Reviews of Reproduction (1998) **3**, 71–76

Immunological analysis of epitopes on hCG

Torben Lund and Peter J. Delves

Immunoprotein Engineering Group and Medical Molecular Biology Unit, Department of Immunology, Windeyer Institute for Medical Sciences, UCL Medical School, London W1P 6DB, UK

The heterodimeric glycoprotein hormone, human chorionic gonadotropin, has been extensively characterized in terms of its recognition by mouse monoclonal antibodies. A number of different approaches have led to the definition of several epitope clusters on the surface of the molecule. These include epitopes located solely on the α- or β-chain, some of which are masked when the two chains associate to form the holo-hormone. Additional epitopes comprise amino acids contributed by both the chains. In contrast to the extensive knowledge regarding B cell epitopes, the characterization of T cell epitopes on hCG has only recently begun to be explored.

Human chorionic gonadotropin (hCG) is a heterodimeric molecule consisting of an α-chain common to all members of the glycoprotein hormone family (luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH)) non-covalently associated with a β-chain unique to each hormone. However, there is extensive sequence homology between the different β-chains, with LH exhibiting 85%, TSH 46%, and FSH 36% homology with the first 114 of the 145 amino acid residues of hCG. Both the α- and the β-chains are composed of three loops held in place by a cystine knot of three disulfide bonds (Lapthorn *et al*., 1994; Wu *et al*., 1994), a structural motif also found in transforming growth factor β (TGF-β), neuronal growth factor (NGF), platelet-derived growth factor β (PDGF-β) and various other growth hormones. One end of both of the subunits comprises loops 1 and 3, which in the β-subunit are stabilized by a fourth disulfide bond, while loop 2 forms the other end of each of the subunits. The α- and β-subunits are oriented opposite to each other in such a way that the paired loops 1 and 3 form each end of a cigar-like molecule (Fig. 1) with the α-subunit held in place by an additional loop structure in the β-subunit containing two disulfide bonds and referred to as the 'seat belt' (β91–110). The seat belt is also important in the receptor binding. The unique C-terminal peptide (CTP, β113–145) of the hCGβ-subunit protrudes from the compact molecule without any obviously constrained structure. The hormone is extensively glycosylated, having two *N*-linked oligosaccharides on each chain and, in addition, four *O*-linked oligosaccharides located on the serine rich CTP of the β-chain. Although receptor binding requires intact holo-hormone, hCG can be extracted from urine and serum in several different molecular species, reflecting different glycoforms, proteolytic fragments and sometimes the presence of free β-chain.

After fertilization, hCG is produced from the eight-cell blastocyst stage onwards, and is initially detectable at days 7–12 after fertilization. Production of hCG is continued by trophoblast cells and promotes the secretion of progesterone from the corpus luteum. After 7 weeks, the synthesis of hCG is switched to the placenta, where the production remains constant until 14–15 weeks, after which time secretion begins to decline. The function of the placenta-derived hCG is not known. While expression of dimeric hCG is associated with trophoblastic neoplasia, non-trophoblastic tumours can express hCG ectopically but, in these tumours, only the β-chain is produced. Therefore, increased serum concentrations of the hormone are used both as an aid to diagnosis and as a marker of established disease.

Generation of immune responses

A prerequisite for the induction of an immune response against a protein antigen is that the antigen is taken up by specialized antigen-presenting cells (APC), such as dendritic cells. After proteolytic degradation, selected peptide fragments (T cell epitopes) are expressed on the cell surface of the APC in association with major histocompatibility complex (MHC)-encoded molecules. When the MHC–peptide complexes are recognized by antigen-specific T cell receptors on $CD4^+$ helper T cells, the cells become activated and begin synthesis and secretion of cytokines that help to stimulate B cells to secrete antigen-specific antibodies. Small molecules containing no suitable T cell epitopes can be made immunogenic by covalently attaching larger carrier proteins, such as tetanus toxoid (TT) or diphtheria toxoid (DT).

