

The Evolution of Viral Diversity

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Abstract

This thesis focuses on the population dynamics of three antigenically diverse RNA viruses: dengue, influenza and HIV-1. It comprises a set of studies highlighting the roles of structural constraints on critical antigenic determinants, interactions between immune responses to different antigenic types, host lifespan, and the degree of mixing between different host populations in determining the epidemiology and within-host dynamics of these pathogen systems.

Dengue exists in humans as a collection of four antigenically related serotypes. Although infection by one serotype appears to convey life-long protection to homologous infection, it is believed to be a risk factor for severe disease manifestations upon secondary, heterologous infection due to the phenomenon of Antibody-Dependent Enhancement (ADE). It is not clear if third or fourth infections are possible, and if so, how they contribute to dengue epidemiology. In this thesis, I investigate the effect of third and fourth infections on the transmission dynamics of dengue.

By contrast with dengue, human influenza viruses are known to be in rapid antigenic flux, manifesting in the sequential replacement of antigenic types. This pattern of evolution does not appear to be the same in shorter-lived hosts such as swine and birds. In this thesis, I have used a simple multi-locus model to explore the relationship between host lifespan and viral evolution, as well as to elucidate the effects of transmission between hosts of different lifespan in effort to capture the cross-species element of influenza transmission.

My final chapter concerns the within-host evolution of HIV-1. I propose a new model for the pathogenesis of HIV-1 where the transition to AIDS is primarily linked to the gradual loss of the ability to make new antibody responses as the CD4⁺ population declines.

Together these studies emphasise that it is the changing profile of immune responses – either at the population level or within the host – that is the principal determinant of the dynamics of the pathogen, rather than the mode and tempo of antigenic innovation.

For my parents

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Table of Contents

Chapter 1. Introduction	1
1.1: Introduction.....	1
1.2: Basic viral biology	2
1.2.1: Viral classification and genome replication	4
1.2.2: Generation of viral diversity	8
1.3: Immunity to viruses in vertebrate hosts	10
1.3.1: Innate immunity	11
1.3.2: Adaptive immunity.....	14
1.4: Viral adaptation to immune responses	21
1.5: Modelling of the virus-host interaction	22
1.5.1: Population models.....	23
1.5.2: Within-host models.....	26
1.6: Overview of thesis	30
Chapter 2. Dengue fever: Do third and fourth infections matter?	33
2.1: Introduction.....	33
2.2: Methods.....	44
2.2.1: Mathematical models	46
2.3: Results.....	51
2.3.1: Serotype synchronisation	52

2.3.2: Sequential replacement of dominant serotypes	53
2.3.3: Inter-epidemic period	55
2.3.4: Serotype persistence.....	56
2.3.5: Age structure at equilibrium	57
2.4: Discussion	60
Chapter 3. Antigenic evolution of influenza in different species.....	67
3.1: Introduction.....	67
3.1.1 Genetic evolution of human influenza.....	70
3.1.3 Influenza outbreaks.....	71
3.1.4 Immunity to influenza	73
3.2.1 Haemagglutination inhibition assay	75
3.2.2 Virus neutralization assays	77
3.2: Antigenic evolution of human influenza	80
3.3: The ‘Limited-Epitope’ model.....	85
3.3.1: Model structure.....	85
3.3.2: Model behaviour.....	87
3.3.3: Relationship with serological data.....	91
3.3.4: Limitations to antigenic space	97
3.4: Antigenic evolution of influenza in other species.....	100
3.5: Exploring discrepancies in the antigenic evolution of influenza in different species	101
3.5.1: Strength of cross-protective effect.....	102

3.5.2: Lifespan.....	103
3.5.3: Infectious period	107
3.5.4: Differences in basic reproduction number, R_0	109
3.5.5: Architecture of Antigenic Space	111
3.5.6: Effect of linking two populations with different life expectancies	114
3.6: Discussion	120
Chapter 4. A new model for HIV-1 pathogenesis	129
4.1: Introduction.....	129
4.1.1 The virus	131
4.2: Adaptive immune responses to HIV-1	135
4.2.1: Antibody responses.....	135
4.2.2: T cell responses.....	136
4.3: Existing models of HIV-1 pathogenesis.....	139
4.4: A multi-locus model for HIV-1 pathogenesis	143
4.4.1: Basic model	143
4.4.2: Linked CD8+ and NAb epitopes on Env	154
4.4.3: Combined model.....	165
4.4.4: Escape from CD8+ responses	167
4.5: Discussion	171
Chapter 5. Conclusion	182
Bibliography.....	191

Chapter 1

Introduction

1.1: Introduction

A virus is an infectious biological agent that must parasitize a living cell in order to replicate. Successfully infected cells are ultimately forced to produce, assemble and release a great multitude of copies of the offending virus. For the virus to survive, its daughters must then find in turn new cells to target, either within the same host or in a new one.

Inevitably, the process of viral replication is far from trivial. It can be thought of as set of challenges, each of which the virus must be able to reliably and repeatedly negotiate in order to ensure its survival. Perhaps chief among these is the struggle against the host immune system. This exists in large part to prevent exploitation of the host by viruses and other pathogens and acts to both block infection of vulnerable cells and eliminate infected ones, thereby seeking to ultimately purge the virus from the host.

This antagonistic relationship between virus and host is dynamic, with each responding to the other on both short and long time scales, both within an individual host and in the wider population. This thesis is concerned with the patterns that these interactions create, specifically how distinct patterns of viral diversity and behaviour emerge from the intricate, multi-scale relationships between virus and host. To this end, I have constructed deterministic mathematical models of three antigenically diverse viral pathogens: dengue, influenza, and human immunodeficiency virus type-1 (HIV-1). I have then used these

relatively simple models to devise novel biological hypotheses about viral spread and pathogenesis. Each of these pathogens, though antigenically diverse, nevertheless harnesses this diversity in different ways. For example, dengue has access to only a very limited range of antigenic variability with just four serotypes, all of which co-circulate. By contrast, influenza seems to be able to generate and shuffle through a much wider variety of antigenic types, yet in human populations only one is ever widely prevalent. HIV-1 meanwhile seems to exhibit a possibly even greater degree of antigenic variation within a host, but is a virus whose pathogenesis, and its relationship to this variation, is not fully understood.

1.2: Basic viral biology

Ultimately, a free virus particle, or virion, has evolved to protect and transport the fragile genetic material it contains from cell to cell, either within or between hosts. As such, each virion essentially consists of a nucleic acid genome that is shielded from the elements by a densely packed coat of protein called the viral capsid. Some viruses are further enclosed by an envelope: a lipid membrane collected as a parting 'gift' from the parasitized cell upon release, giving it an extra layer of protection. Viral envelopes vary in complexity, but surface glycoproteins are universally embedded in and protrude beyond them. These glycoproteins consist of a large external domain together with a smaller internal one, bridged by membrane-spanning α -helices. The internal domain often plays a pivotal role in virus assembly, whilst the external domain contains both the binding sites for cell surface receptors and the components that can subsequently mediate virus-cell membrane fusion and so allow the virus to gain entry to the host cell. Those viruses that do not possess envelopes are referred to as 'naked' and their surface proteins simply extend from the capsid. Once bound, naked viruses can mediate cell entry in one of three ways:

translocation (crossing the cell membrane intact), genome injection (literally injecting the genome through a pore on the cell surface) or endocytosis (where the virus is drawn into the cellular cytoplasm inside a vesicle).

The first challenge in the life cycle of a virus then, is to encounter a suitable host cell. Not only does this require a cell to be susceptible to viral entry, but also that it is permissive of viral replication. Location of an appropriate cell does not consist of a conscious effort on behalf of a virus; rather they diffuse randomly through space via Brownian motion, colliding with cells by chance. An obvious corollary is that producing more progeny, and/or increasing the quantity of receptors for cell binding on the virus surface, will also increase the number of chance encounters with appropriate cells.

Should chance dictate a suitable virus-cell collision, the virus can then bind and attach to receptors on the cell surface. Entry into the cell can then be facilitated as outlined above and, at some point and in some way, viral disassembly needs to take place so as to expose the viral genome. Importantly, each of these steps requires flexibility in the same structures that served to rigidly protect the virus in the extracellular environment. Many viruses are capable of replication in the cytoplasm, but some classes of virus instead require the additional step of being subsequently transported to the cell nucleus for this purpose. A coarse general rule as to the necessity of this latter step can be applied based on whether the viral genome is constructed from either RNA (not necessary) or DNA (necessary).

The next step is to hijack the host cell machinery in order to produce both viral proteins and new viral genomes. These components then need to be collated and assembled to produce viable progeny that ultimately need to be released from the cell. Enveloped viruses tend to be assembled at the plasma membrane of the cell, where the final step in assembly

– collection of cellular membrane to form the envelope – all but coincides with exit from the cell. In effect, as these viruses bud from the cell, they take with them part of the cell membrane. Consequently, if many viruses bud all at once then the cell itself will likely die in a somewhat cataclysmic end to the parasitism. However, in some cases, such as with retroviruses, non-destructive budding is possible and allows a long-lasting exploitation of the cell. The usual exit strategy for naked viruses is more blunt: cell lysis results in the death of the cell and the release of however many virions have been successfully assembled.

1.2.1: Viral classification and genome replication

The genes of cells are encoded in their DNA, but this information needs to be expressed by messenger RNAs (mRNAs) in the cell cytoplasm to allow its translation into protein by ribosomes. The process of creating an mRNA copy from some template is referred to as transcription. During the process of division, cells also copy their DNA in order to pass their genes on to their daughter cells. A virus achieves replication by directing these normal cellular processes to instead produce viral proteins and copies of its own genome. The Baltimore classification system is used to distinguish between groups of viruses on the basis of their type of genome and method of replication (Baltimore, 1971). A visual representation of this system is shown in Figure 1-1, and will be explained below.

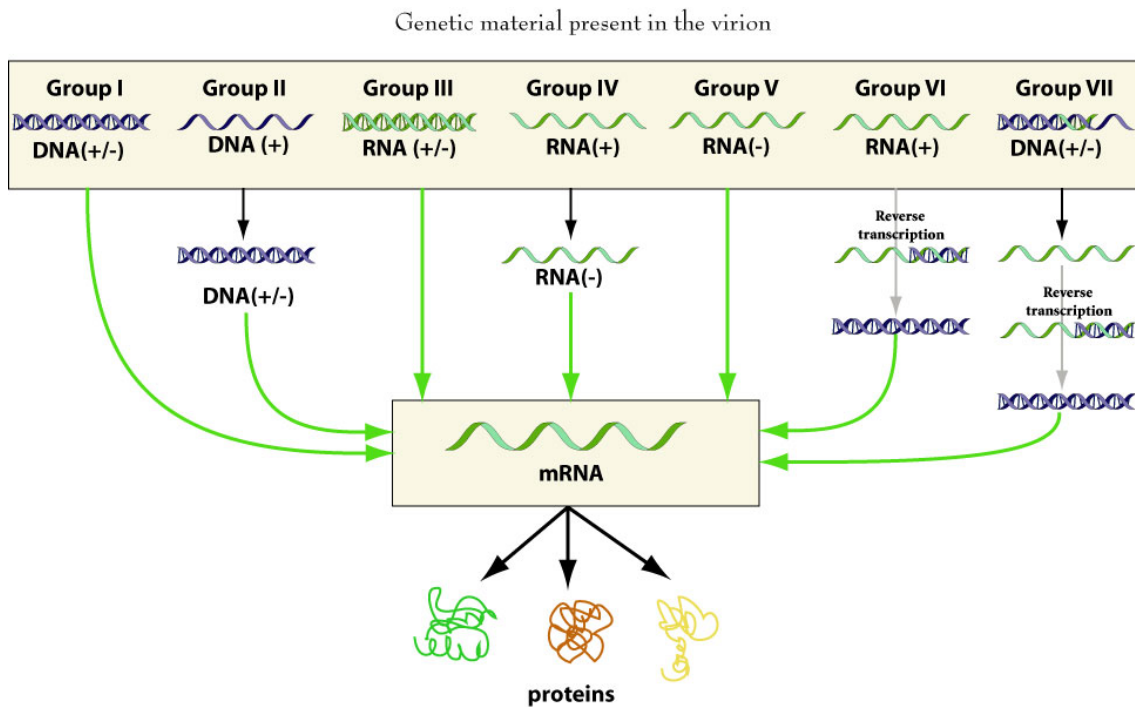


Figure 1-1 The Baltimore classification. *This groups viruses depending on their type of genome and method of replication.*

Understanding the polarity of different forms of RNA and DNA is important. mRNA is defined as being positively (+) stranded because it contains information that can be immediately translated by ribosomes. A strand of RNA is therefore denoted as a (+) strand if it can be immediately translated by the cell. Negatively (-) stranded RNA is complementary to this and must be first converted into (+) strand RNA before translation can take place. Analogously, DNA is also referred to as being (+) (or (-)) stranded which means that if it were mRNA then it could (or could not) be immediately translated. Viral genomes can consist of just a single (+) or (-) strand of DNA or RNA, or as a double strand, with both versions bound together.

The genome of each virus often exists in a single, continuous linear or circular form but can be segmented instead, meaning that the genome is broken up into several smaller parts.

In these viruses, each individual segment generally codes for just a single protein, but there are multiple ways in which this rule can be broken.

Class I: Double stranded DNA viruses (e.g. Adenoviridae, Herpesviridae, Papillomaviridae). The genomes of these viruses are converted into mRNA as the result of transcription by DNA-dependent RNA polymerase (DdRp), which can be found naturally within a host cell although some viruses (e.g. *Adenoviridae*) provide their own version. This mRNA is translated into viral proteins for the construction of new viral particles. It may also need to direct the cell to create copies of the viral genome, although some viruses (e.g. *Herpesviridae*) encode their own replication factors. The general rule is that these viruses can only replicate once inside the cell nucleus, but *Poxviridae* provide the exception. These viruses encode an entire DNA replication system of their own and replicate in the cell cytoplasm instead.

Class II: Single stranded DNA viruses (e.g. Anelloviridae, Circoviridae, Parvoviridae). Importantly, only double stranded DNA can be transcribed by DdRp to create mRNA. Regardless of the polarity of the single strand of DNA, the first thing these viruses must do is to synthesise the complementary DNA strand. This double stranded DNA can then be transcribed into mRNA and replicated as before. However, these double strands also need to be broken down again so that only the single DNA strand with the original polarity is packaged into the new viruses.

Class III: Double stranded RNA viruses (e.g. Birnaviridae, Reoviridae, Totiviridae). Cells do not have a polymerase capable of transcribing the genomes of these viruses into mRNA. These viruses therefore carry with them an RNA-dependent RNA polymerase (RdRp) that transcribes the double stranded RNA into mRNA. Translation of mRNA can then take

place, also whilst viral polymerase copies mRNA to produce the complementary (-) RNA strand necessary for the formation of new double stranded RNA genomes. Notably all of the viral genomes within this class are segmented.

Class IV: (+) Single stranded RNA viruses (e.g. Coronaviridae, Flaviviridae, Togaviridae). By definition, the genomes of these viruses can usually be directly translated into protein by host ribosomes and therefore do not need to carry their own RdRp with them. One of the protein products of this translation is an RNA polymerase that copies the genomic (+) RNA into its complementary (-) strand. This (-) strand is then again copied to produce (+) stranded RNA which can be then in turn either packaged into progeny virions or used as a template for further production of the (-) strand.

Class V: (-) Single stranded RNA viruses (e.g. Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae). The (-) strand RNA of these viruses must first be copied into mRNA by an RdRp, which must again be provided by the virus. This new (+) strand of mRNA is then copied to produce new (-) strand genomes. Viruses within this class may have genomes that are either segmented or continuous. The *Orthomyxoviridae* are notable as they replicate within the host cell nucleus, where they are able to take advantage of alternative splicing mechanisms to produce multiple proteins from some individual segments.

Class VI: (+) Single stranded RNA viruses that replicate through a DNA intermediate (Retroviridae). In contrast to the viruses of Class IV, those of this class use a viral encoded RNA-dependent DNA polymerase (RdDp) called reverse transcriptase to produce a double stranded DNA intermediate of their genome. This DNA intermediate is then integrated into the genome of the host cell itself, whereupon it can be subsequently transcribed as part of the cell's natural life cycle. Consequently, these viruses are among the few RNA viruses

that require transport into the cell nucleus. Infection can be latent, with no viral replication until the advent of some trigger signal.

Class VII: Double stranded DNA viruses that replicate through a single stranded RNA intermediate (e.g. Hepadnaviridae). The genomes of these viruses are usually only partially double stranded. These gaps in the double strand therefore need to be filled in to produce a perfect duplex before mRNA synthesis by host cell DdRp can take place. Again, this mRNA can then be translated to produce viral proteins, but crucially also copied by reverse transcriptase into the double stranded of DNA genome.

1.2.2: Generation of viral diversity

There are three main methods by which virus genomes can be altered during replication: reassortment, recombination and point mutation.

Reassortment can occur when two or more viruses with segmented genomes co-infect the same cell. As the genomes of these viruses are copied, and the individual segments are put together to form new viral genomes, it is possible for new viruses to end up with a mixture of segments from each of the original viruses. This can be a potent tool for generating extensive genetic diversity.

Recombination can occur in either segmented or non-segmented viruses, when nucleotide sequence from one segment or virus is partially overwritten by that from another. This is referred to as homologous recombination when the new genetic information comes from exactly the same location on a similar genome. In the case of a slight mismatch then this is

instead described as aberrant homologous recombination. Non-homologous recombination is used to describe an exchange of totally unrelated genetic information.

An important distinction between RNA and DNA viruses is the extent to which mechanisms exist to prevent errors (point mutations) as the viral genome is copied. Thanks to the proof-reading mechanisms of replicative DNA polymerases, cellular DNA replication is a high-fidelity process. These polymerases are essentially self-correcting enzymes that excise errors as replication progresses. So-called 'mismatch repair' mechanisms subsequently act as an insurance policy to correct errors in the daughter strand of newly replicated DNA that were somehow missed during the proof-reading process. Together, these mechanisms have an impressively small error rate on the order of just one mistake in every 10^9 synthesised base pairs.

Infection of a cell by small DNA viruses does not tend to result in the inhibition of cellular protein synthesis. It may therefore be that small DNA viruses have access to the complete array of cellular proof-reading mechanisms with the presumed consequence that replication of such viruses is as accurate as that of the genome of their host cell. However, although the viral DNA polymerases of many larger DNA viruses have good proof-reading capabilities, infection of cell by a large DNA virus inhibits cellular protein expression. In this case, the insurance policy of 'mismatch repair' may not be available to these viruses; thus, although still low, there may be an ensuing increase in error rate.

Contrastingly, no such mechanism exists for RNA viruses as RdRps do not possess proof-reading capabilities. The net result is a comparatively high error frequency for RNA viruses: around 1 in every 10^3 - 10^4 nucleotides will be polymerised incorrectly during RNA virus

transcription and this is often stated as being roughly 1000 times greater than the equivalent rate for DNA viruses.

The high transcription error rate of RNA viruses has important consequences for their evolution, in particular the fact that they have much smaller genomes when compared with DNA viruses. In theory this high mutation rate means that RNA viruses are generally better equipped to deal with novel impediments to their proliferation. A good example of this is the often observed rapid acquisition of resistance to a new drug. Despite this, restrictions are often imposed by structural constraint: however else these viruses change, they must still be able to carry out the basics of their life cycle. The consequence of this is that RNA viruses are therefore subject to strong purifying selection (Holmes, 2009).

Notably each of the three viruses studied in this thesis uses RNA for its genetic template, although they belong to different classes within the Baltimore classification. In each case, I attempt to consider the balance between high mutation rate and strong purifying selection. For example, even though these viruses can interrogate large portions of genetic space, the results of these interrogations are not always successful and many individual mutations will be at least mildly deleterious. Thus, there is not necessarily a great deal of flexibility for this mutational process to exploit and the virus may be highly constrained in some regions of its genome. A good example of this, which will be explored further in Chapter 2, is that human dengue virus appears incapable of antigenic innovation.

1.3: Immunity to viruses in vertebrate hosts

Left unchecked, a virus will not cease replicating within a host until it is no longer capable of encountering appropriate targets. Consequently, in order to preserve cell function and

survive an infection, vertebrate hosts have evolved an intricate set of defences with which to guard against and, when that fails, eradicate infection. In this section, I provide a brief overview of the anti-viral properties of two key components of this immune system: innate and adaptive immunity. The cells and mechanisms of the innate immune system are always primed and ready to recognise and respond to viruses (and other pathogens) in a generic manner, and innate immune responses therefore form the bulwark of the initial anti-viral response. Some version of this system can be found in all animal and plant life, but it cannot confer long-lasting or protective immunity to the host. In contrast, the adaptive immune system of vertebrates is capable of recognizing and remembering specific pathogens, enabling the development of a tailored response upon an initial encounter with a pathogen and more rapid clearance of repeat infections.

1.3.1: Innate immunity

Anatomical Barriers. Epithelial surfaces, namely the skin and mucosal surfaces of the gut and respiratory tract, present a physical barrier to an invading virus. This primary and literal layer of defence is further complemented by the secretion of peptides with antibiotic properties. Any damage to these epithelial surfaces weakens their protective capacity, making it easier for viruses (and pathogens in general) to enter the body. However, all the viruses considered in this thesis can all breach this barrier regardless. Dengue can be introduced as an infected mosquito pierces the skin in order to take a blood meal, whilst influenza is capable of breaching the mucosal surface in the respiratory tract. HIV-1 can gain access, for example, through injection by a dirty needle directly into the blood or, more usually, through the exposure of the virus to oral, rectal or genital mucosal surfaces. Once these anatomical barriers to infection have been breached, there are many other aspects of the innate immune system that are capable of responding to a virus.

Phagocytes. These are immune cells that engulf, or phagocytose, invading viruses. The normal function of these cells is to clear rubbish such as dead cells from tissue but they can also target virus if their pattern recognition receptors can recognise molecular patterns on the viral surface. One of the reasons for the non-specificity of the innate immune response is because many of these motifs are shared between many different viruses.

Macrophages are one example of a phagocytic cell and mature from blood monocytes that have entered into and become resident in sub-epithelial tissue. These cells are capable of destroying ingested virus, thereby stimulating their proliferation. They also release chemokines and cytokines that impact on the regulation of the ongoing immune response in the tissue and help to attract more macrophages and other components of the immune system to the site of infection.

Another phagocytic cell is the neutrophil. These usually circulate in the blood stream, but can be chemically induced to enter tissue at the site of an infection and participate in the immune response. These cells, however, focus solely on the destruction of phagocytosed virus. Neutrophils are also attracted to the site of an infection more quickly than macrophage-precursors and so dominate the early phagocytic response. However, their shorter life span and inability to proliferate mean that macrophages ultimately dominate this aspect of the anti-viral response.

Dendritic cells form another key group of phagocytic cells. However, unlike macrophages and neutrophils, the primary purpose of these cells is to interact with and prime the adaptive immune system through antigen presentation. Macrophages are also capable of acting as antigen-presenting cells (APCs), and I will explain the importance of this process within the section on adaptive immunity.

Natural Killer Cells. Like neutrophils, Natural Killer (NK) cells usually circulate in the blood stream, only enter tissue when signalled to do so, and ultimately act to destroy. Contrastingly however, the anti-viral behaviour of NK cells is not mediated by phagocytosis but instead by inducing apoptosis in infected cells. Apoptosis is an important anti-viral tool because simple lysis of an infected cell would allow the escape of any virions contained inside. Besides killing, NK cells also release cytokines, chiefly IFN- γ and IL-2, which act to activate macrophages (enhancing their anti-viral activity) and, in the right context, allow NK cells and other lymphocytes to proliferate.

Complement System. Another important, and ancient, part of innate immunity is the complement system. One anti-viral property of this system relies on interactions between various proteins to create membrane attack complexes. These complexes are capable of killing viruses by cutting holes in their surface, causing the degradation of targeted virions. There are three separate pathways that can create these attack complexes, but only two are relevant for viruses. Of these, the 'classical' pathway is activated by host antibodies whilst the 'alternative' pathway acts against cells lacking particular surface proteins. Fragments of complement proteins also act as chemoattractants for phagocytes, whilst the system can also opsonise viral particles and thereby promote their phagocytosis.

This collection of responses is often capable of eliminating a viral infection without further help and phagocytes and the complement system can work well against free virions. However, the ability to respond is much more limited once a virus has managed to infect a target cell. NK cells and activated macrophages can secrete cytokines that reduce the amount of virus produced by infected cells and reduce the susceptibility of those that remain uninfected. NK cells are also capable of causing the apoptosis of some infected cells. It is much harder however, for the innate immune system to control infected cells as

compared with free virus. Consequently, though the innate system is good at helping to contain the early viral infection, this is not always enough to purge the virus from the system and which may require the intervention of the adaptive immune response.

1.3.2: Adaptive immunity

This arm of the immune response is composed of highly specialised cells that are usually capable of eliminating a first infection by a particular virus and doing so even more rapidly upon secondary challenge. Importantly, some viruses, such as HIV-1, are capable of causing a persistent infection. Others, like dengue, are able to exploit previously stimulated adaptive immune responses for their own benefit, whilst influenza is an example of a virus that is capable of evading many of these responses through antigenic variation.

B cells. Naïve B cells originate in the bone marrow. Here, they select gene segments to code for the two proteins that make up their particular B cell receptor (BCR). The B cell surface is then coated with multiple copies of the BCR, together with a variety of other receptors. B cells can be activated either with or without T cell help, but the latter is generally unimportant for viral infections. The former first requires recognition and binding of cognate antigen by the BCR. The B cell can then phagocytose the bound viral particle and present antigen as part of a Class II MHC complex. Activation through binding to T cells, which have been themselves primed by APCs presenting the same antigen, can now proceed, and results in proliferation of the activated B cell. Ultimately, this cell will go on to either manufacture antibodies or transition to memory.

During proliferation, qualitative changes to the B cell may occur. Depending on the current cytokine environment, antibody “class switching” can occur, which alters the functional

capabilities and structure of secreted antibody. At the same time, each round of proliferation results in slight mutations to the BCR. Consequently, mutations that increase BCR affinity for its cognate antigen end up being preferentially selected in a process known as “affinity maturation.” The final possible change is the ultimate decision about whether to return to the bone marrow and become a plasma cell or remain in circulation as a memory B cell.

Plasma cells are the cells that actually produce antibodies – Immunoglobulin (Ig) M, G, A and E. These antibodies are structures of conjoined BCRs, but without the anchor protein sequence required to tether them to the cell surface they end up being secreted into the body. Memory B cells, on the other hand, are high affinity B cells that are consequently capable of rapidly responding to small amounts of antigen upon re-infection to facilitate faster production and proliferation of B cells and, hence, antibody.

IgM tends to be the default, and very large, antibody produced by a plasma cell in the absence of class switching and is essentially 5 BCRs stuck together. In this way, an IgM antibody can simultaneously bind to its cognate antigen on multiple pathogens and in doing so perform two key functions. The more general of these is to activate the “antibody-dependent” pathway of the complement system. Here, the antibody ‘tags’ pathogens by binding to their cognate antigen, and complement proteins can then bind to the antibody. Crucially, if two sets of the complement proteins are brought together (*i.e.* if the antibody manages to tag 2 or more pathogens), the complement proteins can set up a complement cascade that will ultimately destroy the bound pathogens. More specifically, IgM is also quite good at neutralizing viruses; when IgM binds to antigens on viral surfaces, it can hinder or even prevent viral attachment to a cell.

There are multiple subclasses of IgG antibodies all with slightly different ways of functioning. The two main subclasses, however, are IgG1, which acts to opsonize antigens to facilitate ingestion by phagocytes and IgG3, which can bind to NK cells to bring them close to a target and simultaneously augment the NK cell's killing capacity (this is antibody-dependent cellular cytotoxicity or ADCC).

IgA antibodies get transported out of tissue onto mucosal surfaces to guard against penetration by pathogens. These antibodies look like 2 BCRs stuck together and their dimeric structure consequently allows them to more easily bind to multiple antigens at once. In so doing, they allow pathogens to be brought together into clumps big enough that can be swept out of the body with mucus.

Thus, IgM, IgG and IgA all have important roles to play to combat a viral infection. A final class of antibody, IgE, is important in the defence against parasites but does not appear to play a role in immunity to viruses.

MHC molecules. MHC molecules are specialised proteins that bind peptide antigens and display them for recognition by antigen-specific T lymphocytes. These molecules come in two distinct groups: Class I and Class II.

The more ubiquitous of these two, Class I MHC molecules, are displayed on the surface of every cell in the body. Protein products are continually produced within all cells as the result of mRNA translation. These are then sampled by the cell and fragments of these samples are presented as peptide-antigen complexes on Class I MHC molecules on the cell surface. Samples of viral protein products are also presented on the surface of infected cells as part of this natural process. Once on the surface, these peptide-antigen complexes are

inspected by a particular class of T lymphocyte – the CD8⁺ T lymphocyte. These cells are capable of distinguishing between ‘self’ and ‘non-self’ (*i.e.* viral) protein products, and the detection of ‘non-self’ as part of Class I MHC molecules results in the T lymphocyte inducing apoptosis of the infected cell. In response to this, some viruses are capable of down-regulating Class I MHC expression on the surface of infected cells, thereby reducing the risk of foreign protein fragments being exposed. NK cells are in turn believed to target cells that don’t express enough class I MHC.

Class II MHC molecules can only be found on APCs such as the macrophages and dendritic cells of the innate immune system, whilst B cells are another important form of APC. APCs are capable not only of phagocytosis but also the subsequent presentation of ingested viral protein fragments as part of Class II MHC molecules. Recognition of Class II MHC molecule complexes by T cells plays a key role in the development of an adaptive immune response.

Antigen Presenting Cells. Dendritic cells are perhaps the most important class of APC, because they are the only class of cell that can activate naïve T cells. Under normal circumstances, dendritic cells are at ‘rest’ just beneath the epithelia and merely sample the extracellular fluid that surrounds them. In this state they express relatively low levels of both MHC and the important co-stimulatory protein B7 on their surface. However, recognition of stereotypical viral molecules and/or stimulation by tumour necrosis factor (TNF) secreted by activated macrophages can lead to their activation. Activated dendritic cells then leave the infected tissue and migrate through the lymphatic system to the nearest lymph node, concurrently upregulating expression of B7 protein together with Class I and II MHC molecules on their surface. In this way they can now present antigen sampled through Class II MHC and act as efficient activators of naïve helper T cells. If the dendritic

cell has been infected itself, or because it has phagocytosed an infected cell, then it can also present protein fragments through Class I MHC in order to activate naïve killer T cells. The chemical signature of an activated dendritic cell recruits more naïve dendritic cells to the infected tissue, which means that more antigen that is present in tissue (*i.e.* the more severe the infection), the more dendritic and hence T cells that end up activated. This, coupled with the short life span of activated dendritic cells, results in T cell activation that is roughly proportional to the near contemporaneous amount of antigen present in the infected tissue.

Macrophages will only present antigen efficiently after having been activated by a cytokine signal such as IFN- γ , which results in the up-regulation of B7 and MHC-II. However, unlike dendritic cells, activated macrophages remain in the infected tissue. This is in order to ensure that activated T cells continue to remain activated once they arrive in the tissue: an activated T cell requires continuous stimulation in order to avoid pre-programmed cell death. Thus, macrophages serve to ensure that as long as antigen remains in the tissue, the number of activated T cells will continue to accumulate – dendritic cells activate T cells, but macrophages keep them going once they arrive in the tissue.

Although presentation of antigen as part of a Class II MHC complex is itself an important aspect of B cell activation, this is actually done to a much greater degree by activated B cells, which also express increased levels of B7. This method of antigen presentation and T cell activation is particularly proficient during periods when there is relatively little antigen around. Consequently, upon re-infection, antigen presentation by memory B cells allows a much more rapid acceleration of the T cell response than in the original infection.

T cells. There are two main types of T cell – those that kill infected cells (CD8+ T cells) and those that help develop the general immune response (CD4+ T cells). However, as with B cells, both types start life as a naive lymphocyte that must be activated before it can actively participate in eliminating an infection. Activation is again a twin track process that requires recognition of cognate MHC antigen (Class I for killers, Class II for helpers) together with a specific co-stimulatory signal between protein B7 on an APC and CD28 on a T cell.

Activated T helper cells express high levels of CD40L, which is important within the context of B cell activation, as well as now being able to proliferate in response to growth factors. This latter behaviour means that activated T helper cells will respond to growth factors produced largely by other activated T helper cells, but also in part by naive ones, and will proliferate to form a large pool of activated T helper cell clones. Activated T helper cells can also be further stimulated to proliferate again by other dendritic cells in the lymph node, notably with reduced need for co-stimulatory signals, until they eventually mature into effector cells.

Effector T helper cells have two main duties. First, they can remain in the blood and lymphatic circulation to provide help for B cells and/or killer T cells. Second, they can exit the blood vessels at sites of infection/inflammation to provide ‘help’ for the ongoing innate and adaptive responses in tissue. This help is chiefly provided through cytokine secretion, which acts to establish/reinforce local feedback loops that hone the immune system to fight the particular class of invasion.

The initial cytokine profile of an effector T helper cell will be determined by the exact co-stimulatory signals provided during activation. Generally, if the system has been stimulated to fight a viral or bacterial invasion then Th1 cells will be produced. These primarily secrete

IFN- γ (which promotes macrophage activation), IL-2 (a Th1 growth factor) and TNF (which stimulates phagocytosis by macrophages and is a chemoattractant for neutrophils). In turn, IL-12 secreted by activated macrophages serves to encourage T helper cells to adopt the Th1 cytokine profile. In parasitic infections, complementary Th2 cells will be produced instead. These two types of helper T cell, and more specifically their associated cytokine profiles, simultaneously encourage development of their own type and restrict that of the other. Thus, not only does a strong local Th1 response reinforce itself, it also prevents the development of a local Th2 response (and vice-versa); this results in local specialisation of the T helper cells responding to a pathogen.

Activation of killer T cells, or cytotoxic T lymphocytes (CTLs), also requires both recognition of cognate antigen, though as now presented by class I MHC molecules on a dendritic cell, and co-stimulation. The precise mechanism of co-stimulation remains unclear but it seems to require some form of interaction with both T helper and dendritic cells. Following activation, CTLs rapidly proliferate and then leave the lymph node to circulate in the blood and then enter infected tissue. Once there, they can kill infected cells that display cognate antigen by injecting perforin and granzyme B onto the surface or by engaging Fas protein on the cell surface, resulting in apoptosis of the infected cell. Further proliferation is also possible in the infected tissue in response to IL-2, and CTLs also secrete IFN- γ to promote macrophage activity.

As with B cells, some T cells (both helper and killer) eventually end up as memory cells. Exactly how this occurs is also an area of active research, but the important thing here is that such cells exist, can continue to exist in the absence of antigen, and can rapidly respond to a repeat infection. In the absence of antigen and co-stimulation, most T cells rapidly die.

1.4: Viral adaptation to immune responses

Many pathogens, including viruses, have evolved their own counter-measures that mitigate the immune response. The relatively large error rates in RNA virus replication allow them to inadvertently edit their antigens through point mutations, or in more extreme cases, through genome reassortment. Such edits can result in viruses physically altering the antigens that are presented either by MHC molecules or on the surface of the virus itself. In this way, the antigen may no longer be recognised by previously primed antibody and T cell responses. Alternatively, the edited antigen might no longer be sensitive to neutralisation by these responses and yet still cross-react with them. This also has negative implications for the development of an immune response that is capable of neutralising the variant antigen. Examples of this effect have been observed for both T cells and antibodies; in the former it is often the result of altered peptide ligands (APLs), whilst the latter has been implicated in original antigenic sin. Antagonistic interactions between T cells and APLs have also been suggested as a cause of T cell anergy. A similar strategy is for a virus to produce a decoy antigen, such as a mitogen, that can act to stimulate B cell responses that do not hinder viral infectivity.

Mutation might also not actually physically alter an antigen but instead make it less likely to be presented as part of an MHC molecule. Similarly, conformational changes or accumulation of glycosylation sites on the surface of the virus can obscure or hide surface antigens. Some viruses can inhibit MHC class I expression in infected cells, although this leaves the cells more vulnerable to NK-mediated killing. One further strategy is to produce molecules that can inhibit innate and adaptive immunity. For example, poxviruses can produce proteins that can bind to cytokines and act as competitive antagonists. Similarly, cytomegalovirus produces a protein that is homologous to class I MHC and may compete

for binding and presentation of peptide antigens. More aggressive still are those viruses that actually infect and either kill or inactivate immunocompetent cells, of which HIV-1 is a good example. One additional strategy is antibody dependent enhancement (ADE). This allows some viruses, such as some flaviviruses and possibly HIV-1, to more readily infect target cells after they have been bound by antibody and is discussed in more detail in the next chapter.

1.5: Modelling of the virus-host interaction

Given the morbidity and mortality often associated with viral infection, it is hardly a surprise that a substantial body of work has been undertaken in an attempt to model the dynamics of virus populations. These studies most often focus on the spread of a virus within a population, as opposed to within an infected host, but the latter is also an area of active research.

There is often a gap between the known biology of a viral pathogen and the ability to recreate its known dynamics with a mathematical model. From this we can deduce that either the model has failed to accurately portray some aspect of the known biology, or some part of the virus' biology is potentially misunderstood or maybe even missing. In these instances, mathematical models can help us hypothesise about the true/complete nature of the virus; by making additional or revised assumptions we can see if we can better recover the known viral dynamics. If attempts to do this are successful, then experiments can be set up to test the corollaries of these novel assumptions, to hopefully support, or reject, the model's hypotheses. In the latter case, the simple next step is to return to the drawing board.

1.5.1: Population models

Most of these models are ultimately derived from a simple epidemic model originally proposed in 1927 (Kermack and McKendrick, 1927). Here, Kermack and McKendrick divided the host population into three distinct compartments: Susceptible (S), Infected Individuals (I) and those who have Recovered from infection and are now immune to further infection (R). There is thus an irreversible path through the three compartments, from S to I to R. Collectively, this model and its many variants, are known as SIR-models and have been used to try to elucidate the dynamics of epidemics. These models are usually deterministic, assuming that the population is both large and well-mixed, and often, for simplicity, assume that the population size is fixed with constant birth and death rates or ignore demographic effects completely.

Many interesting aspects of pathogen spread have been derived from these simple foundations, but the most pertinent here is the concept of R_0 , the basic reproduction number for a given pathogen. R_0 is best defined as the number of secondary cases resulting from a single infection in a totally susceptible population. In most mathematical descriptions of SIR models, this boils down to a product of a combination of the pathogen's intrinsic transmission coefficients (B) and the length of its infectious period (D). Thus, $R_0 = BD$ and if $R_0 > 1$ then an introduced pathogen will spread whilst an $R_0 < 1$ means that it will usually quickly die out.

The basic SIR model can only deal with the spread of a single viral strain, and is therefore inadequate for efforts that wish to understand how antigenic variation impacts on viral spread. So called 'multi-strain' models are typically based on the tenet that a host infected by one particular viral strain gains not only complete immunity to that strain, but also some

form of partial cross-immunity acting to protect against subsequent infection by other strains.

‘History-based’ models describe host individuals by labelling them with the collection of those strains they have previously been infected by and to which they are therefore immune. The host may have some immunity to other strains if it has similar strains in its history of infection. One of the main problems with this type of model, though, is that as the number of strains modelled increases, so too does the intractability of the system: modelling n strains requires 2^n equations, which quickly becomes problematic for antigenically diverse pathogens.

One possible solution to this, used extensively in this thesis, is to use a multi-locus approach where each pathogen type is visualised as a set of antigenic determinants or epitopes with limited variation at each. Using such a structure assumes that the associated antigenic space, though possibly very large, is ultimately finite but this fundamental assumption can be justified in terms of structural and functional constraints on epitope variability. By allowing host compartments to overlap, this ‘limited-epitope’ framework is much more tractable than a standard proliferation of the ordinary SIR-type model to accommodate strain diversity (Gupta *et al.*, 1996; Gupta *et al.*, 1998).

Alternatively, ‘status-based’ models (initially developed by Gog & Swinton (Gog and Swinton, 2002)) describe each individual host according to their current immune status. This type of model has the advantage of being more tractable for larger numbers of strains, but it is hard to find behaviour that corresponds to antigenic evolution within this type of model (Dawes and Gog, 2002).

Within these various models, cross-immunity between strains affects either the susceptibility of hosts *e.g.* (Adams and Boots, 2006), the transmissibility of future infections *e.g.* (Gupta *et al.*, 1998), or both *e.g.* (Recker *et al.*, 2009). This biological distinction does not however usually have important qualitative effects on the observed model dynamics *e.g.* (Ferguson and Andreasen, 2002; Recker *et al.*, 2009). Infection by one strain can provide partial or full immunity against all others *e.g.* (Andreasen *et al.*, 1997; Ferguson *et al.*, 2003), or to just a subset of other strains as in the ‘limited-epitope’ model (Gupta *et al.*, 1998; Gupta and Galvani, 1999); combinations of both have also been considered (Gupta and Galvani, 1999; Dawes and Gog, 2002). Another way of looking at the acquisition of immunity is through ‘polarized’ immunity. An invention of Gog and Grenfell, in this framework only some recovered hosts gain immunity to antigenically related strains, whilst the rest remain totally susceptible (Gog and Grenfell, 2002). This type of model can only be considered as part of a ‘status-based’ framework (Gog and Swinton, 2002).

The exact dynamics exhibited by each model depends on a number of factors, but can be broadly outlined as follows. Weak immunological interaction between antigenically related variants leads to an absence of strain structuring where all coexist in the host population with equal abundance. Strong interaction causes discrete structuring where a subset of variants co-exist, but have competitively excluded everything else. Both of these scenarios correspond to antigenic stasis because the respective strain structuring is stable with respect to time. One other behaviour that is observed in certain models is oscillatory dynamics, which correspond to antigenic evolution (*e.g.* (Andreasen *et al.*, 1997; Gupta *et al.*, 1998; Gomes *et al.*, 2002)). Importantly, oscillatory dynamics are not universally observed in all models, for example they are extremely rare within ‘status-based’ models (*e.g.* (Dawes and Gog, 2002; Gog and Swinton, 2002)). Patterns of antigenic evolution are also hard to elicit in systems with less than four variants, but this can be done by changing assumptions

about symmetric cross-reactivity (Lin *et al.*, 1999) or through ADE (*e.g.* (Ferguson *et al.*, 1999)). Oscillations may also arise in very simple systems due to stochastic effects (Levin *et al.*, 2004).

1.5.2: Within-host models

Models of within host dynamics build on the predator-prey type dynamics of the models of Lotka (Lotka, 1920) and Volterra (Volterra, 1926) with the viral pathogen seen as the predator and its target cell the prey. The so-called Lotka-Volterra equations exhibit a wealth of different dynamics. At its heart are undamped oscillations around a neutrally stable equilibrium in the basic system (where one species predate upon another). Here, predator and prey dynamics indefinitely follow periodic oscillations that do not converge on the true equilibrium, although their long-term mean prevalence does. This situation is structurally unstable and even small changes to the basic equations can make the equilibrium either stable or unstable, in contrast to its previous neutrality.

Expansion of a basic Lotka-Volterra system to include more than two species can lead to the admission of a multiplicity of equilibrium solutions, of varying stability. Limit cycles, a structurally stable cousin of the basic neutral oscillations, are also possible, as are chaotic attractors, which yield contrastingly aperiodic, unclosed trajectories, resulting in irregular oscillations and the chaos for which they are named. Simple adaptations of the basic equations are therefore capable of generating a whole host of complex phenomena.

Many of the models that have been used to describe within-host dynamics bear striking resemblance to some macroscopic analogue in the form of a model for spread within a population. In essence, the two are fundamentally the same except for the fact that the

population is now a microscopic population of cells, and perhaps the chief sources of difference between the two areas of research are the therefore divergent natural processes governing population birth, interaction and death. Nowak and May have referred consequently to within-host models as “micro-epidemiology” and go on to note that the basic model of epidemiological dynamics is identical to the basic model of virus dynamics “apart from the equation for free virus which hardly ever matters” (Nowak and May, 2000). Indeed, perhaps one could argue that in models of epidemiology, an implicit assumption has always been that free virus dynamics (whose analogue is virus particles persisting within the macroscopic environment) can be ignored because they occur on an extremely rapid timescale relative to the dynamics of the host population in question. This is not necessarily so when instead dealing with viruses and cells.

Several important results have emerged from the endeavours of theoreticians to consider within-host processes. Further, these models have been used not only to investigate proposed models of pathogenesis (*e.g.* (Nowak *et al.*, 1991)) but also to deduce intrinsic biological properties of a pathogen, such as its basic reproductive ratio (*e.g.* (Little *et al.*, 1999; Stafford *et al.*, 2000)) or the half-life of an infected cell (*e.g.* (Bonhoeffer *et al.*, 1997b)). In many ways a direct corollary of the evolutionary history of these models dating back to Lotka-Volterra, one of the earliest of these results was the observation that viral populations (predators) can decline simply as a result of running out of targets (prey) to attack, as opposed to such declines being the sole preserve of an active and effective immune response (Nowak and Bangham, 1996). In the same paper, Nowak and Bangham also showed how viral load and magnitude of an immune response are not necessarily correlated: for example, a strong response will control the virus at low magnitude whereas a weak response may eventually manage to control the virus, but to do so requires a much greater magnitude of response. They further note that cytopathic viruses will achieve a

much lower viral load at equilibrium than non-cytopathic viruses and counter-intuitively irrespective of the rate of production of new virions. On a similar note, others have shown that CD8⁺ mediated cell lysis can reduce viral load by limiting production of new virions but with only small effects on the half-life of an infected cell (Klenerman *et al.*, 1996).

