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Investigations into the Pathogenesis of Dengue Virus Infections of Humans: the Endothelium as a Target.

Tara Anne Kelly

A Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, University of Dublin, Trinity College.

Department of Microbiology November, 2000

…Two roads diverged in a wood and I –

I took the one less travelled by

And that has made all the difference.

Robert Frost (1874-1963).

For my family, with love.

DECLARATION

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SUMMARY

The pathogenesis of the disease Dengue Haemorrhagic Fever/Dengue Shock Syndrome has not been elucidated. This study investigated the effects of Dengue virus, serotype 2 infection on both the human cell line ECV304/T-24 and primary HUVEC, as an *in vitro* endothelial cell model system.

Infection of ECV304/T-24 cells by DEN 2 resulted in a loss of viability of the cells and death by apoptosis. However, the cells recovered and were persistently infected by the virus. Infection was productive, with the release of infectious viral progeny into the supernatant and maximum production was found at 48 hours post infection. Persistently infected cells secreted DEN 2 at lower titres. Viral antigen expression within infected ECV304/T-24 cells and HUVEC was investigated using indirect immunofluorescence analysis.

DEN 2 infection of these cells resulted in the induction of a pro-inflammatory immune response. Elevated levels of Tumour necrosis factor-α and Interleukin-6 (IL-6) mRNA expression were detected, using semi-quantitative RT-PCR. Infection of primary HUVEC resulted in the induction of Interleukin-1 and IL-6 mRNA expression. No TNF-α mRNA expression was detected in these cells.

It was decided to investigate one possible mechanism of action of DEN 2 on the host cell immune response. Infection of ECV304/T-24 cells was found to activate the transcription factor NF-κB, which is known to induce the transcription of the cytokines TNF-α and IL-1. DEN 2 infection of these cells resulted in the phosphorylation and degradation of the inhibitor protein I-κB-α.

It was decided to investigate the effects of DEN 2 infection on monocytic cells and studies included the pro-monocytic cell line U937 and primary adherent monocytes. The effects of infection on these cells were not as pronounced as with either ECV304/T-24 cells or HUVEC.

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ABBREVIATIONS

AP61	*Aedes psuedoscutellaris* mosquito cell line
ATP	Adenosine triphosphate
BPL	β-propiolactone
C6/36	*Aedes albopictus* mosquito cell line
CD	Cluster of differentiation
CMC	carboxymethylcellulose
DEN 2	Dengue virus, serotype 2
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
d.p.i.	days post infection
d.p.s.	days post seeding
dNTP	deoxynucleotide triphosphate
DTT	Dithiothrietol
dUTP	deoxyuridine triphosphate
ECV304/T-24	Human epithelial bladder carcinoma cell line
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
ffu	Focus forming units
G6PD	Glucose-6-phosphate dehydrogenase

HRP	Horse-radish peroxidase
HUVEC	Human umbilical cord vein endothelial cells
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IκB	Inhibitor for NF-κB
IL-1	Interleukin-1
IL-6	Interleukin-6
LDH	Lactic dehydrogenase
MOI	Multiplicity of infection
mRNA	messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NEAA	Non-essential amino acids
NF-κB	Nuclear factor-κB
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TBS	Tris-buffered saline
$TCID_{50}$	Tissue culture infectious dose assay
TGF-β_2	Transforming growth factor-beta

TNF-α	Tumour necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UV	Ultraviolet

TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION 6

1.1.1 Dengue virus 6

1.1.2 *Human diseases caused by infection with Dengue* 8

1.1.3 *Pathology* 9

1.1.4 *Pathogenesis of Dengue infections* 10

1.1.5 *Dengue virus-susceptible cells* 12

1.1.6 *Induction of host cell cytokines by Dengue virus* 13

1.2 Cytokines 14

1.2.1 *TNF-α* 15

1.2.2 *IL-1* 16

1.2.3 *IL-6* 16

1.3 IκB 18

1.4 NF-κB 18

1.5 Endothelial Cells 19

1.6 Apoptosis 20

1.7 Persistent viral infections 21

CHAPTER TWO
MATERIALS AND METHODS

2.1. MATERIALS 23

2.1.1 Cell Culture 23

2.1.2 Cell culture media and additions 23

2.1.3 Cytokines and RT-PCR chemicals 24

2.1.4 Fine chemicals 24

2.2 METHODS 25

2.2.1 Cell Culture 25

2.2.2 Dengue Virus 28

2.2.3 Cell Viability studies 31

2.2.4 Apoptosis studies 32

2.2.5 Cytokine expression studies 33

2.2.6 IκB-α assay 37

2.2.7 NF-κB activation studies 39

CHAPTER THREE

EFFECTS OF DEN 2 INFECTION ON HOST CELL VIABILITY

3.1 INTRODUCTION 41

3.2 RESULTS 41

3.2.1 Detection of intracellular DEN 2 viral antigens 41

3.2.2 Infectious DEN 2 viral progeny production 43

3.2.3 Cell Viability studies 45

3.2.4 Apoptotic studies 49

3.2.5 Persistence/chronicity of DEN 2 infection in ECV304/T-24 cells 52

3.2.6 Investigations into whether phenotypic changes of DEN 2 or adaptations of ECV304/T-24 cells have occurred in persistently infected cells 53

3.2.7 'Superinfection' of ECV304/T-24 cells with DEN 2 54

3.3 DISCUSSION 55

CHAPTER FOUR

EFFECTS OF DEN 2 INFECTION ON PRO-INFLAMMATORY IMMUNE RESPONSE

4.1 INTRODUCTION 57

4.2 RESULTS 58

4.2.1 Pro-inflammatory cytokine gene expression studies using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) 58

4.2.2 Pro-inflammatory cytokine mRNA expression in exponentially growing ECV304/T-24 cells following DEN 2 infection 61

4.2.3 Pro-inflammatory cytokine mRNA expression in DEN 2 infected polarised confluent ECV304/T-24 cells 63

4.2.4 Pro-inflammatory cytokine mRNA expression in DEN 2 infected human umbilical vein endothelial cells (HUVEC) 64

4.2.5 Pro-inflammatory cytokine mRNA expression in the human promonocytic cell line U937, following infection with DEN 2 66

4.2.6 Pro-inflammatory cytokine mRNA expression in DEN 2 infected primary adherent monocytes 66

4.2.7 Effects of exposure to inactivated DEN 2 on the host cell cytokine response 68

4.2.8 Secretion of tumour necrosis factor-α from DEN 2 infected ECV304/T-24 cells 70

4.2.9 Effects of TNF-α pre-activation on pro-inflammatory cytokine induction by DEN 2 infection of ECV304/T-24 cells 71

4.2.10 Effects of IFN-γ pre-activation on pro-inflammatory cytokine induction by DEN 2 infection of ECV304/T-24 cells 74

4.2.11 Detection of intracellular virus following pre-activation with Tumour necrosis factor-α, Interferon-γ or Transforming growth factor-β 75

4.3 DISCUSSION 77

CHAPTER FIVE

MECHANISM OF ACTION OF DEN 2 ON HOST CELL IMMUNE RESPONSE

5.1 INTRODUCTION 80

5.2 RESULTS 81

5.2.1 Effects of stimulation of ECV304/T-24 cells with IL-1α on IκB-α 81

5.2.2 Effects of infection of ECV304/T-24 cells by DEN 2 on IκB-α 82

5.2.3 Activation of the transcription factor NF-κB in DEN 2 infected ECV304/T-24 cells 84

5.3 DISCUSSION 85

CHAPTER SIX

6.1 FINAL PERSPECTIVES 87

BIBLIOGRAPHY 92

CHAPTER ONE

Introduction

1.1 INTRODUCTION

1.1.1 Dengue virus

1.1.1.1 Taxonomy

Dengue viruses are enveloped, positive-strand RNA flaviviruses, belonging to the family Flaviviridae. Other members include Japanese encephalitis, Murray Valley encephalitis, West Nile, Kunjin and Yellow Fever viruses. These enveloped viruses are composed of a lipid bilayer with two or more species of envelope (E) proteins surrounding a nucleocapsid core, which consists of the single-stranded positive sense RNA genome complexed with copies of a small basic capsid (C) protein (1). The envelope proteins E (envelope) and M (membrane) are type I membrane proteins embedded in the lipid bilayer by C-terminal hydrophobic anchors. Flaviviruses are spherical in shape and ring shaped structures have been observed on the surface of Dengue virus. Four serotypes of Dengue virus have been identified; DEN 1, 2, 3 and 4. Infection with any of the viruses confers life-long homotypic, but not heterotypic immunity (2). The virus was first isolated by Sabin and Schlesinger in mice in 1944 and the existence of more than one serotype was established by cross-protection studies in human volunteers (3).

1.1.1.2 DEN entry into host cells, life cycle and replication

The cellular receptor(s) for Dengue virus are as yet unidentified. Different routes of entry of the virus into the cell have been observed which include the uptake of single virions via clathrin-coated pits or uncoated vesicles, the engulfment of virus particles via cytoplasmic processes or invagination of the plasma membrane (4). It has also been shown that infection in the presence of subneutralizing concentrations of antibody bound to virus can mediate attachment and uptake via the Fcγ receptors RI and RII, on the cell surface by receptor-mediated endocytosis. FcγR binds to the Fc portion of Dengue virus-specific IgG antibody. Dengue virus infection has been shown to occur within two hours after adsorption of the virus to the cell membrane (5).

Entry is followed by fusion with virus-induced membrane structures in the cytoplasm of the host cell, in which the translation of the viral genome, polyprotein

processing and viral RNA synthesis occur. These membranes appear as packets of vesicles associated with the sites of viral RNA synthesis and appear to originate from the trans-golgi membranes and the intermediate compartment (6). The replication complex comprises most of the viral non-structural proteins and a double-stranded RNA replicative form which acts as a template for nascent single-stranded RNA transcription (7, 8). Sequence motifs characteristic of a serine protease, RNA helicase and RNA-dependent RNA polymerase (believed to be NS5) are found in similar locations in the polyproteins of all flaviviruses. The cleavage products containing these regions are believed to form the enzymatic components of the RNA replicase (1). RNA replication occurs via synthesis of a full-length negative strand RNA intermediate. This acts as a template for production of additional genome-length positive stranded molecules by a semi-conservative mechanism involving replicative intermediates (duplex RNA molecules) and replicative forms, which contain double-stranded regions and nascent single-stranded RNA molecules (9). An illustration of the Flavivirus life-cycle has been taken from (1) and is given in Figure 1.1.

1.1.1.3 DEN genome

The genome is a single-stranded positive sense RNA genome, which encodes genes for 3 structural and 7 non-structural proteins in the gene order 5'-C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3' (10). Cleavage of the polyprotein to generate individual proteins is mediated either by a host signal peptidase or a virus-encoded two-component protease, NS2b/NS3. The exception is cleavage at the NS1-NS2a junction, which is mediated by an as yet unidentified host cell enzyme, located within the endoplasmic reticulum. A 26 amino acid sequence immediately downstream of NS1, at the N-terminus of NS2a, comprises a domain that is similar to C-terminal signal sequences present in nascent eukaryotic proteins prior to processing to a glycosyl-phosphatidylinositol (GPI)-anchored form. This suggests that NS1 may also undergo post-translational modification to a GPI-anchored form (11, 12).

Virion morphogenesis and viral glycoprotein maturation occur within intracellular vesicles, which follow the secretory pathway and fuse at the plasma membrane resulting in the release of the mature enveloped virions from the cell.

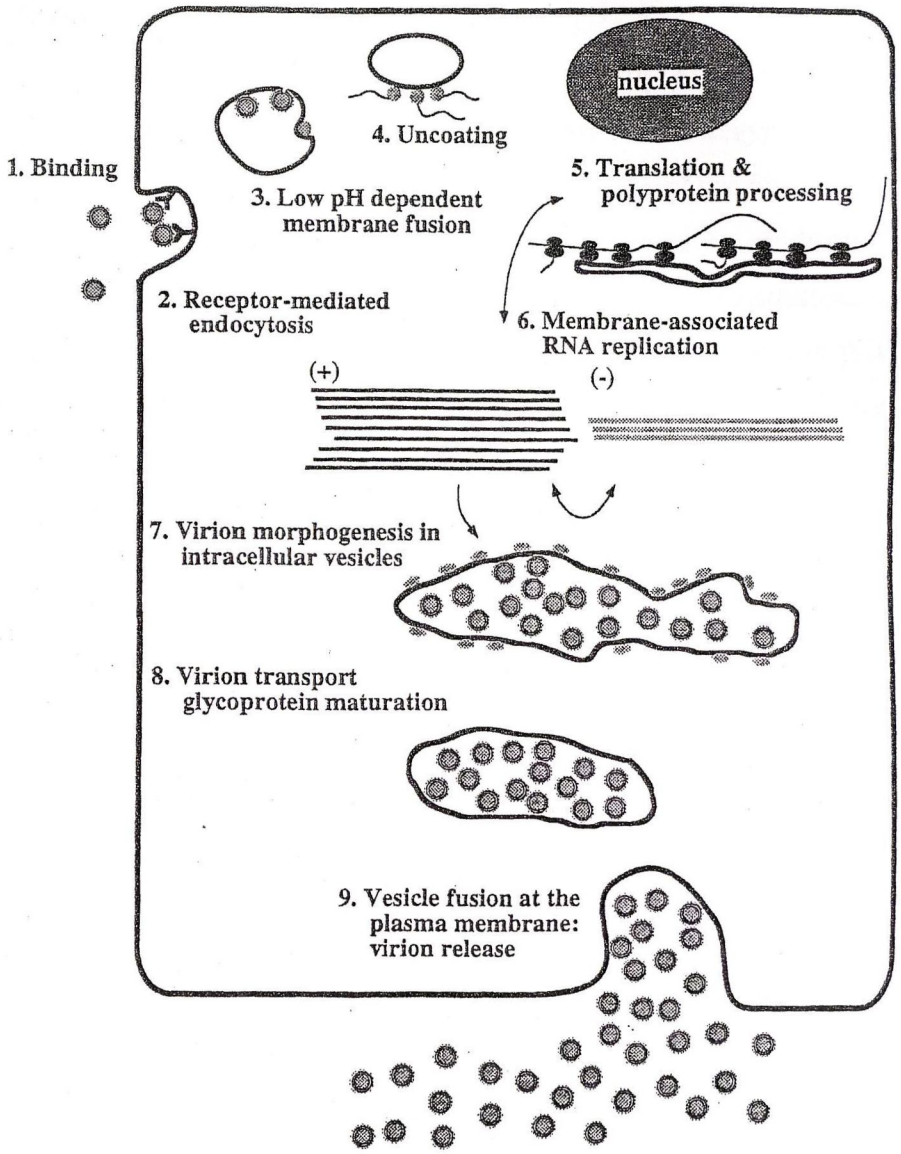

Fig. 1.1 An illustration of the Flavivirus life-cycle, taken from 'Fields Virology' (1).

1.1.1.4 Transmission

Dengue virus is transmitted by the *Aedes* family of mosquitoes, of the subgenus *Stegomyia.* The primary vectors are the mosquitoes *Aedes aegypti* and *Aedes albopictus* (13). *Aedes aegypti* is a highly domesticated, day biting species that lives and breeds around the home. The female mosquitoes often engage in multiple feedings before completing their gonotrophic cycle. Once infected, female mosquitoes remain infective throughout the adult stage, even after repeated bites on humans. Primary larval habitats for the mosquito include domestic water storage containers. There are only three known natural hosts for Dengue virus; mosquitoes, humans and lower primates (14, 15).

Biological transmission of Dengue virus by the mosquito is dependent on the ingestion of an infected blood meal by the mosquito and the infection and replication of the virus within mesenteronal epithelial cells of the animal. Once the virus is released from these cells, it infects the salivary glands either directly or following secondary amplification in other cells or tissues. The virus is released from the epithelial cells of the salivary gland and is transmitted by feeding, where it is deposited in either the extravascular tissues or vascular space of the host (3).

1.1.1.5 Distribution

Dengue viruses have a worldwide distribution in the tropics, including Southeast and South Asia, Central and South America, the Caribbean and Africa (16). Uncontrolled urbanization, global population growth, lack of mosquito control and increased air travel provide the ideal mechanisms for the transport of Dengue between population centres of the world (17). Within countries, the virus spreads along transportation routes and between different countries, it appears first at seaport or airport cities (18).

1.1.2 Human diseases caused by infection with Dengue

Infection of humans with Dengue virus causes a spectrum of illnesses, ranging from a mild febrile illness (Dengue Fever) to a severe and fatal haemorrhagic disease (Dengue Haemorrhagic Fever/Dengue Shock Syndrome). The incubation period varies from 3 to 14 days, with a typical latent period of 4 to 5 days after infection (17, 19). Patients suffering

from Dengue Fever (DF) present with an abrupt onset of fever, severe headache, pain behind the eyes, muscle and joint pains and a rash. A typical saddleback fever is presented in patients (20). Platelet counts are normal and there is no coagulation defect (21).

However, infection may result in the disease Dengue Haemorrhagic Fever (DHF), which ranges in it's degree of pathogenesis from fever (Grade I) to fever with spontaneous haemorrhagic manifestations (Grade II), circulatory failure (Grade III) and deep shock (Grade IV) (21, 22). In Dengue Shock Syndrome (Grades III and IV) hypovolaemic shock occurs which is associated with marked thrombocytopaenia (less than 100,000 platelets/mm^3) and haemoconcentration and frequently leads to death. The hypovolemia follows massive leakage of intravascular fluid and protein through the capillary walls into interstitial serosal spaces (19). If a patient receives intravascular fluid replacement, the capillary leakage syndrome abates quickly, within hours or days and a complete recovery is made. Infact, Dengue haemorrhagic fever is not a good description of the disease, as it is the loss of fluids by increased capillary permeability rather than bleeding, which occurs. Important risk factors for DHF include the strain and serotype of the virus, as DEN 2 and 3 appear to be more pathogenic on average than types 1 and 4 (2), the age, immune status and genetic predisposition of the patient (23).