T cell epitopes on hCG

Purified hCG in the absence of a carrier protein can elicit an antibody response in mice and rabbits, implying that one or both subunits contain appropriate helper T cell epitopes for these species. After immunization of BALB/c mice, Rouas *et al*. (1993) were able to identify two overlapping T cell epitopes on the α-subunit (residues α50–70 and α60–80, respectively) and two distinct epitopes on the β-subunit which included the residues β1–13 and β11–22. As a part of preliminary studies aimed at developing an anti-tumour vaccine, Triozzi *et al*. (1997) immunized non-HLA-matched human subjects with the 37 amino acid CTP covalently linked to DT. Subsequently, T cell proliferative responses could be obtained after stimulation *in vitro* with hCG holo-hormone, but not with the CTP in the absence of a carrier, suggesting that either or both of the α- and the β-subunits possess T cell epitopes able to bind to various HLA class II molecules.

After immunization of BALB/c mice with an expression

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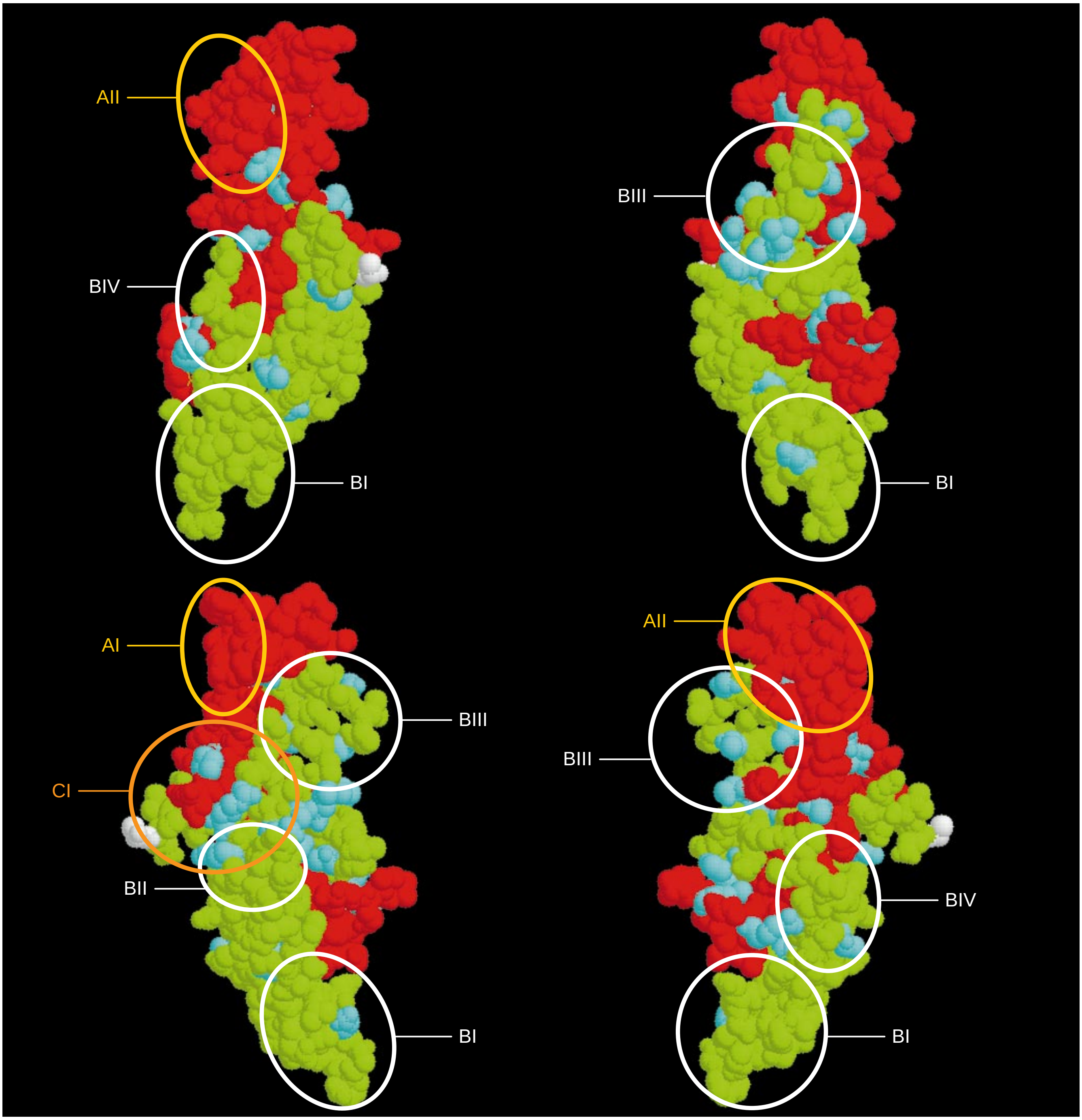