The study of within-host models has also lead to the concept of ‘dynamic elimination.’ This term is used to describe the initial large amplitude oscillation of virus, with viral load reaching a peak and then subsequently crashing, when the viral population size at the trough of the crash is insufficient to maintain the infection, even though the mathematical model permits a stable equilibrium solution. In these circumstances, the probability of a dynamic elimination increase with responsiveness of the immune system and the proportion of target cells used up during the initial phase of viral growth (Nowak and May, 2000). In persistent infection, where dynamic elimination has not occurred, it has been hypothesised that a long-lived CD8⁺ memory response is critical for the elimination of the virus (Wodarz *et al.*, 2000). Further, they also showed that the longevity of CD8⁺ responses is also associated with the probability of an initial dynamic elimination.

Another important area of research in within-host models has been associated with the concept of viral quasispecies as originally considered by Eigen and Schuster (Eigen and Schuster, 1977; Eigen *et al.*, 1989). This theory essentially refers to the equilibrium distribution of viral sequences created by the large mutation rate and strong selective forces acting on a viral population and has important consequences as a concept, many of which have been elucidated through mathematical considerations of the mutational process. Perhaps chief among these consequences are criteria for the emergence of drug resistance within a host. Especially important is whether a drug resistant mutation is present prior to the start of treatment (Ribeiro *et al.*, 1998), as considered by a collection of models

(Bonhoeffer and Nowak, 1997; Bonhoeffer *et al.*, 1997a; Bonhoeffer *et al.*, 1997b). In the instance of presence, these have revealed a positive correlation between sensitivity of wild type virus to the drug and the emergence of the resistant variant, but with the interesting corollary that, as with the strength of CD8+ response above, the total benefit (*i.e.* total increase in susceptible cells or reduction in viral load) is independent of drug dosage in the case of the drug being impotent against the variant. Nevertheless, the size of total benefit is dependent on the pre-treatment frequency of the resistant mutant. On the other hand, if the resistant mutant is not present prior to the induction of therapy, then if the drug is potent enough to eliminate the infection then a mutation that is unlikely to pre-exist within a large viral population is even less likely to emerge on a short time scale of eradication. However, if the drug cannot eliminate infection, then resistance is sure to eventually emerge albeit at a pace commensurate with its fitness advantage.

Models have also focussed on the immunodominance of an immune response against multiple epitopes during an infection ((Nowak *et al.*, 1995a; Nowak *et al.*, 1995b; Nowak and Bangham, 1996)), which can be thought of as being analogous to competitive exclusion between predators that target the same prey (Nowak and May, 2000), and has also been used in epidemiological models (*e.g.* (Gupta *et al.*, 1998)). Among these insights is the fact that it is not essential that *de novo* antigenic material is constantly generated during the course of infection – the dynamics of the basic predator-prey interaction between immune system and virus are sufficient to cause so-called antigenic oscillations of perpetually present variants. Production of new antigenic material can however shift dominance between epitopes; in particular, the emergence of a variant epitope that is poorly targeted at an immunodominant epitope will not necessarily come to dominate the viral population but will shift the focus of the immune response elsewhere. In turn, shifting

immunodominance to intrinsically weaker epitopes in this manner has been shown to increase viral load.

Typically then, these models follow the viral population within a single host and consider how processes such as mutation and immune responses act to shape the course of an infection. Consequently, often either they are to begin with or they end up being multi-strain models, and highlight the role of cross-reactive responses on the within-host dynamics of viruses (see, for example, (Nowak *et al.*, 1991)).

1.6: Overview of thesis

This thesis focuses on the population dynamics of three antigenically diverse RNA viruses: dengue, influenza and HIV-1. It comprises a set of studies highlighting the roles of structural constraints on critical antigenic determinants, interactions between immune responses to different antigenic types, host lifespan, and the degree of mixing between different host populations in determining the epidemiology and within-host dynamics of these pathogen systems.

Dengue exists in humans as exactly four antigenically distinct serotypes, apparently without the possibility of further antigenic innovation. Although infection by one serotype appears to convey life-long protection to homologous infection, it is believed to be a risk factor for severe disease manifestations upon secondary, heterologous infection due to the phenomenon of Antibody-Dependent Enhancement (ADE). It is not clear if tertiary or quaternary infections are possible, and if so, how they contribute to dengue epidemiology. In this thesis, I investigate the effect of third and fourth infections on the transmission dynamics of dengue and show that the system more readily exhibits desynchronised

serotype oscillations and multi-annual epidemic outbreaks upon their inclusion. I also show that age-prevalence patterns of dengue and seroconversion rates are easier to reconcile with its likely low transmissibility (R_0) if allowing for more than two infections.

By contrast with dengue, human influenza viruses are known to be in rapid antigenic flux, manifesting in the sequential replacement of antigenic types. There are several competing hypotheses that seek to explain the antigenic evolution of influenza within a single host species. However, none of these have yet been used to explicitly consider the impact of lifespan on evolutionary dynamics. In this thesis, I have used a simple multi-locus model to explore the relationship between host lifespan and viral evolution, as well as to elucidate the effects of transmission between hosts of different lifespan in effort to capture the cross-species element of influenza transmission. My results challenge the notion that influenza viruses infecting hosts with short life expectancy will exhibit antigenic stasis due to the absence of selective pressure from herd immunity. I also show that linking populations of different lifespans seems to restrict, rather than promote, the possibility of antigenic evolution.

My final chapter concerns the within-host evolution of HIV-1. I propose a new model for the pathogenesis of HIV-1 where the transition to AIDS is primarily linked to the gradual loss of the ability to make new antibody responses as the CD4⁺ population declines. This simple model is able to account for a wide range of features of HIV-1 pathogenesis and the genetics of host susceptibility to disease progression, and implies that progression towards disease may facilitate rather than result from CD8⁺ T cell escape.

Together these studies emphasise that it is the changing profile of immune responses – either at the population level or within the host – that is the principal determinant of the dynamics of the pathogen, rather than the mode and tempo of antigenic innovation.

Chapter 2

Dengue fever: Do third and fourth infections matter?

2.1: Introduction

Dengue viruses belong to the *Flavivirus* genus of the family *Flaviviridae*. Flaviviruses are positive-sense single-stranded RNA viruses and most, but not all, require mosquitoes or ticks to complete their horizontal transmission (one such counter-example being Entebbe bat virus). Significant human diseases caused by flaviviruses include yellow fever, Japanese encephalitis and tick-borne encephalitis, but dengue is the most important with respect to incidence of human mortality and morbidity and is a growing global health concern.

Dengue viruses are transmitted from person to person mainly by the mosquito vector *Aedes aegypti* (and to a lesser extent by *A. albopictus*) and infect roughly 50 million people every year. Although most of those infected will remain asymptomatic or develop a self-limiting febrile infection known as dengue fever (DF), a significant number (roughly 500,000) progress to a severe disease characterised by plasma leakage usually coupled with haemorrhage. This is generally known as dengue haemorrhagic fever (DHF), but the most serious manifestations are sometimes referred to as dengue shock syndrome (DSS), which is also associated with extremely low blood pressure. DHF is a potentially lethal disorder where case fatality rates can be as high as 20% in the absence of adequate treatment. With

treatment, this rate drops to around 1%. In total, around 2.5% of those who develop DHF will die (TDR/WHO, 2009).

The mosquito vectors become infected when they feed on humans during the roughly 5 day period of infection. The virus then spreads systemically from the mosquito intestinal tract over the next 8-12 days, and the mosquito will remain infected for the remainder of its 2-4 week existence (TDR/WHO, 2009). Virus that migrates to mosquito salivary glands can be transmitted to humans when skin is broken during a blood meal. In the skin, the dengue virus may initially infect immature dendritic cells (Wu *et al.*, 2000), but is also capable of replication in a variety of other cells (Jessie *et al.*, 2004), such as macrophages and peripheral blood monocytes.

Each dengue virion comprises a spherical particle about 40-50 nm in diameter, with a lipid envelope. The dengue genome is about 11kb in length and consists of a single open reading frame that encodes three structural – the capsid (C), precursor membrane/membrane (prM/M) and envelope (E) – and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Guzman *et al.*, 2010) (see Figure 2.1). The E protein is structurally conserved among flaviviruses and consists of three distinct domains (DI, DII and DIII) that between them are responsible for viral assembly, budding and entry into target cells, although DIII in particular seems to be the primary target of neutralizing antibodies (Beltramello *et al.*, 2010). Following genome replication and protein translation, virions are initially coated with prM-E proteins (Zhang *et al.*, 2003). These immature particles must have prM cleaved into the M protein to become infectious, but the inefficiency of this process means that many virions never attain maturity. The prM protein is targeted by host antibody and this inefficiency may therefore represent a strategy of immune evasion (Rodenhuis-Zybert *et al.*, 2011) and exploitation (Dejnirattisai *et al.*, 2010) by the virus.

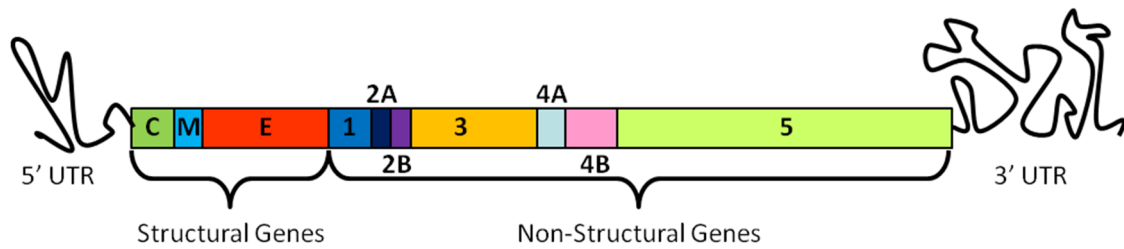


Figure 2-1: Dengue genome. *The single open reading frame of dengue encodes 3 structural proteins and 7 non-structural proteins.*

Dengue viruses are organised into four antigenically distinguishable serotypes: DENV-1, DENV-2, DENV-3, DENV-4 (Calisher *et al.*, 1989). Each serotype is believed to be descended from different viral lineages that were present in non-human primates and which independently entered humans between 100 and 1500 years ago, probably in Asia (Gubler, 1997; Wang *et al.*, 2000). Long term epidemiological data reveal multi-annual cycles in disease prevalence and sequential replacement of dominant serotypes (for example, see Figure 2-2). Within each serotype, a number of distinct genotypes, or clusters of virus, have been defined based on a limited degree of within-cluster sequence divergence in the relatively small E/NS1 junction of the genome (Rico-Hesse, 1990; Vasilakis and Weaver, 2008). One can also distinguish among serotypes in this manner.

Primary infection induces lifelong protective immunity to the infecting serotype (Halstead, 1974) and is generally asymptomatic in children, though adults often develop DF (Guzman *et al.*, 2010). There is a suggestion that recovery from infection leads to a short period of cross-immunity to all dengue strains (Sabin, 1952), but it is unclear if this means being merely clinically asymptomatic (as is often the case in dengue infection), or being incapable of transmitting disease. Nevertheless, a distinguishing feature of infection by dengue is that subsequent heterotypic infections are consistently associated with instances of severe disease (*e.g.* (Halstead *et al.*, 1967; Halstead *et al.*, 1970; Sangkawihba *et al.*, 1984; Thein *et al.*,

1997; Guzman *et al.*, 2000; Fried *et al.*, 2010)), along with primary infections in infants < 1 year old born to dengue-immune mothers (Kliks *et al.*, 1988; Hung *et al.*, 2004).

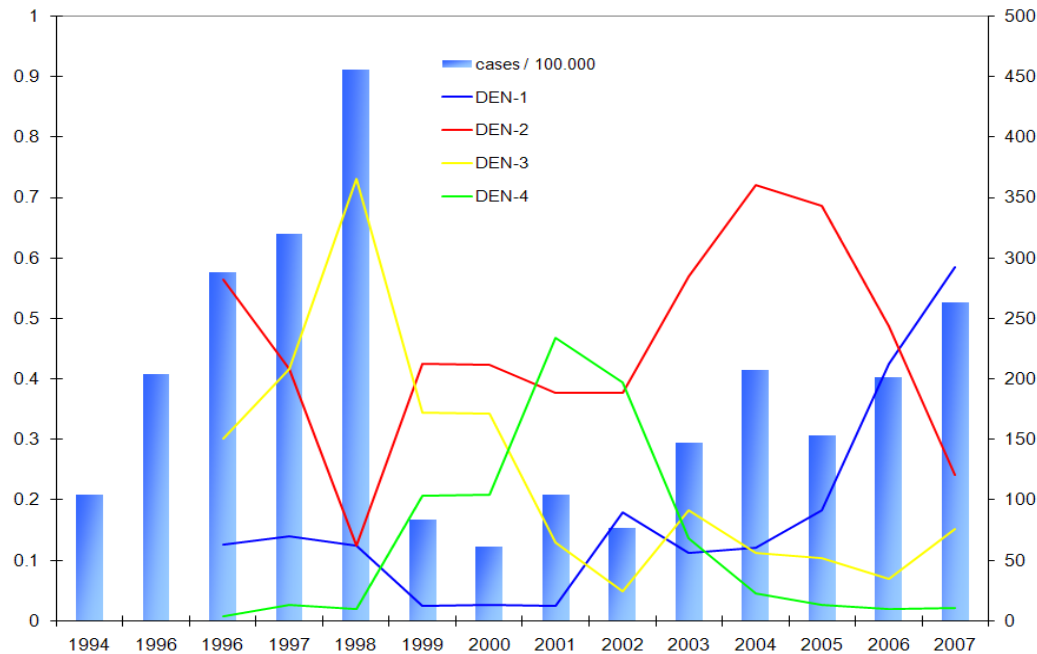


Figure 2-2: Dengue epidemiology in South Vietnam. *The total annual number of dengue cases (blue bars) and relative serotype prevalence (lines) over the period 1994–2008 in the southern 20 provinces of Vietnam show the characteristic fluctuation in disease incidence and sequential replacements of dominant serotypes. Source of data: Vietnamese Ministry of Health Dengue passive surveillance scheme and kindly provided by the Pasteur Institute, HCMC, Vietnam. The Hospital for Tropical Diseases is a tertiary referral hospital for infectious diseases.*

A link between second heterotypic infections and disease has not always been observed *e.g.* (Guilarde *et al.*, 2008) and other authors have suggested alternative risk factors for severe disease, such as intrinsic genotypic virulence (Harris *et al.*, 2000), gender & age (Guzman *et al.*, 1984; Halstead *et al.*, 2001; Guzman *et al.*, 2002), nutrition (Nimmannitya *et al.*, 1969; Kalayanaroj and Nimmannitya, 2005) and host genetic predisposition (Bravo *et al.*, 1987). T cell immunopathology has also been associated with severe disease, with the suggestion that it may be the immune response to the virus that causes severe disease, as opposed to

the virus itself (Bethell *et al.*, 1998; Green *et al.*, 1999; Mongkolsapaya *et al.*, 2006; Duangchinda *et al.*, 2010).

An explanation for increased severity of later heterologous infections is a phenomenon described as Antibody Dependent Enhancement (ADE) (Halstead *et al.*, 1973). In effect, non-neutralizing antibodies, either cross-reactive from a previous infection or transiently acquired from an immune mother, bind to epitopes on the surface of the virus and consequently inadvertently promote viral entry into Fc receptor-binding cells such as dendritic cells, macrophages and monocytes. Such enhancing antibodies have been found to target both prM (Dejnirattisai *et al.*, 2010) and E (Schiefflen *et al.*, 2010) in humans. The former suggests that immature virions with uncleaved prM may be able to exploit ADE to become indirectly infectious.

Fc receptor-binding by antibody is normally crucial for aspects of cell activation, endocytosis and phagocytosis (Daeron, 1997). As the result of ADE however, it results in suppression of the innate immune response, which in turn leads to increased intracellular infection, viral replication and viraemia. T cell immunopathology may then directly follow due to egregious secretion of inflammatory cytokines and, ultimately, the endothelial damage and altered haemostasis characteristic of severe disease (Nielsen, 2009; Guzman *et al.*, 2010; Halstead *et al.*, 2010).

Original antigenic sin could also play a role in ADE by boosting poorly-neutralizing, low-avidity antibody in secondary infection. A recent study demonstrated an inverse relationship between the avidity of an anti-dengue antibody response and enhancement (Midgley *et al.*, 2011). Antibodies recalled from memory to deal with heterologous infection

may therefore both promote entry into Fc receptor bearing cells and be intrinsically inferior at neutralization.

One key question, not yet definitively answered, is whether ADE is simply an important clinical phenomenon, or whether it also results in increased susceptibility to and/or transmission of an infection. In some seroepidemiological studies there have been suggestions of higher infection rates among those primed by a previous infection when compared to the completely immune (Halstead *et al.*, 1970; Guzman *et al.*, 1990; Guzman *et al.*, 2000). Those authors note that this could be due to spatial heterogeneity in dengue transmission but, as mentioned, experimental studies seem to indicate that host cells are more permissive to secondary infection (Dejnirattisai *et al.*, 2010; Schiefflen *et al.*, 2010; Midgley *et al.*, 2011).

With respect to transmissibility, it has been suggested that both peak viral titres (Vaughn *et al.*, 2000a; Libraty *et al.*, 2002; Wang *et al.*, 2003; Wang *et al.*, 2006) and slow viral clearance (Wang *et al.*, 2003; Wang *et al.*, 2006) correlate with disease severity and enhancement. Others have found that viraemia may last longer in primary, as opposed to secondary, infections (Gubler *et al.*, 1981; Vaughn *et al.*, 1997; Vaughn *et al.*, 2000a). If true, this could act to balance out any increase in transmissibility due to an elevated peak viral titre in secondary infection. However, it has been suggested that that *A. aegypti* has limited oral susceptibility to infection (Vasilakis and Weaver, 2008). Thus, although the total duration of viraemia may be shorter in heterologous infection, the period of time during which viraemia is sufficient to infect a feeding mosquito could be increased (see Figure 2-3) rendering secondary infections more transmissible.

From a more rigorous mathematical perspective, one can consider competition between two viral strains (1 & 2, say) within a simple SIR model, where recovery from one strain imparts immunity to both. One can suppose that both viral strains have the same basic reproductive number, R_0 , and recall that $R_0 = B_1D_1 = B_2D_2$ is a product of each virus' transmissibility (B) with its duration of infection (D). Interesting dynamics arise when, say, strain 2 is more infectious but for a shorter duration of infection *i.e.* $B_2 > B_1$ but $D_2 < D_1$. This could be true of secondary dengue infections. In this case of equal R_0 , strain 2 is able to competitively exclude strain 1: there is a selective advantage to being more transmissible for a shorter period of time as opposed to vice-versa. Thus, enhancement can remain beneficial for secondary infections even if the associated viraemia is cleared more rapidly than in primary infection.

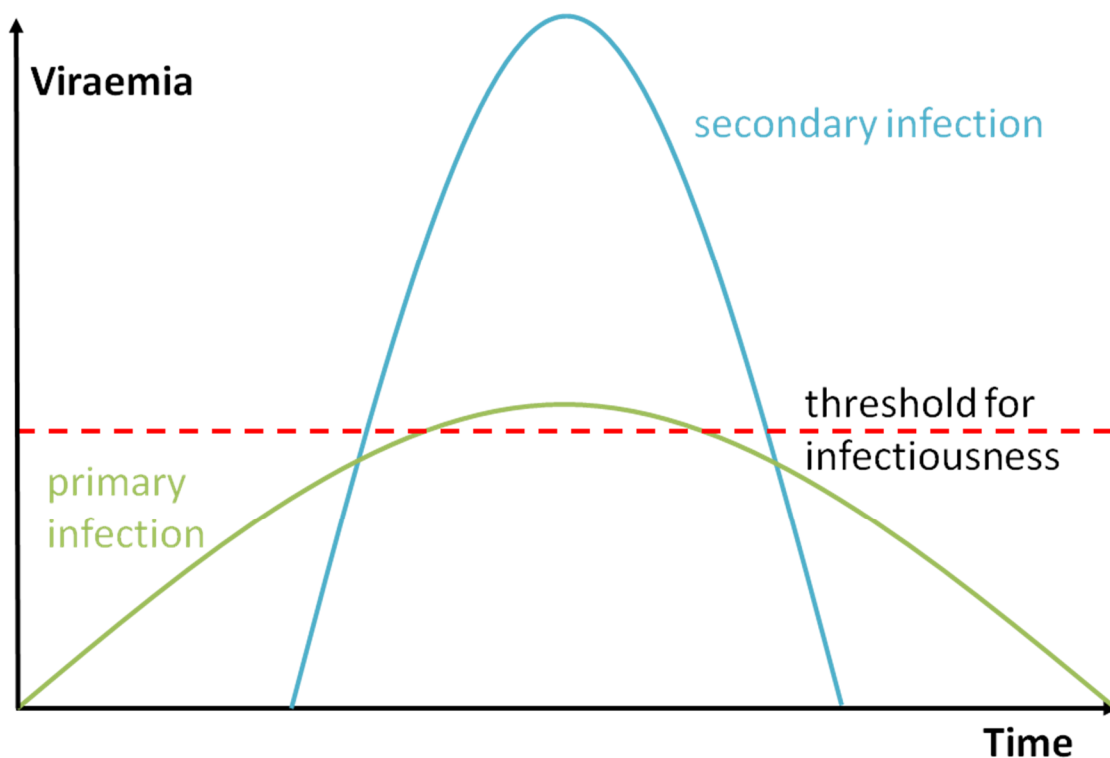


Figure 2-3: Theoretical relationship between viraemia and infectiousness. *Viraemia may be shorter but with a higher peak in secondary infection (blue line) when compared with primary infection (green line). However, if there is a threshold viraemia (dotted red line) required for virus to be transmissible to a mosquito, then the period of time in which a host is infectious may be longer in secondary infection. This effect would be magnified further if increasing excess of the threshold correlated with increased transmissibility.*

Uncertainty has not discouraged modellers from investigating the potential effects of a link between enhancement and infectiousness/susceptibility. In 1970, Fischer and Halstead showed that assuming secondary, but not subsequent, infection was a risk factor for severe disease provided the best theoretical fit to their age-stratified data (Fischer and Halstead, 1970). Much later, Ferguson *et al.* showed that enhancement of transmission of secondary infections could generate desynchronised, epidemic behaviour in a model of two interacting serotypes (Ferguson *et al.*, 1999). They also showed that ADE could act to favour persistence of both strains by acting as a rescue effect: as one strain circulated in the population, it paved the way for the enhancement and later success of the other. This

hypothesis was revisited by another set of authors who demonstrated that accounting for all four serotypes further augments this effect (Cummings *et al.*, 2005). They showed also that this relationship between enhancement and persistence is non-linear. Too much enhancement results in very large amplitude oscillations in single serotype prevalence, which increases the likelihood of stochastic extinction of individual serotypes. ADE can therefore encourage the persistence of serotypes that interact in this manner, but only up to a point. In another paper, the same authors revealed that enhancement of transmissibility also allowed the recovery of the characteristic 3-5 year period of dengue epidemics (Schwartz *et al.*, 2005). Soon after, Adams and Boots made an attempt to consider aspects of enhancement other than just increased transmissibility, as well as cross-immunity, in a two serotype model (Adams and Boots, 2006). They showed that increased susceptibility to secondary infection could also generate cyclical dynamics, though the same was not true of enhanced mortality. In contrast to enhancement and its promotion of persistence, cross-immunity encouraged competitive exclusion. More recently, the level of enhancement required for epidemic behaviour has been shown to be reduced by taking account of enhancement of both susceptibility and transmission (Recker *et al.*, 2009). This allows the model to exhibit the typical 3-5 year period of dengue epidemics before enhancement is sufficient to threaten global persistence.

Dengue models that incorporate ADE are not the only models that generate irregular epidemic behaviour that corresponds to data. Seasonal forcing, together with either temporary (Wearing and Rohani, 2006) or permanent (Adams *et al.*, 2006) cross-protection, can also do the trick. There are also a number of models that synthesise ADE with other effects. For example, Nagao and Koelle have suggested that taking account of both ADE and cross-protection can account for changing patterns in the average age of infection in

Thailand (Nagao and Koelle, 2008). Subsequent work however, has shown that this data can be alternatively explained by demographic changes alone (Cummings *et al.*, 2009).

It is widely recognised that a high proportion of dengue infections are asymptomatic and clinically imperceptible (Burke *et al.*, 1988; Endy *et al.*, 2002; Shepard *et al.*, 2004; Porter *et al.*, 2005; Anderson *et al.*, 2007; Cordeiro *et al.*, 2007). To highlight just one example, a 1996 study in Haiti demonstrated that over 85% of children had antibodies to 2 or more dengue serotypes despite no child having been hospitalized or dying of clinical symptoms or signs suggestive of DHF/DSS for at least 16 years (Halstead *et al.*, 2001). A major obstacle in determining the underlying nature of dengue epidemiology lies in the fact that most data are based on clinically-reported cases.

A further complication is the effect of original antigenic sin, between dengue serotypes and also other flaviviruses. This has been exploited to allow deduction of the serotype of the primary infecting virus in secondary cases (Halstead *et al.*, 1984), but it also means that it is often impossible to distinguish among second, third and fourth infections (Innis *et al.*, 1989; De Paula and da Fonseca, 2004; Alvarez *et al.*, 2006; Gibbons *et al.*, 2007; Guzman *et al.*, 2010). Consequently, “secondary infection”, particularly in clinical studies, often means that a patient is believed to have recovered from at least one prior dengue infection *e.g.* (Nimmannitya *et al.*, 1969; Halstead *et al.*, 1970; Ming *et al.*, 1974; Nisalak *et al.*, 2003; Pervin *et al.*, 2004; Fried *et al.*, 2010). One clinical study that tried to resolve this was conducted following a dengue epidemic in Cuba in 2001-2002. Of the 17 adults hospitalised with DHF/DSS whose data is fully presented, the authors suggested that 11/17 (65%) were cases of secondary infection, whereas 6/17 (35%) may have been due to a tertiary infection (Alvarez *et al.*, 2006). In the same study, among 40 adults who had been hospitalised with DF, 6 (15%) may have been tertiary infections, 30 (75%) secondary, and 4 (10%) primary

infections. Another clinical study that tried to estimate the incidence of third and fourth infections in hospitalisation was undertaken through analysis of 11 years of admissions of children to two hospitals in Thailand (Gibbons *et al.*, 2007). Here the authors considered only those children for whom two separate admissions for dengue were recorded – 191/15825 (0.01%). There were complete records on vaccination against Japanese encephalitis and serologic data from both admissions for only 125/191 (65%) of these children. Of these, 36/125 (29%) represented a primary infection at first admission followed by a secondary infection at second admission and 13/125 (10%) were attributed to third or fourth infection. The authors could not be sure of the remaining 76/125 (61%) – antibody cross-reactivity was again the confounding factor. Unfortunately, they do not report how many of the remaining 15634/15825 (99.9%) cases for which just one admission for dengue was recorded may have been caused by primary, secondary or tertiary infections.

Longitudinal studies are better placed to uncover tertiary dengue infections as they repeatedly sample sera from the same individuals. Sera from different time points can then be compared to infer dengue infections, especially when participants are otherwise asymptomatic. One such study based in Rayong, Thailand provides evidence of further (*i.e.* third or fourth) infections of children already in possession of antibodies to at least two dengue serotypes (Sangkawihba *et al.*, 1984). This study also uncovered instances of initially seronegative children who had antibodies to at least two dengue serotypes 12 months later. A later study in Yogyakarta, Indonesia uncovered similar results; in particular, 103/401 (26%) of children with pre-existing antibodies to at least 2 dengue serotypes got infected by a heterologous serotype during the 12 month study (Graham *et al.*, 1999).

Overall then, it seems reasonable to conclude that third and fourth infections do occur, often asymptotically, although repeat incidences of hospitalisation with dengue are rare. One key area of uncertainty is the transmissibility of such clinically-silent dengue and this is reflected in the way in which various theoretical models have represented the epidemiology of the virus. Some models explicitly include the possibility of third and fourth infection (Wearing and Rohani, 2006; Nagao and Koelle, 2008; Chikaki and Ishikawa, 2009). Others assume complete immunity after a second heterologous infection (Ferguson *et al.*, 1999; Cummings *et al.*, 2005; Cummings *et al.*, 2009; Recker *et al.*, 2009) or model only two serotypes (Ferguson *et al.*, 1999; Schwartz *et al.*, 2005; Adams and Boots, 2006; Adams *et al.*, 2006). Wearing and Rohani have compared 2- and 4- strain versions of their model and find that there is little difference between them. However, this does not tell us what effect the ‘twice-infected, protected’ assumption has on the epidemiology of dengue.

In this chapter, I explicitly examine the effect of third and fourth dengue infections by contrasting the epidemiological dynamics of a previously analysed ‘twice-infected, protected’ model that includes ADE (Recker *et al.*, 2009), to one that permits further infections. I will show that whilst relaxing this assumption leads to qualitatively similar behaviour, third and fourth infections significantly affect the *per capita* risk of infection and the ensuing age-dependent incidence rates of DHF in the population.

2.2: Methods

I present two models to compare the effect of third and fourth infections on the epidemiology and transmission dynamics of four co-circulating dengue serotypes. The models are based on previously published epidemiological frameworks (Ferguson *et al.*, 1999; Cummings *et al.*, 2005; Recker *et al.*, 2009) and differ in their assumptions about

acquired immunity. In the reduced model (RM), I assume that recovery from a secondary heterologous infection renders the host completely immune against subsequent challenges. The full model (FM) assumes that exposure to all four serotypes is necessary for complete protection.

A previous study of the RM scenario has shown that increased transmissibility and susceptibility can both independently and synergistically explain the observed desynchronised serotype dynamics and irregular epidemic behaviour of dengue (Recker *et al.*, 2009). Thus, for simplicity, I will assume that enhancement manifests solely in terms of increased onward transmission. In both models I therefore assume that ADE acts to increase infectiousness of all non-primary infections. In RM, this means that only the second infection is enhanced, after which the host becomes completely immune to all four serotypes. However, in FM, an individual's second, third and fourth infections are all enhanced.

As noted above, repeat hospital admissions for dengue are rare. Allowing enhancement of multiple infections in FM therefore implies that enhancement of transmission is not directly correlated with severity of disease. In some way this may reflect the fact that peak viraemia occurs significantly earlier than the peak of the clinical syndrome; viraemia has abated by the time the disease is at its most severe (Libraty *et al.*, 2002). As outlined earlier, the severity of secondary infections is likely related to a combination of increased susceptibility of Fc-receptor bearing cells to infection and the recall from memory of cross-reactive but inappropriate T- and B-cell responses. If so, then an infected host must face the same problems during the, say, third encounter with dengue as they did at the second. Viraemia may again attain a much higher peak than in primary infection, be subsequently rapidly cleared and have had its transmission enhanced as before. By this encounter,

though, the immune system may now be mature enough to rein in its response as viraemia declines and avoid the immunopathology implicated in severe disease. A corollary of this more mature immune response could be that transmission is not as enhanced during third and fourth infections as it is in the second.

2.2.1: Mathematical models

In order to describe the RM and FM models I need to divide a given population into the following classes, where each represents the fraction of the population that:

$\hat{z}_i$ - has recovered from infection by serotype i only;

z_i - has recovered from infection by at least serotype i ;

y_i - is infectious with a primary infection with serotype i ;

$\tilde{y}_i$ - is infectious with serotype i , having previously recovered from infection by a heterologous serotype;

r - is completely protected against further infection;

w - has recovered from infection by at least one serotype;

s - is completely susceptible to infection;

I assume in both models that the host population size is constant, with $1/\mu$ denoting the average host life expectancy and $1/\sigma$ representing the average duration of infection. I assume that hosts recover at the same rate from primary and subsequent infections. Since dengue infection typically lasts for less than a week (Kuno, 1997), I also assume the incidence of multiple infections is negligible. I also take note of the fact that the dynamics of the vector population occur on a much faster time scale than those of the host. Thus, although dengue is a vector-borne disease, I choose to model its transmission through direct contact between susceptible and infectious individuals. This in line with most other authors *e.g.* ((Ferguson *et al.*, 1999; Cummings *et al.*, 2005; Adams and Boots, 2006; Adams *et al.*, 2006; Nagao and Koelle, 2008; Cummings *et al.*, 2009; Recker *et al.*, 2009)) and allows the models to be greatly simplified.

The force of infection of serotype i , λ_i , is defined as $\lambda_i = \beta y_i + \varphi \beta \tilde{y}_i$, where β is the transmission coefficient of individuals suffering from a first-time dengue infection. φ describes the increase in transmissibility during secondary (or subsequent) infections due to the action of ADE, with $\varphi = 1$ denoting no enhancement and a value of $\varphi = 1.5$, for example, corresponding to a 50% increase in transmissibility.

Finally, although differences in virulence and an effect of infection order on disease outcome have been suggested by some authors *e.g.* (Halstead, 1970; Sangkawihba *et al.*, 1984; Vaughn *et al.*, 2000a), I assume that there are no differences among the serotypes in terms of transmissibility or duration of infection.

I can now write down the models as the following sets of Ordinary Differential Equations:

RM – secondary infection leads to complete protection

$$\frac{d\hat{z}_i}{dt} = \lambda_i s - \left(\sum_{k \neq i} \lambda_k \right) \hat{z}_i - \mu \hat{z}_i \quad (2:1)$$

$$\frac{dy_i}{dt} = \lambda_i s - \sigma y_i \quad (2:2)$$

$$\frac{d\tilde{y}_i}{dt} = \lambda_i \left(\sum_{k \neq i} \hat{z}_k \right) - \sigma \tilde{y}_i \quad (2:3)$$

$$\frac{dr}{dt} = \left(\sum_{i=1}^4 \left(\sum_{k \neq i} \lambda_k \right) \right) - \mu r \quad (2:4)$$

with the proportion susceptible to any infection simply given as $s = 1 - r - \sum_{i=1}^4 \hat{z}_i$.

FM – full protection only gained by exposure to all 4 serotypes

$$\frac{dz_i}{dt} = \lambda_i (1 - z_i) - \mu z_i \quad (2:5)$$

$$\frac{dy_i}{dt} = \lambda_i (1 - w) - \sigma y_i \quad (2:6)$$

$$\frac{d\tilde{y}_i}{dt} = \lambda_i(w - z_i) - \sigma\tilde{y}_i \quad (2:7)$$

$$\frac{dw}{dt} = \left(\sum_{i=1}^4 \lambda_i \right) (1 - w) - \mu w \quad (2:8)$$

Figure 2-4 provides a visualisation of the transfer among compartments in both models. For simplicity, only three serotypes are considered in the figure.

In order to compare and contrast the dynamics of the two models, I perform a series of simulations. I explore the effects of both R_0 and the level of ADE (φ) within the two models. The basic reproduction number R_0 is defined here as β/σ and in all simulations I vary R_0 from 2.5 - 4 solely through changes in transmissibility β . For simplicity I keep all other parameter values constant and set one unit of time equal to one year and set $\mu = 0.02$, equivalent to an average host life expectancy of $1/\mu = 50$ years, and $\sigma = 73$ equivalent to an infectious period of $1/\sigma = 1/73$ of a year ≈ 5 days.

Unless stated otherwise, all results are taken and qualitative analyses performed after running the models for sufficient time to remove the effect of transient dynamics and to allow the system time to settle on a particular dynamical behaviour.

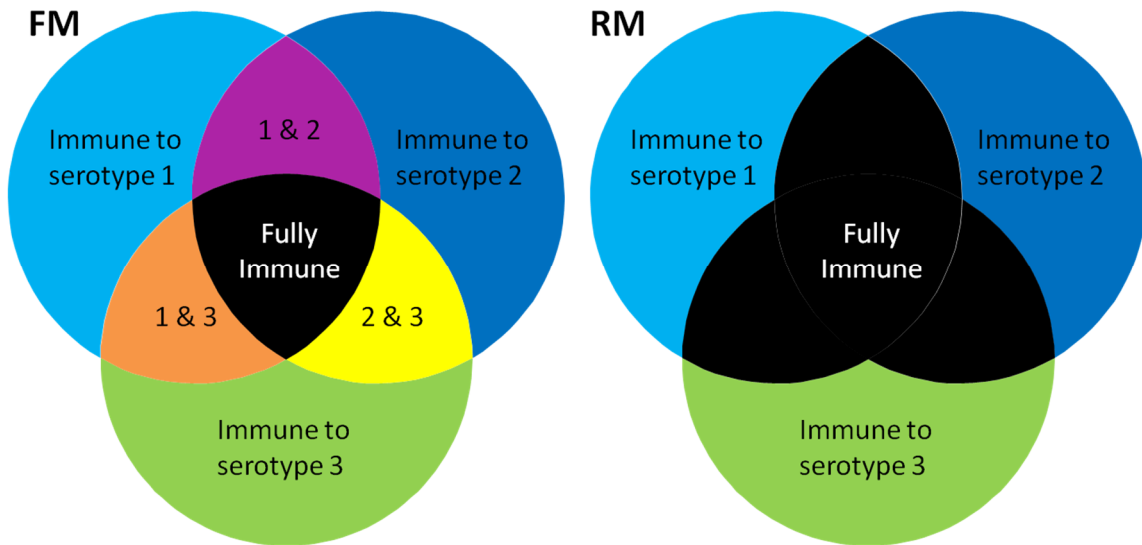


Figure 2-4: Comparison between FM and RM. *Note that only three serotypes are considered for the purposes of this figure; the general principle is entirely analogous to the reality of 4 dengue serotypes. FM: an individual needs to be infected by each serotype in order to become fully immune, and undergoes ADE at each infection subsequent to the first. For example, an individual is originally completely susceptible to dengue and outside the Venn diagram. Infection by serotype 1, say, leaves the individual immune to future infection by serotype 1 but at risk of an (enhanced) infection by serotype 2 or 3 (light blue). If infected by serotype 2, the individual is now immune to serotypes 1 and 2 (pink) but at risk of an (enhanced) infection by serotype 3. Following infection by serotype 3, the individual becomes fully immune (black). RM: an individual becomes completely immune to dengue infection after having been exposed to any two serotypes. Infection by serotype 1, say, leaves the individual immune to future infection by serotype 1 but at risk of an (enhanced) infection by serotype 2 or 3 (light blue). Following infection by either of these two serotypes, the individual becomes completely immune to all future dengue infection (black).*

2.3: Results

Figure 2-5 shows a selection of time series (for fixed R_0) that represent typical model output, highlighting the wide range of dynamical behaviours observed in both models under increased levels of enhancement φ . In line with previous studies (Ferguson *et al.*, 1999; Cummings *et al.*, 2005; Recker *et al.*, 2009), the following pattern of behaviour emerges. For low levels of enhancement there is steady-state equilibrium with all serotypes persisting at equal frequencies. As the degree of enhancement, φ , is increased, this equilibrium is replaced by synchronized oscillations. Further augmentation of φ desynchronises these oscillations, leading to the emergence of a more ‘chaotic’ strain structure where individual serotypes are sequentially replaced. Finally, the model approaches a state where periods of low disease prevalence are interspersed with semi-regular epidemic outbreaks. In both models this stage appears to be further characterised by the sequential replacement of the dominant serotype.

Notably, however, the time-series presented in Figure 2-5 suggest that the progression between these stages is much faster in FM and also suggest that the amplitude of oscillations in this case is around 5-10 times larger than in RM. To qualitatively and quantitatively compare both models and thus highlight the effect of third and fourth infection I use a variety of methods for examining serotype and incidence dynamics.

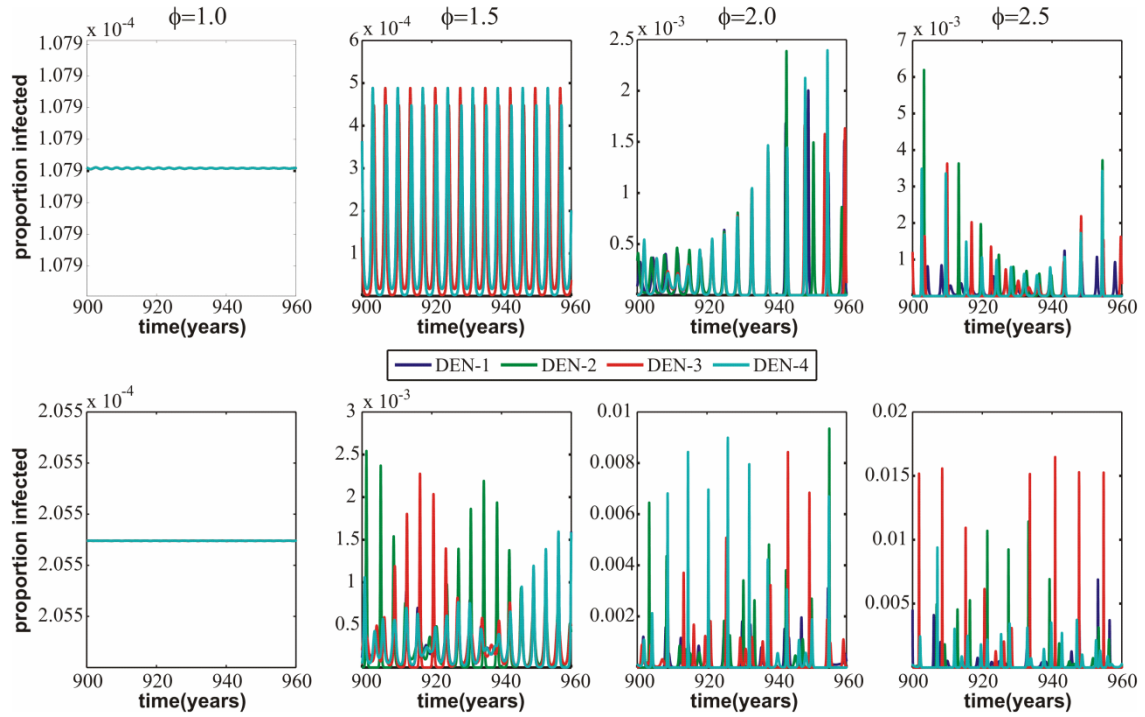


Figure 2-5: General model dynamics under parameter changes. Here I observe the fluctuations in the total proportion of the population that has been infected by each serotype against time for different levels of transmissibility enhancement φ (from left to right: $\varphi = 1.0$, $\varphi = 1.5$, $\varphi = 2.0$, $\varphi = 2.5$; no enhancement is represented by $\varphi = 1.0$; increasing φ corresponds to augmented levels of enhancement). The top row is for RM whilst the bottom row is for FM. The individual serotype dynamics seem to become increasingly desynchronised as φ increases in both models, though this effect appears stronger in FM.

2.3.1: Serotype synchronisation

First I analyse the synchronisation pattern between two arbitrary serotypes over a period of 1000 years; I choose serotype 1 and 2 but since the dynamics of each serotype are independent, any pair would give the same results. Complete synchronisation indicates that the prevalence of each serotype is equal for the entirety of this period; partial synchronisation defines a regime in which the serotypes are not fully synchronised their prevalence coincides for at least one period of more than 100 years; and the dynamics are described as desynchronised in all other cases. Figure 2-6a shows that in both models desynchronised oscillations characterise a large and roughly equivalent portion of

parameter space. However, there is a tendency in RM for some partial synchronisation at low R_0 and high φ , which is not the case in FM.

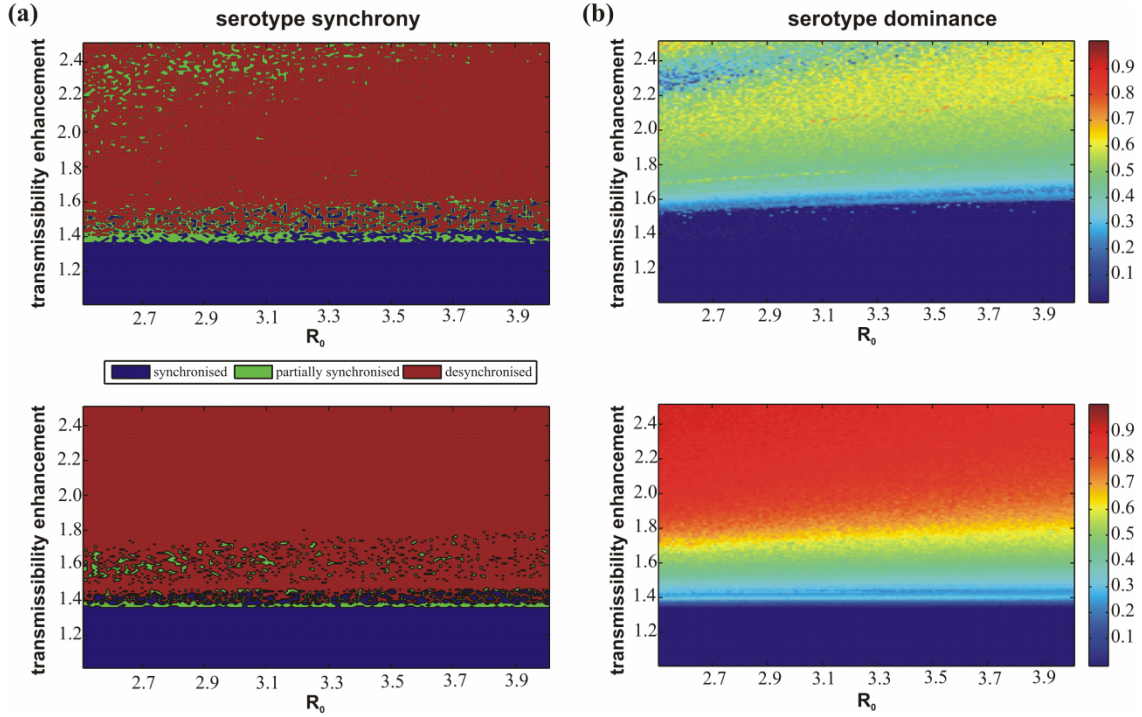


Figure 2-6: Comparison of synchronisation and single-serotype dominance between RM (top) and FM (bottom). (a) The synchronisation pattern between serotypes 1 and 2 indicates that, for both models, most of parameter space is characterised by desynchronised behaviour i.e. the dynamics of the two serotypes are not ‘locked’ together. (b) Using a measure of single serotype dominance (where 0 (blue) corresponds to at least two serotypes being simultaneously dominant and higher values (more red) indicate a greater tendency for one serotype to be dominating at any given time), one can observe that in both models the trend is for increasing levels of dominance with increasing enhancement; this trend is more pronounced in FM than RM.

