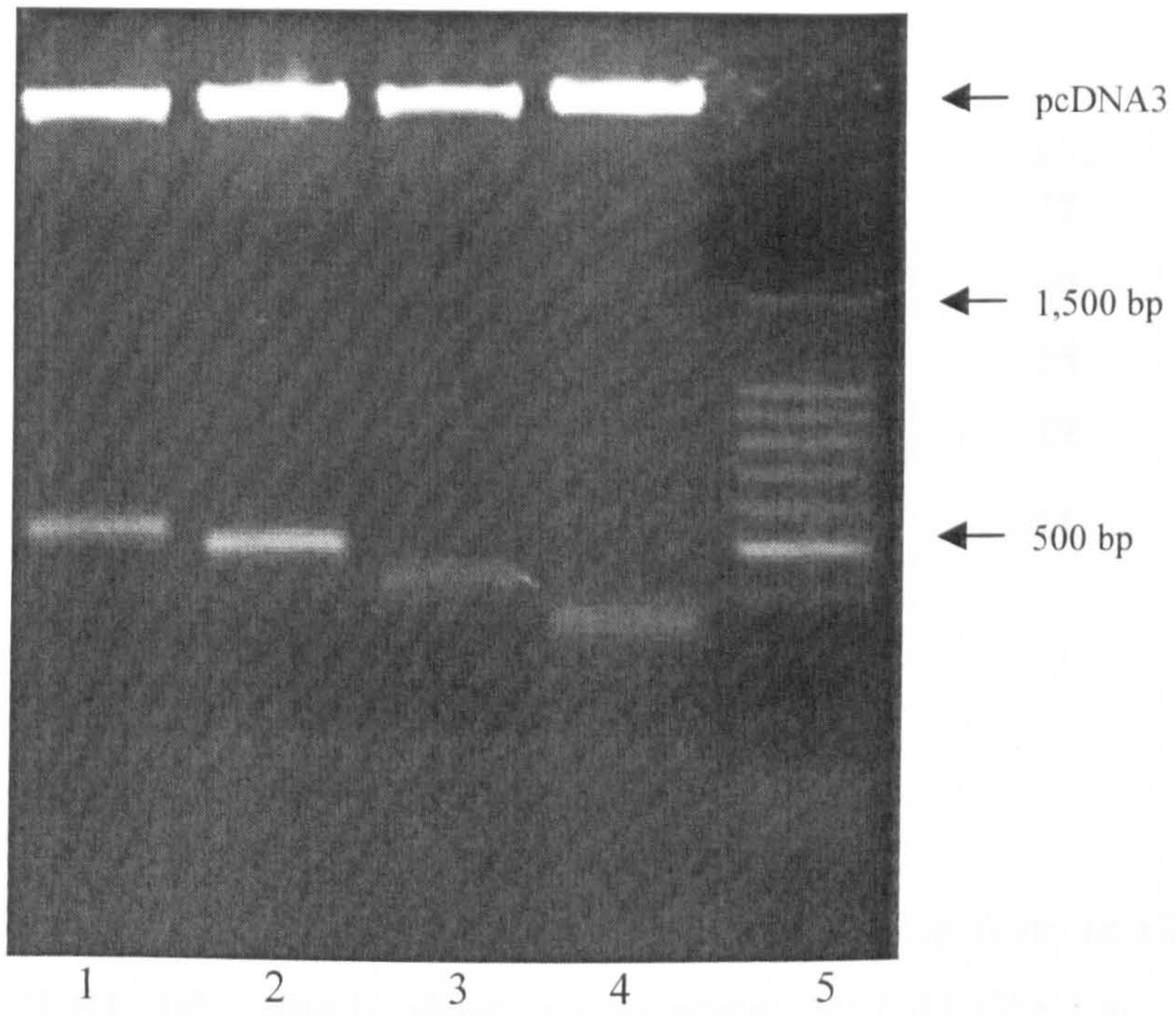


Figure 3.2b Agarose gel electrophoresis of MCHR1 cDNA deletion constructs. Plasmid DNA was restricted with *Xba*I and *Eco*RI and the DNA fragments generated were analysed by electrophoresis in a 0.8% (w/v) agarose gel as described in Section 2.11. The above gel shows: pMCHR1-178 (*lane 1*); pMCHR1-139 (*lane 2*); pMCHR1-138 (*lane 3*); pMCHR1-299 (*lane 4*); 100 base pair ladder (*lane 5*).

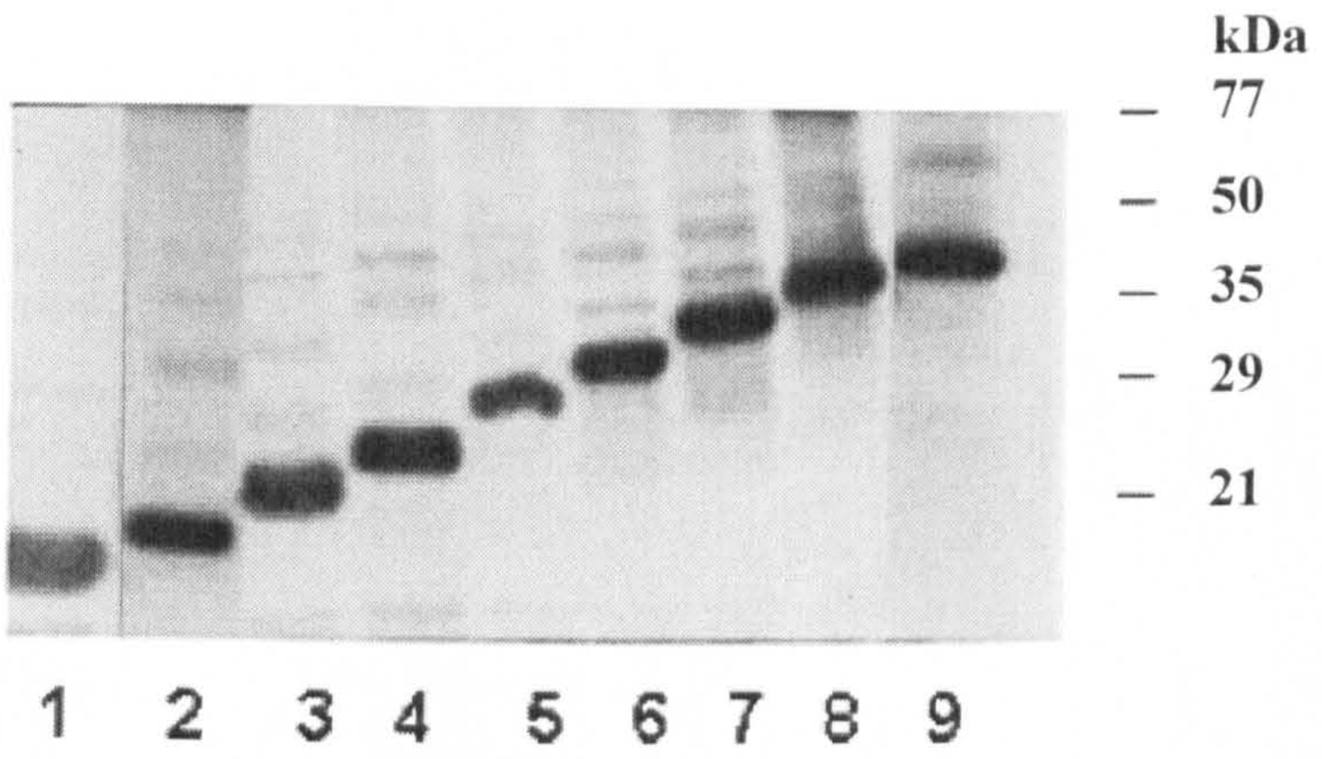


Figure 3.3 SDS-PAGE and autoradiography of products arising from *in vitro* translation of MCHR1 cDNA and its deletion derivatives. MCHR1 cDNA and its deletion derivatives were translated *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System and the radio-labelled products analysed by electrophoresis in a 12.5% (w/v) SDS-polyacrylamide gel followed by autoradiography as described in Section 2.19. Deletion derivatives of MCHR1 *in vitro* translated from: pMCHR1-138 (*lane 1*); pMCHR1-139 (*lane 2*); pMCHR1-178 (*lane 3*); pMCHR1-218 (*lane 4*); pMCHR1-258 (*lane 5*); pMCHR1-298 (*lane 6*); pMCHR1-338 (*lane 7*); pMCHR1-378 (*lane 8*); pcMCHR1 (*lane 9*). Protein molecular weight markers are shown on the right-hand side of the gel.

Table 3.5 Reactivity of MCHR1 antibody-negative vitiligo sera to MCHR1 deletion derivatives.

MCHR1 deletion derivative	Amino acids encoded	Reactivity of sera[1]										Upper level of normal for the assay[2]
		V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	
pcMCHR1	1-402	0.74	0.95	0.77	0.96	1.05	0.99	1.02	0.99	0.89	1.06	1.12
pMCHR1-138	1-138	0.80	0.80	0.83	0.73	0.77	1.06	0.96	1.03	0.75	1.01	1.18
pMCHR1-178	1-178	0.97	1.06	1.00	1.04	1.01	0.98	1.01	1.04	0.98	1.05	1.28
pMCHR1-218	1-218	0.95	1.01	1.02	0.97	0.99	1.00	0.98	0.96	0.95	1.01	1.13
pMCHR1-258	1-258	0.87	1.03	1.03	1.05	1.05	0.94	0.96	1.16	0.98	0.98	1.14
pMCHR1-298	1-298	0.91	0.98	0.98	0.95	1.01	0.94	1.00	0.91	0.97	0.99	1.21
pMCHR1-338	1-338	0.92	1.04	0.92	0.89	0.98	0.93	1.02	0.97	0.92	1.03	1.18
pMCHR1-378	1-378	0.97	1.04	0.97	0.91	0.98	1.03	0.99	0.94	1.00	0.80	1.09
pMCHR1-139	139-298	0.91	0.95	0.97	0.88	0.92	0.96	0.96	0.96	0.88	0.90	1.18
pMCHR1-299	299-402	0.98	0.99	0.95	0.94	1.00	0.95	1.00	1.02	0.97	0.95	1.57

[1] The binding reactivity of each of the sera to the full-length MCHR1 and each deletion derivative was expressed as an antibody index calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 20 healthy control sera. Each serum was analysed in at least two experiments and the mean antibody index was calculated from these.

[2] The upper level of normal for each assay with a different ligand was calculated using the mean antibody index + 3 SD of 20 control sera. Patient sera with an antibody index lower than the upper level of normal were regarded as negative for binding to the radiolabelled ligand used in the assay.

Table 3.6 Reactivity of MCHR1 antibody-positive vitiligo sera to MCHR1 deletion derivatives.

MCHR1 deletion derivative	Amino acids encoded	Reactivity of sera[1]									Upper level of normal for the assay[2]
		V1	V2	V3	V4	V5	V6	V7	V8	V9	
pcMCHR1	1-402	**1.94**	**1.48**	**1.85**	**2.49**	**1.47**	**1.41**	**1.70**	**1.49**	**3.00**	1.12
pMCHR1-138	1-138	**1.88**	**1.52**	**1.91**	**2.35**	0.97	1.01	**1.74**	**1.46**	**2.89**	1.18
pMCHR1-178	1-178	**1.87**	**1.48**	**1.83**	**2.41**	1.01	1.04	**1.63**	**1.32**	**2.71**	1.28
pMCHR1-218	1-218	**2.01**	**1.64**	**1.94**	**2.32**	0.98	1.02	**1.74**	**1.46**	**2.91**	1.13
pMCHR1-258	1-258	**2.06**	**1.58**	**1.95**	**2.48**	0.96	1.07	**1.75**	**1.44**	**2.93**	1.14
pMCHR1-298	1-298	**2.08**	**1.62**	**1.84**	**2.40**	**1.57**	**1.59**	**1.85**	**1.43**	**3.01**	1.21
pMCHR1-338	1-338	**1.82**	**1.56**	**1.90**	**2.31**	**1.32**	**1.41**	**1.66**	**1.42**	**3.03**	1.18
pMCHR1-378	1-378	**1.93**	**1.61**	**1.89**	**2.38**	**1.50**	**1.50**	**1.74**	**1.52**	**3.05**	1.09
pMCHR1-139	139-298	**1.80**	1.00	**1.78**	**2.48**	**1.41**	**1.48**	0.97	1.00	1.05	1.18
pMCHR1-299	299-402	0.97	1.08	1.06	0.84	1.05	1.00	0.98	1.03	1.00	1.57

[1] The binding reactivity of each of the sera to the full-length MCHR1 and each deletion derivative was expressed as an antibody index calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 20 healthy control sera. Each serum was analysed in at least two experiments and the mean antibody index was calculated from these.

[2] The upper level of normal for each assay with a different ligand was calculated using the mean antibody index + 3 SD of 20 control sera. Patient sera with an antibody index greater than the upper level of normal (in bold-type face) were regarded as positive for binding to the radiolabelled ligand used in the assay.

On this basis, none of the healthy individuals was positive for antibodies to any of the MCHR1 deletion derivatives used in the assay. In addition, none of the MCHR1 antibody-negative vitiligo patient sera reacted with any of the radio-labelled MCHR1 deletion derivatives tested (Table 3.5). Of the nine MCHR1 antibody-positive vitiligo (V) patient sera analysed, all were positive in the radio-binding assay when full-length MCHR1 positive was used as the radio-labelled antigen (Table 3.6). Seven of the nine (78%) sera (V1, V2, V3, V4, V7, V8, V9) were positive for binding to radio-labelled ligands containing amino acids 1-378, 1-338, 1-298, 1-258, 1-218, 1-178 and 1-138 (Table 3.6). Five of nine (56%) sera (V1, V3, V4, V5, V6) were found to react with a MCHR1 deletion derivative comprising amino acids 139-298 (Table 3.6). Using a cDNA construct encoding the C-terminal domain of the receptor (amino acids 299-402), none of the vitiligo patient sera was positive for reactivity to the deletion protein encoded therein (Table 3.6).

Longitudinal serum samples taken at 8, 3, 12 and 5 months after the initial tested sample from patients V2, V4, V6 and V9, respectively, were analysed for reactivity to the panel of MCHR1 deletion derivatives. The sera displayed a pattern of reactivity with the radio-labelled ligands identical to the initial serum sample obtained from the patient (data not shown).

SDS-PAGE and autoradiography indicated that major protein bands equating to the size of the radio-labelled ligands were immunoprecipitated by MCHR1 antibody-positive vitiligo patient sera and that the sera did not react with any other proteins. The results obtained for three MCHR1 antibody-positive vitiligo patient sera with five of the receptor deletion derivatives and full-length MCHR1 are illustrated in Figure 3.4. As determined by SDS-PAGE and autoradiography, normal control sera and MCHR1 antibody-negative vitiligo patient sera did not immunoprecipitate any of the radio-labelled ligands (data not shown).

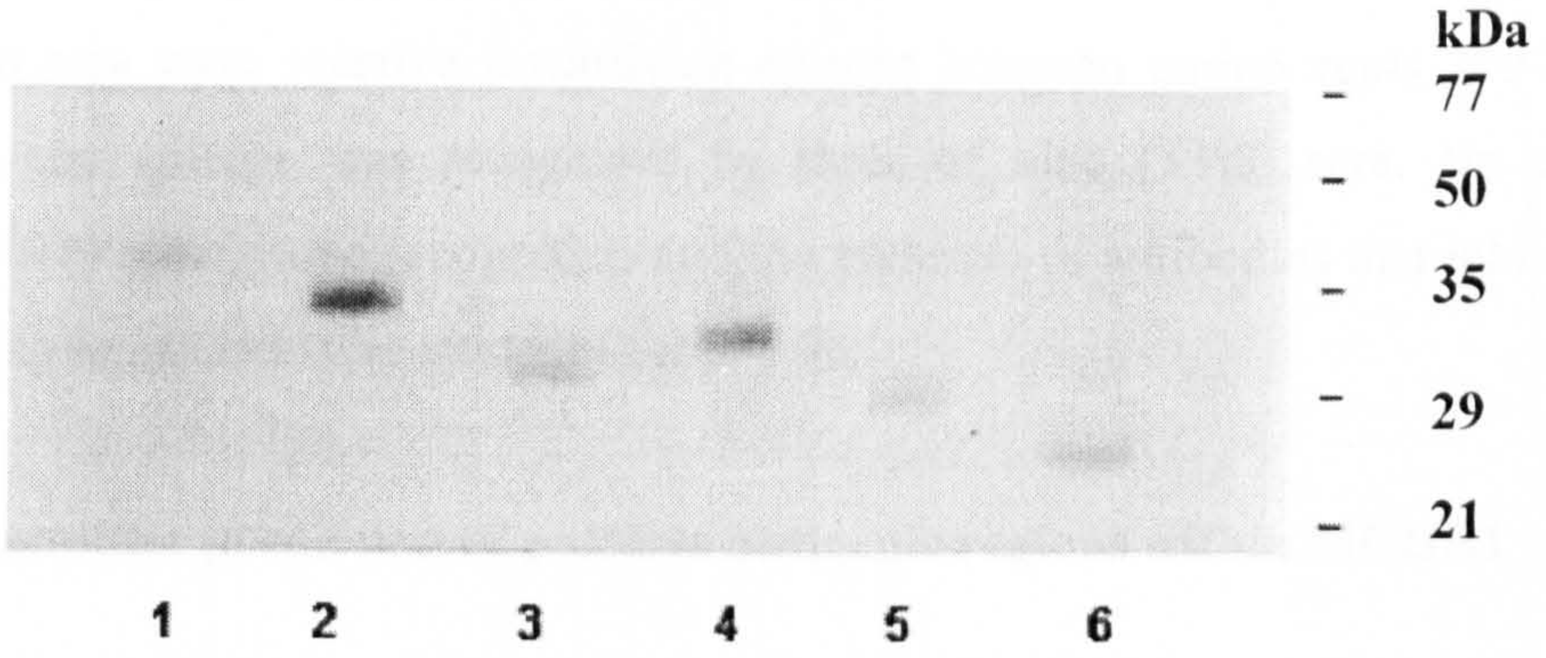


Figure 3.4 SDS-PAGE and autoradiography of radio-labelled proteins following immunoprecipitation with vitiligo patient sera. *In vitro* translated radio-labelled proteins were used in radio-binding assays with MCHR1 antibody-positive vitiligo patient as detailed in Section 3.2.4 prior to analysis by SDS-PAGE. MCHR1 proteins encoded by: pcMCHR1 (*lane 1*) and pMCHR1-378 (*lane 2*) immunoprecipitated by vitiligo patient serum (V1); pMCHR1-338 (*lane 4*) and pMCHR1-298 (*lane 3*) immunoprecipitated by vitiligo patient serum (V2); pMCHR1-258 (*lane 5*) and pMCHR1-218 (*lane 6*) immunoprecipitated by vitiligo patient serum (V3). Protein molecular weight markers are shown on the right-hand side of the gel.

3.3.3 Analysis of the antibody binding sites on MCHR1

The results of the radio-binding assays indicate the existence of multiple antibody binding sites on MCHR1: between amino acids 1-138 located at the N-terminal of the receptor, and between amino acids 139-298 found towards the centre of the MCHR1 molecule (Table 3.7). No epitopes were identified between amino acid residues 299-402. Of the sera examined, seven of nine (78%) reacted with the epitope domain comprising amino acids 1-138. Five of the nine (56%) sera recognised the epitope region containing amino acids 139-298 and within this domain two sera were reactive towards an epitope between amino acids 259-298. More than one epitope was recognised by three of nine (33%) sera. No clear correlation between epitope recognition and the presence of antibodies that inhibited MCH binding to MCHR1was evident (Table 3.7).

3.3.4 Computer prediction of putative antigenic regions within MCHR1

Computer prediction analysis revealed several putative antigenic regions within MCHR1 including amino acids 1-75, 110-115, 165-175, 190-205, 245-250, 275-300 and 365-402 (Figure 3.5). The epitope domains identified on the receptor were found to overlap, at least in part, with the areas on the protein that were predicted to be highly antigenic. Amino acid homologies between the identified epitope domains and either microbial or viral polypeptides were not demonstrated.

Table 3.7 Epitope regions on MCHR1 that are recognised by vitiligo sera.

Vitiligo patient sera	MCH binding-inhibition reactivity[1]	Reactivity of sera with MCHR1 epitope domains[2]	
		1-138	139-298
V1	+	+	+
V2	+	+	-
V3	+	+	+
V4	+	+	+
V5	ND	-	+[3]
V6	+	-	+[3]
V7	+	+	-
V8	ND	+	-
V9	ND	+	-

[1]MCH binding-inhibition reactivity of antibodies taken from Kemp *et al.* (2002): + positive for MCH binding-inhibition reactivity; -, negative for MCH binding-inhibition reactivity; ND, not determined.

[2]Reactivities with epitope regions are indicated as: +, reactivity with the specified epitope region; -, unreactive with the specified epitope region.

[3]Sera V5 and V6 recognize an epitope between 259 and 298.

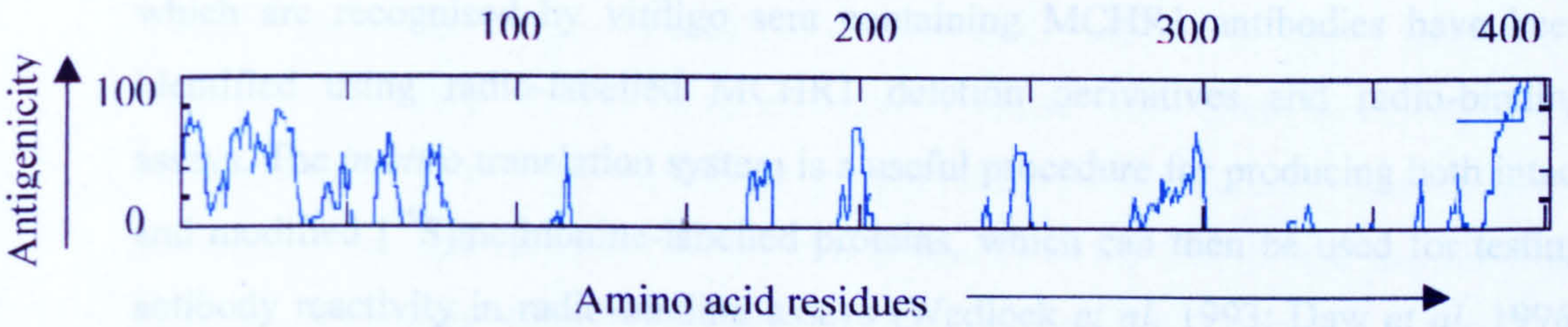


Figure 3.5 Antigenicity profile of the deduced amino acid sequence of MCHR1. Local antigenicity values, calculated as previously detailed (Parker *et al.* 1986), are plotted against the amino acid residues of MCHR1. The *y*-axis from 0-100 reflects increasing antigenicity.

3.4 Discussion

Recently, MCHR1 has been identified as an autoantigen in patients with vitiligo (Kemp *et al.* 2002). Immunoreactivity against the receptor was demonstrated in 16.4% of vitiligo patient sera, but not in the sera of either normal subjects or individuals with other autoimmune disorders, including systemic lupus erythematosus (Kemp *et al.* 2002). In this chapter, B cell epitopes on the receptor which are recognised by vitiligo sera containing MCHR1 antibodies have been identified using radio-labelled MCHR1 deletion derivatives and radio-binding assays. The *in vitro* translation system is a useful procedure for producing both intact and modified [^{35}S]methionine-labelled proteins, which can then be used for testing antibody reactivity in radio-binding assays (Wedlock *et al.* 1993; Daw *et al.* 1996; Volpato *et al.* 1998). Indeed, this method of epitope mapping has been successfully used to identify antibody-binding sites on the vitiligo autoantigens tyrosinase (Kemp *et al.* 1999a) and Pmel17 (Kemp *et al.* 2001b).

Analysis of results obtained in radio-binding assays with receptor deletion derivatives suggested the existence of multiple antibody binding sites on MCHR1, including regions between amino acids 1-138 and 139-298. These domains overlapped with areas on the receptor that were predicted to be highly antigenic. As autoimmune responses may be triggered by an infection with a pathogenic agent (Wucherpfennig 2001), homologies of the identified epitope regions with either microbial or viral polypeptides were searched for. Potentially cross-reacting epitopes were not demonstrated, however, and, therefore, no evidence for molecular mimicry in causing the humoral immune response to MCHR1 was apparent.

Mapping of B cell epitopes can provide an understanding of the association of an autoantigen with a particular autoimmune disease. For example, in myasthenia gravis autoantibodies are directed to an epitope in the acetylcholine-binding site in the acetylcholine receptor (Hoedemaekers *et al.* 1997). No clear correlation, however, between epitope recognition and the presence of antibodies that inhibited MCH binding to MCHR1 (Kemp *et al.* 2002) was found. Although epitope 139-298 contains an aspartic acid residue reported to be involved in the binding of MCH to its receptor (Macdonald *et al.* 2000), antibodies from two patients (V2 and V7) that inhibited hormone interaction with MCHR1 did not appear to recognise this epitope domain, at least in the context of the radio-binding assays used in this study.

Our findings indicate that the humoral response to MCHR1 in vitiligo is heterogeneous in nature with several patients exhibiting autoantibodies to more than one MCHR1 epitope. Similarly, autoantibodies in autoimmune thyroid disorders and type 1 diabetes have been reported to react against multiple epitopes on thyroid peroxidase (Zanelli *et al.* 1992) and tyrosine phosphatase-like IA-2 autoantigen (Lampasona *et al.* 1996), respectively. This has been explained by the intramolecular spreading of an autoimmune response from a single or few epitope(s) to multiple epitopes during the progression of the disease. For example, in type 1 diabetes, a temporal spreading of the autoantibody response has been described from immunodominant epitopes in an early preclinical phase to lesser immunogenic domains at the manifestation of disease (Naserke *et al.* 1998). We did not find evidence of epitope spreading in longitudinal serum samples, however, taken from four of the patients included in this study. Possibly, these four sequential patients may have been studied too late in the disease to see an indication of epitope spreading (Naserke *et al.* 1998).

The use of human sera in the mapping of MCHR1 epitopes can be problematic because sera contain multiple antibody species against the autoantigen. It is difficult, therefore, to examine the reactivity of a specific MCHR1 autoantibody in isolation and it is not possible to discriminate between a single MCHR1 antibody targeted at an epitope and a set of closely related MCHR1 antibodies directed at the same epitope. To gain information on the array of autoantibodies present in a particular serum and the epitope specificity of a particular autoantibody usually requires the production of human monoclonal antibodies from the patient. Indeed, monoclonal antibodies isolated from individuals with insulin-dependent diabetes mellitus have been successfully employed in identifying the antibody binding sites on glutamic acid decarboxylase, an autoantigen in this disease (Syren *et al.* 1996). Although the monoclonal antibody approach can allow the precise characterization of epitopes recognized by a specific antibody, there are drawbacks. For example, it is difficult to know the relative frequencies of the different monoclonal antibodies in the patient under investigation. Despite this, the isolation of monoclonal antibodies from vitiligo patients would allow a more complete and detailed analysis of the epitopes recognized by MCHR1 autoantibodies.