Fig. 1. Computer-generated space-filling model of hCG highlighting differences between surface-exposed amino acid residues on hCG and LH. The α-subunit is in red; the residues shared between hCGβ and LHβ are in green; the hCGβ specific residues are in blue and the N-terminus of the hCGβ chain is highlighted in white. The projection of the crystal structure is visualized in four different orientations.

plasmid containing the hCGβ gene, Geissler *et al.* (1997) demonstrated that the β-subunit can induce a cytotoxic T lymphocyte (CTL) response, suggesting that the hormone also contains sequences that can bind to MHC class I molecules. Furthermore, they found that the induced CTL response could prevent the growth of a myeloma cell line transfected to express hCGβ. While hCGβ CTL epitopes in humans have yet to be defined, their characterization would clearly facilitate the development of an hCGβ-specific anti-tumour vaccine.

B cell epitopes on hCG

Unlike T cell epitopes, which are short linear peptides derived from processed antigen, antibodies recognize structures on the

Table 1. Methods used for epitope mapping of hCG

Chemical modification of the hormone
Enzymatic fragmentation
Peptide scanning with overlapping peptides
Binding to hormones from different species
Binding to related hormones (FSH, LH, TSH)
Binding to LH/CG chimeric polypeptide chains
Site-specific mutagenesis
Competition binding with two or more mAbs
Synergistic binding with two or more mAbs
Interference with hormone–receptor interaction

surface of the native antigen. Several groups have isolated and characterized panels of monoclonal antibodies (mAbs) raised in mice against purified or recombinant hCG, hCG fragments, isolated subunits or specific peptides (Kofler *et al*., 1981; Kofler *et al*., 1982; Bidart *et al*., 1985; Hojo and Ryan, 1985; Norman *et al*., 1985; Berger *et al*., 1988; Krichevsky *et al*., 1988; Bottger *et al*., 1993; Furui *et al*., 1994). Since antibodies to globular proteins most commonly interact with 14–21 amino acids on the surface of the protein, most of the epitopes on hCG are, by necessity, discontinuous, made up of amino acids that juxtapose in the native conformation. This has been confirmed by the loss, or considerable decrease, of antibody binding after reduction and alkylation of the free subunits. While some of the mAbs can bind to short linear peptides, they usually do so with considerably reduced affinity compared with their binding to the native hormone. The epitope specificities of the antibodies have been determined using a variety of methods (Table 1). Distinct epitope clusters have been identified that consist of several overlapping epitopes with subtle differences recognized by individual mAbs.