2.3.2: Sequential replacement of dominant serotypes

I now look at the serotype dynamics in terms of the sequential replacement of a dominant serotype. To quantify sequential dominance I use a measure previously introduced by (Recker *et al.*, 2007), given as:

$$\varepsilon = \frac{1}{P} \sum_{i=1}^P \frac{Y_{max}^i - Y_{sub}^i}{Y_{max}^i} \quad (2:9)$$

where P = number of epidemic peaks observed in some time interval (1000 years in this instance), Y_{max} = the prevalence of the peaking dominant serotype and Y_{sub} = the prevalence of the serotype with the second-highest peak. In other words, I analyse where epidemic peaks occurred in the model and then consider whether these epidemics consisted of one or more dengue serotypes being prevalent. Effectively, I calculate the proportion of disease caused solely by the most prevalent serotype during each epidemic and then average this value across all epidemics within each model run. These values are given in Figure 2-6b. Thus, in this definition $\varepsilon = 0$ (dark blue in the figure) corresponds to at least two strains always being simultaneously dominant, and $\varepsilon = 1$ (dark red) indicates that every epidemic observed in the model consisted of just a single, and therefore totally dominant, serotype. Intermediate values of ε correspond to varying levels of single serotype dominance – smaller values (blue-green) indicate that epidemics tend to consist mostly of at least two serotypes, whereas larger values (yellow-red) indicate that epidemics tend to consist of one serotype much more than any other. In practice a value of $\varepsilon = 1$ is unattainable, but the higher the value of ε , the more the dynamics of the system correspond to epidemics that consist solely of a single serotype.

Figure 2-6b demonstrates how ε varies with R_0 and φ . In both models there is a trend of increasing dominance by - and thus replacement of - a single serotype with increasing levels of enhancement, φ . However, this trend is substantially more pronounced when allowing for third and fourth infection. Further, for $\varphi \geq 1.8$ in FM, the values of ε are much larger than the maximum value of ε observed anywhere in RM. In other words, in FM, it is much easier to recover the dynamic of a single dominant serotype in a dengue epidemic.

2.3.3: Inter-epidemic period

I next use a standard mathematical approach (spectral analysis of the total dengue prevalence) to determine the inter-epidemic period. These results are shown in Figure 2-7a. Here, blue colouring corresponds to short epidemic cycles of roughly 2 years duration and the transition to green and then yellow and red corresponds to a lengthening of the inter-epidemic period. Both models readily exhibit the characteristic 3-5 year (sky blue, approximately) epidemic cycles of disease incidence associated with dengue for a reasonably wide range of enhancement, φ . Notably, however, the cycles are generally longer and increase more quickly with increasing levels of enhancement in FM compared to RM, where full immunity is gained after two infections. I also observe a trend towards longer cycles at high- φ , low- R_0 ; again, this tendency is much less pronounced in RM. The consequence is that cycles in FM that are twice as long as those observed in RM. Indeed, it is very rare in RM to observe an inter-epidemic period of longer than 6-7 years or so, but in FM I observe such periods of 12 years for a significant region of parameter space.

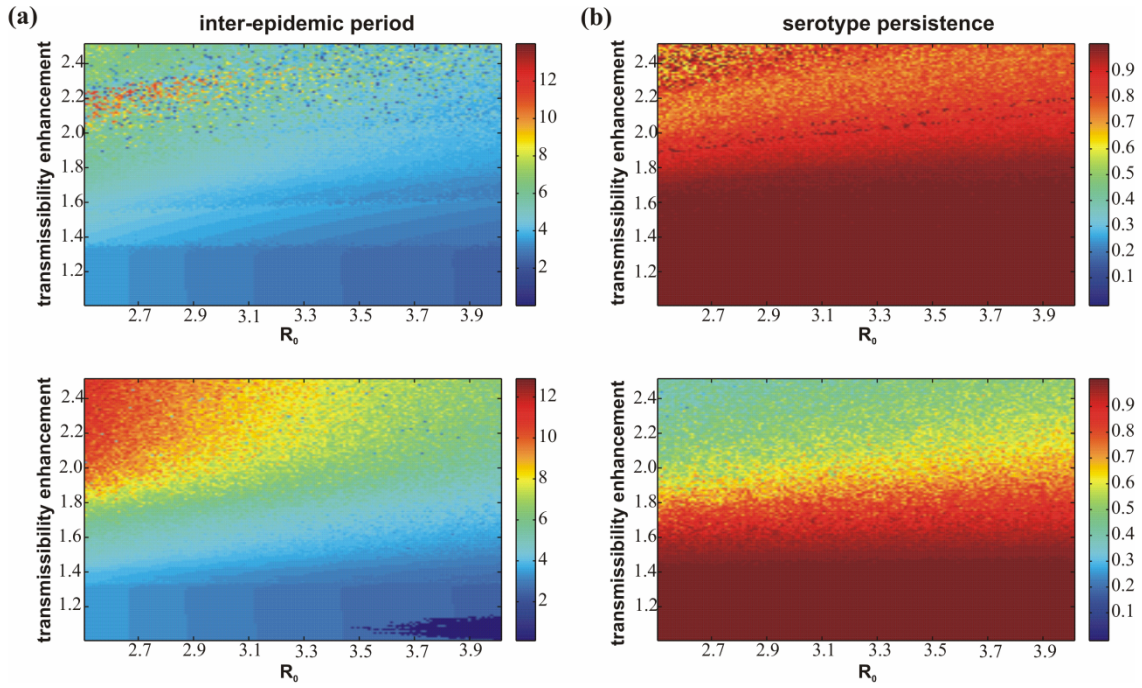


Figure 2-7: Comparison of inter-epidemic period and serotype persistence between RM (top) and FM (bottom). (a) As enhancement increases so too does the epidemic period observed in each model. There is also a trend towards longer periods at lower R_0 . However, these trends both appear to be stronger in FM. (b) The risk of stochastic extinction within the model is shown as the proportion of time in each model that the prevalence of a particular serotype exists above a specific threshold. In both models there is a low risk of extinction but the risk increases with enhancement; again, this trend is stronger in FM.

2.3.4: Serotype persistence

It has previously been shown that high levels of enhancement increase the risk of serotype extinction due to large epidemic outbreaks leading to extended periods of low transmission (Cummings *et al.*, 2005). In line with previous work I measure persistence as the proportion of time a particular serotype persists above a given threshold level of 10^{-8} (Cummings *et al.*, 2005; Recker *et al.*, 2009). The larger this value, the less likely a serotype is to go extinct through small random population fluctuations. Figure 2-7b shows that there is a low risk of stochastic extinction, corresponding to the dark red colouring, for low levels of enhancement in both models. As in previous studies, high levels of enhancement

significantly increase this risk of extinction (yellow-green colouring) and much more so when allowing for third and fourth infections in FM, due to its higher propensity of exhibiting large amplitude oscillations as observed in Figure 2-7b.

2.3.5: Age structure at equilibrium

The only notable effect of third and fourth infections, so far, has been an overall higher propensity for desynchronised, large amplitude oscillations. This can be directly attributed to an overall higher level of transmission in FM, resulting from a larger proportion of the population being susceptible to infection and onward transmission. One ought to therefore also expect an effect on age-structured prevalence and incidence rates. To explicitly compare the age structure of prevalence within each model, I adapt the methods of (Gupta and Day, 1994). I consider in both models the distinct (unstable) equilibrium solutions where all strains have equal forces of infection in the case of ‘medium’ levels of enhancement ($\psi = 2$). This equilibrium serves as an approximation of the mean prevalence of each serotype over a long period of time. In this case, the proportion of the population that has experienced exactly i different strains is given by I_i . The probability of acquiring a new infection is then proportionate to the number of as yet un-encountered strains. In this case with four co-circulating dengue serotypes individuals enter state I_{i+1} from state I_i at a rate $(4 - i)\hat{\lambda}$, where $\hat{\lambda}$ is the average per capita force of infection per strain. The dynamics of this system with respect to time, t , and age, a , may then be described by the following set of partial differential equations for $i=0..3$:

$$\frac{\delta I_{i+1}}{\delta t} + \frac{\delta I_{i+1}}{\delta t} = (4 - i)\hat{\lambda}I_i - (4 - i - 1)\hat{\lambda}I_i \quad (2:10)$$

where the proportion of individuals yet unexposed is given by

$$I_0 = 1 - \sum_i I_i \quad (2:11)$$

I can solve these equations (noting, in this instance, that the time derivative is zero) to approximate how the number of infections varies with age in each model.

In Figure 2-8a I plot this data for each model in order to compare and contrast age structure. In Figure 2-8b I make a minor adaptation to the models, in that I consider what would happen if there were only 2 or 3 co-circulating serotypes of dengue. To generate the figure, I have repeated the above but show only how the age of first infection, average age of disease and total force of infection changes for each scenario in both models. Finally, Figure 2-8c shows how the average age of first infection changes with increasing R_0 in both models.

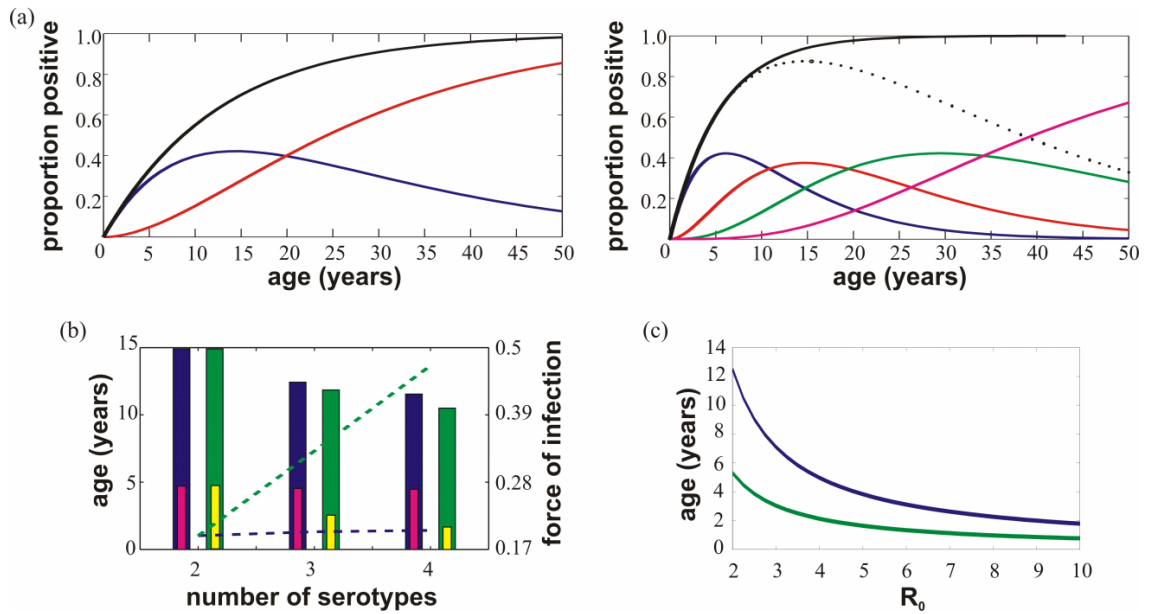


Figure 2-8: Comparison of age structured dynamics between RM and FM. (a) The lines show the proportion of the population at each age (for RM (top) and FM (bottom)) who have suffered one (solid dark blue line), two (solid red), three (solid green), four (solid magenta) and any (solid black) dengue infections. For FM the proportion of the population that is at risk of disease (defined as having seen 1, 2 or 3 serotypes) is also plotted (dotted black) for comparison to the equivalent in RM (solid dark blue). Generally, people are exposed to dengue at an earlier age in FM, experience heterologous infections younger, and take much longer to become completely immune. (b) For RM (FM), the blue (green) bar shows how the average age of disease (DHF), determined as heterologous infection, changes with the number of serotypes present whilst the small bars show the change in age of first infection. The increase in the total force of infection with the number of serotypes is shown as dotted lines (RM: blue, and FM: green). (c) For RM (blue line) and FM (green line) I observe that increasing R_0 acts to decrease the average age of first infection (here estimated as $1/\text{total force of infection}$) and that for all levels of R_0 this value is significantly lower when allowing for third and fourth infection (FM). Parameter values: $\varphi = 2.0$ ((a), (b) and (c)) and $R_0 = 2$ (a), $R_0 = 4$ (b).

Figure 2-8 thus demonstrates that including the possibility of third and fourth infection results in a higher force of infection (2-8b) which generally exposes hosts to dengue at an earlier age (2-8c) but also delays the acquisition of full immunity (2-8a). As an example, it takes roughly twice as long for 50% of the population to become completely immune compared to the case where two infections are sufficient for complete protection (2-8a). Consequently, allowing for more than two infections increases the pool of people able to onwardly transmit dengue, which in turn explains the observed higher force of infection. I

also observe in FM that the proportion of the population that has experienced at least one heterologous infection is greater at all ages, which could translate to an increased risk of DHF/DSS (2-8a). Finally, the increased force of infection due to third and fourth infections marks a much higher level of seroconversion: for example, in RM, by the age of 10 years, only 30% have seen two infections and 30% are still completely susceptible, whereas by the same age in FM around 60% have seen at least two infections and only 10% remain completely susceptible (2-8a).

2.4: Discussion

I have constructed a framework to examine the effect of third and subsequent infections on dengue epidemiology. My results indicate that the qualitative nature of the behaviour observed in models with and without third and fourth infections is similar. In both, there is a transition from synchronised serotype oscillations to their desynchronisation and an increased tendency towards single serotype dominance and replacement as I increase the level of ADE. I also observe an augmented risk of stochastic extinction and longer inter-epidemic periods due to large amplitude epidemic outbreaks that leave the majority of the population immune. Consequently, I can argue that the fundamental properties of both systems remain invariant to the introduction of possible third and fourth infections.

However, there are certain critical quantitative ways in which the results of the models differ from each other. From my analysis, markedly different patterns of the age structure of infections can be observed depending on the number of subsequent infections allowed (Figure 2-8). A notable feature of dengue is that it can reach very high seroprevalence rates at a relatively early age. For example, in Haiti, a 2001 study of 210 6-13 year olds revealed that 98% of the cohort had been previously exposed to dengue (Halstead *et al.*, 2001),

similarly a 2006 study of 4-16 year old Nicaraguans found an overall seroprevalence of 91%, with 80% of the children exposed by age 5 (Balmaseda *et al.*, 2006) and a comparable study in Thailand revealed a notable, though lower, overall seroprevalence of 66% (Thai *et al.*, 2005). My results show that in order to obtain seroprevalence rates that are consistent with epidemiological data one has to assume values of the basic reproductive number, R_0 , that are very much higher in a ‘twice infected, protected’ framework than if third or fourth infections are allowed (Figure 2-8c). This is because relaxing the assumption that two heterologous infections are sufficient to achieve protection from further challenges leads to an overall higher force of infection which in turn causes a significant drop in the age of first infection and thus higher levels of seroprevalence across all ages (Fig 2-8a).

Estimates of the basic reproductive number range from $R_0 < 2$ to $R_0 > 11$, depending on location and the methods used (see, for example, (Koopman *et al.*, 1991; Ferguson *et al.*, 1999; Massad *et al.*, 2003; Favier *et al.*, 2006; Nagao and Koelle, 2008)). However, there are a number of reasons to believe that high values of R_0 are unlikely for dengue. Many other vector borne pathogens have evolved sophisticated immune evasion mechanisms to prolong their infectious period, in order to overcome the uncertainty in transmission and vector abundance; dengue, by contrast, has a very short period of infection (Vaughn *et al.*, 2000b). Another issue is the limited oral susceptibility of its principle vector *A. aegypti* to infection. It has been suggested that it may require high human viraemia levels for the virus to be transmitted effectively (Vasilakis and Weaver, 2008). Thus, two of the crucial components of R_0 – infectivity and the duration of infectiousness – of dengue are likely to be low; indeed a degree of enhancement of later infections may be crucial to ensure a continued chain of transmission. Furthermore, the average lifespan and dispersal of *A. aegypti*, are limited (Trpis and Hausermann, 1986; Harrington *et al.*, 2005) which also acts to reduce the likelihood of sustained transmission. Therefore, third and fourth infections

might be critical for reconciling the R_0 of dengue with the observed high seroprevalence in younger age groups.

As a corollary to the above, increasing the number of co-circulating serotypes in a particular transmission setting has a much greater effect on both the force of infection and age of first infection when allowing for tertiary and quaternary infections. As shown in Fig 2-8b, and in line with previous work (Gupta *et al.*, 1994), the overall risk of infection in this case increases linearly with the number of serotypes whereas the average age at first infection declines in inverse proportion. By contrast, increasing the number of serotypes has a negligible effect on overall force of infection when assuming full protection after two infections only. Looking at the epidemiology of dengue in regions where two serotypes have co-circulated for extended periods before the introduction of the remaining two could therefore provide a means of testing the two hypotheses. If the ‘twice-infected, protected’ hypothesis is correct then the average age of first infection should not decline with the introduction of third and fourth serotypes.

The average age of presentation with DHF has seen a steady increase over the last few decades in some parts of SE Asia (Kongsomboon *et al.*, 2004; Halstead, 2005), possibly in response to reduction in transmission through vector control programs (Nagao and Koelle, 2008) or as a consequence of demographic transitions (Cummings *et al.*, 2009). In these models I have assumed that enhancement manifests in terms of increased transmission of heterologous infection. Since third and fourth infections generally occur in older hosts, assuming further that any heterologous infection also carries a risk of severe disease means that the average age of clinical disease would be higher in FM. However, if I assume that other immune responses are able to confer clinical immunity in third and fourth infection, then including transmission of third and fourth infections will simply lower the average age

of DHF as it does the average age of first infection. Thus, my results show that a low age of seroconversion and a high age of DHF are more easily reconciled by relaxing the assumption of clinical protection after secondary infection.

The two models analysed in this work represent the two extreme ends of a cross-immunity spectrum. At one extreme, two infections are sufficient to protect from further infections and at the other all four serotypes have to be experienced. Importantly, in the latter scenario third and fourth infections are enhanced by pre-existing, cross-reactive antibodies. One can equally imagine the scenario where only secondary infections are enhanced but subsequent infections are possible. Here, however, there would still be an increase in force of infection and a drop in the age of both first infection and DHF. Reducing the transmission potential of third and fourth infections in FM only serves to slightly weaken the trends observed in switching between the two models. The important component is the assumption that third and fourth infections are in some way transmissible, as this has important effects on the epidemiology of infection and age patterns of disease.

Another important aspect of dengue transmission and R_0 that I have not considered here is the effect of spatial heterogeneity and the role of human movement (see for example (Adams and Kapan, 2009; Stoddard *et al.*, 2009)). Although my results are based on simple mass-action principles (i.e. random mixing between individuals), I expect my general conclusions to hold true when considering explicit spatial or contact structures. In fact, the possible constraints on dengue transmission imposed by spatial structure should benefit from high levels of third and subsequent infections.

As noted in this chapter's introduction, it is suspected that each of the 4 human serotypes is the result of 4 independent cross-species transmissions from primates. However, there

seems to have been a long lag between the first known transmissions of each serotype into humans, before the sudden and rapid emergence of first DENV-2 and the other three as epidemic strains (Twiddy *et al.*, 2003). The emergence of sustained dengue transmission in human populations is largely attributed to the Second World War and subsequent globalisation ultimately leading to large concentrations of humans in urban environments living in conditions perfectly suited to growth of *A. aegypti* populations (as outlined in (Vasilakis and Weaver, 2008)). This theory is supported by experimental data that tentatively suggests that sylvatic DENV-2 has not adapted to replicate more efficiently in humans (Vasilakis *et al.*, 2007). However, the critical human/mosquito densities required for sustained dengue transmission of multiple serotypes will have been reduced if ADE does enhance infectiousness of heterologous infection. Dengue may have consequently emerged as problem earlier and easier than it otherwise might have done.

ADE could also have been responsible for the rapid sequential emergence of all four serotypes. Aided by urban expansion, DENV-2 could have finally managed to become established in human populations and so facilitate through ADE the cross-species transmission and establishment of the other three serotypes. Enhancement of transmission could have compensated for a lack of fitness for human replication in sylvatic DENV-1, 3 and 4. More studies of the ability of sylvatic dengue to replicate in human cells would therefore be informative. Once established, ADE can then act to ensure co-existence by rescuing serotypes that would otherwise be in danger of disappearing, ensuring persistence of all four (Ferguson *et al.*, 1999; Cummings *et al.*, 2005). If it eventually turned out that there were more than 4 sylvan serotypes, it could be that those now co-circulating in humans are the only ones capable of exploiting ADE.

That each of the 4 circulating serotypes is believed to have been independently introduced into humans in Asia has important implications for the existence of third and fourth infections. Transmission of one serotype could have originally occurred, which would then have been well placed, via ADE, to aid and abet the subsequent transmission of a second. However, if humans become completely immune to dengue after two infections, then it becomes difficult to see how the third and fourth serotypes could have overcome strong herd immunity to serially spread from primates to humans in the same geographic regions.

Distinct sylvan dengue lineages are believed to have emerged as the result of allopatric (rather than sympatric) and/or ecological partitioning of ancestral sylvatic dengue in different non-human primate hosts (Holmes and Twiddy, 2003; Vasilakis and Weaver, 2008). This means that ADE could be seen as a consequence rather than the cause of the evolution and emergence of diversity. However, the sylvatic serotypes seem to now exploit similar non-human primate hosts. Consequently, if dengue serotypes did evolve allopatrically, they probably then later expanded into overlapping regions after their chance divergence into distinct, yet antigenically interactive, entities (Holmes and Twiddy, 2003; Vasilakis and Weaver, 2008). As suggested in the human case, ADE could have been crucial in the establishment of multiple serotypes in the same primate species.

The vast majority of selection acting on dengue appears to be purifying, or negative, selection. In fact, although some weak positive selection has been observed on putative B- and T- cell epitopes on the E protein (Twiddy *et al.*, 2002a; Twiddy *et al.*, 2002b), pretty much all of the observed positive selection has been implicated in increased transmission and virulence (Sanchez and Ruiz, 1996; Twiddy *et al.*, 2002a; Twiddy *et al.*, 2002b). This suggests that dengue is therefore unable to generate substantial antigenic diversity, in contrast to influenza and HIV-1. Indeed, there is a high degree of antigenic cross-reactivity

and structural conservation of the E protein among all flaviviruses. Strong purifying selection therefore seems to maintain certain integral structural features of the dengue E protein, but which are nonetheless highly immunogenic. This could be due to its need, as with most other flaviviruses, to maintain separate transmission cycles in vector and host (see, for example, the altered affinity for binding lectins of virus produced by insects and humans (Dejnirattisai *et al.*, 2011)). The consequence is close antigenic relationships between multiple serotypes, but which can nonetheless be maintained in a single population through the action of ADE.

These results demonstrate that it is not possible to determine whether a ‘twice-infected, protected’ hypothesis is a realistic and appropriate description of dengue from the observed epidemiology and its four serotypes alone; both models exhibit the same qualitative behaviour. However, allowing more than two infections has a significant impact on the overall force of infection of dengue and could be a more parsimonious explanation for the observed epidemiology than inducing high transmission rates. Crucially, the fact that low values of R_0 are perfectly compatible with high seroconversion rates is an important consideration for future vaccine strategies. On the other hand though, if third and fourth infections do have a significant contribution to the overall level of dengue transmission, any control strategies based on drug treatment of clinical cases alone can only be expected to have a minor effect. More data on the transmissibility of third and fourth infections is therefore of major importance. Finally, ADE may not be the only factor that governs the severity of disease, but perhaps ADE, together with the possibility of third and fourth infections were crucial for the emergence and persistence of dengue in humans as four discernible serotypes.

Chapter 3

Antigenic evolution of influenza in different species

3.1: Introduction

Influenza viruses are segmented, single-stranded, negative sense RNA viruses of the family *Orthomyxoviridae*. Three phylogenetically and antigenically distinct viral types – A, B and C – circulate globally in human populations, but type A viruses are responsible for the vast majority of influenza mortality. Human influenza is in a state of rapid genetic evolution and antigenic flux. Each dominant strain is periodically superseded by some variant that is sufficiently different so as to escape host immune defences and trigger a seasonal epidemic. These epidemics predominantly afflict the very young and the elderly, with little clinical disease in young adults. Occasionally, influenza pandemics also break out. There have been a number of these in the last 150 years, most notably the "Spanish flu" of 1918. This is estimated to have killed around 50 million people, with unusually high absolute and relative prevalence in young adults (Johnson and Mueller, 2002).

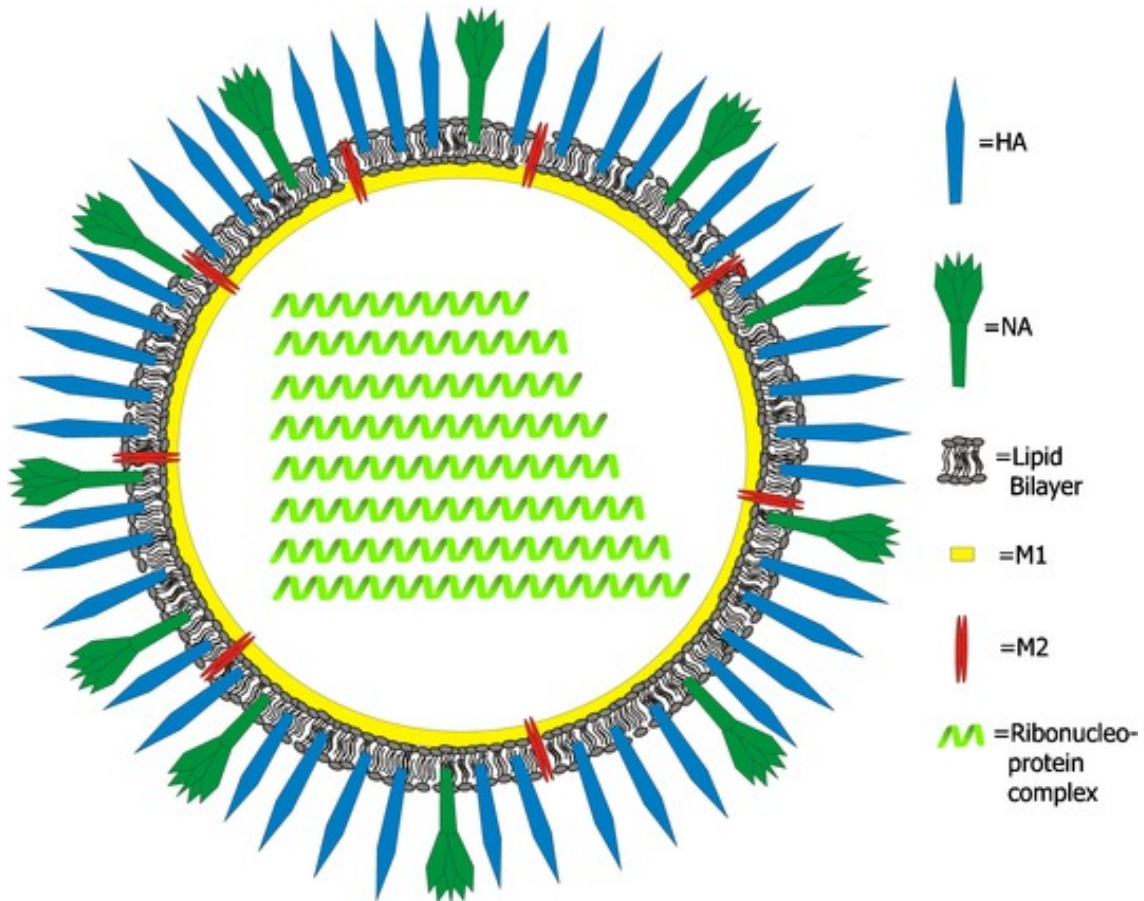


Figure 3-1: The Influenza Virus. A schematic of an influenza virus taken from Amorij *et al.*, (2008), reprinted with kind permission of Springer Science and Business Media.

An influenza A virus particle is largely spherical, around 80-120nm in diameter, and is encased by a viral envelope (Figure 3-1). The eight RNA segments within the viral genome encode 10, or sometimes 11, proteins. Segment 2 (which encodes polymerase basic 1, PB1) is the source of the discrepancy, as it sometimes also encodes a second short protein, PB1-F2, from an additional open-reading frame (Conenello and Palese, 2007). Slightly confusingly, the other polymerase basic protein (polymerase basic 2, PB2), is encoded by segment 1. Together with the polymerase acidic protein (PA), these form the trimeric RNA polymerase complex which binds to the 5' and 3' ends of the viral RNA to form a noncovalent circular complex (Klump *et al.*, 1997). The RNA fragments themselves are bound and protected by the viral nucleoprotein (NP) (Compans *et al.*, 1972). Collectively,

the viral RNA, polymerase complex and NP, form the ribonucleoprotein complex, RNP. The matrix (M) and non-structural (NS) segments each encode two proteins critical for viral replication: M1/M2 and NS1/NS2 respectively. The viral envelope consists of M2, which acts as an ion channel, and the two remaining proteins, haemagglutinin (HA) and neuraminidase (NA). These latter two are the main targets of the host humoral immune response.

Mature HA consists of two sub-units: the globular surface unit HA1, which physically binds to target cell receptors, and the stalk-like transmembrane protein HA2, which mediates fusion of the viral envelope with the host cell membrane (Skehel and Wiley, 2000). It is initially synthesised as HA0, but this precursor protein is non-functional. Maturity is achieved through cleavage of HA0 into HA1 and HA2 by host cell proteases (Garten and Klenk, 1999; Steinhauer, 1999). Many viruses (such as human and non-pathogenic avian viruses) are cleaved extracellularly, which limits their spread to those tissues where the appropriate proteases are available. The acquisition of a polybasic cleavage site is implicated in the pathogenesis of avian influenza as this allows a virus to be cleaved intracellularly by ubiquitous host cell proteases and thereby enables systemic replication (as reviewed in (Steinhauer, 1999)). HA1 binds to terminal sialic acids of cell glycoproteins and glycolipids (Gottschalk, 1957; Wiley and Skehel, 1989). Two major linkages between sialic acid and carbohydrate side chains are found in nature: $\alpha(2,3)$ and $\alpha(2,6)$ (Skehel and Wiley, 2000) and different HAs have different specificities for each linkage. Human viruses recognise the $\alpha(2,6)$ linkage, avian and equine viruses recognise $\alpha(2,3)$ and swine viruses recognise both (Rogers and Dsouza, 1989; Connor *et al.*, 1994; Gambaryan *et al.*, 1997; Matrosovich *et al.*, 1997; Ito *et al.*, 1998). These differences may be important in limiting the cross-species transmission of influenza *e.g.* (Matrosovich *et al.*, 1999; Matrosovich *et al.*, 2004b). To date, 16 variants, or subtypes, of HA have been

identified, but of these only H1, H2 and H3 have achieved sustained transmission in human populations.

NA is responsible for cleaving terminal sialic residues from carbohydrate moieties on the surfaces of the host cell and virus (Gottschalk, 1957), thus assisting in virus cell entry by mucus degradation (Matrosovich *et al.*, 2004a) and the release and spread of progeny virions (Palese *et al.*, 1974). There are 9 known NA subtypes, though again only a subset of these, N1 and N2, have achieved sustained transmission in humans. It is customary to refer to influenza A strains by their HA and NA subtype combinations (thus the 1918 strain was an H1N1).

3.1.1 Genetic evolution of human influenza

Large quantities of sequence data have facilitated extensive studies of the genetic evolution of influenza. One of the principal driving factors behind this evolution is the generation of, and subsequent selection acting on, amino acid variants that occur as the result of errors during genome replication. Accordingly, influenza virus evolution is generally said to be characterised by “cactus-like” phylogenetic trees, best described as a ‘trunk’ of fixed, advantageous mutations together with short ‘branches’ that proved only temporarily successful (Fitch *et al.*, 1997; Bush *et al.*, 1999b). However, these studies tend to focus on H3N2 viruses, and in particular the HA1 domain of its HA. By contrast, trees of the HA1 of human H1N1 (Ferguson *et al.*, 2003) and equine H3N8 (Daly *et al.*, 1996), for example, tend to exhibit much longer branches, indicating much more extensive and frequent co-circulation of different lineages. Furthermore, in contrast to HA1, whole genome phylogenies of H3N2 show co-existence of multiple viral lineages (Holmes *et al.*, 2005).

The segmented genome of influenza facilitates reassortment between viruses that co-infect the same cell. Those viruses then produced by this cell exhibit novel combinations of gene segments from parental strains and this process may therefore play an important role in generating both genetic and antigenic diversity, particularly in pigs and birds (Nelson and Holmes, 2007). Contrastingly, the extent of the role of recombination in influenza evolution is unclear, but may be low as is often the case with negative sense RNA viruses (Chare *et al.*, 2003). There is some evidence of non-homologous recombination between different viral segments (Khatchikian *et al.*, 1989; Orlich *et al.*, 1994), but there is little evidence for that which is homologous (Nelson and Holmes, 2007).

3.1.3 Influenza outbreaks

The general pattern of influenza occurrence is characterised by annual epidemics with clear signals of multi-annual cycles in strain abundance. These epidemic strains can cause severe disease in all age groups, but the groups at highest risk are those under 2 or over 65 years old, and those with underlying medical conditions such as asthma or diabetes. Worldwide, annual epidemics result in about 3-5 million cases of severe illness and about 250-500 thousand deaths. Antiviral drugs can effectively prevent and treat the illness, but the potential for the emergence of drug resistance is always there. These epidemics are highly seasonal in temperate climates, but the virus appears capable of all-year round transmission in the tropics, particularly in South East Asia, which may result in these regions acting as the source of seasonal strains (Rambaut *et al.*, 2008; Russell *et al.*, 2008). Epidemic human influenza exhibits rapid antigenic evolution, which means that recovery from infection in one epidemic does not confer life-long protection against infection by the causative viruses of future epidemics.

Occasionally (as in 1918, 1957, 1968, 1977 and 2009), a pandemic influenza virus emerges. The first of these viruses, the “Spanish” influenza of 1918, may have killed upwards of 50 million people (Johnson and Mueller, 2002), but subsequent pandemics have not been anywhere near as deadly (Taubenberger and Morens, 2010). Pandemic viruses are believed to be generated through reassortment events whereby human influenza strains acquire novel genes that allow them to assume a form to which a significant proportion of the world's human population have no clinical protection. This process is known as ‘antigenic shift.’ It is likely that the emergence of a new pandemic strain involves a constellation of changes in the virus proteins, but changes to the HA surface protein seem paramount. Accordingly, the first three recorded influenza pandemics of the 20th centuries each involved the emergence of new HA subtypes, namely H1 (1918), H2 (1957) and H3 (1968). This is in contrast to NA, with N1 (1918) being replaced by N2 (1957), which was then maintained in 1968.

Until 1977, only one of these subtypes had ever circulated at once. Seemingly as a result of the accidental release of an archived laboratory strain (Zimmer and Burke, 2009), H1N1 reemerged as a pandemic virus in 1977. This pandemic was not particularly severe, almost certainly because it was genetically identical to virus from 1950 and much of the population had therefore been exposed to it before. Since this pandemic, both H3N2 and H1N1 have co-circulated, although typically only one of these achieves widespread transmission in a given year.

In 2009 H1N1 was again responsible for the outbreak of a pandemic, notably despite the widespread circulation of this subtype in the population for the last 30 years. This pandemic H1N1 virus has been shown to be of swine origin and now seems to have replaced the prevailing H1N1 viruses (Neumann and Kawaoka, 2011).

3.1.4 Immunity to influenza

The first line of defence towards influenza infection is mediated by the innate immune system. Early responses from alveolar macrophages, dendritic cells and respiratory epithelial cells lead to the secretion of Type I interferons to stimulate macrophages and NK cells into an anti-viral response (Koyama *et al.*, 2007), and the secretion of chemokines to attract additional populations of potential effector cells (Herold *et al.*, 2006). The exact nature of the subsequent response by resident and recruited cell populations is less well understood (Valkenburg *et al.*, 2011). For example, the arrival of neutrophils in the lung has been reported in correlation with both disease control (Tate *et al.*, 2009) and severity (Crowe *et al.*, 2009). Generally though, innate immunity is believed to act in the usual way: adapting the inflammatory response, killing infected epithelial cells, priming dendritic cells for antigen presentation in the lymph node, and regulating CD8+ T cell proliferation and survival.

Interaction between antigen-presenting dendritic cells and B cells in lymphoid tissue results in division and production of antibody by B cells. These antibodies primarily target HA, but also NA. Sufficiently high titres of anti-HA antibody can provide sterilizing immunity and prevent virus binding and invasion of epithelial cells in the respiratory tract (Gerhard, 2001). NA-specific antibody contrastingly cannot provide sterilizing immunity but can operate to reduce morbidity and mortality by inhibiting the release of newly produced viruses (Gerhard, 2001). Recovery from infection by a particular influenza strain leads to the development of antibody-mediated lifelong protection against re-infection by that strain, as illustrated by the presence of NAb responses against 1918 H1N1 more than 90 years after infection (Yu *et al.*, 2008).

Original antigenic sin may be an important phenomenon with respect to B cell memory to influenza in humans. Indeed, the theory of original antigenic sin itself emerged from the observation that convalescent sera from an infected individual could react more strongly against virus responsible for a previous infection than against that from which the individual was recovering (Francis Jr. *et al.*, 1953; Fazekas de St. Groth and Webster, 1966; Webster, 1966). An important consequence is that pre-existing immunity can interfere with the response to immunization (Gross *et al.*, 1999; Sasaki *et al.*, 2008), although the epidemiological significance of the phenomenon is debatable *e.g.* (Haaheim, 2003).

CD8⁺ T cells are believed to be important in the clearance of influenza infection through the production of pro-inflammatory cytokines (La Gruta *et al.*, 2004) and direct killing of infected cells (Topham *et al.*, 1997). CD8⁺ T cell responses tend to recognise peptides from the internal proteins of the virus (Wahl *et al.*, 2009), which are often conserved across strains and subtypes (Assarsson *et al.*, 2008; Kreijtz *et al.*, 2008; Lee *et al.*, 2008). In mice, the absence of CD8⁺ T cell responses leads to a delay in virus clearance and more severe disease (Eichelberger *et al.*, 1991; Bender *et al.*, 1992) and this pattern seems to be replicated in humans (McMichael *et al.*, 1983b; Epstein, 2006; Kreijtz *et al.*, 2008). However, excessive secretion of cytokines and chemokines may also result in immunopathology and severe disease (La Gruta *et al.*, 2007; Peiris *et al.*, 2010). Upon clearance, the CD8⁺ T cell population contracts to leave a pool of long-lived, antigen-specific cells capable of rapid recall upon re-infection. From age 15, all human adults have circulating influenza specific memory CD8⁺ T cells (Lawson *et al.*, 2001). However, the protective effect of these responses may wane over a period of 3 years, and they may therefore require periodic re-stimulation (McMichael *et al.*, 1983a). The role of CD4⁺ T cells in immunity to influenza is less well characterised. CD4⁺ T cell responses seem important in the generation of effective CD8⁺ T cell memory responses (Riberdy *et al.*, 2000; Belz *et al.*, 2002), but an

absence of CD4+ T cell help in the lymph node does not preclude the development of primary CD8+ T cell responses (Riberdy *et al.*, 2000).

3.2: Methods for detecting antigenic change in influenza

Serologic assays play critical roles in various aspects of influenza surveillance, antigenic characterization, vaccine development and evaluation. Of these, the Haemagglutination Inhibition (HI) and Virus Neutralization (VN) assays are the most reliable and specific serologic methods for the antigenic characterization of influenza viruses.

3.2.1 Haemagglutination inhibition assay

The HI assay is the most widely used assay for the detection of antibody to the influenza virus. The test is based on the ability of the influenza virus to agglutinate the red blood cells of chickens (Hirst, 1941). The discovery that immune sera could prevent this agglutination then swiftly lead to the establishment of the earliest versions of this assay (Hirst, 1942; Salk, 1942).

Modern HI data is generated by challenging a host, often a ferret, with a particular isolate. Post-infection antisera is then taken from the animal and titrated against other viruses; dilution is achieved by adding more blood. The level of dilution at which the antisera stops being able to prevent agglutination of red blood cells by a virus determines the HI titre and higher titres indicate better prevention of haemagglutination. Originally, the primary purpose of these assays was to classify the subtype of an influenza virus. This may be achieved by testing against a panel of 16 reference sera harvested from infected ferrets – one for each of the 16 distinct HA subtypes. The subtype is then determined to

correspond with the serum that managed to inhibit the agglutination of erythrocytes; this usually amounts to having a titre of 1:8 (Pedersen, 2008). However, because the result of the assay is quantitative, it has also been used as a tool to measure the antigenic relatedness of different influenza strains. In this case, titres are assumed to correlate with antigenic relatedness between strains. An HI titre of 40 or more against another virus is regarded as a correlate of a 50% reduction in the risk of influenza infection by it (de Jong *et al.*, 2003).

Published HI tables have typically exhibited one of two patterns. The first, generally associated with antigenic evolution, is usually found when examining human HI data – here we see a pattern of very high titres to the strain used to generate the antisera, significant though highly variable titres to temporally close isolates and, usually, a complete loss of reactivity against strains isolated more than a few years distant from the considered strain. Contrastingly, the second pattern, generally associated with antigenic stasis, has been of reactivity extending over much longer, even decades, but also often with significant variations in titre. This has been most commonly observed in swine.

It is worth noting that the HI assay is not a functional test of immunity and merely measures the ability of a virus to agglutinate red blood cells. Given that the primary target of influenza infection is non-ciliated epithelial cells in humans (Matrosovich *et al.*, 2004b), it may therefore be that the ability of a mammalian virus to agglutinate avian red blood cells is not actually a very good correlate of protection and cross-reactivity. This argument is strengthened by evidence suggesting that the cross-reactive response of sera to an isolate raised in ferrets can be different to that of sera to the same isolate raised in mice and rabbits (Johansson and Kilbourne, 1992; Gillim-Ross *et al.*, 2008). To my knowledge, no comparative study has been done between the cross-reactive response of post-infection sera taken from ferrets and humans, or ducks and chickens or indeed for many of the pairs

of animals used such that post-infection sera from the former are used to infer antigenic relationships in the latter. Given the known differences between ferrets, mice and rabbits, it seems likely that we might also observe discrepancies between, say, ferrets and humans, possibly distorting the truth of whatever antigenic relationships can be gleaned from the assay.

There are, in any case, a large number of serious, technical drawbacks associated with the HI assay (de Jong *et al.*, 2003; Katz *et al.*, 2011). Firstly, the antisera used in such assays often contain non-specific inhibitors of HA, which cannot always be reliably removed by pre-treatment. HI titres depend also on the source of the red blood cells used in the assay and it is acknowledged that the observed titre may depend on the passage history of the viral isolate, as well as the species and individual immune response of the animal used for the production of antisera (Katz *et al.*, 2011). In particular, these difficulties compound the problem of the varying and evolving ability of different influenza viruses to agglutinate different red blood cells *e.g.* (Medeiros *et al.*, 2001). Furthermore, the analysis of the test itself is often difficult and is highly subjective; a comparative study of 5 laboratories in 1994 showed that the between lab variability in titres was as high as 112% (Wood *et al.*, 1994).

In the 60 years since the test was originally developed, it is perhaps disappointing that these concerns over its effectiveness remain, and one would perhaps have to question whether the test would have persisted if it were not so convenient.

3.2.2 Virus neutralization assays

Partly because of the issues with the HI assay, other techniques such as the VN assay have been developed and used to characterise antigenic relationships between circulating viruses

and reference or vaccine viruses (Katz *et al.*, 2011). The VN assay is a highly sensitive and specific method for detecting the strain-specific antibodies responsible for hindering viral replication. To do this, virus is first mixed with varying dilutions of serum, and then inoculated into culture where the extent of viral production is measured. The presence of virus-specific antibodies in the serum is therefore indicated by impaired or absent viral replication.

Originally, these assays were carried out by measuring the inhibition of viral growth and/or reduction in viral plaque formation in primary cells or embryonated chicken eggs. The current procedure is to instead conduct a microneutralization (MN) assay using cultured Madin Darby canine kidney (MDCK) cells. This process is highly standardised, but variation in titre arises from different measures of viral growth thereafter (Katz *et al.*, 2011). Most commonly, ELISA is used to detect the NP expressed in infected cells following overnight culture (Harmon *et al.*, 1988). This allows calculation of an end-point titre, as determined by the reciprocal of the serum dilution exhibiting at least 50% neutralization.

A problem with these assays is that the intra- and inter- laboratory variation in them is worse than the equivalent for HI assays (Stephenson *et al.*, 2007). VN and MN assays also take longer to carry out than HI assays and it is only possible to characterise just a handful of viruses within one test, which means that they are only irregularly used as a supplement to the latter. Despite these problems however, MN assays detect functional antibodies to HA, whilst modification to detect both HA and NA in aggregate is possible *e.g.* (Hassantoufighi *et al.*, 2010). As such, they represent a potentially better tool for measuring actual immunity to influenza than is provided by HI assays. This could explain why reports differ as to whether there is a consistent correlation between VN and HI assays. Within laboratories, HI titres tend to correlate well with the results of VN assays *e.g.* (Okuno *et al.*,

1990). However, it has been observed on multiple occasions that antisera to avian influenza viruses can have a high virus neutralization titre but a low or even absent HI titre (Yoden *et al.*, 1982; Rowe *et al.*, 1999). Figure 3-2 shows that such discrepancies could also be observed in the 2009 H1N1 pandemic. Others have discovered monoclonal antibodies which neutralize an influenza virus but do not inhibit haemagglutination and vice versa (Kida *et al.*, 1982; Gitelman *et al.*, 1986).