Although only a limited number of MCHR1 epitopes were identified, it is possible that more autoreactive epitopes could be present on the receptor. The

methodology used here for epitope mapping, employing recombinant proteins produced from deletion mutants generated by PCR amplification, is useful to detect linear/continuous epitopes but is not effective for the identification of conformational/discontinuous antigenic domains (Petterson 1992). In Graves' disease, for example, it is well documented that the autoantibodies to the thyrotropin receptor, which are responsible for disease activity, recognize a number of different conformational epitopes (Morgenthaler *et al.* 1999). Autoantibody epitopes on MCHR1 dependent upon the conformation of the receptor were not, therefore characterised, in this study. In so far as short linear sequences may contribute to conformational epitopes, it may be possible to identify at least part of some conformational epitopes using the methodology applied in this study. Nevertheless, it would be of interest to apply phage-display technology (Table 3.1; Scott 1992; Williams *et al.* 2001) to mapping epitopes on MCHR1. Phage-display is more suited to the study of conformational epitopes since expressed proteins are able to fold into their correct three-dimensional structures in the periplasmic space of the bacterial host, and can maintain a native arrangement once displayed on the surface of a phage particle (Wilson and Finlay 1998). The identification of conformational epitopes on MCHR1 by phage-display technology is the subject of Chapter 4.

4 B Cell Epitope Mapping of the Melanin-Concentrating Hormone Receptor 1 using Phage-display Technology

4.1 Introduction

Traditional methods of epitope mapping are to use deletion mutants of the antigen or to work at the peptide level by screening small synthetic peptides derived from the antigen. Indeed, autoantibody epitopes on autoantigens in vitiligo have been identified in this and previous studies by the use of deletion derivatives (Kemp *et al.* 1999a; Kemp *et al.* 2001b; Gottumukkala *et al.* 2003). However, these methods have proved labour intensive, expensive and require the antigen to be cloned and expressed (Petterson 1992). In addition, the traditional methods of epitope mapping can only detect linear epitopes and short sequences that may constitute part of the conformational epitopes. A recent development in the identification of epitopes is that of phage-display technology (Williams *et al.* 2001).

4.1.1 Phage-display technology

Filamentous phage, so called because of their narrow rod-shaped appearance (Figure 4.1), comprise three families of bacterial viruses that depend on the F-pilus for infection of their *E. coli* host (Wilson and Finlay 1998). Each phage particle consists of a single-stranded DNA genome packaged in a tube made up of 2700 copies of a major coat protein, pVIII, closed at the ends by four or five copies of each of four species of minor coat proteins, including pIII (Wilson and Finlay 1998). The discovery that insertion of foreign DNA fragments into the filamentous phage gene gIII results in the display of the encoded foreign polypeptide as a fusion protein with the minor coat protein pIII on the phage surface (Smith 1985), led to the development of phage-display technology. Libraries of phage-displayed fusion proteins can be screened for peptides based on their binding properties, with the advantage that the selected peptide sequences are physically linked to the genetic material that encodes them.

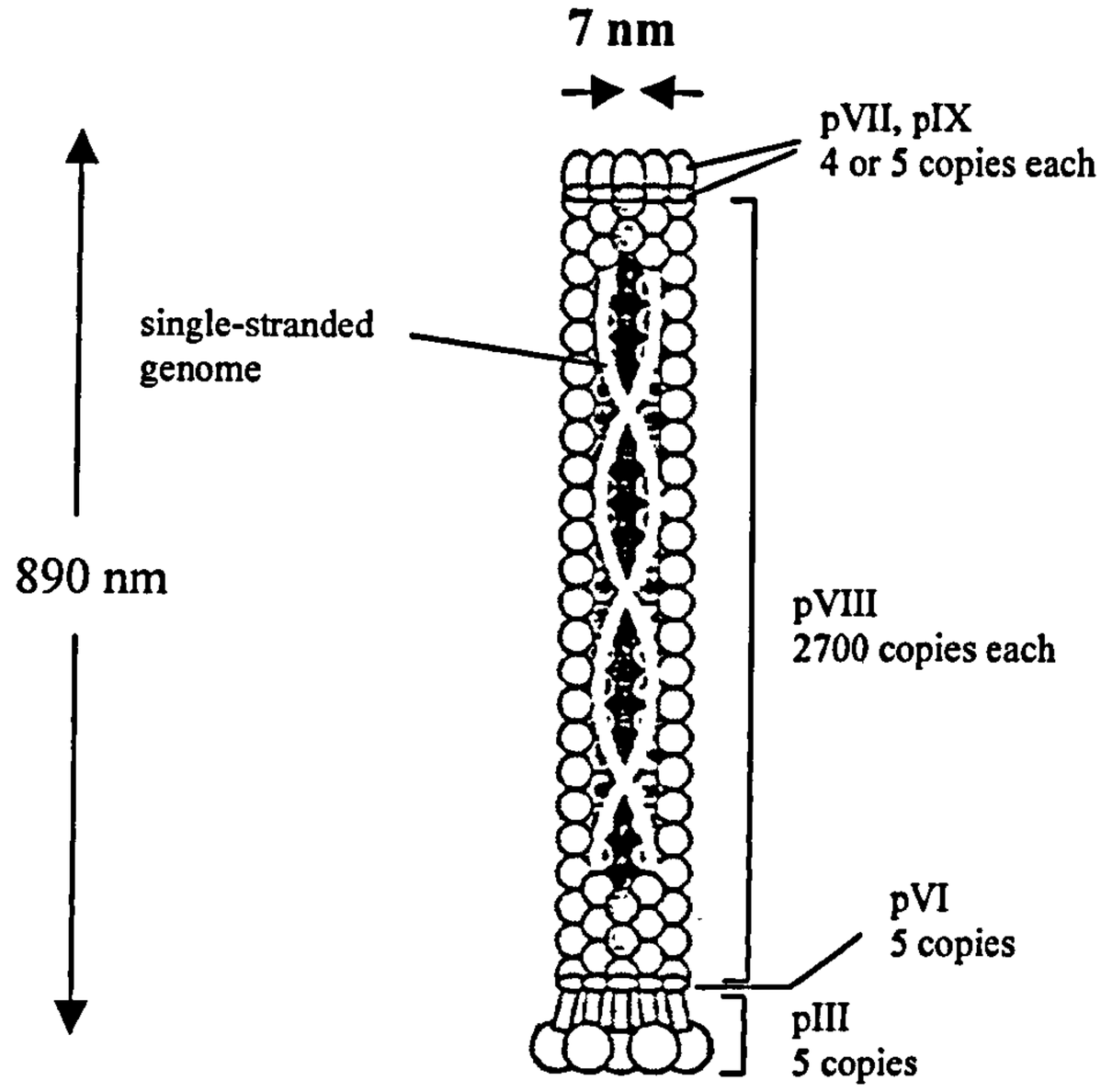


Figure 4.1 The filamentous phage. A schematic representation of the structure of a filamentous phage particle reproduced from (Wilson and Finlay 1998). A single-stranded circular genome is surrounded by ~2700 copies of the major coat protein pVIII, and 4 or 5 copies of each of four types of minor coat proteins, including pIII. Part of the coat sheath, formed by copies of the major coat protein, is not shown in order to reveal the phage genome.

The technique has become a powerful tool for studying protein-ligand interactions and has been applied to the study of receptor-ligand (Bass *et al.* 1990), enzyme-substrate (Redl *et al.* 1999), transcription factor-ligand (Butteroni *et al.* 2000), and antibody-antigen (Barbas 1993) interactions. Previous immunological applications of phage-display include the immunoscreening of phage libraries displaying antibody fragments to identify antibodies that interact with a specific antigen (Barbas 1993). More recently, immunoscreening of proteins expressed on phage from cDNA libraries has been utilised to identify novel autoantigens (Kemp *et al.* 2002). Furthermore, phage-display technology has been used to map B cell epitopes on autoantigens by using antibodies to screen libraries displaying random peptide sequences generated from a selected antigen (Table 4.1). Phage-display provides for the possibility of detecting conformational epitopes: larger peptides can be displayed that may adopt structural conformations, which are important for the binding of some antibodies. In addition, an advantage of this method is the large library size that can be generated ensuring that each clone in the library is well represented and can be rapidly screened.

4.1.2 Aim

The aim of this part of the study was to identify B cell epitopes on MCHR1 using phage-display technology. Initially, this involved the construction of a phage-display MCHR1 cDNA fragment library in the vector pComb3 (Figure 4.2). In this library, peptides encoded by the MCHR1 cDNA fragments were expressed as fusions with coat protein pIII and subsequently exposed on the surface of phage particles (Figure 4.3). The phage-display library was then immunoscreened with IgG samples purified from vitiligo patients in order to identify immunoreactive peptides that are recognised by MCHR1 autoantibodies (Figure 4.4).

Table 4.1 Examples of B cell epitope mapping of autoantigens using phage-display technology.

Autoantigen	Disease	Reference
Glutamic acid decarboxylase	Autoimmune polyendocrine syndrome type II and Stiff-man syndrome	Al-Bukhari *et al.* 2002
Glutamic acid decarboxylase	Type 1 diabetes mellitus	Myers *et al.* 2000
Tyrosinase phosphatase-like protein IA-2/ICA512bdc	Type 1 diabetes mellitus	Farilla *et al.* 2002
Tyrosinase phosphatase-like protein IA-2	Type 1 diabetes mellitus	Dromey *et al.* 2004
Smith autoantigen	Systemic lupus erythematosus	del Rincon *et al.* 2000
Gliadin	Celiac disease	Osman *et al.* 2000
Pyruvate dehydrogenase complex E2	Primary biliary cirrhosis	Rowley *et al.* 2000
Platelet autoantigen	Autoimmune thrombocytopenic purpura	Gevorkian *et al.* 1998

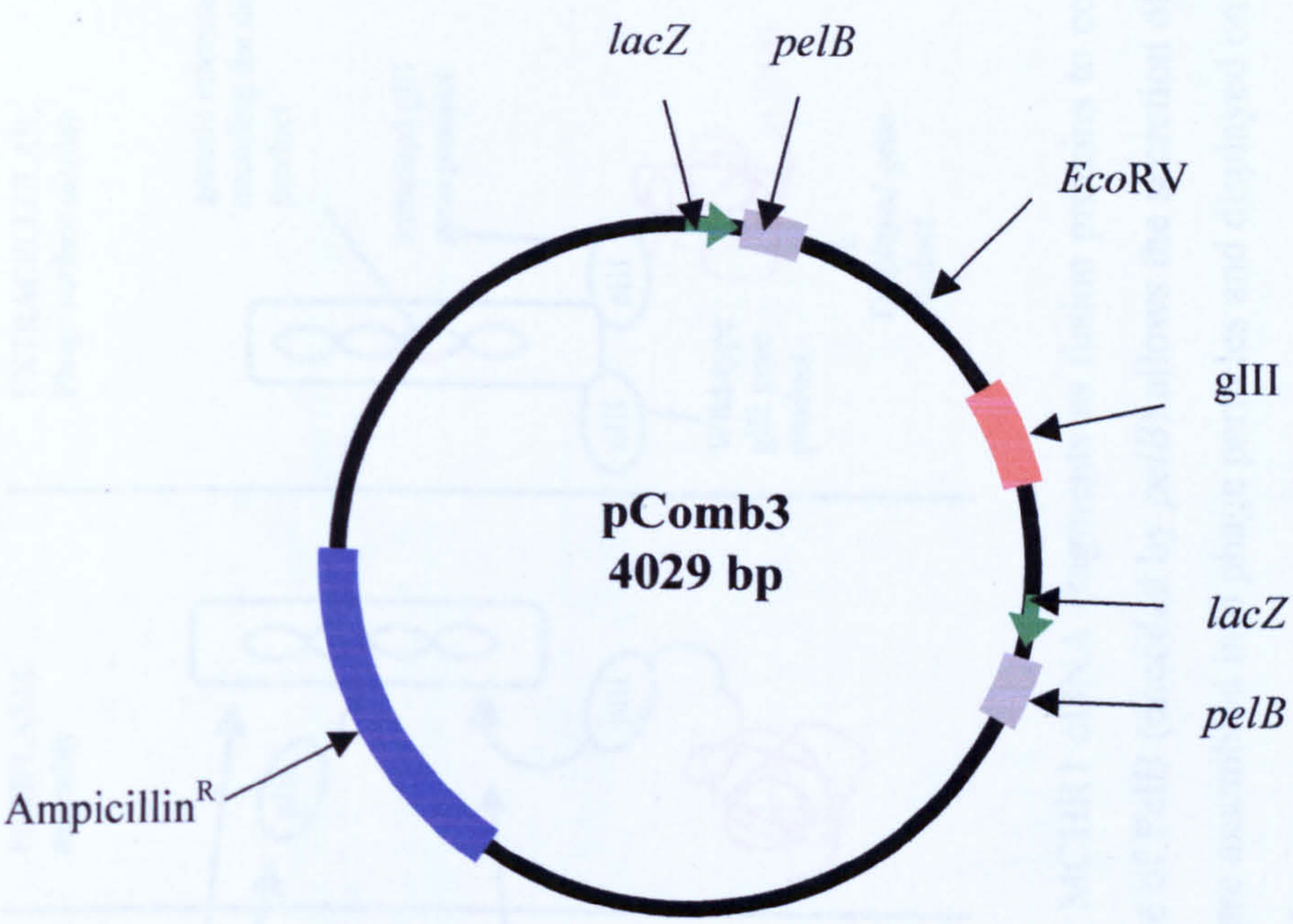


Figure 4.2 A map of the pComb3 vector. MCHR1 cDNA fragments were generated by digestion of PCR amplified MCHR1 cDNA. The cDNA fragments were cloned into the *Eco*RV restriction site of pComb3.

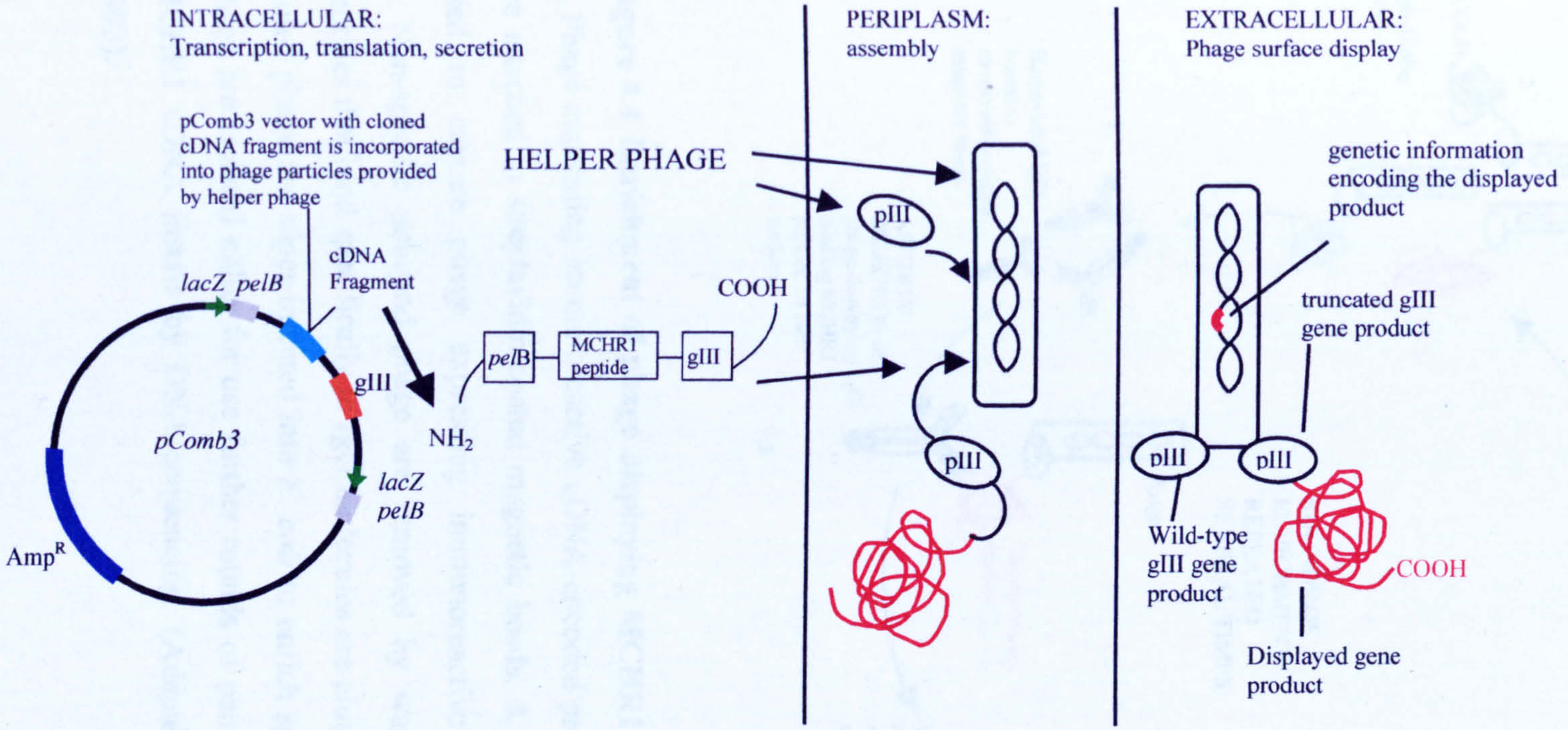


Figure 4.3 The pComb3 phage-display system. Expression of the MCHR1 cDNA fragments as fusion proteins to coat protein pIII (encoded by gIII) is controlled by the *lacZ* gene promoter. The leader sequence of PelB (encoded by *pelB*) allows the secretion of the fusion proteins into the periplasm of the bacterial cell. Subsequently, the fusion proteins are assembled into phage particles and displayed on the surface of the phage by virtue of coat protein pIII. (Adapted from Crameri and Suter 1993).

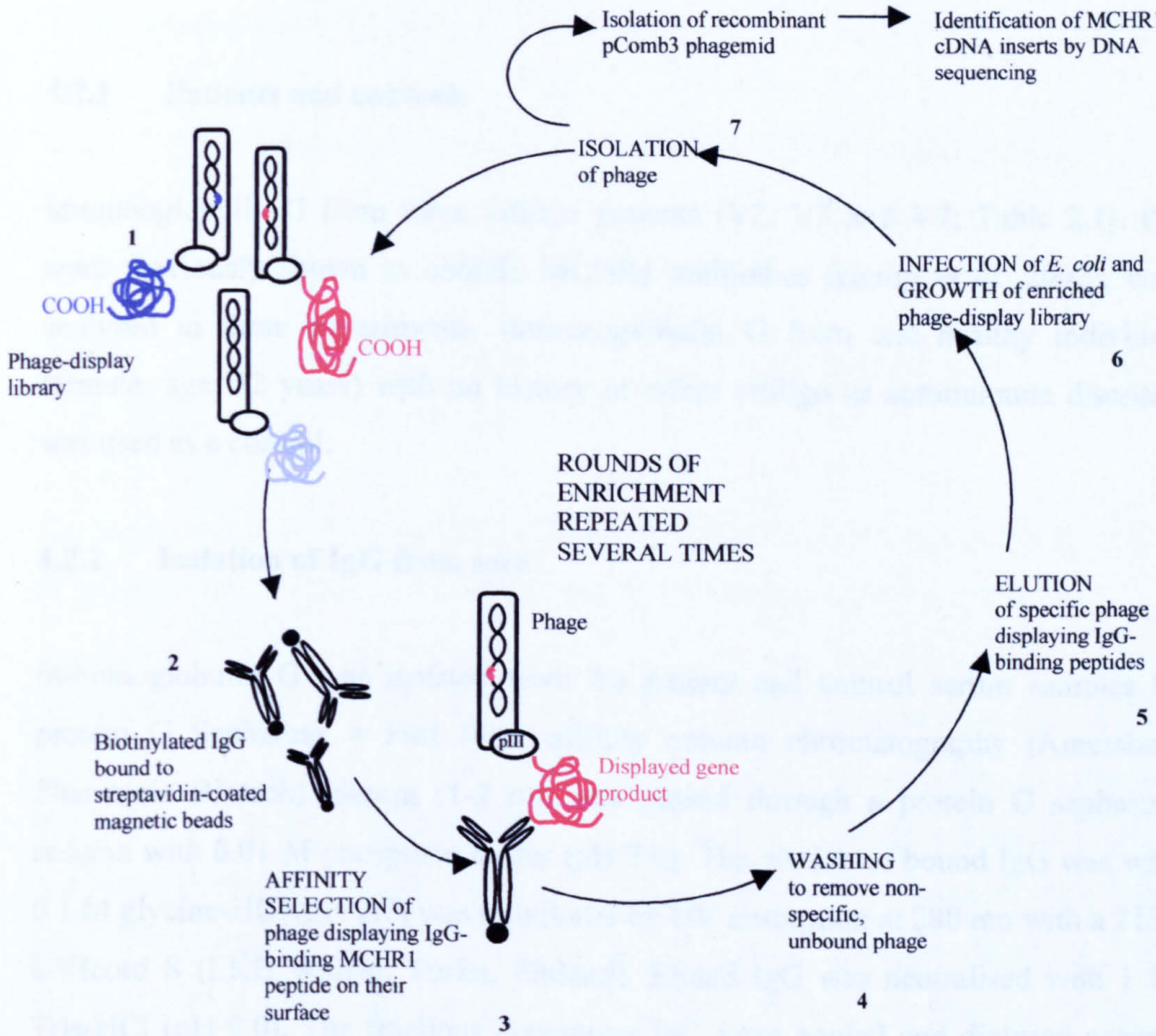


Figure 4.4 Enrichment of phage displaying MCHR1 peptides on their surface. **1.** Phage expressing immunoreactive cDNA-encoded products. **2.** Biotinylated IgGs are attached to streptavidin-coated magnetic beads. **3.** Biotinylated IgGs are then used to capture phage expressing immunoreactive cDNA-encoded products. **4.** Non-specific unbound phage are removed by washing. **5.** Phage displaying peptides that bind specifically to IgG molecules are eluted with glycine-HCl. **6.** The eluted phage are super-infected into *E. coli* to enrich specific-bound phage. **7.** The phage are isolated either for use further rounds of panning or for identification of MCHR1 cDNA inserts by DNA sequencing. (Adapted from Crameri and Suter 1993).

4.2 Materials and Methods

4.2.1 Patients and controls

Immunoglobulin G from three vitiligo patients (V2, V3 and V7; Table 2.1), that were previously shown to contain MCHR1 antibodies (Kemp *et al.* 2002), were analysed in these experiments. Immunoglobulin G from one healthy individual (female; age: 32 years) with no history of either vitiligo or autoimmune disorders was used as a control.

4.2.2 Isolation of IgG from sera

Immunoglobulin G was isolated from the patient and control serum samples by protein G Sepharose 4 Fast Flow affinity column chromatography (Amersham Pharmacia Biotech). Serum (1-2 ml) was passed through a protein G sepharose column with 0.01 M phosphate buffer (pH 7.0). The elution of bound IgG was with 0.1 M glycine-HCl (pH 2.7) was monitored by UV absorption at 280 nm with a 2138 UVIcord S (LKB Wallac, Turku, Finland). Eluted IgG was neutralised with 1 M Tris-HCl (pH 9.0). The fractions containing IgG were pooled and dialysed against PBS at 4°C overnight. After dialysis, the eluted IgG fractions were concentrated using an Amicon Concentrator (Amicon Inc., Beverley, MA, USA). The concentrated IgG was filter-sterilised with a Millex® Filter Unit (Millipore UK, Watford, UK) and the final IgG concentration measured by spectrophotometry at 280 nm with a Unicam 8625 UV/VIS spectrometer (Unicam, Cambridge, UK). All IgG samples were stored at -20°C until required and were at a concentration of 1-5 mg/ml.

4.2.3 Anti-MCHR1 antibody

Rabbit polyclonal anti-human MCHR1 antibody C11CB (Hervieu *et al.* 2000) was used as a positive control in biopanning experiments and was provided by Professor G. Hervieu (Neurology and Gastro-Intestinal Centre for Drug Discovery, GlaxoSmithKline Beecham, Harlow, UK). The C11CB antibody was raised against

the C-terminal intracellular tail of the receptor between amino acid residues 387-402 with sequence SNAQTADEERTESKGT.

4.2.4 Construction of a phage-display MCHR1 cDNA fragment library

4.2.4.1 Preparation of MCHR1 cDNA fragments .

Initially, MCHR1 cDNA was prepared by PCR amplification of pcMCHR1 (Kemp *et al.* 2002) using the following primers (Invitrogen):

Forward primer: 5' TTGAATTCGCCGCCATGTTGTGTCCTTCCAAG 3'
Reverse primer: 5' AATCTAGACGCCTATCAGGTGCCTTTGCTTTC 3'

Samples (50 ng) of pcMCHR1 plasmid DNA were subjected to 38 cycles of PCR amplification in a DNA Thermal Cycler using the following conditions: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and 72°C for 10 minutes to terminate the reaction. The composition of each 50-µl PCR amplification reaction was as previously described (Section 2.15). Random 100-300 base pair fragments of MCHR1 cDNA were then prepared by digestion with DNAseI (Promega) in a reaction containing: 1 µg of MCHR1 cDNA, 5 µl of 10x digestion buffer (500 mM Tris HCl, pH 7.6; 100 mM $MnCl_2$; 1 mg/ml BSA; Promega) and 1.5 µl of DNAseI (diluted 1:65 from the stock enzyme in 50 mM Tris HCl, pH 8.0). The reaction was left at room temperature for 1 hour and then terminated by the addition of EDTA (pH 8.0) to a final concentration of 50 mM.

The DNA fragments were cleaned by phenol-chloroform extraction. Firstly, the DNAseI digestion reaction was made up to 200 µl in TE buffer and then mixed with 200 µl of phenol:chloroform (1:1 v/v). Following centrifugation for 10 minutes at 10,000 *g*, the top layer was removed and transferred to a fresh tube. An equal volume of 100% ethanol was added and the precipitated DNA pelleted by centrifugation at 10,000 *g* for 20 minutes. The DNA pellet was washed in 70% (v/v) ethanol and then air-dried before resuspension in 100 µl of TE buffer.

The ends of the DNA fragments were repaired to allow subsequent cloning into the blunt-end *Eco*RV restriction site of pComb3. The end-filling reaction contained: 20 µl of DNA fragments, 3 µl of 10 mM dNTPs, 5 µl of 25 mM $MgCl_2$ and 1.5 µl of T4 polymerase (Promega). The reaction was left at room temperature for 15 minutes prior to the addition 1 µl of Klenow fragments (Promega) and incubated at room temperature for a further 15 minutes. The DNA fragments were loaded onto a 0.8% (w/v) agarose gel (Section 2.11) and, following electrophoresis, DNA fragments of 100-300 base pairs were excised from the gel and purified using a Wizard PCR Prep DNA Purification Kit (Section 2.12).

4.2.4.2 Vector preparation

Vector pComb3 (Figure 4.2) was digested with *Eco*RV in a 20-µl reaction containing: 1 µg of pComb3 DNA, 2 µl of 10x restriction buffer (Promega) and 1 µl of *Eco*RV (Promega). The reaction was incubated at 37°C for 2 hours. Two units alkaline phosphatase (Promega) and 10 µl of alkaline phosphatase buffer (Promega) were then added and the reaction left at 37°C for a further 30 minutes. The DNA was loaded onto a 1% (w/v) agarose gel (Section 2.11) and, following electrophoresis, the vector fragment was excised from the gel and purified using a Wizard PCR Prep DNA Purification Kit (Section 2.12).