Epitopes on hCGα

Characterization of antibodies derived from immunization with hCG, FSH, TSH and LH identified three distinct dominant epitope clusters on the α-subunit, two of which are accessible on the holo-hormone and a third that is only accessible on the free α-subunit (Norman *et al*., 1985; Krichevsky *et al*., 1988; Berger *et al*., 1990; Dirnhofer *et al*., 1994a). Peptide scanning for antibody binding sites maps one of these clusters to loop 1 of the α-chain and includes sequences within α13–22. This cluster, AI, reacts with several different mAbs that recognize overlapping amino acid residues. The second, spatially distinct epitope cluster on the intact hormone, AII, is conformation-sensitive, with no linear peptide stretch contributing significantly to the antibody recognition. It is also iodination-sensitive and is located on the third loop involving residues α65–80. Immunization with the α-subunit has identified another epitope cluster, AIII, which is masked by the β-subunit in the native hormone. Since a linear stretch of amino acids contributes to the AIII epitope, it has been mapped to include the region α32–41.

Epitopes on hCGβ

There is extensive sequence homology between hCGβ and LHβ (Fig. 1). Therefore, it is not surprising that many hCGβ-reactive mAbs also bind to LH, although a number of mAbs specific for either hCGβ or LHβ have been produced (Norman *et al*., 1985; Berger *et al*., 1990; Moyle *et al*., 1990; Bottger *et al*., 1993; Dirnhofer *et al*., 1993, 1994b). We have used site-specific mutagenesis to characterize the immunogenicity of hCGβ and have found that a single amino acid substitution βR68E completely eliminates binding of all the hCG/LH cross-reactive mAbs in the panel of hCG mAbs used (Jackson *et al*., 1996). Since each crossreactive mAb has unique but overlapping contact residues (Fig. 2) located on either loop 1 (residues β24 and β25) or loop 3 (residues β68, β74, β75 and β79), the whole of the tip of hCGβ (and of LHβ; Fig. 1) formed by loops 1 and 3 probably constitutes a single large epitope cluster, BI. This is perhaps not so surprising when the degree to which this region protrudes from the rest of the molecule is considered.

Several distinct hCGβ-specific conformation-dependent epitope regions have been characterized. Two of them are exposed only on the free hCGβ subunit and include amino acids β20–22/β75 (BV) and β89 (BVI) among their contact residues. Three distinct hCGβ-specific epitope regions (BII–BIV) are accessible on the native hormone. One of these, BII, maps close to the cysteine knot and includes residue β10. BIII includes amino acids in loop 2 of hCGβ (β38–56) and may have a linear stretch of amino acids as part of the conformational epitope. The last epitope cluster, BIV, has been mapped to the seat belt (β100–109). Finally, four independent linear epitopes have been characterized on the CTP. However, because of the high entropy resulting from the flexible conformation of the CTP, these epitopes may be relatively weakly immunogenic when intact hCGβ is encountered by the immune system.

Epitopes present only on the holo-hormone

A number of mAbs have been described that recognize native hCG but do not react with either of the free subunits. Antibody competition with subunit-specific mAbs has defined a single epitope cluster in the junction between the cysteine knot, loop 2 of the β-subunit and loop 1 of the α-subunit (Norman *et al*., 1985; Schwartz *et al*., 1986; Krichevsky *et al*., 1988; Bottger *et al*., 1993). This epitope cluster, CI, includes the recognition sequences for several different mAbs, which bind to distinct but overlapping epitopes.

Topographic relationship of the epitopes

We have devised a method to relate the spatial location of different epitope clusters to each other using a synergistic binding assay where two or three mAbs are cross-linked by immobilization on the same surface (Klonisch *et al*., 1996a,b). Simultaneous binding of radiolabelled hCG to the cross-linked mAbs results in binding constants greater than the sum of the binding constants for the individual mAbs. This synergistic binding occurs when the epitopes on the surface of the hormone are spatially distinct and orientated in the same plane so as to cause minimal torsion in antibody–antigen interaction. For example, a 50-fold increase in the binding constant was observed for a BI (mAb 3E2)–AI (mAb INN-hFSH-123) combination, suggesting that the Fab arms of the antibodies could interact with their epitopes with maximal loss of free energy. Some, but not all, members of the BI cluster could synergize with certain mAbs binding to the AI epitope cluster, to the CI cluster and to