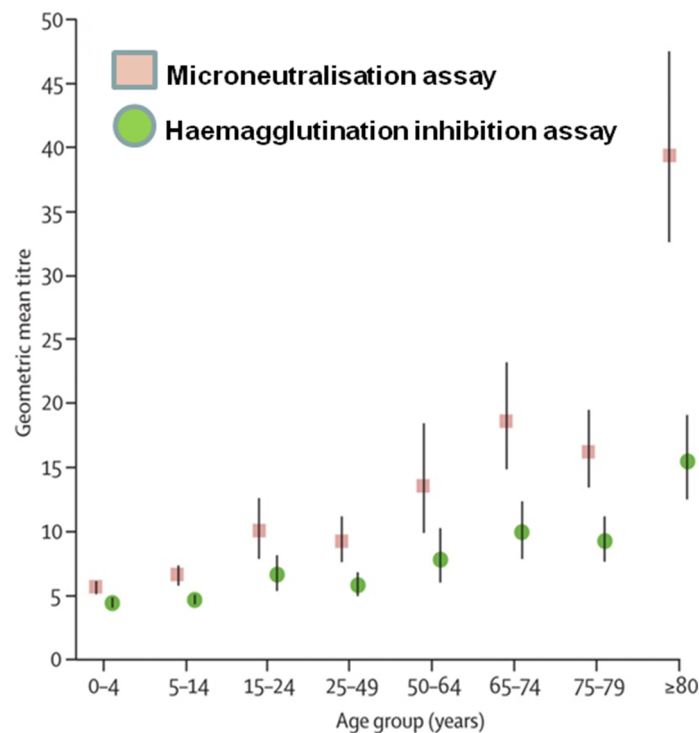


Figure 3-2: Example of discordance between HI and MN titre. *Geometric mean titre by age group against pandemic H1N1 as measured by the haemagglutination inhibition and microneutralisation assays. Error bars represent 95% CIs. The ≥80 year old age group have an extremely large MN titre, but a comparatively small HI titre. Reprinted from Miller et al. 2010, with permission from Elsevier.*

3.2: Antigenic evolution of human influenza

Each annual influenza epidemic consists of a set of viruses that are extremely homogenous antigenically. However, the antigenic profile of the viruses that emerge from season to season is not consistent. This observation, and the consequent ability of influenza to continually generate annual epidemics, has been generally attributed to the process known as ‘antigenic drift.’ Here, the virus incrementally accumulates mutations that render it less and less ineffective against the prevailing herd immunity, eventually allowing it to breach this barrier. This ‘model’ of antigenic evolution has gained wide acceptance, featuring in almost every virology textbook and regularly appearing as the opening sentence of any scientific paper on this subject. However, as I will demonstrate in this chapter, a formal mathematical exploration of this hypothesis shows its link to patterns of influenza dynamics and genetic and antigenic diversity to be far more tenuous.

The cactus-like trees of the HA1 domain of H3N2 viruses have been held by some to articulate the definitive proof of this linear antigenic drift. Although the majority of epitopic regions known to be targeted by host antibody responses can be found on HA1, these still represent only a subset of the entire domain and, consequently, a phylogeny estimated from full-length HA gene sequences will accurately represent shared ancestry among the sampled sequences but it will not necessarily accurately portray their antigenic relationships.

This is illustrated by discordance between patterns of genetic and antigenic evolution in humans. In particular, small genetic changes can have disproportionately large effects on antigenicity and the antigenic evolution of H3N2 viruses seems more clustered than continuous (Smith *et al.*, 2004). Although phylogenies of the internal genes of both H3N2

and H1N1 can also be described as ‘cactus-like’, the simile is stretched by frequent co-circulation of multiple lineages of these genes (Rambaut *et al.*, 2008). Whole genome phylogenies of H3N2 are likewise much less ‘cactus-like’ in nature and show co-existence of multiple viral lineages (Holmes *et al.*, 2005), which in itself implies that transition between antigenic types does not proceed in a simple linear manner.

Importantly also, Gray *et al.* have recently shown that such cactus-like trees can be recovered under neutral evolution as a straight-forward consequence of sampling sequences serially through time; positive selection is consequently just one force capable of creating such phylogenies and such a phylogeny does not therefore necessarily tell us much about the evolutionary forces acting on a given virus. Thus, it may be that there is much less to be gleaned about the antigenic evolution of influenza from these trees than has been originally supposed.

In truth however, the fundamental problem with the model of antigenic drift is that each influenza epidemic appears to comprise a virus population of very limited genetic and antigenic diversity. Under the action of antigenic drift, this can only be rationally expected if the antigenic space of influenza is one-dimensional, which it is not (Lapedes and Farber, 2001; Smith *et al.*, 2004). Consequently, the basic model of antigenic drift does not prevent a multiplicity of viral types from simultaneously acquiring antigenic characteristics that will allow them to escape the pressures of herd immunity and emerge to cause influenza epidemics. Thus, in order to adequately explain the antigenic evolution of influenza, either the basic model of “antigenic drift” needs careful refinement or an altogether rather different mechanism is required. To date, there have been 3 significant efforts to describe a plausible alternative mechanism.

The earliest of these models (Ferguson *et al.*, 2003) invokes a short-term strain-transcending immunity following recovery from infection. Essentially, this allows a single strain to give rise to an epidemic within the window of opportunity created by the rapid decay of cross-immunity. The waning of this immune response creates the opportunity for a new strain to invade the population provided it is sufficiently antigenically different from its predecessors. Simultaneously, the door is shut on the emergence of other such strains through the rapidly ensuing re-establishment of cross-immunity. The biological basis for this would have to be a conserved determinant to which a brief but powerful immune response was established upon infection. One plausible such mechanism could therefore be temporary T cell responses to influenza. Such responses are unlikely to prevent infection by a new strain, but are likely to promote viral clearance and reduce the likelihood of developing severe disease. However, the model requires a form of immunity that contrastingly prevents infection. Further, though seemingly temporary, these responses last much longer (a few years (McMichael *et al.*, 1983a; McMichael *et al.*, 1983b)) than those assumed by the model (6 months) and the potential effect of this is unclear. An additional problem is that the mutation rate must be restricted, and arguably excessively so, in order for this system to reliably reproduce single-strain epidemics.

The ‘neutral-network antigenic evolution’ model (Koelle *et al.*, 2006) suggests that the emergence of a single strain is essentially due to the failure of most genetic changes to produce an adequate phenotypic change. Here, most mutations are antigenically neutral, thereby limiting the probability that a mutated strain will have the capability of evading herd immunity. However, eventually a mutation that results in altered antigenicity inevitably arises, and with it, an antigenic cluster jump as observed in reality. Again though, the ability of this model to recreate the pattern of single strain dynamics ultimately depends on the rate of mutation and the architecture of its antigenic space.

A third model (Recker *et al.*, 2007) turns the notion of an infinitely large antigenic space, with unlimited possibilities for evolution, on its head. This approach instead argues that the antigenic space of influenza is relatively small and tightly constrained, with the virus capable of continually generating all of its possible antigenic variations through mutation. Consequently, the spectrum of epitope-specific immunity within the population prevents the rise of all but a particular antigenic type consisting of a set of variants to which the population has not recently been exposed. Each epidemic increases the proportion of individuals with specific immune responses to the epitopes contained by that strain; the kaleidoscope of herd immunity thus shifts and favours a new strain, leading to a succession of 'single strain' epidemics.

Unlike the other two models, this 'limited epitope' model therefore relies on neither the mode nor the tempo of mutation to generate the observed dynamics of influenza. This is a potentially significant advantage: influenza is an RNA virus with correspondingly enormous error rates during replication. It is often said that HIV-1 can generate every single possible point mutation within an individual in a given day and, indeed, it has been estimated that HIV-1 has a mutation rate of 0.22 mutations/genome/replication (Drake *et al.*, 1998), but the analogue for influenza is nearly 5 times larger at ≥ 1 mutation/genome/replication (Drake, 1993). Further, this is not due to a large discrepancy in genome size: HIV-1 is roughly 10kb in size compared to 12kb for influenza. Thus, influenza ought to be able to spew out a large collection of mutants and rapidly explore the available antigenic space. This extensive exploration of antigenic space is not compatible with the basic idea of antigenic drift, nor possibly with any model that puts brakes on mutation.

The 'limited-epitope' model thus visualizes the antigenic space as always largely occupied by pre-existing immune responses in the population; it predicts that future epidemics will

consist of different combinations of a limited set of antigenic variants or epitopes, rather than entirely novel antigenic types. This is precisely why this model can recreate the single strain dominance characteristic of influenza epidemics even though the model assumes that a tremendous amount of antigenic diversity is being continually generated. Finally, it is also worth noting that with a sufficiently large, yet finite, repertoire of epitopes, over short periods of time the antigenic evolution of the virus would seem linear, but as the period of time over which antigenic evolution is considered is increased, so too would the departure of antigenic evolution from being strictly linear. One way to visualise this would be to consider it as a vaguely directional progression around a sphere; whilst the apparent consequence of antigenic evolution is a progression away from what once was, in reality the epitopes are gradually evolving back toward what they once were.

I will now briefly review the 'limited-epitope' model, before using it to study the antigenic evolution of influenza in pigs and birds.

3.3: The ‘Limited-Epitope’ model

3.3.1: Model structure

The basic model can be described as follows (Gupta *et al.*, 1998; Recker *et al.*, 2007).

Consider the case of two antigens each encoded by a distinct locus with two alleles, namely ‘a’ and ‘b’ at one locus and ‘x’ and ‘y’ at the other. For a given strain i , representing one of the four possible genotypes (‘ax’, ‘ay’, ‘bx’ and ‘by’), I can then write down the following.

The proportion immune to strain i , z_i , is given by:

$$\frac{dz_i}{dt} = \lambda_i(1 - z_i) - \mu z_i \quad (3:1)$$

Where λ_i is the risk or force of infection associated with strain i , μ is the per-capita death rate and life-long immunity is assumed.

One then defines an additional compartment w_i that represents the proportion of the population immune to any strain j that shares alleles with i (including i itself):

$$\frac{dw_i}{dt} = \left(\sum_{j \sim i} \lambda_j \right) (1 - w_i) - \mu_i w_i \quad (3:2)$$

where $j \sim i$ denotes the collection of strains j that share alleles with strain i .

Individuals who have never been exposed to any strain sharing alleles with strain i (that is: $1 - w_i$) are completely susceptible to strain i . However, those that have been exposed to a strain that shares alleles with strain i but not strain i itself (that is: $w_i - z_i$) will become infectious with probability $[1 - \gamma]$ when infected by strain i ; thus the protective effect corresponds to reduced viral replication in an infection and manifests itself as a reduction in the transmission potential of those strains that share alleles with strain i , as measured by $0 < \gamma < 1$. With σ being the rate of loss of infectiousness by the host, the dynamics of the proportion of the population infectious with strain i , y_i , are then given by:

$$\begin{aligned} \frac{dy_i}{dt} = & \lambda_i((1 - w_i) + (1 - \gamma)(w_i - z_i)) \\ & - \sigma y_i \end{aligned} \quad (3:3)$$

In the absence of any form of connection with another population, the force of infection for strain i , λ_i , is then given simply by:

$$\lambda_i = \beta y_i \quad (3:4)$$

where β is a constant and β/σ defines the basic reproductive number R_0 for each strain.

This simple 2 locus system with just 2 alleles expressed at each locus easily scales to as many loci with as many and as variable numbers of alleles as desired. To refer to an n -locus system, with m_k alleles at each locus k ($1 \leq k \leq n$) I will use the following notation: $[m_1, m_2, m_3, \dots, m_n]$. Thus the system described above is a $[2, 2]$ system.

3.3.2: Model behaviour

It has been shown previously (Gupta *et al.*, 1998), that a strong cross-protective effect, i.e. high γ and a correspondingly large reduction in transmission of strains similar to those previously encountered, causes pathogen populations to stably segregate into discrete strains with non-overlapping antigenic repertoires (termed Discrete Strain Structure or DSS). Contrastingly, a weak protective effect leads to the absence of strain structure and the co-existence of all variants at equal frequencies (No Strain Structure or NSS). Both of these behaviours correspond to antigenic stasis because there is no antigenic turnover with time, although there is greater antigenic diversity in NSS than in DSS.

Nevertheless, for a broad range of intermediate levels of immune selection, strain structure is unstable, varying in a manner that is either cyclical or presumed chaotic (Cyclical/Chaotic Strain Structure or CSS), leading to low antigenic diversity at any one time point coupled with rapid rates of antigenic turnover, as illustrated in Figure 3-3. See also Figure 3-3 for an illustration of how the dynamical behaviour depends on γ . It was this CSS behaviour that inspired Recker *et al.* to use this model as a caricature for the antigenic evolution or flux of influenza in humans (Recker *et al.*, 2007), and to argue for its superiority over the conventional theory of antigenic drift.

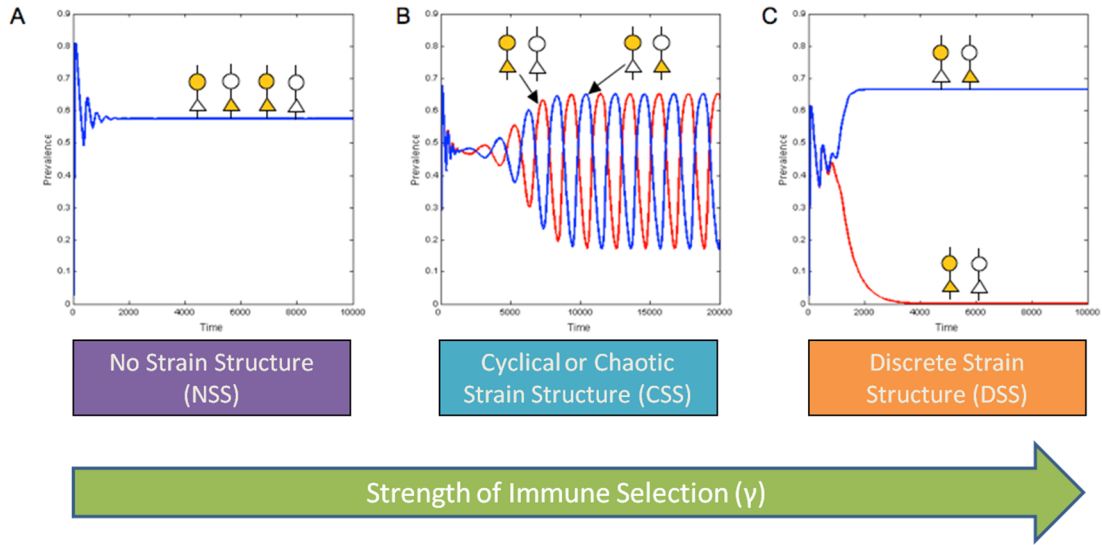


Figure 3-3: Illustration of the three dynamical regimes in a [2, 2] structure. (a) For weak levels of immune selection, there is an absence of strain structuring and all variants co-exist at equal prevalence. (b) For intermediate levels of immune selection, variants oscillate in prevalence. (c) For strong levels of immune selection, variants stably segregate into groups with non-overlapping antigenic repertoires.

Recker *et al.* also extended the two-locus version of the model to consider the impact of allowing multiplicative cross-protective effects. Take again for example, the [2, 2] system (with strains ‘ax’, ‘ay’, ‘bx’ and ‘by’). They considered the immune status of an individual who had seen both strains ‘ay’ and ‘bx’, but not ‘ax’. Under the original model, this host would have a reduction in transmission potential of an ‘ax’ infection, as measured by $[1 - \gamma]$. However, they postulated that this host might have a further such reduction thanks to having previously seen both ‘a’ and ‘x’ even though because they had not seen them together as ‘ax’ then they would not be fully immune. Thus the total reduction in transmission potential was facilitated by a new parameter, δ , and measured by the product $[(1-\delta)(1-\gamma)]$. δ is therefore a measure of the additional cross-immunity arising from cumulative exposure to more than one allele. Figure 3-4 illustrates the effect of including δ . For very high δ *i.e.* where the additive effects of exposure to multiple alleles is high, the region of parameter space in which CSS can be observed is much reduced. For smaller

values of δ , the chief effect is to alter the precise range of γ for which CSS is observed. Because of this, and for simplicity, I have decided not to include δ in my subsequent simulations. I therefore assume that the additive effect of exposure to multiple alleles is not sufficiently large so as to alter the qualitative nature of my results. This is supported by Recker *et al.*'s argument that the effect of original antigenic sin would be to reduce δ .

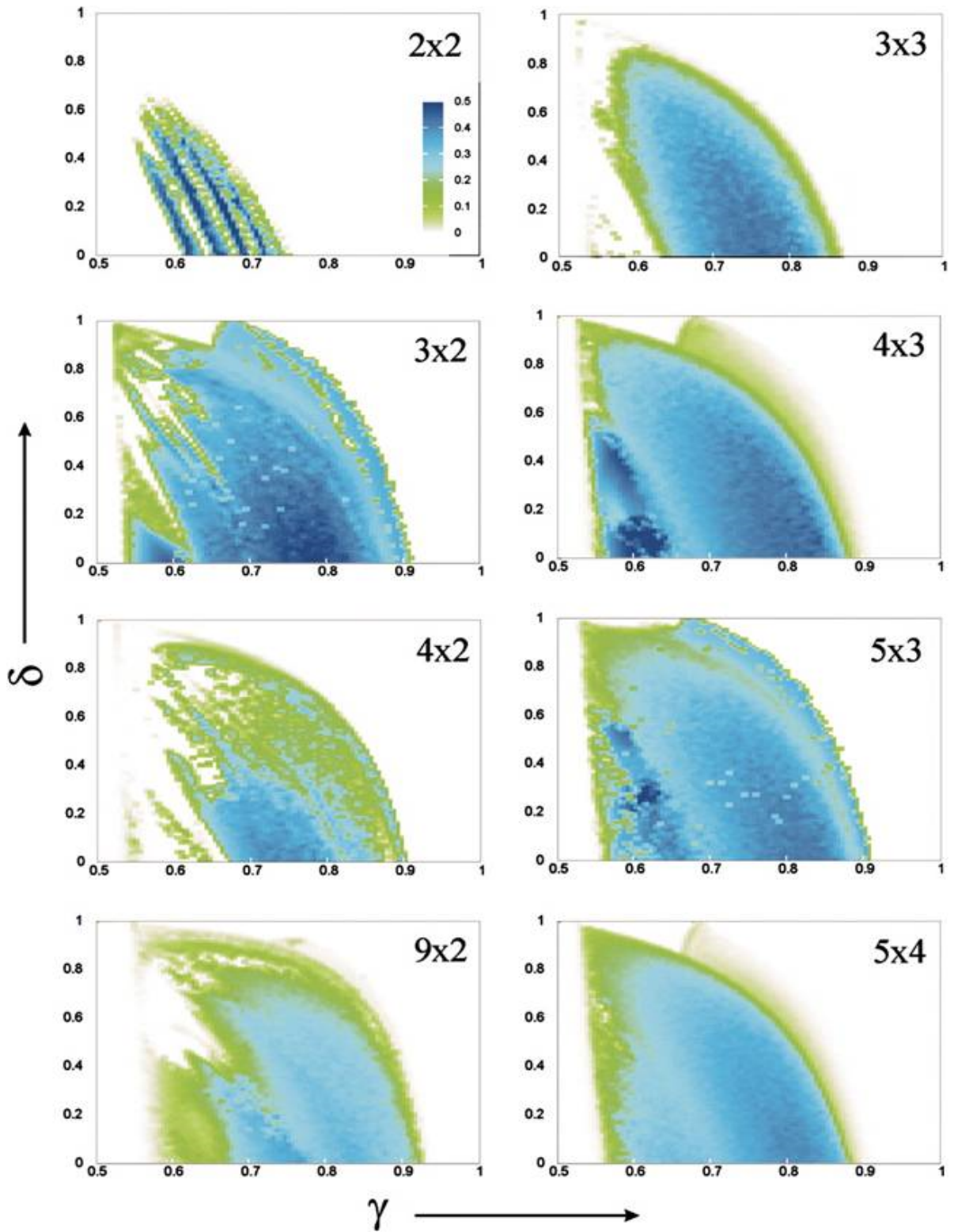


Figure 3-4: Effect of altering γ and δ within the 'limited-epitope' model. Regions where CSS (blue-green colouring) can be observed are shown for a variety of combinations of numbers of alleles ($n \times m$) at two loci within the (γ, δ) parameter space. The specific hue corresponds to the strength of single-strain dominance (ϵ). Reproduced from Recker et al., 2007 with permission. Copyright (2007) National Academy of Sciences, USA.

3.3.3: Relationship with serological data

Antigenic cartography

Figure 3-5 shows the results of an exercise in antigenic cartography based on HI assays performed on samples of influenza H3N2 collected between 1968 and 2002 (Smith *et al.*, 2004). At first glance this seems to imply that antigenic distance does increase with time; however, upon applying the same sampling method and multivariate analysis to a [2, 2, 2, 2, 2] system (32 strains in total), Recker *et al.* (2007) obtained a similarly zigzagging trajectory for antigenic distance within the ‘limited-epitope’ model. From these analyses, they contend that the linear element of antigenic distance in the Smith *et al.* (2004) data is generated by the replacement of antigenic distances between isolates from nonadjacent time points by censored data and may not be observed if accurate measures of antigenic distance were available for all the isolates studied. In any case, the close similarity of the behaviour observed in both the real data and in the ‘limited-epitope’ model, and the fact that antigenic drift cannot adequately explain such a pattern, is suggestive of this model being a superior caricature for influenza’s antigenic evolution.

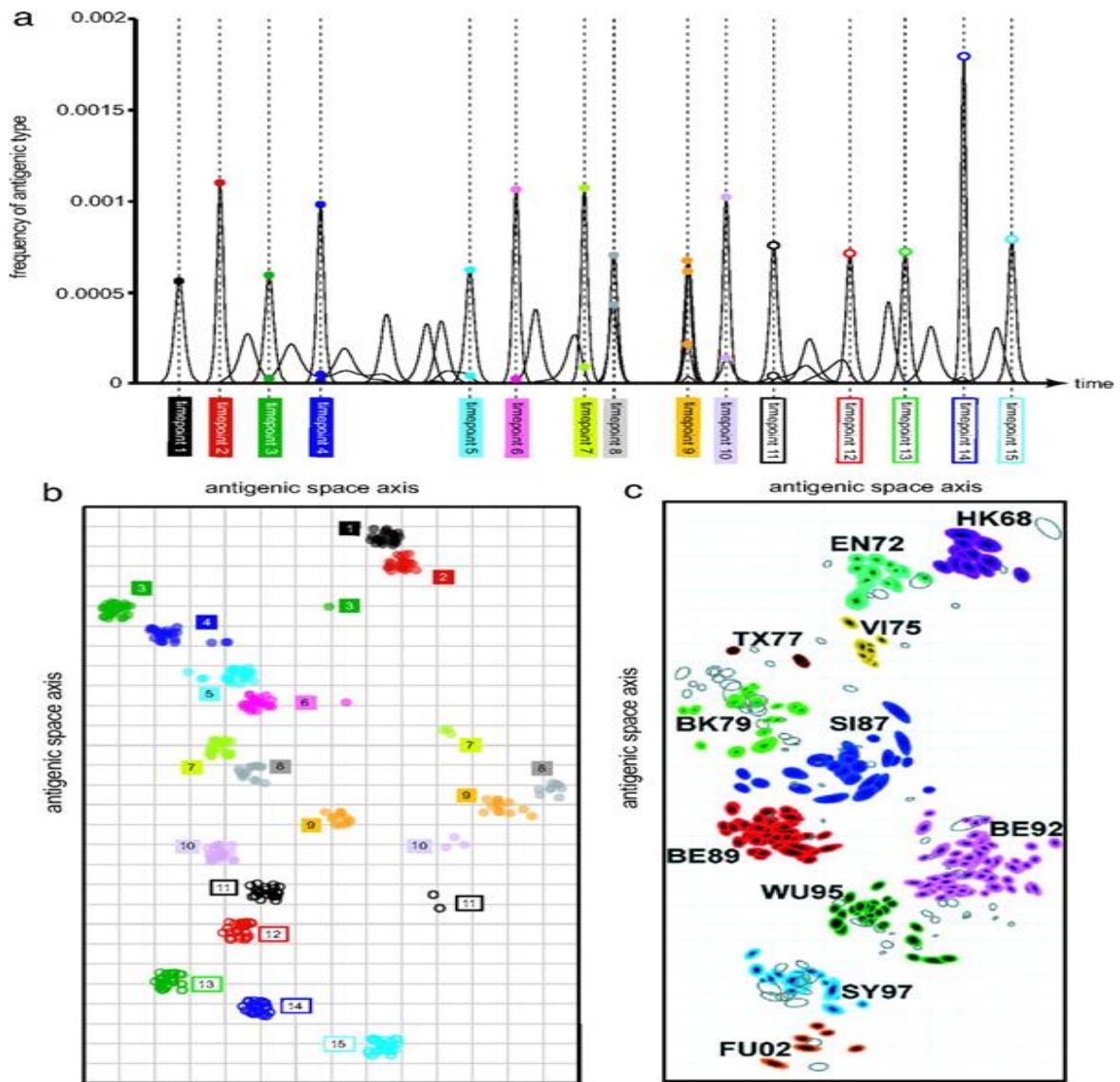


Figure 3-5: Antigenic map of influenza. (a) Changes in the proportions of hosts that are infectious for the 32 different strains within a $[2, 2, 2, 2, 2]$ system with $\delta = 0$ and $\gamma = 0.8$. Other model parameters used were $\mu = 0.014$, $\beta = 400$, and $\sigma = 100$. Twenty-five infected individuals were randomly sampled from the model population at 15 time points, corresponding to the peaks of 15 successive epidemics (dotted lines). Circles highlight the relative frequencies of the strains that were sampled at each time point. (b) The antigenic map of the sampled isolates, calculated by using multivariate analysis (see Recker et al., 2007 for further details). Each circle represents one of the 375 sampled infected individuals, coloured and labelled by time point. Each point was subjected to a small amount of random noise to simulate measurement error. (c) The antigenic map of human influenza A isolates sampled between 1968 and 2002 (adapted from Smith et al. with permission from AAAS), calculated by using the same multivariate statistical analysis. Reproduced from Recker et al., 2007 with permission. Copyright (2007) National Academy of Sciences, USA.

Within the population, the initial pattern of immunity against pandemic influenza seems to be similar, with cross-reactive antibodies present in the sera of the elderly. For example, there is ample evidence that the elderly had pre-existing antibodies to the emerging 1968 H3 pandemic strains, almost certainly due to exposure that occurred in the 19th century. Figure 3-6 shows the results of a study conducted on sera collected from a care home in 1964, split into two groups – sera belonging to those born pre- and post- 1892 – and compared with sera collected from young adults in 1967-68 (Marine *et al.*, 1969). Both the geometric mean HI titre level against, and the percentage of the group with antibodies to, the 1968 H3 strain were extremely elevated in the group of older residents. In fact, these were at levels comparable to those observed among all three groups when tested against H2 strains that had been circulating just before their sera had been collected. It is widely held that there was a period of H3 dominance in the late nineteenth century in which case one would expect that the elderly proportion of the population would have a measure of protection against the H3 strains of 1968. However, the reason why we need to regularly develop new vaccine strains is because of antigenic changes within a subtype that abrogate recognition by antibody. It is therefore surprising that these titres are higher than those against recently circulating strains.

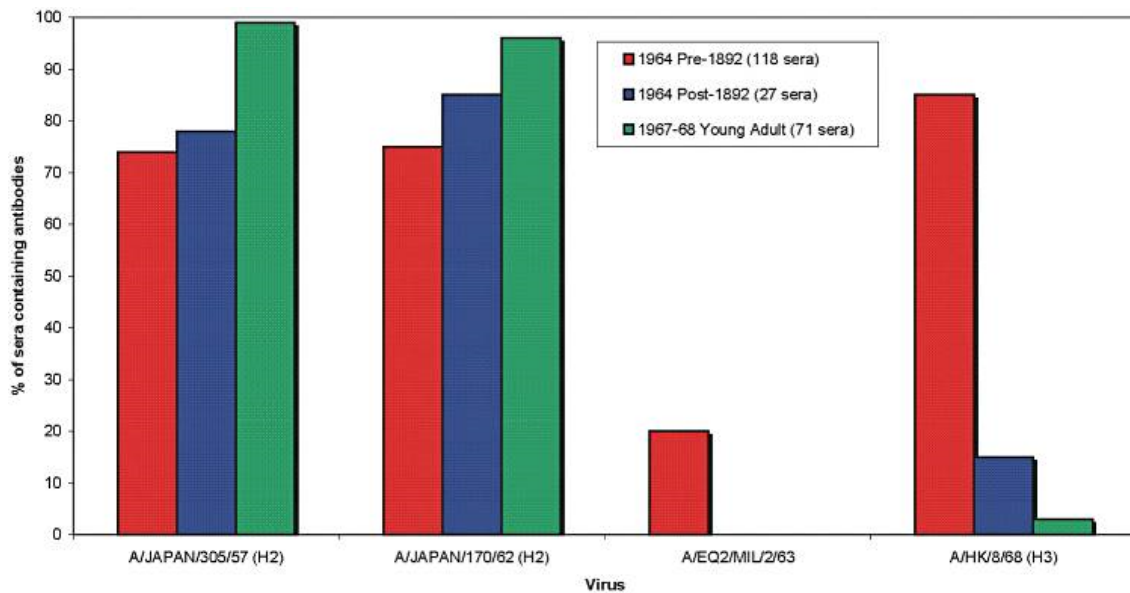


Figure 3-6: Frequency of HI titres of selected influenza A viruses in the 1964 nursing home population born before and after 1892 and in the 1967–1968 young adult population. *In the nursing home studied, the difference in immune response to the 1968 H3 strain is clearly marked between those born before and after 1892. Moreover, the immune response in the older group would suggest that they had been previously primed to A/HK/8/68; contrast this with the lack of response in both the younger group of residents and the young adults studied. Taken together this seems to indicate that an influenza virus antigenically similar to A/HK/8/68 circulated in or around the early 1890s (based on data from Marine et al., 1969).*

Similar observations were made by other authors (Davenport *et al.*, 1969; Fukumi, 1969; Masurel, 1969). Davenport *et al.* present HI assay data on the presence of antibodies to the 1968 Hong Kong strain in human sera collected in 1958 and 1966 and show that people born before 1890 had significantly elevated titres to the 1968 strain when compared to the titres of those born later. Masurel analyses similar data, this time collected in 1956-7, which shows that at least 70% of those born before 1890 had HI antibody against the newly (re)emergent strain. The data collected by Fukumi from Japanese care homes in 1967 indicates that not only did this population have a “residue” of antibody against the main antigen of the 1968 Hong Kong virus, but also that this immunity was not correlated with immunity to the H2 strains of the preceding period.

In the 1977 pandemic, the genetic and antigenic similarity to a strain from 1950 resulted in a general lack of severe disease, especially in the elderly (Scholtissek *et al.*, 1978). Further, Masurel and Heijntink showed that people born between 1896-1907 tended to have much higher HI titres against H1N1 from 1977-8 than those born between 1908-1930 (Masurel and Heijntink, 1983). Likewise, sera collected in Holland immediately before the pandemic outbreak of H2 strains in 1957, where it was found that “pre-pandemic...antibody shows a concentration in number and rise in titre in people aged 71-94” (Mulder and Masurel, 1958).

Arguably, much of this cross-reactivity in the elderly could be explained by original antigenic sin. In this case, however, cross-reactivity should not correspond to clinical protection, of which there is strong evidence in these groups. In the 1968 pandemic for example, the attack rate for people born before 1890 was roughly one third that of people born after 1899 (Schoenbaum *et al.*, 1976), whilst the associated ‘excess mortality’ was drastically lower than would normally be expected, with none occurring in people born before 1885 (Housworth and Spoon, 1971).

Figure 3-7 illustrates how such observations have also been mirrored in the recent 2009 H1N1 pandemic. Multiple serological studies have found evidence of high levels of cross-reactive antibodies solely in the elderly (Hancock *et al.*, 2009; Chi *et al.*, 2010; Ikonen *et al.*, 2010; Miller *et al.*, 2010). Others have demonstrated the protective effects in mice of inoculation with various previously circulating influenza strains against the 2009 strain (Kash *et al.*, 2010; Skountzou *et al.*, 2011), or how the usual age distribution of hospitalisation with seasonal influenza was shifted away from the elderly and towards young adults (Karageorgopoulos *et al.*, 2011).

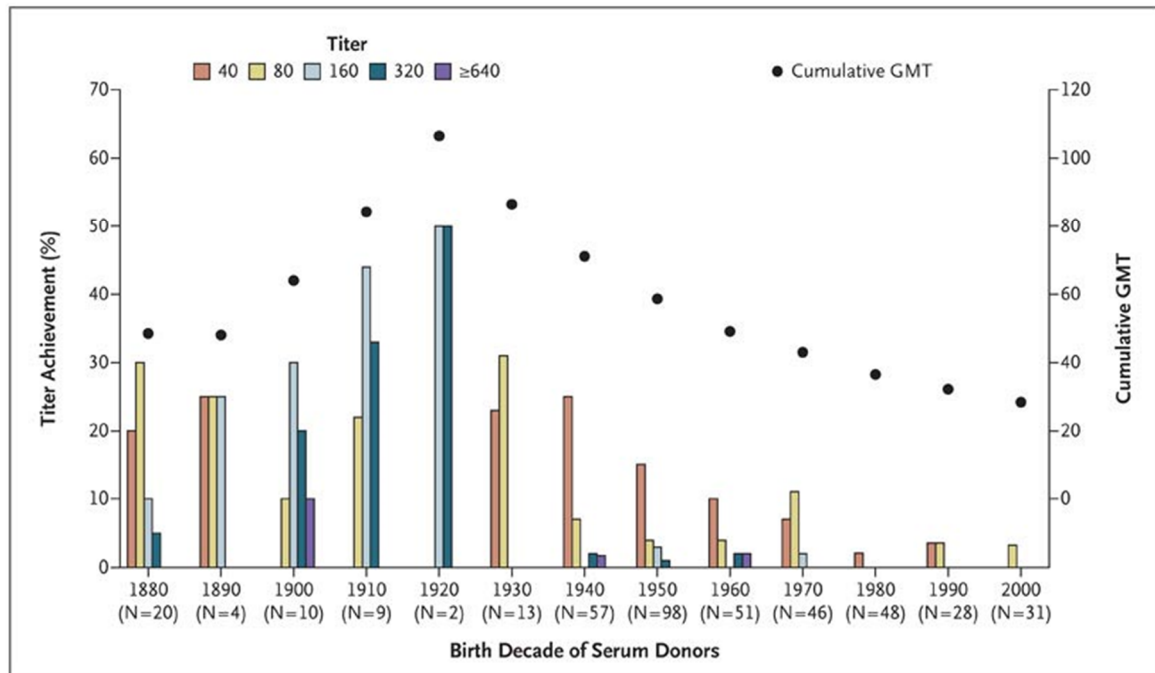


Figure 3-7: Neutralizing Antibody Titers against the 2009 Pandemic H1N1 Virus among Serum Donors, According to Birth Decade (1880–2000). Serum samples that were collected in 1971 and between 2002 and 2009 were tested by microneutralization assay. The proportion of subjects with neutralizing antibody titers of 40, 80, 160, 320, and ≥ 640 are plotted on the left ordinate, according to the birth decade of the serum donor. The cumulative geometric mean titer (GMT) for all subjects in a birth decade and in all preceding birth decades is plotted on the right ordinate and is shown with black circles. Reproduced with permission from Hancock et al., 2009, Copyright Massachusetts Medical Society.

Thus, patterns of immunity to pandemic influenza indicate that it is possible for emergent viruses to share antigenic characteristics with viruses that have previously circulated. Further, this is possible despite the fact that significant portions of the population are protected against these viruses, although there is presumably some threshold size of cohort that prevents antigenically re-emergent viruses from spreading widely. Unless there is some genetic reason why the mutations that lead to these antigenic types cannot ever be repeated, we might expect the re-emergence of the same or similar antigenic types over time. Establishing whether this is also apparent in seasonal influenza is therefore of major importance. One way to do this might be through a longitudinal birth-cohort study that

examines the cross-reactivity of these new-born's sera with a panel of old influenza viruses as they age. This might be especially useful given that clinical incidence seems to represent just a fraction of the true incidence of infection in the population: see, for example (Miller *et al.*, 2010).

3.3.4: Limitations to antigenic space

In models of 'antigenic drift', the antigenic space of influenza is thought of as being large, effectively limitless, and generally available to any virus that has accumulated a sufficient degree of said drift. As noted however, this concept cannot produce single-strain epidemics and it is worth noting that all of the previously outlined alternatives abandon this concept. The 'short-term cross-immunity' model specifies a temporary occlusion of the total space for a particular period of time, the 'neutral-network antigenic evolution' model envisages that most of the mutations achieved by the virus leave it effectively in the same place on this landscape, and the 'limited-epitope' model posits that life-long epitope-specific responses render most of the area out of bounds to any new mutant.

I remarked in the previous chapter on the fact that the flavivirus E protein is generally structurally conserved, resulting in extensive antibody cross-reactivity between all flaviviruses. This, together with the more specific example of how an individual is able to generate indefinite immunity against previously encountered dengue serotypes, illustrates that although RNA viruses have extraordinary mutational capabilities, they are not always able to escape strong immune responses. Most plausibly, this is because the targeted antigenic determinants are crucial for the structural and/or functional integrity of the virus, and hence any change is strongly deleterious. Influenza, of course, is an RNA virus that is able to continually evade a pre-existing immune response and seemingly without much cost

(hence the possibility of re-infection and, in part, the desperate vulnerability of the elderly despite a lifetime's experience of the virus). However, there are reasons to suspect that, although clearly substantially more extensive than the four options available to dengue, there may be limits to the array of variation accessible to influenza.

As noted, changes to HA are believed to be the primary correlate of immune evasion by the influenza virus. Nevertheless, I've already mentioned how HA plays a crucial role in cell invasion and therefore must be subject to strong constraint: each HA protein must be capable of binding to sialic acid on the surface of a target cell and then inducing virus-cell membrane fusion. Amino acid alterations giving rise to escape from neutralising antibodies appear to cluster in 4 or 5 epitopes on the HA1 domain (Wiley *et al.*, 1981; Caton *et al.*, 1982) depending on virus subtype. These antigenic domains are likely to be under strong purifying selection, particularly those that are closely associated with the binding site. It has been shown that the ability of viruses to produce new antigenic variants can be severely compromised by targeted mutation of the immuno-dominant sites (Temoltzin-Palacios and Thomas, 1994), which implies that new mutations can only survive within a narrow band of structural constraints. It is worth noting also that both the 1972 and 1975 strains of H3 (the two major disease causing viruses of the 1970s) had at least one mutation in each of the four sites described by Wiley *et al.*, indicating that the resistance imposed by antibodies to one antigenic site extends widely to other strains that are similar at that site.

Evidence that the antigenic space may be limited also arises from a consideration of the variation in a set of H3 codons associated with influenza isolates that give rise to epidemics (Fitch *et al.*, 1997; Bush *et al.*, 1999a). These authors identified 18 codons that were subject to positive selection to change and hence identified as possible genetic determinants of epidemic emergence. All of these codons were additionally associated with antibody-

combining sites and 5 of them also helped form the receptor-binding site of HA. Further, 14 of these 18 codons contained just 2 or 3 different amino acids at >1% frequency, suggesting strong evolutionary constraint. An analysis of the haplotype distribution shows that just 25 possible patterns account for roughly half of these isolates. This suggests that the antigenic space may be constrained to a much higher degree than suggested even by the restrictions on amino acid usage, particularly since many of the singletons may represent transient slightly deleterious variants or could be attributable to sequencing error.

3.4: Antigenic evolution of influenza in other species

Influenza seems to exhibit contrastingly different, and varying, patterns of antigenic evolution in other hosts such as birds and swine.

In swine, the virus is known to genetically evolve roughly as rapidly as in humans (Nerome *et al.*, 1995; Lindstrom *et al.*, 1998; de Jong *et al.*, 2007), but HI tests with polyclonal sera and monoclonal antibody (mAb) studies show a variety of patterns. In North American swine, so called classical H1N1 (cH1N1) viruses are said to have been antigenically stable for the 80 years or so from their introduction in 1918 (Sheerar *et al.*, 1989; Vincent *et al.*, 2006). However, this picture began to change in 1998 with it becoming possible to antigenically distinguish ‘modern’ cH1N1 from the ‘classical’ varieties, as well as antigenic diversity within the ‘modern’ group (Vincent *et al.*, 2006; Thacker and Janke, 2008). Antigenically distinct populations of H3N2 (Richt *et al.*, 2003; Thacker and Janke, 2008) and H1N2 (Vincent *et al.*, 2006) have also begun to circulate in North American swine since this time. A study of H1N1 viruses in United States swine herds has suggested that as many as 5 antigenically distinct clusters may have been co-circulating during 2008 (Lorusso *et al.*, 2011). In Europe, co-circulation of antigenically distinct H1N1 and H1N2 viruses has also been observed (Van Reeth *et al.*, 2004), together with what is described as slow antigenic evolution of H3N2 (de Jong *et al.*, 1999; de Jong *et al.*, 2007) or periods of a “lack of significant antigenic drift” in classical H1N1 (Kuntz-Simon and Madec, 2009).

Avian influenza was long held to be in a state of evolutionary stasis (Gorman *et al.*, 1990; Gorman *et al.*, 1991; Hatchette *et al.*, 2004; Widjaja *et al.*, 2004), but this has been disproved by a more robust analysis of nucleotide substitution that revealed evolutionary rates on a par with mammalian influenza viruses (Chen and Holmes, 2007). The picture of antigenic

evolution is again complex. The results of studies with mAbs seem to indicate antigenic stasis, with mAbs binding consistently to viruses over a long period of time (Bean *et al.*, 1985; Kida *et al.*, 1987; Horimoto *et al.*, 1995; Shortridge *et al.*, 1998; Sturm-Ramirez *et al.*, 2004), although on rare occasions particular mAbs have been able to distinguish between groups of viruses *e.g.* (Shortridge *et al.*, 1998). Very few of these older studies considered HI data, but one more recent study used HI data to suggest antigenic conservation of H5N1 in Hong Kong between 1997 and 2001 (Sturm-Ramirez *et al.*, 2004). In this paper however, HI data also suggested the emergence of antigenically distinct strains in 2002. This is similar to the pattern observed in Korean H9 viruses with antigenic stasis between 1996 and 2003 followed by the emergence and dominance of a novel antigenic type from late-2003 until 2007 (Lee *et al.*, 2007; Moon *et al.*, 2010). Only a handful of other studies have looked at the antigenic relationship between avian influenza strains using HI data. These have often revealed the co-circulation of antigenically distinct lineages in, for example, low pathogenic H5N2 in Mexico (Lee *et al.*, 2004), H5N1 in China (Li *et al.*, 2010), and H9N2 in Korean chickens from 2007 onwards (Park *et al.*, 2011). The antigenic evolution of H9 viruses in Korea therefore neatly illustrates the general complexity of patterns of antigenic evolution of influenza in birds. Here there have been periods of dominance by distinct antigenic types (1996-2003 and 2004-2007) before the eventual emergence and co-circulation of several antigenically distinct variants (2007 onwards).

3.5: Exploring discrepancies in the antigenic evolution of influenza in different species

Currently, there are few hypotheses that seek to explain why there is such heterogeneity in the patterns of antigenic evolution in birds and pigs. It is widely held that birds and pigs are

generally too short-lived to exert immune pressure on the virus, with the justification being that the rapid turnover in susceptible hosts results in a lack of immune pressure on the virus and hence a lack of antigenic evolution *e.g.* (Lee *et al.*, 2004; de Jong *et al.*, 2007; Kash *et al.*, 2010). This theory continues by justifying the emergence of antigenic evolution as a consequence of vaccination policy increasing this pressure. However, this idea doesn't really make much sense since an absence of immune selection ought to lead to an increased expression of antigenic diversity, unless all possible antigenic changes are deleterious which seems unlikely.

This question is not one that has been previously rigorously explored within a theoretical framework. Here, I use this model to explore a number of different hypotheses that could alternatively explain why the mode and tempo of antigenic evolution, and the ability of our methods to detect it, may differ between species.

3.5.1: Strength of cross-protective effect

Perhaps the most obvious explanation for the different patterns of antigenic evolution in different species could be simply that the strength of immune selection, γ , varies between them. As a concept, this is in itself plausible since we can imagine that the intrinsic immunological difference between species might manifest in varying abilities to respond to antigenically related strains. Thus as previously illustrated in Figure 3-3, immune selection in humans could be such that we observe antigenic evolution through CSS, but in other species it is either too strong (and so we see DSS) or too weak (leading to NSS) to elicit this behaviour. Both of the latter correspond to antigenic stasis because there is no change in the observed antigenic diversity with time. That said, in NSS we would expect to see substantial antigenic diversity within a population, with complex patterns of cross-reactivity

between isolates from similar time-points. This contrasts with complete discordance between temporally related isolates under DSS.

It would however be difficult to use this to construct a hypothesis that could explain periods of both antigenic stasis and evolution within a single species as to do so would require a plausible argument for variations in γ (immune selection) over short periods of time in that species. Therefore, although differences in γ could explain some aspects of general discrepancies between species, it seems unlikely that it could explain the wide variety of modes of antigenic evolution that have been observed in pigs and birds.