It is estimated that at least 100,000,000 cases of DF and more than 250,000 cases of DHF occur annually (24, 25). DF and DHF/DSS are the most important arthropod-borne diseases of humans in the world today. The largest number of DHF cases occur within children aged 4 to 12 years. Most of these children are fully immunocompetent, well developed and nourished.

1.1.3 Pathology

In general, autopsy samples of fatal DHF/DSS cases show no gross or microscopic evidence of sever organ pathology, which could explain the cause of death. Dengue antigens have been found to be localized in cells of reticuloendothelial origin. There is no evidence of bacterial infection in the terminal stages of infection, suggesting that the immune system of the patients had not been compromised. Biopsy studies are very difficult to obtain and are generally considered too risky to the patient and those rare samples which have been investigated have shown that the microvasculature located in the dermal papillae

is the main site of injury. The basic changes which are detected include a swelling of the endothelial cells and the perivascular tissue. However, DHF/DSS is unlike the haemorrhagic fever observed with Ebola or Marburg infections, as there is no massive destruction of the blood vessels visible (26-28). An increase in the number of cells such as lymphocytes, mononuclear phagocytes and other large unidentified cells of lymphoblastoid origin, has been observed around the microvasculature (29).

The huge loss of plasma volume and lack of major organ injury, suggest that the general vascular system is the major site of injury. Due to the speed of fluid loss and in survivors, the speed of subsequent recovery (about 24 to 48 hours) from shock, without severe changes or destruction to the body, it is suspected that biological mediators, such as cytokines or complement proteins are involved (29, 30). It is interesting to note that the capillaries of children are more prone to cytokine-mediated permeability and are more permeable to water and plasma proteins than those of adults. Children are also known to have a larger microvascular surface area per unit volume of skeletal muscle than adults (31).

1.1.4 Pathogenesis of Dengue infections

The pathogenesis of DHF/DSS is not understood. A number of hypotheses have been described to explain the prevalence of DHF/DSS in children, the lack of gross pathology on autopsy and the speed and degree of recovery after illness. It has also been documented that a higher incidence of DHF/DSS is found in children presenting with a secondary infection by a heterologous serotype of virus (32, 33). Halstead (1988) believes that antibody is a risk factor in converting Dengue from a mild to a severe disease (32, 34). Figure 1.2 illustrates Halstead's theory of antibody dependent enhancement (ADE). Residual sub-neutralizing amounts of antibody following a primary Dengue infection with DEN 1 for example, could recognise and bind to a heterologous serotype upon a second infection (DEN 2), without neutralizing DEN 2. The virus-immune complexes could then bind to Fcγ receptors (FcγR) on the surface of cells and the virus could enter the cell via receptor-mediated endocytosis. FcγRs bind to the Fc fragment of Dengue virus-specific IgG antibody and are found on mononuclear phagocytes, polymorphonuclear leukocytes,

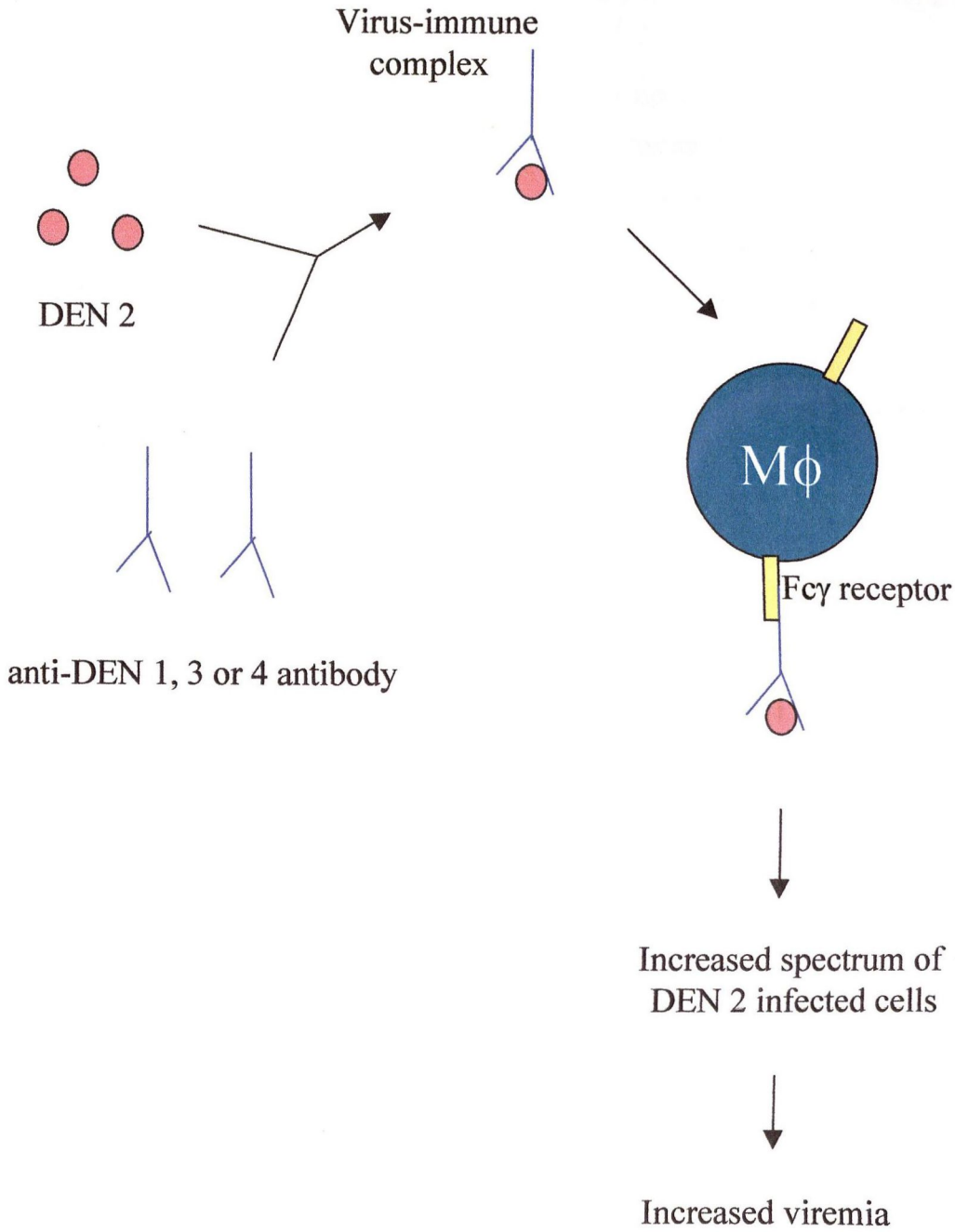

Fig. 1.2 Schematic representation of Halstead's hypothesis of antibody dependent enhancement (ADE)

platelets, natural killer cells, B and T lymphocytes (35, 36). The spectrum of cells which are susceptible to Dengue infection, increases.

This theory can be challenged with respect to a number of unanswered aspects. Several DHF/DSS cases have occurred in patients suffering their first infection with Dengue virus. This theory also implicates the macrophage as the primary target cell, whereas the primary target cell has not really been identified. The theory predicts that a second infection should result in a higher viraemia than in a primary infection and so far evidence for this has been negative.

Kurane and Ennis (37) have added to this hypothesis to explain the immunopathogenesis of DHF, which is illustrated in Figure 1.3. As described by Halstead, the infection of monocytes and macrophages is enhanced by the presence of virus-antibody complexes. Dengue virus-specific helper CD4+ T cells are stimulated by encountering infected monocytes/macrophages and secrete lymphokines such as Interleukin-2 (IL-2) and Interferon-gamma (IFN-γ). It has been shown that Dengue-specific CD4+ T cells appear to be mainly types T_{H1} and T_{H0}. IFN-γ can up-regulate the expression of Fcγ receptors, which would increase the susceptibility of these cells to infection. A high level of T cell activation ensues, which results in the production of increased levels of lymphokines. IFN-γ activated monocytes can also produce high levels of Tumour necrosis factor-alpha (TNF-α), Interleukin-1 (IL-1) and platelet activating factor (PAF) upon infection, or as a result of lysis by cytotoxic T cells, or contact with virus-specific T cells. A cycle of cytokine and chemical mediator induction can follow and a high level of these proteins could be produced in a short time. This may result in the malfunction of vascular endothelial cells which can lead to plasma leakage and shock (37).

It has also been suggested that the severity of the disease DHF could be correlated with the intrinsic biological properties of the infecting strains of virus (38, 39). Differences in the ability of two strains of DEN 1 to fuse to the membranes of mosquito cell lines in culture, have been linked to differences in the amino acid sequences of the two strains and in particular within two overlapping antigenic domains of the envelope protein E (40). A study of the genome sequences of eleven Dengue viruses isolated from DF and DHF patients revealed structural differences which were consistent with the potential for each virus to cause DHF. In this study, the primary determinants of DHF were found to reside in

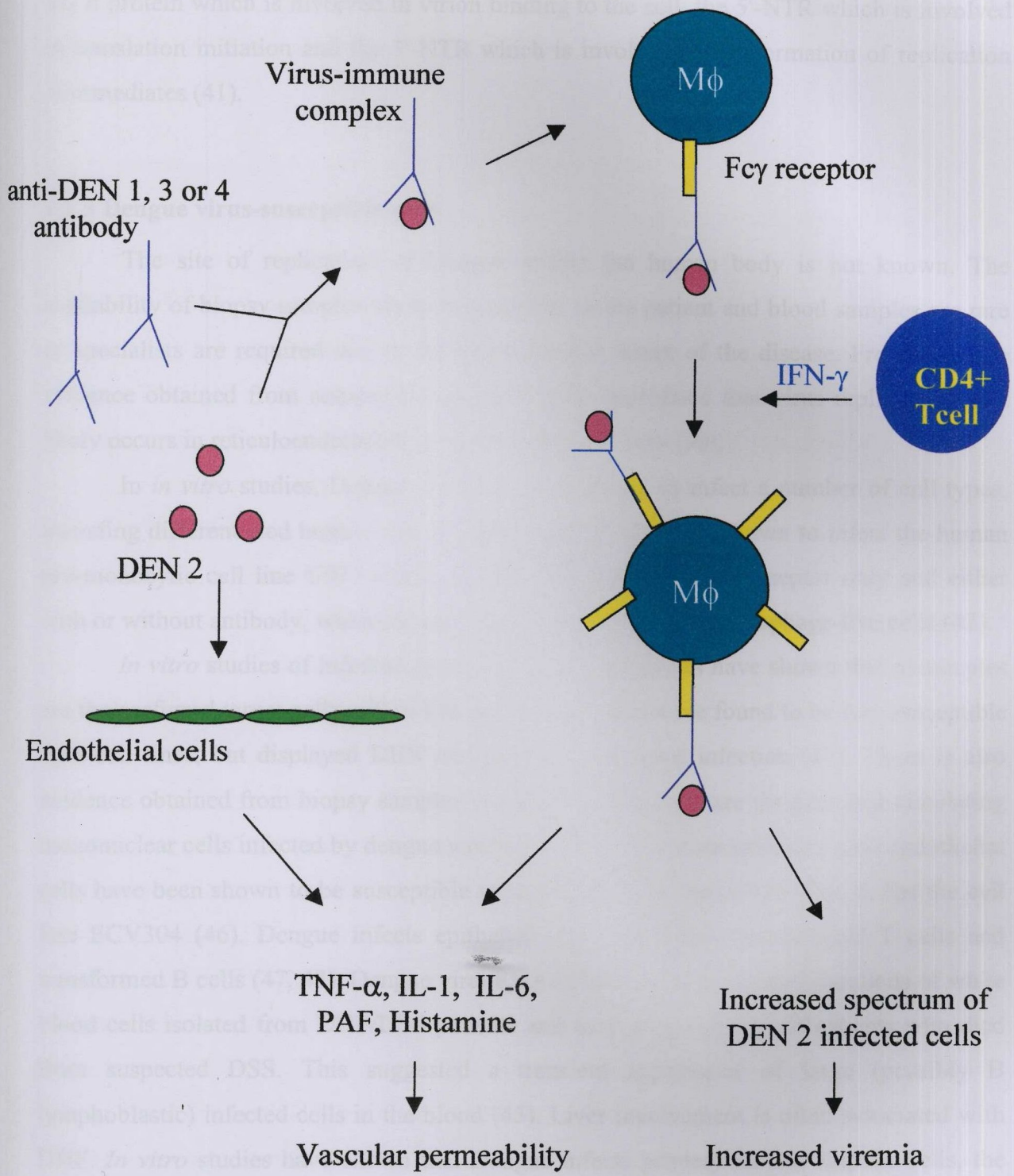

Fig. 1.3 Schematic representation of the Immunopathogenesis hypothesis of DHF as proposed by Kurane and Ennis

the E protein which is involved in virion binding to the cell, the 5'-NTR which is involved in translation initiation and the 3'-NTR which is involved in the formation of replication intermediates (41).

1.1.5 Dengue virus-susceptible cells

The site of replication of Dengue within the human body is not known. The availability of biopsy samples involves great risk to the patient and blood samples are rare as specialists are required due to the haemorrhagic nature of the disease. From the little evidence obtained from autopsy cases, it has been postulated that virus replication most likely occurs in reticuloendothelial cells, fibroblasts or both (30).

In *in vitro* studies, Dengue virus has been shown to infect a number of cell types, including differentiated human macrophages. Dengue has been shown to infect the human pro-monocytic cell line U937 when undifferentiated, via the Fc receptor only and either with or without antibody, when the cells are differentiated into macrophage-like cells (42).

In vitro studies of infected peripheral blood leukocytes have shown that monocytes are the preferred target cells within that population. They were found to be less susceptible than cell lines, but displayed DEN antigens five days post infection (43). There is also evidence obtained from biopsy samples to suggest that B cells are the principal circulating mononuclear cells infected by dengue virus (44). Primary human umbilical vein endothelial cells have been shown to be susceptible to infection by Dengue virus (45), as has the cell line ECV304 (46). Dengue infects epithelial cells, fibroblasts, transformed T cells and transformed B cells (47, 48). Dengue viral RNA is detectable in some preparations of white blood cells isolated from DHF/DSS patients and in thymus samples of patients who died from suspected DSS. This suggested a transient appearance of large (possibly B lymphoblastic) infected cells in the blood (45). Liver involvement is often associated with DHF. *In vitro* studies have shown that Dengue infects primary human Kupffer cells, the macrophages residing in the liver (49) and the human hepatoma cell line HepG2.

There is no murine model for DHF/DSS and intracerebral injection of mice with strains adapted for replication in mouse brain result in encephalitis, which is not seen in human Dengue infections. The capillary leakage associated with human infections is not

observed in mice (50). All non-human primates that have been tested are susceptible to infection with DEN, but none exhibit signs of illness (2).

1.1.6 Induction of host cell cytokines by Dengue virus

Viruses alter haemostasis in two ways, through direct effects on cellular function and through activation of immune and inflammatory pathways. Both mechanisms may lead to variable degrees of cellular injury, including cell death (26). Elevated levels of the cytokines TNF-α and Interleukin-6 (IL-6) have been detected in the serum of children suffering from DHF. TNF-α levels corresponded directly to the severity of the disease. IL-6 levels increased in general, after the induction of TNF-α. Only in some adults an induction of IL-6 was detected in their serum (51). In peripheral blood mononuclear cells (PBMCs) which were isolated from patients suffering from DHF, elevated levels of Interleukin-8 were detected. The level of severity of the disease showed a direct relationship with the level of cytokine induced (52). A cytotoxic cytokine, human cytotoxic factor (hCF), which has the ability to increase vascular permeability in mice reversibly, has been isolated from the sera of patients suffering from DHF. hCF did not show homology to any other cytokine or Dengue-specific proteins (53).

The infection of human cells by Dengue virus *in vitro,* has resulted in the induction of cytokines. DEN infection of primary human Kupffer cells, the macrophages residing in the liver results in the induction of Nitric oxide (NO), TNF-α, IL-6 and IFN-γ (49). Interferon-alpha (IFN-α) has been found to be produced by Dengue-infected monocytes isolated from PBMCs *in vitro* and the culture fluids of these infected cells was found to inhibit further infection of human monocytes by the virus (54). Studies have shown that the treatment of cells with Interferon inhibits DEN replication by preventing the accumulation of negative-strand viral RNA (55). In PBMCs prepared from patients suffering from DHF, Dengue has been shown to induce IFN-γ, IL-2, soluble cluster of differentiation marker 4 (sCD4), sCD8 and soluble Interleukin-2 receptor (sIL-2R), which are all markers of T cell activation (56). Infection of human peripheral blood leukocytes (PBL) by DEN 2 *in vitro*, results in an initial T_{H1} response with a corresponding increase in the levels of cytotoxic factor, TNF-α, IL-2, IL-6 and IFN-γ. A T_{H2}-type response was then observed from four

days post infection, with a reduction in these cytokines and an increase in the levels of Interleukins-10, 5 and 4 (57).

This study aims to investigate the ability of Dengue virus, serotype 2, to elicit an immune response in infected endothelial cells, using primary HUVEC and the cell line ECV304/T-24. The cytokines to be investigated include the pro-inflammatory cytokines TNF-α, IL-1 and IL-6.

1.2 Cytokines

Cytokines are a group of proteins, which act as messengers of the immune system. The immune system is a highly regulated and adapted defense system in vertebrates whose primary role is the protection of the host against invading foreign pathogens. It can generate a huge diversity of cells and molecules, which can specifically recognise the invaders. Immune responses can be divided into humoral and cell-mediated responses. The effector molecule of the humoral responses is the antibody and the cells involved are the B lymphocytes. The cell-mediated response is mediated by a number of cells, including cytotoxic T lymphocytes, macrophages and natural killer cells. Both arms of the immune system require cytokines to communicate between cells. Cytokines act locally on target cells which bear receptors specific to the given protein. The actions of many can be pleiotropic, where different effects are elicited on different target cells, or redundant, where a number of different cytokines elicit the same effects.