mAb	Specificity		WT	K20N E21R G22E	P24H V25Y	R68E R74S G75H V79H	K20N E21R G22E P24H V25Y	P24H	V25Y	R68E	R74S G71R	G75H	V79H	R74S
INN-hCG-2	CG	BII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
INN-hCG-32	CG	BII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
INN-hCG-22	CG/LH	BI	Green	Green	Green	Red	Green	Green	Green	Red	Green	Green	Green	Green
INN-bLH-1	CG/LH	BI	Green	Green	Red	Red	Red	Red	Orange	Red	Red	Green	Green	Green
INN-hCG-111	CG/LH	BI	Green	Orange	Red	Red	Red	Orange	Red	Red	Red	Green	Green	Green
INN-hCG-24	CG/LH	BI	Green	Green	Green	Red	Green	Green	Green	Red	Green	Green	Green	Green
INN-hCG-58	CG/LH	BI	Green	Green	Green	Red	Green	Green	Green	Red	Green	Green	Green	Green
INN-hCG-51	CG/LH	BI	Green	Green	Green	Red	Green	Green	Green	Red	Green	Green	Green	Green
INN-hCG-20	CG/LH	BI	Green	Green	Green	Red	Green	Green	Green	Red	Green	Green	Green	Green
3E2	CG/LH	BI	Green	Orange	Orange	Red	Yellow	Orange	Orange	Red	Red	Green	Orange	Red
INN-hCG-64	CGβ	BV	Green	Red	Green	Red	Red	Green	Green	Green	Green	Red	Green	Green
INN-hCG-22	CGβ	BVI	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
OT3A	CTP	BVII	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green

Fig. 2. Binding of a panel of mAbs (Schwartz *et al.*, 1986; Berger *et al.*, 1990; Dirnhofer *et al.*, 1994a,b) to wild type (WT) and mutant hCGβ chain (Jackson *et al.*, 1997) expressed on the surface of COS cells. Amino acid substitutions in the mutants are indicated using the single amino acid code to indicate the wild type amino acid, the position in the hCGβ sequence, and the amino acid in the mutant. The binding of the mAbs is expressed relative to the binding of mAb OT3A, which recognizes a linear epitope (β133–39) on the C-terminus. Green >70% binding; orange, 50–70% binding; yellow, 20–30% binding; red <10% binding.

the BII cluster. The BII cluster allowed synergistic binding to mAbs from the AI cluster. This suggests that the AI, and part of the BI, BII and CI clusters are orientated in the same topographic plane. mAbs recognizing epitopes in the AI and AII clusters could also bind synergistically. One of the mAbs recognizing a linear (although not fully characterized) epitope on the CTP (mAb 3G12) and which has very little innate binding affinity towards the intact hormone was able to bind synergistically with mAbs from many epitope clusters on both the α- and β-subunits and on the holo-hormone by substantially lowering the dissociation constant for the antigen–antibody interaction.

The hCG/LH receptor interacts with sequences on both subunits of hCG, and a model has been proposed in which the receptor may bind the hormone like a horseshoe (Jiang *et al.*, 1995). Receptor binding involves residues that contribute to the formation of epitope clusters CI, BII, AI and part of BIII on loop 2 and to residues β93–100 on the seat belt, which may contribute to the cluster BIV. In addition, part of the sequences on the hCG-unique CTP can interact with the receptor and are needed for transmission of the hCG-specific signal. Therefore, the receptor interaction will block the binding of a number of the hCG mAbs, but some epitopes still remain accessible (Schwarz *et al.*, 1991; Cosowsky *et al.*, 1995). Binding of mAbs directed to most of the BI cluster is unaffected by ligation of the hormone to its receptor, implying that hCG binds in an orientation that results in the β-loops 1 and 3 projecting from the cell surface. Consistent with our observation that the AI and AII clusters allow synergistic binding is the fact that mAbs to the AII cluster, but not to the AI cluster, bind to the hormone–receptor complex. Evidence has been presented that an antibody that recognizes amino acids within the AI cluster (mAb HT13) can bind to hCG complexed with a soluble form of the receptor but that the epitope recognized is not accessible when the hormone is bound to the full length receptor, suggesting that the epitope recognized by this particular antibody may be in close contact with the transmembrane region of the receptor (Pantel *et al.*, 1993).