3.5.2: Lifespan

Another source of obvious difference between species is their life expectancy. An adult human might reasonably expect to live until well into their seventies, but for pigs and birds much will depend on whether they are living in the wild or in captivity. Wild pigs can live for up to 25 years, but this is unlikely for a farmed pig. Indeed, most piglets won't live much beyond six months, although those female piglets that survive to become breeding sows can live for up to 15 years. Similarly, battery farmed chickens would be doing well to last more than a few months and domestic poultry a few years, but some wild birds have life expectancies of up to 20 years. It is therefore conceivable that at least some of the variation in the antigenic evolution of the virus could reflect variety in longevity. Figure 3-8 illustrates however how in the 'limited-epitope' model, the ratio of life expectancy and infectious period of the host population does seem to play a role in shaping the observed dynamical regime (Gupta *et al.*, 1998), but this has not been considered in any detail.

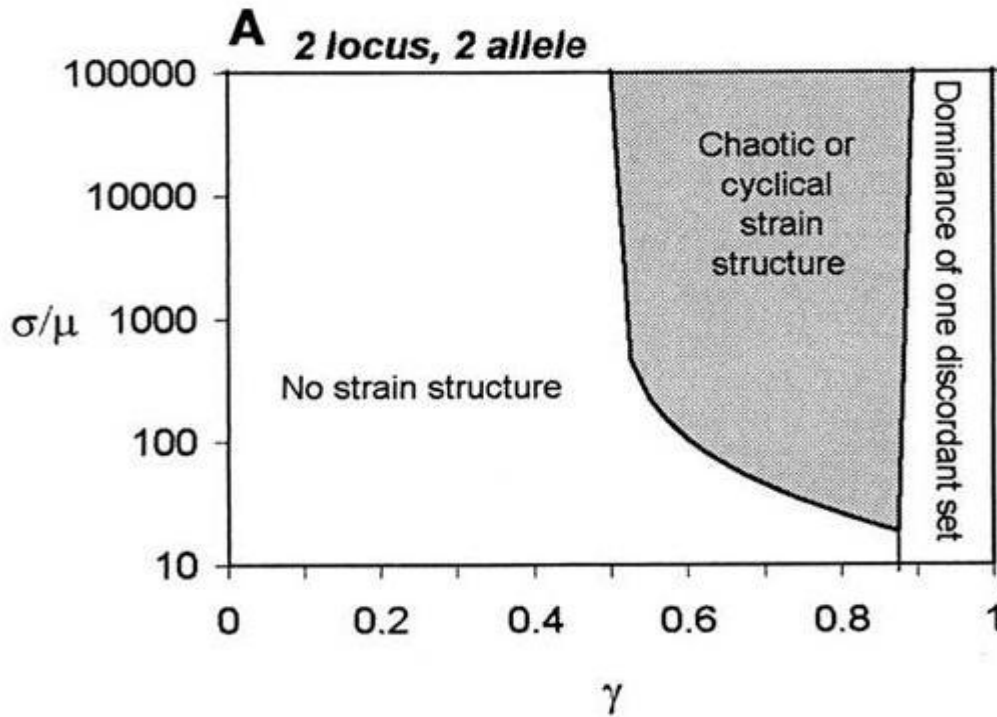


Figure 3-8: Dependency of strain structuring on model parameters. *Strain structure type is shown as a function of the degree of immune selection (γ) and the ratio of host life-span to pathogen infectious period (σ/μ) for pathogens in a [2, 2] system (from Gupta et al. 1998, reprinted with permission from AAAS).*

To further explore the dependence of the dynamics on lifespan, I first fix an infectious period of 5 days ($\sigma = 73/\text{year}$) and vary the key model parameters life-expectancy [$1/\mu$] and immune selection [γ], to determine the proportion of (γ - μ) parameter space that is characterised by CSS (antigenic evolution) within an arbitrary locus-allele system: [2, 3, 5] (30 strains in total). This variation is achieved by varying life expectancy in 0.5 year intervals between 0.5 and 50 years, and immune selection in intervals of 0.01 between 0 and 1. Simulations are carried out in MATLAB using a numerical ODE solver. I keep the sole remaining parameter, pertaining to the force of infection, constant: $\beta = 292$ (and, hence, $R_0 = \beta/\sigma = 4$). Thus, within (γ - μ) space, I can find parameter combinations that lead to antigenic evolution. Further, where antigenic evolution is found to occur, I can assess its qualitative nature by reapplying the methods of Chapter 2.3.2 and 2.3.3 to consider

respectively (i) the extent of single strain dominance and (ii) the inter-epidemic period of influenza.

Figure 3-9 illustrates the regions in parameter space where the three distinct dynamical behaviours exhibited by the basic model are found to occur. The first point to note here is that immune selection can have an effect even at very short life expectancies, leading to either stable or unstable antigenic structure. However, the qualitative nature of antigenic evolution varies considerably with life span, with shorter lived hosts typically exhibiting higher levels of antigenic diversity with fluctuations in frequency of antigenic types occurring on a faster timescale.

Taking a slice, for example, at $\gamma = 0.8$, shows that a reduction in lifespan can transform the dynamics from a sequential appearance of new antigenic types every 2 to 3 years (as observed in human populations) to a situation where many more antigenic types can coexist but rise and fall on a much shorter timescale. This is in accordance with the observed co-circulation of antigenically distinct viruses from the same subtype in pigs (*e.g.* (Van Reeth *et al.*, 2004; Thacker and Janke, 2008)) and birds (*e.g.* (Park *et al.*, 2011)), in contrast to humans. Similarly, the data presented in Figure 3-10 suggests that perhaps a diminished inter-epidemic period is responsible for the relative ease of constant isolation of influenza from pigs and birds (*e.g.* (Webster *et al.*, 1992; Campitelli *et al.*, 1997; Olsen *et al.*, 2006; Kyriakis *et al.*, 2009)), along with the generally increased prevalence in shorter-lived hosts.

Another important result to emerge from this analysis is that the qualitative nature of antigenic evolution of influenza in a short lived host has the potential to be radically altered by relatively small changes in host life expectancy. Thus, a small increase in host life

expectancy in, say, swine, associated perhaps with a change in farming practices and a move towards greater longevity in herds, could lead to a reduction in the currently observed antigenic diversity and more epidemic-like behaviour.

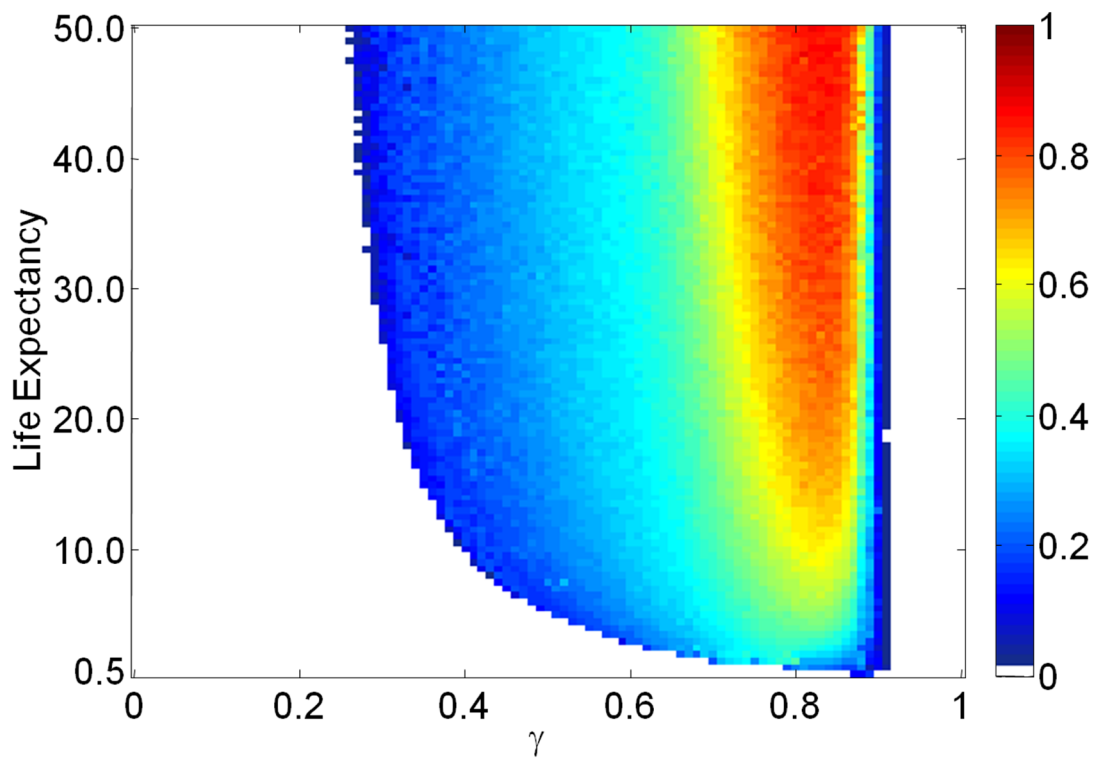


Figure 3-9: Dependency of strain structuring and single strain dominance on immune selection (γ) and life expectancy. Coloured regions correspond to CSS (antigenic evolution) and white areas to NSS (left) and DSS (right) (antigenic stasis) in a [2, 3, 5] system. CSS is therefore possible for life expectancy as low as 6 months. Where CSS occurs, I have used a measure of single strain dominance (where 0 corresponds to at least two strains being simultaneously dominant and higher values indicate a greater tendency for one serotype to be dominating at any given time). The strength of single strain dominance near monotonically increases with life expectancy.

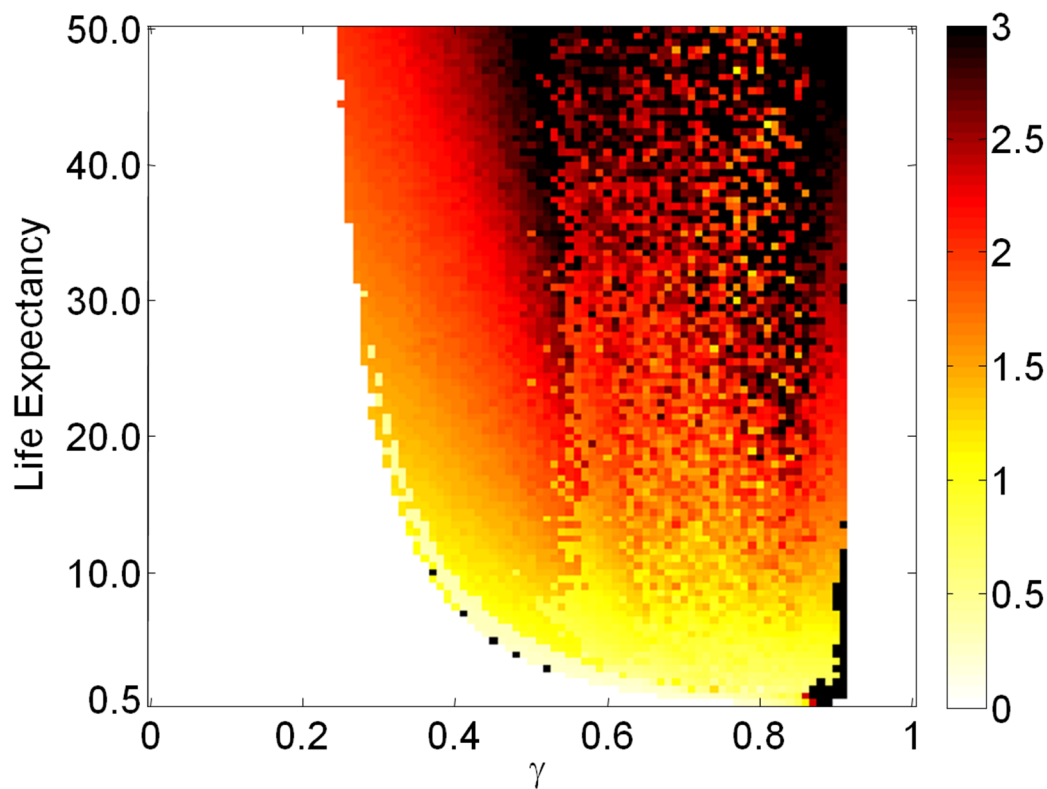


Figure 3-10: Dependency of inter-epidemic period on immune selection (γ) and life expectancy. *Where CSS occurs in a [2, 3, 5] system, I have looked at the inter-epidemic period. This period generally increases with life expectancy, but for very short host life expectancy, the virus essentially becomes endemic.*

3.5.3: Infectious period

Although an infectious period of 5 days (as used above) roughly corresponds to the length of time that humans and swine are presumed to spread influenza through respiratory excretions, the main route of transmission in birds is faecal-oral. Consequently, due to the resilience of avian virus in the environment, particularly in lake water (as reviewed in (Stallknecht *et al.*, 2010)), it may be that the effective infectious period is actually much longer for avian influenza, or at least for aquatic birds.

To simulate environmental persistence of shed virus, I repeat the previous experiment, but for the much longer infectious period of roughly 1 month ($\sigma = 12/\text{year}$). Notably, here I choose to fix $R_0 = 4$ and so $\beta = 48$; the force of infection is therefore notably lower. However, what I am trying to compare is how life expectancy and infectious period impact on antigenic evolution for equivalently transmissible viruses i.e. for viruses with equivalent R_0 , and so this is appropriate here.

Figure 3-11 illustrates the effect of increasing the infectious period. The key result to take away from this figure is that increasing the infectious period has a big effect on the range of parameter space in which we can observe antigenic evolution. This region collapses both in terms of the level of immune selection (γ) required to observe antigenic evolution and also the threshold life expectancy. It is still possible to observe antigenic evolution at relatively short life expectancies, but the minimum has now increased to 2.5 years. Consequently, although aquatic birds tend to be longer lived than poultry, my model suggests that an effectively longer infectious period for the virus in the former group may lead to antigenic stasis even though the virus would exhibit rapid antigenic evolution in equivalently long-lived poultry. Given the already observed impact of lifespan, it might even be possible to reconcile antigenic evolution in short-lived poultry with antigenic stasis in long-lived aquatic birds. Further, a longer infectious period has a big effect on single strain dominance, with much more diversity apparent in epidemics for equivalent life expectancy. Therefore, although the virus could manifest as single-strain epidemics in a swine herd with a life expectancy of around 15 years, we could not observe this in equivalently long-lived populations of aquatic birds.

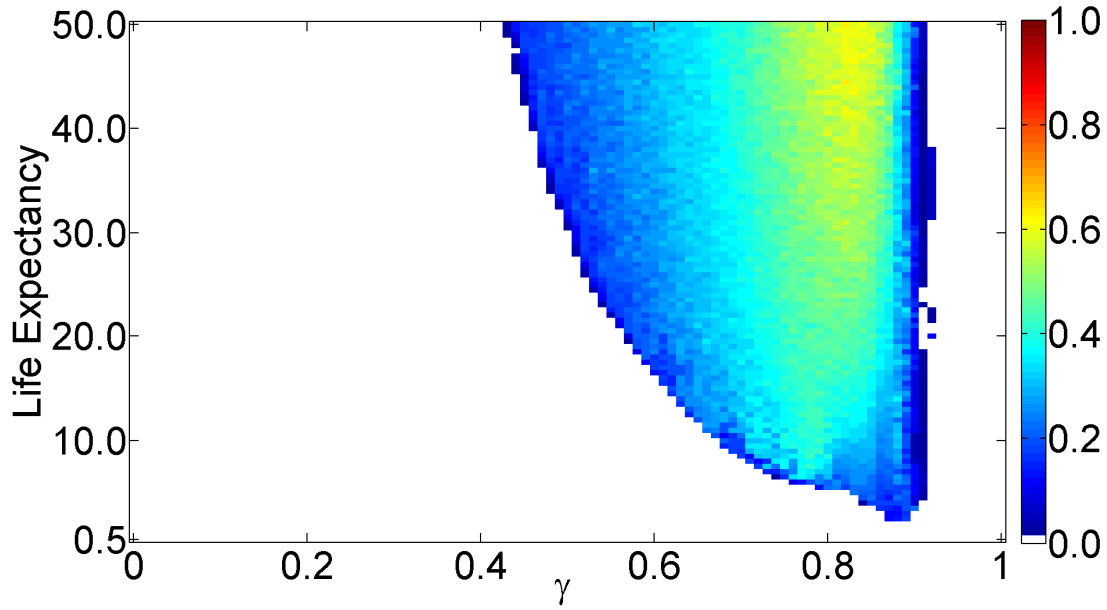


Figure 3-11: Effect of increasing the infectious period. Coloured regions correspond to Single Strain Dominance in CSS (antigenic evolution) and white areas to NSS (left) and DSS (right) (antigenic stasis) in a $[2, 3, 5]$ system with an infectious period of 1 month. Compared to its analogue Figure 3-9 with a shorter infectious period, the threshold for CSS is higher and there is much less dominance by a single strain.

3.5.4: Differences in basic reproduction number, R_0

It is not hard to envisage a scenario where R_0 varies between influenza viruses in different species, or even in different subtypes within a species. I therefore next consider how changes in R_0 affect the behaviour of the model. Thus, I repeat the experiment of section 3.5.2 for the smaller value of $R_0 = 2$.

Figure 3-12 shows that changing R_0 seems to have a limited effect on the observation of antigenic evolution when compared with Figure 3-9. In fact, the sole effect seems to be a shift of the right-hand boundary of the region in which antigenic evolution is observed to the left with decreasing R_0 . Importantly, the left-hand boundary remains unchanged. The qualitative nature of the antigenic evolution within this region also seems to be altered. The

region of maximal single strain dominance now occurs for weaker immune selection (γ), and with less strong signals of single strain dominance in this region.

Consequently, if influenza were to become more transmissible (develop a higher force of infection) with time as a result of genetic changes, thereby increasing R_0 but holding other parameters constant, then we could see human influenza move from its current state of rapid antigenic evolution, to a state with shorter epidemic cycles and greater antigenic diversity. Similarly, an increase in transmissibility could lead to the emergence of antigenic evolution, and as further typified by single-strain epidemics, in a species which may previously have been characterised by antigenic stasis and limited antigenic diversity. Conversely, if human influenza became less transmissible then this would have the same effect as increased immunological competition between strains and we could see an end to the antigenic flux that has so far characterised our relationship with the virus, with the emergence of a DSS regime.

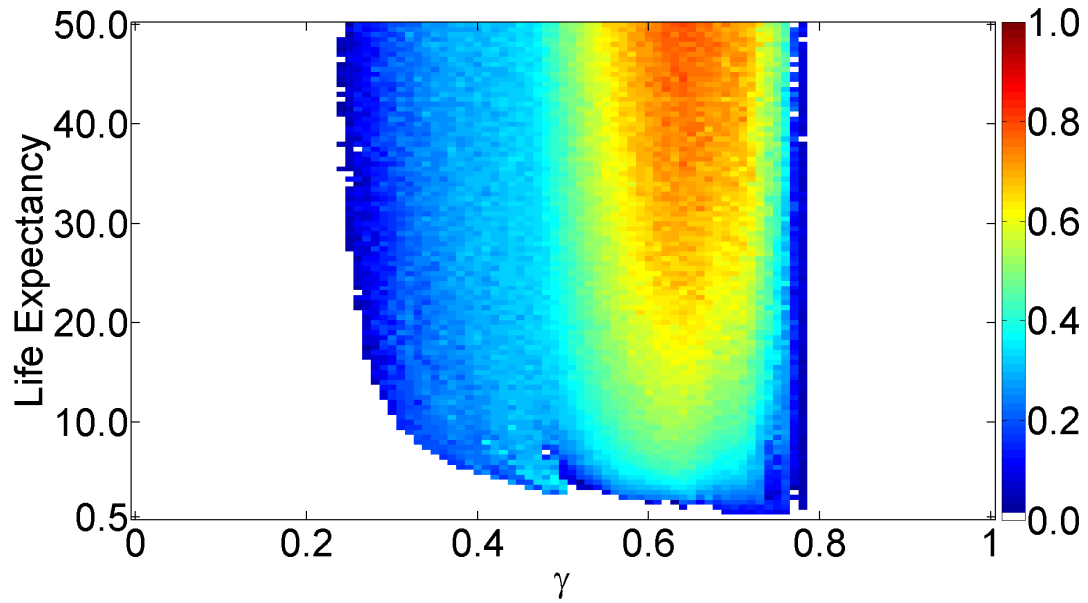


Figure 3-12: Effect of decreasing R_0 . Coloured regions correspond to Single Strain Dominance in CSS (antigenic evolution) and white areas to NSS (left) and DSS (right) (antigenic stasis) in a $[2, 3, 5]$ system with an R_0 of 2. Compared to its analogue Figure 3-9 with larger R_0 , DSS emerges for weaker immune selection between antigenically similar variants (γ), and there is less single-strain dominance at longer host life expectancies. It is almost as though the right hand-side of the CSS region has been squashed into a smaller space, but with no change to the left hand-side boundary.

3.5.5: Architecture of Antigenic Space

Another possibly difference between influenza in different species that may be crucial to its antigenic evolution is the degree of variation in its antigenic determinants.

I can determine this by comparing different structures of antigenic space. I compare a $[2, 2, 2, 2, 2, 2, 2, 2, 2, 2]$ system (128 strains in total) with a $[10, 10]$ system (100 strains in total). The first corresponds to an antigenic space where, although there are many sites at which antigenic variation is possible, the range of allowed changes is quite restricted. Conversely, the second system has fewer sites that can vary, but the range of possible changes is larger.

To make this comparison, I generate a time series for each of two systems keeping all other parameters constant: an infectious period of 5 days ($\sigma = 73/\text{year}$); $\beta = 292$ (and, hence, $R_0 = \beta/\sigma = 4$); life expectancy of 50 years ($\mu = 0.02/\text{year}$). I then select the same arbitrary time period of 25 years within each model, isolate the most prevalent strain from each year of that period, and compare the number of epitopes that are shared between strains. Given that sharing one allele is the unit of immunological cross-reactivity in the model, I assume that strains that share at least one allele would cross-react in an HI test. I then plot a heat map to illustrate this cross-reactivity and I subsequently refer to these heat maps as pseudo-HI tables.

The pseudo-HI tables clearly demonstrate that both of the two classical patterns observed in HI tables can be associated with antigenic evolution. In the [10, 10] model (Figure 3-13a), a pattern similar to human HI reactivity can be recovered from the model. Here, an isolate from a given year quickly loses cross-reactivity with isolates from subsequent years. Part of the reason for this is simply because the antigenic space is extremely flexible – with a high degree of variation possible at the considered epitopes it is not particularly likely that two arbitrary strains will cross react. In fact, an individual strain only cross-reacts with 19/99 or 19.2% of the other strains in this antigenic space. By contrast, if I assume that antigenic space is much more restricted as in the [2, 2, 2, 2, 2, 2, 2] model, any given strain within the system of 128 will cross-react with every single other strain bar the single type it does not share any alleles with (*i.e.* 126/127 or 99.2%) leading to an HI table where cross-reactivity is maintained over long periods of time (Figure 3-13b). Hence, although antigenic evolution occurs in both models, the flexibility of the associated antigenic space determines the extent of the observed cross-reactivity. Figure 3-13 therefore provides a stark visual illustration of the fact that a high rate of antigenic turnover does not necessarily manifest itself in a rapid loss of reactivity with time in an HI assay.

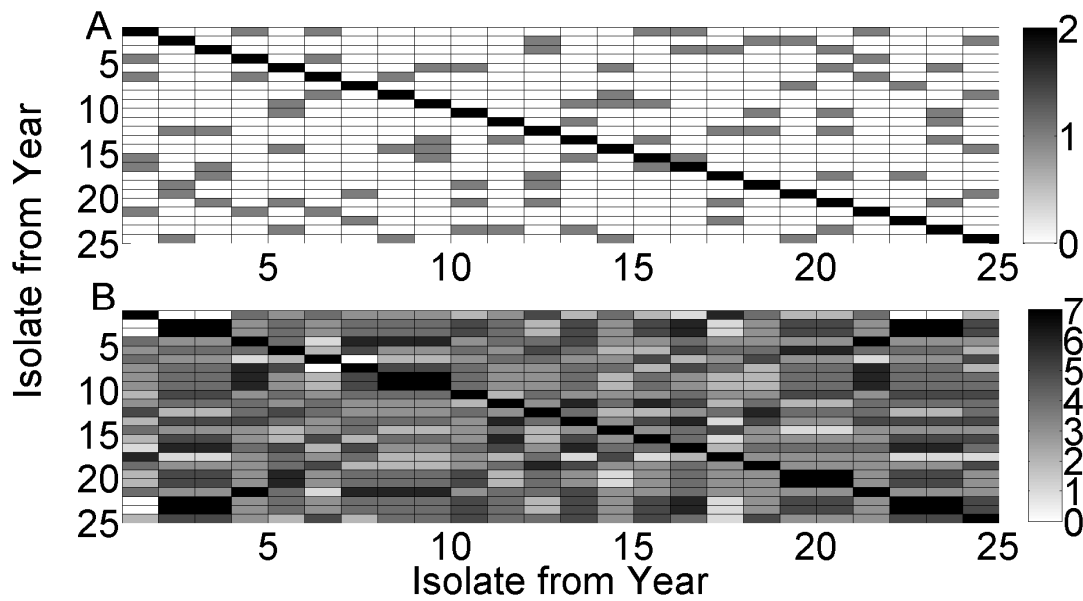


Figure 3-13: Pseudo HI tables within the limited-epitope model for (a) [10, 10] system and (b) [2, 2, 2, 2, 2, 2, 2] system. *Strains quickly lose cross-reactivity in an antigenic space with more flexibility at its epitopes, in contrast to extreme cross-reactivity between isolates in an antigenic space of a similar size but with much less flexibility at each epitope.*

These results suggest that more care may need to be taken in future when analysing HI data: the patterns of general cross-reactivity typical of many HI assays in birds and swine could actually be representative of rapid antigenic evolution rather than overall stasis. In these species, although there may be many sites at which antigenic change could occur, the range of possible changes at those sites may be relatively restricted. Contrastingly, the typical human HI pattern of limited cross-reactivity with time is present in a space where the range of possible changes at antigenic sites is much larger. Given that the natural host of the virus is often said to be birds (*e.g.* (Webster *et al.*, 1992)), perhaps the avian immune system has had enough time to evolve to efficiently target epitopes where fidelity is integral to successful replication. This requirement for fidelity could then correspond to a relatively restricted antigenic space, together with the associated pattern of HI cross-reactivity. Human immune systems, more recently exposed to the virus, may be unable to target these

epitopes, resulting in a more flexible antigenic space and the typical HI pattern of human isolates.

The idea that the antigenic space accessible to influenza varies between species is supported by efforts to map human and swine H3N2 viruses using antigenic cartography methods (de Jong *et al.*, 2007). It was discovered in that study that the evolution of human and swine viruses could both be plotted in (separate) two dimensional planes, but that to map both together required three dimensions. As the authors themselves note, this is indicative of exclusive antigenic changes being provoked and, hence, separate evolutionary paths being followed in each species.

3.5.6: Effect of linking two populations with different life expectancies

Another important feature of influenza ecology is the degree to which we observe transmission both between species and between different population subgroups of a species. These interactions significantly enhance the complexity of the virus' epidemiological behaviour. In particular, and as noted, among global avifauna there is a wide range in life span – from a period of just a few weeks for a battery farmed chick to perhaps 20 years for a wild swan – with ample evidence of cross-species transmission (Xu *et al.*, 1999; Widjaja *et al.*, 2004; Spackman *et al.*, 2005; Olsen *et al.*, 2006). Thus, perhaps it would be better to consider avian influenza in wild birds exists as a large pool of functionally equivalent and interchangeable gene segments (Dugan *et al.*, 2008). With swine, industrial practices lead also to the effective partitioning of the species into relatively long-lived sows and short-lived grower pigs with likely much transmission of influenza between these two groups. Thus, here we also have complex influenza ecology, with a link between 2 subgroups of pig; one short-lived and the other longer-lived.

It has been argued that the key to understanding viral evolution is to understand the physical interaction between and evolutionary processes within each species (Caron *et al.*, 2009). Consequently, perhaps discrepancies between reported rates of antigenic evolution in different studies and species could also arise as a result of dynamical shifts in our model due to epidemic links between both different species sub-populations and different species themselves.

In order to explore this idea, I need to make some small adaptations to the model. For the sake of simplicity, I assume that the same antigenic types, with identical transmissibility and infectious periods, circulate in each of two populations: population 1 and population 2. I only consider the most basic model, the [2, 2] system with just four antigenic types: ‘ax’; ‘ay’; ‘bx’; and ‘by’. The same equations (numbers) can be used to describe this system, with additional subscripts to designate the host species: thus the proportion immune to strain i in population 1 becomes $z_{1,i}$, similarly the proportion immune to strain i in population 2 becomes $z_{2,i}$.

To model the mixing process between two populations, I assume that it is relatively harder for an individual in population 1 to be infected by an individual in population 2; this difficulty is represented by the force of infection multiplier, $0 \leq \epsilon_2 \leq 1$, which could cover a variety of interactions between two populations including the scale of inter-population mixing and the difficulty of infecting an individual in the other population. I can now write, for each strain i :

$$\lambda_{1,i} = \beta y_{1,i} + \epsilon_2 \beta y_{2,i} \quad (3:5)$$

And

$$\lambda_{2,i} = \beta y_{2,i} + \varepsilon_1 \beta y_{1,i} \quad (3:6)$$

thus $\varepsilon_1 = \varepsilon_2 = 0$ corresponds to no cross-population infection, whilst $\varepsilon_1 = \varepsilon_2 = 1$ corresponds to a homogenous, single population where an individual has one of two equally distributed life expectancies.

I now consider two populations, one with a fixed life expectancy of 1 year (population 1) and the other with a fixed life expectancy of 50 years (population 2), and observe the qualitative effects in $(\gamma-\varepsilon)$ parameter space of varying ε within three different dynamics: (a) allowing the longer-lived population to infect the shorter-lived population but not vice-versa ($\varepsilon_1 > 0, \varepsilon_2 = 0$), (b) allowing the shorter-lived population to infect the longer-lived population but not vice-versa ($\varepsilon_1 = 0, \varepsilon_2 > 0$) and (c) allowing both populations to infect each other to the same degree ($\varepsilon_1 = \varepsilon_2 > 0$). The other parameters are fixed as: $\beta = 292$; $\sigma = 73/\text{year}$ (an infectious period of 5 days).

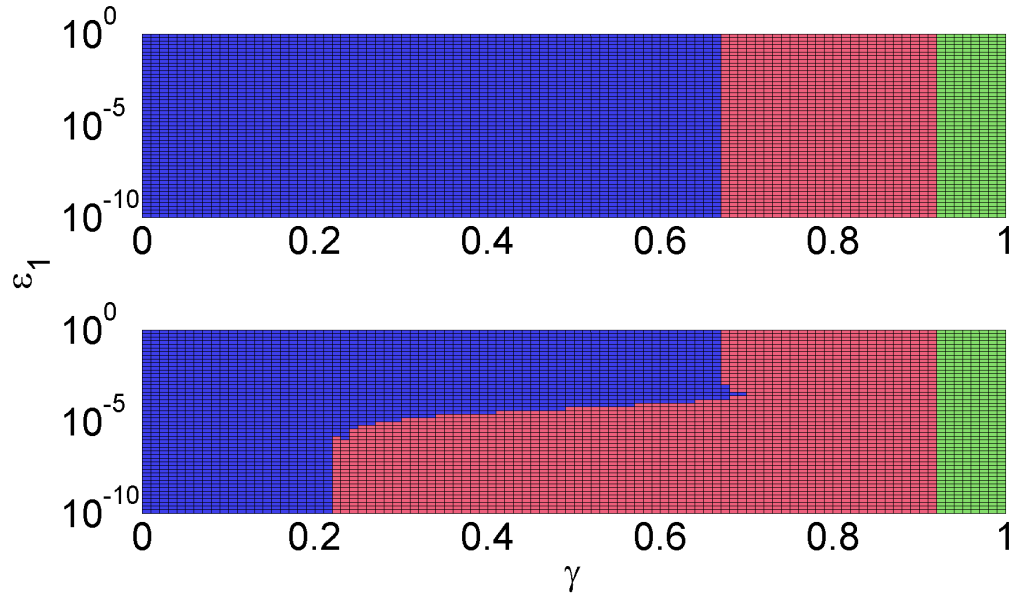


Figure 3-14: Allowing a short-lived population to infect a long-lived population within two otherwise equivalent [2, 3, 5] systems. *The top panel shows the relationship between the strength of immune selection (γ) and antigenic evolution for a population where host life expectancy is 1 year: blue (NSS); red (CSS); green (DSS). The bottom panel shows the equivalent relationship in a longer-lived population where host life-expectancy is 50 years, and how this relationship alters with increasing levels of exclusive transmission from the short-lived to long-lived population (ϵ_1). In essence, the qualitative nature of antigenic evolution in the long-lived population is progressively altered with increasing transmission from the short-lived population.*

This extension of the model to consider epidemiological interactions between two populations – one short-lived and the other long-lived – yields intriguing results. I have considered what happens if we allow just one (dominant) population to transmit virus to the (dominated) other and also the effect of allowing both to transmit to each other. For low levels of cross-population transmission, the dynamics within each population remain invariant. However, as I increase the strength of the interaction, then the more the qualitative dynamics of the dominated population shift to look like those of the dominant. Figures 3-14 and 3-15 show that the strength of interaction required for the short-lived population to dominate the long-lived population is much smaller than in the antagonistic interaction.

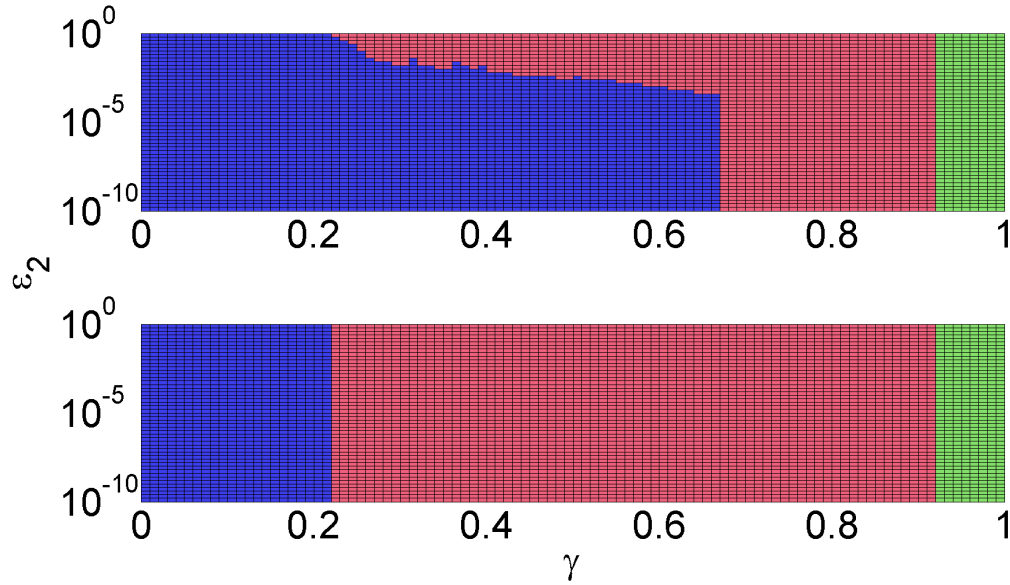


Figure 3-15: Allowing a long-lived population to infect a short-lived population within two otherwise equivalent [2, 3, 5] systems. *The bottom panel shows the relationship between the strength of immune selection (γ) and antigenic evolution for a population where host life expectancy is 50 years: blue (NSS); red (CSS); green (DSS). The top panel shows the equivalent relationship in a shorter-lived population where host life-expectancy is 1 year, and how this relationship alters with increasing levels of exclusive transmission from the long-lived to short-lived population (ϵ_2). In essence, the qualitative nature of antigenic evolution in the short-lived population is progressively altered with increasing transmission from the long-lived population.*

Figure 3-16 shows what happens if, on the other hand, I allow both populations to infect each other at equal rates then I observe little change in the dynamics of the short-lived population, whereas those of the longer-lived population shift qualitatively to match those of the short-lived population. For intermediate levels of transmission in Figure 3-16, I also observe a region of parameter space where, without cross-transmission, CSS would occur in both populations but, with cross-transmission, this is replaced by NSS in the longer-lived population. This odd behaviour serves to highlight how even a relatively simple epidemiological interaction can lead to counter-intuitive dynamics that do not always behave in a qualitatively simple fashion.

These results indicate that, for linked influenza ecologies, the evolutionary dynamics of the virus in the shortest lived population may be key to understanding the dynamics in the system as a whole. This dominance of the shortest-lived population may be at least in part attributable to an inversely proportional link between life expectancy and viral prevalence. Indeed, the effect of a reduced lifespan is the large, continual influx of susceptible hosts and a resultant increase to the overall level of viral prevalence. Consequently, when each population is as capable of transmitting virus to the other, there is greater transmission from the shorter-lived group and hence the evolutionary behaviour of the virus in that group dominates. This would also explain why it is easier to alter the dynamics of the long-lived population in the one-way interaction – for the same ease of cross-population transmission, more virus is transmitted from the short- to long-lived population than vice-versa. Thus, instances of antigenic stasis in relatively long-lived species could be explained by a close epidemiological link to short-lived aquatic birds or a host that is very short-lived indeed. Similarly, where we see antigenic evolution of the virus in very short lived hosts such as land-based poultry, this could either be because the population in question is actually long-lived enough for antigenic evolution of the virus to occur, or because there is a lot of transmission from a longer-lived species.

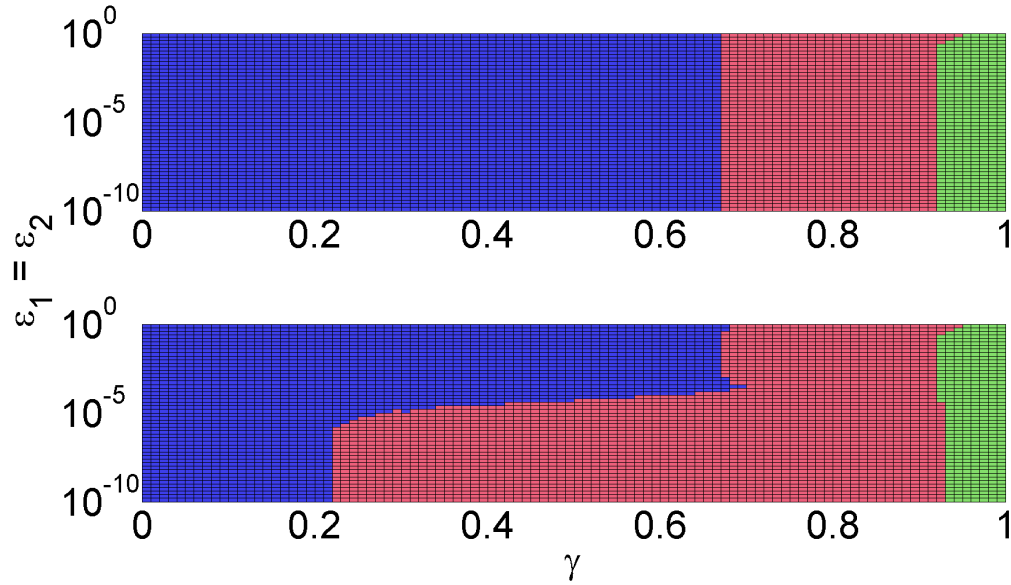


Figure 3-16: Allowing transmission between otherwise equivalent short-lived and long-lived populations in a [2, 3, 5] system. *The top and bottom panels respectively show the relationship between the strength of immune selection (γ) and antigenic evolution (blue (NSS); red (CSS); green (DSS)) for each of two populations where host life expectancy is 1 and 50 years and how this relationship changes with increasing transmission ($\varepsilon = \varepsilon_1 = \varepsilon_2$) between the two populations. The qualitative nature of antigenic evolution in the short-lived population is largely unchanged by this interaction, but is progressively altered with increasing transmission in the long-lived population.*

3.6: Discussion

The fundamental mechanisms by which influenza epidemics arise has important consequences for its control. If antigenic change is incremental, as suggested by the conventional ‘antigenic drift’ model, tracking these changes allows us to anticipate - albeit only haphazardly - the nature of future epidemics. If, however, the virus is bouncing around from point to point within a confined antigenic space, as suggested by the ‘limited-epitope’ model, the prediction of future antigenic types is no longer feasible due to the fundamentally unpredictable nature of this process. On the other hand, a limited set if

antigenic types affords the possibility of comprehensive vaccine coverage against all variants. This alternative model for the antigenic evolution of influenza could therefore herald a sea-change in our approach to mitigating influenza mortality, if it stands up to rigorous empirical testing. Certainly, short-term patterns of antigenic evolution, at least as understood based on antigenic cartography, can readily be replicated by the model. Further, pre-existing immune responses have been found, or at least suggested, to protect the elderly from severe disease in each of the recorded influenza pandemics. This pattern of protection illustrates both how the viruses of today can indeed be antigenically similar to the virus of the past, in itself giving the lie to the idea that influenza evolves along a linear antigenic path and with anything related to its archived antigenic history strictly out of bounds. However, such a phenomenon has yet to be demonstrated for epidemic influenza. This may be due to biases in the Haemagglutination Inhibition (HI) assays typically used to define the antigenic relationships between successive epidemics. The use of micro-neutralization (MN) and other new assays may enable us to identify neutralising antibodies to shared epitopes of limited variability which are both crucial to the antigenic dynamics of influenza and could form the basis of a new vaccine.

An important strength of the ‘limited-epitope’ model is that it totally eschews any sort of constraint on the rate of mutation governing the antigenic evolution of influenza. In so doing, the model avoids ransoming itself to possibly inappropriate restrictions on the ability of an RNA virus to churn out mutations and is the only proposed model that can make this claim. The ‘short-term strain-transcending immunity’ of Ferguson *et al.* requires strenuous restrictions on the mutation rate so as to avoid the simultaneous emergence of multiple antigenic types. Meanwhile, the ‘neutral-network’ model of Koelle *et al.* essentially proposes that it is hard for the virus to switch between alternative antigenic configurations. This model implicitly assumes that very much more than a few mutations are required to

switch between antigenic types; this is contradicted by the observation that mutations at some key sites are always sufficient, though not necessary, for the emergence of new antigenic clusters (Smith *et al.*, 2004).

In assuming that all possible antigenic types are constantly accessible, the ‘limited-epitope’ model demonstrates how nothing more complicated than immunological cross-reactivity between similar antigenic types can account for the single strain epidemics and antigenic cluster jumps that have characterise the population dynamics of influenza. At the heart of this model is the notion that that the antigenic flexibility of influenza is finite. This concept is currently well supported by the literature, but much further work is necessary to establish this model as a more plausible alternative to the prevailing ‘antigenic drift’ paradigm. Although it has not been explicitly shown to be capable of generating the known phylogenetic tree of type A influenza, the sequential dominance of antigenic types exhibited by the model is very likely to translate into a sequential replacement of genotypes. This framework, thus, is able to capture many features of influenza epidemiology and evolution under a minimum of assumptions concerning mutation rate, duration of non-specific immunity, and variability of antigenic determinants.

In this chapter, I have attempted to use the ‘limited-epitope’ model to gain an understanding of why we seem to observe different patterns of antigenic evolution in different species. A key feature of the limited epitope model is that it is capable of generating different forms of strain dynamics. Under low levels of immune selection, all possible antigenic types will co-exist and we effectively have antigenic stasis. Contrastingly, at high levels of immune selection, only those viruses that are utterly antigenically discordant can co-exist (see Figure 3-3) – this, too, is a form of antigenic stasis with reduced antigenic diversity. For intermediate levels of immune selection, we enter a

dynamical regime exemplified by cyclical or chaotic dynamics where antigenic types (as defined by particular combinations of epitope variants) wax and wane with time. While the epidemic behaviour of human influenza seems to fall within this latter regime, influenza in birds and swine cannot be described as neatly fitting into any one of these three patterns of behaviour, with periods of seemingly slow antigenic evolution and/or the co-circulation of multiple antigenic types. It seems unlikely that there would be sufficiently rapid variations in the degree of immune selection such that we could ascribe these complicated patterns to flitting between these regimes. It is not hard to see how this ability might vary between species (and, as noted, there is evidence of this from HI data), but it is hard to see how it would vary substantially within a species over very short periods of time.

One crucial difference between humans, pigs and birds is, of course, their average life expectancy. Indeed, when either antigenic stasis or slow antigenic evolution has been observed in the avian and swine populations, this has often been attributed to an absence of selective pressure due to a shorter host-lifespan. However, such an absence of selective pressure is more likely to manifest in observations of large amounts of antigenic diversity, rather than the maintenance of a single antigenic type over long periods of time. Indeed, only extremely strong selective pressure could conceivably select for the scenario of limited antigenic diversity over extended periods of time. My investigations suggest that antigenic evolution can be observed even for extremely short host life expectancies within the 'limited-epitope' model. Instead, the critical effect of reductions in host life expectancy within the model is on the qualitative nature of the observed antigenic evolution. For short life expectancies, not only does the inter-epidemic period drastically shorten, but the dynamics are typified by much greater antigenic diversity at any particular time point. Instead of single strain epidemics as observed in humans, we ought to expect influenza in shorter-lived species to be endemic and to present itself simultaneously in a number of

antigenic forms. Further, in some instances, small changes in host life expectancy could have a big impact on the observed antigenic diversity. Thus, for example, even a small increase in the life expectancy of swine, arising perhaps from changing farming practices, could manifest in a reduction in the observed antigenic diversity, together with more epidemic-type outbreaks.