4.2.4.3 Ligation of MCHR1 cDNA fragments into pComb3

A ligation reaction was set up containing *Eco*RV-digested pComb3 and MCHR1 cDNA fragments. The ratio of insert:vector (10:1) was much higher than in a standard ligation reaction (Section 2.13), as ligations using blunt-ended DNA are less efficient than those using DNA with protruding ends. The ligation reaction contained: 2 µl of *Eco*RV-digested pComb3, 20 µl of MCHR1 cDNA fragments, 2 µl of 10x ligase buffer and 1 µl of T4 DNA ligase. The reaction was incubated at 16°C overnight. The ligated DNA was cleaned by phenol-chloroform extraction as described in Section 4.2.4.1 and finally resuspended in 20 µl of sterile water.

4.2.4.4 Preparation of the phage-display library

The total ligation reaction was electroporated into *E. coli* XL-1 Blue cells as detailed earlier (Section 2.14.2). A 10-µl aliquot of the electroporated culture was plated onto a LB agar plate containing 10 µg/ml tetracycline and 100 µg/ml ampicillin, which was incubated at 37°C overnight. The colonies obtained after incubation were used to assess the total number of transformants obtained from electroporation of the ligation reaction. In addition, individual transformants were picked, added to 10 ml of LB medium containing 10 µg/ml tetracycline and 100 µg/ml ampicillin and grown overnight with shaking at 37°C. Subsequently, phagemid DNA was prepared from each culture using a Wizard Minipreps DNA Purification System (Section 2.8). Each phagemid DNA sample was then analysed by PCR amplification (Section 4.2.9) in order to assess the frequency of recombinants in the initial library, and by DNA sequencing (Section 4.2.7) in order to identify the MCHR1 cDNA fragments.

The remainder of the electroporated cells were added to 10 ml of LB, containing 10 µg/ml tetracycline and 100 µg/ml ampicillin, and grown at 37°C. When turbid, $1x10^{11}$ plaque-forming units of VCS-M13 helper phage (Stratagene) were added to the culture, which was then left at room temperature for 15 minutes. The culture was then transferred to 100 ml of LB containing 10 µg/ml tetracycline, 100 µg/ml ampicillin and 10 µg/ml kanamycin, and grown overnight with shaking at 37°C.

The 100 ml culture was centrifuged at 2000 *g* for 15 minutes to remove all bacterial cells. Phage particles were precipitated from the supernatant with 15 ml of 40% polyethylene glycol 4000 and 15 ml of 5 M NaCl (PEG/NaCl). Following overnight incubation at 4°C, the phage were harvested by centrifugation at 2000 *g* for 30 minutes and the pellet resuspended in 1-2 ml of PBS before storage at -20°C.

The phage titre was determined by infecting 2 ml of log-phase *E. coli* XL-1 Blue with an aliquot of the phage-display library, incubating at room temperature for 30 minutes and then plating out samples of the culture onto LB agar containing 10 µg/ml tetracycline and 100 µg/ml ampicillin. A titre of $1x10^{11}$ colony-forming units /ml was estimated.

4.2.5 Immunoscreening of the phage-display library

4.2.5.1 Biotinylation of IgG

Biotinylation of IgG was performed using EZ-Link™ Sulfo-NHS-LC-LC-Biotin (Pierce, Rockford, IL, USA), according to the manufacturer's protocol. Briefly, a 1 mg/ml solution of Sulfo-NHS-LC-LC-Biotin was made up immediately prior to setting up the biotinylation reaction, which comprised 2 mg of IgG sample in 1 ml of PBS and 75 µl of the Sulfo-NHS-LC-LC-Biotin solution. The reaction was mixed gently and allowed to proceed for 2 hours on ice, after which time any unreacted biotin was removed by extensive dialysis against fresh PBS. All IgG samples were stored at 4°C until required and were at a concentration of 2 mg/ml.

4.2.5.2 Paramagnetic bead preparation

Dynabeads® M-280 Streptavidin (Dynal Biotech, Oslo, Norway) beads were used to immobilise biotinylated IgGs during the isolation of phage particles that displayed IgG-binding peptides. For each experiment, a 20-µl (10 mg/ml) sample of beads was washed extensively with 500-µl aliquots of PBS containing Tween 20 at 0.05% (v/v). A Dynal Magnetic Particle Concentrator (Dynal Biotech) was used to separate the beads after each wash and the beads were finally resuspended in 235 µl of sterile water.

4.2.5.3 Isolation of phage displaying IgG-binding peptides

A 15-µl aliquot of either biotinylated rabbit anti-MCHR1 antibody, biotinylated IgG from a vitiligo patient (n=3) or biotinylated IgG from a healthy control (n=1) was incubated with 235 µl of prepared Dynabeads® on a rotating wheel at 4°C for 30 minutes. To block any non-specific binding to the beads later in the procedure, 300 µl of 2% (w/v) dried milk powder in PBS were added to the bead-IgG suspension and incubation continued for 1 hour at 4°C. After blocking, the bead-IgG complexes were separated from the blocking buffer using a Dynal Magnetic Particle Concentrator, washed twice with PBS/0.05% Tween 20 and finally resuspended in 150 µl of PBS/0.05% Tween 20. A 100-µl sample of the phage-display library was

then added to the bead-IgG suspension, which was then incubated overnight at 4°C to allow the interaction of antibody-bead complexes with peptides diplayed on the surface of the phage particles.

The bead-IgG complexes were washed extensively with PBS/0.05% Tween 20 and resuspended to remove any unbound phage. Bound phage were eluted from the bead-IgG complexes with 150 µl of 100 mM HCl (adjusted to pH 2.2 with solid glycine), and the beads then magnetically separated from the supernatant that contained the phage particles. Neutralisation of the supernatant was accomplished by the addition of 9 µl of 2 M Tris-HCl (pH 8.0). The eluted phage were then used to infect 2 ml of exponentially growing *E. coli* XL-1 Blue cells at room temperature for 15 minutes. Aliquots of the infected cells were then plated onto LB agar containing 10 µg/ml tetracycline and 100 µg/ml ampicillin and the plates incubated overnight at 37°C. This allowed the assessment of the number of eluted phage following each round of biopanning.

To generate a phage-display library for the next round of selection, the infected *E. coli* XL1-Blue culture was made up to 10 ml with LB medium and incubated for 1 hour with shaking at 37°C before superinfection with of $1x10^{11}$ plaque-forming units of VCS-M13 helper phage. Following incubation at room temperature for 30 minutes, the cells were transferred to 100 ml LB medium containing 10 µg/ml tetracycline, 100 µg/ml ampicillin and 10 µg/ml kanamycin and grown overnight with shaking at 37°C. Phage were prepared from the infected culture as described above (Section 4.2.4.4) and then stored at -20°C. This first round library, enriched in phage particles displaying IgG-binding peptides, was used in a second round of biopanning in the same way as the original library. In all, five rounds of biopanning were undertaken.

In the final round of biopanning, individual colony-forming units, isolated by plating out the culture infected with eluted phage, were picked and grown overnight with shaking at 37°C in 10 ml of LB medium containing 10 µg/ml tetracycline and 100 µg/ml ampicillin. Subsequently, phagemid DNA was prepared from each culture using a Wizard Minipreps DNA Purification System (Section 2.8) and each phagemid DNA sample was analysed by DNA sequencing (Section 4.2.7) in order to identify any immunoreactive MCHR1 peptides that had been enriched during the biopanning procedure.

4.2.6 Analysis of recombinant pComb3 phagemid by PCR amplification

Phagemid DNA (50 ng) was subjected to 38 cycles of PCR amplification in a standard reaction as described previously (Section 2.15) with primers (Invitrogen):

Forward primer PetComb1: 5' GGCTCGAGGTCGACGGTATCGATAAG 3'
Reverse primer PetComb2: 5' GGCTCGAGGCCACCACTAGTGGATCC 3'

The forward and reverse primers anneal to pComb3 (Figure 4.2) upstream and downstream, respectively, of the *Eco*RV restriction site thereby amplifying any cloned DNA fragments. The following reaction conditions were used: 94°C, 1 minute; 55°C, 1 minute; 72°C, 2 minutes; 72°C for 10 minutes. An aliquot of each PCR amplification product was analysed by electrophoresis in a 0.8% (w/v) agarose gel (Section 2.11) to confirm the presence of a fragment of MCHR1 cDNA.

4.2.7 DNA sequencing of pComb3 phagemid

Automated DNA sequencing of phagemid DNA was performed as detailed in Section 2.17.2. The sequencing primer (Invitrogen), 5' GGTGGCGGCCGCAAATTC 3', annealed to pComb3 (Figure 4.2) upstream of *pelB*. The insert DNA sequences were compared with the full-length MCHR1 cDNA sequence using the BLAST network service of the National Centre for Biotechnology Information.

4.3 Results

4.3.1 Construction of the MCHR1 cDNA phage-display library

The size of the MCHR1 cDNA fragment library was 1 x 10^6 independent clones. In order to determine the frequency of recombinant phagemid in the library, ten individual colony-forming units obtained after electroporation of the library ligation reaction into *E. coli* XL1-Blue were picked and grown. Phagemid DNA was prepared from each cultured transformant and analysed by PCR amplification with primers Petcomb1 and Petcomb2. This indicated that 15/15 (100%) of phagemids in the MCHR1 cDNA fragment library contained a DNA insert (Figure 4.5). The inserts ranged from 100-500 base pairs. Ten phagemid were also sequenced to determine if the library contained random MCHR1 cDNA fragments (Table 4.2). The results indicated that the library inserts were varied in their sequence. Following amplification with helper phage, an initial phage-display library with a titre of 1 x 10^{11} colony-forming units/ml was produced.

4.3.2 Screening of the phage-display MCHR1 cDNA fragment library with anti-human MCHR1 antibody C11CB

The phage-display MCHR1 cDNA fragment library was screened for immunoreactive peptides with rabbit polyclonal anti-human MCHR1 antibody C11CB. Five rounds of enrichment were carried out. A significant increase in the number of phage eluted was observed by the third round of biopanning (Table 4.3). Phagemid DNA from ten bacterial clones isolated from the final round of biopanning was analysed by DNA sequencing in order to identify if particular MCHR1 peptides had been selected during the enrichment process. Table 4.4 summarises the peptide sequences from the ten phagemid. The results demonstrated that specific MCHR1 peptides were indeed enriched by biopanning of the phage-display library with C11CB antibody. Seven of the ten MCHR1 peptide sequences included the amino acid region (residues 387-402), or part there of, that is recognised by C11CB. All the peptide sequences were in-frame with both the PelB leader sequence and the gIII

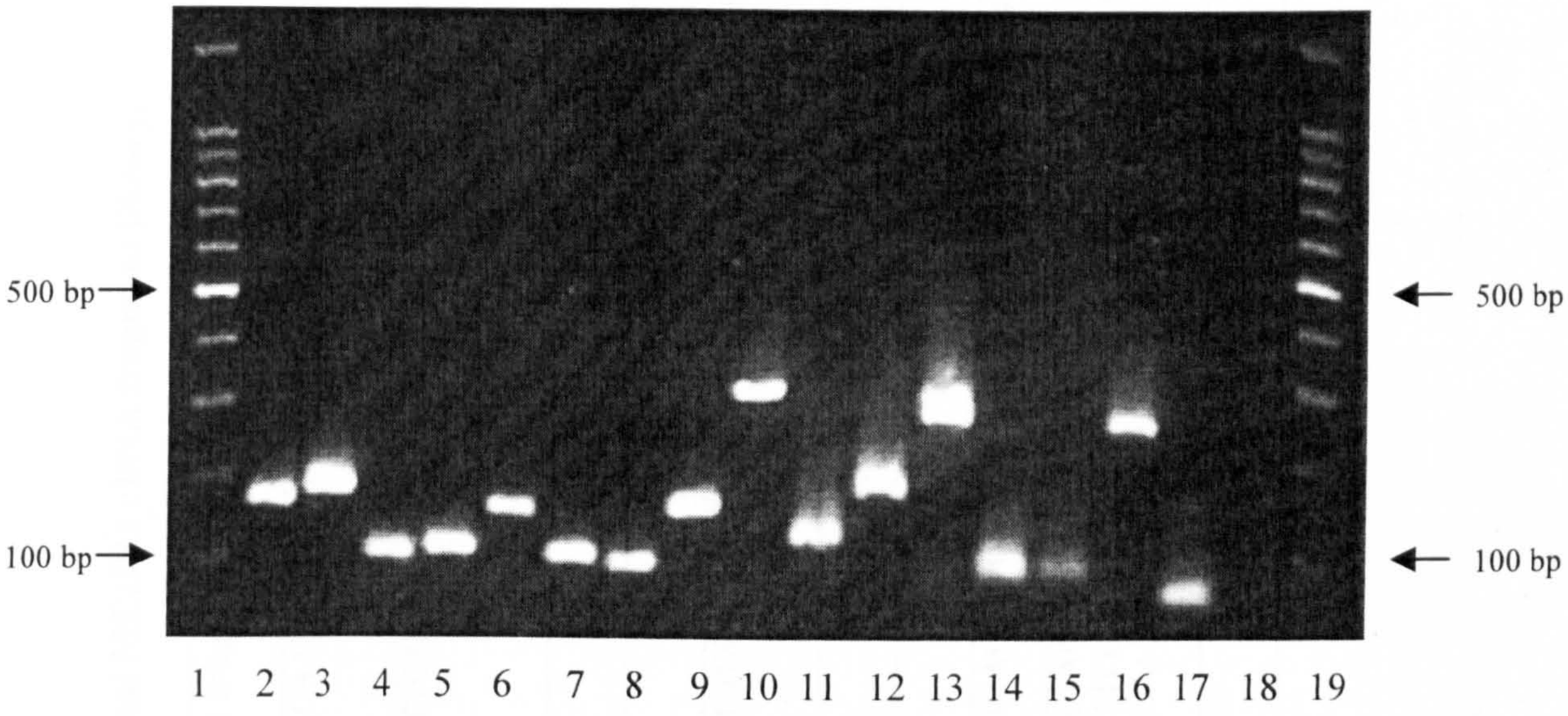


Figure 4.5 PCR amplification of the DNA inserts from 15 phagemid from the initial MCHR1 cDNA fragment library. PCR amplification products show a size distribution of between 100-500 base-pairs. Individual phagemid (lanes 2-16); non-recombinant pComb3 vector (lane 17); negative control containing no DNA (lane 18); 100 base-pair ladder (lanes 1 and 19).

Table 4.2 Sequences of MCHR1 cDNA fragments contained in the initial MCHR1 cDNA fragment library.

Clone number	Sequences encoded by the fragment of MCHR1 cDNA
1	GGACCAAGCGTTTGCGGAACGTCTCACAGAGCACGATGTACACAAAGGGGTTGAGGCAGCTGTTGGCATAGCCCAAGCTGA TGGCCGCA
2	TGCGGCATACGCCTGCCCAACCCAGACACTGACCTCTACTGGTTCACCCTGTACCAGTTTTTCCTGGCCTTTGCCCTGCCTTT TGTGGTCATCACAGCCGCATACG
3	CGGGGAGCATCTCCTACATCAACATCATCATG
4	TGCACTGGTGCAACAACGTCCCCGACATCTTCATCATCA
5	TGCTCCTTCTGTCCCCAGGATCACCTCCTCGCACGGGGAGCATCTCCTACATCAACATCATCATGCCTTCGGTGTTCGGCA
6	CCAAAGTGCCACACCCCATTGCCCATGAGCTGGT
7	GGACAACTGGGTCAGCTGTAGCA
8	TGATCCACCAGCTCATGGGCAATGGGGTGTGGCACTTTGGGGAGACCATGTGCACCCTCATCACGGCCATGGATGCCAATA
9	AGCTGACCCAGTTGTCCATCAGCCGCCCGACCCTCACCTTTGTCTACTTATACAATGCGGCCATCAGCTTGGGCTATGCCAA CAGCTGCCTCAACCCCTTTGTGTACATCGTGCTCTGTGAGACGTTCCGCAAACGCTTGGTCCTGTCGGTGAAGCCTGCAGCC CCAGGGGCAGCTTCGCGCTGTCAGCAACGCTCAGACGGCTGACGAGGAGAGGACA
10	CCTGGGCATGCCCTTCATGATCCACCAGCTCATGGGCAATGGGG

Table 4.3 Enrichment of phage by biopanning of the phage-display MCHR1 cDNA fragment library with anti-MCHR1 antibody C11CB.

Biopanning round	Amount of infected bacteria plated out onto selective LB[1] agar		Total number of phage eluted
	1 µl	10 µl	
Round 1	11	129	$2.58x10^4$
Round 2	73	TMTC[2]	$1.46x10^5$
Round 3	783	TMTC	$1.56x10^6$
Round 4	831	TMTC	$1.67x10^6$
Round 5	905	TMTC	$1.81x10^6$

[1]LB, Luria Bertani.

[2]TMTC, Too many to count.

Clone number	Amino acid sequences recognised by polyclonal anti-MCHR1 antibody	Amino acid number
1	GYANSCLNPFVYIVLCETFRKRLVLSVKPAAQGQLRAVSNAQTADEERTESKGT	349-402
2	AAQGQLRAVSNAQTADEERTESKGT	378-402
3	CETFRKRLVLSVKPAAQGQLRAVSNAQTADEERTESKGT	364-402
4	QGQLRAVSNAQTADEERTESKGT	380-402
5	RKRLVLSVKPAAQGQLRAVSNAQTADEERTES	368-399
6	VLCETFRKRLVLSVKPAAQGQLRAVSNAQTADEERTESKGT	362-402
7	FRKRLVLSVKPAAQGQLRAVSNAQTADEERT	367-397
8	ETMCTLITAMDANSQFTSTYILTAMA	162-187
9	KTDGSGHSGRIHQETHGEG	6-24
10	NSQRLLLLSPGSPPRTGSISYINIIMPSVFGTICLLGIIGNSTVIF	67-112

Table 4.4 Peptide sequences displayed on the surface of phage particles enriched by biopanning of the phage-display MCHR1 cDNA fragment library with anti-MCHR1 antibody C11CB. The consensus amino acid sequence between the peptides is shown in red and the sequence **SNAQTADEERTESKGT** is recognised by C11CB antibody.

protein product which would result in the expression of the peptides as fusion proteins on the surface of phage.

4.3.3 Screening of the phage-display MCHR1 cDNA fragment library with IgG from vitiligo patients and a healthy control

The phage-display MCHR1 cDNA fragment library was screened for immunoreactive IgG from three vitiligo patients (V2, V3 and V7) and one control. Five rounds of enrichment were carried out for each IgG sample. In experiments with two of the vitiligo patient IgGs (V2 and V3), a moderate level increase in the number of eluted phage was observed, by the final round of biopanning (Table 4.5) suggesting that some enrichment had occurred. In experiments with the remaining vitiligo patient (V7) and control IgGs, no increase in the number of eluted phage was observed by the final round of biopanning (Table 4.5), suggesting that no significant enrichment had taken place.

Phagemid DNA from twenty bacterial clones isolated from the final round of biopanning with IgG from three vitiligo patients and one control was analysed by DNA sequencing in order to identify if particular MCHR1 peptides had been selected during the enrichment process. The results demonstrated that biopanning of IgG from one vitiligo patient (V2) had enriched MCHR1 peptides containing a consensus sequence of MCHR1 amino acid residues 49-82 in 11/20 of the phagemid analysed (Table 4.6). The remaining nine clones contained MCHR1 cDNA fragments that could not be expressed as fusion proteins as they were not in the correct reading frame with respect to the PelB leader sequence and the gIII protein product. Biopanning with IgG from a second vitiligo patient (V3) enriched phage expressing MCHR1 peptides containing a consensus of amino acid residues 156-194 (Table 4.7). This was found in 9/20 of the phagemid sequenced. Again, the remaining eleven clones contained MCHR1 cDNA fragments that could not be expressed as fusion proteins as they were not in the correct reading frame with respect to the PelB leader sequence and the gIII protein product. Immunoglobulin G from the third vitiligo patient (V7) and the control did not enrich any particular MCHR1 peptide sequences and the majority of the phagemid carried MCHR1 cDNA fragment inserts that would not be expressed as fusion proteins.

Table 4.5 Enrichment of phage by biopanning of the phage-display MCHR1 cDNA fragment library with vitiligo patient and control IgG.

Biopanning round	Total number of phage eluted			
	Patient V2	Patient V3	Patient V7	Control
Round 1	$2.72x10^4$	$1.96x10^4$	$3.02x10^4$	$1.24x10^4$
Round 2	$2.76x10^4$	$3.18x10^4$	$1.56x10^4$	$1.56x10^4$
Round 3	$3.46x10^4$	$4.08x10^4$	$1.96x10^4$	$1.84x10^4$
Round 4	$4.70x10^4$	$3.72x10^4$	$2.52x10^4$	$1.90x10^4$
Round 5	$1.12x10^5$	$1.22x10^5$	$3.10x10^4$	$1.93x10^4$

Clone number	Displayed amino acid sequence	Amino acid number in MCHR1
1	GGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINIIMPSVFG TICLLGIIGNSTVIFAVVKKSKLHWCNNVPDIFIINLSVVD	38-138
2	TDGSGHSGRIHQETHGEGKRDKISNSEGRENGGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTG	7-83
3	NGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINIIMPSVFG TICLLGIIGNSTVIFAVVKKSKLHWCNNVPDIFIINLSVVDLLFLLGMPFMIHQLMGNGVWHF	45-160
4	ENGGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISY	36-87
5	GGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISY	46-87
6	LEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINIIMPSVF	49-96
7	PSKTDGSGHSGRIHQETHGEGKRDKISNSEGRENGGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINI	4-90
8	HGEGKRDKISNSEGRENGGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRT	21-82
9	HQETHGEGKRDKISNSEGRENGGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINIIMPSVFGTICLL GIIGNSTVIFAVV	17-115
10	FQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINIIMP	42-93
11	RDKISNSEGRENGGRGFQMNGGSLEAEHASRMSVLRAKPMSNSQRLLLLSPGSPPRTGSISYINIIMPSVFGTICLL	26-102

Table 4.6 Peptide sequences displayed on the surface of phage particles enriched by biopanning of the phage-display MCHR1 cDNA fragment library with vitiligo patient (V2) IgG. The consensus amino acid sequence (residues 49-82) between the peptides is shown in red.

Clone number	Displayed amino acid sequence	Amino acid number in MCHR1
1	GIIGNSTVIFAVVKKSKLHWCNNVPDIFIINLSVVDLLFLLGMPFMIHQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKFRKPSVATLVI CLLWA	103-218
2	FMIHQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKFRKPSVA	147-209
3	GNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKFRKPSVA	156-209
4	HQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKFRKPS	150-207
5	GIIGNSTVIFAVVKKSKLHWCNNVPDIFIINLSVVDLLFLLGMPFMIHQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLAT	103-194
6	MPFMIHQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKFRKP	145-206
7	GIIGNSTVIFAVVKKSKLHWCNNVPDIFIINLSVVDLLFLLGMPFMIHQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKFRKPSVATLVI CLLWALSFISITPVWLYARLIPFPGGAVGCGIRLPNPD	103-251
8	MPSVFGTICLLGIIGNSTVIFAVVKKSKLHWCNNVPDIFIINLSVVDLLFLLGMPFMIHQLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVH	92-196
9	QLMGNGVWHFGETMCTLITAMDANSQFTSTYILTAMAIDRYLATVHPISSTKF	151-203

Table 4.7 Peptide sequences displayed on the surface of phage particles enriched by biopanning of the phage-display MCHR1 cDNA fragment library with vitiligo patient (V3) IgG. The consensus amino acid sequence (residues 156-194) between the peptides is shown in red.

4.4 Discussion

In this study, a phage-display MCHR1 cDNA fragment library was screened with an anti-MCHR1 antibody C11CB and with vitiligo patient IgGs in order to map B cell epitopes on MCHR1. On biopanning of the library with C11CB antibody, which is raised against amino acid residues 387-402 of the receptor and served as an experimental positive control, high levels of enrichment were observed. Sequence analysis of ten phagemid from bacterial clones isolated following the final round of biopanning revealed that seven of them contained an amino acid consensus sequence of residues 380-397. This consensus contained the target amino acid sequence, or part thereof, for C11CB. This initial control experiment demonstrated that the library could be successfully screened with a specific antibody.

Biopanning of the phage-display library with IgG from three vitiligo patients that were positive for MCHR1 autoantibody reactivity, resulted in a moderate level of enrichment in 2/3 cases. For one patient (V2), sequence analysis of twenty phagemid from bacterial clones isolated following the final round of biopanning revealed that eleven of them contained an amino acid consensus sequence of residues 49-82. This epitope was located within the autoantigenic domain (amino acid residues 1-138; Table 3.7) that had been previously determined for this vitiligo patient (Gottumukkala *et al.* 2003). Biopanning with IgG from a second vitiligo patient (V3) enriched by the final round phage expressing MCHR1 peptides containing a consensus of amino acid residues 156-194 and this was found in 9/20 of the phagemid sequenced. This epitope was located within one of the autoantigenic domains (amino acid residues 139-298; Table 3.7) that had been previously determined for this vitiligo patient (Gottumukkala *et al.* 2003). In contrast to the screening of these two vitiligo patients, IgG from the third vitiligo patient (V7), which had previously been shown to react with MCHR1 epitope domain 1-138 (Table 3.7; Gottumukkala *et al.* 2003), and the control did not enrich any particular MCHR1 peptide sequences.

The presence of significant numbers of peptide sequences in the final round of biopanning with patient IgGs, which were not in the correct reading frame for expression, is probably due to the low titres of MCHR1 autoantibodies in vitiligo patients compared with the titre of the anti-MCHR1 C11CB antibody. So, although enrichment takes place with patient IgGs, it is at a lower level and the selected phage

probably do not completely dominate the binding of non-specific phage that is inherent in this technique.

Each of the consensus sequences identified may represent a linear epitope that is recognised by patient MCHR1 autoantibodies. Conversely, the consensus sequences could have been selected due to them being part of a conformational epitope as the biopanning method is carried out in liquid phase and might therefore facilitate folding of the peptide. To differentiate, Western blot analysis could be applied to the expressed peptides, which would identify linear epitopes due to the denatured state of the protein in SDS-polyacrylamide gels.

Phage-display technology has been used previously to identify linear and conformational autoantigenic epitopes in several autoimmune conditions. For example, a random peptide phage-display library was used to identify discontinuous epitopes in autoantigens that are recognised by anti-platelet autoantibodies in patients with autoimmune thrombocytopenic purpura (Gevorkian *et al.* 1998). Similarly, four prominent epitopes in α-type gliadin and one prominent and repetitive epitope in γ-type gliadin have been demonstrated in patients with celiac disease, an autoimmune disorder caused by the indigestion of gluten (Osman *et al.* 2000). In addition, the major conformational epitope for the pyruvate dehydrogenase complex E2 in primary biliary cirrhosis patients has been predicted by comparing epitopes derived by phage-display screening with the known nuclear magnetic resonance structure of the lipoyl domain (Rowley *et al.* 2000) and phage-display technology has been used to identify conformational epitopes on tyrosinase phosphatase-like IA-2/ICA512bdc autoantigens in type 1 diabetes (Farilla *et al.* 2002; Dromey *et al.* 2004). Autoantibodies to 65 kDa glutamic acid decarboxylase (GAD65) are produced in many patients with autoimmune polyendocrine syndrome type II, stiff-man syndrome and type 1 diabetes mellitus (Myers *et al.* 2000; Al-Bukhari *et al.* 2002). The major linear epitopes of GAD65, which are recognised by patient autoantibodies, occur in the N-terminal and C-terminal regions of the protein and these epitopes have been mapped using random peptide phage-display libraries (Al-Bukhari *et al.* 2002).