During inflammation, caused by infection or tissue injury, an acute phase response follows, which is mediated by three principal cytokines which act synergistically; Tumour necrosis factor-alpha, Interleukin-1 and Interleukin-6. The response is initiated by among many cells, dendritic cells, macrophages, helper T lymphocytes and endothelial cells, which secrete these and other recruiting cytokines at the site of infection. TNF-α, IL-1 and IL-6 act locally on fibroblasts and endothelial cells to induce coagulation and increased vascular permeability. TNF-α and IL-1 induce among many other responses, the expression of adhesion molecules, including the endothelial leukocyte adhesion molecule (ELAM-1), the vascular cell adhesion molecule (VCAM-1) and the intercellular adhesion molecule (ICAM-1) on the vascular endothelial cells. This in turn, facilitates the adherence of monocytes, neutrophils and circulating lymphocytes to the endothelium prior to their transport across the endothelium and into the infected tissues, by diapodesis.

The inflammatory response is far more detailed than this simple overview presents and involves the interaction of numerous cytokines and immune cells. Pro-inflammatory cytokines are linked together in a cascade, that is the binding of one cytokine to a receptor will elicit the secretion of a second cytokine and so on (58, 59). Most receptors for cytokines belong to the immunoglobulin superfamily. Studies have shown that haemorrhagic shock without massive tissue trauma is associated with elevated plasma levels of the cytokines TNF-α, IL-1 and IL-6 (60).

1.2.1 TNF-α

TNF-α is a non-glycoprotein and exists as both a transmembrane and secreted form. This cytokine plays a key role in many infectious and inflammatory diseases. High concentrations of the cytokine have been detected in a variety of infectious and inflammatory diseases (61). A major site of action of TNF-α is the vascular endothelium, where it induces inflammatory responses by enhancing adhesion molecule expression and cytokine secretion (62).

The major source of TNF-α is activated monocytes/macrophages. RNA and DNA viruses have been shown to be potent inducers of TNF-α (63, 64). TNF-α has also been shown to display antiviral effects against both RNA and DNA viruses, by synergizing with interferons and inhibiting viral replication (65). TNF-α is involved in endotoxin-induced septic shock induced during a bacterial infection.

Agents known to inhibit TNF-α production include immunosuppressive drugs such as cyclosporin A and some cytokines including IL-6. It has been shown that the pathways involved in the induction of TNF-α may also be involved in it's downregulation.

TNF-α is directly toxic to endothelial cells and increases blood vessel permeability (66, 67). The cytokine acts on the endothelium to induce other cytokines, adhesion molecules, inducible Nitric Oxide synthase and coagulation factors (61).

No cell type has been reported that is TNF receptor deficient. There are two types of receptor, TNF-receptor 1 (p55), which is expressed on almost all cell types and TNF-receptor 2 (p75), which is primarily expressed on endothelial cells. Both receptors when activated lead to the activation of the transcription factor NF-κB, which in turn induces pro-inflammatory cytokines, including IL-6 and can result in the death of the cells by apoptosis

(68). The binding of TNF-α to the TNFR1 receptor on endothelial cells results in rapid translocation of NF-κB from the cytoplasm to the nucleus (62, 69). The actions of TNF-α following receptor binding, are mediated through a number of protein kinases within stimulated cells, such as c-Jun NH_2-terminal kinase (JNK) and mitogen activated protein kinase (MAPK). The expression of TNF-α is regulated at the transcriptional, translational and post-translational levels (70).

The other pro-inflammatory cytokines IL-1 and IL-6 display partially overlapping activities with TNF-α (61).

1.2.2 IL-1

IL-1 exists as two independent polypeptides; the membrane-bound IL-1α and the secreted protein IL-1β constitute this cytokine family. IL-1 is not produced from healthy cells. Along with TNF-α, IL-1 has been shown to be a determining cytokine in the pathogenesis of many inflammatory diseases (71). The IL-1 receptor family respond to infection by the activation of the transcription factor NF-κB (72). During inflammation, it has been shown to be secreted from monocytes, macrophages, B lymphocytes, endothelial cells, epithelial cells, dendritic cells, fibroblasts, keratinocytes, Langerhans cells, neutrophils and astrocytes (73). IL-1 causes chemotaxis by inducing IL-8 production.

The cytokine acts on a wide variety of cells including monocytes/macrophages, where it induces the expression of TNF-α, IL-6 and itself. IL-1 stimulation of T cells results in IL-2 and IL-4 expression and on B lymphocytes, the cytokine is responsible for the proliferation and maturation of these cells (74).

1.2.3 IL-6

IL-6 is produced by lymphoid and non-lymphoid cells, including activated helper T cells, monocytes, macrophages and fibroblasts. Several tumour cell lines produce IL-6 constitutively (73). It functions primarily to stimulate cell proliferation, the maturation of B lymphocytes and the secretion of immunoglobulin from these cells. With IL-1, it acts as a co-stimulator of T_H cells.

It has been shown to rescue cells which are dying by apoptosis. It has been shown that T cells express *bcl-2* immediately following isolation from the host, but after 24 hours in culture, expression is undetectable and the cells die. If, on the other hand, T cells are incubated with endothelial cell supernatants containing IL-6, *bcl-2* is not downregulated and the cells survive. IL-6 did not induce proliferation of these cells and therefore, it appears to be involved in the survival of cells (75).

IL-6 has been shown to increase the permeability of endothelial cells *in vitro* (76). The cytokine is upregulated at the transcriptional level by activation of the transcription factors AP-1, c-fos, cyclic AMP and NF-κB.

1.3 IκB

The IκB family of proteins are located in the cytosol of cells and one of their functions is in the regulation of the nuclear transcription factor NF-κB. A number of IκB genes exist, encoding the proteins IκB-α, β, γ and ε, all of which contain multiple copies of an ankyrin repeat motif (77). The DNA binding activity and cytoplasmic sequestration derive from protein-protein interactions between the ankyrin repeats of IκB and the Rel homology domain of the transcription factor NF-κB. IκB-α preferentially binds RelA of NF-κB. The degradation of IκB-α is associated with phosphorylation at Ser 32 and Ser 36 by the IκB–kinase complex (IKK) and NF-κB inducing complex (NIK) of proteins (71). Phosphorylation is followed by ubiquitination by a ligase complex, which adds ubiquitin groups to lysine residues 21 and 22 on IκB-α. Persistent activation of NF-κB is associated with IκB-β activation, while more transient signals involve the activation of IκB-α.

1.4 NF-κB

NFκB is a dimeric transcription factor which exists in a latent form in the cytoplasm of unstimulated cells, complexed to an inhibitor protein IκB. Five DNA binding subunits are currently known, RelA(p65), RelB, c-Rel, p50 and p53. The subunits are all highly related over an approximately 300 residue amino terminal DNA binding and dimerization domain, termed the Rel homology domain (RHD). The predominant form of NF-κB is a complex of p50/RelA (78). Upon stimulation, it is rapidly released from IκB, where it is free to translocate to the nucleus and activate target genes, by binding to κB elements in their promoters. NF-κB activates a wide array of genes in response to a diversity of stimuli, including those that promote inflammation and apoptosis.

In endothelial cells, translocation of NF-κB to the nucleus is important for stimulated transcription of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 and for the cytokines IL-6 and IL-8. Each of these molecules possess NF-κB promoter/enhancer elements in their gene sequence (62). The regulated expression of these proteins initiates the inflammatory cascade. In endothelial cells, TNF-α stimulation has been shown to result in the translocation of the RelA subunit of NF-κB to the nucleus following IκB

phosphorylation and degradation. RelA forms a functional RelA-p50 complex in the nuclei (62).

One method of inducing NF-κB may be due to endoplasmic reticulum (ER) overload caused by an excess of proteins in the membrane of the ER. This may result in the release of intracellular Ca^{2+} ions which leads to the accumulation of reactive oxygen intermediates (ROIs) which can lead to NF-κB activation by an unknown method.

The activation of the transcription factor NF-κB was detected in the human hepatoma cell line HepG2 infected with DEN 1. Activation coincided with viral protein synthesis. Infectious virus particles were released into the culture medium (79). NFκB has been shown to be activated in the cell line ECV304 (80).

1.5 Endothelial Cells

DEN 2 infection of the endothelial cell and it's effects on the cell's immune response and viability is one of the objects of this study. Endothelial cells are an epithelial tissue, which originate from the mesoderm during development. The endothelium lining blood vessels and the mesothelium, lining certain body cavities are examples of simple squamous epithelium. Both have boundary layer properties, which include inducible, reversible permeability increases. These cells line blood vessels and regulate the passage of molecules across the vessel wall into the surrounding tissues (81). Endothelial cells play an active role in hemostasis, inflammatory reactions and immunity. When exposed to cytokines, the cells become activated and the induction of gene expression and protein synthesis follows (82). They are also a source of cytokines, including TNF-α, IL-1 and IL-6 (81). TNF-α and IL-1 increase the permeability of endothelial monolayers to macromolecules (83). Cytokines can also initiate angiogenesis, or the formation of new blood vessels. *In vitro* studies will often involve the culture of primary human umbilical vein endothelial cells, as these cells are more easily obtained than endothelial cells from other sites of the body. However, in culture, organ-specific differences in endothelial cells are retained and as a result they may be a poor model for microvascular endothelium (59, 84). Primary and transformed endothelial cells have also been shown to have a differential response to stimuli such as cytokines (85-87). HUVEC have been shown to be susceptible to infection by DEN 2, as have the cell line ECV304/T-24 (46).

In addition, endothelial cell activities are modified by the state of confluency of the cells. The functional state of interendothelial cell junctions can be changed by activation of the cells. In the adult, under normal physiological conditions, the endothelium is a confluent monolayer (88).

1.5.1 ECV304/T-24 cells

The cell line ECV304 was originally described as a spontaneously transformed endothelial cell line derived from the umbilical cord of a new-born Japanese female and was the only commercially available endothelial cell line. The cells bore many of the characteristics of endothelial cells including the responsiveness to pro-inflammatory cytokines and the resulting increase in vascular permeability. These cells were found to be more easily handled and rendered highly reproducible results (46). These cells, along with many transformed cell lines lacked the ability to produce the pro-inflammatory, growth-inhibiting cytokine, IL-1 or the expression of Factor VIII (89). ECV304 cells express ICAM-1, VCAM-1, MHC class I, Lymphocyte function associated antigen-3 (LFA-3) and Granule membrane protein-140 (GMP-140) (90-92). The ECV304 cell line has recently been shown to have an identical DNA fingerprint to the squamous carcinoma epithelial cell line T-24 and so is herein referred to as ECV304/T-24 (93, 94). All publications to date which refer to either cell line apply to ECV304/T-24.

1.6 Apoptosis

The controlled or programmed death of cells is an essential mechanism for the growth of multicellular organisms and is used to eliminate unnecessary or harmful cells (95). Upon activation of the programmed cell death pathway, many cells undergo characteristic changes in morphology, including condensation of cytoplasm and chromatin within the nucleus, fragmentation of DNA into nucleosome-length fragments, blebbing of the cytoplasmic membrane and the eventual fragmentation of the cell into membrane-bound bodies. This process is referred to as apoptosis. In the body, these bodies are engulfed by adjacent cells (96, 97). Necrosis, or death due to trauma of the cell, is characterised by an

early loss of membrane integrity, random degradation of DNA and the release of cytoplasmic material into surrounding tissue.

Proteins known to induce apoptosis in cells include the TNF receptor family, when bound by the ligands Fas and TNF. In a simplified way, a signal transduction pathway is induced which involves the binding of the cytoplasmic domain of the receptor, termed the death domain (TRADD) which can bind other cytoplasmic proteins and eventually activate NF-κB or Fas-associated death domain (FADD) (98).

Many viruses have been associated with the killing of host cells by apoptosis, including members of the herpesvirus, adenovirus, poxvirus, baculovirus and alphavirus families (96). It has also been suggested that apoptosis is a protective host response for eliminating virus-infected cells. Dengue virus is no exception and has been shown to induce apoptosis in a variety of cell types *in vitro*.

Infection of Neuro2a cells with either of two isolates of DEN 1, which differed in their membrane fusion properties in mosquito cells lines, induced apoptotic DNA degradation 25 hours post infection, with different kinetics. It was found that the accumulation of viral proteins in the endoplasmic reticulum, rather than virus release, induced stress and led to the death of the cells by apoptosis (99).

Some cases of DHF are accompanied by liver injury. Infection of the human hepatoma cell line HepG2, by DEN 1 resulted in cell death by apoptosis, late in the virus cycle.

1.7 Persistent viral infections

At a cellular level, for viruses to cause persistent infection, the virus must not kill the infected cell and it must prevent the elimination of the cell by the immune response. It has been demonstrated that infection of cells with viruses which induce apoptosis, can be converted to persistent infection by the expression of apoptotic-blocking genes. The alphavirus Sindbis virus, induces apoptosis in non-neuronal cells. However, transfection of these cells with the *bcl-2* gene resulted in a long-term persistent and productive infection. Viral production has also been found to be less in persistent infections than in acute lytic infections (100).

Bcl-2 is an integral membrane protein, localised primarily to the inner mitochondrial membrane (96, 100) and protects a wide variety of cell types from cell-death inducing stimuli. The *bcl-2* gene belongs to a family of genes including *bcl-x, bcl-w* and *mcl-1*. Bcl-2 can inhibit the activation of NF-κB and block the induction of pro-inflammatory genes (101). Two other members of the family of proteins, Bax and Bak have been shown to exhibit protective effects and pro-apoptotic effects (102). A number of hypotheses have been proposed to explain how Bcl-2 blocks apoptosis. Bcl-2 can form heterodimers with the proteins Bax and Bak and sequester them, thereby blocking their death-promoting activity, or alternatively, by mediating downstream events. Bcl-2 has also been postulated to regulate intracellular calcium and a Ca^{2+}-dependent endonuclease is often involved in DNA fragmentation and calcium ionophores can induce apoptosis (96). In viral infections, it has been shown that the *bcl-2* gene functions at an early stage of the virus life cycle; at entry, pretranscriptional events, or transcription, to inhibit virus replication (103).

CHAPTER TWO

Materials and Methods

2.1. MATERIALS

2.1.1 Cell Culture

2.1.1.1 Human cells

The human bladder carcinoma cell line ECV304/T-24, was kindly provided by Dr. L. J. O'Neill, University of Dublin, Trinity College. HUVEC were purchased from TCF Biologicals and also given as a generous gift by Dr. G. Mackerel, University College Dublin. The pro-monocytic cell line U937, was donated by Dr. Tom Cotter, Dept. of Immunology, St. Patrick's College, Maynooth. Primary adherent monocytes were obtained from H. Croxon, Irish Blood Transfusion Service Board. Buffy coats were prepared from fresh whole blood donations group A+B+ and maintained at 22°C for 48 hours prior to use for completion of pathogen testing.

2.1.1.2 Mosquito cells

C6/36 *Aedes albopictus* mosquito ovarian cell line was supplied by Dr. Colin Leake, London School of Hygiene and Tropical Medicine. AP61 *Aedes pseudoscutellaris* mosquito cells were obtained from Dr. V. Deubel, Institut Pasteur, Paris.

2.1.2 Cell culture media and additions

Dulbecco's Modified Eagle's medium, Leibovitz-15 and RPMI-1640 were obtained from Gibco BRL, Paisley, Scotland. Medium-199 was obtained from Sigma Diagnostics Ltd., St. Louis, MO, U.S.A. All media additions were obtained from Gibco BRL. Heparin was purchased from Leo Laboratories Ltd., Bucks., U.K. Tryptose phosphate broth was purchased from Oxoid, Unipath Ltd., Hampshire, U.K. Benoxypenicillin was obtained from Britannia Pharmaceuticals Ltd., Surrey, England and Streptomycin Sulphate BP was obtained from Evans Medical Ltd., Sussex, U.K. Trypan Blue was obtained from Flow Laboratories Inc., VA, U.S.A.

Cell culture plastics were obtained from Corning Costar, Bucks., U.K. Falcon chamber slides were purchased from Becton Dickinson Labware, NJ, U.S.A.

2.1.3 Cytokines and RT-PCR chemicals

Recombinant IL-1α, TNF-α, IFN-γ and TGF-β were obtained from R and D Systems Inc., MN, U.S.A. All enzymes used in molecular biology studies were purchased from Promega Corporation, WI, U.S.A or New England BioLabs, MA, U.S.A. G6PD primers were purchased from Stratagene, CA., U.S.A. ART tips used in RT-PCR studies were obtained from Molecular Bio-Products Inc., San Diego, CA, U.S.A. [γ-^{32}P]ATP was obtained from Amersham International plc, Amersham, U.K.

2.1.4 Fine chemicals

All chemicals for general use were of analytical grade and were obtained from BDH Laboratories, Poole, England and Sigma Chemical Co., St. Louis, MO, U.S.A.

2.2 METHODS

2.2.1 Cell Culture

2.2.1.1 Human ECV304/T-24 bladder carcinoma cell line

ECV304/T-24 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) supplemented with 10% Foetal Calf Serum (FCS), 3.29mM L-Glutamine, non-essential amino acids and Penicillin(100U/mL)/Streptomycin(100µg/mL) and were incubated at 37°C with 5%CO_2.

Passage of ECV304/T-24 cells

Cells were washed twice in 1X phosphate buffered saline (PBS), pH 7.4 and incubated in 0.25% Trypsin/EDTA in PBS for 10 min at 37°C. DMEM containing 10% Foetal Calf Serum was added to neutralize the solution and the cells were centrifuged at 1,300 r.p.m. for 10 min. The pellet was resuspended in 1 mL of DMEM containing 10% Foetal Calf Serum and the cells were counted using a Neuerbach haemocytometer.