Of course, there are many other differences between these species than just lifespan. For example, avian influenza is transmitted primarily through the faecal-oral route. Consequently, when deposited in lake water, it may have a much longer infectious period than influenza in humans and pigs, which must rely instead on airborne transmission. Within this model, such increases in the infectious period, whilst maintaining overall transmissibility, have the effect of increasing the threshold life expectancy at which antigenic evolution can occur. Thus, influenza may exhibit antigenic stasis in aquatic bird species even though these species are relatively long-lived. These boundaries are also sensitive to the intrinsic transmissibility (R_0) of influenza. As a result, if there is a positive correlation between R_0 and pathogenicity, then the evolution of a more pathogenic virus could alter the pattern of antigenic evolution of influenza within a species. For example, populations where immune selection is strong and the virus was therefore previously subject to antigenic stasis as exemplified by DSS could be shifted into CSS and antigenic evolution. For human populations, which already exhibit CSS, the emergence of more pathogenic viruses could alter the qualitative nature of the observed antigenic evolution and result in increased antigenic diversity.

None of the scenarios outlined above can explain why we might observe just a single antigenic type being preserved as dominant within a population over an extended period of time as has been suggested, for example, with cH1N1 in North American swine between

1918 and 1998. It is possible, however, that this is a misconception, and that the data from HI assays is not actually representative of antigenic stasis. As I've remarked several times already, the 'limited-epitope' model requires some form of restriction on the total possible antigenic diversity, even though the virus is free to mutate continually to any of these forms. I have demonstrated within this chapter how the exact composition of that restriction impacts on the observed relatedness between antigenic variants. If the antigenic epitopes are very flexible, then I can observe the pattern of cross-reactivity classically described as antigenic evolution: the predominant strain at any one point will quickly lose its cross-reactivity with subsequent isolates. On the other hand, if any of these epitopes are relatively restricted then cross-reactivity between isolates will be preserved with time under the guise of antigenic stasis in an HI assay, even though in truth the dominant antigenic type is changing over time. Consequently, it may be that periods of supposed antigenic stasis are in truth representative of a restricted antigenic space in which rapid antigenic evolution is nonetheless occurring, and not due to a short life spans leading to a lack of selection pressure being exerted by a host's immune system. Given that, for example, this pattern of evolution is not observed in human influenza, but has been observed in birds, this could reflect the fact that birds are regarded as the 'natural' host for influenza, and have consequently co-evolved with the virus over a much longer period of time than humans have. Perhaps therefore, avian immune systems have evolved to be better at recognising inherently less flexible antigenic determinants than human immune systems have yet managed.

I have also attempted to understand how the complex global ecology of influenza might impact on the virus' antigenic evolution. In particular, the virus is readily transmissible between different species and populations of birds that have widely varying life expectancies. It appears that if I link two populations – one short-lived and the other long-

lived – by allowing one to transmit disease to the other, then as I increase the degree of connectivity, then the more the qualitative dynamics of the latter population shift to look like those of the former. If, on the other hand, I allow both populations to infect each other at equal rates then I observe little change in the dynamics of the short-lived population, whereas those of the longer-lived population shift qualitatively to match those of the short-lived population. This is almost certainly because the virus attains higher prevalence within the shorter lived population, precisely because of the greater availability of freshly susceptible hosts in shorter lived populations. Consequently, in a linked influenza ecology, it seems that the evolutionary dynamics of influenza within the shortest-lived species are key to understanding the evolutionary dynamics of the network as a whole. If there is antigenic stasis in the shortest lived population, this may extend to the longer-lived population, even though the latter would exhibit antigenic evolution in isolation. In this instance, I would predict that if the link between the two species was broken, then I would observe antigenic evolution of influenza in the longer-lived species. Thus, the nature of the mode and tempo of the antigenic evolution of influenza within a population is in part a product of its role in the wider influenza ecology, which of course might also change substantially, and rapidly, with time.

These ideas may explain the rapid antigenic evolution of H5N1 in Hong Kong between 2001 and 2002 as observed by Sturm-Ramirez *et al.* – namely that the epidemiological link between long-lived wild birds and shorter-lived market birds in Hong Kong may have been broken. Evidence for this comes from the fact that when antigenically similar isolates to the highly pathogenic 1997 virus emerged in Hong Kong in 2001 (Guan *et al.*, 2002; Sturm-Ramirez *et al.*, 2004), stringent control measures were introduced that resulted in just one isolate being recovered between June and December 2001 (Guan *et al.*, 2002; Sims *et al.*, 2003). H5N1 viruses then emerged in Hong Kong at multiple live bird markets in January

2002 and local farms in February (Sims *et al.*, 2003), before an outbreak of highly pathogenic H5N1 in November and December 2002 in wild birds in two Hong Kong parks (Sturm-Ramirez *et al.*, 2004). Sturm-Ramirez *et al.* then revealed that these isolates were antigenically distinct from the 2001 viruses and their predecessors. It is possible that the extensive poultry culling and stringent control measures introduced in 2001 to combat the re-emergence of H5N1 could have isolated it in the longer-lived wild bird population of Hong Kong. This could have broken the epidemiological link to short-lived birds and restored the virus to a state of antigenic evolution in the wild birds, thereby leading to the antigenically distinct virus that was eventually introduced into poultry.

In conclusion, the 'limited-epitope' model of the antigenic evolution of influenza can shed light on the complex observed patterns of such evolution in species other than humans. My studies have highlighted why we should not accept the reasoning that hosts with short life spans cannot exert enough immune pressure on the virus for it to exhibit antigenic evolution. I have shown that that weaker immune pressure is more likely to manifest in increased, not reduced, antigenic diversity, whether directly through reduced immune selection or indirectly as a result of decreased life span, increased duration of infection or increased transmissibility. The main impact of varying host life span is on the qualitative nature of the observed antigenic evolution: in shorter lived hosts we should expect to see a pattern of prevalence that is more endemic than epidemic and characterised by increased antigenic diversity.

Observations, in pigs and birds, of the dominance of just a single antigenic type over long periods of time could be partially, but not entirely satisfactorily, explained by the HI test. This assay, though very good at differentiating between different subtypes, may not be truly fit for the purpose of inferring antigenic relationships among isolates from within a

subtype. A more satisfying explanation, I believe, may be that the structure of antigenic space is different for different species. Consequently, in those species where this presumed picture of antigenic stasis has been observed, it may simply be an echo of the underlying limitations to influenza's antigenic repertoire within that species. Thus, in demonstrating that both of the classical patterns of HI assays can be reconciled with antigenic evolution, this work challenges the assumption that influenza in swine and birds is generally in a state of antigenic stasis, or simply evolving less rapidly than in humans.

Finally, all of these things no doubt combine to ultimately have different effects on different populations of different species. However, in a first step at trying to model the wider influenza ecology within this model, I have shown how the basic evolutionary behaviour within the shortest lived population plays a dominant role in the evolutionary behaviour of influenza within the network as a whole. These network connections, perhaps more than anything else, are the most likely to change extensively in short periods of time, possibly say, as the result of drastic interventions of our own species to either contain a worrisome disease (highly pathogenic H5N1) or produce more food (farming practices). Such changes in network connectivity could consequently result in highly complicated patterns of antigenic evolution of influenza within a single species, as it flits between its default behaviour in isolation and the behaviour imposed on it by the wider network. Thus, radical changes in the nature of antigenic evolution over short periods of time almost certainly reveal a change in the associated host population's role within the wider influenza ecology, whilst observations of stasis to date reflect the strictures under which influenza within that population can operate.

Chapter 4

A new model for HIV-1 pathogenesis

4.1: Introduction

Human Immunodeficiency Virus 1 (HIV-1) causes an infection that is ultimately almost universally fatal. The virus was originally identified in the early 1980s as the causative agent of Acquired Immunodeficiency Syndrome (AIDS). Most infected patients eventually develop this condition, preventing them from mounting effective immune responses to HIV-1 and opportunistic infections.

The virus is spread predominantly by exposure to infected genital fluids, blood and blood products, and through mother-to-child transmission. Today, around 35 million people are living with HIV infection and the pandemic is still growing. Around 2 million will develop AIDS and die in the next 12 months, but they will be replaced by another 2.5 million newly infected individuals. Within the last decade, antiviral therapies have become available which have proven to be effective in arresting the development of AIDS. Long-term therapy is, however, not always possible as a result of cost, toxic drug side-effects and the evolution of drug resistance.

There are, in fact, two viruses capable of causing AIDS – HIV-1 and HIV-2. Whereas HIV-1 can be found globally and is responsible for the vast majority of infections, HIV-2 infection is not widely seen outside of West Africa. HIV-1 is also more pathogenic than HIV-2. Both viruses can be further subdivided into groups and subtypes on a genetic basis, each themselves varying in prevalence and geographic spread. One important and closely related virus is Simian Immunodeficiency Virus (SIV). However, this virus does not usually cause disease in its natural hosts. My work in this chapter pertains primarily to HIV-1, but its potential application to HIV-2 and SIV will be discussed.

The typical course of HIV infection in the host can be divided into three phases: (i) an initial phase of about 2 weeks with a large peak in viral prevalence; (ii) a chronic phase of indeterminate length with low viral prevalence during which CD4⁺ T cell counts decline but without otherwise immediately serious additional complications and (iii) a transition to Acquired Immunodeficiency Syndrome (AIDS), with a sharp increase in viral load and, eventually, death. This incubation period varies widely from person-to-person although, in the absence of therapy, around 50% of individuals will develop AIDS within 10 years of infection (Levy, 2009). Some are capable of remaining healthy and maintaining their CD4⁺ T cell count for more than 10 years without receiving antiretroviral therapy. These long-term non-progressors (LTNPs) account for around 5% of infected individuals. A separate group of LTNPs, called Elite Controllers (ECs), consists of those individuals who are known to be infected but without detectable plasma viral loads (Saez-Cirion *et al.*, 2007). Many factors, such as infection by an attenuated strain, likely play a role in the control of disease by LTNPs and ECs. One factor that is definitely important is the genetic background of an infected host; there are links between MHC Class I polymorphism and the pace of progression to AIDS (Goulder and Watkins, 2008). However, little is truly understood about the mechanism responsible for the ultimate development of disease or

how and which immune responses delay progression. By applying a multi-locus framework similar to that used in the last chapter, here I focus on the mechanisms that lead to pathogenesis; my results suggest the loss of control of viraemia arises primarily from the loss of the ability to make neutralising antibody responses rather than escape from or degeneration of cytotoxic CD8⁺ T cell (CTL) responses.

4.1.1 The virus

HIV-1 is a lentivirus, a genus of the family *retroviridae*, with a spherical virion around 120nm in size (Figure 4-1). Each virion contains two copies of a positive single-stranded RNA genome, which encodes nine genes (*gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*) and 19 proteins in total. HIV-1 genes, and their products, can be broadly organised into 3 groups. First, there are those that encode the structural proteins/viral enzymes that are essential components of any retroviral particle, namely *gag* (capsid proteins), *pol* (viral enzymes) and *env* (envelope precursor, gp160). Second, *tat* and *rev* encode regulatory proteins that modulate transcriptional and post-transcriptional steps of virus gene expression – without them construction of viral progeny is impossible. The remaining genes *nef*, *vif*, *vpr* and *vpu* encode auxiliary proteins. Expression of *nef* (along with *tat* and *vpu*) within an infected cell seems to downregulate MHC molecules (Kamp *et al.*, 2000) and may therefore be implicated in immune evasion. *Vif* protects the virus against host APOBEC-protein induced hypermutation by marking these proteins for proteasomal degradation (Sheehy *et al.*, 2002; Zheng *et al.*, 2004).

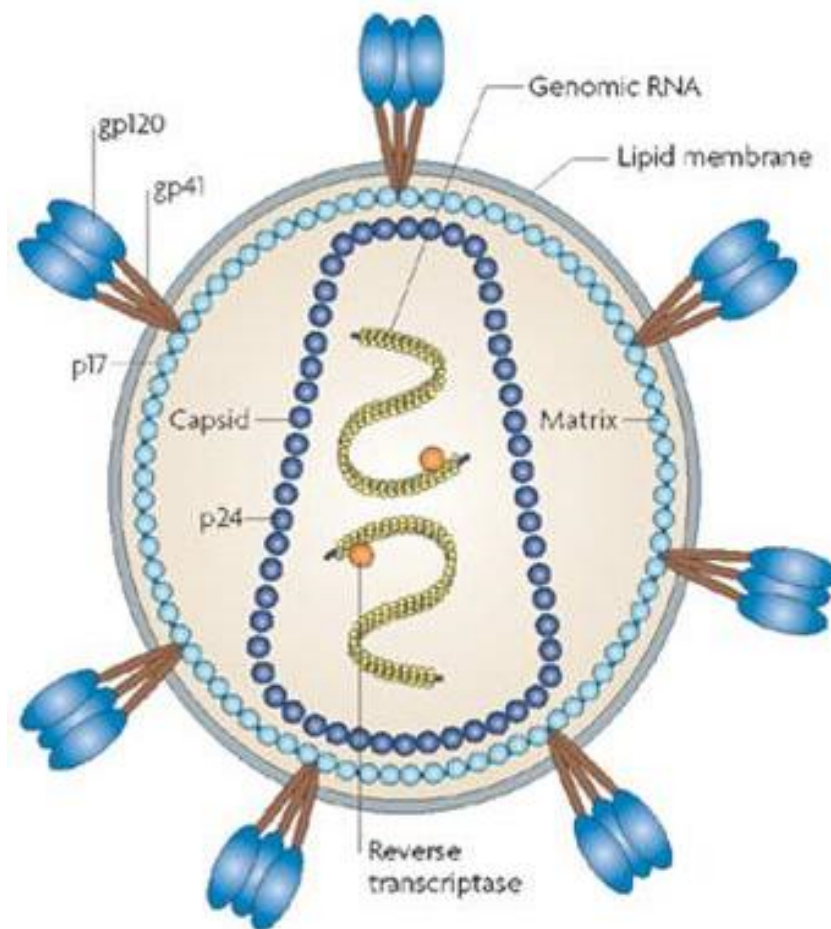


Figure 4-1: The HIV-1 virion. A schematic of an HIV-1 virion. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology* Karlsson Hedestam et al., 2008, copyright.

Each virion contains two copies of the genome, surrounded first by around 2,000 copies of the conical viral capsid. In turn, the capsid is enclosed by several copies of the matrix protein which help anchor the protein Env, roughly 70 copies of which are embedded in the lipid membrane of the viral envelope. The *env* gene encodes a precursor protein gp160, but this is later cleaved by the host cell's own enzymes to produce a non-covalent complex of the external glycoprotein gp120 'spike' and the trans-membrane protein gp41 'stem'; mature gp120-gp41 proteins are then thought to associate as a trimer on the cell surface (Roux and Taylor, 2007). Entry into host cells is instigated by the high-affinity binding of

gp120 to CD4 receptors on the target cell's surface. This triggers a series of conformational changes and leads to exposure of both chemokine co-receptor binding sites and gp41, which can then initiate host-cell virus fusion.

Different HIV viruses use different chemokine co-receptors for binding. A large number of these can act as primary or secondary attachment sites for both HIV-1 and HIV-2 isolates, but are not commonly involved in infection (Berger *et al.*, 1999). In contrast, the two most important chemokine co-receptors, CCR5 and CXCR4, broadly define the cell tropism of a virus: R5 viruses use CCR5, X4 use CXCR4 and R5X4 viruses can use either. CCR5 is expressed on the vast majority of mature and immature dendritic cells, along with most CD4⁺ memory T cells (Lee *et al.*, 1999). Within the CD4⁺ memory phenotype, effector cells much more commonly express CCR5, and typically at much higher quantities, than resting cells, although both are productively infected *in vivo* (Lee *et al.*, 1999). CXCR4, on the other hand, is predominantly found in high concentrations on B cells, mature dendritic cells and naive CD4 T cells (Lee *et al.*, 1999). R5 viruses have an enormous yet poorly understood selective transmission advantage over their X4 counterparts (Cohen *et al.*, 2011), although later mutations can result in the emergence of X4 and R5X4 viruses (*e.g.* (Bunnik *et al.*, 2011)), often in association with the development of disease (Connor *et al.*, 1997; Scarlatti *et al.*, 1997).

The vast majority of HIV infections seem to be initiated by just a single virus breaching a mucosal barrier (Keele *et al.*, 2008; Salazar-Gonzalez *et al.*, 2009; Fischer *et al.*, 2010). The major initial targets of infection are believed to be mucosal Langerhans' and CD4⁺ T cells (Hladik *et al.*, 2007), but within a few days the virus manages to reach the gut associated lymphoid tissue (GALT) wherein swift expansion of viral populations commences. This expansion is at first local (Schacker *et al.*, 2001) but then likely systemic (Mattapallil *et al.*,

2005). The net result, either directly or indirectly, is the depletion of the majority of memory CD4⁺ T cells in the GALT and mucosa (Veazey *et al.*, 1998; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Mattapallil *et al.*, 2005). Another consequence of extensive viral replication is the destruction of many of the germinal centres crucial for B cell maturation, leading to the impairment of B cell responses (Levesque *et al.*, 2009).

Plasma viraemia, often referred to as viral load, thus increases initially exponentially, before reaching a peak of around a million RNA copies of virus per ml of blood, about 3-4 weeks after infection (McMichael *et al.*, 2010). At this juncture, CD4⁺ T cell numbers are low both in blood and the GALT, but levels in blood will go on to return to near normal (Veazey *et al.*, 1998; Brenchley *et al.*, 2004; Mattapallil *et al.*, 2005). Subsequent to achieving its peak, viral load declines over the following 12-20 weeks to reach a plateau known as the viral setpoint. There can be subsequent strong transient fluctuations in viral load during chronic infection, but it generally increases only gradually from this setpoint (Schacker *et al.*, 1998; Sabin *et al.*, 2000). The most striking feature of chronic infection is a generally continuous decline in CD4⁺ T cell count. A patient is defined as having AIDS once their CD4⁺ T cell count drops below 200 cells/ μ l blood or if CD4⁺ T cells represent less than 15% of their total lymphocyte population. This is accompanied by a significant, and sudden, increase in viral load. A patient with AIDS is much more susceptible to infection by various pathogens, many of which are opportunistic and would be routinely cleared by a healthy immune system. Eventually, a patient with AIDS will succumb to one of these opportunistic infections and die.

4.2: Adaptive immune responses to HIV-1

4.2.1: Antibody responses

The first B cell associated immune complexes can be detected around 8 days after transmission, with a further 5 days (*i.e.* 13 days post-transmission) before detection of free antibody, specific to gp41, in plasma and another 14 days (27 days post-transmission) before antibody to gp120 can be found (Tomaras *et al.*, 2008). Notably these antibody responses are non-neutralizing and further do not appear to select for escape mutants, and are thus believed to be ineffective against HIV-1 (McMichael *et al.*, 2010). Neutralizing antibody (NAb) can first be detected around 12 weeks after infection, and are generally targeted at variable epitopes (*e.g.* (Moore *et al.*, 2008)); extensive possibilities for variation within the *env* gene allow the virus to continually indulge in cat-and-mouse escape from these responses (Richman *et al.*, 2003; Wei *et al.*, 2003; Gray *et al.*, 2007). Antibodies that target relatively conserved regions of Env, and which are therefore capable of neutralizing a broad spectrum of heterologous viruses, develop only rarely and very slowly (Gray *et al.*, 2009; Shen *et al.*, 2009; Stamatatos *et al.*, 2009). The severe early loss of germinal centres may be partly responsible for the inability to develop such presumed high-affinity responses and may also account for the delay in the appearance of autologous neutralizing antibody (McMichael *et al.*, 2010). It is also interesting to note that, like dengue, HIV-1 can exploit antibody dependent enhancement to gain entry into cells with Fc-receptors (Robinson Jr. *et al.*, 1988; Homsy *et al.*, 1989). The maintenance of a strong antibody response has been implicated in avoiding progression to AIDS (Ljunggren *et al.*, 1987; Karpas *et al.*, 1988; Ljunggren *et al.*, 1990; Cao *et al.*, 1995; Scarlatti *et al.*, 1996; Pilgrim *et al.*, 1997; Cecilia *et al.*, 1999). In particular, Cecilia *et al.* detected no difference in NAb titres in early infection when matching LTNPs with infected patients who progressed rapidly to

disease but found that when again compared later on in infection, LTNP had significantly higher titres. The potential importance of B cells in controlling viral load is also suggested by a study of an infected patient showing that B cell depletion lead to a decline in NAb titres and an increase in viral load (Huang *et al.*, 2010).

4.2.2: T cell responses

The first T cell responses to HIV-1 infection emerge just before peak viraemia (*e.g.* (Lichterfeld *et al.*, 2004), and themselves peak 1-2 weeks later as viraemia declines (Borrow *et al.*, 1994; Koup *et al.*, 1994; Pantaleo *et al.*, 1994; Wilson *et al.*, 2000; McMichael *et al.*, 2010). The earliest responses are often specific for Nef and Env, but responses to other proteins soon follow (Goonetilleke *et al.*, 2009; Turnbull *et al.*, 2009). The selective pressure imposed by these CD8⁺ T cell responses is demonstrated by the ensuing and recurrent pattern of rapid positive selection of escape mutants (Phillips *et al.*, 1991; Borrow *et al.*, 1997; Goulder *et al.*, 1997; Price *et al.*, 1997; Bernardin *et al.*, 2005; Goonetilleke *et al.*, 2009; Salazar-Gonzalez *et al.*, 2009). Mutations conferring escape may affect binding of the epitope to the HLA molecule, preventing presentation of the epitope at the surface *e.g.* (Couillin *et al.*, 1995; Goulder *et al.*, 1997; Yusim *et al.*, 2002), or more generally interfere with the cellular processes that lead to epitope presentation *e.g.* (Draenert *et al.*, 2004a; Allen *et al.*, 2005a; Milicic *et al.*, 2005). Within the epitope itself, mutation may affect the interaction between T cell receptor and the epitope-HLA complex *e.g.* (Allen *et al.*, 2000; Leslie *et al.*, 2004; Oxenius *et al.*, 2004; Allen *et al.*, 2005b). A specific example of this is the generation of altered peptide ligands (APLs) within CD8⁺ T cell epitopes, which may act as T cell receptor antagonists (Klenerman *et al.*, 1994; Klenerman *et al.*, 1995; Purbhoo *et al.*, 1998). Usually, CD8⁺ T cell responses decline rapidly once an escape mutant emerges (Ogg *et al.*, 1998; McMichael and Rowland-Jones, 2001; Rowland-Jones *et al.*, 2001;

Jamieson *et al.*, 2003; Allen *et al.*, 2005b; Goonetilleke *et al.*, 2009; Turnbull *et al.*, 2009; Liu *et al.*, 2011), indicating some form of dependency on antigen. The breadth of the CD8+ T cell response is believed to change with time, with increased numbers of epitopes targeted in later infection (Addo *et al.*, 2003).

Several studies have indicated a relationship between mutations that confer escape from CD8+ T cell responses and progression to AIDS (Goulder *et al.*, 1997; Kelleher *et al.*, 2001; Kemal *et al.*, 2008; Crawford *et al.*, 2009), although the direction of causality is unclear. Escape from CD8+ T cell responses has been shown to increase viral load (Barouch *et al.*, 2002; Feeney *et al.*, 2004), but can also have no impact (Jamieson *et al.*, 2003). CD8+ T cell depletion studies in SIV-infected monkeys have demonstrated that CD8+ T cell responses help control viral replication *in vivo* (Jin *et al.*, 1999; Schmitz *et al.*, 1999; Mueller *et al.*, 2009; Okoye *et al.*, 2009). There is also evidence that LTNPs and ECs are more likely to have strong CD8+ T cell responses than those who progress to disease (Borrow *et al.*, 1994; Cao *et al.*, 1995; Klein *et al.*, 1995; Rinaldo *et al.*, 1995; Harrer *et al.*, 1996; Kloosterboer *et al.*, 2005; Lambotte *et al.*, 2005; Betts *et al.*, 2006). Presence of HIV-1 CD8+ T cell responses in an individual prior to infection has been implicated in lower viral load once infected (Rowland-Jones *et al.*, 2001; Kaul *et al.*, 2010). Together with the correlation of the appearance of CD8+ T cell responses with the decline from peak viraemia in acute infection (Borrow *et al.*, 1994; Koup *et al.*, 1994), all this has led to a focus on escape from or impairment of CD8+ T cell responses as a mechanism for pathogenesis *e.g.* (Rowland-Jones *et al.*, 2001; Goulder and Watkins, 2008; Freel *et al.*, 2011; Munier *et al.*, 2011). One hypothesis is that CD4+ T cell help plays an integral role in the generation of fully functional memory CD8+ T cell responses, and these are therefore weakened as a result of HIV infection (Sun *et al.*, 2004; Wherry and Ahmed, 2004).

Further support for a central role of CD8+ T cells in the control of HIV-1 infection come from associations between HLA Class I alleles and rate of progression to AIDS (Carrington and O'Brien, 2003; Martin and Carrington, 2005; Fellay *et al.*, 2007; Goulder and Watkins, 2008; The International HIV Controllers Study, 2010). Individual HLA class I alleles may influence pathogenesis depending on their ability to bind and present different CD8+ T cell epitopes (The International HIV Controllers Study, 2010), although HLA-B alleles seem to dominate this effect (Kiepiela *et al.*, 2004). For example, B*27 and B*57 have been consistently associated with a slower rate of developing disease (Gao *et al.*, 2005; Fellay *et al.*, 2007), whilst HLA-B*35Px alleles associate with rapid progression to AIDS (Gao *et al.*, 2001), as does HLA class I homozygosity (Carrington *et al.*, 1999; Tang *et al.*, 1999). Association between HLA class I and the immunoglobulin-like receptors on NK cells (KIRs) may also have an impact on the rate of progression to disease (Bashirova *et al.*, 2011).

Several epitopes for CD4+ T cells have also been identified, predominantly in Gag and Nef (Kaufmann *et al.*, 2004). Importantly, there seem to be differences in CD4+ T cell function between ECs and those with progressive disease that cannot be explained simply by controlling for viral load and T cell count (as reviewed by (Porichis and Kaufmann, 2011)). For example, ECs seem to carry specific HLA Class II molecules that elicit more robust and polyfunctional mucosal CD4+ T cell responses (Ferre *et al.*, 2010). This study by Ferre and colleagues also therefore suggests that there could be more general HLA class II allele associations with progression to disease, as with HLA Class I. Secretion of IL-21 by CD4+ T cells may be critical for maintaining CD8+ T cell function (Elsaesser *et al.*, 2009; Frohlich *et al.*, 2009; Yi *et al.*, 2009) and impaired as HIV-1 infection progresses (Iannello *et al.*, 2008; Iannello *et al.*, 2010). Secretion of IL-21 is also vital for differentiation of B cells into antibody-secreting plasma cells (Ettinger *et al.*, 2005; Kuchen *et al.*, 2007). The possible

relationship between the general decline of CD4+ T cell count during chronic infection and anti-HIV-1 antibody responses is the main focus of the model for pathogenesis in this chapter.

4.3: Existing models of HIV-1 pathogenesis

A significant amount of quantitative work, in conjunction with experimental data, has enhanced our understanding of many of the basic features of HIV-1 infection. Mathematical models have provided us with insights into, for example, the multiphasic nature of viral decay upon treatment, the lifespan of an infected cell, the dynamics of the reservoir of latently infected cells and the optimal timing for that start of antiretroviral therapy (Ho *et al.*, 1995; Perelson *et al.*, 1996; Perelson *et al.*, 1997a; Perelson *et al.*, 1997b; Stafford *et al.*, 2000; Muller *et al.*, 2002; Perelson, 2002; Rong and Perelson, 2009). However, many aspects of the virus's pathogenesis remain puzzling. What is known, or at least believed, is that the virus 'wears down' the immune system during the chronic phase of infection, eventually reaching a point where the infected hosts cease to be immunocompetent and consequently develop AIDS. There is a strong correlation between low CD4+ T cell count and the development of AIDS, but the precise way(s) in which the immune system fails and how this is related to CD4 count remain unknown. A number of mathematical models have been proposed in effort to try to better understand HIV-1 pathogenesis, although far fewer than have been devoted to understanding basic viral dynamics. One consistent problem for models of pathogenesis has been capturing both the dynamic of a steady, stable viral setpoint and a later transition to AIDS represented by substantially elevated viraemia.

The most well-known of these models is the ‘Antigenic Diversity Threshold’ model of Nowak and colleagues (Nowak *et al.*, 1991). In this model, the virus begins as a single antigenic type, against which the immune system can manufacture independent responses that target both highly variable and conserved regions of the virus. This leads to a classical SIR-type dynamic for this strain, which initially increases in prevalence but is then brought under control and suppressed by the immune system. However, the model assumes that replication errors periodically produce antigenically novel strains of HIV-1. They model two arms of the immune response: one cross-reactive against all strains (targeted against a conserved region) and one that is strain specific (targeted against a variable region). They further assume that all viral strains are capable of killing the CD4+ T cells which are necessary for both types of immune response. Crucially, in this model, strains are not cleared from the system but instead suppressed to low prevalence. The background viral load therefore increases as more strains are introduced, thereby also increasing the rate of CD4+ killing by the virus. Eventually, the number of strains in the system reaches a threshold (the eponymous Antigenic Diversity Threshold) where the rate of killing of CD4+ T cells is no longer balanced by the generation of specific immune responses against each variant and viraemia explodes. By these means, this model is able to reproduce the two-peaked pattern of viral abundance (*i.e.* acute infection followed by chronic infection and then AIDS), but with small sporadic upsurges in the asymptomatic phase (due to new strains emerging and then being brought under control). It is characterised by the coexistence of an increasing number of antigenically distinct viral strains over the incubation period and the dominance of fast replicating strains once AIDS develops. Although extremely elegant in its formulation, this model relies on the sequential introduction of new antigenic types, and their persistence at low levels within the host. It is thus highly sensitive to assumptions about both the mutation rate of HIV-1 and, particularly, the rate at which it generates new antigenic variants. Indeed, a critical

dependency on some mutational process is common to most models of HIV-1 pathogenesis.

Other attempts have been made to construct models that simulate gradual immune escape by the virus. Korthals Altes *et al.* have proposed a framework where the rate of escape is proportional to viral load: this interaction creates a feedback loop that eventually leads to a transition to AIDS (Korthals Altes *et al.*, 2006). However, this requires that no mutation can occur before the virus has reached setpoint, since AIDS would otherwise develop all but instantaneously. Galvani (2005) has constructed a model that seeks to explain the sudden emergence of AIDS as the ultimate product of deleterious mutations in thymocytes due to over-exertion of the immune system (Galvani, 2005). However, this model relies on the biologically-inaccurate requirement that there is a homeostatic link between thymocyte production and the number of circulating mature lymphocytes. Further, the model implicitly assumes that this is the only possible source of new lymphocytes. Alternatively, others have hypothesised that the virus could gain replicative capacity with time (Stilianakis *et al.*, 1997; Stilianakis and Schenzle, 2006)), but instead of a sudden shift to AIDS, this leads to linear increases in viraemia. Another model has proposed progressive dendritic cell dysfunction as the eventual cause of AIDS (Hogue *et al.*, 2008). However this model also requires sudden, large variations in this dysfunction to capture the dynamic of sudden progression to AIDS.

One model that does completely eschew mutation as an explanation for the transition to AIDS is that of Fraser *et al.* (Fraser *et al.*, 2001). These authors assume that there is a homeostatic mechanism that acts to balance CD4⁺ and CD8⁺ T cell numbers. Killing of CD4⁺ T cells by virus disrupts this balance in favour of CD8⁺ T cells and results in a linear decline in CD4⁺ T cell count. Eventually this count drops below a threshold level

and the patient develops ‘AIDS.’ Importantly however, the anti-viral immune responses considered in this model are totally independent of both CD4+ and CD8+ T cells. Consequently, the development of AIDS in their model has no impact on viral replication.

In summary, no currently published model can produce the appropriate twin-peaked pattern of viral load associated with HIV-1 infection without also requiring a staged emergence of antigenic variants. This presents much the same conundrum as that encountered for influenza virus in Chapter 3: unless the network of possible antigenic changes is 1-dimensional then such an assumption is unrealistic. Further, given the tremendous mutational capabilities of the virus, it should be able to rapidly explore the extent of antigenic diversity available to it. There is likely also to be some limit to the possibilities for antigenic change as a result of structural constraints. In the next section, I attempt to outline a model that can generate this twin-peaked pattern of viral load, despite the continual generation of all possible antigenic variants.

4.4: A multi-locus model for HIV-1 pathogenesis

I have constructed a set of mathematical models to consider the combined effect of short-lived CD8+ T cell responses and long-lived neutralising antibody responses on the pathogenesis of HIV. These build on the “limited epitope” multi-locus framework discussed in the previous chapter, but applied to the dynamics of the viral population within an individual. It should be noted that throughout this section, I have considered the qualitative relationship between variables and all units are therefore arbitrary.

4.4.1: Basic model

I assume that HIV-1 displays neutralising antibody (NAb) epitopes on Env, each eliciting a highly specific immune response, and that the virus contains CD8+ T cell epitopes in various other viral proteins, such as Gag. These may be represented by an n -locus, m -allele system, where each locus describes a different epitope and each allele a different variant therein. As in the last chapter, to refer to an n -locus system, with m_k alleles at each locus k ($1 \leq k \leq n$), I will use the following notation: $[m_1, m_2, m_3, \dots, m_n]$.

I further assume that both the Nab and the CD8+ responses are highly epitope-specific. For example, if we consider the $[2, 2]$ system illustrated by the epitope combinations: ‘ax’, ‘ay’, ‘bx’, ‘by’, the response primed by strains displaying ‘ax’ will also cross-protect against strains displaying ‘ay’ and ‘bx’ but not those displaying ‘by’.

Figure 4-2 provides a visual representation of this system for the case where there are two NAb variants and the CD8+ epitopes conform to the $[2, 2]$ system. In essence, each of

these CD8+ epitopes can be considered as Altered Peptide Ligands (APLs), but which do not exhibit any form of antagonism.

In this basic model, I assume free association between NAb and CD8+ T cell epitopes. This means that for p antibody epitope variants and q APL epitope combinations, there are a total of the product pq strains or antigenic variants, all of which are constantly generated by the high mutation rate of HIV-1. This implicitly assumes that the mutational distance “divided” by the mutational rate between variants is not that large. Each strain is labelled $v_{i,j}$ where i refers to the NAb epitope and j to the CD8+ epitope combination; the NAb response to NAb epitope i is denoted z_i and the CD8+ T cell response to CD8+ epitope combination j is denoted w_j .

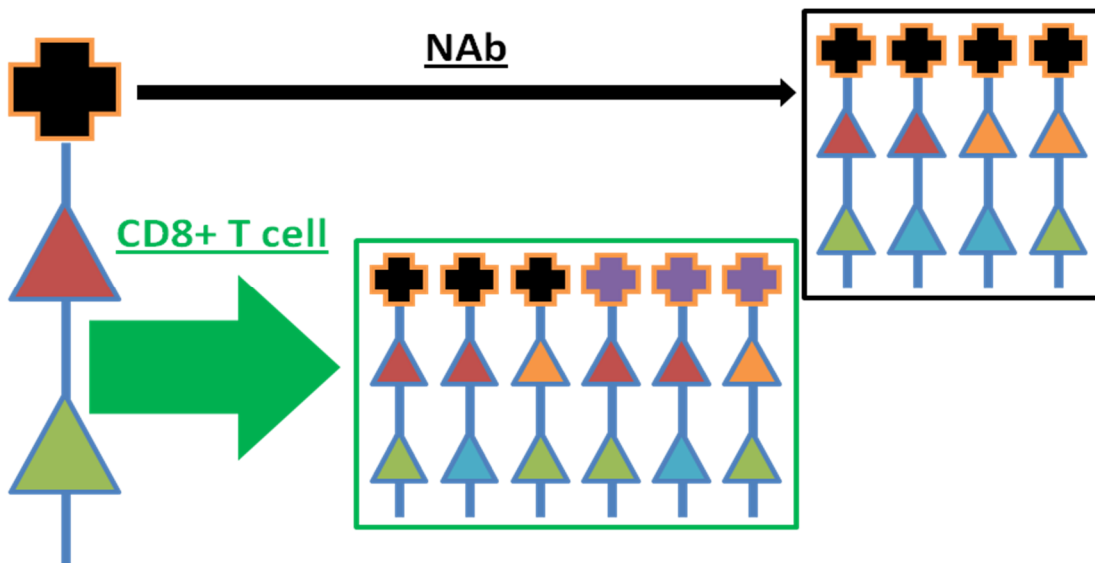


Figure 4-2: Visual representation of the basic model. Black and purple “pluses” represent two different NAb epitope variants. Each HIV-1 virion expresses one of the two. Coloured “triangles” represent the various CD8+ epitope combinations. At one epitope, each virion expresses either red or orange, whilst the options are blue or green at the other epitope. Thus, there are a total of 4 possible CD8+ epitope combinations. Free association between NAb and CD8+ epitope variants leads to a total of $4 \times 2 = 8$ antigenic variants of HIV-1 (not all shown). Specific immune responses protect against all strains carrying the same NAb epitope (in this example, black) while the CD8+ response raised against the epitope combination shown here is able to also protect against all variants that express either the red CD8+ epitope or the green CD8+ epitope or both.

The rate of change in these variables may be described by the following set of equations:

$$\frac{dv_{i,j}}{dt} = (\rho - \kappa z_i - \gamma w_j) v_{i,j} \quad (4:1)$$

where ρ is the viral growth rate, with κ and γ respectively the strength of NAb and CD8+ killing of virus;

$$\frac{dz_i}{dt} = \varphi \left(\sum_j v_{i,j} \right) - \mu_z z_i \quad (4:2)$$

where φ represents the strength of the induction of the NAb response and μ_z its longevity;

$$\frac{dw_j}{dt} = \eta \left(\sum_{k \sim j} \sum_i v_{i,k} \right) - \mu_w w_j \quad (4:3)$$

where η represents the strength of the induction of the CD8+ T cell response, μ_w its longevity and I sum over all strains that share alleles at the CD8+ epitopes with strain j .

CD8+ T cell responses have been demonstrated to be short-lived in the absence of antigen *e.g.* (Ogg *et al.*, 1998; McMichael and Rowland-Jones, 2001; Rowland-Jones *et al.*, 2001; Allen *et al.*, 2005b; Liu *et al.*, 2011). Specific NAb responses do wane over time, but appear to be maintained in the absence of antigen, possibly as the result of antibody secretion by long-lived plasma or memory B cells (Moore *et al.*, 2009). I therefore assume that, in the absence of antigen, a neutralising antibody response to a particular epitope declines much more slowly than does a CD8+ T cell response ($\mu_z < \mu_w$).

During the course of an HIV-1 infection, there is a slow, continual decline of CD4+ effector memory cells that correlates with progression to AIDS (Okoye *et al.*, 2007). To begin with, I simply assume that CD4+ T cell count declines linearly with time, primarily due to the effects of persistent infection and targeting of CD4+ T cells by the virus. I further assume that the NAb response relies on CD4+ T cell help for induction, as represented by φ in the above equations. It therefore becomes harder to generate NAb responses as infection progresses in the model. The early loss of germinal centres and B cell exhaustion will also contribute to a loss of ability to generate antibody responses (Levesque *et al.*, 2009). This is represented by the following additional equation, which changes φ (the strength of induction of the NAb response) from a parameter to a variable:

$$\frac{d\varphi}{dt} = -\alpha \quad (4.4)$$

such that $\varphi = \varphi(t)$ is a monotonic function that decreases linearly with time, at rate α , reflecting the drop in CD4+ T cell count. The drop in CD4+ T cell count may also affect the longevity of the NAb response rather than its induction, but the results of the model are not sensitive to these specific assumptions.

In its simplest form, the model does not include an interaction between CD4+ count and CD8+ response. CD4+ help is not always essential for proliferation of CD8+ cells, for example in cases where there is a strong inflammatory response (Malek, 2002). During the course of HIV-1 infection, there is a lack of correlation between CD4+ count and both circulation of anti-HIV CD8+ T cells (Ogg *et al.*, 1998) and CD8+ T cell-mediated lysis of infected cells (Klenerman *et al.*, 1996). Further, Mollet *et al.* have shown expansion of CD8+ responses even in the absence of detectable CD4+ T cell help (Mollet *et al.*, 2000). In any case, as we shall see later, the behaviour of the model is again largely insensitive to

assuming that the function of the CD8+ responses is also impaired by the drop in CD4+ T cell count.

To summarise, the critical features of this model are

- virus production is proportional to the amount of circulating virus
- circulating virus and virus-infected cells are killed by NAb and CD8+ T cell responses, respectively
- CD8+ T cell responses to the APL epitopes are short-lived in the absence of antigen, but NAb responses last much longer
- the induction of Nab responses is dependent on the CD4+ T cell count

The model is seeded with a small initial prevalence of each individual variant, no pre-existing immune responses and $\varphi(0) > 0$. Additionally, I assume that the high mutation rate of the virus is capable of constantly generating each of these variants. In the following simulations shown, unless otherwise stated, the values assigned to each parameter are: $\rho = 7.5$; $\kappa = \gamma = 1$; $\eta = \varphi(0) = 1$; $\mu_z = 1000^{-1}$ (such that the NAb response decays over 1000 time units); $\mu_w = 1^{-1}$ (such that the CD8+ response decays over 1 time unit); and $\alpha = 4000^{-1}$.

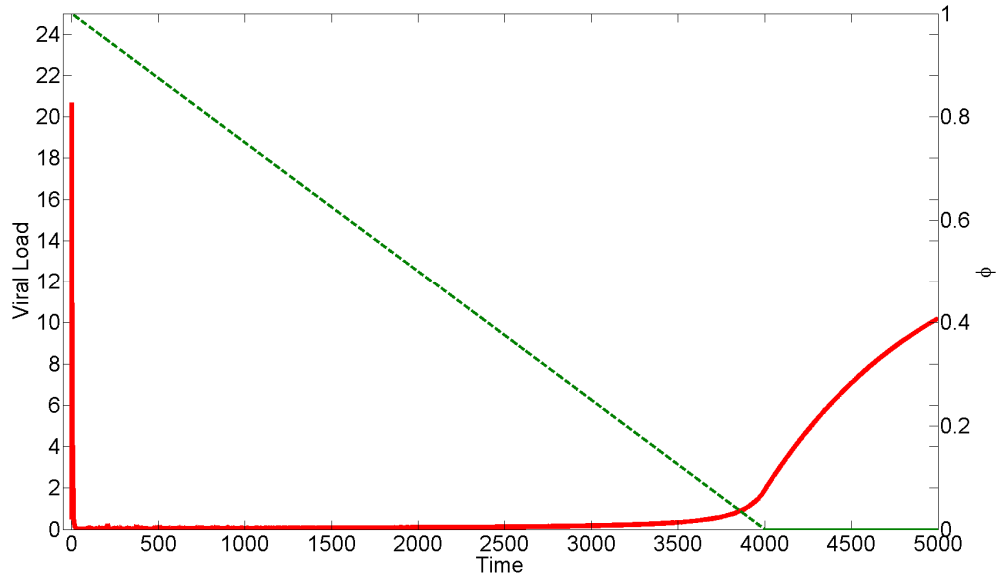


Figure 4-3: Viral load and strength of induction of the neutralising antibody response with time. *Viral Load (red line) initially rises but is then brought under control by the immune system. Chronic infection then persists until the strength of induction of the Neutralising Antibody Response (φ ; dashed green line) reaches a critical threshold, at which point control of the virus is no longer possible and the host develops AIDS. In this figure, the rate of decline of the antibody response is not linked to viral load.*

Figure 4-3 represents a typical time course for total viral load within this model with, in this particular case, 1 NAb epitope with 6 variants and a [2, 3] system for the CD8+ T cell epitopes. Initially, there is a sharp rise in viral load, which is then brought back under control by a combination of immune responses. Viral load is then held relatively constant, with only a slight increase occurring over the period of chronic infection. Importantly, this occurs despite a steady decline in the strength of induction of the NAb response (φ) due to loss of CD4+ T cells, as shown in Fig 4-3. However, once φ falls to very low levels, the system exhibits a stark dynamical transition to regime where the control is entirely effected by CD8+ T cells with a sharp increase in viral load.