The examples given above indicate the usefulness of phage-display as a method of mapping autoantigenic epitopes in various autoimmune disorders. From this study, the phage-display MCHR1 cDNA fragment library proved a useful tool in mapping the epitopes recognised by MCHR1 autoantibodies in vitiligo patients. This

method enabled the more precise identification of epitopes that were located within larger autoantigenic domains determined previously using deletion derivatives of MCHR1 (Chapter 3; Gottumukkala *et al.* 2003).

5 Functional Blocking of the Melanin-Concentrating Hormone Receptor 1 by Vitiligo Patient Autoantibodies

5.1 Introduction

5.1.1 Receptor autoantibodies in autoimmune disease

In several autoimmune disorders, including autoimmune thyroid disease, myasthenia gravis, Addison's disease and pernicious anaemia, autoantibodies have been demonstrated to affect the functioning of cell receptors. TSHR is one of the major target autoantigens in autoimmune thyroid disease. TSHR autoantibodies are functionally heterogeneous: they include both thyroid-stimulating autoantibodies (TSAb) and thyroid-blocking autoantibodies (TBAb). Thyroid-stimulating autoantibodies activate TSHR and elicit hyperthyroidism in Graves' disease by mimicking the ability of TSH to bind to TSHR. Antibody binding to the receptor is more prolonged thereby inducing a chronic overproduction of thyroid hormone (Zakarija and McKenzie 1987; McGregor 1990; Nagayama and Rapoport 1992; Weetman and McGregor 1994; Song *et al.* 1996; Watanabe *et al.* 1997; Akamizu 2001). In contrast, receptor occupancy by non-stimulatory TBAb prevents TSH action and may result in atropic thyroiditis, which is often accompanied by hypothyroidism (Arikawa *et al.* 1985; McKenzie and Zakarija 1992; Kosugi *et al.* 1993).

In myasthenia gravis, autoantibodies directed against the acetylcholine receptor block the acetylcholine-binding site and provoke accelerated receptor degradation (Hara *et al.* 1993; Eymard and Chillet 1997; Hoedemaekers *et al.* 1997; Ferrero *et al.* 1997; Fabien *et al.* 2001).

Pernicious anaemia is characterised by cobalamine (Vitamin B12) deficiency, autoimmune gastritis and anti-intrinsic factor autoantibodies that block the cobalamine-binding site of intrinsic factor, a gastric protein that is required for the assimilation of Vitamin B12 (Wahlstedt 1990; Gueant *et al.* 1997). In addition, it has been demonstrated that serum immunoglobulin in patients with pernicious anaemia can block the gastrin-stimulation of acid secretion and it has been suggested that this

blocking activity is mediated by competition with gastrin for the surface receptors on parietal cells (de Aizpurua *et al.* 1985; Burman *et al.* 1989).

In Addison's disease, patient IgGs can block the *in vitro* action of ACTH on adrenal growth and cortisol production by binding to its receptor (Wulffraat *et al.* 1989). In contrast, subsequent studies did not demonstrate the blocking actions of IgGs from Addison's disease patients on ACTH receptor (Wardle *et al.* 1993).

5.1.2 Intracellular calcium signalling

The calcium ion is an important intracellular messenger, controlling a diverse range of cellular processes, such as gene transcription, muscle contraction, cell growth, cell survival and cell proliferation (Clapham 1995; Berridge *et al.* 1998). In most cells, calcium has its major signalling function when it is elevated in the cytosolic compartment. The calcium concentration inside cells is regulated by the interplay of several processes, which can be divided into calcium 'on' and calcium 'off' mechanisms depending on whether they serve to increase or decrease cytosolic calcium, respectively (Bootman and Lipp 2001). The calcium 'on' mechanisms include channels located at the plasma membrane (PM) which regulate the inexhaustible supply of calcium from the extracellular space, and channels on the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR) which release the finite intracellular calcium stores. A more diverse set of 'off' mechanisms is employed by cells to remove calcium from the cytoplasm. These include calcium ATPases on the PM and ER/SR, in addition to exchangers that utilize gradients of other ions to provide the energy to transport calcium out of the cell (Bootman and Lipp 2001).

When cells are at rest, the balance lies in favour of the 'off' mechanisms, thus yielding an intracellular calcium concentration of approximately 0.1 μM. However, when cells are stimulated by various means such as hormones the 'on' mechanisms are activated and the cytosolic calcium concentration increases to levels of 1 μM or more. The binding of hormones, such as TSH and MCH, to their specific receptors on the PM leads to the activation of phospholipase C which catalyses the hydrolysis of phospholipids to produce the intracellular messenger IP_3. Although derived from a lipid, IP_3 is water-soluble and diffuses into the cell interior where it can encounter IP_3 receptors on the ER/SR. The binding of IP_3 changes the conformation of IP_3

receptors such that an integral channel is opened, thus allowing the calcium stored at high concentrations in the ER/SR to enter the cytoplasm (Bootman and Lipp 2001).

It has been reported that melanocyte growth, differentiation, dendricity and pigmentation have all been linked to the induction of the IP_3/diacylglycerol/phospholipase C cascade that yields increased levels of intracellular calcium (Yaar and Gilchrest 1991). Since MCH can stimulate the release of intracellular calcium on binding to MCHR1, this receptor might have a role in regulating melanocyte function. Indeed, a recent study demonstrated that stimulation of cultured human melanocytes with MCH reduced α-MSH-induced increases in cyclic AMP production (Hoogduijn *et al.* 2002) suggesting that MCH can down regulate the actions of α-MSH, a well-known stimulator of melanogenesis, via MCHR1 expressed on pigment cells (Hoogduijn *et al.* 2002). Recently, it has been reported that binding of MCH to MCHR1 can be inhibited significantly by vitiligo patient IgG: the mean percentage of inhibition (± SD) of MCH binding was 45% (± 4%) and 16% (± 14%), respectively, for vitiligo patient and control groups (p=0.001; Kemp *et al.* 2002). We were, therefore, interested in studying the effects that vitiligo patient MCHR1 autoantibodies might have upon the functioning of the receptor. As MCHR1 is exposed on the cell surface, it could be accessible to autoantibodies that might adversely affect receptor function as in other autoimmune disorders (Section 5.1.1). This might disrupt normal pigment cell behaviour resulting in vitiligo.

5.1.3 Aim

The present study aimed to examine the effects of MCHR1 autoantibodies from vitiligo patients on the functioning of the receptor by using fluorimetry to measure changes in intracellular calcium levels in response to MCH-stimulation in a CHO-K1 cell line expressing MCHR1. Various *in vitro* studies have shown that CHO-K1 cells and human embryonic kidney (HEK)-293 cells expressing MCHR1 respond to MCH resulting in an increase in intracellular calcium levels, the inhibition of forskolin-stimulated cyclic AMP production, an increase in IP_3 production and the activation of mitogen-activated protein kinase (Chambers *et al.* 1999; Lembo *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999; Hawes *et al.* 2000; Verlaet *et al.* 2002). Initially, a CHO-K1 stable cell line expressing MCHR1 was established by transfection of CHO-K1 cells with pcMCHR1 and then characterised by: flow cytometry with a rabbit polyclonal anti-human MCHR1 antibody, measuring changes in intracellular calcium levels in response to MCH-stimulation by fluorimetry and analysis of changes in forskolin-stimulated cyclic AMP levels in response to MCH-stimulation using a cyclic AMP immunoassay. The MCHR1-expressing cell line (CHO-MCHR1) was then used to investigate the effect of MCHR1 autoantibodies on receptor function.

5.2 Materials and Methods

5.2.1 Transfection of CHO-K1 cells with pcMCHR1

A stable CHO-K1 cell line expressing MCHR1 was isolated by transfection of CHO-K1 cells with pcMCHR1. A liposome-mediated transfection technique was employed using Tfx-50 reagent (Promega) prepared according to the manufacturer's instructions. Approximately $2x10^6$ of CHO-K1 cells in 10 ml of CHO-K1 culture medium (Section 2.20) were plated in 10-cm dishes and the cells incubated at 37°C in a 5% CO_2 incubator until 80% confluent. For each 10-cm dish, 6 ml of Nutrient Mixture F-12 (Ham) medium containing 2 mM L-glutamine was pre-warmed to 37°C in a 5% CO_2 incubator and then added to a sterile tube along with 20 µg of plasmid pcMCHR1. After mixing, 60 µl of Tfx-50 reagent was added, vortexed, and incubated at room temperature for 10-15 minutes. Subsequently, the medium was removed from the plated CHO-K1 cells and 6 ml of the Tfx-50 reagent /DNA mix was added to each dish before incubation at 37°C in a 5% CO_2 incubator for 1 hour. Cells were overlaid with 10 ml of CHO-K1 culture medium, pre-warmed to 37°C in a 5% CO_2 incubator, and incubated for a further 48 hours. To select transfected cells at the end of the incubation period, the cultures were re-plated in 10-cm dish at 1:20, 1:200, and 1:500 dilutions, in CHO-K1 culture medium containing 1 mg/ml geneticin sulphate. The cells were incubated at 37°C in a 5% CO_2 incubator for approximately 2 weeks, with a change of culture media every 3-4 days. When complete cell death had occurred in any untransfected cells, the small islands of transfected cells remaining were removed from the dish, transferred to wells on a 24-well plate and grown in CHO-K1 culture media containing geneticin sulphate at 1 mg/ml. Each geneticin-resistant cell line was then analysed for expression of MCHR1 by flow cytometry.

5.2.2 Flow cytometry

Flow cytometry was used to detect MCHR1 expression in pcMCHR1-transfected CHO-K1 cells. Forty-eight geneticin-resistant CHO-K1 cell lines were grown to confluence in T75 tissue culture flasks in CHO-K1 culture medium containing geneticin sulphate at 1 mg/ml. Subsequently, the cells were washed once with PBS and detached from the tissue culture flask using Cell Dissociation Solution (Sigma). After centrifugation at 1000 *g* for 5 minutes, cells were resuspended in PBS and 100-µl samples aliquoted into LP4 tubes (Becton Dickinson Labware, Oxford, UK). One hundred microlitres of Reagent A of a CALTAG Fix and Perm® Cell Permeabilization Solution Kit (Caltag Laboratories, Burlingame, CA, USA) were added to the cells which were then incubated at room temperature for 20 minutes. Following incubation, the cells were washed with 2 ml of PBS containing 5% FBS and then centrifuged at 2000 *g* for 5 minutes. Expression of MCHR1 was detected using C11CB, a rabbit polyclonal anti-human MCHR1 antibody (Section 4.2.3). Three microlitres of a 1:10 dilution of C11CB stock antiserum were added to the cells along with 50 µl of Reagent B of a CALTAG Fix and Perm® Cell Permeabilization Solution Kit (Caltag Laboratories) and the cells then incubated at 4°C for 30 minutes. Cells were washed again with 2 ml PBS/5% FBS and then centrifuged at 2000 *g* for 5 minutes. Cells were resupended in 50 µl of Reagent B of a CALTAG Fix and Perm® Cell Permeabilization Solution Kit along with 1 µl of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma) prior to incubation at 4°C for 30 minutes in the dark. After a final wash with 2 ml PBS/5% FBS, cells were analysed using a FACScan fluorescence activated cell sorter running CELLQuest acquisition and analysis software (Becton Dickinson, Oxford, UK). Overall, each cell line was analysed in duplicate in two separate experiments along with untransfected CHO-K1 cells.

5.2.3 Measurement of intracellular calcium levels by fluorimetry

The changes in intracellular calcium levels in untransfected CHO-K1 cells and pcMCHR1-transfected CHO-K1 cells when stimulated with MCH were measured using the fluorescent dye FURA-2/AM (Merck Biosciences, Nottingham, UK). FURA-2/AM is able to transverse the lipid bilayer of cells and, once inside, esterases react with the dye to produce the active derivative FURA-2 that is not longer membrane-permeable. The active dye FURA-2 is then able to bind intracellular calcium that can then be measured by fluorimetry (Metcalfe *et al.* 1998).

Initially, cells were plated out on glass coverslips in 6-well plates at $5.0x10^5$ cells per well in 2.5 ml of CHO-K1 culture medium and incubated at 37°C in a 5% CO_2 incubator for two days. Geneticin sulphate was included in the medium for culturing CHO-MCHR1 cells at 1 mg/ml. Subsequently, intracellular calcium was measured using a Kontron SFM 25 fluorimeter (Kontron Instruments Ltd, Watford, UK) as previously described (Metcalfe *et al.* 1998). Monolayers of cells (used at around 80% confluence by eye) were incubated with 4 μM FURA-2/AM for 30 minutes at 37°C in a 5% CO_2 incubator. The coverslips were then rinsed twice in balanced salt solution (BSS; 135 mM NaCl; 4.5 mM KCl; 1.5 mM $CaCl_2$; 0.5 mM $MgCl_2$; 5.6 mM glucose; 10 mM HEPES; pH 7.4) to remove any excess dye. The coverslips were placed across the diagonal of a 1-cm quartz cuvette being supported on a plastic bridge within the cuvette that allowed a small magnetic stirrer to be used to give a continuous mixing of the cuvette contents.

To ensure that the cells were loaded with FURA-2/AM, they were excited at 335 nm and emissions recorded between the wavelengths of 400 nm and 600 nm (Figure 5.1). The emission scan peaked at around 490 nm, indicating that the cells had taken up the dye and were fluorescing at this wavelength. Changes in intracellular calcium levels were detected by measuring changes in relative fluorescence intensity versus time by exciting cells at 335 nm and collecting emissions at 490 nm. Once a stable baseline of fluorescence was obtained by adding BSS to the cells, acute bolus additions of MCH (Alpha Diagnostics, San Antonio, TX, USA) were made at different concentrations ranging from 10^{-12} M to 10^{-7} M along with an adenosine

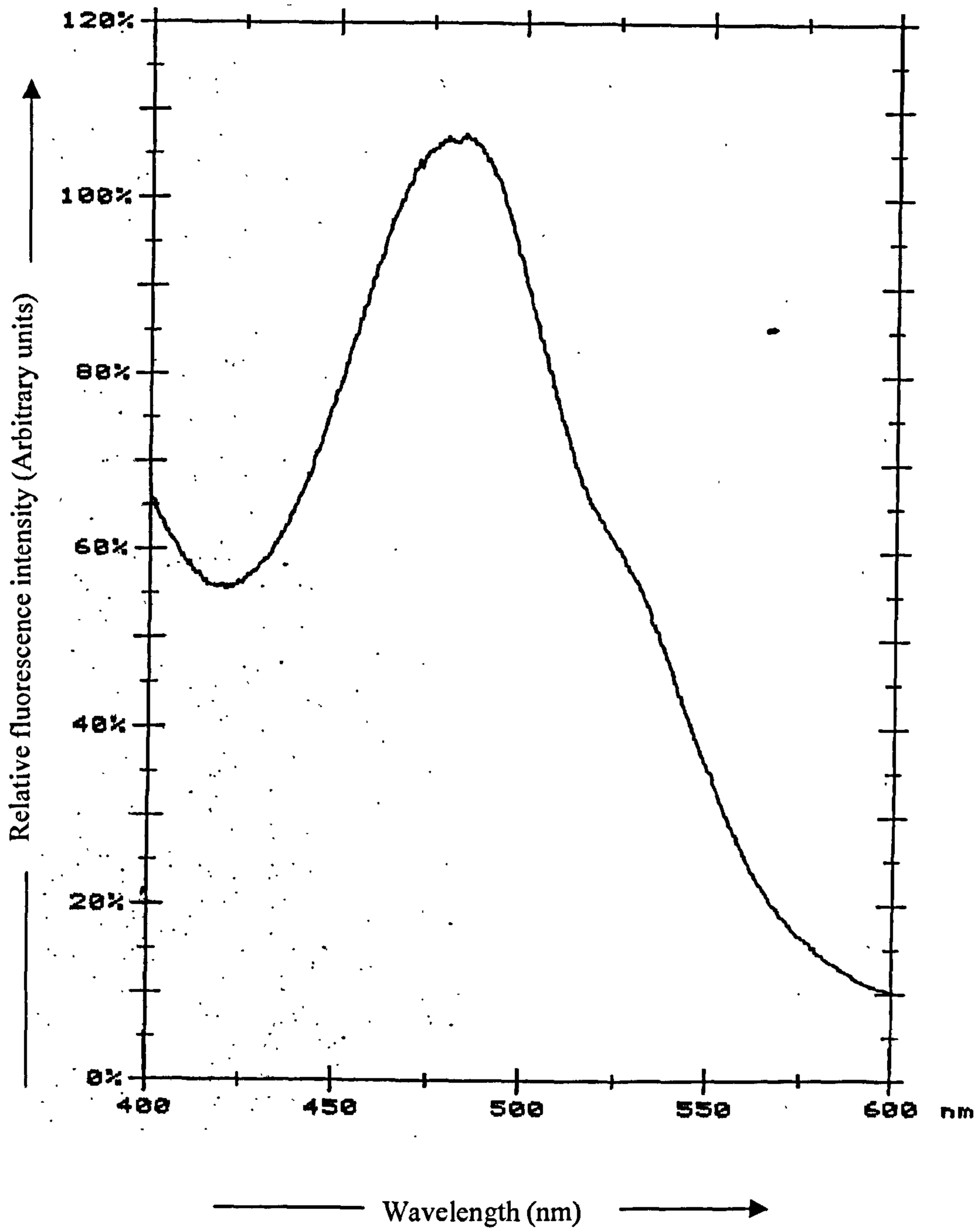


Figure 5.1 Emission scan for FURA-2/AM-loaded CHO-K1 cells. Cells cultured on glass coverslips were loaded with FURA-2/AM for 30 minutes and excess dye removed by washing with BSS. Cells were excited at 335 nm and emissions recorded between the wavelengths of 400 nm and 600 nm. Emissions are in arbitrary units of relative fluorescence intensity (y-axis). The emission scan peaks at 490 nm, indicating that cells have taken up the dye and are fluorescing at this wavelength.

derivative N6-(L-2-phenylisopropyl adenosine) (PIA) at 0.1 M (Sigma). In all cases, additions represented no more than 1-2% of the cuvette contents (3 ml).

In all, eight pcMCHR1-transfected cell lines that were shown to express the receptor by flow cytometry were analysed for changes in intracellular calcium levels when stimulated with MCH. Each cell line was tested in duplicate in at least two experiments with untransfected CHO-K1 cells as a control.

5.2.4 Measurement of cyclic AMP levels

Eight pcMCHR1-transfected cell lines that were shown to express the receptor by flow cytometry were analysed for changes in forskolin-stimulated cyclic AMP levels in response to MCH-stimulation. Each cell line was tested in duplicate in three experiments with untransfected CHO-K1 cells as a control. Initially, cells were plated out in 24-well plates at $5x10^4$ cells per well in 1 ml of CHO-K1 culture medium containing geneticin sulphate at 1 mg/ml and incubated at 37°C in a 5% CO_2 incubator for two days. Subsequently, the cells were washed once with PBS and incubated for 20 minutes at 37°C in 1 ml of cyclic AMP buffer comprising Hanks balanced salt solution (HBSS; 1.26 mM $CaCl_2$; 5.53 mM KCl; 0.44 mM KH_2PO_4; 0.5 mM $MgCl_2$; 0.41 mM $MgSO_4$ $7H_2O$; 4 mM $NaHCO_3$; 0.30 mM NaH_2PO_4; 5.6 mM glucose; 222 mM sucrose; 20 mM HEPES; pH 7.2), 0.1 % bovine serum albumin (Sigma) and 1 mM 3-isobutyl-1-methyl xanthine (Sigma). At the end of the incubation period, the cells were treated with either 20 μM forskolin (Sigma) alone or 20 μM forskolin in combination with a series of concentrations of MCH (10^{-10} to 10^{-7} M) and then incubated for 60 minutes at 37°C in a 5% CO_2 incubator. Finally, 100 μl of supernatant were taken from each well and cyclic AMP levels measured using a cyclic AMP Immunoassay Kit (R & D Systems, Abingdon, UK) according to the manufacturer's instructions.

One hundred microlitres of supernatant were diluted in 300 μl of assay buffer ED2 prior to running the assay. Standard dilutions (200, 50, 12.5, 3.12 and 0.78 pmoles/ml) of cyclic AMP were also made in ED2 assay buffer from the cyclic AMP standard stock (2000 pmoles/ml) provided with the kit. All reagents and samples were kept at room temperature before use. Each sample and standard was assayed in

duplicate. To a 96-well microtitre plate coated with a goat anti-rabbit polyclonal antibody, 100 µl of cyclic AMP standard dilutions (200, 50, 12.5, 3.12 and 0.78 pmoles/ml) and of diluted test sample were added. Fifty microlitres of cyclic AMP-conjugated to alkaline phosphatase were added to each well followed by 50 µl of rabbit polyclonal anti-cyclic AMP antibody. The 96-well plate was covered with an adhesive strip and incubated at room temperature for 2 hours on a horizontal microtitre plate shaker set at 500 rpm. At the end of the incubation, each well was decanted and washed three times with 200 µl of wash buffer. Two hundred microlitres of *p*-nitrophenyl phosphate substrate were applied to all wells and the plate incubated for one hour at room temperature on the bench without shaking. Fifty microlitres of stop solution were added to each well and the optical density of each well was measured immediately using a Labsystems Integrated EIA Management System (Labsystems, Basingstoke, UK) set to 405 nm.

5.2.5 Patients and controls

Immunoglobulin G samples from nine vitiligo patients (V1-V9; Table 2.1), that were previously shown to contain MCHR1 autoantibodies in a radiobinding assay (Kemp *et al.* 2002), were analysed in this study. In addition, IgG from nine vitiligo patients (V10-V18; Table 2.1), which were previously shown not to contain MCHR1 autoantibodies (Kemp *et al.* 2002), were analysed. In addition, IgG samples from ten SLE patients (1 male, 9 females; mean age: 45 years with range 21-55 years) and from 20 healthy individuals (9 males, 11 females; mean age: 32 years with range 24-48 years) with no history of either vitiligo or autoimmune disorders were analysed.

5.2.6 Isolation of IgG from sera

Immunoglobulin G was isolated from the serum samples by protein G Sepharose 4 Fast Flow affinity column chromatography as described in Section 4.2.2.

5.2.7 Cell culture

Untransfected CHO-K1 cells and CHO-MCHR1 cells were grown in CHO-K1 culture media as described in Section 2.20. The CHO-MCHR1 cells were grown in

culture media containing 1 mg/ml of geneticin sulphate. Initially, cells were plated out on glass coverslips in 6-well plates at $5.0x10^5$ cells per well in 2.5 ml of CHO-K1 culture medium and incubated at 37°C in a 5% CO_2 incubator for two days. Cells were then pre-incubated with either patient or control IgG as described below.

5.2.8 Effect of different concentrations of vitiligo patient and control IgG on the functioning of MCHR1

In order to study the effect of vitiligo patient autoantibodies on the functioning of the MCHR1, preliminary experiments were carried out to determine the minimum concentration of vitiligo patient IgG that can block the functioning of MCHR1. Initially, untransfected CHO-K1 cells and CHO-MCHR1 cells were pre-incubated with different concentrations of IgG (10, 50, 100, 150 and 300 µg/ml) from either vitiligo patients (n=4) or controls (n=4) for 30 minutes at 37°C in a 5% CO_2 incubator. Vitiligo patient IgGs used in the preliminary experiments contained MCHR1 autoantibodies (Kemp *et al.* 2002). Intracellular calcium levels were measured in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions using fluorimetry as described in Section 5.2.3. Each IgG sample at each concentration was tested in duplicate in at least two separate experiments with untransfected CHO-K1 cells as a control.

5.2.9 Effect of vitiligo patient, SLE patient and control IgG on the functioning of MCHR1

Once the minimum concentration of IgG (150 µg/ml) that could block the functioning of MCHR1 was determined, further experiments were carried out to examine the effects of vitiligo patient IgG on the functioning of MCHR1. CHO-MCHR1 cells were pre-incubated with 150 µg/ml of purified IgG from either vitiligo patients (n=18), SLE patients (n=10) or controls (n=20) for 30 minutes at 37°C in a 5% CO_2 incubator. Intracellular calcium levels were measured in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) addition using fluorimetry as described in Section 5.2.3. Each sample was tested in duplicate in at least two separate experiments.

5.2.10 Statistical analysis

The frequency of MCHR1 function-blocking autoantibodies was compared between vitiligo patients and controls using 2 x 2 contingency tables and chi-squared tests. Yates' correction was applied and p values <0.05 (two-tailed) were regarded as significant.

5.3 Results

5.3.1 Transfection of CHO-K1 cells with pcMCHR1

Plasmid pcMCHR1 was transfected into CHO-K1 cells as described in Section 5.2.1. Transfected cells were selected by growth in culture media containing geneticin at 1 mg/ml. Forty-eight geneticin-resistant cell lines were isolated and analysed by flow cytometry following staining with a rabbit polyclonal anti-human MCHR1 antibody C11CB, in order to confirm the expression of MCHR1. Receptor expression was also assessed by measuring changes in intracellular calcium levels following stimulation with MCH using fluorimetry and by measuring changes in forskolin-stimulated cyclic AMP levels in response to MCH-stimulation using a cyclic AMP immunoassay.

5.3.2 Flow cytometry analysis

Both untransfected CHO-K1 cells and 48 geneticin-resistant pcMCHR1-transfected cell lines were grown to confluence in T75 tissue culture flasks. The cells were permeabilized and receptor expression assessed by staining with rabbit polyclonal anti-human MCHR1 antibody C11CB and subsequently analysing the stained cells by flow cytometry as explained in Section 5.2.2. Each cell line was tested in duplicate in two separate experiments along untransfected CHO-K1 cells. The results revealed that 8/48 geneticin-resistant pcMCHR1-transfected CHO-K1 cell lines exhibited a higher level of MCHR1 expression compared to untransfected CHO-K1 cells (Figure 5.2). Transfected cells did not show any expression of MCHR1 if stained without permeabilization (data not shown) suggesting that antibody C11CB recognises an intracellular epitope only present in MCHR1-expressing cells.