Confluence of endothelial cells

Exponentially growing ECV304/T-24 cells were seeded at 0.05 times the density of confluent cells to render a final density of $1.33x10^5$cells/cm^2, unless otherwise stated and infected by virus 24 hours post seeding.

Polarised confluent cells were seeded onto 24-well tissue culture plates (Costar), pretreated with Fibronectin at a density of $6.1x10^5$cells/cm^2, as previously described (46). The cells were gently washed with medium 3 hours post addition to the wells, to remove any non-adherent cells. The monolayer was washed daily with medium and infected 3 days post seeding.

2.2.1.2 Human umbilical vein endothelial cells (HUVEC)

Pooled HUVEC were obtained from two sources. Cells were purchased from TCF Biologicals and were cultured in the recommended manufacturer's EGM-2 without hydrocortisone. Pooled HUVEC which had been kindly provided by Dr. G. Mackerel,

University College Dublin, were grown in Medium-199 containing Earle's salts and Hepes buffer, which was supplemented with 20% Foetal Calf Serum, endothelial cell growth factor (Sigma), 70U/mL Heparin and Penicillin(100U/mL)/Streptomycin(100µg/mL) and were incubated at 37°C with 5% CO_2.

Passage of HUVEC

HUVEC were washed twice in M-199 without FCS and trypsinized using 0.25% Trypsin/EDTA in PBS for 10 min at 37°C. Exponentially growing HUVEC were never grown to more than 80% confluence, which facilitated the passage of the cells. HUVEC were passaged at one-third the density of these 'confluent' cells and infected at 24 hours post seeding.

2.2.1.3 Primary adherent monocytes

Human buffy coats were prepared from random healthy whole blood donations by the Irish Blood Transfusion Service Board. Samples were never exposed to temperatures lower than room temperature, which prevented lysis of the erythrocytes present. Two buffy coats from different donors were pooled and diluted with an equal volume of serum-free RPMI-1640 medium containing 2.5mM Hepes and Penicillin(100U/mL)/ Streptomycin(100µg/mL). Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats by isopyknic cushioning. An aliquot of diluted buffy coat was layered onto an equal volume of Ficoll-Isopaque (density 1.077g/mL) and centrifuged at 900 x *g* for 20 min in 17x100mm Elkay tubes. Two volumes of the resulting PBMCs were added to one volume of Ficoll-Isopaque and centrifuged as above. The resulting PBMCs were washed to remove contaminating platelets, counted using a haemocytometer and seeded at a density of $2x10^6$cells/mL into fibronectin-treated 24-well tissue culture plates. Cells were cultured in RPMI-1640 containing 10% Foetal Calf Serum, 2.5mM Hepes, Penicillin(100U/mL)/Streptomycin(100µg/mL) and incubated at 37°C and 5% CO_2. The medium was changed daily to remove any non-adherent lymphocytes or platelets.

Elution of primary adherent monocytes

When counting monocytes, ice cold EDTA (0.67%) in PBS pH 7.4, was added to the well for 10 min and cells were counted using a haemocytometer.

2.2.1.4 Fluorescence activated cell sorting (FACS) analysis of primary adherent monocytes

Monocytes were seeded onto Ultra Low-Cluster 24-well plates (Costar) and incubated overnight at 37°C with 5% CO_2. Cells were collected, centrifuged at 1,300 r.p.m. and resuspended in PBS. The solution of cells was divided into three groups. The first group was incubated with murine anti-IgG1 antibody (as a negative control); the second with human anti-CD11b antibody and the remaining cells were incubated with human anti-CD14 antibody. All cells were shielded from light and incubated at room temperature for 10 min. CD11b and CD14 are cell surface ligands, found in conjunction only on the surface of human monocytic cells. All antibodies were labeled with the fluorescent molecule, phytoerythrin. The cells were washed in PBS (free from Ca^{2+} or Mg^{2+}), containing sodium azide and centrifuged at 2,000 r.p.m. for 5 min. This was repeated and the cells were resuspended in 100µL of PBS. The cells were then fixed with 3% paraformaldehyde (PFA) in PBS and the fluorescence was detected using a Becton Dickinson cell sorter.

2.2.1.5 Human U937 pro-monocytic cell line

U937 cells were cultured in RPMI-1640 medium containing 10% FCS, 2.5mM Hepes buffer and Penicillin(100U/mL)/Streptomycin(100µg/mL). Cells were seeded at a density of $3x10^5$ cells/mL and cultured at 37°C in 5% CO_2.

2.2.1.6 C6/36 mosquito cells

C6/36 *Aedes albopictus* mosquito cells were grown in Leibovitz L-15 medium, supplemented with 5% Foetal Calf Serum, 10% Tryptose Phosphate Broth and Penicillin (100U/mL)/Streptomycin (100µg/mL). C6/36 cells were cultured at 28°C. L-15 containing 1% FCS was used during infection studies, which impeded the growth of the cells.

2.2.1.7 AP61 mosquito cells

AP61 *Aedes pseudoscutellaris* mosquito cells were propogated in Liebovitz L-15 medium, supplemented with 10% Foetal Calf Serum, and 0.55% tryptose phosphate broth. L-15 containing 2% FCS was used during infection studies.

Passage of mosquito cells

Confluent cells were washed twice in 1X PBS and dislodged from the surface using a cell scraper (Costar). C6/36 and AP61 cells were passaged at 0.25 times the density of confluent cells.

2.2.2 Dengue Virus

Clinical Dengue virus serotype 2 (DEN 2) isolates (1232 from Jakarta, Indonesia), were kindly provided by Dr. Rico Hesse, Yale University School of Medicine. Stock DEN 2 was passaged twice in mosquito cells prior to use in infection studies.

2.2.2.1 DEN 2 infection of human cells

ECV304/T-24 cells were infected at a multiplicity of infection (MOI) of 1, 4 or 10 $TCID_{50}$ per cell. Cells were washed with PBS, pH 7.4 and incubated for 90 min in a minimum volume of DMEM supplemented as above, containing DEN 2, at 37°C in 5%CO_2. Cells were gently agitated every 15 min. At 90 min post addition of DEN 2 to the cells, the virus was eluted from the cells and the optimal volume of medium was replaced. HUVEC were infected with DEN 2 in the same manner as ECV304/T-24 cells, replacing DMEM with Medium-199.

Primary adherent monocytes and U937 cells were infected with DEN 2 viral-immune complexes in RPMI-1640. DEN 2 was incubated with pooled hyperimmune antiserum to all four serotypes of DEN (1:40,000) at 37°C for 15 min prior to addition of the virus to the cells. The antiserum used was kindly provided by Dr. B. Innis, AFRIMS, Bangkok.

2.2.2.2 Titre calculations as $TCID_{50}$/mL

A ten-fold dilution series of whole supernatants containing DEN 2, was prepared in Leibovitz L-15 containing 1% FCS and was added in duplicate to monolayers of C6/36 cells grown in 96-well tissue culture plates. Cells were incubated for 8 days post infection at 28°C. As the concentration of virus decreased, the final well showing a cytopathic effect was given a nominal value of having 1 virus present. The concentration of the original virus solution per mL was determined from the dilution of virus within this well. Titres of stock DEN 2 were of the order of 10^7 or 10^8 $TCID_{50}$/mL.

2.2.2.3 Titre calculations as focus forming units/mL

The titre of infectious viral progeny secreted into the supernatant, as the number of focus forming units observed in infected AP61 mosquito cell monolayers, was determined as previously described (40, 104). AP61 cells were seeded at 1/10 the density of confluent cells into tissue culture-treated 24-well plates.

Confluent AP61 cell monolayers were exposed to supernatants containing DEN 2 diluted in Leibovitz L-15 medium containing 2% FCS. Infected monolayers were incubated for 2 hours at 28°C. The cells were then overlayed with an equal mixture of carboxymethylcellulose (CMC) and Leibovitz L-15 medium and incubated at 28°C for 6 days. The cells were then fixed in 3% PFA in PBS and incubated with DEN 2-specific hyperimmune mouse ascitic fluid (1:150), which was kindly provided by Mme. M.-T. Drouet, Institut Pasteur, Paris, France. Foci of infected cells were detected using HRP-conjugated anti-murine IgG antibody (1:100).

Results of stock DEN 2 titres have shown that a titre of 10^7 $TCID_{50}$/mL using the C6/36 viability assay, was equivalent to a titre of $5x10^6$ ffu/mL on AP61 cells.

2.2.2.4 Inactivation of DEN serotype 2, using β-propiolactone

DEN 2 inactivation was performed by exposing the virus to the inactivating agent β-propiolactone (BPL) (Sigma Diagnostics Ltd., MO, U.S.A.), as previously described (105, 106). To ensure the virus was inactivated, with the minimum of toxicity to cells, titration

curves of BPL concentration with time of exposure and buffer composition were prepared. The levels of safety and inactivity were determined as those, which did not result in a cytopathic effect to exposed C6/36 monolayers, as described in section 4.2.7. The optimal conditions were found to be 48 hours incubation of DEN 2 at room temperature in 0.06% BPL resuspended in Tris buffer, pH 7.5.

2.2.2.5 Detection of intracellular DEN 2 antigen expression using indirect immunofluorescence analysis

Exponentially growing and polarised confluent ECV304/T-24 cells and exponentially growing HUVEC were cultured in 4-chamber culture slides (Falcon) and infected with DEN 2 at a MOI of 1 or 4 $TCID_{50}$/mL. Cells were washed with PBS, fixed with 3% PFA in PBS for 20 min at room temperature, allowed to air dry and frozen at –80°C until analysis. At various intervals after infection, supernatants were collected and stored at –80°C, for use in infectious virus progeny production studies.

Cells were treated with 50mM NH_4Cl in PBS and permeabilized with 0.5% Triton-X-100 in PBS. DEN 2 viral antigen expression in infected cells was detected as previously described (40, 104), by incubating the cells with DEN 2-specific hyperimmune mouse ascitic fluid (1:150) and fluorescein-conjugated goat anti-murine IgG (1:150) in 1% Foetal Calf Serum, which prevented non-specific binding to the cells. Cells were then double-labeled with propidium iodide (see section 2.2.4.1) to examine the nuclei of infected cells. Fluorescence was detected using a fluorescent microscope (Leitz, Rockleigh, N.J.) or confocal microscope (Zeiss Axiovert 100TV).

2.2.3 Cell Viability studies

2.2.3.1 Trypan Blue exclusion assay

Exponentially growing and polarised confluent ECV304/T-24 cells were grown on either tissue culture treated flasks ($25cm^2$), mini petri-dishes ($9.5cm^2$) or 24-well plates (Costar) and infected with DEN 2 virus. Control groups consisted of 'mock infected' counterparts. ECV304/T-24 cells were trypsinized as described in section 2.2.1.1 and an aliquot of the resuspended cells was diluted in an equal volume of filter-sterilized 0.4% Trypan Blue in 0.85% saline. The mixture was placed onto a haemocytometer and the number of viable cells (which excluded the dye) was counted.

2.2.3.2 Lactate Dehydrogenase (LDH) assay

Exponentially growing and polarised confluent ECV304/T-24 cells and primary adherent monocytes were cultured as described above. Cells were infected with DEN 2 and at regular intervals post infection, the supernatants were removed and any detached cells were collected by centrifugation at 1,300 r.p.m. The monolayer was washed with PBS, pH 7.4 and the cells were lysed by the addition of $50\mu L/cm^2$ of lysis buffer (0.1%Triton-X-100/0.1%BSA in PBS) to the cells. Lysis buffer was also added to the pelleted cells, which had been released into the medium. 50μL of the resulting cell lysate was added to 1mL of the Lactate Dehydrogenase reagent LD-L (Sigma) and incubated in a quartz cuvette at 30°C. The activities of the cytosolic enzyme LDH in both infected and 'mock infected' cells were determined spectrophotometrically using a Cecil CE 2020 spectrophotometer. The absorbance of the solution was calculated at 2 min intervals at a wavelength of 340nm and the activity of the enzyme in U/L was determined.

2.2.4 Apoptosis studies

2.2.4.1 Propidium iodide staining

Following staining for intracellular DEN 2 antigen expression (section 2.2.2.5), the cells were then treated with 200µg/mL of RQ1 DNAse-free RNAse A, covered with ApopTag plastic coverslips and incubated in a humidified chamber at 37°C for 1 hour. Nuclei were stained with 20µg/mL propidium iodide in PBS and incubated at room temperature for 10 min. The slides were washed in PBS, mounted in mounting medium (Sigma) and observed under a fluorescent microscope.

2.2.4.2 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis

Exponentially growing ECV304/T-24 cells were seeded into 4-chamber culture slides (Falcon) and infected with DEN 2. Cells were washed with PBS, fixed in 3% paraformaldehyde in PBS, air dried and frozen at –80°C until use. Cells in each chamber were permeabilized with 0.5% Triton-X-100 in PBS rinsed with PBS and incubated with 50µL TUNEL reaction mixture (Boehringer Mannheim, GmbH) in a humidified chamber at 37°C for 30 min, according to the manufacturer's instructions. The reaction mixture consisted of 5µL of TUNEL enzyme with 45µL of reaction buffer, which contained fluorescein-labeled dUTP and unlabeled d-NTPs. Slides were rinsed with PBS, mounted using mounting medium and observed under a flourescent microscope.

2.2.4.3 Differential staining of cytoplasm and nucleus

Exponentially growing ECV304/T-24 cells were seeded onto tissue culture treated dishes and infected with DEN 2. Supernatants were collected and any detached cells were centrifuged at 1,300 r.p.m. for 10 min and resuspended in 100µL of DMEM containing 10% FCS. Monolayers were washed with PBS and trypsinized as described in section 2.2.1.1. The pelleted cells were counted and the volume of medium adjusted, to render a final concentration of $3x10^5$cells/mL. A 100µL aliquot of the monolayer cells and all of the

resuspended detached cells were spun onto poly-L-Lysine coated slides (Sigma) using a cytospin at 500 r.p.m. for 2 min.

The cells were fixed in methanol and stained with Eosin Y and Methylene Blue and mounted using DPX mountant. Cells were observed using a light microscope and photographed using Kodak Gold 200 film.

2.2.5 Cytokine expression studies

2.2.5.1 Total cellular RNA isolation

Adherent cells such as exponentially growing and polarised confluent ECV304/T-24 cells, HUVEC and primary adherent monocytes were grown on 9.5cm^2 tissue culture treated mini petri-dishes. At fixed time intervals post infection, the supernatants were collected and the monolayers were washed with PBS, pH 7.4. A 1mL aliquot of RNA Isolator fluid (Genosys) which contains guanidinium isothiocyanate and phenol was added to each petri-dish and the total cellular RNA was isolated according to the manufacturer's instructions. The homogenate was transferred to a microcentrifuge tube (Costar) and incubated for 5 min at room temperature to ensure complete lysis of the cells had occurred. Samples were then frozen at –80°C until use.

Upon defrost, 200µL of chloroform was added to the lysate and vigorously mixed. Samples were incubated at room temperature for 15 min and subsequently centrifuged at 12,000 x *g* for 15 min at 4°C. Following centrifugation, 400µL of the upper aqueous layer was transferred to a new tube, mixed with 500µL of isopropanol, incubated at room temperature for 7.5 min and centrifuged at 12,000 x *g* for 10 min at 4°C.

The pelleted RNA was washed with 1mL of 75% RNAse-free ethanol and centrifuged at 7,500 x *g* for 5 min at 4°C. The pellet was then air-dried and resuspended in 50µL of 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated water. To ensure that the RNA was completely in solution, the solution was incubated at 55-60°C for 10 min, pipetted repeatedly and subjected to cycles of freezing at –80°C and thawing at 37°C. RNA was then frozen at –80°C until use.

For non-adherent U937 cells, a maximum of $5x10^6$cells were centrifuged at 1,300 r.p.m. for 10 min and 1mL of RNA Isolator was added to the pelleted cells. The lysate was transferred to a microfuge tube and the RNA was isolated as above.

Standardisation of total cellular RNA

The concentration of RNA isolated was calculated by spectrophotometric analysis at a wavelength of 260nm, adopting an OD_{260} reading of unity to be equivalent to 40μg/mL for denatured RNA. Volumes were adjusted with Nuclease free water (Promega) to render a final concentration of RNA of 4ng/mL.

2.2.5.2 Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Four nanograms of total cellular RNA was reverse transcribed and the mRNA of the gene of interest was amplified, using the one-step Access Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) system (Promega) as shown in Fig. 2.1. Primers for the genes β–Actin, Tumour Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6) and Interleukin-1α (IL-1α) were purchased from King's College, London. Glucose-6-Phosphate Dehydrogenase (G6PD) primers were purchased from Stratagene. Primers were chosen which crossed splice sites, to ensure that any possible contaminating genomic DNA would not be amplified. See Table 2.1. Each reaction mix contained TAE reaction buffer, 0.2mM of each dNTP, 50pmol of each primer, 0.5mM $MgSO_4$, 5U AMV Reverse Transcriptase and 5U *Tfl* Polymerase and nuclease-free water, to render a final volume of 50μL. One drop of mineral oil (Sigma) was added to each tube to prevent evaporation of the samples during amplification in a thermocycler (Perkin Elmer).