So far, I have taken the clinically described decline in CD4+ T cell count as given and made no assumptions about why this decline occurs. I now explore the implications of assuming that this rate of killing is proportional to the viral load by changing the equation for $\varphi(t)$ to the following:

$$\frac{d\phi}{dt} = -\alpha \sum_i \sum_j v_{i,j} \quad (4:5)$$

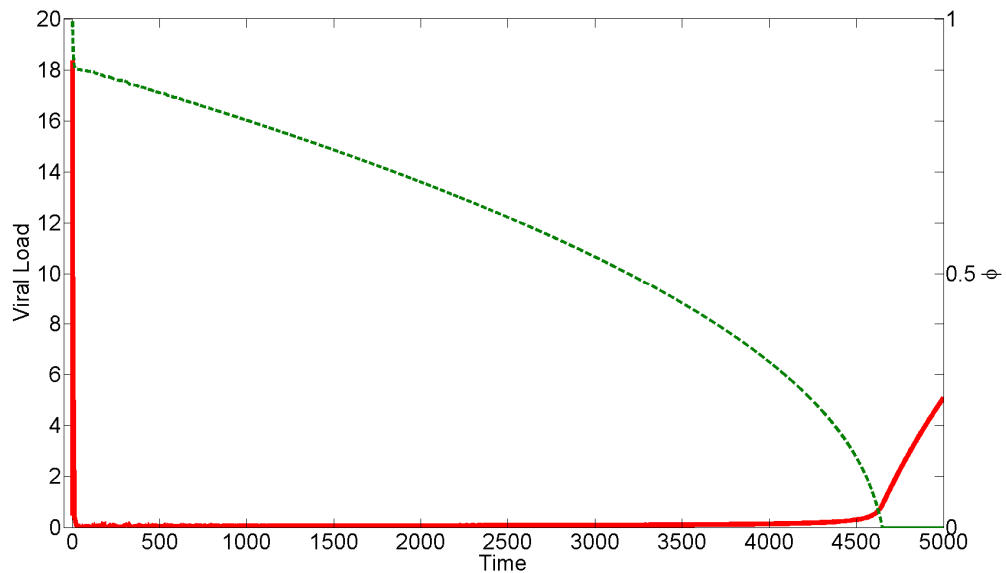


Figure 4-4: Viral load and strength of induction of the neutralising antibody response with time. *Viral Load (red line) initially rises but is then brought under control by the immune system. Chronic infection then persists until the strength of induction of the Neutralising Antibody Response (φ ; dashed green line) reaches a critical threshold, at which point control of the virus is no longer possible and the host develops AIDS. In this figure, the rate of decline of the antibody response is proportional to viral load. The only other difference with Figure 4-3 is that α has been increased to 500^{-1} .*

As shown in Figure 4-4, linking the loss of CD4+ T cells to viral load makes no change to the two-peaked pattern of viraemia. There is initially a steep drop in φ : this may accurately capture the severe events of acute infection where the CD4+ T cell count drops in the blood, but there is also widespread depletion of mucosal CD4+ T cells and irreversible destruction of the GALT (Brenchley *et al.*, 2006; Levesque *et al.*, 2009). Subsequent to this, there is a much slower decline in φ once the chronic phase has been established. Note that φ never goes back to its original levels even though CD4+ T cell counts might (Veazey *et al.*, 1998; Brenchley *et al.*, 2004; Mattapallil *et al.*, 2005). This discrepancy may reflect, for example, a destruction of GALT and depletion of mucosal CD4+ T cells that is not reflected in the CD4+ T cell count. The pace of decline then accelerates in late chronic infection, ultimately resulting in the failure of the NAb response and the development of AIDS. Making this assumption also results in a negative correlation between the viral load at setpoint and the time it takes to progress to AIDS; any mechanism that acts to reduce viral load will therefore be positively associated with a good long-term prognosis in the model. This may explain why a number of clinical studies have found a link between fast progression and high viral load in the blood (Mellors *et al.*, 1996; O'Brien *et al.*, 1996; de Wolf *et al.*, 1997; Mellors *et al.*, 1997; Stein *et al.*, 1997). The existence of long-term non-progressors (LTNPs) and elite controllers (ECs) may be explained within this framework by their ability to maintain low levels of viraemia either through the action of the immune responses represented in this model as well as others not explicitly included (for example, CD8+ T cell responses against conserved epitopes). Alternatively, LTNPs could be infected with an attenuated virus, or the quality of their CD4+ T cell help may be less adversely affected by viral replication. In this model, ECs would constitute the small fraction of infected patients able to suppress viral load to the point where its impact on CD4+ T cell count is negligible (O'Connell *et al.*, 2010).

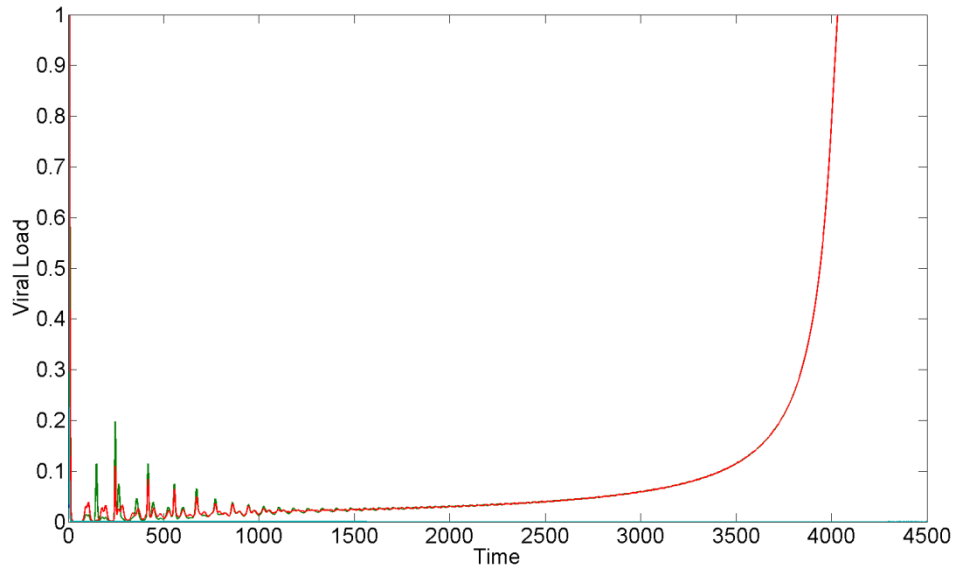


Figure 4-5: Pattern of evolution at the CD8+ T cell epitopes. *This is shown for a $[2, 2]$ system where the CD8+ T cell epitopes are illustrated as ‘ax’ (dark blue), ‘ay’ (red), ‘bx’ (green), ‘by’ (light blue). Each coloured line tracks the prevalence of a different variant through time. The epitope combinations quickly settle into discordant sets ($\{ax; by\}$ and $\{ay; bx\}$) and one set ($\{ay; bx\}$) competitively excludes the other. This form of variant structuring is maintained at the transition to AIDS at Time $t = 4000$.*

The type of antigenic evolution that occurs among the epitopes in this model is determined by whether it is targeted by NAbs or CD8+ T cells. The CD8+ T cell epitopes rapidly assume a form of structuring similar to Discrete Strain Structure (DSS) described in the previous chapter. Thus, arbitrary (in the sense that they are not physically determined) associations will arise and be maintained between epitope variants, such that the dominant types will share no variants, as shown in Figure 4-5. This situation is not altered by the transition to AIDS: the dominant discordant set increases in prevalence and still competitively excludes all the other such sets.

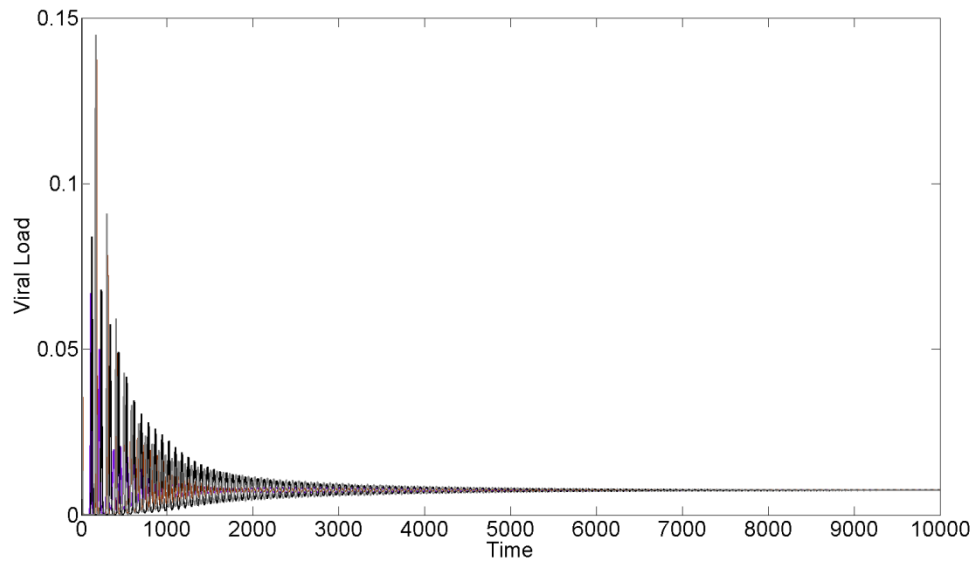


Figure 4-6: Long-term evolution at the NAb epitope, in the absence of immune decay. *This is shown for a model with 4 NAb variants; each coloured line tracks the prevalence of a different variant through time. There are initial oscillations in the prevalence of each variant, but this is only transient. Eventually, the oscillations break down and an absence of strain structure emerges where each variant co-exists at equal prevalence.*

Variants at the NAb epitope on Env initially exhibit oscillations in prevalence but, since there is no direct competition among them, this is only a transient artefact of the initial conditions. Figure 4-6 shows that this structuring inevitably eventually breaks down and reaches a state where each variant co-exists at equal prevalence – equivalent to the state of NSS (no strain structure) described in the previous chapter. If the transition to AIDS occurs after this state has been reached, then this absence of strain structure is preserved in the new regime. However, the pattern of variant structuring at the NAb epitope does change if the system has not yet fully settled at the transition to AIDS, as seen in Figure 4-7. In this case, the single variant that has the lowest Nab response directed against it at the point of transition will outcompete all others by virtue of strong sustained cytotoxic responses directed against their shared CD8+ T cell epitopes.

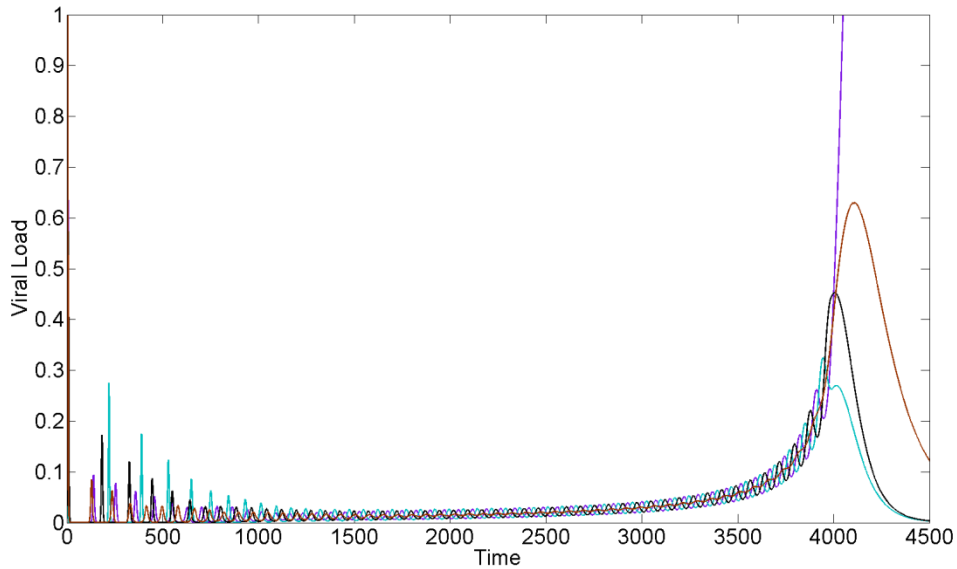


Figure 4-7: Structuring of NAb epitope variants on transition to AIDS. *This is shown for a model with 4 NAb variants; each coloured line tracks the prevalence of a different variant through time. At the transition to AIDS (Time $t = 4000$), the variant (purple) against which the NAb response is weakest increases in prevalence, in a background of the dominant set of APL epitopes (say D). The cross-protective immune responses against these epitopes prevent the growth of the other NAb-D combinations.*

In reality, there may be a limit to the degree of expansion of CD8+ T cell responses that has, for simplicity, been ignored here. Nevertheless, this will not have a critical impact on the model. If these responses became saturated during the ‘normal’ course of chronic infection in the model, then since the NAb response is still functioning, the net result would still be control of viraemia, albeit at a higher level. This would then lead to more rapid development of AIDS. Alternatively, they could become saturated in the final stages of disease, explaining why there does not appear to be a difference in the magnitude of CD8+ T cell response when comparing chronically infected patients with those that have developed AIDS (Draenert *et al.*, 2004b). In this case, viral load would remain uncontrolled as opposed to being eventually curtailed at some exceedingly high value. Even if saturation of CD8+ T cell responses did not occur, this eventual curtailment is something that might not be observed *in vivo* before a patient’s death.

In summary, this simple model demonstrates that neutralising antibody and CD8+ T cell responses can combine to control HIV-1 as a chronic infection with a roughly constant viral setpoint. However, if the effect of continued viral replication is depletion of CD4+ count and a consequent weakening of the NAb response, then eventually a sudden non-linear transition will occur to a new equilibrium state with a much higher viral setpoint in which the viral population is only under CD8+ control. Further, if viral load is explicitly linked to the rate of weakening of CD4+ T cell quality, and hence NAb response, then I can recover the known link between viral setpoint and the time it takes for a patient to develop AIDS.

4.4.2: Linked CD8+ and NAb epitopes on Env

I next consider the effects of including CD8+ T cell epitopes on Env itself that are linked to Nab epitopes. I assume that each CD8+ epitope combination defines a unique NAb variant. In other words, again taking the [2, 2] system illustrated by: ‘ax’, ‘ay’, ‘bx’, ‘by’, the NAb response against ‘ax’ is different from the NAb response to ‘ay’ but the CD8+ T cell response against ‘ax’ cross-protects against the antigenic Env variants ‘ay’ and ‘bx’.

There are several biological justifications for making this assumption. Escape from NAb can occur as the result of just a few amino acid mutations, including those outside the antibody target site (Moore *et al.*, 2009), indicating that Nab epitopes are highly sensitive to alterations in the Env protein. It is thus reasonable to assume that mutations at CD8+ T cell targeted epitopes on Env will alter any associated conformational B cell epitopes on the virus surface (Sattentau, 2008). Mutations in the CD8+ epitopes could also disrupt binding: it has been shown that conformational masking of receptor binding sites disrupts binding of NAb to the virus (Kwong *et al.*, 2002). Alternatively, it could be that the targeted CD8+

T cell epitopes are the basis of some form of glycan shield for NAb epitopes and therefore act in a slightly different way to obscure or reveal NAb epitopes (Wei *et al.*, 2003).

This version of the model is actually less mathematically complicated since I can now identify each viral Env variant, v , by just a single epitope combination, j , and can write the analogous equations as:

$$\frac{dv_j}{dt} = (\rho - \kappa z_j - \gamma w_j)v_j \quad (4:6)$$

$$\frac{dz_j}{dt} = \varphi v_j - \mu_z z_j \quad (4:7)$$

$$\frac{dw_j}{dt} = \eta \left(\sum_{k \sim j} v_k \right) - \mu_w w_j \quad (4:8)$$

$$\frac{d\phi}{dt} = -\alpha \sum_j v_j \quad (4:9)$$

The model is again seeded with a small initial prevalence of each individual strain, no pre-existing immune responses, and $\varphi(0) > 0$. Unless otherwise stated, the values assigned to each parameter are: $\rho = 7.5$; $\kappa = \gamma = 1$; $\eta = \varphi(0) = 1$; $\mu_z = 1000^{-1}$ (such that the NAb response decays over 1000 time units); $\mu_w = 1^{-1}$ (such that the CD8+ T cell response decays over 1 time unit); and $\alpha = 1250^{-1}$ and the following simulations are presented for a [4, 4] structure at the CD8+ T cell epitopes. In considering that different Env variants elicit both short- and long- lasting immune responses, this model is similar to one used to describe antigenic variation in malaria (Recker *et al.*, 2004).

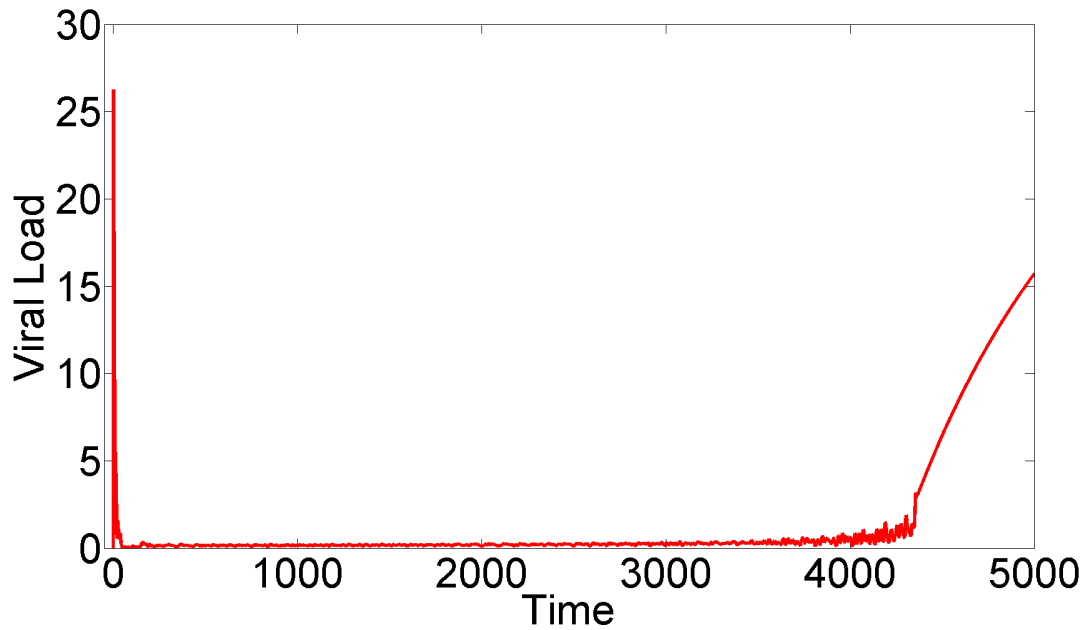


Figure 4-8 Viral load with time. *Viral Load (red line) initially rises but is then brought under control by the immune system. Chronic infection then persists until the strength of induction of the NAb Response reaches a critical threshold, at which point control of the virus is no longer possible and the host develops AIDS.*

As shown in Figure 4-8, linking the CD8+ T cell epitopes with the Nab epitopes has no effect on the fundamental property of the model that immune responses against them can combine to control HIV-1 as a persistent infection but that the weakening of the NAb response with time results in progression to AIDS.

Importantly, however, it does result in a very different form of antigenic evolution, with the model now exhibiting true cyclical or chaotic dynamics (CSS) as shown in Figure 4-9, resulting in the sequential emergence of antigenic variants during chronic infection. In this regard, it is a better model for the HIV system, as it inherently captures the observed sequential appearance of Env variants in acute and chronic infection (Wei *et al.*, 2003; Bunnik *et al.*, 2008; Moore *et al.*, 2009; Alter and Moody, 2010), and not as an artefact of the initial conditions as in the Basic Model.

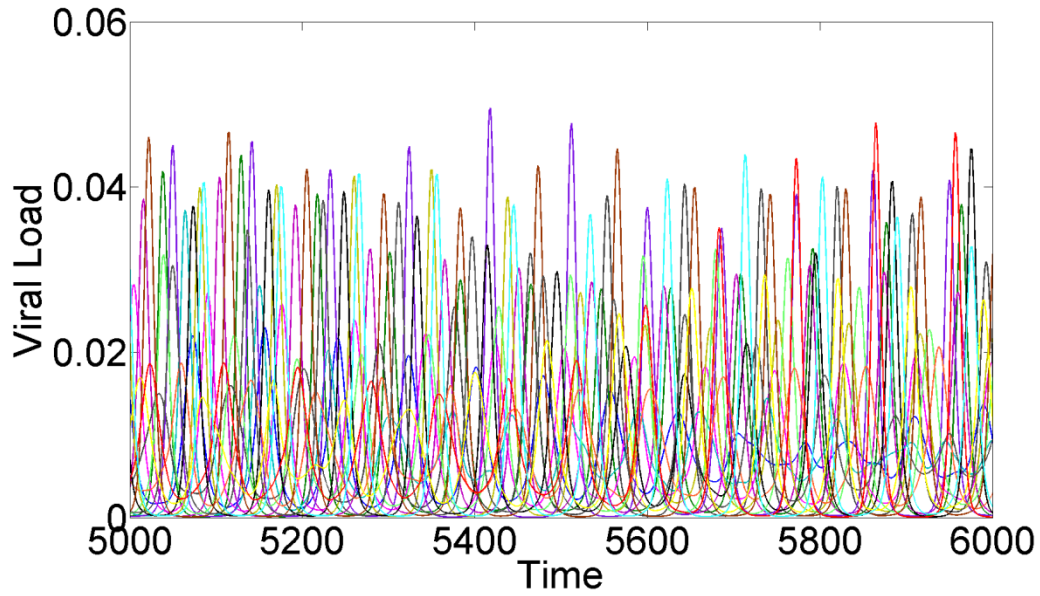


Figure 4-9: Long-term evolution of Env in the absence of immune decay. *Each coloured line tracks the prevalence of a different variant through time. This pattern continues indefinitely in the absence of immune decay.*

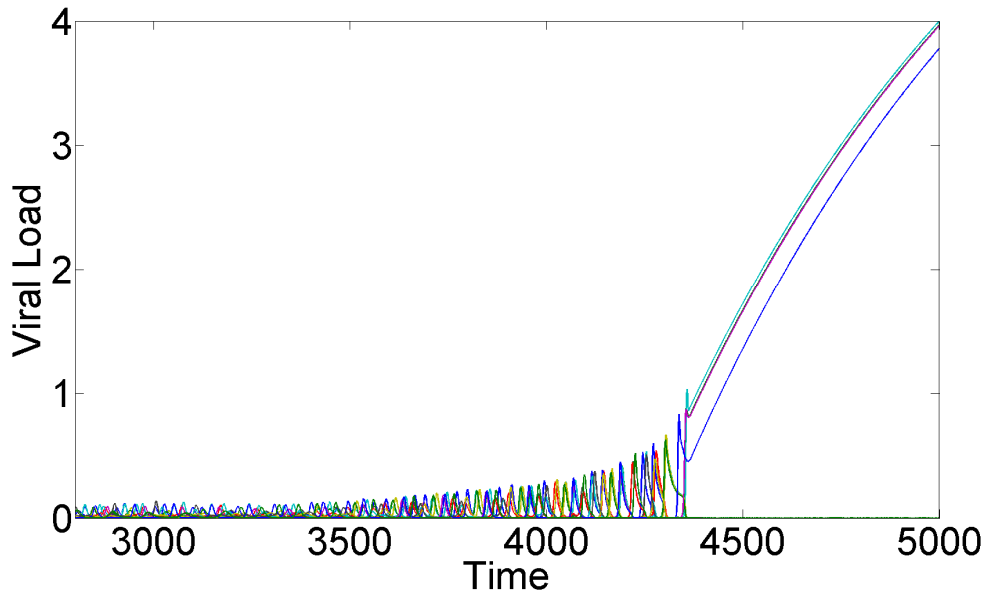


Figure 4-10: Qualitative evolution of Env changes at the transition to AIDS. *Each coloured line tracks the prevalence of a different Env variant through time, here shown for late chronic infection and at the transition to AIDS. The CSS dynamic of chronic infection is replaced by DSS in AIDS, with a reduction in single strain dominance.*

As shown in Figure 4-10, there is a major qualitative shift in Env diversity when the transition to AIDS occurs, with an arrest in the rate of antigenic turnover, and just a handful of strains (characterised by non-overlapping combinations of CD8+ epitope variants) growing to high levels. This is supported by data indicating a slowdown in the turnover of hypervariable regions of Env when CD4⁺ counts are sustained at low levels (Riddle *et al.*, 2006), and evidence of an association between progression and a slow-down in Env adaptation in longitudinal data sets (Williamson *et al.*, 2005).

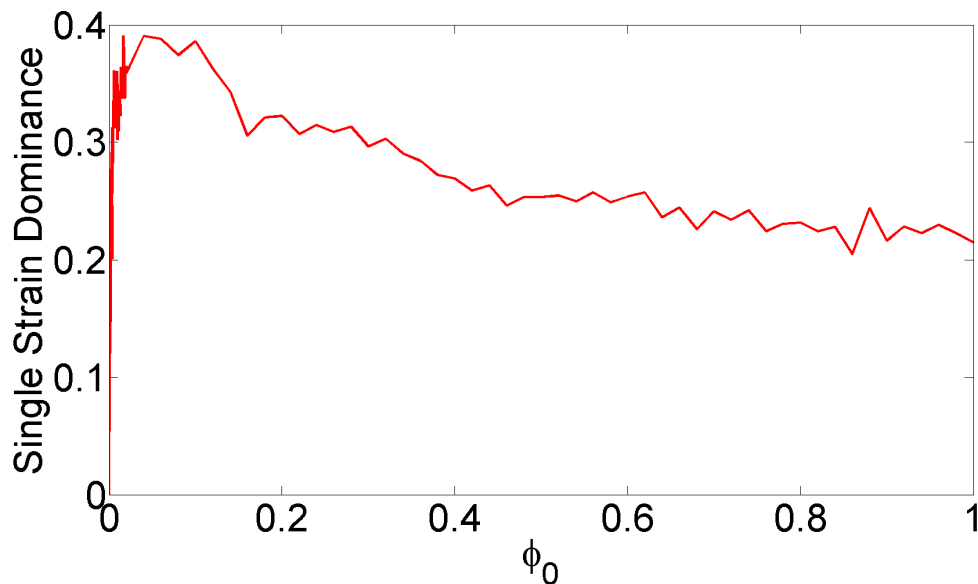


Figure 4-11: Effect of ϕ_0 on dominance by single Env variants in the absence of immune decay. Dominance by Env variants is measured as in Chapter 2.3.2. The closer the value of this metric is to 1 then the more a single Env variant dominates the viral population. Single strain dominance generally increases with decreasing ϕ but collapses with small ϕ_0 and the dynamical transition to DSS during the development of AIDS.

I next consider how the strength of antibody induction (ϕ) affects the extent to which the viral population is dominated by a single Env variant, and how this may change during the course of infection. This is done by adapting the methods of (Recker *et al.*, 2007) as in Chapter 2.3.2 to obtain a value of Single Strain Dominance for different values of ϕ (by

fixing $\frac{d\varphi}{dt} = 0$). The results indicate that the degree of single strain dominance has a non-linear relationship with strength of antibody induction (φ), as shown in Figure 4-11. The general trend is for decreasing φ to result in increasing single strain dominance, but this is reversed for small φ . This means that the virus population is more likely to be dominated by single variants as chronic infection progresses. The inversion of this pattern for small φ corresponds to the development of AIDS, which leads to the co-dominance of variants that do not trigger overlapping CD8+ T cell responses.

I can also look, in the absence of CD4+ T cell decay (i.e. fix $\frac{d\varphi}{dt} = 0$), at how other parameters affect viral load in the model and determine whether variations in other parameters could also lead to the non-linear dynamics observed for declining φ (Figures 4-12, 4-13 and 4-14). This analysis reveals four important features of the model. First, that decline in any of the parameters governing the antibody response leads to a non-linear relationship with viral load in time: in other words, that a reduction in the strength of antibody killing of virus (κ) or antibody lifespan (μ_z) would have had the same effect a reduction in the strength of induction of the antibody response (φ). Second, that changing the strength of either CD8+ killing (γ) or induction (η) has only a limited effect on viral load – variation of these parameters would not lead to the development of AIDS. Third, that relaxing the antigen-addicted nature of the CD8+ response (decreasing μ_z) has a comparatively much bigger effect on reducing viral load (although not to the same extent as any of the antibody-associated parameters). Fourth, and unsurprisingly, increasing the basic viral replication rate (ϱ) has a strong positive correlation with viral load.

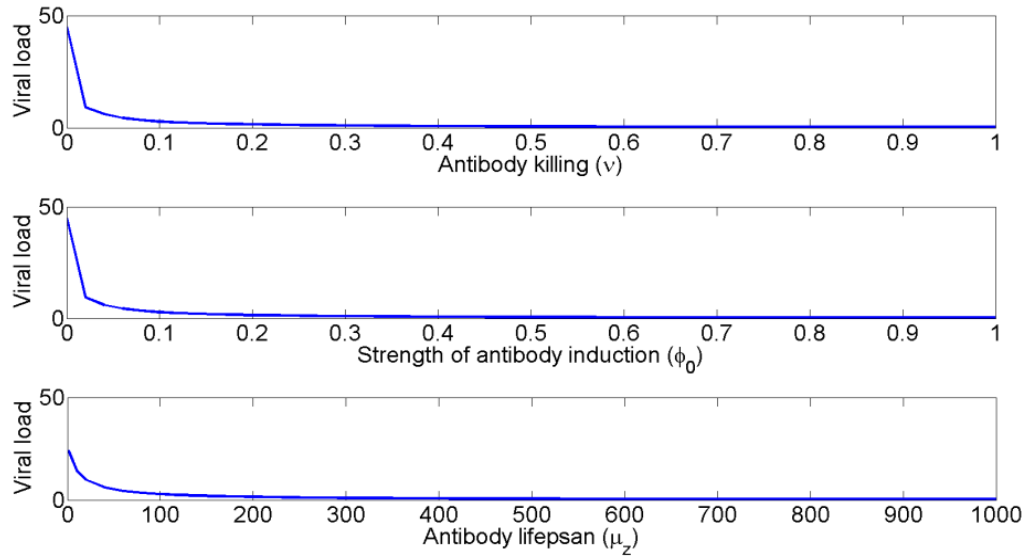


Figure 4-12: Effect on viral load of altering NAb responses in the absence of immune decay. *Decreasing any of the parameters associated with the neutralising antibody response has a non-linear effect on viral load. As these values become very small, virus load increases exponentially.*

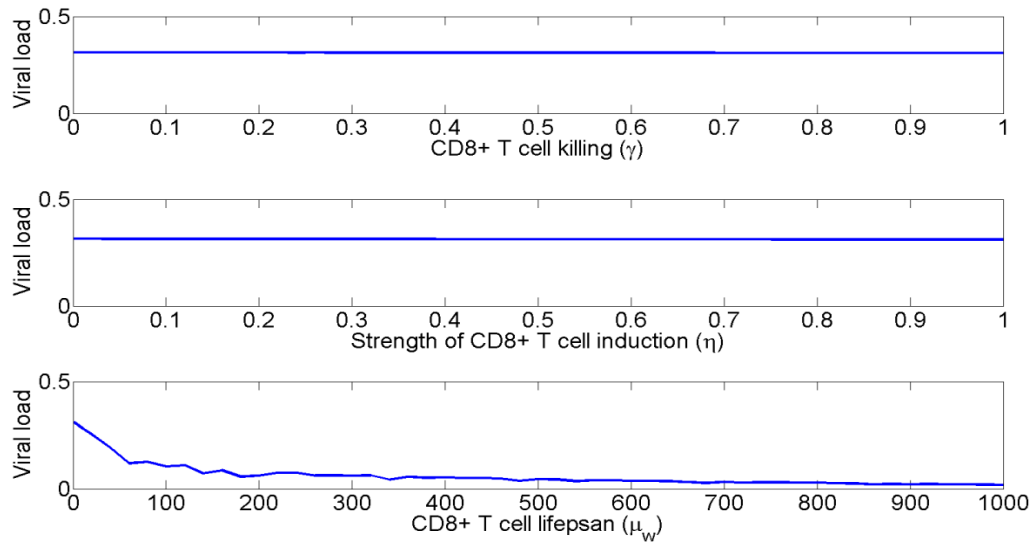


Figure 4-13: Effect on viral load of altering CD8+ T cell responses in the absence of immune decay. *Changing CD8+ T cell response killing or induction has only a small, linear effect on viral load. As these values get smaller, viral load increases slightly. Increasing lifespan from the usually modelled 1 time unit has a larger, non-linear effect and depresses viral load. Note the difference in scale between this and Figure 4-12.*

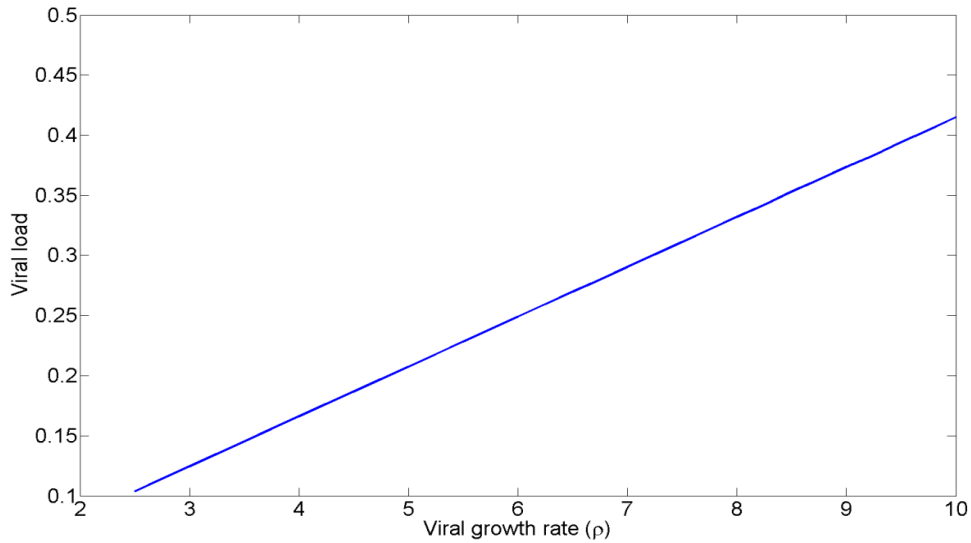


Figure 4-14: Effect on viral load of changing viral growth rate in the absence of immune decay. *Viral growth rate and viral load are positively correlated.*

Figures 4-15, 4-16 and 4-17 also highlight the relative importance of the antibody response is also apparent. In these figures, I consider the effects of parameter variation on the time to progression in models where the CD4+ count does decay. Here it becomes clear that weakening any aspect of the antibody response has a strong accelerative effect on the time it takes to develop AIDS in the model, as does increasing viral replication. Relaxing the antigen-addicted nature of the CD8+ response also significantly decreases the rate of progression. Viral growth rate also has a strong influence on the time it takes to progress. However, although enhancing CD8+ induction or killing of the virus lengthens the time it takes for the disease to progress, these effects are much smaller than observed for their antibody counterparts.

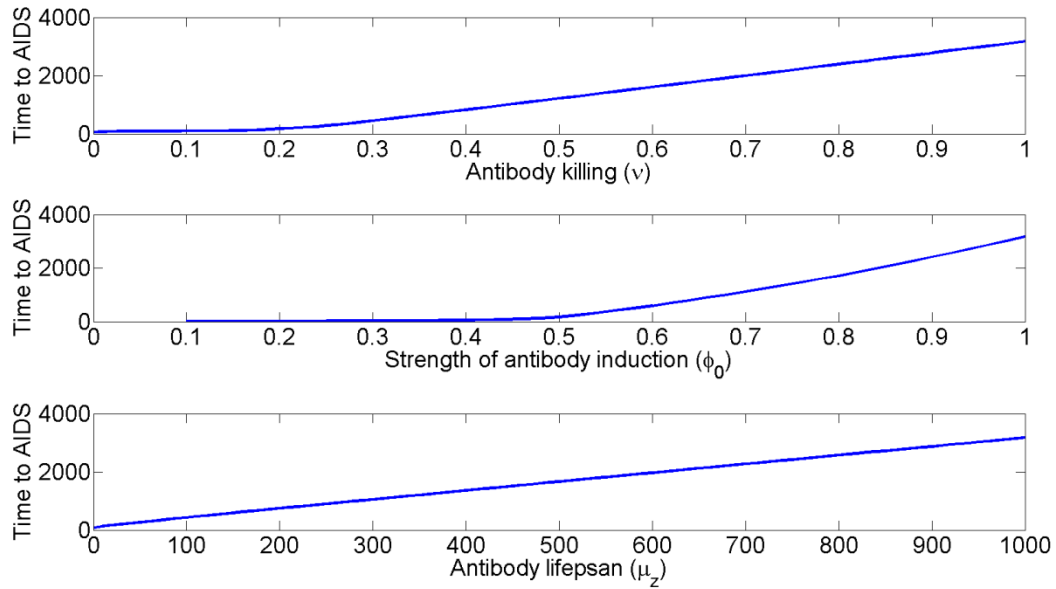


Figure 4-15: Relationship between antibody response and time to develop AIDS. *Weakening any aspect of the antibody response leads to much faster progression to AIDS. Notably however, the relationship is only non-linear for antibody killing and induction.*

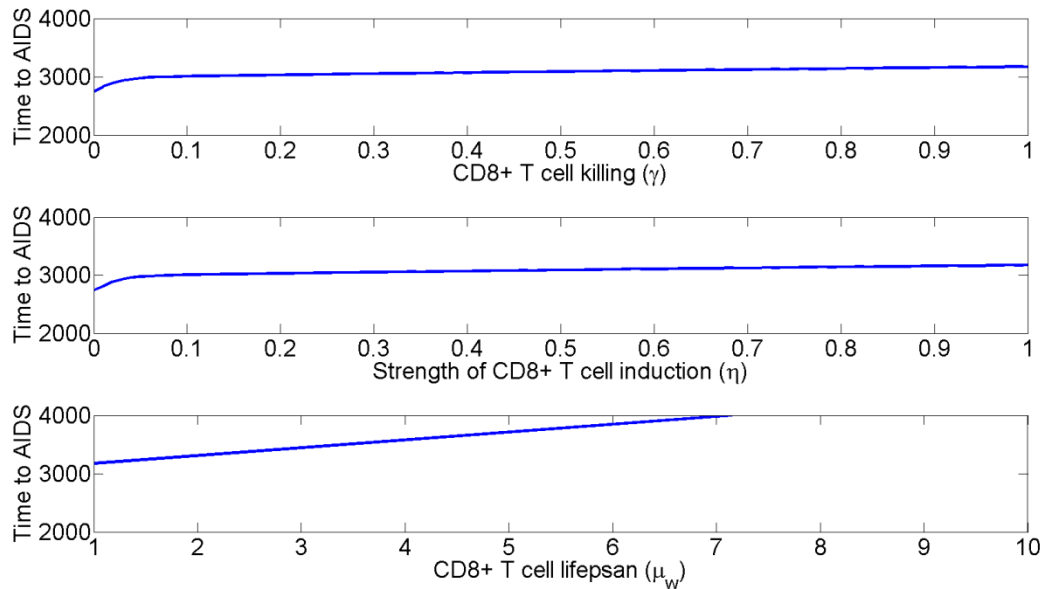


Figure 4-16: Relationship between CD8+ T cell response and time to develop AIDS. *Weakening any aspect of the CD8+ T cell response leads to faster progression to AIDS. This trend is very strong with respect to the longevity of the CD8+ T cell response in the absence of antigen, but extremely weak with respect to CD8+ T cell killing and induction.*

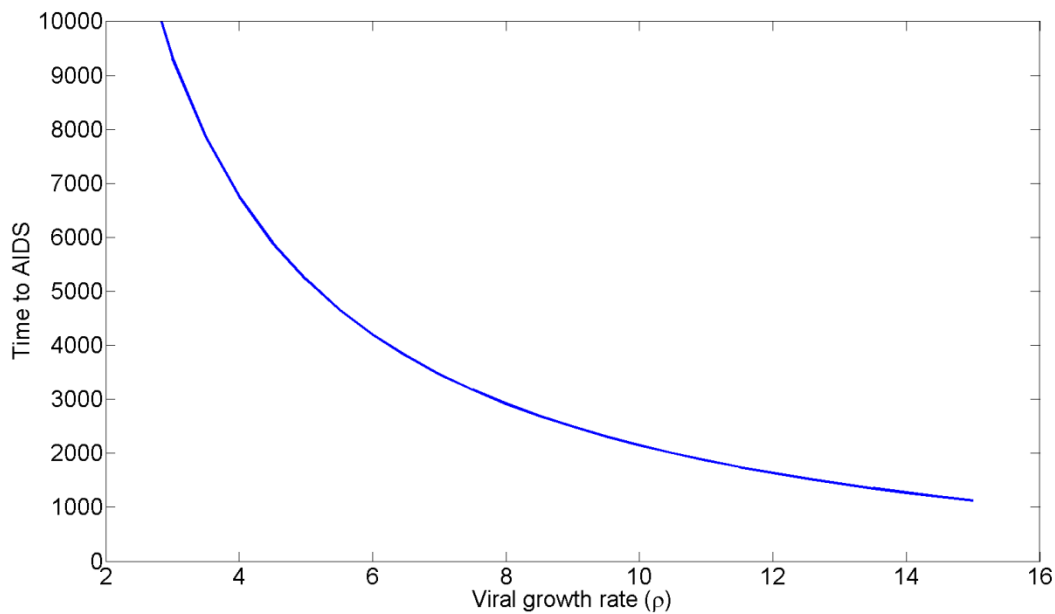


Figure 4-17: Relationship between viral growth rate and time to develop AIDS. *There is a non-linear, negative correlation between viral growth rate and time to AIDS in the model.*

From these analyses, I conclude that the key property of this model that governs progression to AIDS is the weakening of the antibody response. Other factors, such as killing by CD8+ T cells, play a role in keeping viral replication in check and increasing the time it takes to progress, but are not the critical factors that govern the observed non-linear transition between chronic infection and AIDS. Therefore, including an effect of CD4+ T cell loss on CD8+ T cell function or induction will act to accelerate progression but will not alter the fundamental three-phase pattern of viraemia observed here.

There is also a strong association between antigenic architecture of the system and viral load and (hence) disease progression. Figure 4-18 shows the effects of increasing the possibilities for variation at two CD8+ T cell epitopes, from 2 to 7 ‘alleles’ at each of these ‘loci’. As shown, greater flexibility at either epitope corresponds to the patient progressing

more rapidly to AIDS. The observed link between HLA Class I type and disease progression (Goulder and Watkins, 2008) could therefore also be rationalised in this model by considering HLA Class I type as directly related to variability of targeted epitopes in this model. The vast majority of sites in Env are under strong purifying selection, with few sites displaying a consistently high degree of turnover (Woo *et al.*, 2010), although there is a high rate of insertion and deletion in the variable regions. Further, studies of CD8+ T cell responses to Env often reveal a response that targets only a few epitopes *e.g.* (Goonetilleke *et al.*, 2009). Consequently, if the CTL epitopes associated with different HLA types have varying degrees of flexibility then Figure 4-18 indicates that those HLA types that target CD8+ epitopes on Env that can least tolerate variability will have the best prognosis.

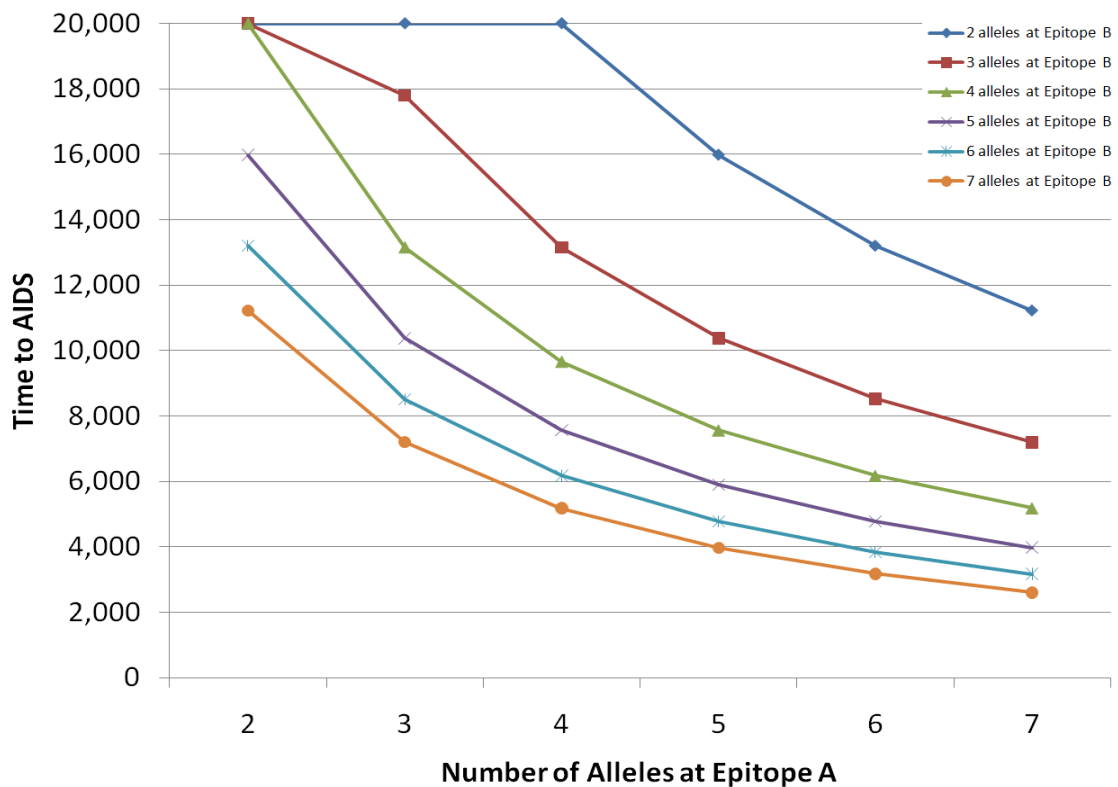


Figure 4-18: Effect of Varying Epitope Structure on Time to Develop AIDS. For each combination in an $[A, B]$ system where $2 \leq A, B \leq 7$, this plot shows the time it takes for an infected host to develop AIDS. Each line represents a fixed number of variants at epitope B, each point on the line represents a different number of variants at epitope A. The general effect is decreasing time to AIDS with increasing levels of possible variation.

4.4.3: Combined model

I now consider a hybrid of the basic model and the linked model, representing the reality that each viral variant will contain both CD8+ T cell epitopes (generally on Env) that determine the specificity of NAb epitopes and CD8+ T cell epitopes that are unlinked to Nab epitopes (such as on Gag or Pol). Using w to denote the CD8+ responses to epitopes on Env, ψ to denote the CD8+ responses to epitopes on other proteins and $v_{j,k}$ to denote the strain with Env epitope combination j and, say, Gag epitope combination k , I can write:

$$\frac{dv_{j,k}}{dt} = (\rho - \kappa z_j - \gamma w_j - \gamma \psi_k) v_{j,k} \quad (4:10)$$

$$\frac{dz_j}{dt} = \varphi v_j - \mu_z z_j \quad (4:11)$$

$$\frac{dw_j}{dt} = \eta \left(\sum_{m \sim j} \sum_k v_{m,k} \right) - \mu_w w_j \quad (4:12)$$

$$\frac{d\psi_k}{dt} = \eta \left(\sum_{n \sim k} \sum_j v_{j,n} \right) - \mu_w \psi_k \quad (4:13)$$

$$\frac{d\varphi}{dt} = -\alpha \sum_j \sum_k v_{j,k} \quad (4:14)$$

For simplicity, I assume that, aside from targeting different sets of epitopes, the two sets of CD8+ responses are otherwise identical. Below I present the results of a system with a [3,

3] structure at the Env epitopes and another, separate [3, 3] structure for the CD8+ targeted epitopes on other proteins. Unless otherwise stated, the values assigned to each parameter are: $\varrho = 7.5$; $\kappa = \gamma = 1$; $\eta = \varphi(0) = 1$; $\mu_z = 1000^{-1}$ (such that the NAb response decays over 1000 time units); $\mu_w = 1^{-1}$ (such that the CD8+ response decays over 1 time unit); and $\alpha = 1250^{-1}$.