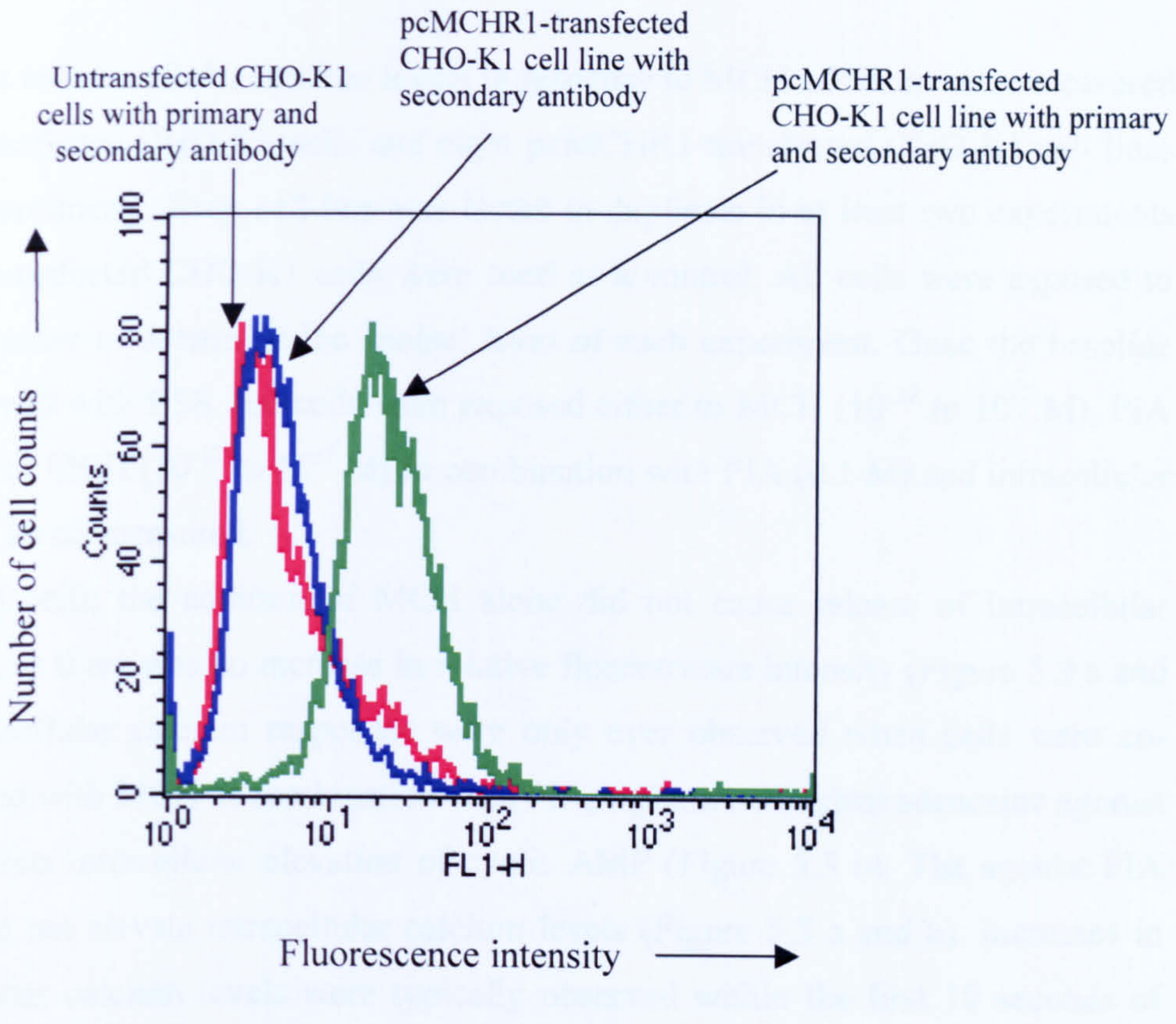


Figure 5.2 Flow cytometry analysis of CHO-K1 cells transfected with pcMCHR1. Assessment of MCHR1 expression was carried out by staining pcMCHR1-transfected cell lines and untransfected CHO-K1 cells with rabbit polyclonal anti-human MCHR1 antibody C11CB (primary antibody) and then with FITC-conjugated anti-rabbit IgG (secondary antibody). Subsequently, the stained cells were analysed by flow cytometry as described in Section 5.2.2. Results are shown for one transfected cell line and untransfected CHO-K1 cells. The increasing fluorescence intensity (*x*-axis) indicates that the geneticin-resistant pcMCHR1-transfected CHO-K1 cell line (green) stained with primary and secondary antibody exhibits a higher level of MCHR1 expression compared to geneticin-resistant pcMCHR1-transfected CHO-K1 cell line (blue) stained with secondary antibody alone and untransfected CHO-K1 cells (red) stained with primary and secondary antibody.

5.3.3 Measurement of intracellular calcium in untransfected CHO-K1 cells and pcMCHR1-transfected CHO-K1 cells

Changes in intracellular calcium levels in response to MCH additions were measured in untransfected CHO-K1 cells and eight pcMCHR1-transfected CHO-K1 cell lines using fluorimetry. Each cell line was tested in duplicate in at least two experiments and untransfected CHO-K1 cells were used as a control. All cells were exposed to BSS in order to determine the 'noise' level of each experiment. Once the baseline was formed with BSS, the cells were exposed either to MCH (10^{-12} to 10^{-7} M), PIA (0.1 M) or MCH (10^{-12} to 10^{-7} M) in combination with PIA (0.1 M) and intracellular calcium levels measured.

In all cells, the addition of MCH alone did not cause release of intracellular calcium, as there was no increase in relative fluorescence intensity (Figure 5.3 a and b). Intracellular calcium responses were only ever observed when cells were co-stimulated with MCH in combination with PIA, a pharmacological adenosine agonist that inhibits intracellular elevation of cyclic AMP (Figure 5.3 b). The agonist PIA alone did not elevate intracellular calcium levels (Figure 5.3 a and b). Increases in intracellular calcium levels were typically observed within the first 10 seconds of MCH and PIA addition to the transfected cells and responses returned to the original baseline level approximately 30 to 60 seconds thereafter (Figure 5.3 b). Changes in intracellular calcium responses were observed in pcMCHR1-transfected CHO-K1 cells in response to MCH addition at different concentrations ranging from 10^{-12} to 10^{-7} M along with PIA addition at 0.1 M concentration as indicated by an increase of relative fluorescence intensity (Figure 5.3 b). In contrast, changes in intracellular calcium levels were not observed in untransfected CHO-K1 cells in response to the addition of MCH and PIA, as there was no increase of relative fluorescence intensity (Figure 5.3 a). These results clearly indicate that pcMCHR1-transfected CHO-K1 cells express the receptor.

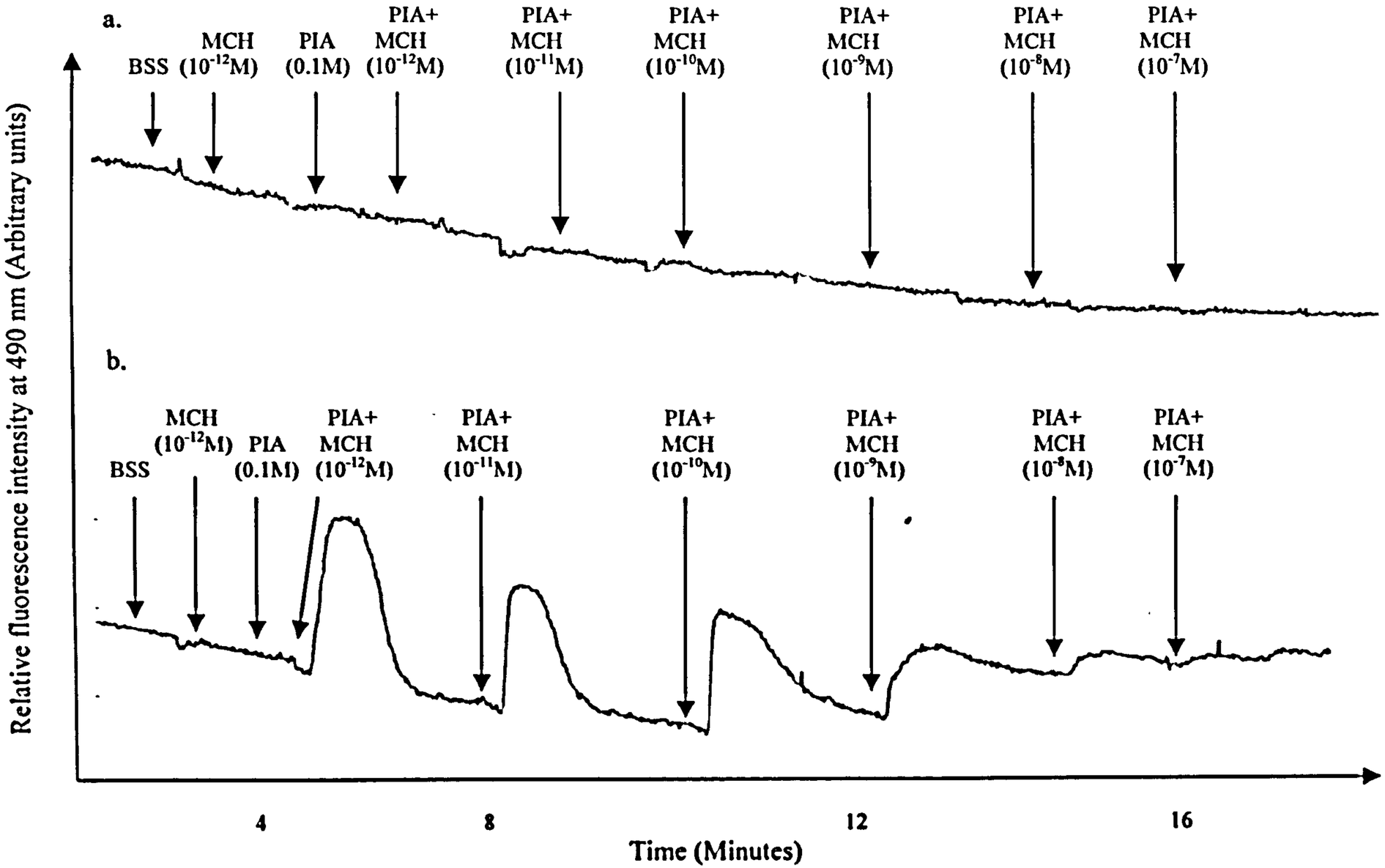

a.
BSS
MCH (10^{-12}M)
PIA (0.1M)
PIA+ MCH (10^{-12}M)
PIA+ MCH (10^{-11}M)
PIA+ MCH (10^{-10}M)
PIA+ MCH (10^{-9}M)
PIA+ MCH (10^{-8}M)
PIA+ MCH (10^{-7}M)
b.
BSS
MCH (10^{-12}M)
PIA (0.1M)
PIA+ MCH (10^{-12}M)
PIA+ MCH (10^{-11}M)
PIA+ MCH (10^{-10}M)
PIA+ MCH (10^{-9}M)
PIA+ MCH (10^{-8}M)
PIA+ MCH (10^{-7}M)
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (Minutes)

Figure 5.3 Changes in intracellular calcium levels in response to MCH-stimulation measured by fluorimetry in untransfected CHO-K1 cells and pcMCHR1-transfected CHO-K1 cells. (a) Measurement of intracellular calcium levels in untransfected CHO-K1 cells. There was no release of intracellular calcium in untransfected CHO-K1 cells in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as there was no increase in relative fluorescence intensity. (b) Measurement of intracellular calcium levels in pcMCHR1-transfected CHO-K1 cells. The results are shown for one transfected cell line. There was a release of intracellular calcium in pcMCHR1-transfected CHO-K1 cells in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as indicated by the increase in relative fluorescence intensity.

5.3.4 Measurement of cyclic AMP levels

To assess the functional activity of MCHR1, the effect of stimulation by MCH on forskolin-stimulated cyclic AMP levels in both untransfected and pcMCHR1-transfected CHO-K1 cells was analysed. Eight pcMCHR1-transfected CHO-K1 cell lines were examined with each cell line being tested in duplicate in three experiments with untransfected CHO-K1 cells as a control. Both untransfected CHO-K1 cells and pcMCHR1-transfected CHO-K1 cells were incubated with either 20 μM forskolin or 20 μM forskolin in combination with MCH at different concentrations (10^{-10} to 10^{-7} M). Cyclic AMP levels were then measured using a cyclic AMP Immunoassay Kit as described in Section 5.2.4. Forskolin directly activated adenylate cyclase to produce an increase in cyclic AMP in pcMCHR1-transfected CHO-K1 cells (Figure 5.4). Co-incubation of pcMCHR1-transfected CHO-K1 cells with forskolin and MCH inhibited forskolin-stimulated cyclic AMP production in a concentration-dependent manner (Figure 5.4). A maximum inhibition of cyclic AMP production was observed at 10^{-7} M MCH (Figure 5.4). In contrast, untransfected CHO-K1 cells did not show any inhibition of cyclic AMP levels when the cells were co-incubated with forskolin (20 μM) and MCH at different concentrations (10^{-10} to 10^{-7} M) (Figure 5.5).

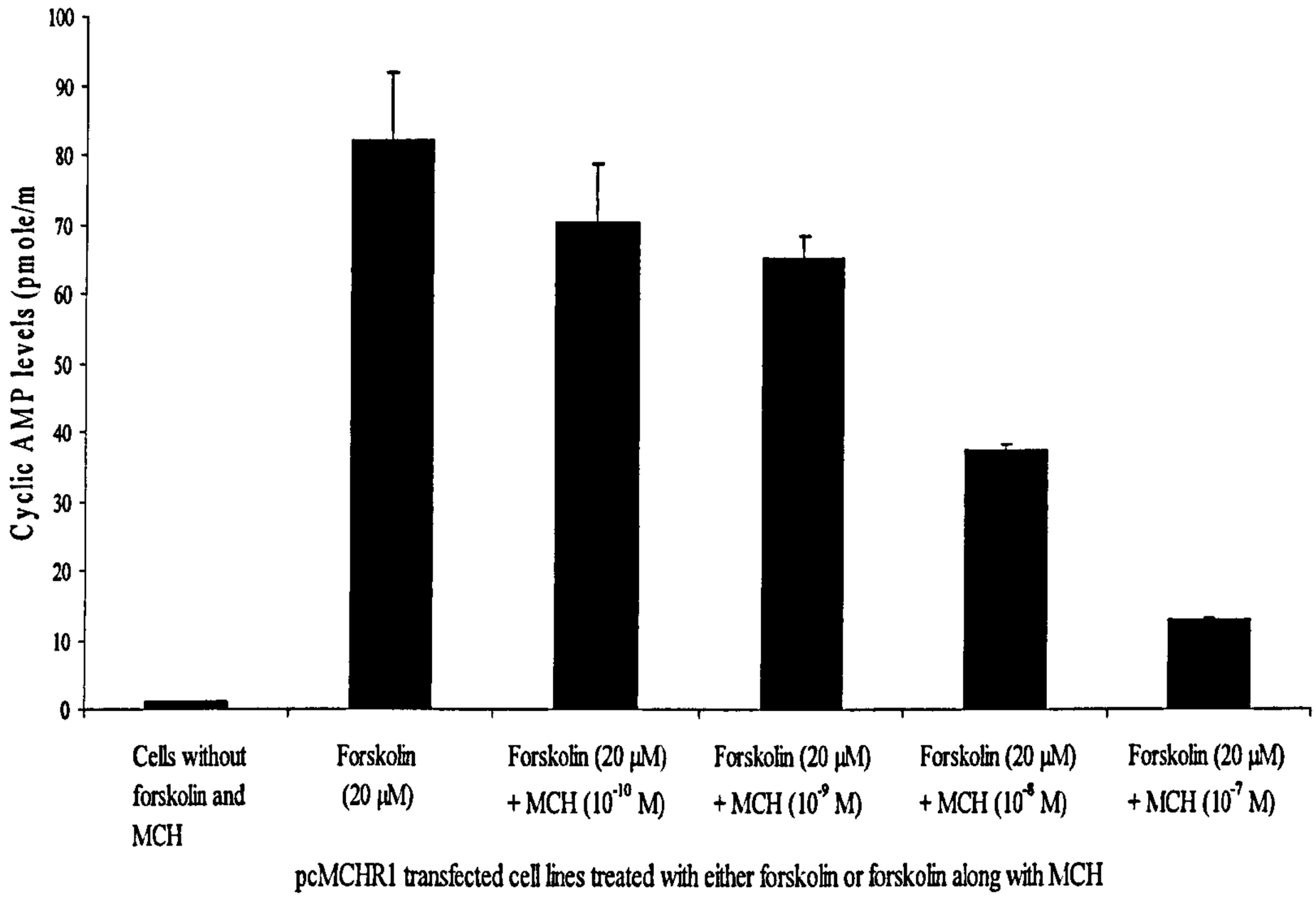
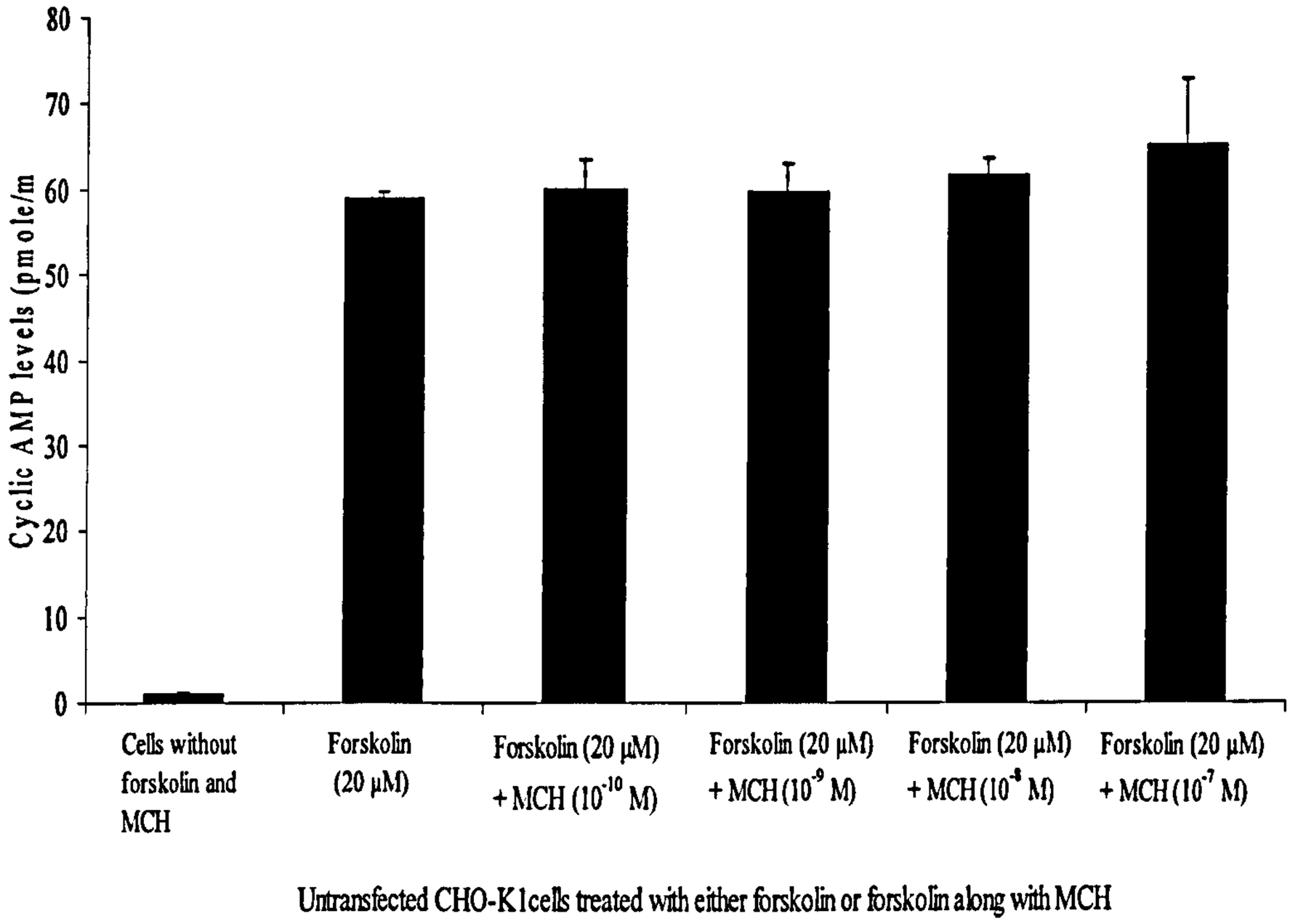


Figure 5.4 Effect of MCH on forskolin-stimulated cyclic AMP levels in pcMCHR1-transfected CHO-K1 cells. Forskolin was used as a positive control at a concentration of 20 μM. The cells were incubated with either forskolin alone or forskolin in combination with MCH at different concentrations (10^{-10} to 10^{-7} M). Forskolin directly activated adenylate cyclase to produce an increase in cyclic AMP in pcMCHR1-transfected CHO-K1 cells. Co-incubation of pcMCHR1-transfected CHO-K1 cells with forskolin and MCH inhibited forskolin-stimulated cyclic AMP production in a concentration-dependent manner. A maximum inhibition of cyclic AMP production was observed at 10^{-7} M MCH. The results show the mean values (± SE) of duplicate samples in one experiment for one pcMCHR1-transfected CHO-K1 cells.



Untransfected CHO-K1cells treated with either forskolin or forskolin along with MCH

Figure 5.5 Effect of MCH on forskolin-stimulated cyclic AMP levels in untransfected CHO-K1 cells. Forskolin was used as a positive control at a concentration of 20 μM. The cells were incubated with either forskolin alone or forskolin in combination with MCH at different concentrations (10^{-10} to 10^{-7} M). Forskolin directly activated adenylate cyclase to produce an increase in cyclic AMP in untransfected CHO-K1 cells. Co-incubation of untransfected CHO-K1 cells with forskolin and MCH did not inhibit forskolin-stimulated cyclic AMP production. The results show the mean values (± SE) of duplicate samples in one experiment for one untransfected CHO-K1 cells.

5.3.5 Effect of different concentrations of vitiligo patient and control IgG on the functioning of MCHR1

Preliminary experiments were carried out to determine the minimum concentration of vitiligo patient IgG that blocked the functioning of MCHR1. For this, both untransfected CHO-K1 cells and CHO-MCHR1 cells were pre-incubated with either control IgG (n=4) or vitiligo patient IgG (n=4) at different concentrations. Changes in intracellular calcium levels in response to MCH and PIA addition were then measured using fluorimetry. Each IgG sample at each concentration was tested in duplicate in at least two separate experiments with untransfected CHO-K1 cells as a control.

None of the untransfected CHO-K1 cells pre-incubated with different concentrations of either control or vitiligo patient IgG released any intracellular calcium in response to MCH and PIA addition, as there was no increase in relative fluorescence intensity (Figures 5.6 and 5.7). In contrast, when CHO-MCHR1 cells were pre-incubated with different concentrations of control IgG, there was a release of intracellular calcium in response to MCH and PIA addition, as there was an increase of relative fluorescence intensity (Figure 5.8).

Pre-incubation of CHO-MCHR1 cells with vitiligo patient IgG at 10, 50 and 100 µg/ml resulted in the release of intracellular calcium in response to MCH and PIA addition, as there was an increase of relative fluorescence intensity (Figure 5.9 a to c). In contrast, CHO-MCHR1 cells did not release any intracellular calcium in response to MCH and PIA addition when the cells were pre-incubated with vitiligo patient IgG at concentration of 150 and 300 µg/ml, as there was no increase of relative fluorescence intensity (Figure 5.9 d and e). These results indicated that pre-incubation of CHO-MCHR1 cells with vitiligo patient IgG at concentrations of 150 and 300 µg/ml blocked the functioning of MCHR1.

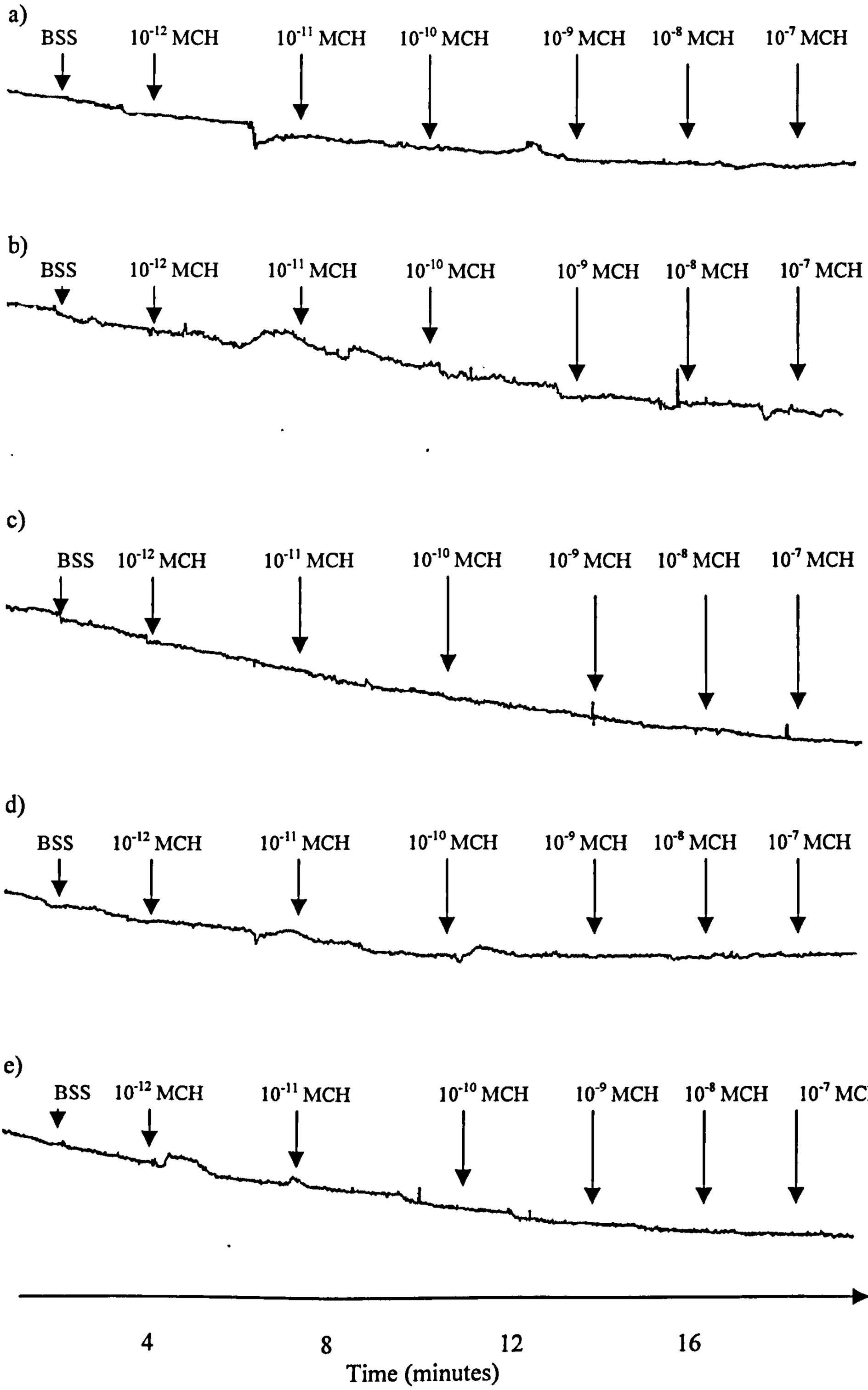
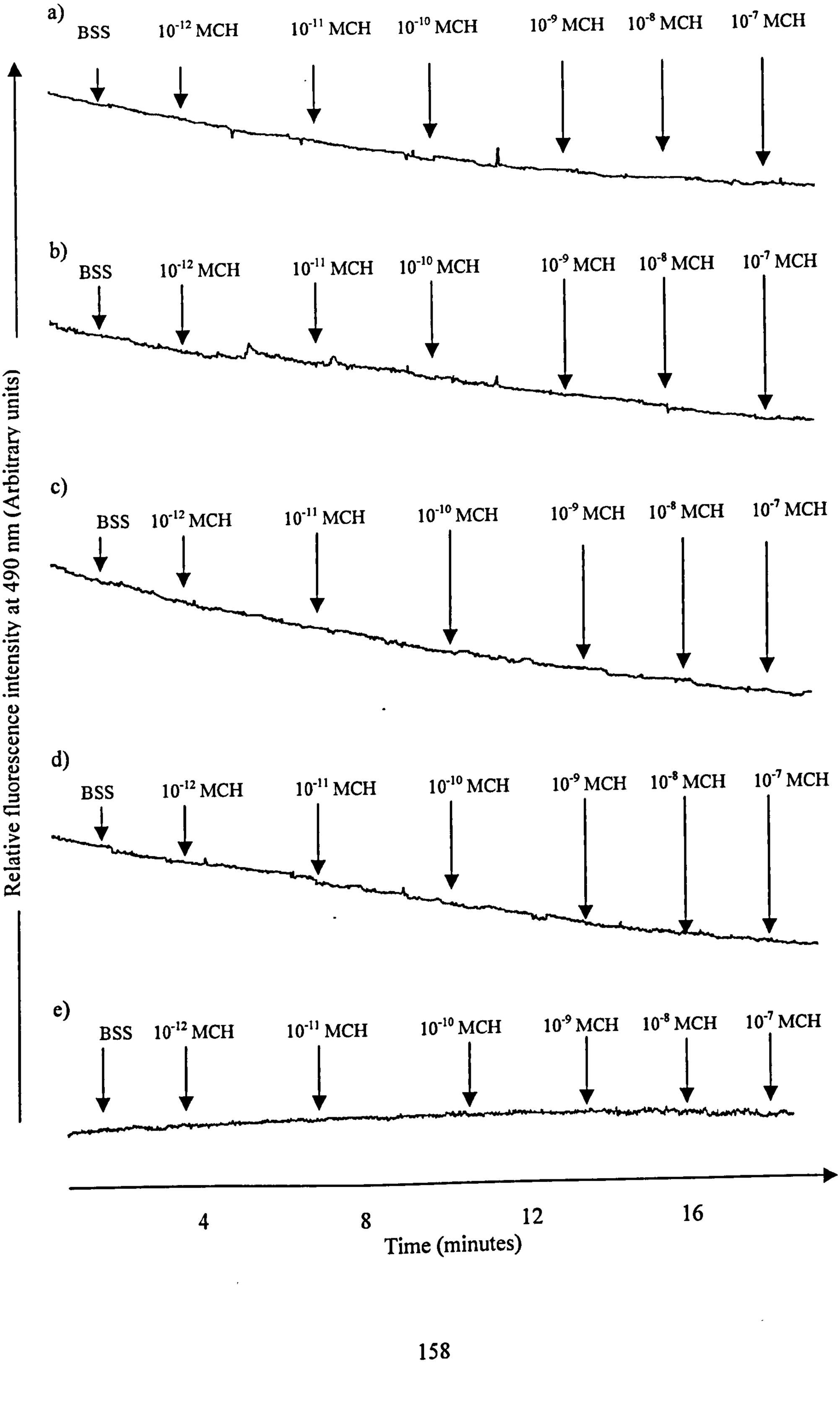

a)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
e)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.6 Effect of different concentrations of control IgG on untransfected CHO-K1 cells. Measurement of intracellular calcium levels in response to MCH in untransfected CHO-K1 cells pre-incubated with different concentrations of control IgG at (a) 10 μg/ml (b) 50 μg/ml (c) 100 μg/ml (d) 150 μg/ml (e) 300 μg/ml. The results are shown for one control IgG sample. Each concentration of IgG was tested in duplicate in at least two experiments. There was no release of intracellular calcium in untransfected CHO-K1 cells pre-incubated with different concentrations of IgG (10, 50, 100, 150 and 300 μg/ml) in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions, as there was no increase in relative fluorescence intensity.