Optimal thermocycler conditions

For reverse transcription and amplification of the mRNA of the genes TNF-α, IL-6 and IL-1α, each reaction mix was subjected to one cycle each of 48°C for 45 min, 94°C for 5 min and 60°C for 5 min. This was followed by cycles of amplification of 70°C for 2 min, denaturation at 94°C for 45 sec and annealing at 55°C for 1 min 15 sec. The number of cycles differed for each primer pair, with TNF-α and IL-1α each requiring 33 cycles and IL-6 requiring 29 cycles. The G6PD primer pair was subjected to one cycle each of 48°C

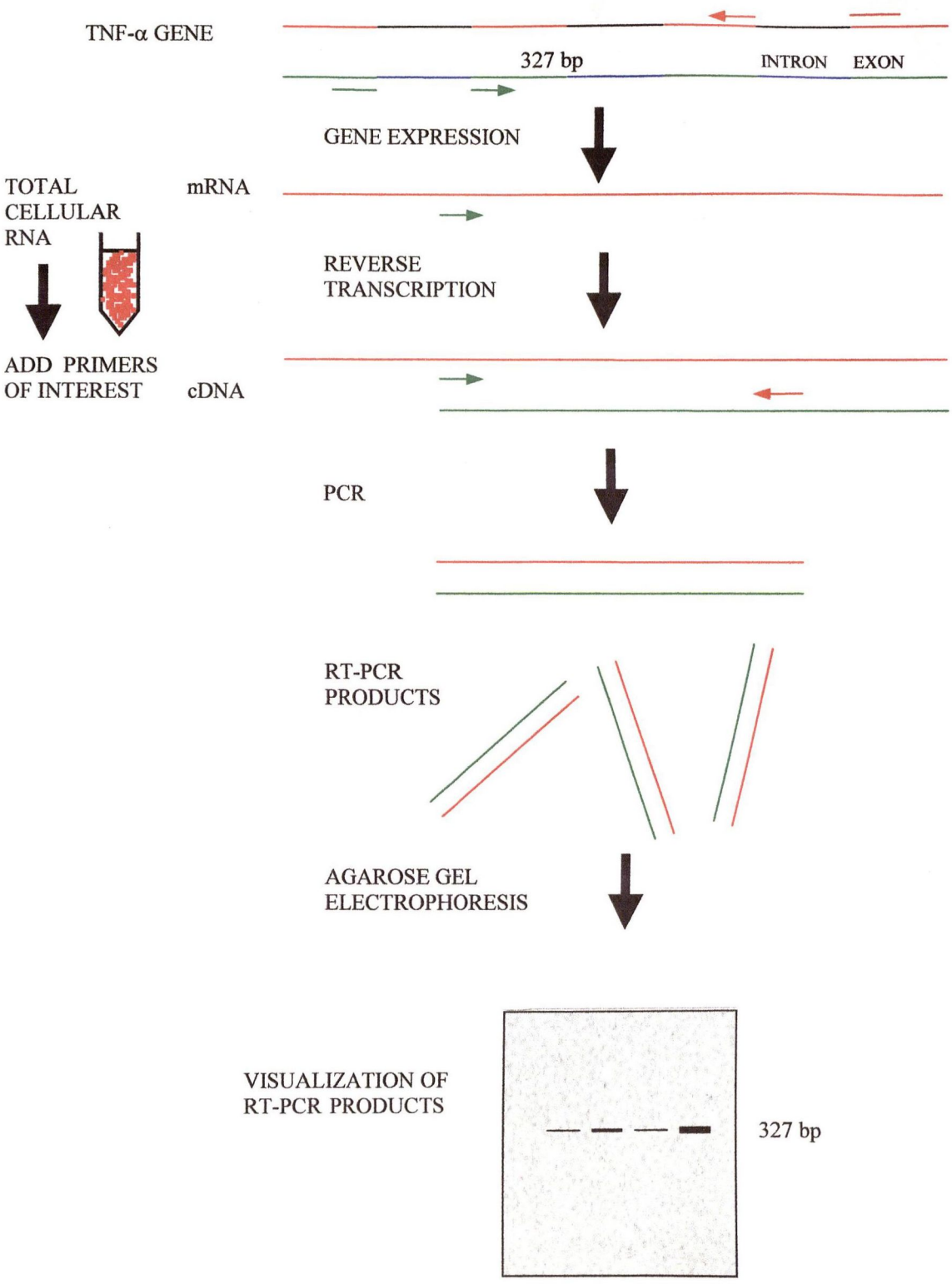

Fig 2.1 Schematic representation of semi-quantative Reverse Transcriptase Polymerase Chain Reaction.

Table 2.1. Sequences of primers used to amplify the genes of interest in the reverse transcriptase-polymerase chain reaction.

Gene	Oligonucleotide sequence	
β-Actin	Sense:	5'-CCTTCCTGGGCATGGAGTCCT-3'
	Antisense:	5'-GGAGCAATGATCTTGATCTTC-3'
G6PD	Sense:	5'-TTCTTCAACCCCGAGGAGT-3'
	Antisense:	5'-GGGAAGGAGGGTGGCCGTG-3'
TNF-α	Sense:	5'-CAGAGGGAAGAGTTCCCCAG-3'
	Antisense:	5'-CCTTGGTCTGGTAGGAGACG-3'
IL-6	Sense:	5'-CCTTCTCCACAAGCGCCTTC-3'
	Antisense:	5'-GGCAAGTCTCCTCATTGAATC-3'
IL-1α	Sense:	5'-GAGAGCATGGTGGTAGTAGCAACC-3'
	Antisense:	5'-CCCTGCCAAGCACACCCAGTAGTC-3'

for 45 min, 94°C for 5 min and 60°C for 5 min. This was followed by 33 cycles of amplification of 72°C for 1 min 30 sec, 94°C for 45 sec and 60°C for 45 sec. An extension step of 72°C for 10 min for each primer pair completed the reaction.

2.2.5.3 Agarose gel electrophoresis

RT-PCR products were visualized by electrophoresis on a 2% agarose gel. Two grams of agarose (SeaKem LE, FMC Bioproducts, ME, U.S.A.) was added to 100mL of a 1X TBE buffer and melted using a bunsen burner. The solution was cooled and re-heated to optimize the cross-linking of the agarose. This was then cooled to 60°C and ethidium bromide at a final concentration of 500ng/mL was added. The gel was cast in a horizontal tray (BioRad) and a 1.5 mm thick, 20-well comb (BioRad) was used to prepare the wells into which 10μL of each sample mixed with 2μL of loading dye (Promega) was added. 10μL of a 100 base pair DNA ladder (Promega) was added to each gel as an indicator of the fractionated product sizes. The gels were electrophoresed at 150V for 90 min in a 1X TBE running buffer.

2.2.5.4 Visualization of RT-PCR products

The gel was then viewed using a UV transilluminator and photographed onto thermal imaging paper (UVP imaging system). The density of each product was determined using a densitometer (GelWorks software). Negative images were often used to obtain better contrast during densitometric analysis.

2.2.5.5 Southern Hybridization

To ensure the amplified products were specific to the genes of interest, a control experiment was performed in which oligonucleotide probes for the genes G6PD (MWG-Biotech), β-Actin, TNF-α and IL-6 (King's College, London) were hybridized to a nylon membrane onto which the RT-PCR products had been transferred. The probe sequences are given in Table 2.2.

Table 2.2. Sequences of oligonucleotide probes using during Southern hybridization.

Gene	Oligonucleotide sequence
β-Actin	5'-ACGTGGACATCCGCAAAGAC-3'
G6PD	5'-CACTGCTGCACCAGATTGAG-3'
TNF-α	5'-GAAGAGGACCTGGGAGTAGAT-3'
IL-6	5'-CACAGACAGCCACTCACCT-3'

After photographing the gel, it was subjected to partial DNA hydrolysis (100mM HCL) for 20 min at room temperature. The gel was then denatured in a denaturation solution (1.5M NaCl/0.5M NaOH) for 1 hour at room temperature with constant agitation. This was followed by agitation of the gel in neutralization buffer (1M Tris, pH 8/1.5M NaCl) for 1 hour at room temperature. The DNA was transferred onto a nylon membrane (Micron Separations Inc.) overnight by capillary transfer, as previously described (107). The membrane was then baked at 80°C for 2 hours. The membrane was wetted in 10X SSC and then incubated in a pre-hybridization solution (200μL/cm^2), which consisted of 6X SSC, 0.5% SDS, 5X Denhardt's solution and 100μg/mL Salmon sperm DNA at 37°C for 3 hours.

5'-end labeling of the oligonucleotide probes with 50 pmol [γ-^{32}P]ATP at 10,000 c.p.m., was performed using T4 polynucleotide kinase (Promega) and the labeled probe was fractionated in 100μL aliquots using a NAP-5 column (Pharmacia LKB, Uppsala, Sweden). The three hottest fractions were added to the membrane in 50μL/cm^2 pre-hybridization solution and incubated at 37°C for 1 hour.

The membrane was exposed to Kodak diagnostic film (X-OMAT RP), in autoradiography cassettes with intensifying screens for 48 hours at –70°C and any bound probe was detected.

Linearity of RT-PCR

The PCR reaction was halted within the linear phase of amplification, before the plateau effect occurred. Non-competitive RT-PCR relies on the observation that prior to the onset of the plateau effect, there is a linear relationship between the quantity of input RNA and the output of amplified DNA product (108). It was necessary to ensure that an increase in RNA concentration by a factor of 2 or a single increase in the amplification cycle number resulted in a two-fold increase in PCR product visualized on an agarose gel. A full account of the measures taken for the optimization of the conditions necessary for semi-quantitative RT-PCR is given in chapter 4.

2.2.5.6 Enzyme-linked immunosorbant assays (ELISA)

Secreted levels of the human cytokine TNF-α were detected using the DuoSet human TNF-α ELISA (Genzyme Diagnostics, Cambridge, MA, USA) according to the manufacturer's instructions. Supernatants of DEN 2 infected and 'mock' infected cells were collected at regular time intervals post infection and stored at –80°C until use. The levels of secreted TNF-α were compared to a standard curve of recombinant TNF-α, which ranged in concentration from 1000pg/mL to15.625pg/mL as described in section 4.2.8.

2.2.6 IκB-α assay

2.2.6.1 Preparation of whole cell extracts for immunoblot analysis

Immunoblot analysis was performed for the detection of IκB-α and IκB-α which has been phosphorylated at serine 32. ECV304/T-24 cells were seeded at a density of $2.25x10^5$ cells into mini petri-dishes (9.5 cm^2). Three days post seeding, cells were infected with DEN 2 or stimulated with the cytokine Interleukin -1α at a concentration of 10ng/mL.

Whole cell lysates were prepared at regular intervals following addition of the virus or cytokine to the cells, as described previously (109, 110). The mini petri-dishes were placed onto a tray of ice and the medium was aspirated from the monolayers. The cells were washed with ice cold PBS and 100μL of Lysis buffer (62.5mM Tris-HCl pH6.8, 2%(w/v) SDS, 10%(v/v) glycerol, 0.1% bromophenol blue, with 50mM DTT) was added to each well. Cells were removed from the surface using a cell scraper, transferred to a microfuge tube and homogenized by passage through a 21-guage needle. Samples were placed into a boiling water bath for 5 min and frozen at –20°C until use.

2.2.6.2 SDS-polyacrylamide gel electrophoresis and electrotransfer

20μL of each sample was loaded onto a 5% stacking gel and a 10% resolving gel, as described in Table 2.3 and subjected to SDS-PAGE, using a discontinuous gel system. The gel was immersed in an electrophoresis running buffer (25mM Tris-HCl pH 8.3, 0.1%(w/v) SDS and 0.192M glycine) and run for approximately 90 min at 160V. A 6μL aliquot of prestained broad range SDS-PAGE molecular weight markers was run alongside the samples.

Table 2.3. Composition of stacking and resolving gels for SDS-PAGE during IκB-α analysis.

Chemical	Volume added (5% stacking gel)	Volume added (10% resolving gel)
Acrylamide:bisacrylamide (37.5:1)	1mL	5mL
1.5M Tris-HCl, pH 6.8	-	3.8mL
1.0M Tris-HCl, pH 6.8	750µL	-
10% (w/v) SDS	60µL	150µL
10% Ammonium persulphate	60µL	150µL
N,N,N',N'-Tetramethylethylenediamine (TEMED)	15µL	20µL
H_2O	4.1mL	5.9mL

The proteins were electrotransferred onto a nitrocellulose membrane at 18V for 1 hour using a semi-dry transfer apparatus (BioRad). The membrane was soaked in semi-dry transfer buffer (25mM Tris-HCl pH 8.3, 0.192M glycine, 20%(v/v) methanol) for 20 min at room temperature prior to transfer. The membrane and gel were sandwiched by 6 layers of 3M whatman filter paper, which had also been soaked in transfer buffer.

Following transfer, proteins were visualized by staining the membrane with Ponceau-S stain (3%(w/v) trichloroacetic acid, 3%(v/v) sulphosalicyclic acid). The stain was removed by washing the membrane in PBS.

To ensure even loading of each sample, in all experiments, a duplicate gel was run alongside each gel and stained with Coomassie Brilliant blue stain (50%(v/v) methanol, 10%(v/v) glacial acetic acid, 0.25%(w/v) Coomassie Brilliant Blue G-250) for 1 hour at room temperature. This was followed by destaining in destain (50%(v/v) methanol, 10%(v/v) glacial acetic acid) overnight at room temperature with gentle agitation.

2.2.6.3 Western immunoblot detection of IκB-α and phospho-IκB-α (Ser 32)

Non-specific sites were blocked by incubating the membrane overnight at 4°C in TBS (10mM Tris-HCl pH 7.4 and 150mM NaCl) containing 5%(w/v) nonfat dried milk. The membrane was then incubated with primary antibody. Polyclonal anti-human phospho-IκB-α (Ser 32) antibody raised in rabbit, was diluted 1:1,000 times in TBS, 0.05% Tween-20 and 5%(w/v) nonfat dried milk and incubated with gentle agitation overnight at 4°C. Monoclonal anti-human IκB-α antibody raised in mice, was diluted 1:100 times and incubated with gentle agitation overnight at 4°C.

The primary antibody was removed from the membrane and the secondary antibody was added. Goat anti-rabbit IgG, conjugated to HRP was diluted 1:10,000 times in TBS containing 0.05% Tween-20 and 5%(w/v) nonfat dried milk and incubated with the membrane for 1 hour at room temperature, with gentle agitation to detect phospho-IκB-α. Goat anti-murine IgG, conjugated to HRP was diluted 1:1,000 times and used as a secondary antibody for the detection of IκB-α.

The membrane was then washed three times for 5 min intervals with gentle agitation in TBS and 0.05%(v/v) Tween-20 at room temperature.

2.2.6.4 Detection of IκB-α and phospho-IκB-α (Ser 32)

1 mL of SuperSignal Substrate working solution (Pierce) was prepared according to the manufacturer's instructions and incubated with each membrane for 2 min at room temperature. The excess was removed and the membrane was wrapped in saran wrap and autoradiographed using Kodak diagnostic film (X-OMAT RP) at room temperature.

2.2.7 NF-κB activation studies

2.2.7.1 Preparation of nuclear extracts

ECV304/T-24 cells were seeded at a density of $2.25x10^5$ cells into mini petri-dishes (9.5 cm^2). Three days post seeding, cells were infected with DEN 2. At regular intervals post infection, the cells were washed with ice cold PBS and kept on ice. Washed cells were then scraped in 800μL hypotonic buffer (10mM Hepes, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM phenylmethylsulphonylfluoride (PMSF), 0.5mM DTT). Nuclear extracts were prepared as previously described (109, 110).

All subsequent steps were performed on ice using ice cold buffers. Cells were pelleted in hypotonic buffer by centrifugation at 13,000 x *g* for 15 min at 4°C, then lysed in 20μL hypotonic buffer containing 0.1%(v/v) Nonidet P-40 (NP-40) for 10 min on ice. Lysates were centrifuged as above and the pelleted nuclei were resuspended in 15μL nuclear lysis buffer (20mM Hepes, pH 7.9, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25%(v/v) glycerol, 0.5mM PMSF) and incubated for 15 min on ice. The pellet was resuspended in 20μL of Buffer A containing the protein inhibitors PMSF and DTT and 0.1% NP40 and incubated on ice for 10 min. Lysed nuclei were centrifuged as before and resuspended in 50μL storage buffer (10mM Hepes, pH 7.9, 50mM KCl, 0.2mM EDTA, 25%(v/v) glycerol, 0.5mM DTT, 0.5mM PMSF).

Protein concentration was determined according to the Bradford dye binding assay.

2.2.7.2 Labelling of oligonucleotides for electrophoretic mobility shift assay (EMSA)

5'-end labeling of the 22 base pair oligonucleotide containing the NF-κB consensus sequence (5'-GGGACTTTCC-3') was carried out as described (107). A 10pmol aliquot of

the DNA probe which was kindly provided by Dr. K. Fitzgerald, Dept. of Biochemistry, Trinity College, was incubated with 50pmol [γ-^{32}P]ATP at 10,000 c.p.m., 20U T4 polynucleotide kinase and 5μL of 10X kinase buffer (100mM $MgCl_2$, 50mM DTT, 1mM spermidine, 500mM Tris-HCl, pH 7.5). H_2O was added to render a final volume of 50μL and incubated at 37°C for 10 min. The reaction was terminated with 2μL of 0.5M EDTA.

2.2.7.3 κ-light chain consensus site binding

Four micrograms of each nuclear extract was incubated with 10,000 c.p.m. of radiolabeled probe, binding buffer (16%(v/v) glycerol, 10mM EDTA, 50mM DTT, 100mM Tris, pH 7.5, 1M NaCl, 1mg/mL BSA) and non-specific competitor DNA, poly dIdC, at room temperature for 30 min. The reaction was terminated by adding 2μL of storage buffer (see section 2.2.7.1). A 5% polyacrylamide gel was prepared with a composition as described in Table 2.4. The gel was run at 140 V for 30 min prior to the addition of the samples in a 0.5X TBE running buffer. Following the addition of the samples, the gel was run at 140 V for 90 min at room temperature.

After electrophoresis, the gels were placed on whatman filter paper and dried for 30 min at 80°C on a slab gel dryer (Savant SGD 4050). The gel was autoradiographed using Kodak diagnostic film (X-OMAT RP) in autoradiography cassettes with intensifying screens for 48 hours at –70°C.