Epitopes on hCG recognized by the human immune response

The glycoprotein hormones are 'self' antigens. Although normally expressed only during pregnancy, it appears that hCG is very effective at establishing immunological tolerance. There are hardly any reports of circulating autoantibodies to the hormone being detected in humans, even in patients with a history of recurrent spontaneous abortion (Tulppala *et al*., 1992). However, it is also clear that this tolerance is not absolute, because when it is administered coupled to a potent carrier and in the presence of adjuvant, hCG can break tolerance and elicit an immune response.

The important function of the hormone as an inducer of progesterone in the corpus luteum has prompted a number of laboratories to develop potential antifertility vaccines based on hCGβ. In addition, the ectopic expression of hCGβ in certain tumours makes the hormone a suitable candidate for a cancer vaccine (Acevedo *et al*., 1995; Geissler *et al*., 1997). Two separate strategies have been pursued in which the antifertility vaccine candidates have undergone phase I and II trials. The World Health Organisation (WHO) has for many years supported a vaccine development programme based upon the unique hCG CTP (Jones *et al*., 1988). The antibodies elicited do not appear to block hormone–receptor interaction, suggesting that the antifertility effect relies essentially on Fc receptor-mediated clearance of the antibody–hCG complexes. The Indian Government has promoted a vaccine programme based upon a heterospecies dimer (HSD) composed of an ovine α-chain associated with the hCGβ-subunit (Talwar *et al*., 1994). Eighty-five per cent of women immunized with this vaccine were transiently protected against pregnancy. Immunization with the HSD induces an LH crossreactive antibody response. Although no adverse biological side effects have so far been observed in the vaccinees, it is not known if long-term exposure to the HSD will induce undesired autoimmune responses to LH in immunized women. Therefore, we have embarked upon a programme aimed at selectively deleting the crossreactive epitopes so that the antifertility or antitumour vaccine contains only the relevant hCGβ specific epitopes (Jackson *et al*., 1996; Delves *et al*., 1997).

Although the epitopes recognized by a very large number of mouse mAbs have been characterized, less is known regarding which hCG epitopes dominate the human polyclonal antibody response *in vivo*. In mice, the epitopes used are determined by the antigen receptors on the mouse B cells, which, owing to sequence differences, are unlikely to have the same fine specificity as the antigen receptors in humans. Therefore, the epitope usage in humans cannot be predicted on the basis of the epitope characterization using mouse-derived mAbs. However, an indication of the epitope usage in humans has been obtained using sera from the vaccinees in the Indian phase I and II trials. In competition ELISAs using four mouse mAbs (B206, a LH-crossreactive mAb binding to BI; 357-2, which binds to the BIII cluster on hCGβ; 218, binding to CI; and P_23_3 specific for A1), only B206 was able to significantly (40–90%) inhibit the binding of the human antisera to hCG (Deshmukh *et al*., 1993). This suggests that the crossreactive BI epitope cluster is the most dominant in humans *in vivo*, although additional epitope clusters may also be used.

The work done in the authors' laboratory was supported by The UCL and Middlesex Special Trustees, The Sir Jules Thorn Charitable Trust, The Medical Research Council of the UK and The Wellcome Trust. The authors would like to thank I. M. Roitt for numerous helpful discussions.

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