a)
BSS
10-12 MCH
10-11 MCH
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10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
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10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
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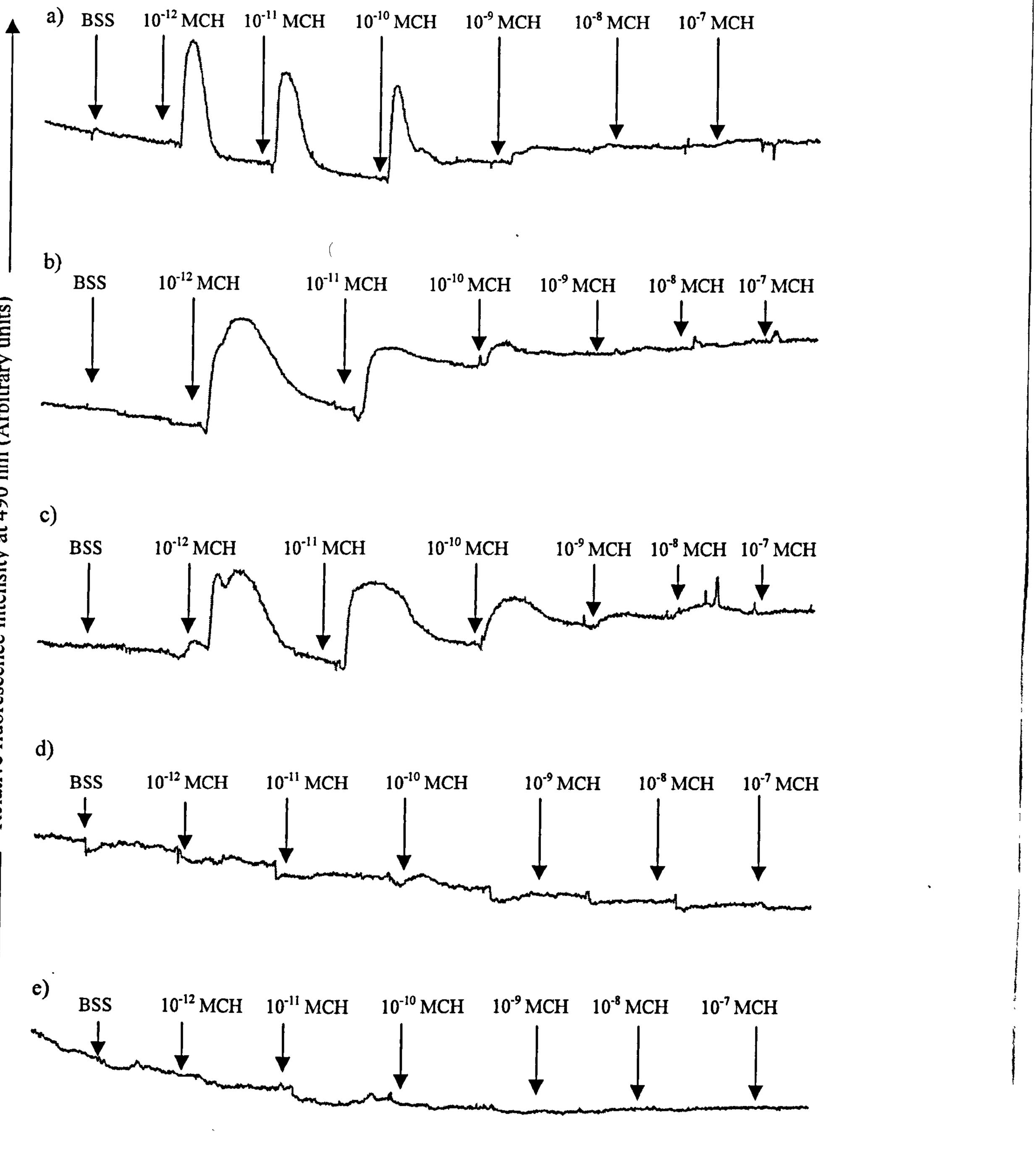
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
e)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.7 Effect of different concentrations of vitiligo patient IgG on untransfected CHO-K1 cells. Measurement of intracellular calcium levels in response to MCH in untransfected CHO-K1 cells pre-incubated with different concentrations of vitiligo patient IgG at (a) 10 μg/ml (b) 50 μg/ml (c) 100 μg/ml (d) 150 μg/ml (e) 300 μg/ml. The results shown are for one vitiligo patient IgG sample. Each concentration of IgG was tested in duplicate in at least two experiments. There was no release of intracellular calcium in untransfected CHO-K1 cells pre-incubated with different concentrations of IgG (10, 50, 100, 150 and 300 μg/ml) in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as there was no increase in relative fluorescence intensity.

a)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
e)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.8 Effect of different concentrations of control IgG on the functioning of MCHR1 in CHO-MCHR1 cells. Measurement of intracellular calcium levels in response to MCH in CHO-MCHR1 cells pre-incubated with different concentrations of control IgG at (a) 10 μg/ml (b) 50 μg/ml (c) 100 μg/ml (d) 150 μg/ml (e) 300 μg/ml. The results shown are for one control IgG sample. Each concentration of IgG was tested in duplicate in at least two experiments. There was a release of intracellular calcium in CHO-MCHR1 cells pre-incubated with different concentrations of IgG (10, 50, 100, 150 and 300 μg/ml) in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as indicated by the increase in relative fluorescence intensity.


a)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
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10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
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10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
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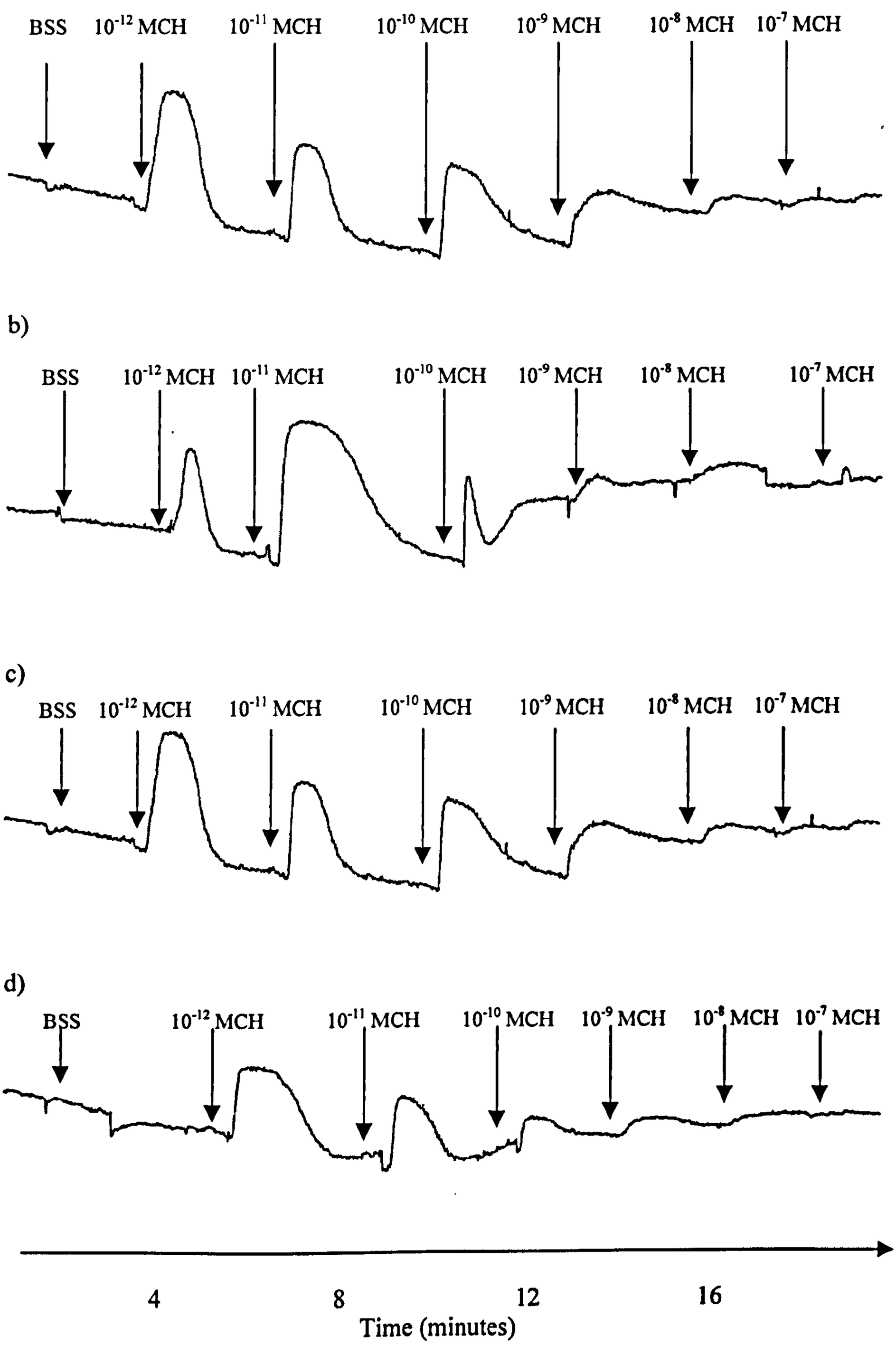
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
e)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.9 Effect of different concentrations of vitiligo patient IgG on the functioning of MCHR1 in CHO-MCHR1 cells. Measurement of intracellular calcium levels in response to MCH in CHO-MCHR1 cells pre-incubated with different concentrations of vitiligo patient IgG at (a) 10 µg/ml (b) 50 µg/ml (c) 100 µg/ml (d) 150 µg/ml (e) 300 µg/ml. The results shown are for one vitiligo patient IgG sample. Each concentration of IgG was tested in duplicate in at least two experiments. There was a release of intracellular calcium in CHO-MCHR1 cells pre-incubated with different concentrations of IgG (10, 50 and 100 µg/ml) in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as indicated by the increase in relative fluorescence intensity. There was no release of intracellular calcium in CHO-MCHR1 cells pre-incubated with different concentrations of IgG (150 and 300 µg/ml) in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions, as there was no increase in relative fluorescence intensity.

5.3.6 Effect of vitiligo patient and control IgG on the functioning of MCHR1

Once the minimum concentration of IgG required to block the functioning of MCHR1 was determined as 150 µg/ml, IgG samples from eighteen vitiligo patients and twenty healthy controls were tested for their ability to block the functioning of MCHR1. CHO-MCHR1 cells were pre-incubated with IgG at 150 µg/ml and changes in intracellular calcium levels in response to MCH and PIA additions were measured by fluorimetry. Each IgG sample was tested in duplicate in at least two experiments.

Of twenty control IgG samples analysed, none blocked the release of intracellular calcium in response to MCH and PIA additions, as there was an increase of relative fluorescence intensity (Figure 5.10; Table 5.1). Of eighteen vitiligo patient IgG samples tested, eight had no effect upon the release of intracellular calcium in response to MCH and PIA additions, as there was an increase in relative fluorescence intensity (Figure 5.11; Table 5.2). In contrast, 10/18 vitiligo patient IgG samples blocked the release of intracellular calcium in response to MCH and PIA additions, as there was no increase in relative fluorescence intensity (Table 5.2; Figure 5.12). These results indicated that 10/18 (56%) of the vitiligo patient IgG samples tested were able to block the functioning of MCHR1. Seven (V1 to V4 and V10 to V12) of the vitiligo patient IgGs that blocked MCHR1 function were able to do so at a concentration of 150 µg/ml (Table 5.2), and three vitiligo patient IgGs (V5 to V7) blocked MCHR1 function only at a concentration of 300 µg/ml (Table 5.2). Among the nine vitiligo patients that contained MCHR1 binding autoantibodies as measured in a radiobinding assay (Kemp *et al.* 2002), seven (V1 to V7; Table 5.2) contained MCHR1 autoantibodies that blocked receptor function. In addition, of nine patients that were previously shown not to contain MCHR1 binding autoantibodies (Kemp *et al.* 2002), three (V10 to V12; Table 5.2) were demonstrated to have MCHR1 function blocking autoantibodies.


a)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
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10-11 MCH
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10-7 MCH
c)
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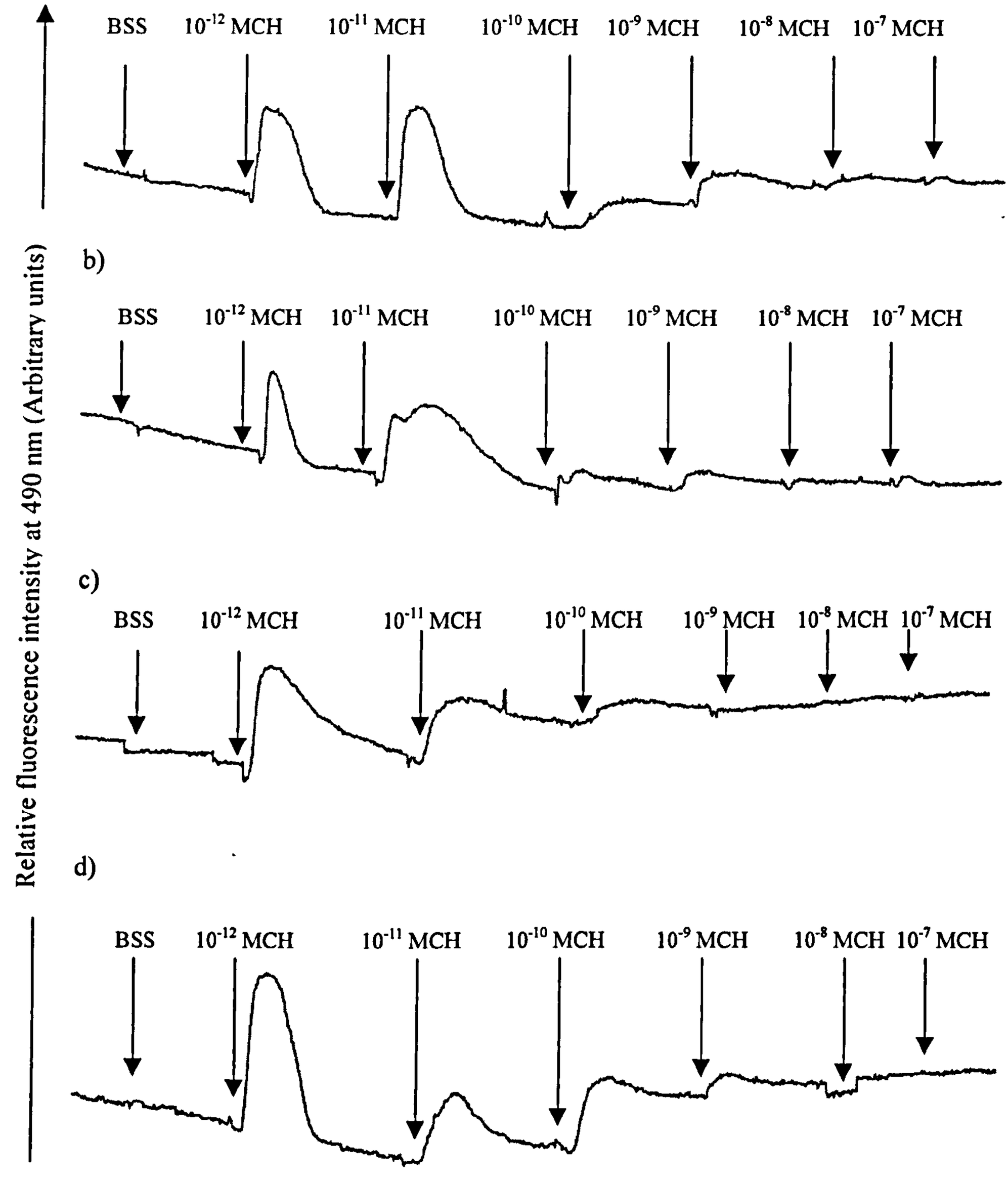
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.10 Effect of control IgG on the functioning of MCHR1 in CHO-MCHR1 cells. Measurement of intracellular calcium levels in response to MCH in CHO-MCHR1 cells pre-incubated with control IgG at 150 μg/ml. The results shown are for one control IgG sample. Each IgG sample was tested in duplicate in at least two experiments. Results (a) and (b) represent one control IgG sample in duplicate. Results (c) and (d) represent the same sample in duplicate in a separate experiment. There was a release of intracellular calcium in CHO-MCHR1 cells pre-incubated with control IgG in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions, as indicated by the increase in relative fluorescence intensity.

Table 5.1 Effect of control IgG on the functioning of MCHR1.

Sample	Concentration of IgG used (µg/ml)	Number of times sample tested	Number of times intracellular calcium released after pre-incubation with IgG	IgG blocking MCHR1 function
C1	150	4	4	No
C2	150	4	3	No
C3	150	4	3	No
C4	150	4	4	No
C5	150	4	4	No
C6	150	4	4	No
C7	150	4	3	No
C8	150	4	3	No
C9	150	4	3	No
C10	150	4	3	No
C11	150	4	3	No
C12	150	4	3	No
C13	150	4	4	No
C14	150	4	4	No
C15	150	4	4	No
C16	150	4	4	No
C17	150	4	4	No
C18	150	4	4	No
C19	150	4	4	No
C20	150	4	4	No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BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
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10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.11 Effect of vitiligo patient IgG on the functioning of MCHR1 in CHO-MCHR1 cells. Measurement of intracellular calcium levels in response to MCH in CHO-MCHR1 cells pre-incubated with vitiligo patient IgG at 150 µg/ml. The results shown are for one vitiligo patient IgG sample. Each IgG sample was tested in duplicate in at least two experiments. Results (a) and (b) represent one vitiligo IgG sample in duplicate. Results (c) and (d) represent the same sample in duplicate in a separate experiment. There was a release of intracellular calcium in CHO-MCHR1 cells pre-incubated with vitiligo patient IgG in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M), additions as indicated by increase in relative fluorescence intensity.

Table 5.2 Effect of vitiligo IgG on the functioning of MCHR1.

IgG Sample[1]	Concentration of IgG used (µg/ml)	Number of times sample tested	Number of times intracellular calcium released after pre-incubaion with IgG	IgG blocking MCHR1 function
V1	150	4	0	Yes
V2	150	4	0	Yes
V3	150	4	1	Yes
V4	150	4	0	Yes
V5[2]	150	4	3	No
	300	4	0	Yes
V6[2]	150	4	3	No
	300	4	1	Yes
V7[2]	150	4	2	Yes/No
	300	4	0	Yes
V8	150	4	4	No
V9	150	4	4	No
V10	150	4	0	Yes
V11	150	4	0	Yes
V12	150	4	0	Yes
V13	150	4	3	No
V14	150	4	4	No
V15	150	4	4	No
V16	150	4	3	No
V17	150	4	4	No
V18	150	4	4	No

[1]IgG from vitiligo patients containing MCHR1 autoantibodies (V1 to V9); IgG from vitiligo patients negative for autoantibody reactivity to MCHR1 (V10 to V18).

[2]Vitiligo patient IgG samples that blocked MCHR1 function only at an IgG concentration of 300 µg/ml.

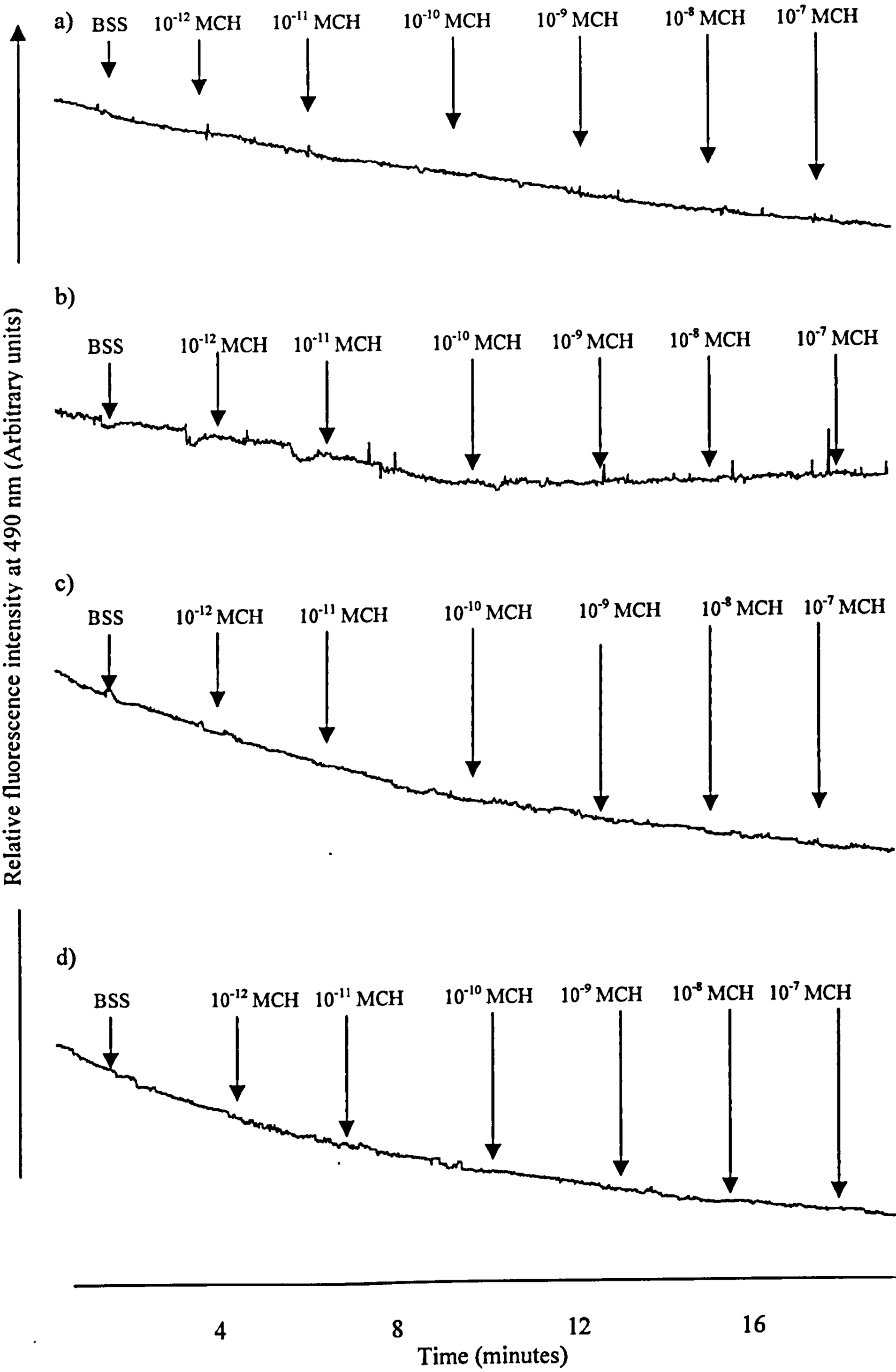
a)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.12 Effect of vitiligo patient IgG on the functioning of MCHR1 in CHO-MCHR1 cells. Measurement of intracellular calcium levels in response to MCH in CHO-MCHR1 cells pre-incubated with vitiligo patient IgG at 150 µg/ml. The results shown are for one vitiligo patient IgG sample. Each IgG sample was tested in duplicate in at least two experiments. Results (a) and (b) represent one vitiligo IgG sample in duplicate. Results (c) and (d) represent the same sample in duplicate in a separate experiment. There was no release of intracellular calcium in CHO-MCHR1 cells pre-incubated with vitiligo patient IgG in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as there was no increase in relative fluorescence intensity.

5.3.7 Effect of SLE patient IgG on the functioning of MCHR1

Systemic lupus erythematosus patient IgG samples were used as controls. These individuals generally display a wide variety of autoantibody reactivity. Ten SLE patient IgG samples were tested for MCHR1 function blocking reactivity. Each sample was tested in duplicate in at least two separate experiments (Table 5.3). CHO-MCHR1 cells were pre-incubated with SLE patients IgG at 150 µg/ml. Changes in intracellular calcium levels in response to MCH and PIA additions were then measured using fluorimetry. None of SLE patients IgG samples tested were able to block the release of intracellular calcium in response to MCH and PIA additions, as there was an increase in relative fluorescence intensity (Figure 5.13). These results demonstrate that SLE IgG do not block the functioning of MCHR1 and show that MCHR1 function blocking autoantibodies are specific to patients with vitiligo.