Table 2.4. Composition of a 5% polyacrylamide gel for use in NF-κB activation studies.

Chemical	Volume added (5% polyacrylamide)
Accugel (40% solution)	3.1mL
10X TBE	2.5mL
DTT (1M)	5μL
N,N,N',N'-Tetramethylethylenediamine(TEMED)	15μL
Ammonium persulphate	50 mg
H_2O	19.3mL

CHAPTER THREE

Effects of DEN 2 Infection on Host Cell Viability

3.1 INTRODUCTION

The aim of this study was to investigate the ability of DEN 2 to infect and replicate in the human ECV304/T-24 bladder carcinoma cell line and primary HUVEC. These cells had been shown to be susceptible to infection (45, 111) and it was decided to determine whether the infection was productive and if the cells became persistently infected. The presence of intracellular virus was investigated using indirect immunofluorescence analysis and titre calculations of infected cells, which were snap-frozen. Secreted infectious viral progeny was detected as $TCID_{50}$/mL or focus forming units/mL on confluent mosquito cell monolayers.

The viability of these cells following DEN 2 infection was studied using the Trypan Blue exclusion dye and Lactate Dehydrogenase assay. The effects of infection on the rate of cell growth and confluence of the cells, was also compared. It was decided to determine whether DEN 2 infection of ECV304/T-24 cells induced programmed cell death/apoptosis in these cells, using differential staining, propidium iodide staining and TUNEL analysis.

3.2 RESULTS

3.2.1 Detection of intracellular DEN 2 viral antigens

3.2.1.1 Exponentially growing ECV304/T-24 cells

Exponentially growing ECV304/T-24 cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 1 or 4 $TCID_{50}$/cell. Infected cells and 'mock' infected negative control counterparts were fixed with 3% paraformaldehyde in PBS at 0, 48, 72, 96, 122 and 145 hours post infection. The presence of intracellular viral antigen was visualized using an indirect immunofluorescence assay.

Cells were incubated with DEN 2-specific hyperimmune mouse ascitic fluid and a fluorescein-conjugated anti-murine IgG, as described in section 2.2.2.5. Viral antigen-containing cells were detected using a fluorescent microscope. The results were obtained from two independent experiments, using two different imaging systems. In the first, 35-mm slides were prepared following observation under a fluorescent microscope (Leitz, Institut Pasteur, Paris) and the second experimental system involved the production of

digital images (BioRad MRC 1024), which were generated using a confocal fluorescent microscope (Zeiss Axiovert 100 TV, Biotechnology Institute, Trinity College, Dublin).

No fluorescence was observed in the 'mock' infected negative control cells, at any of the time points chosen. See Figures 3.1(a), (c), (e) and (g).

At time zero or 90 min post addition of the virus to the cells, no fluorescence was observed within ECV304/T-24 cells infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell, see Fig. 3.1(b). Forty-eight hours post infection, approximately 30% of cells showed intense fluorescence, throughout the cytoplasm of the cell as demonstrated in Fig. 3.1(d). This number had increased to 50% by seventy-two hours shown in Fig. 3.1(f). The maximum number of infected cells was found ninety-six hours post infection with almost 80% of the cells in the monolayer displaying fluorescence, however the pattern of fluorescence had changed to a speckled appearance with the greatest intensity localized to the perinuclear area of the cell. See Fig. 3.1(h). A speckled pattern of fluorescence was seen at 122 hours post infection in almost 80% of the cells. See Fig. 3.1(i). At 145 hours however, the proportion of fluorescent cells had fallen to forming approximately 30% of the monolayer. The fluorescence was diffuse and patchy and restricted to the nuclear region of these cells. The positive cells were observed as foci of fluorescence. See Fig. 3.1(j). A graph depicting the expression of DEN 2 viral antigens within infected cells with respect to time is given in Fig. 3.2.

When ECV304/T-24 cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell, the kinetics of viral antigen expression followed that of cells infected with a MOI of 4 $TCID_{50}$/cell, but the number of infected cells was lower.

3.2.1.2 Polarised confluent ECV304/T-24 cells

Polarised confluent ECV304/T-24 cells were prepared as described in section 2.2.1.1 and were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Cells were fixed in 3% paraformaldehyde in PBS at 48 and 72 h post infection and observed under a fluorescent microscope.

'Mock' infected cells did not display fluorescence as seen in Fig. 3.3(a), whereas approximately 10% of the cells were fluorescent at 48 hours, see Fig. 3.3(b) and this percentage increased to 25% of the monolayer at 72 hours post infection, as shown in Fig.

Detection of intracellular DEN 2 viral antigens in infected exponentially growing ECV304/T-24 cells

Fig. 3.1. Detection of intracellular DEN 2 viral antigens in infected ECV304/T-24 cells using indirect immunofluorescence. Exponentially growing cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. Cells were fixed with 3% PFA, at regular intervals post infection and observed under a fluorescent microscope. (a), (c), (e) and (g) are 'mock' infected control cells at 0, 48, 72 and 96 hours post infection. (b), (d), (f), (h), (i) and (j) are DEN 2 infected cells at 0, 48, 72, 96, 122 and 145 hours post infection. Figs. 3.1 (i) and (j) have been photographed using a digital imaging system (BioRad). The presence of DEN 2 antigens was detected using a fluorescein-conjugated antibody to DEN 2 hyperimmune mouse ascitic fluids.

Fig. 3.1(a)

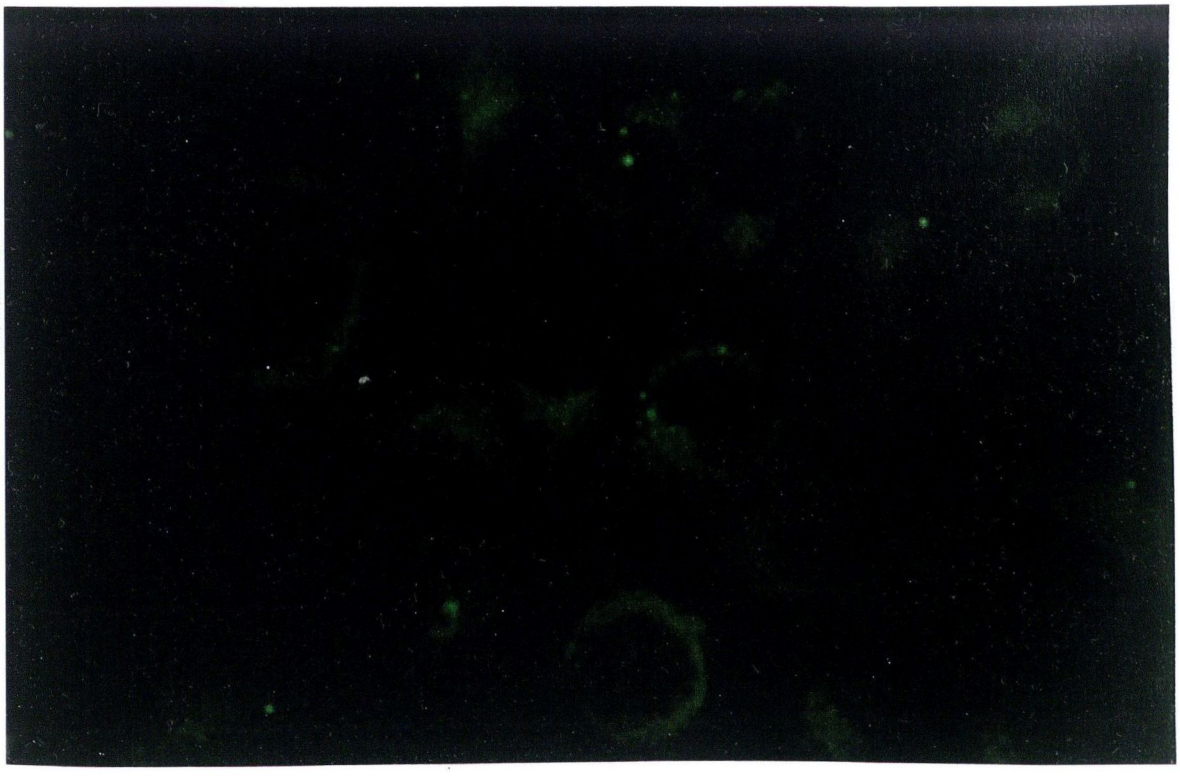

Fig.3.1(b)

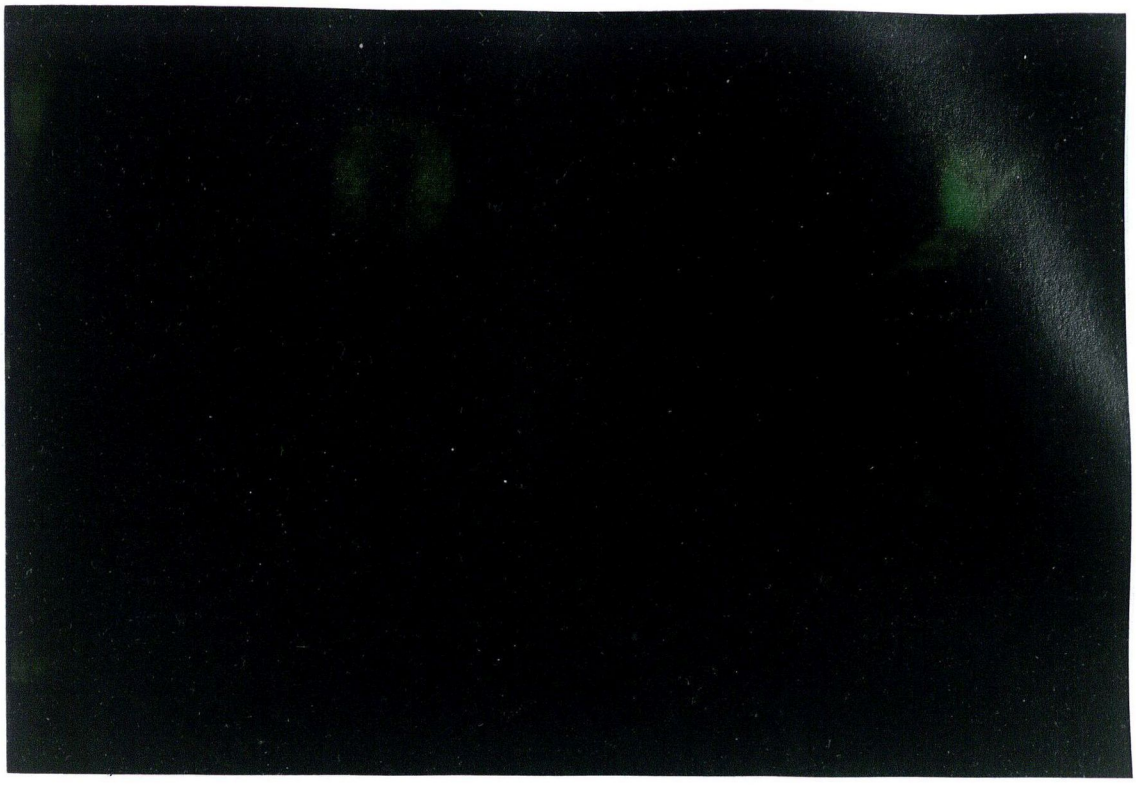

Fig. 3.1(c)

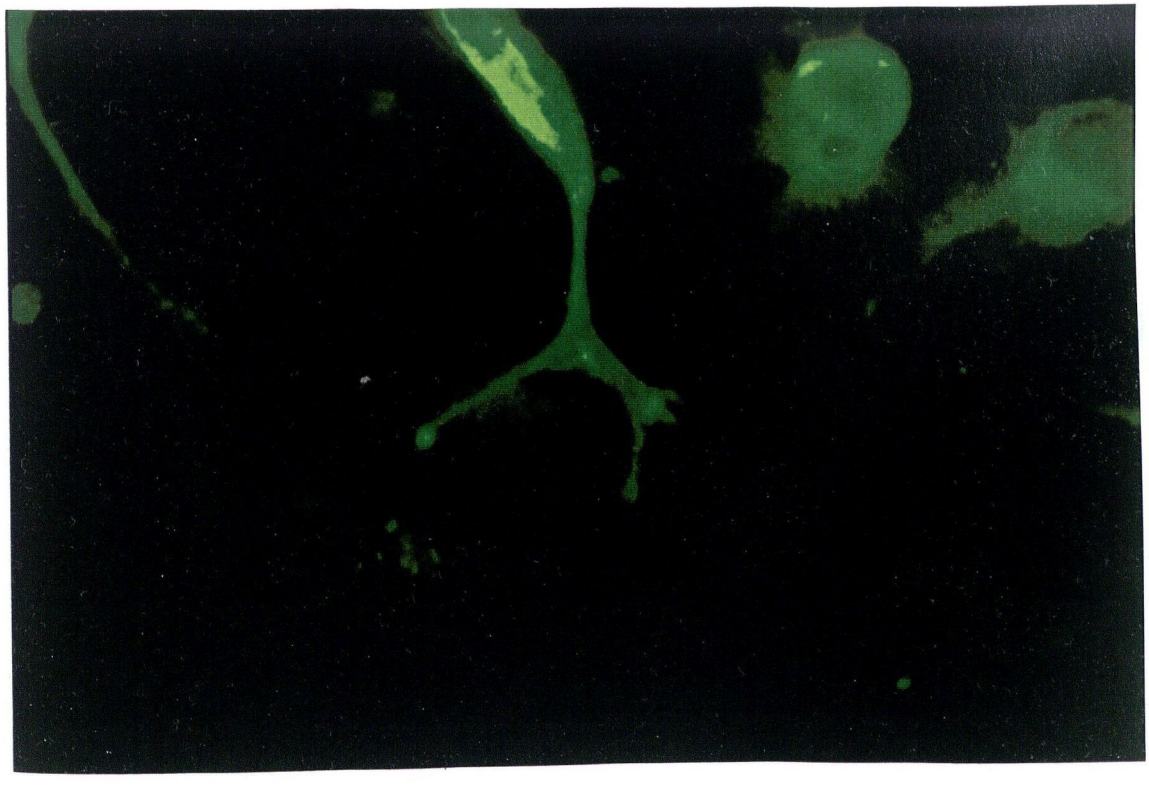

Fig.3.1(d)

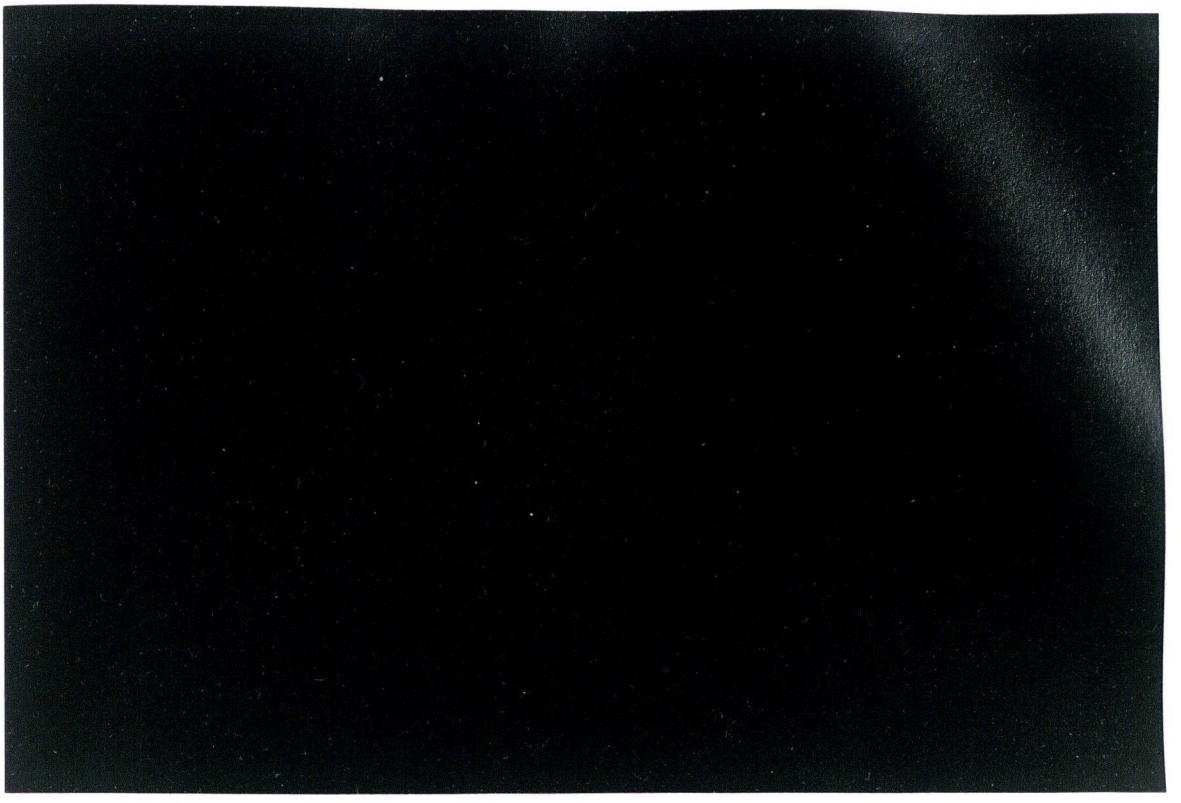

Fig. 3.1(e)