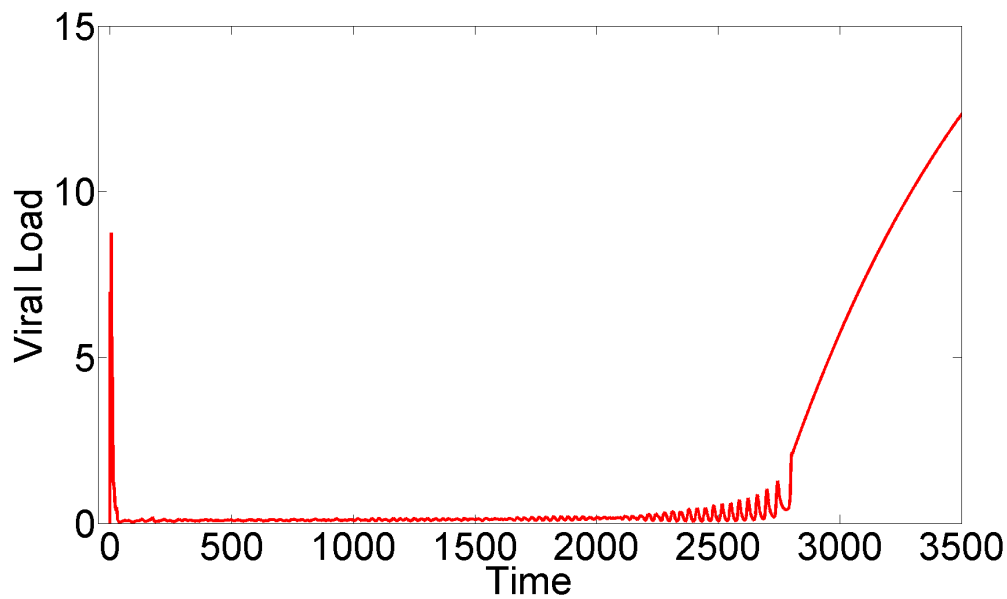


Figure 4-19: Viral load with time. *Viral Load (red line) initially rises but is then brought under control by the immune system. Chronic infection then persists until the strength of induction of the Neutralising Antibody Response reaches a critical threshold, at which point control of the virus is no longer possible and the host develops AIDS.*

As illustrated in Figure 4-19, there is little qualitative difference in the pattern of viraemia with the inclusion of additional CD8+ epitopes. There is also no difference to the pattern of evolution of linked Nab and CD8+ T cell epitopes on Env. The unlinked CD8+ epitopes exhibit a different pattern of evolution as shown in Figure 4-20. In chronic infection, each variant rises and falls in line with overall viral load but, although there is a hierarchy of variants at each time point, no single variant dominates. The hierarchy of variants changes with time, but becomes fixed after the transition to AIDS.

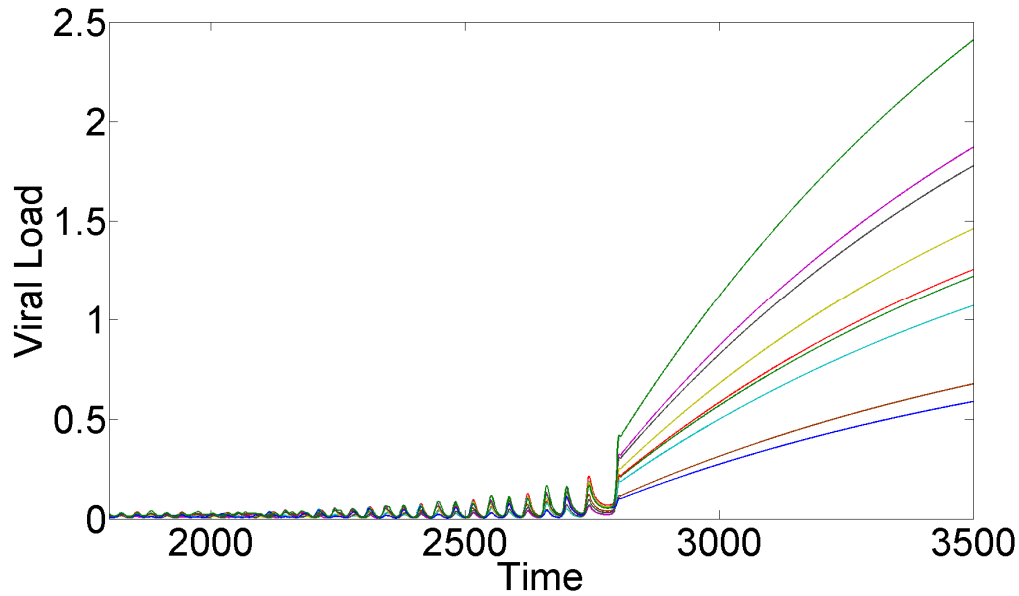


Figure 4-20: Qualitative evolution of CD8+ epitopes on other proteins changes at the transition to AIDS. *Each coloured line tracks the prevalence of a different variant, located on proteins other than Env, through time, here shown for late chronic infection and at the transition to AIDS.*

4.4.4: Escape from CD8+ responses

It is clear that including additional CD8+ responses to the linked CD8+/NAb model does not alter the fundamental non-linear model dynamic, but none of these have so far been allowed to sustain variation that would lead to full escape from the T cell response. I now consider the impact of viral escape by adding a single unlinked CD8+ epitope which can mutate to be no longer recognised by the host. In line with previous assumptions, I assume that this escape mutation is generated at a high rate during viral replication, but carries some cost to viral fitness. In the following equations, I designate strains without the escape mutation as v_i , as before, and strains with the same epitope combination on Env but carrying the escape mutation by e ; I incorporate an additional CD8+ T cell response, g , targeting the ‘escape’ epitope with a killing rate β . The system can now be described as:

$$\frac{dv_j}{dt} = (\rho - \kappa z_j - \gamma w_j - \beta g)v_j \quad (4:15)$$

$$\frac{de_j}{dt} = \rho_e e_j - \kappa z_j - \gamma w_j \quad (4:16)$$

$$\frac{dz_j}{dt} = \varphi(v_j + e_j) - \mu_z z_j \quad (4:17)$$

$$\frac{dw_j}{dt} = \eta \left(\sum_{k \sim j} (v_k + e_k) \right) - \mu_w w_j \quad (4:18)$$

$$\frac{dg}{dt} = \eta \left(\sum_j v_j \right) - \mu_w g \quad (4:19)$$

$$\frac{d\varphi}{dt} = -\alpha \sum_j (v_j + e_j) \quad (4:20)$$

Note that I assume that the induction, killing strength and life-span of the two sets of CD8+ immune response are equal and that $\varrho > \varrho_e$ to reflect the loss of fitness in the escape mutant. In the following simulations, unless otherwise stated, the values assigned to each parameter are: $\varrho = 20$; $\varrho_e = 19$; $\kappa = \gamma = 1$; $\beta = 3$; $\eta = \varphi(0) = 1$; $\mu_z = 1000^{-1}$ (such that the NAb response decays over 1000 time units); $\mu_w = 1^{-1}$ (such that the CD8+ response decays over 1 time unit); and $\alpha = 4000^{-1}$.

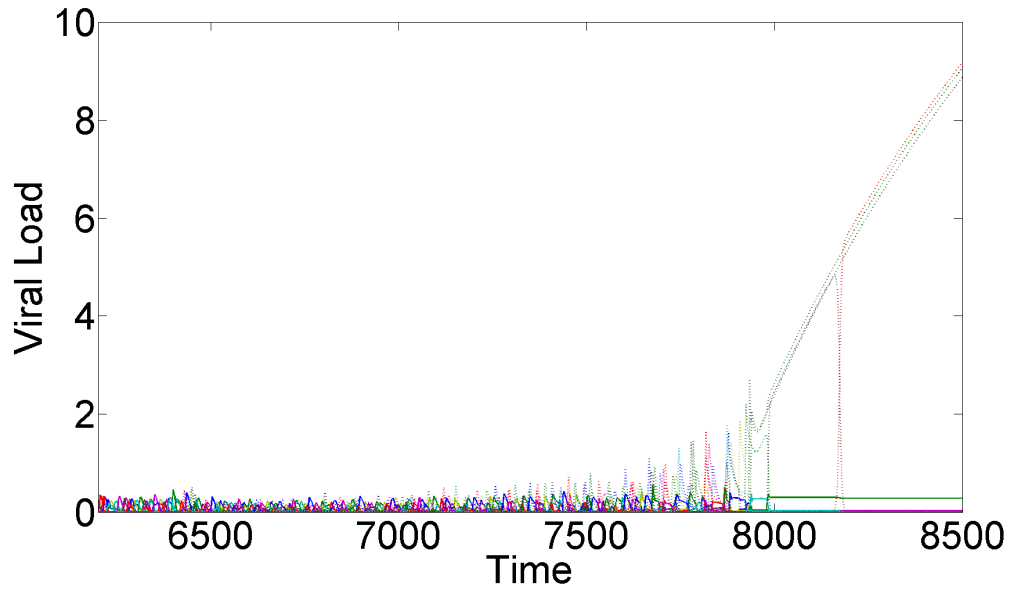


Figure 4-21: Including possible escape mutations. *Each coloured line represents a different Env variant; solid lines represent the wild-type, dashed lines represent those variants with the escape mutation. Even though the escape mutation is constantly generated within the model, it does not instantly take hold but eventually emerges and takes over.*

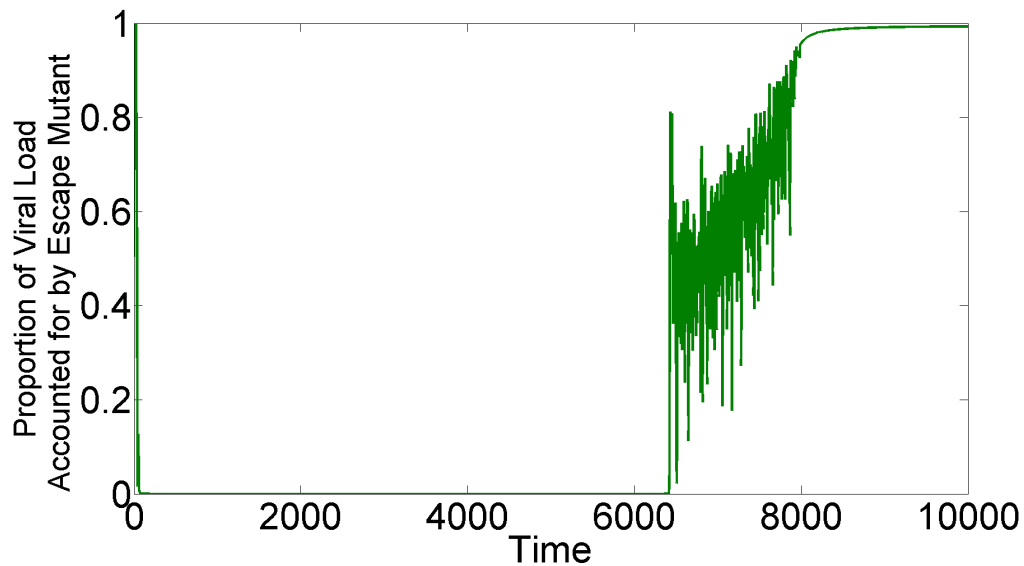


Figure 4-22: Relative prevalence of escape mutants with time. *Initially, the escape mutant is outcompeted by the wild-type, but eventually the increasing strength of the immune response against the wild-type allows the escape mutant to emerge and dominate.*

Model simulations indicate that basic properties of progression in the model are unaltered by the inclusion of epitopes at which escape may occur. The virus is initially controlled by antibody and T cell responses as a chronic infection, but the later loss of the antibody response results in the development of AIDS. However, depending on the relative fitness of the escape mutant and strength of the associated immune response, escape may or may not happen. Figure 4-21 presents part of a time-series for a [4, 3] system, with a single additional CD8+ T cell epitope from which escape is possible. Variants with this escape mutation are shown with a dashed line. In this example, escape does not occur instantly, despite the fact that relevant mutations are being constantly generated. Rather, there is a gradual emergence of the escape variant before it eventually outcompetes the wild type as explicitly shown in Figure 4-22. This is because, initially, viral load is relatively low and so too is the strength of the CD8+ response against the wild type epitope. Consequently, the slightly deleterious escape mutant does not have sufficient advantage to propagate. However, the slight rise in viraemia associated with the weakening of the NAb response leads to an eventual and gradual shift in the balance of competition between wild-type virus and the escape mutant. The latter becomes ever more viable and as viraemia rises, we see it become the dominant type. At the point of escape there is also a small blip in viraemia, as the initially escaped mutant is brought back under control by enhanced levels of the other responses. My results therefore indicate that escape from CD8+ T cell responses is neither necessary nor sufficient for transition to AIDS in this model. Figure 4-23 shows that it does however lead to a faster progression to AIDS as often observed in the literature (Koup *et al.*, 1991; Klein *et al.*, 1995; Goulder *et al.*, 1997; Miura *et al.*, 2009) since, even though the virus is less intrinsically fit, escape leads to an increase in viral load (Ammananond *et al.*, 2011) or set-point.

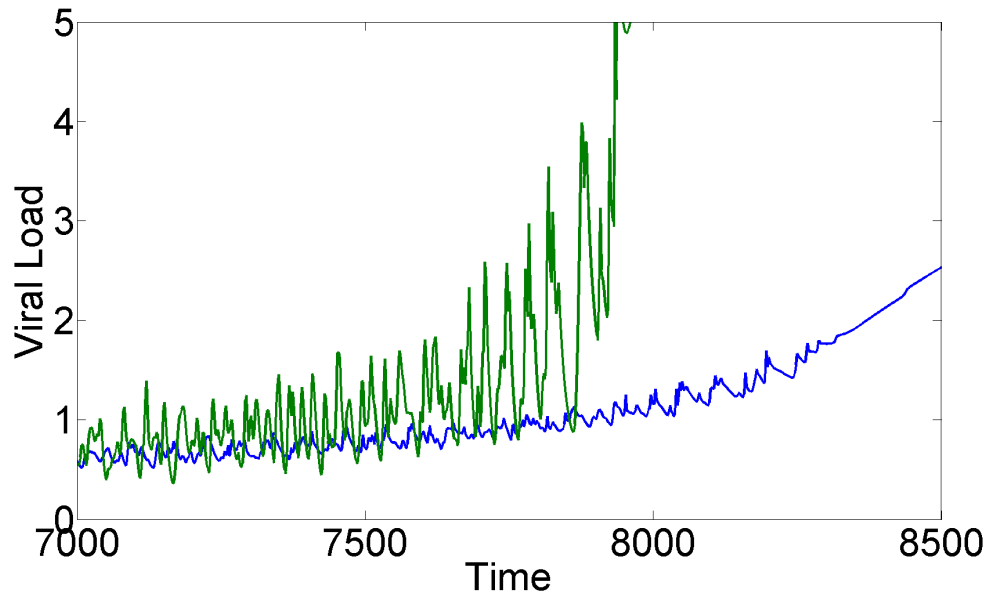


Figure 4-23: Effect of escape on viral load. *In this figure, the green line shows the viral load with time for a virus that can escape from CD8+ T cell responses to, say, Gag. The blue line, in contrast, shows viral load where such escape is not possible. AIDS occurs more quickly if escape is possible.*

4.5: Discussion

Despite significant advances in characterising the immune response against HIV-1 and the evolution of the virus within the host, its pattern of disease progression remains poorly understood. It is clear that CD8+ T cell responses play an important role in the control of chronic infection, but these are sustained among individuals who develop AIDS; furthermore escape from these responses appears to only make a small contribution to the time to progression. My studies implicate the (CD4+ T cell help dependent) antibody response in being crucial to the control of HIV-1 despite a lack of correlation between viral levels and CD4+ count. I also show that progression towards disease may facilitate rather than result from CD8+ T cell escape; pathogenesis is accelerated by CD8+ T cell escape but is fundamentally caused by the weakening of the antibody response.

In this work, I visualise each HIV-1 virion as containing two classes of epitope. First, there are CD8+ T cell targeted epitopes of limited variability that elicit short-lived cytotoxic responses and could be located in any one of the viral proteins. Second, highly variable epitopes specifically in the Env glycoprotein that elicit much longer-lived neutralising antibody (NAb) responses. I assume that the NAb response in my model relies on CD4+ help for induction, but that CD8+ responses can be mobilised in the absence of CD4+ help. Finally, I assume that CD4+ cell counts decline at a rate proportional to viral load.

This simple interaction between viral load, CD4+ count and NAb generation is able to account for a wide range of features of HIV-1 pathogenesis and the genetics of host susceptibility to disease progression. Under these few assumptions, I can reproduce the general pattern of viraemia observed during the natural course of HIV infection. Initially very high levels of viral replication lead to a sharp drop in CD4+ count (for which I consider the parameter φ as a proxy), but viral load is soon brought under control by a combination of CD8+ and NAb responses and hovers around a set-point that is determined by the strength of both these responses as well as the range of epitopes recognised. Because infection is not eliminated, I observe a continual decline in CD4+ count but very little change in the viral set-point; thus, even though the generation of neutralising antibodies is critical to the control of the virus, the setpoint remains insensitive to the decline in CD4+ count. However, once the CD4+ T cell count drops below a critical threshold, a stark dynamical transition occurs to a state where the virus is controlled entirely by CD8+ responses at a significantly higher set-point, mimicking the development of AIDS. A mechanistic link between viral load and decline in CD4+ is not strictly essential here (*i.e.* this 3-phase pattern may be generated simply by assuming there is a decline in

CD4+ count), but this additional assumption provides the link between viral set-point and time to AIDS that has been widely observed in HIV-1 infection.

My results can explain why depletion of CD8+ T cells in monkeys (Jin *et al.*, 1999; Schmitz *et al.*, 1999; Mueller *et al.*, 2009; Okoye *et al.*, 2009) and of B cells in humans (Huang *et al.*, 2010) both lead to an increased viral load that is brought under control again upon termination of the respective depletion treatment, and why the maintenance of a strong antibody response has previously been implicated in avoiding progression to AIDS (Ljunggren *et al.*, 1987; Karpas *et al.*, 1988; Ljunggren *et al.*, 1990; Cao *et al.*, 1995; Scarlatti *et al.*, 1996; Pilgrim *et al.*, 1997; Cecilia *et al.*, 1999). In the model, the T cell and NAb arms of the immune response are both important for control and maintenance of setpoint and initially work together to control the virus. Sudden changes in either would therefore lead to increases in viraemia akin to those seen in the above depletion experiments. Nevertheless, in my model it is the failure of the antibody response that ultimately leads to the fatal transition to AIDS. Contrastingly, if the virus were able to permanently escape the CD8+ T cell response, then a transition to AIDS would not automatically occur: viral load would increase but would be curtailed by antibody. Nevertheless, the resulting increase in viral load would substantially hasten progression. The model therefore highlights how an antibody response can help maintain control of the virus despite a declining CD4 count: only when the count is very low is antibody no longer capable of being stimulated to the titres necessary for the control of emerging variants. Further, although T cell responses do not have the same critical impact, they nonetheless act to restrict viral growth and setpoint and hence lengthen the time it takes to progress to AIDS.

This behaviour is largely independent of the manner in which I choose to model the interaction between NAb and CD8+ epitopes. The 3-phase pattern of viraemia can be

recovered whether or not they are linked in any way. However, connections between NAb and CD8+ epitopes on Env promote the sequential emergence of Env variants during chronic infection. Within this framework, there is a major qualitative shift in Env diversity and evolution at the transition to AIDS: the sequential dominance of Env variants is replaced by a small subset of strains reaching high prevalence. Notably, these strains are all antigenically discordant with respect to the CD8+ T cell response since immune selection prevents the coexistence of strains sharing these epitopes. Thus, an explicit connection between CD8+ and NAb epitopes on Env is not required for the observed pathogenesis but it can explain why the waxing and waning of Env variants within a general soup of viral diversity has been observed (Alter and Moody, 2010), before the pace of Env evolution slows in progressive disease.

Adding CD8+ epitopes on other proteins, such as Gag, which are unlikely to be linked to the NAb epitopes, does not alter either the pattern of sequential emergence of Env variants, or overall pattern of disease progression within the model. In particular, taking account of the possibility of escape from CD8+ responses to unlinked epitopes demonstrates how escape will not necessarily occur just because an appropriate escape mutant is generated – any such mutant needs to be generated in an environment that allows it to overcome the potentially deleterious nature of its escape.

The observed link between HLA Class I type and disease progression (Goulder and Watkins, 2008) can also be explained by this model. Different epitope structures, with varying degrees of flexibility could be thought of as corresponding to different HLA types. In the model, more flexible epitope structures lead to more rapid progression. Accordingly, those HLA types that correspond to, say, a good prognosis for control, could match to a

CD8+ T cell response that target epitopes on Env that are inherently less flexible than those targeted by HLA types corresponding to a poor prognosis.

The link between epitope variability and progression also possibly explains experimental results that show a positive correlation among chronically infected patients between their level of NAb responses to heterologous virus and their own viraemia (Deeks *et al.*, 2006). In this study and as expected, NAb responses against autologous virus were contrastingly negatively correlated with viraemia. It is tempting to speculate that a higher degree of epitope variability, which is associated with higher viraemia in our model, also manifests in increased recognition of heterologous sera by virtue of a broader antibody response. This is similar to the suggestion of a mathematical model for malaria that broad recognition of heterologous isolates is correlated with the presence of parasitaemia (Recker *et al.*, 2004).

Deeks and colleagues also note that in recently infected patients, titres against current autologous virus are often low compared to titres against virus sampled several months previously (Richman *et al.*, 2003; Wei *et al.*, 2003). They confirm that this is also true for patients who have recently discontinued a successful therapy regime. However, in chronically infected patients, the ability to respond to arbitrary laboratory strains and older versions of their own virus does not change with time. These observations match the behaviour of neutralising antibody responses in my model. As shown in Figure 4-24, once a NAb response has been induced, it remains detectable until AIDS develops, although its strength will oscillate slightly in response to the presence or absence of antigen. These dips in Nab levels are what allow the corresponding Env variants to periodically re-emerge throughout infection. In chronic infection, it is therefore always possible to detect a NAB response to any previously seen variant, even if at the particular point of sampling the targeted Env variant is not itself widely prevalent. Conversely, in acute infection it takes

time for a NAb response to emerge and so the ability to respond to autologous virus grows with time.

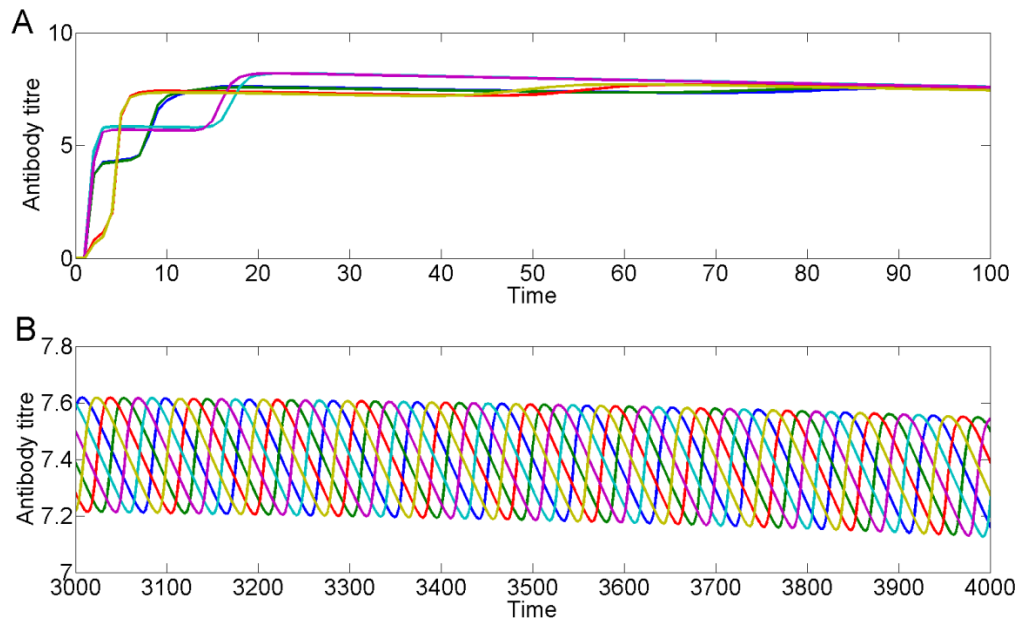


Figure 4-24: Antibody titre with time. *Figure shows antibody titre in (a) acute infection and (b) chronic infection in a model with 6 Env variants. It takes time for the NAb response to develop in acute infection, but once fully developed the NAb response can always be subsequently detected in chronic infection. Note how the achieved antibody titre declines gradually with time in chronic infection.*

Therefore, this model cannot, in its current form, accurately capture patterns of viral evolution in early infection, as all strains are introduced at the outset (to remove any assumptions concerning their rate of generation). This could be rectified by including some form of stochastic process for generating new variants in early infection, or relaxing the assumption that the mutational distance between variants is very small. In this way, until all the possibilities have been generated, autologous sera from a particular time point will likely largely consist of ‘new’ NAb variants that have only just emerged and to which the NAb response is growing. Eventually all the possibilities for Env innovation would be exhausted,

and the total set of possible Nab responses would be established within the host; these would oscillate in frequency as shown in Figure 4-24b, thereby promoting the sequential emergence of the different antigenic types. Deeks *et al.* interpret their observations as evidence of an absence of viral evolution in these epitopes, despite NAb responses. In truth, all they are failing to observe is a change in the NAb response; my model shows how their results are entirely compatible with rapid ongoing viral replication and evolution.

A sequential emergence of a finite number of Env variants could also tie in with an important study into how the diversity of Env changes as infection progresses (Shankarappa *et al.*, 1999). In a study of nine men with moderate or slow progression to disease, Shankarappa and colleagues showed that Env diversity initially increases with time, before eventually stabilising in chronic infection and then possibly decreasing once a patient has developed AIDS. The sequence data collected in this study has also informed later statistical analyses that try to estimate the rate of adaptation in Env as infection progresses (Ross and Rodrigo, 2002; Williamson, 2003)(Shankarappa *et al.*, 1999). These studies both suggest that the adaptation rate of Env is negatively correlated with the rate of progression to AIDS. It remains to be seen if the patterns of Env adaptation in my model also follow this trend, but preliminary work suggests that this might be the case.

Another study, this time looking at the specificity of the cellular immune response in chronic infection, also provides support for the behaviour of my model. This work, looking at these responses at a population level, found a positive relationship between the breadth of CD8+ responses to Gag and control of viraemia, but the opposite when considering the breadth of CD8+ responses to Env (Kiepiela *et al.*, 2007). This is exactly what the model suggests – CD8+ responses to proteins other than Env act to lower viraemia in the model,

whereas a broader response to epitopes on Env is synonymous with a more complicated epitope structure which, as outlined above, is associated with increased viraemia.

This model predicts that progressors will have a strong CD8+ T cell response that may increase in strength in late disease. This is in line with evidence that a strong, broadly directed and high-avidity gamma-interferon positive CD8+ T-cell response persists into late-stage disease and is driven by persistent exposure to antigen (and therefore despite low CD4 count and uncontrolled viraemia) (Draenert *et al.*, 2004b). However, these authors did not find a difference in the breadth of regions targeted by the CD8+ T cell response when comparing progressors with a cohort of chronically infected individuals. This also consistent with the model as it suggests that the transition to chronic infection does not also result in changes to the spectrum of epitopes targeted by the CD8+ T cell response. In essence, these T cells are as effective and as broadly targeted as they were in chronic infection but are now no longer able to control viraemia, in the absence of help from the NAb response.

This model also provides a rational explanation for observations of viral replication and evolution even in elite controllers (Bailey *et al.*, 2006; Bailey *et al.*, 2007; Bailey *et al.*, 2009; O'Connell *et al.*, 2010). It suggests that if viraemia can be sufficiently suppressed then the virus will still be able to replicate and evolve in response to selection pressure, but low level replication will not greatly impair the performance of the immune system. In particular, the CD4+ T cell count will not be adversely affected and the NAb response will remain strong. Progression to disease will therefore take an extremely long time.

HIV-2 is a pathogen that is closely related to HIV-1 but perplexingly causes AIDS in only a minority of infected individuals. In fact, viral load in HIV-2 infection tends to be lower

than in HIV-1 infection, with patients having generally superior CD4+ T cell responses (de Silva *et al.*, 2008). There also appears to be less rapid turnover of Env variants with time and greater sensitivity to NAb in an HIV-2 infection (Shi *et al.*, 2005; MacNeil *et al.*, 2007a). Interestingly though, there is little difference in observed immune dysfunction in those who go on to develop AIDS as a result of infection by either virus, and the rate of CD4+ T cell decline is the same for both viruses when matched for baseline viral load (Gottlieb *et al.*, 2002). Within the context of the model, I could speculate that the underlying cause of pathogenesis is identical within either infection – if the NAb response eventually fails, then the patient progresses to AIDS, and the causes of this failure are the same in each case, namely, high viral load leading to rapid CD4 decline. The source of this difference could be that HIV-2 may have a lower replication rate than HIV-1 *in vivo* (MacNeil *et al.*, 2007b) or, perhaps the range of possible mutation in Env is smaller for HIV-2 (Shi *et al.*, 2005), corresponding to a more restricted epitope structure in the model. The result of either would be a generally lower viral load in HIV-2 infection and so progression is generally much slower or, if viral load is low enough, simply not going to happen within the host's natural lifespan.

Such ideas could also be extended to explain the largely non-pathogenic nature of SIV infection. As a quick example, in a longitudinal study of sooty mangabeys over 5 years, Taaffe *et al.* showed that the CD4 count in infected animals did decline gradually over time (Taaffe *et al.*, 2010). However, not only was this not predicted by viral load, as in HIV-1, but similar declines were observed in uninfected controls and may just be a result of the aging process. Thus, perhaps SIV is non-pathogenic in these animals because, unlike HIV-1, it does not adversely affect CD4 T cell count and (as presumed in my model) the ability to generate NAb responses and these monkeys are therefore able to control SIV as a chronic infection.

An important feature of my model of HIV pathogenesis is that it manages, under a minimum of realistic assumptions, to capture both a steady viral set point and a transition to AIDS (represented by substantially elevated viraemia). Previous models of HIV pathogenesis have attributed the transition to AIDS to the accrual of viral antigenic diversity (Nowak *et al.*, 1991), immune escape (Korthals Altes *et al.*, 2006), increasing intrinsic viral growth rate (Stilianakis *et al.*, 1997; Stilianakis and Schenzle, 2006), accumulation of deleterious mutations in the progenitors of lymphocytes (Galvani, 2005), dendritic cell dysfunction (Hogue *et al.*, 2008), and the alteration of a homeostatic balance between CD4+ and CD8+ T cell populations (Fraser *et al.*, 2001). However, these models have, in general, struggled to capture the 3-phase pattern of infection and typically rely on sequential introduction of antigenic variants to draw out the duration of infection. I have assumed instead that, because the virus has such a high mutation rate, it effectively has continuous access to all possible antigenic variants. Adding restrictions to the mutational process would not, however, affect the underlying dynamic of a 3-phase infection, which emerges solely as a result of virus-mediated disruption of NAb, and not CD8+ T cell, responses. Instead, restricting the generation of each Env variant would probably act to spread out the emergence of these variants in chronic infection, reinforcing the dominance of a single variant at a time. Thus, it is the host immune response that sculpts the apparent pattern of viral evolution as opposed to viral diversity sprouting along a path of least resistance through the immune landscape.

Within my framework, qualitatively different immune responses to CD8+ T cell and NAb epitopes initially combine to ‘control’ the virus as a chronic infection. However, the ruinous effects of infection on one arm of this response leads to the ultimate loss of this control. Importantly therefore, the results of my modelling approach suggest that the T cell response may still be effective in late-stage disease and that the key to improving the

prognosis of an HIV infection is the preservation of the NAb response. Additional immune responses not explicitly modelled here might act to suppress viraemia but will not directly determine the course of disease, unless they act to preserve CD4 count and hence the NAb response. Pathogenesis may also be accelerated by CD8+ T cell escape, but it is fundamentally caused by the loss of CD4+ T cell help for the generation of neutralising antibodies.

Chapter 5

Conclusion

RNA viruses produce extremely large quantities of progeny during each round of viral replication. Moreover, they are also incapable of correcting the frequent mistakes that occur whilst copying the parent genome. The net result is the generation of an enormous amount of genetic diversity within a population of replicating RNA viruses. This genetic flexibility allows RNA viruses to overcome challenges posed by the immune system or drug therapy interventions, provided the targeted residues can tolerate escape-enabling change. In this thesis, I have explored the impact of immune selection on viral diversity both on the scale of an individual infection and within the population at large.

When speaking of viral diversity, there is an important distinction to be made between the processes of genetic and antigenic evolution. Antigenic determinants only ever represent a small subset of the whole viral genome; thus, whilst patterns of antigenic evolution will contribute to the aggregate picture of genetic evolution, they need not necessarily follow the same pattern. Nevertheless, it is important to try to understand the root cause of any difference between the two, and the precise impact and limitations in the extent of antigenic evolution and diversity. In this thesis, I have been primarily concerned with the antigenic diversity of three different viral pathogens – dengue, influenza and HIV.

The most widely known theory of antigenic evolution is that referred to as antigenic drift. This theory postulates that a given viral population currently occupies some niche within a large and limitless antigenic space, but mutation allows the viral population to traverse this

space, usually in some form of random walk. Herd immunity against past antigenic variants renders many of these paths futile, but new epidemics occur each time the virus is able to breach this barrier. Under the action of antigenic drift however, there are multiple directions within antigenic space in which the virus can travel to evade herd immunity. Thus, this ought to lead to an explosion in antigenic diversity with multiple variants breaching this barrier at roughly the same time. The only circumstance in which this is not the inevitable outcome of a basic drift-like process is if antigenic space is one-dimensional. When we do not observe this extensive antigenic diversity, we must therefore look to explain this behaviour by some mechanism other than antigenic drift.

Dengue provides one such example – it is a virus that generates a large amount of genetic diversity and yet, with only four, there are just a handful of antigenic types (serotypes) that circulate in humans. Furthermore, each of these types are sufficiently antigenically similar that antibodies to one serotype cross-react with each of the others. This most likely reflects limited flexibility of certain key residues or structural features; only small degrees of variation are tolerable so as to ensure successful completion of dengue's life cycle which requires successful replication in both human and vector. Indeed, antibodies against one flavivirus commonly cross-react against others, and this has been suggested to result in, for example, competition between dengue and yellow fever. Cross-reactivity between dengue serotypes may have played an important role in both the original emergence of dengue into human populations from sylvan reservoirs and continued persistence thereafter. In dengue, prior exposure to one serotype is paradoxically a risk factor for disease upon a second infection. Evidence that cross-reactive antibodies from a previous infection plays a role in this is convincing and is referred to as the theory of Antibody Dependent Enhancement (ADE). It is not clear whether this propensity for severe disease at secondary infection also correlates with either an enhanced transmissibility of or susceptibility to secondary

infection, but several mathematical models of the epidemiology of dengue have investigated one or other assumption. These investigations have collectively revealed that moderate levels of ADE may play a role in maintaining transmission cycles of dengue, and may help to rescue from extinction those serotypes to which there are high levels of herd immunity.

One additional assumption that has often been made is that immunity to all four serotypes is gained upon exposure to any two or a system where just two serotypes are considered. Such assumptions are often based on the difficulty of clinically determining the true and full extent of a patient's prior experiences with the virus, not least because of cross-reactivity between both dengue serotypes and flaviviruses in general. One thing that does seem clear however is that repeat clinical admissions with dengue infection are rare – lending weight to the hypothesis that enhanced disease is a one-off occurrence. However, the high prevalence of asymptomatic dengue infection indicates that disease is not always necessarily correlated with infection and transmission. Thus, to ignore third and fourth dengue infections as a source of transmission is to potentially overlook an important component of the virus' epidemiology.

Inspired by this point, I used Chapter 2 to look at the impact of third and fourth infections on a model of dengue that also incorporated Antibody Dependent Enhancement (ADE). As it turns out, including third and fourth infections has little effect on the qualitative behaviour of this model, which explains why models without it have been able to reproduce features of the known epidemiology. Importantly however, including these infections acts to lower the levels of enhancement and virus transmissibility (R_0) that are required to observe the complex, desynchronised oscillatory behaviour believed to correspond to serotype co-circulation, and the very high seroprevalence observed in the

young. Thus, including third and fourth infections means that a high value of R_0 is no longer required to match a model to data, which may be important given both the short infectious period of the virus, and its transmission by vector. Further, dengue is believed to have emerged in humans as the result of independent introductions of sylvatic progenitors of each of the four serotypes within South East Asian populations. If infection by two serotypes really is sufficient to allow complete protection against all four, then it becomes difficult to see how the two latterly introduced serotypes could have achieved sustained transmission in humans (the action of ADE notwithstanding), given that a large proportion of the population into which they were introduced would have been consequently immune.

Thus, in the case of dengue case we see only a limited degree of antigenic variation because of stringent constraints on certain highly immunogenic surface residues. However, the reason why dengue is such an important problem, and not extinct, may be due to the nuances of cross-reactivity between its four serotypes. In particular, sequential infection by all four serotypes acts, in combination with ADE, to reduce the required R_0 for persistence and co-circulation of all four, and explains the high levels of seroprevalence in the very young.

Type A influenza is another viral pathogen that exhibits limited antigenic diversity in humans, albeit in a different fashion, with epidemics typically consisting of just a single antigenic variant. Thus, in contrast to dengue which displays a degree of antigenic diversity at any one time point, but with conservation of antigenic types through time, influenza displays very little antigenic diversity at any one point, but with a large amount of antigenic flux through time. This pattern of evolution is therefore very different again from the idea

of antigenic drift which predicts both antigenic evolution with time and antigenic diversity among isolates.

One way in which this pattern of antigenic evolution can be explained is by inverting the idea of a limitless antigenic space to which the influenza virus has limited access. The dynamic of sequential, single-strain influenza epidemics can be easily recovered if we suppose instead that the antigenic space of influenza is restricted, perhaps due to functional constraints, but that the virus' large mutation rate means that it has access to all of this space at any one time (Recker *et al.*, 2007). Within this framework, immune selection at a population level is sufficient to generate the sequential emergence of unique antigenic types observed in humans. However, this particular pattern appears to contrast with those observed in other hosts such as pigs and birds, where periods of seemingly slow antigenic evolution and/or high degrees of antigenic diversity have been observed. In these species, lifespan has often been cited as the root cause – particularly in the case of slow antigenic evolution, with a short lifespan suggested to exert less pressure for antigenic variation. I have demonstrated in Chapter 3, however, shortening host lifespan does not necessarily result in the virus no longer exhibiting antigenic flux. Instead, within the model of Recker *et al.*, a shortened lifespan results in an increase in the extant antigenic diversity at any one time point and a reduced inter-epidemic period. The latter observation may in part explain the endemic nature of influenza within birds and swine, as might also the effect of an effectively longer infectious period in aquatic birds.

Antigenic stasis can also be achieved by epidemiologically linking populations with different life expectancies, as may occur, for example, within a global network of avian influenza. My studies indicate that the dynamics of influenza in the shortest-lived population within a linked epidemiological network is key to understanding the dynamics

of the system as a whole. This is presumably because decreased lifespan leads to increased prevalence within a population due to faster turnover of susceptible hosts, and enables the short-lived population to contribute disproportionately to the overall dynamic. Accordingly, interruptions to pan-population transmission could result in complex patterns of evolution with shifts in longer-lived populations between antigenic evolution and stasis.

However, antigenic stasis has often been reported for various swine and avian viruses in terms of long-term persistence of a single antigenic type. This is hard to rationalise, especially within a model of antigenic drift, unless there are very strong constraints on antigenic evolution. My work with the 'limited-epitope' model does however provide an alternative explanation, albeit one also based on greater structural constraint. Within the model, flexible antigenic epitopes, result in a human-like pattern of evolution with little cross-reactivity between isolates from year to year. On the other hand, more stringent restrictions with consequently limited flexibility at a handful of epitopes can result in apparent antigenic stasis of just a single antigenic type. However, this appearance is illusory; antigenic evolution is still occurring at a fervent pace but since most strains are now antigenically related, tests of cross-reactivity seem to indicate stasis.

To summarise, immunity to influenza at the level of the population can act to sculpt a large amount of generated antigenic diversity so that we only observe a very small subset of this diversity at any one time in long-lived hosts. However, increased diversity may be observed in hosts with shorter life expectancies. Turnover in this diversity may be halted in populations with very short life expectancies, but a single antigenic type cannot persist as the solely dominant type for extended periods within these populations. Rather, where this has been observed it is likely a reflection of limited possibilities for variation

within the critical epitopes, which results in the appearance of antigenic stasis because most variants are related, although importantly not identical.

By contrast with human type A influenza, HIV-1 seems to exhibit a tremendous amount of both genetic and antigenic diversity both within and between hosts. The virus seems to be able to adapt quickly to evade cellular and humoral immune responses within an infected individual. However, the impact of this presumably extensive antigenic repertoire on the ultimate pathogenesis of the virus remains poorly understood. What is known is that the virus has a devastating effect on the body's population of memory CD4⁺ cells, almost certainly with knock-on effects for the body's immuno-competence. Although the body is able to initially control HIV-1 as a chronic infection, eventually this control breaks down and the patient progresses to AIDS. To date, there is no satisfactory explanation of this process. In particular, it has proved difficult to reconcile the stark three-phase pattern of viraemia with a gradual weakening of the immune system. The most significant effort, so far, is the 'Antigenic Diversity Threshold' model of Nowak and colleagues. This model suggests that it is the gradual accumulation of antigenic diversity that eventually overwhelms the immune system and causes the progression to AIDS. However, this model requires the same drift-like mutational process that is inappropriate for dengue and influenza. This is not to say that this means that this caricature should also therefore be flawed, but given that HIV-1 is also an RNA virus, it seems worthwhile to explore the possibility that the virus instead evolves within the host in the same manner as Recker et al. suggested for influenza within a population. Chapter 4 therefore considers the possibility that HIV-1 is capable of continually generating a large, though ultimately finite, amount of antigenic diversity, within the context of gradual immune dysfunction.

I have assumed here that the host is able to mount both short-lived CD8+ and long-lived neutralising antibody responses against the virus. Together these two responses are able to control HIV-1 as a chronic infection. Further, depending on the assumptions one makes about the relationship between CD8+ and antibody epitopes, strain structuring can emerge as the virus is able to continually escape from both types of responses by rifling through its antigenic repertoire. In the model however, as a result of infection and the resultant depletion of CD4+ T cells, the ability to mount an antibody response wanes with time. Once this ability ultimately drops to a critical level, control of viral replication is lost and the host develops AIDS.

As with the influenza model, unbounded possibilities for antigenic diversity (characteristic of antigenic drift) are therefore not necessary to generate key aspects of the known biology. A gradual weakening of the antibody response leads to a multi-phasic pattern of viraemia. CD8+ T cell responses act to control viral load, restricting viral replication and prolonging the chronic phase of disease; escape from CD8+ T cell responses is associated with faster progression and can happen suddenly, even if the escape mutant is continually generated throughout infection. Viral evolution is ongoing, even in those hosts who are able to more efficiently restrict viral replication. Finally, if there are connections between mutations at CD8+ T cell and NAb epitopes on Env then I can recover a link between HLA Class I type and pathogenesis. In my model, HLA alleles that focus the immune system on more highly conserved epitopes delay the development of AIDS.

A common theme in my thesis is the restriction of antigenic diversity within RNA virus populations, given their natural tendency to accumulate variation. In the case of dengue, we can surmise that there are strict structural constraints, possibly as the result of needing to maintain the ability to infect both human and mosquito, and this is reflected in the

antigenic repertoire. An extension of this idea to consider that there may also be limits to the antigenic repertoire of influenza allows us to recover the dynamic of single-strain epidemics in humans without imposing unrealistic constraints on the mode and tempo of mutation. I have shown here that these concepts can also allow us to recover the divergent patterns of evolution in pigs and birds. Moreover, depending on the nature of these structural constraints, the virus can appear to be in a form of antigenic stasis, even though in truth it is evolving rapidly. Immune responses to HIV-1 can likewise shape a large amount of generated diversity into a smaller amount of observed diversity, but the toll of continued viral replication within the body eventually results in death of the host. I have also shown however, that classical viral escape does not fundamentally cause this pathogenesis although it can accelerate it.

A key feature of all three modelling exercises is that antigenic diversity is not unlimited as it has to obey a set of structural and functional constraints. Instead of presuming that the mutational process is what limits the interrogation of this antigenic space, I offer the viewpoint that immune selection is the principal force in shaping this diversity. This framework contrasts with current dogma that the virus population is slowly travelling through a vast antigenic space, whether within the host (HIV) or in a community (influenza), and decouples their dynamics and pathogenesis from the rate at which new mutations or antigenic types are generated. As shown in this thesis, this 'limited-epitope' approach based on immune selection acting upon a highly constrained antigenic space provides a powerful and flexible conceptual framework for re-evaluating our ideas about viral evolution.

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