5.3.8 Comparison of the frequency of MCHR1 function blocking autoantibodies in patient and control groups

Overall, MCHR1 function blocking IgGs were found at a significantly increased frequency in the vitiligo patients compared with healthy controls (p=0.0004; Table 5.4). Comparison of healthy controls with vitiligo patient IgG samples containing MCHR1 autoantibodies indicated that there was a significantly increased frequency of MCHR1 function blocking IgGs in the vitiligo patient group (p<0.0001; Table 5.4). Similarly, when comparing healthy controls and vitiligo patient IgG samples not containing MCHR1 autoantibodies, a significant increase in the frequency of function blocking IgGs was noted in the vitiligo patient cohort (p=0.0387; Table 5.4). In contrast, there was no apparent difference in the frequency of MCHR1 function blocking autoantibodies in SLE patients compared with controls (Table 5.4).

Table 5.3 Effect of SLE IgG on the functioning of MCHR1.

Sample	Concentration of IgG used (µg/ml)	Number of times sample tested	Number of times intracellular calcium released after pre-incubation with IgG	IgG blocking MCHR1 function
S1	150	4	4	No
S2	150	4	4	No
S3	150	4	4	No
S4	150	4	4	No
S5	150	4	4	No
S6	150	4	4	No
S7	150	4	4	No
S8	150	4	3	No
S9	150	4	3	No
S10	150	4	3	No

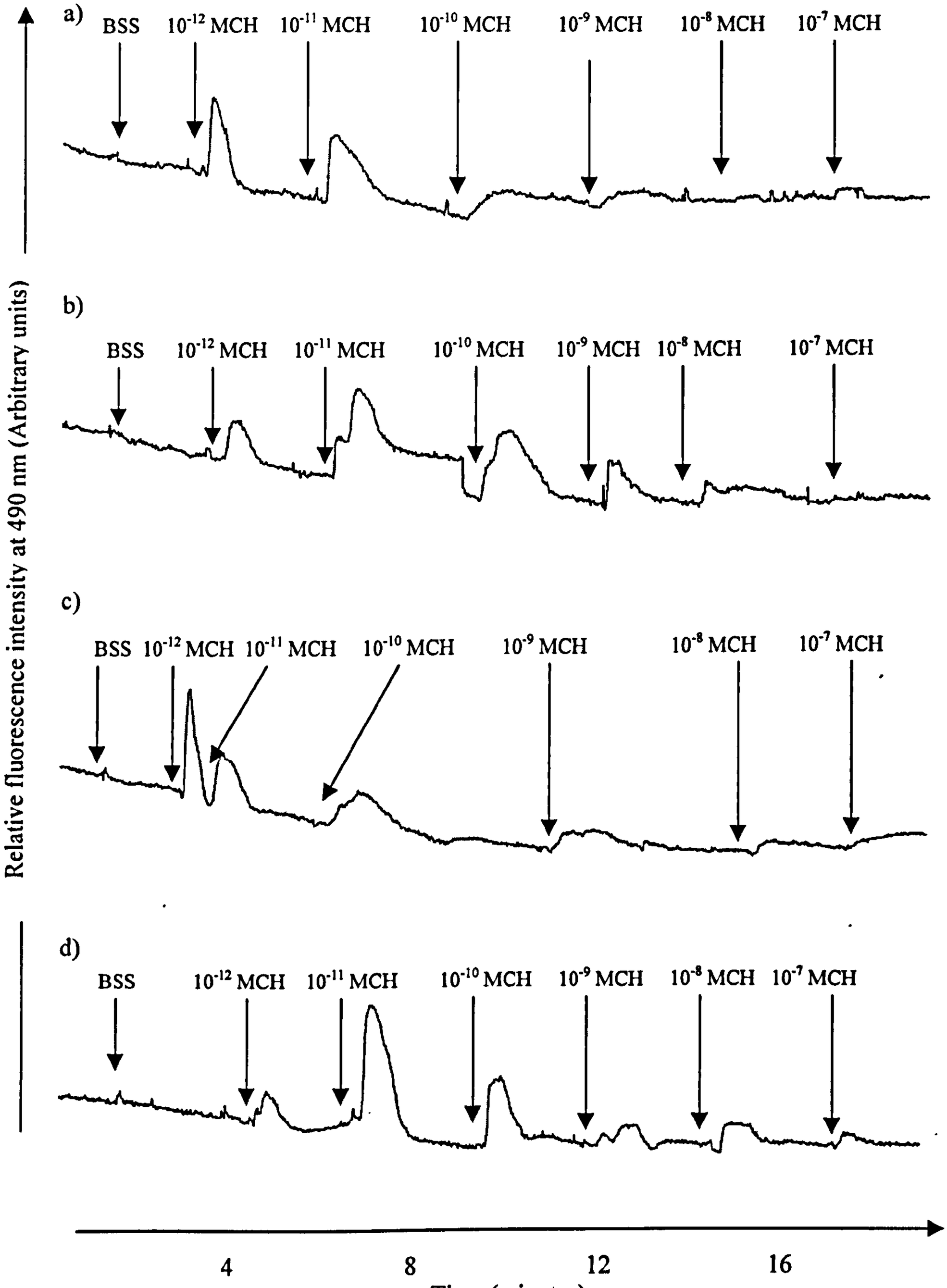
a)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
b)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
c)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
d)
BSS
10-12 MCH
10-11 MCH
10-10 MCH
10-9 MCH
10-8 MCH
10-7 MCH
Relative fluorescence intensity at 490 nm (Arbitrary units)
4
8
12
16
Time (minutes)

Figure 5.13 Effect of SLE patient IgG on the functioning of MCHR1 in CHO-MCHR1 cells. Measurement of intracellular calcium levels in response to MCH in CHO-MCHR1 cells pre-incubated with SLE patient IgG at 150 µg/ml. The results shown are for one SLE patient IgG sample. Each IgG sample was tested in duplicate in at least two experiments. Results (a) and (b) represent one SLE IgG sample in duplicate. Results (c) and (d) represent the same sample in duplicate in a separate experiment. There was a release of intracellular calcium in CHO-MCHR1 cells pre-incubated with SLE patient IgG in response to MCH (10^{-12} to 10^{-7} M) and PIA (0.1 M) additions as there was an increase in relative fluorescence intensity.

Table 5.4 Summary of results of the effects of IgG on the functioning of MCHR1.

IgG samples	Number of samples tested	Number of samples blocking MCHR1 function	*p* value[3]
Total vitiligo	18	10	0.0004
Vitiligo[1]	9	7	<0.0001
Vitiligo[2]	9	3	0.0387
Controls	20	0	-
SLE	10	0	-

[1]IgG samples from vitiligo patients containing MCHR1 autoantibodies (Kemp *et al.* 2002).

[2]IgG samples from vitiligo patients that do not contain MCHR1 autoantibodies (Kemp *et al.* 2002).

[3]The frequency of MCHR1 function-blocking autoantibodies was compared between patient and control groups using 2 x 2 contingency tables and chi-squared tests. Yates' correction was applied and p values <0.05 (two-tailed) were regarded as significant.

5.4 Discussion

In the current study, initially a stable CHO-K1 cell line has been established that expresses functional MCHR1. The results of flow cytometry analysis clearly showed the expression of MCHR1 in pcMCHR1-transfected CHO-K1 cells. To verify if the receptor was functional, the effects of MCH on forskolin-stimulated cyclic AMP production and calcium signalling pathways were analysed.

Intracellular calcium levels were increased in pcMCHR1-transfected CHO-K1 cells following the addition of MCH at different concentrations (10^{-12} to 10^{-7} M) and PIA (0.1 M). In contrast, the untransfected cells did not release any intracellular calcium in response to MCH and PIA additions. Although previous *in vitro* studies demonstrated the release of intracellular calcium by MCH addition alone in MCHR1 transfected cells (Saito *et al.* 1999; Chambers *et al.* 1999; Hawes *et al.* 2000; Hoogduijn *et al.* 2002; Verlaet *et al.* 2002), in the current study, the transfected cells only responded to MCH in combination with PIA, a very specific adenosine agonist that inhibits the activation of adenylate cyclase and elevation of cyclic AMP. These findings essentially confirm the co-operativity first reported by Yanagita *et al.* (1996) for adenosine and TSH in studies on individual human thyroid cells. Later, Metcalfe *et al.* (1998) followed the same approach in CHO-K1 cells expressing the TSHR and demonstrated a calcium response to TSH when using PIA to inhibit the TSH-stimulated elevation of cyclic AMP. In their study, the majority of cells gave a small increase in calcium in response to TSH alone, and this was greatly enhanced by prior addition of PIA illustrating mutual co-operativity in the behaviour of these agonists. Recently, a similar method was used to measure intracellular calcium levels in human keratinocytes in response to α-MSH, MSH 11-13 KPV and ACTH (Elliott *et al.* 2004). Keratinocytes only responded and released intracellular calcium following the addition of PIA along with the peptide.

As previously reported (Chambers *et al.* 1999; Lembo *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999; Hawes *et al.* 2000; Verlaet *et al.* 2002), forskolin-stimulated cyclic AMP production was decreased in MCHR1-expressing cells, in response to MCH. This is in contrast to the effects of α-MSH that acts via the MC-1R to increase the production of cyclic AMP and subsequently melanogenesis (Hunt *et al.* 1994). The down-regulation of the cyclic AMP pathway by MCH in transfected cells is, therefore, antagonistic to the actions of α-MSH on

melanogenesis. In this respect, MCH resembles the agouti protein that acts as an inhibitor of melanogenesis in rodents by blocking the action of α-MSH (Lu *et al.* 1994; Graham *et al.* 1997). The MCH could function in a similar way to regulate melanogenesis in human melanocytes, but this has yet to be determined.

Taken together, the functional studies indicate that the pcMCHR1-transfected CHO-K1 cells express the receptor. The stable cell line was used for further studies to investigate whether the MCHR1 antibodies in vitiligo patients have any effects upon the functioning of the receptor.

Vitiligo patient IgGs that blocked the functioning of MCHR1 were detected in 56% (10/18) of the vitiligo patient IgGs tested. Overall, when comparing healthy controls and vitiligo patients, MCHR1 function blocking IgGs were found at a significantly increased frequency in the vitiligo patient group (p=0.0004). Comparison of healthy controls and vitiligo patients containing MCHR1 autoantibodies (Kemp *et al.* 2002), indicated that there was a significantly increased frequency of receptor function blocking IgGs in the vitiligo patient group (p<0.0001). Similarly, the comparison of healthy controls and vitiligo patients that did not to contain MCHR1 autoantibodies (Kemp *et al.* 2002), demonstrated that there was a significantly increased frequency of function blocking IgGs in the vitiligo patient group (p=0.0387).

Among the nine vitiligo patients that contained MCHR1 binding autoantibodies as measured in a radio-binding assay (Kemp *et al.* 2002), seven contained MCHR1 autoantibodies that blocked receptor function. In the two patients that only contained receptor-binding autoantibodies, it is probable that the MCHR1 autoantibodies bind at a site on the receptor that has no effect upon its functionality. In addition, of nine patients that were previously shown not to contain MCHR1 binding autoantibodies (Kemp *et al.* 2002), three were demonstrated to have MCHR1 function blocking autoantibodies. The existence of antibodies that appear to inhibit receptor function but do not bind MCHR1 could be due to a number of reasons. It may be that the *in vitro*-translated protein used in the radio-binding assay (Kemp *et al.* 2002) fails to bind these antibodies due to incorrect folding of the molecule, the absence of a correct pattern of glycosylation or may simply reflect differential detection sensitivity in the two assays. This is not unprecedented, as similar findings have been documented for TSAb. In one study, one third of Graves' disease sera positive in a

TSH binding inhibiting immunoglobulin assay (TBII) failed to bind to *in vitro*-translated TSH receptor (Morgenthaler *et al.* 1999).

None of the control IgG samples or SLE patient IgGs analysed was able to block the function of MCHR1, indicating that MCHR1 function blocking autoantibodies are specific to patients with vitiligo and are not present in patients with another autoimmune disease.

Similar to our finding that MCHR1 autoantibodies in vitiligo patients can block the function of MCHR1, non-stimulatory TBAb can prevent TSH action resulting in hypothyroidism (Arikawa *et al.* 1985; McKenzie and Zakarija 1992; Kosugi *et al.* 1993). In Addison's disease, patient IgGs can block the *in vitro* action of ACTH on adrenal growth and cortisol production by binding to its receptor (Wulffraat *et al.* 1989). Furthermore, autoantibodies in myasthenia gravis patients directed against the acetylcholine receptor block the acetylcholine-binding site and provoke accelerated receptor degradation (Hara *et al.* 1993; Eymard and Chillet 1997; Hoedemaekers *et al.* 1997; Ferrero *et al.* 1997; Fabien *et al.* 2001). In addition, it has been demonstrated that serum immunoglobulin in patients with pernicious anaemia can block the gastrin-stimulation of acid secretion and it has been suggested that this blocking activity is mediated by competition with gastrin for the surface receptors on parietal cells (de Aizpurua *et al.* 1985; Burman *et al.* 1989).

Here, we have shown that MCHR1 autoantibodies in vitiligo patients can have a blocking effect upon the functioning of the receptor. However, the effect of MCHR1 autoantibodies on the behaviour of melanocytes has yet to be determined and the relevance of MCHR1 autoantibodies to the aetiology of vitiligo needs to be further investigated.

6 An Investigation into the Immunological Functions of Melanin-Concentrating Hormone Receptor 1 Autoantibodies

6.1 Introduction

Although the exact role of anti-melanocyte autoantibodies in the pathogenesis of vitiligo has not been determined, possible mechanisms by which they can destroy pigment cells have been investigated including complement activation and ADCC. The complement system has been described in Section 1.4.4.2 and examples of autoimmune disorders in which complement can have a pathogenic role have been discussed previously (Section 1.4.4.2). Complement-fixing autoantibodies that bind to melanocytes have been reported in patients with mucocutaneous candidiasis, multiple endocrine insufficiencies, alopecia areata and vitiligo (Hertz *et al.* 1977). However, these autoantibodies were not found in patients with isolated vitiligo (Howanitz *et al.* 1981). Deposition of complement component C3 can occur in the basement membrane of vitiliginous skin (Uda *et al.* 1984) and the sera of vitiligo patients can cause direct cytotoxic damage of cultured melanocytes by complement fixation (Norris *et al.* 1988b). In addition, a reduced expression of the membrane regulators of complement activation in both lesional and perilesional keratinocytes and melanocytes has been reported, possibly indicating an increased vulnerability of these cells to autologous complement attack (van den Wijngaard *et al.* 2002). The mechanism of ADCC and examples of autoimmune disorders in which this can play a pathogenic role are discussed in Section 1.4.4.3. With respect to vitiligo, there is evidence that the sera of patients with the disease can induce cultured human melanocytes to detach from their cell matrix and can cause direct cytotoxic damage by ADCC (Norris *et al.* 1988b).

6.2 Aim

The aim of the current study was to examine the immunological reactivities of autoantibodies to MCHR1 using a stable CHO-K1 cell line expressing the receptor.

The ability of MCHR1 autoantibodies to fix complement and mediate ADCC were assessed by flow cytometry and in chromium-release assays, respectively.

6.3 Materials and Methods

6.3.1 Patients and controls

In the complement-fixation assay, sera from the following subjects were analysed: nine vitiligo patients (V1-V9; Table 2.1), that were previously shown to contain MCHR1 autoantibodies in a radio-binding assay (Kemp *et al.* 2002), nine vitiligo patients (V10-V18; Table 2.1), that were previously shown not to contain MCHR1 autoantibodies (Kemp *et al.* 2002) and eighteen healthy controls (7 males, 11 females; mean age: 34 years with range 24-48 years) with no history of either vitiligo or autoimmune disorders. In the ADCC assay, sera from nine vitiligo patients (V1-V9; Table 2.1), that were previously shown to contain MCHR1 autoantibodies (Kemp *et al.* 2002), and three vitiligo patients (V10-V12; Table 2.1), that were previously shown not to contain MCHR1 autoantibodies (Kemp *et al.* 2002) were analysed. Eleven healthy controls (4 males, 7 females; mean age: 36 years with range 24-48 years) with no history of either vitiligo or autoimmune disorders were also investigated.

6.3.2 Specific antisera

Rabbit polyclonal anti-CHO-K1 antibody was kindly provided by Professor B.P. Morgan (Department of Biochemistry and Immunology, University of Wales College of Medicine, Cardiff, UK) and was used as a positive control in the complement-fixation assay.

6.3.3 Complement-fixation

Untransfected CHO-K1 cells and the CHO-MCHR1 cell line were grown to confluence in T75 tissue culture flasks in CHO-K1 culture medium (Section 2.20). Geneticin sulphate was included in the medium for culturing CHO-MCHR1 cell line at 1 mg/ml. Subsequently, the cells were washed once with PBS and detached from

the tissue culture flask using Cell Dissociation Solution. After centrifugation at 1000 *g* for 5 minutes, cells were resuspended in 1 ml of PBS and 100-µl samples were aliquoted into LP4 tubes. Cells were then incubated with either 5 µl of anti-CHO-K1 antibody (positive control), 10 µl of control sera (*n*=18) or 10 µl of vitiligo patient sera (*n*=18) at 4°C for 30 minutes. Before use, serum samples were heat-inactivated by incubation at 56°C for 30 minutes. Cells were washed twice with 2 ml of PBS and then incubated with 50 µl of human complement C7-deficient serum (Sigma), which had been diluted 1:5 in veronal-buffered saline (Sigma), for 15 minutes at 37°C in a 5% CO_2 incubator. Cells were then washed twice with 2 ml of PBS and resuspended in 50 µl of PBS with 1 µl goat anti-human C3 antibody (ICN Biomedicals Inc, Aurora, OH, USA) for 30 minutes at 4°C. At the end of the incubation period, the cells were washed twice with 2 ml of PBS and resuspended in 50 µl of PBS with 1 µl of FITC-conjugated rabbit anti-goat IgG antibody (Sigma) for 30 minutes at 4°C. Following the final wash with 2 ml of PBS, cells were analysed for C3 deposition using a FACScan fluorescence activated cell sorter running CELLQuest acquisition and analysis software. Overall, each serum sample was tested in duplicate in two separate experiments with untransfected CHO-K1 cells and the CHO-MCHR1 cell line. In total, eighteen vitiligo patient sera and eighteen control sera were tested.

6.3.4 Purification of peripheral blood mononuclear cells

Ten millilitres of blood from a normal human donor were collected over heparin. The blood was layered over 6 ml of Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 2800 rpm for 20 minutes. Peripheral blood mononuclear cells (PBMC) present at the interface were collected and washed twice with PBS by centrifugation at 2000 rpm for 5 minutes. The pellet of PBMC was resuspended in 2 ml of CHO-K1 cell culture medium (Section 2.20) and the cells counted in a haemocytometer before making any final adjustments to obtain the required concentration of cells.

6.3.5 Antibody-dependent cell-mediated cytotoxicity

The CHO-MCHR1 cell line were grown to confluence in T75 tissue culture flasks in CHO-K1 culture medium (Section 2.20) containing geneticin sulphate at 1 mg/ml. Subsequently, the cells were washed once with PBS and detached from the tissue culture flask after incubation for 5 minutes at 37°C in a 5% CO_2 incubator with trypsin/EDTA. After centrifugation at 1000 *g* for 5 minutes, cells were resuspended in PBS at a concentration of 10^6 cells/ml. The cells were then labelled with 7.4 MBq of chromium-51 (^{51}Cr; Amersham Pharmacia Biotech) by incubation at 37°C for 2 hours in a 5% CO_2 incubator. ^{51}Cr labelled cells were washed twice with PBS and diluted to 10^5 cells/ml in CHO-K1 cell culture medium (Section 2.20). Fifty microlitres (5000 cells) of cells were then added to each well of a round-bottomed 96-well microtitre plate (Nalge Nunc International). To the wells, either 100 µl of CHO-K1 cell culture medium, 100 µl of control sera (*n*=11) or 100 µl of vitiligo patient sera (*n*=12) were added in triplicate and the plate incubated for 1 hour at 37°C in a 5% CO_2 incubator. Sera were heat-inactivated by incubation at 56°C for 30 minutes and diluted at 1:10 in CHO-K1 cell culture medium before use. Overall, each serum sample was tested in triplicate in at least two separate experiments.

The plate was then centrifuged at 1000 *g* for 5 minutes and the supernatant removed from each well. A 150-µl aliquot of PBMC (Section 7.3.4) suspension containing $4x10^5$ cells/ml was added in to each well. The same normal donor was used in all experiments to ensure consistency. The plate was incubated for 4 hours at 37°C in a 5% CO_2 incubator after which it was centrifuged at 1000 *g* for 5 minutes. One hundred and fifty microlitres of the supernatant were removed and the release of ^{51}Cr was counted in an LKB 1470 Rackgamma liquid scintillation analyser (Wallac UK). Spontaneous chromium-release was estimated from incubation of cells with CHO-K1 cell culture medium alone. Maximum chromium-release was estimated from incubation of cells with 100% Triton X-100. Percentage lysis by each serum sample was expressed as:

$$\% \text{ Lysis} = \frac{\text{Sample } ^{51}\text{Cr release value} - \text{Spontaneous } ^{51}\text{Cr release value}}{\text{Maximum } ^{51}\text{Cr release value} - \text{Spontaneous } ^{51}\text{Cr release value}} \times 100$$

Percentage lysis values above the set arbitary level of 20% are considered as a positive indication of ADCC in this assay (Metcalfe *et al.* 1997). Differences in the percentage lysis as a measure of ADCC activity in control and vitiligo patient samples were analysed using the Student's *t* test and *p* values less than 0.05 (two-tailed) were regarded as significant.

6.4 Results

6.4.1 Complement-fixation by MCHR1 autoantibodies in vitiligo patients

The complement-fixing activity of MCHR1 autoantibodies in vitiligo patients was tested on the CHO-MCHR1 cell line and untransfected CHO-K1 cells by flow cytometry. Both the CHO-MCHR1 cell line and untransfected CHO-K1 cells were incubated with the serum sample to be tested before the addition of human serum containing all complement factors except C7. If MCHR1 autoantibodies fix complement, a higher intensity of C3 deposits would be detected by flow cytometry on the CHO-MCHR1 cell line compared to untransfected CHO-K1 cells. In this method, cell lysis is prevented by the use of serum deficient in terminal complement factor C7.

In total, eighteen vitiligo patient sera and eighteen control sera were analysed along with a positive control anti-CHO-K1 antibody. Each sample was tested in duplicate at least in two separate experiments. When untransfected CHO-K1 cells and the CHO-MCHR1 cell line were incubated with polyclonal anti-CHO-K1 antibody, C3 deposition was detected on both cell types, as there was an increase in fluorescence intensity (Figures 6.1). This positive control indicated that complement-fixation could be detected by the method used here. In contrast, when untransfected CHO-K1 cells and the CHO-MCHR1 cell line were incubated with vitiligo patient sera, C3 deposition could not be detected, as there was no increase in fluorescence intensity (Figure 6.2). Similarly, C3 deposition on untransfected CHO-K1 cells and on the CHO-MCHR1 cell line could not be detected with the control sera tested, as again there was no increase in fluorescence intensity (Figure 6.3).

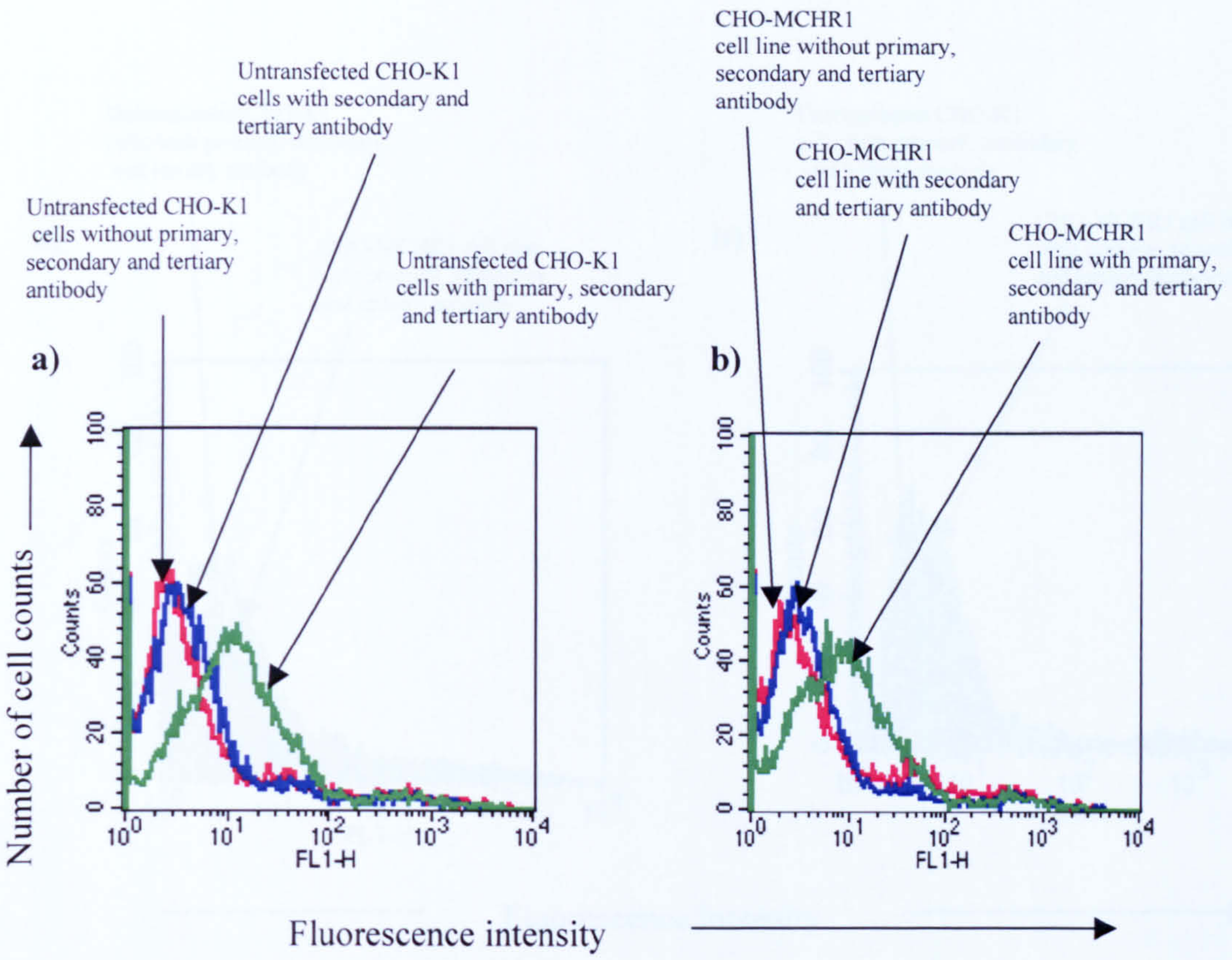

Figure 6.1 Complement-fixation on untransfected CHO-K1 cells and the CHO-MCHR1 cell line by rabbit anti-CHO-K1 antibody using flow cytometry. Untransfected CHO-K1 cells and the CHO-MCHR1 cell line were initially incubated without and with rabbit anti-CHO-K1 antibody (primary antibody) and then with human C7-deficient serum. Cells were then incubated with goat anti-human C3 antibody (secondary antibody) and were finally stained with FITC-conjugated rabbit anti-goat IgG antibody (tertiary antibody). The deposition of C3 was analysed by flow cytometry as described in Section 6.3.3 and the results are shown for untransfected CHO-K1 cells (a) and one CHO-MCHR1 cell line (b). The increasing fluorescence intensity (*x*-axis) indicates C3 deposition on untransfected CHO-K1 cells and a CHO-MCHR1 cell line incubated with rabbit anti-CHO-K1 antibody (green). In contrast, no increase of fluorescence intensity (blue and red) and hence no C3 deposition is apparent on untransfected CHO-K1 cells and the CHO-MCHR1 cell line without incubation with anti-CHO-K1 antibody (red) and with only secondary and tertiary antibody (blue).