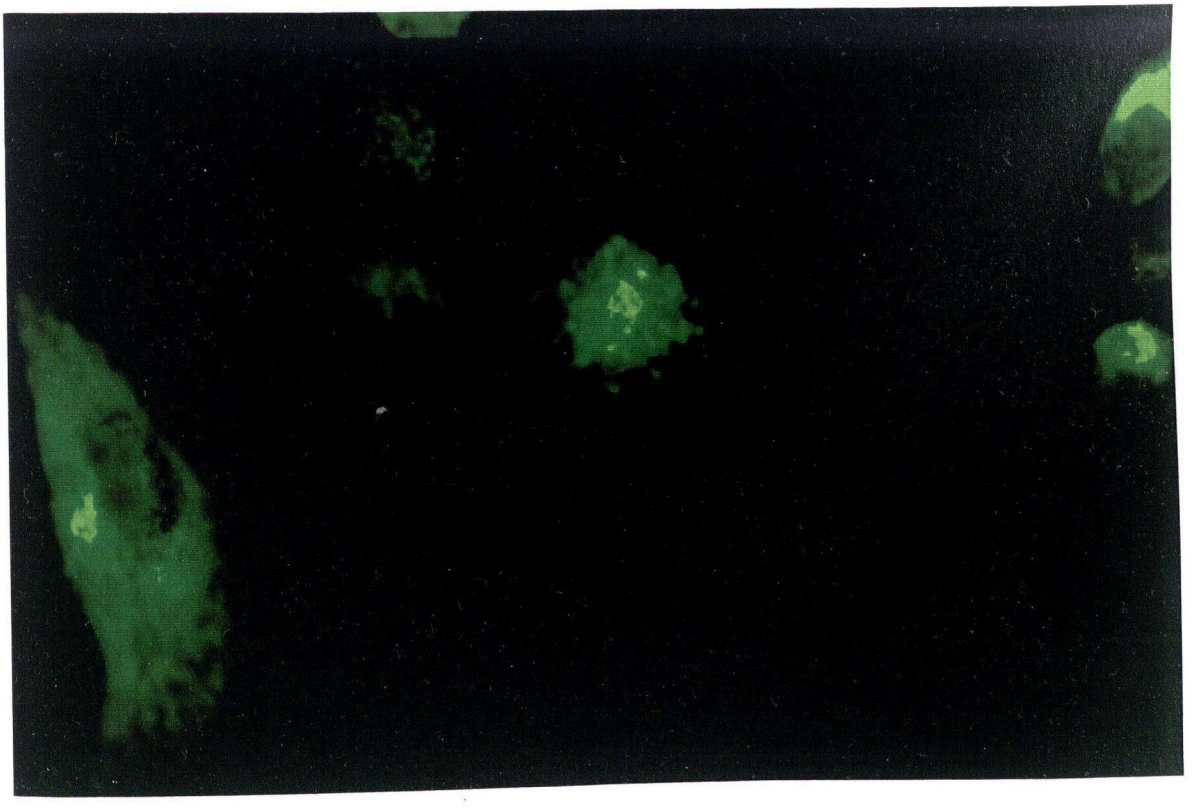

Fig.3.1(f)

Fig. 3.1(g)

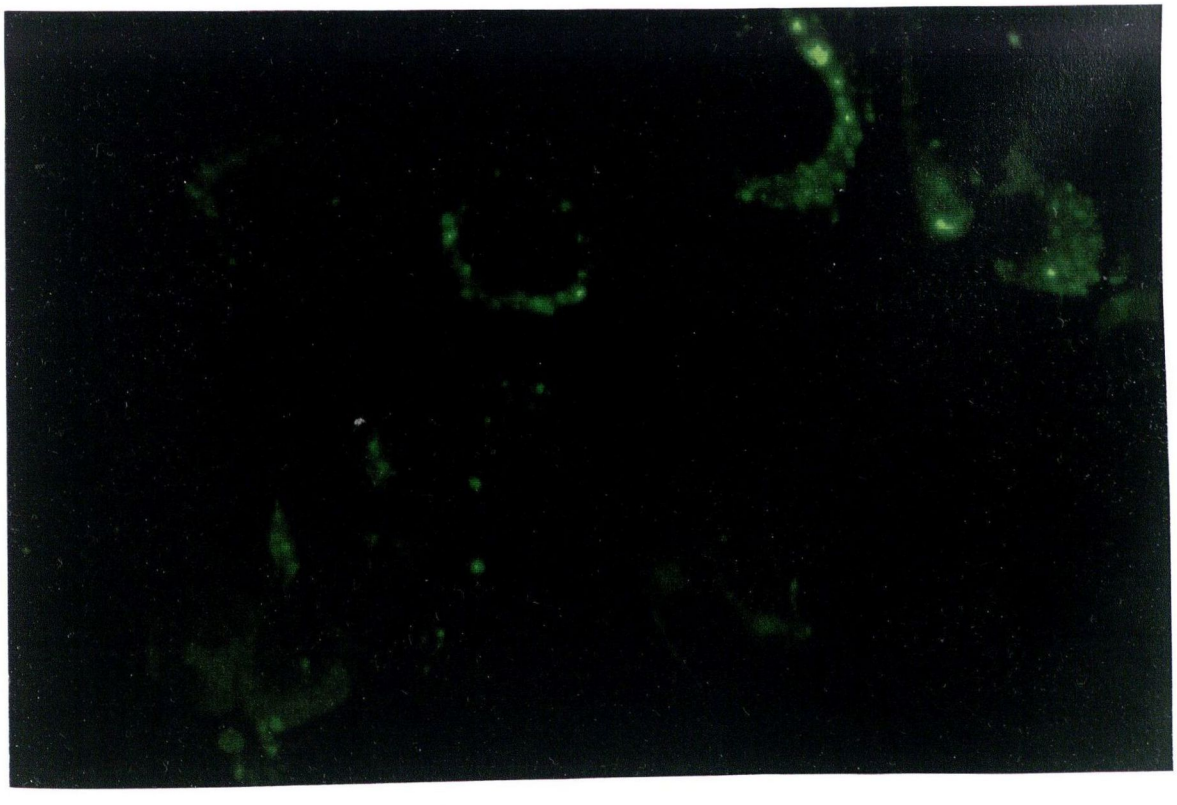

Fig.3.1(h)

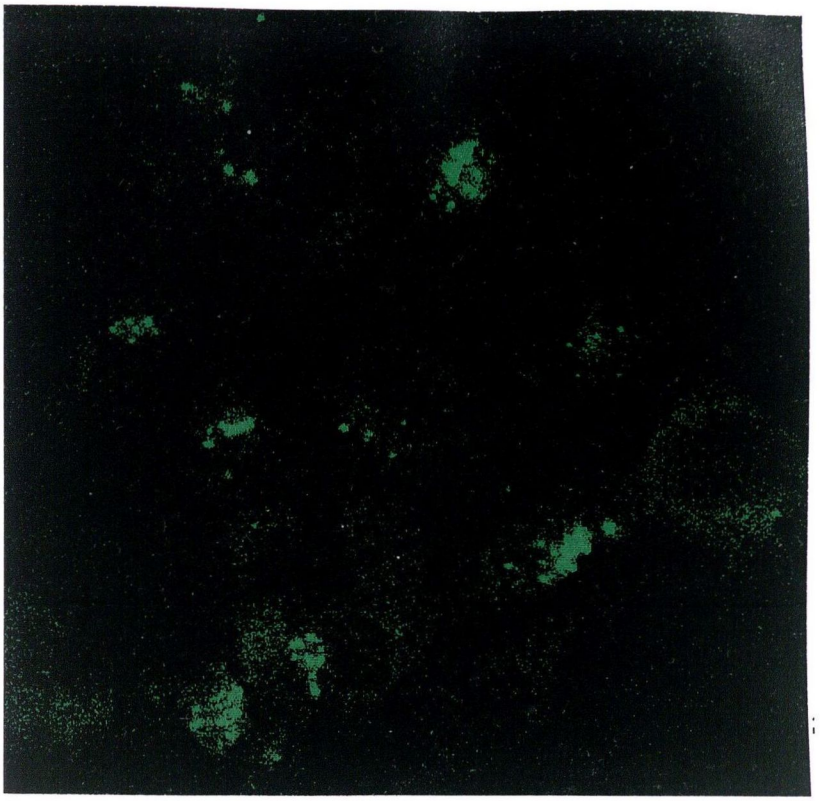

Fig. 3.1(i)

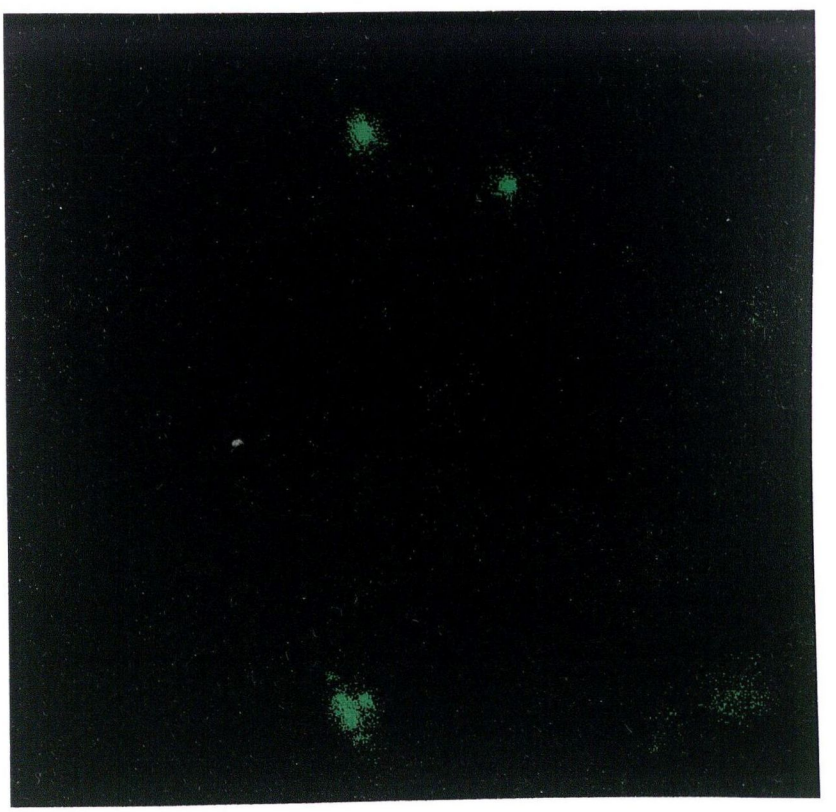

Fig.3.1(j)

Kinetics of DEN 2 viral antigen detection within infected ECV304/T-24 cells

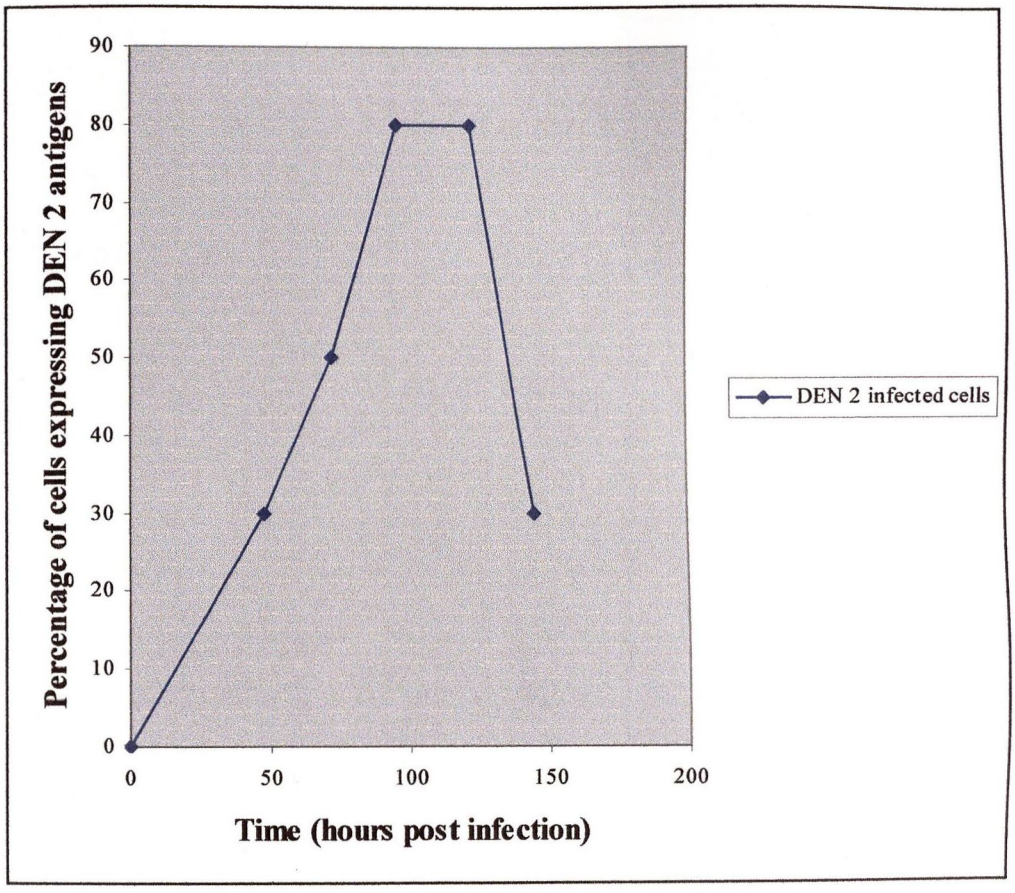

Fig. 3.2. Graphical representation of the percentage of DEN 2 infected, exponentially growing ECV304/T-24 cells expressing viral antigens following indirect immunofluorescence analysis, as depicted in Fig. 3.1.

Detection of intracellular DEN 2 viral antigens in infected polarised confluent ECV304/T-24 cells

Fig. 3.3. Detection of intracellular DEN 2 viral antigens in infected, polarised confluent ECV304/T-24 cells using indirect immunofluorescence. Cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Cells were fixed with 3% PFA, at regular intervals post infection and observed under a fluorescent microscope. 'Mock' infected cells are shown in (a) at 48 hours post infection. Figs. 3.3(b) and (c) represent DEN 2 infected cells at 48 and 72 hours post infection, respectively. The presence of DEN 2 antigens was detected using a fluorescein-conjugated antibody to DEN 2 hyperimmune mouse ascitic fluids.

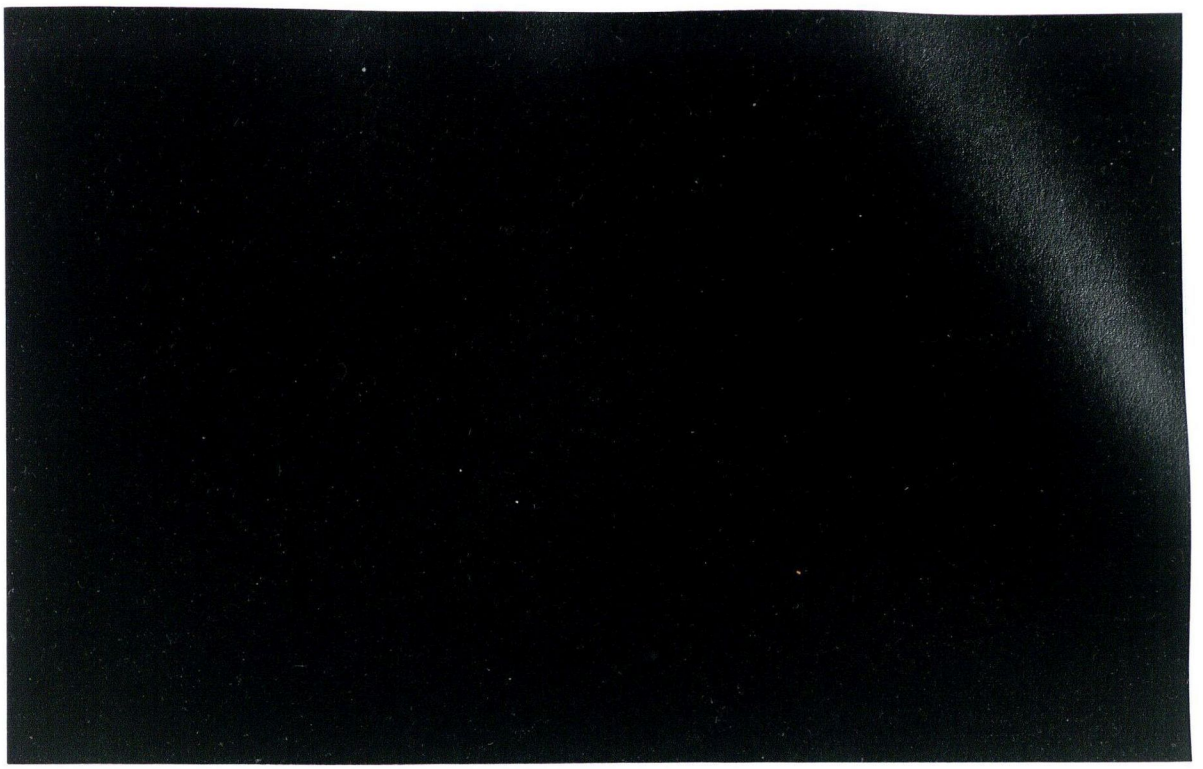

Fig. 3.3(a)