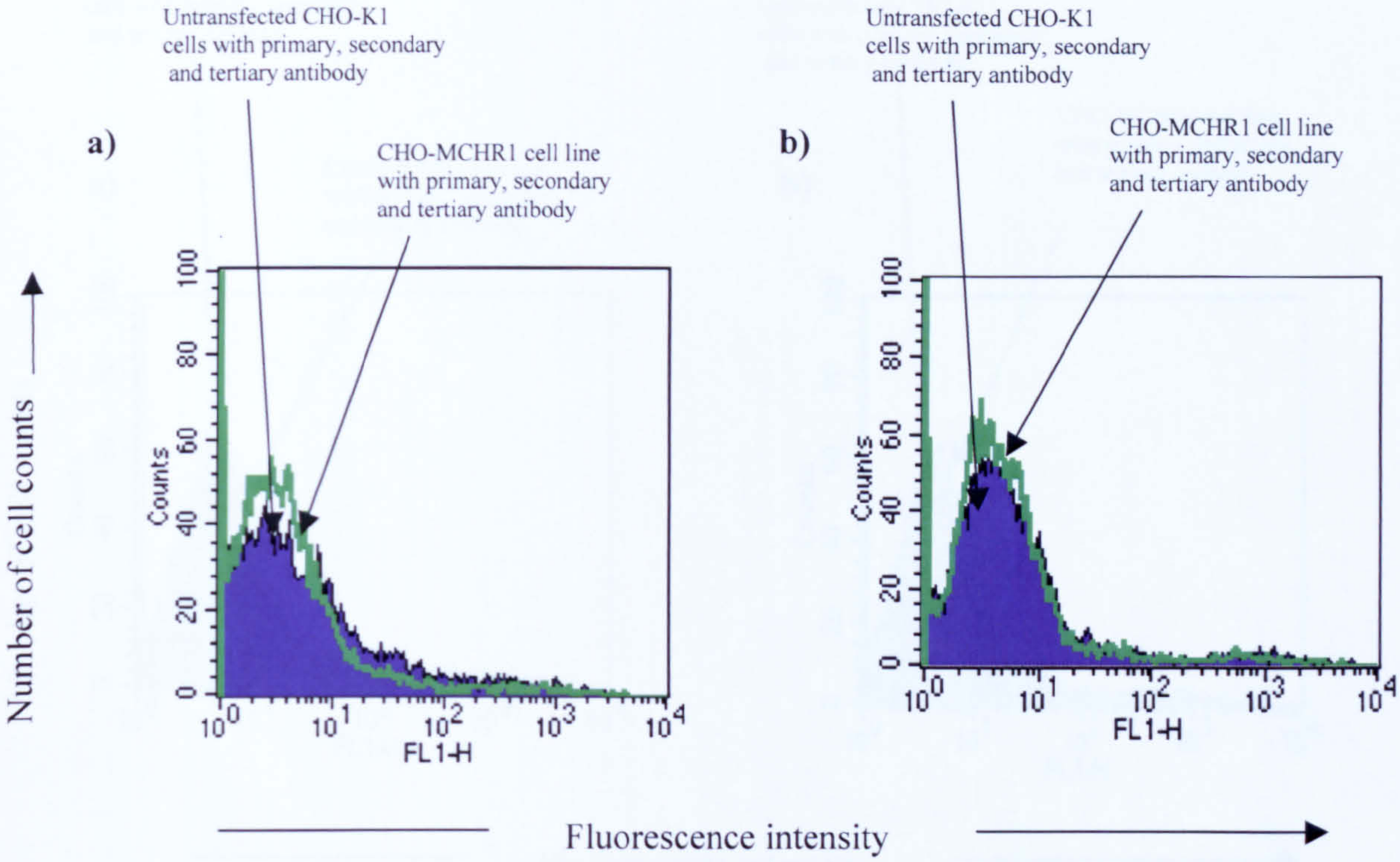

Figure 6.2 Detection of complement C3 deposition on CHO-K1 cells and the CHO-MCHR1 cell line incubated with vitiligo patient sera. Both untransfected CHO-K1 cells and the CHO-MCHR1 cell line were incubated with vitiligo patient sera (primary antibody) and then with human C7-deficient serum. Cells were then incubated with goat anti-human C3 antibody (secondary antibody) and were finally stained with FITC-conjugated rabbit anti-goat IgG antibody (tertiary antibody). The deposition of C3 was analysed by flow cytometry as described in Section 6.3.3 and the results are shown in duplicate (a and b) for one vitiligo patient serum sample. There was no complement C3 deposition on either untransfected CHO-K1 cells (blue) or the CHO-MCHR1 cell line (green), as there was no increase of fluorescence intensity on the *x*-axis. No staining was done with secondary antibody alone.

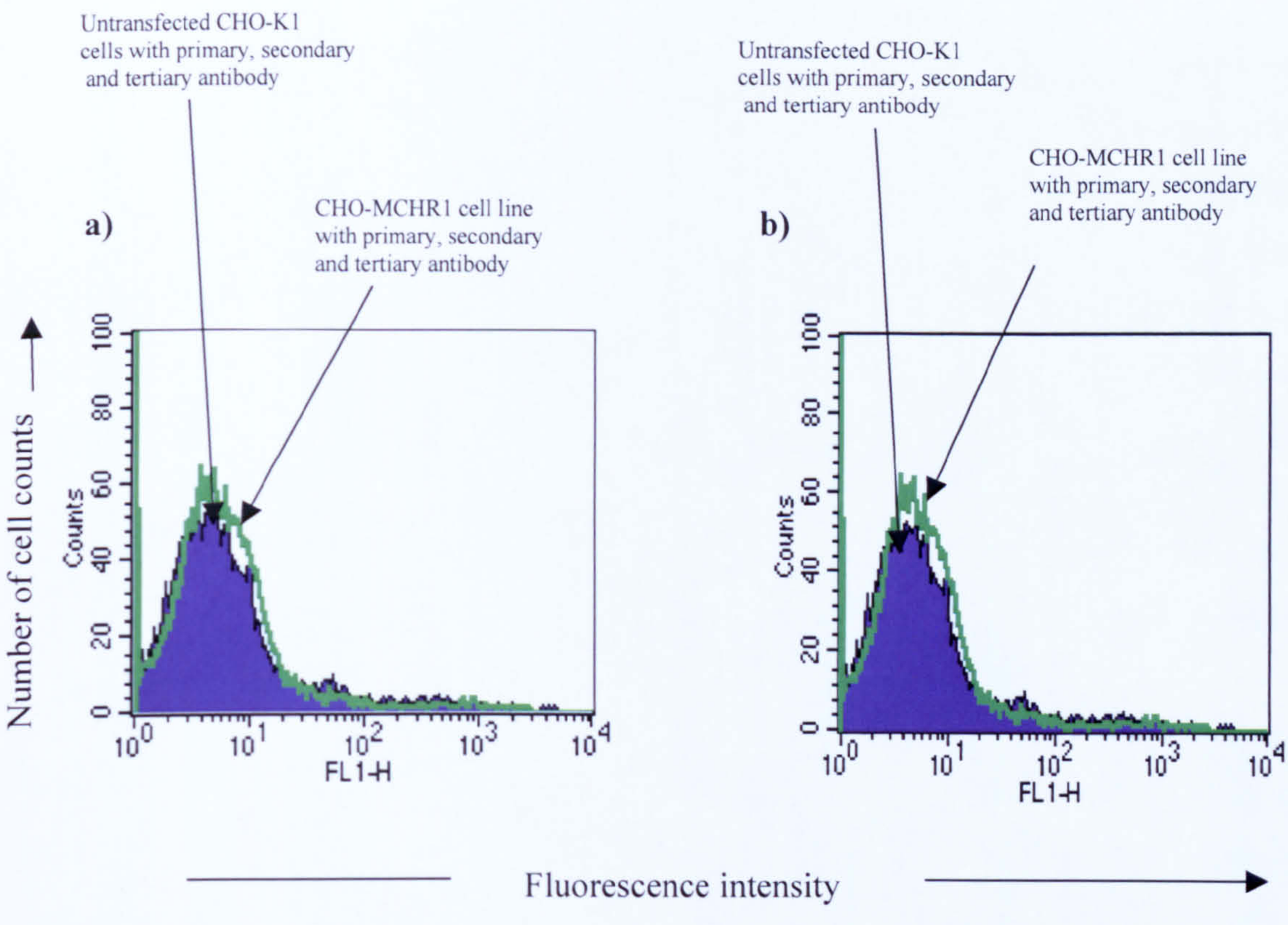

Figure 6.3 Detection of complement C3 deposition on CHO-K1 and the CHO-MCHR1 cell line incubated with control sera. Both untransfected CHO-K1 cells and the CHO-MCHR1 cell line were incubated with control sera (primary antibody) and then with human C7-deficient serum. Cells were then incubated with goat anti-human C3 antibody (secondary antibody) and were finally stained with FITC-conjugated rabbit anti-goat IgG antibody (tertiary antibody). The deposition of C3 was analysed by flow cytometry as described in Section 6.3.3 and the results are shown in duplicate (a and b) for one control serum sample. There was no complement C3 deposition on either untransfected CHO-K1 cells (blue) or the CHO-MCHR1 cell line (green), as there was no increase of fluorescence intensity on the *x*-axis. No staining was done with secondary antibody alone.

6.4.2 Antibody-dependent cell-mediated cytotoxicity by MCHR1 autoantibodies in vitiligo patients

Antibody-dependent cell-mediated cytotoxicity activity of MCHR1 autoantibodies in vitiligo patients was tested in ^{51}Cr-release assays with the CHO-MCHR1 cell line. Initially, both control and vitiligo patient sera were incubated with ^{51}Cr-labelled CHO-MCHR1 cell line. After washing to remove any unbound antibody, the labelled cells were incubated with PBMC. These act as effector cells causing lysis and ^{51}Cr-release on interaction with any MCHR1 antibody that might be bound to the labelled cells via MCHR1. Spontaneous ^{51}Cr-release and maximum ^{51}Cr-release were estimated in each experiment. In total, eleven control sera and twelve vitiligo patient sera (nine with and three without MCHR1 autoantibodies, as previously found in a radio-binding assay; Kemp *et al.* 2002), were tested in triplicate in two separate experiments.

The results indicated that the mean percentage lysis initiated by the control serum samples was 7.37% with a range of 2.04-12.12% (Table 6.1). None of the control sera was, therefore, considered positive for ADCC. Equally, none of the vitiligo patient sera tested contained positive ADCC activity against ^{51}Cr-labelled CHO-MCHR1 cell line as all percentage lysis values were below the arbitary set level of 20% (Table 6.2). The mean percentage lysis in this patient group was 6.28% with a range of 3.79-11.4% (Table 6.2). No significant difference was evident between the mean percentage lysis values of the control sera and those of the vitiligo patient sera (p=0.295).

Table 6.1 Percentage lysis values of control sera in the ADCC assay.

Control sample number	Percentage lysis values	Mean percentage lysis value[1]
C1	5.30 5.42	5.36
C2	2.10 1.98	2.04
C3	6.92 6.12	6.52
C4	10.87 10.17	10.52
C5	6.8 8.0	7.40
C6	9.27 8.67	8.96
C7	6.49 7.27	6.88
C8	8.04 7.56	7.80
C9	11.88 12.36	12.12
C10	5.86 5.82	5.84
C11	7.84 7.56	7.68

[1]The percentage lysis value shown for each control serum sample is the mean of the percentage lysis values from two separate experiments.

Table 6.2 Percentage lysis values of vitiligo patient sera in the ADCC assay.

Vitiligo sample number[1]	Percentage lysis values	Mean Percentage lysis value[2]
V1	7.21 7.11	7.16
V2	4.02 3.56	3.79
V3	6.55 6.97	6.76
V4	6.51 6.53	6.52
V5	6.02 6.06	6.04
V6	9.03 8.49	8.76
V7	4.09 4.15	4.12
V8	6.56 5.36	5.96
V9	6.24 6.32	6.28
V10	11.01 11.79	11.40
V11	4.19 4.13	4.16
V12	4.87 4.17	4.52

[1]Vitiligo patient sera containing MCHR1 autoantibodies (V1 to V9); vitiligo patient sera negative for autoantibody reactivity to MCHR1 (V10 to V12).

[2]The percentage lysis value shown for each vitiligo patient serum sample is the mean of the percentage lysis values from two separate experiments.

6.5 Discussion

Although the exact role of anti-melanocyte autoantibodies in the pathogenesis of vitiligo has not been determined, possible mechanisms by which they can destroy pigment cells have been investigated including complement activation and ADCC.

Complement-fixing autoantibodies that bind to melanocytes have been reported in patients with mucocutaneous candidiasis, multiple endocrine insufficiencies, alopecia areata and vitiligo (Hertz *et al.* 1977). However, these autoantibodies were not found in patients with isolated vitiligo (Howanitz *et al.* 1981). Deposition of complement component C3 can occur in the basement membrane of vitiliginous skin (Uda *et al.* 1984) and the sera of vitiligo patients can cause direct cytotoxic damage of cultured melanocytes by complement-fixation (Norris *et al.* 1988b). In addition, a reduced expression of the membrane regulators of complement activation in both lesional and perilesional keratinocytes and melanocytes has been reported, possibly indicating an increased vulnerability of these cells to autologous complement attack (van den Wijngaard *et al.* 2002).

In contrast to these studies, MCHR1 autoantibodies from vitiligo patients did not appear to fix complement as there was no deposition of complement C3 on the CHO-MCHR1 cell line following incubation with vitiligo patient sera. This difference could be related to the different assays used between studies. In order to assess the reliability of our complement-fixation assay, both untransfected CHO-K1 cells and the CHO-MCHR1 cell line were initially incubated with rabbit anti-CHO-K1 antibody as a positive control and then with human complement with C7-deficient serum. Finally, C3 deposition was analysed by flow cytometry. These control experiments clearly showed the deposition of C3 on the cells and confirmed that the assay was working. However, it is possible that low expression of MCHR1 on the surface of the CHO-MCHR1 cell line and/or low levels of MCHR1 autoantibodies in vitiligo patient sera might mean that any complement-fixing activity of autoantibodies to the receptor remains undetected in the assay system used.

With respect to ADCC, there is evidence that the sera of patients with vitiligo can induce cultured human melanocytes to detach from their cell matrix and can cause direct cytotoxic damage to pigment cells by ADCC (Norris *et al.* 1988b). However, in contrast to the aforementioned study, we were unable to detect any ADCC activity in vitiligo patient sera. Again, the difference between this and the previous study

may be related to the different methodologies used to measure ADCC. Here, the most popular method for assessing ADCC, that is the labelling of target cells with radioisotope chromium-51 (Brunner *et al.* 1968), which binds to cytoplasmic proteins that are subsequently released during cell lysis, was employed. This method has been successfully used to analyse the ADCC activity of thyroid peroxidase autoantibodies in several autoimmune thyroid diseases (Bogner *et al.* 1984; Rodien *et al.* 1992; Bogner *et al.* 1995; Metcalfe *et al.* 1997). Again, it is possible that low expression of MCHR1 on the surface of CHO-MCHR1 cells and/or low levels of MCHR1 autoantibodies in vitiligo patient sera might mean that any ADCC activity of receptor autoantibodies remains undetected in our experiments.

Taken together, the immunological studies indicate that MCHR1 autoantibodies from vitiligo patients do not fix complement and do not mediate ADCC, at least in the assay systems used here with the CHO-MCHR1 cell line. However, the effects of MCHR1 autoantibodies on melanocytes through complement-fixation and ADCC activity have yet to be determined.

7 General Discussion

7.1 Discussion of results

Several hypotheses have been proposed to explain the destruction of cutaneous melanocytes in vitiligo. Persuasive observational and experimental evidence suggests that autoimmune mechanisms play a role in the development of the disease including aberrant humoral immune reactivites (Section 1.3.6). However, only a few autoantigens have been identified as targets of autoantibodies in vitiligo patients (Section 1.5.2.2). Characterisation of autoantigens in vitiligo may be beneficial in the advancement of more appropriate therapies and diagnostic tools and, perhaps, in determining the aetiology of the disease.

Recently, MCHR1 has been identified as a novel autoantigen in 16.5% of vitiligo patients (Kemp *et al.* 2002). In the present work, the autoantibodies that recognise MCHR1 were characterised. Briefly, the aims of the study were (i) to identify B cell epitopes on the MCHR1 using recombinant protein and phage-display technology (ii) to isolate a stable CHO-K1 cell line expressing MCHR1 (iii) to examine the effects of MCHR1 autoantibodies on the functioning of the receptor using the CHO-MCHR1 cell line (iv) to investigate complement-fixation and ADCC with respect to MCHR1 autoantibodies again using the CHO-MCHR1 cell line.

The mapping of B cell autoepitopes can provide an understanding of the association of an autoantigen with a particular autoimmune disease and may give an insight into the mechanisms involved in the initiation and pathogenesis of the disease. Few studies have been carried out to identify B cell epitopes on vitiligo autoantigens (Kemp *et al.* 1999a; Kemp *et al.* 2001b). In this study, molecular analysis of the B cell epitopes on MCHR1 was performed using recombinant proteins in radio-binding assays (Chapter 3) and phage-display technology (Chapter 4). In radio-binding assays, multiple regions of MCHR1 were found to be targets for vitiligo patient MCHR1 autoantibodies, including regions between amino acids 1-138 and 139-298. These domains overlapped with areas on the receptor that were predicted to be highly antigenic.

It has been speculated that the autoimmune responses may arise from cross-reactivity between self-proteins and those of infecting micro-organisms

(Wucherpfennig 2001). However, no similarity was apparent between the amino acid sequences of the identified epitope regions and those of microbial proteins. The current study results indicated that the humoral response to MCHR1 in vitiligo is heterogeneous in nature as several patients exhibiting autoantibodies to more than one MCHR1 epitope. Similarly, autoantibodies in autoimmune thyroid disorders and type 1 diabetes mellitus have been reported to react against multiple epitopes on thyroid peroxidase (Zanelli *et al.* 1992) and tyrosine phosphatase-like IA-2 autoantigen (Lampasona *et al.* 1996), respectively. This has been explained by the intramolecular spreading of an autoimmune response from a single or few epitope(s) to multiple epitopes during the progression of the disease. In type 1 diabetes mellitus, a temporal spreading of the autoantibody response has been described from immunodominant epitopes in an early preclinical phase to lesser immunogenic domains at the manifestation of disease (Naserke *et al.* 1998). In contrast, in the current study we did not find any evidence of epitope spreading in longitudinal serum samples, however, taken from four patients. Possibly, these four sequential patients may have been studied too late in the disease to see an indication of epitope spreading (Naserke *et al.* 1998).

In addition, biopanning using IgG from vitiligo patients to screen a phage-display MCHR1 cDNA fragment library, identified epitopes located within the larger autoepitope domains between amino acids 49-82 and 156-194 (Chapter 4). Each of the consensus sequences identified may represent a linear epitope that is recognised by patient MCHR1 autoantibodies. Conversely, the consensus sequences could have been selected due to them being part of a conformational epitope as the biopanning method is carried out in liquid phase and might therefore facilitate folding of the peptide. However, it was not determined here if the recognised MCHR1 epitopes were linear or conformational. Phage-display technology has been successfully used previously to identify linear and conformational autoantigenic epitopes in several autoimmune diseases such as autoimmune thrombocytopenic purpura (Gevorkian *et al.* 1998), celiac disease (Osman *et al.* 2000), primary biliary cirrhosis (Rowley *et al.* 2000), type 1 diabetes mellitus (Myers *et al.* 2000; Farilla *et al.* 2002; Dromey *et al.* 2004), autoimmune polyendocrine syndrome type II and stiff-man syndrome (Al-Bukhari *et al.* 2002). To differentiate the epitopes on the MCHR1, Western blot analysis could be applied to the expressed peptides, which would identify linear epitopes due to the denatured state of the protein in SDS-polyacrylamide gels.

A stable CHO-K1 cell line expressing the MCHR1 (Chapter 5) was isolated for studying the effects of MCHR1 autoantibodies on the functioning of the receptor. Flow cytometry clearly showed the expression of MCHR1 in transfected CHO-K1 cells and the cell line responded to MCH with a reduction in forskolin-stimulated cyclic AMP levels and increases in intracellular calcium, both characteristic of MCHR1 function.

Subsequently, CHO-K1 cells expressing MCHR1 were used to investigate whether or not MCHR1 autoantibodies in vitiligo patients have any effects upon the functioning of the receptor (Chapter 5). In these experiments, CHO-MCHR1 cells were pre-incubated with vitiligo patient IgG and then any subsequent release in intracellular calcium in response to MCH measured by fluorimetry. Of the vitiligo patient IgG samples tested, 56% (10/18) were able to block the receptor function. In contrast, none of the control IgG or SLE IgG samples tested was able to block the functioning of MCHR1. Among the eighteen vitiligo IgGs analysed, nine were from patients previously shown to contain MCHR1 autoantibodies in radio-binding assays (Kemp *et al.* 2002). The remaining nine IgG samples were from patients shown in the same study not to contain MCHR1 autoantibodies. Of the nine vitiligo patient IgG samples that contained MCHR1 binding autoantibodies, seven contained MCHR1 autoantibodies that blocked receptor function. In the two patients that only contained receptor-binding autoantibodies, it is probable that the MCHR1 autoantibodies bind at a site on the receptor that has no effect upon its functionality. In addition, of nine patients that were previously shown not to contain MCHR1 binding autoantibodies, three were demonstrated to have MCHR1 function blocking autoantibodies. The existence of autoantibodies that appear to inhibit receptor function but do not bind MCHR1 could be due to a number of reasons. It may be that the *in vitro*-translated protein used in the radio-binding assay (Kemp *et al.* 2002) fails to bind these antibodies due to incorrect folding of the molecule or the absence of an normal pattern of glycosylation, or it may simply reflect differential detection sensitivity in the two assays. Similar to our current findings in that MCHR1 autoantibodies in vitiligo patients can block the function of MCHR1, non-stimulatory TBAb can prevent TSH action resulting in hypothyroidism (Arikawa *et al.* 1985; McKenzie and Zakarija 1992; Kosugi *et al.* 1993). In Addison's disease, patient IgGs can block the *in vitro* action of ACTH on adrenal growth and cortisol production by binding to its receptor (Wulffraat *et al.* 1989). Furthermore,

autoantibodies in myasthenia gravis patients directed against the acetylcholine receptor block the acetylcholine-binding site and provoke accelerated receptor degradation (Hara *et al.* 1993; Eymard and Chillet 1997; Hoedemaekers *et al.* 1997; Ferrero *et al.* 1997; Fabien *et al.* 2001). In addition, it has been demonstrated that serum immunoglobulin in patients with pernicious anaemia can block the gastrin-stimulation of acid secretion and it has been suggested that this blocking activity is mediated by competition with gastrin for the surface receptors on parietal cells (de Aizpurua *et al.* 1985; Burman *et al.* 1989). Although MCHR1 autoantibodies in some vitiligo patients were found to have a blocking effect upon the functioning of the receptor, the effect of MCHR1 autoantibodies on the behaviour of melanocytes has yet to be determined. Equally, the relevance of MCHR1 autoantibodies to the aetiology of vitiligo needs to be further investigated.

Finally, MCHR1-expressing cells were used to investigate complement-fixation activity and ADCC with respect to MCHR1 autoantibodies (Chapter 6). The complement-fixing capability of MCHR1-specific autoantibodies was measured by the incubation of heat-inactivated vitiligo patient sera with MCHR1-expressing cells, followed by the addition of serum containing all complement factors except C7. The level of deposition of C3 on the cells was subsequently measured by flow cytometry. In this *in vitro* study model, deposition of C3 on MCHR1-expressing cells by vitiligo patient MCHR1-specific autoantibodies was not detected. In contrast, previous studies have reported the deposition of C3 in the basement membrane of vitiliginous skin (Uda *et al.* 1984) and the detection of anti-melanocyte antibodies from vitiligo patients that display complement-fixation and damage pigment cells (Norris *et al.* 1988b). For ADCC experiments, MCHR1-expressing cells were radiolabelled with ^{51}Cr and then incubated with either heat-inactivated vitiligo patient sera or control sera to allow binding of any MCHR1 autoantibodies to the receptor. After the addition of effector cells, the percentage of cell lysis was determined by measuring the amount of ^{51}Cr released. No difference in the percentage lysis was found between the vitiligo and control serum samples tested. In contrast, previous research has demonstrated that vitiligo patient sera are able to cause direct cytotoxicity to melanocytes by ADCC (Norris *et al.* 1988b). The contrasting results obtained here to those in previous reports, with respect to complement-fixation and ADCC, could be due to differences in the techniques used or that MCHR1-specific autoantibodies do not mediate complement-fixation and ADCC. Alternatively, the expression levels of

MCHR1 on the isolated cell line may not be sufficient to detect MCHR1-specific autoantibodies or low MCHR1-specific autoantibody titres in the patients tested may preclude the detection of complement-fixation and ADCC.

7.2 Future studies

The radio-binding assay originally used to detect MCHR1 autoantibodies (Kemp *et al.* 2002) would not necessarily identify autoantibodies that react with conformational epitopes on the receptor. This is an important area for further study as it is well-documented that autoantibodies to TSHR that are responsible for disease activity in Graves' disease recognise several conformational epitopes (Morgenthaler *et al.* 1999). To address this, work is currently in progress to isolate a cell line expressing high levels of MCHR1 that can be used in flow cytometry to screen vitiligo patient sera for autoantibodies that bind to native receptor.

The isolation of purified receptor would be useful for a number of studies. This would probably be achieved by the expression of histidine-tagged MCHR1 in either a mammalian or insect cell expression system. The MCHR1 protein could subsequently be used for:

(i) Development of an ELISA format for routinely detecting autoantibodies in vitiligo patients.
(ii) Detection of T cell reactivity to the receptor in vitiligo patients.
(iii) Preparation of a MCHR1 affinity column that could be used to purify MCHR1 autoantibodies from vitiligo patient sera. The effects of purified receptor autoantibodies on the growth and pigmentation of melanocytes could then be studied.
(iv) Development of an animal model of vitiligo by inoculation of mice with MCHR1 protein. As well as studying any immune responses to the antigen, it would be interesting to investigate any effects on pigmentation.
(v) Biopanning of antibody libraries prepared from vitiligo patients in order to isolate MCHR1 Fabs that could be used in the fine-mapping of MCHR1 epitopes. These would also allow the gene usage of MCHR1 autoantibodies to be determined.

7.3 Conclusion

This thesis has concentrated on characterising MCHR1 autoantibody reactivity in vitiligo patients. However, the relationship between MCHR1 autoantibodies, and indeed all vitiligo autoantibodies, and the pathogenesis of the disease has yet to be established. The possibility that MCHR1 autoantibodies play no part in vitiligo aetiology but rather indicate the presence of autoreactive anti-MCHR1 T lymphocytes that are cytotoxic to pigment cells, should be investigated. Even if they prove not to be directly pathogenic, MCHR1 autoantibodies may serve as useful markers of cellular disruption in the epidermis of vitiligo patients so further characterisation of them will continue.

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