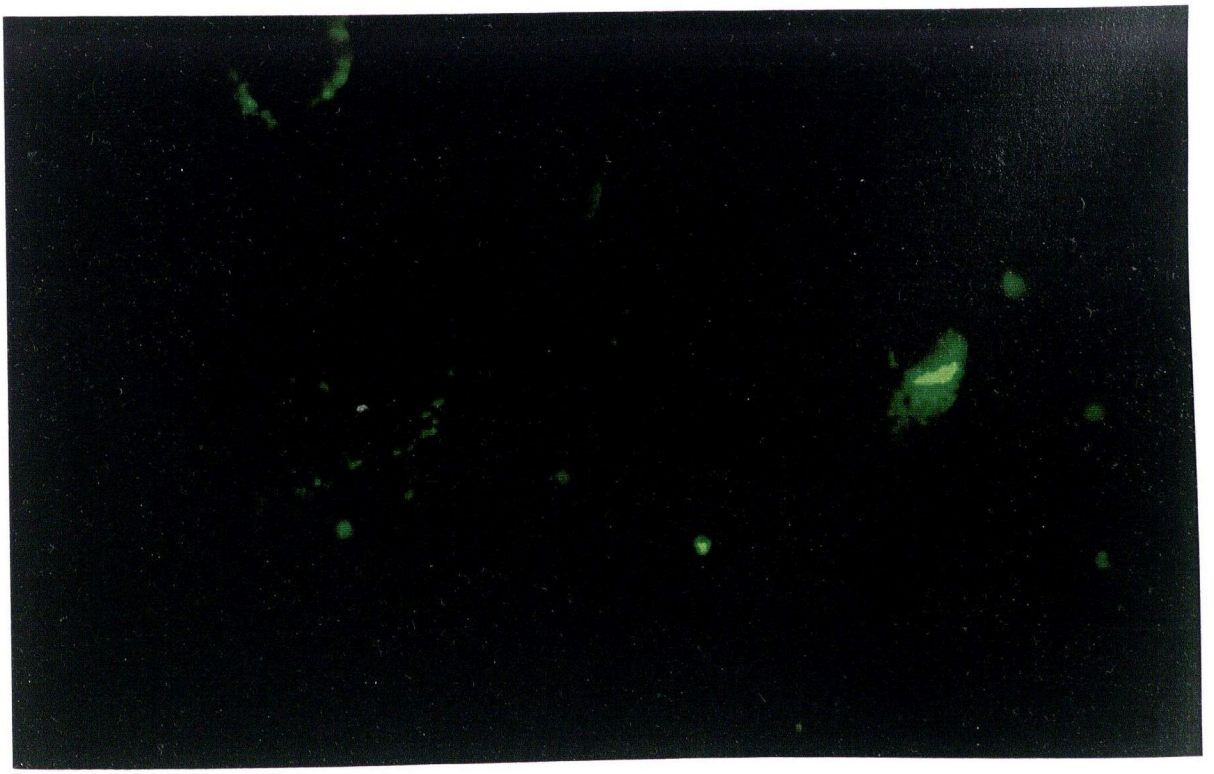

Fig.3.3(b)

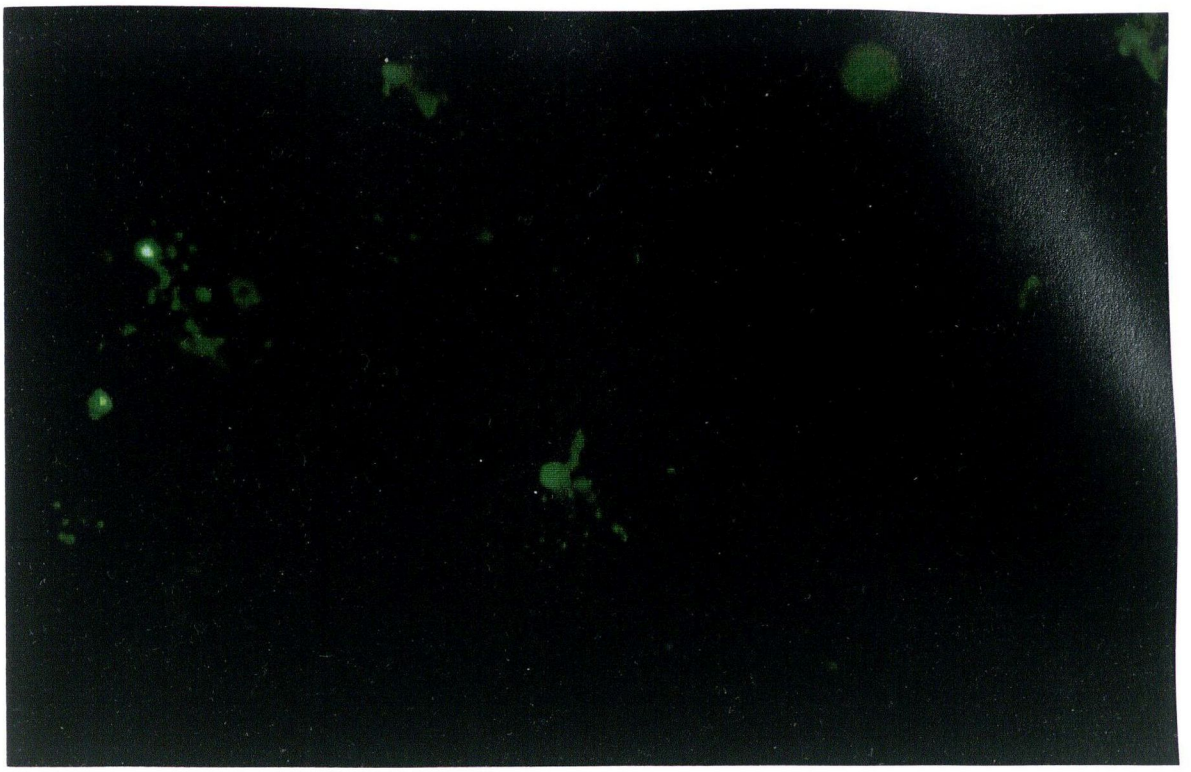

Fig. 3.3(c)

3.3(c). In exponentially growing cells at 48 (Figs. 3.1(d)) and 72 hours post infection (Fig. 3.1(f)), the fluorescence was displayed throughout the cytoplasm, whereas in these cells the fluorescence displayed a speckled pattern.

This cell line is not contact inhibited and once confluent, continues to grow forming more than one layer of cells, which restricted the light from penetrating the slides during observation with the fluorescent microscope. This resulted in a less intense signal than with exponentially growing cells.

The results show that DEN 2 infection of ECV304/T-24 cells is productive, with an increase in the synthesis of viral antigens detected, with time. The maximum number of infected cells was detected at 96 hours post infection.

3.2.1.3 Exponentially growing HUVEC

Exponentially growing HUVEC were cultured on Falcon chamber slides which had been coated with 25µg/mL fibronectin at 37°C for 3 hours. Those cells which did adhere, were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell, fixed with 3% paraformaldehyde and the fluorescence was observed as above. 'Mock' infected cells did not display fluorescence as seen in Fig. 3.4(a). Intense fluorescence was detected throughout the cytoplasm of infected cells at 48 hours post infection which became more diffuse and slightly speckled at 72 h. See Figs. 3.4(b) and (c) respectively. Following the failure of HUVEC to adhere to fibronectin coated glass slides, the experiment was repeated by seeding HUVEC onto thermanox plastic cover slips coated with 1% gelatin in PBS in 24-well plates. HUVEC adhered and were infected. DEN 2 infected HUVEC displayed fluorescence, unlike their 'mock' infected counterparts. However, it was found impossible to focus onto the cells using a fluorescent microscope with these cover slips and glass slides.

3.2.2 Infectious DEN 2 viral progeny production

Two independent experiments were performed in which exponentially growing or confluent ECV304/T-24 cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. The supernatants were collected at regular intervals post infection and the titres of secreted virus were determined as $TCID_{50}$/mL or focus forming units/mL. All results are the average of

Detection of intracellular DEN 2 viral antigens in infected exponentially growing HUVEC

Fig. 3.4. Detection of intracellular DEN 2 viral antigens in infected, polarised confluent ECV304/T-24 cells using indirect immunofluorescence. Cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Cells were fixed with 3% PFA, at regular intervals post infection and observed under a fluorescent microscope. 'Mock' infected cells are shown in (a) at 48 hours post infection. Figs. 3.3(b) and (c) represent DEN 2 infected cells at 48 and 72 hours post infection, respectively. The presence of DEN 2 antigens was detected using a fluorescein-conjugated antibody to DEN 2 hyperimmune mouse ascitic fluids.

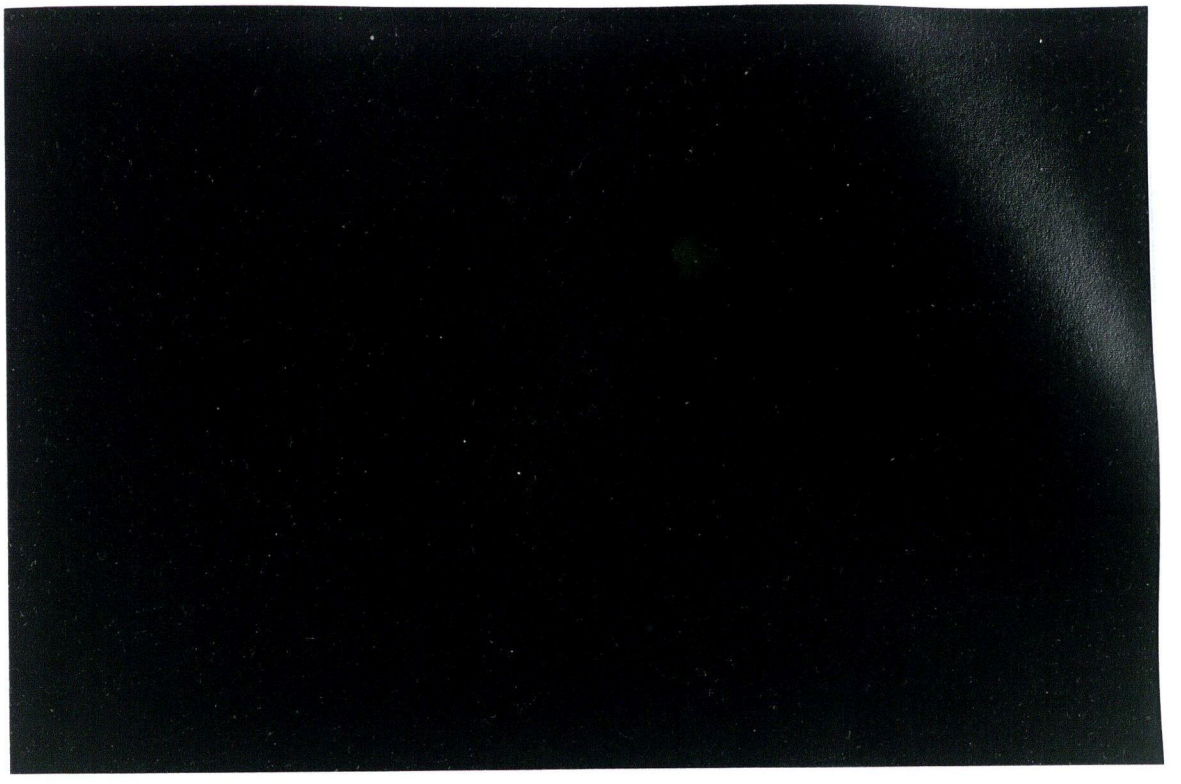

Fig. 3.4(a)

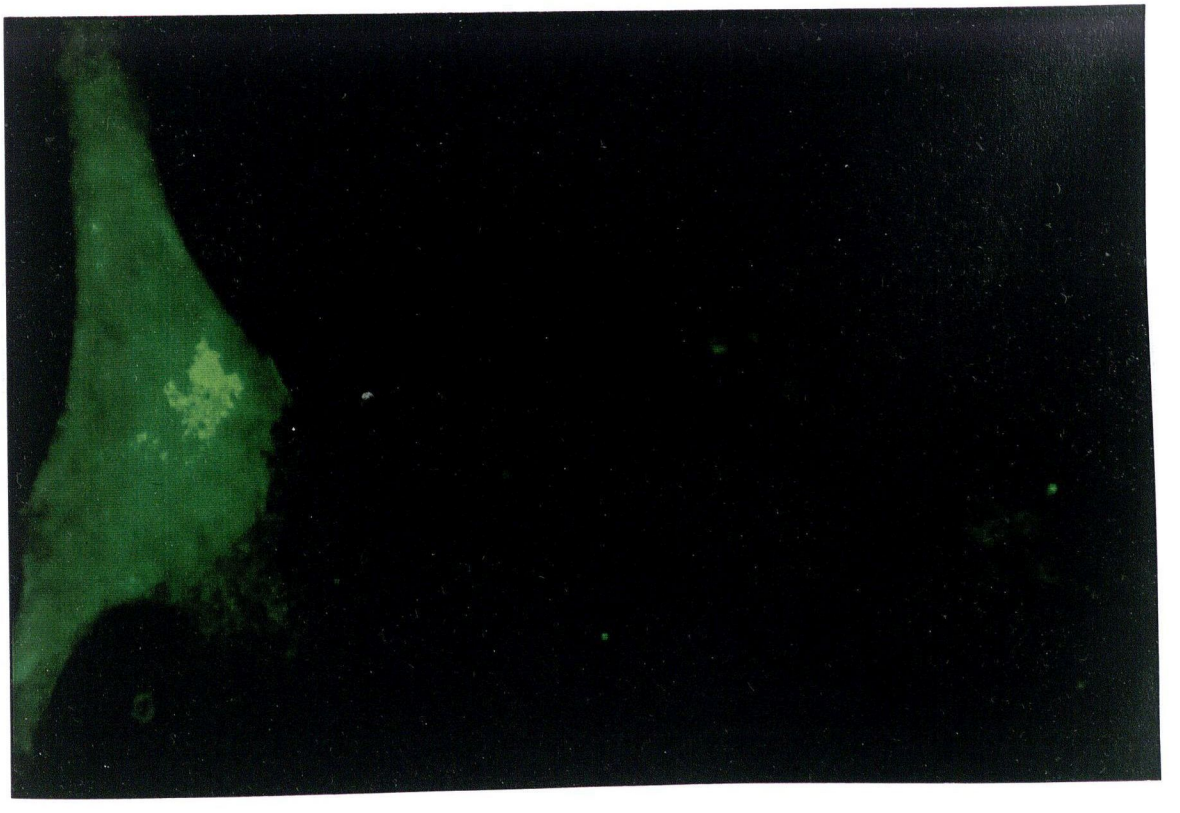

Fig.3.4(b)

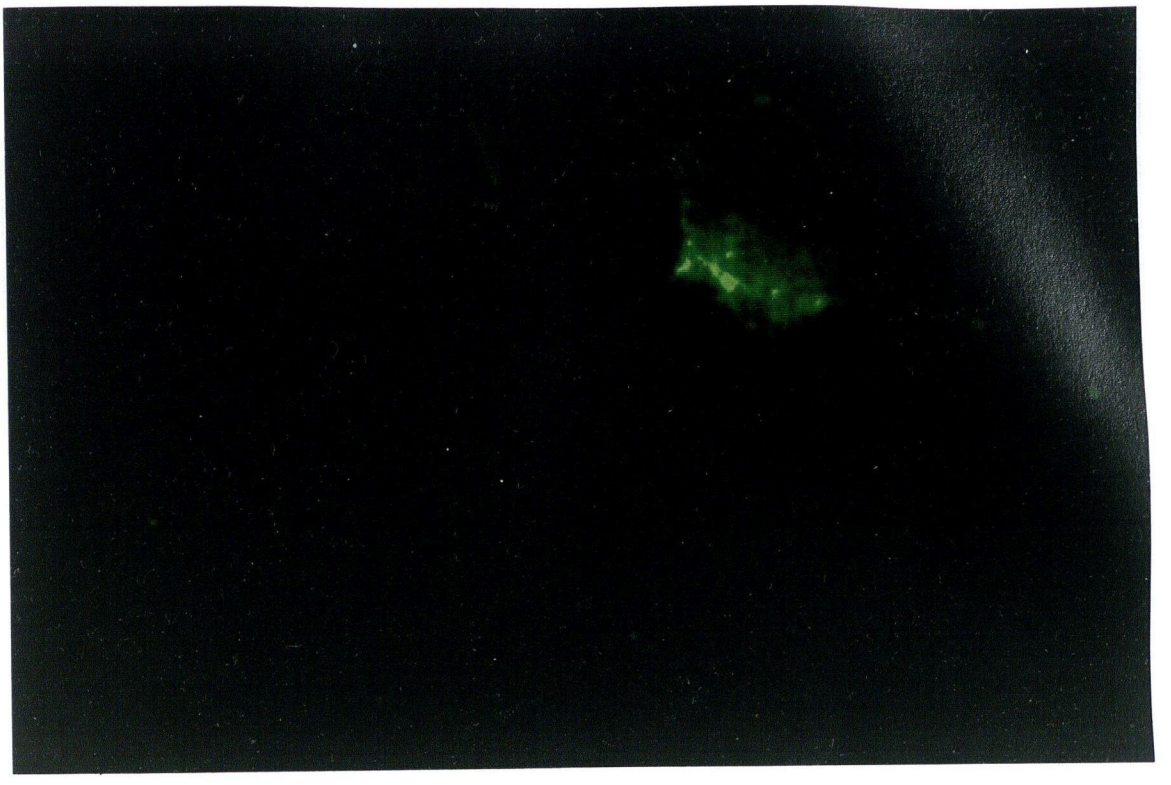

Fig. 3.4(c)

two independent tests. Supernatants from 'mock' infected counterparts were collected at each time point as negative controls.

3.2.2.1 Detection of secreted DEN 2 from ECV304/T-24 cells as $TCID_{50}$ per mL

ECV304/T-24 cells were seeded at a density of $2.5x10^4$ cells per $9.5cm^2$ tissue culture treated mini petri-dish. Forty-eight hours post seeding, the cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Supernatants were collected at regular intervals post infection and frozen at –80°C. A ten-fold dilution series of each time point was prepared in Leibovitz L-15 medium containing 1% FCS, in duplicate. This was added to confluent C6/36 monolayers as described in section 2.2.2.2. The $TCID_{50}$/mL was calculated for each time point and the results are given in Table 3.1.

From the table, it is evident that DEN 2 infects ECV304/T-24 cells in a productive manner with a curve of infectious viral progeny secreted from the cells. The maximum titre of secreted virus was detected at 46 hours post infection.

3.2.2.2 Detection of secreted DEN 2 from ECV304/T-24 cells as focus forming units per mL

ECV304/T-24 cells were seeded at a density of $2.25x10^5$ cells per $9.5cm^2$ tissue culture treated mini-petri dish and grown for 3 days until confluent. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell and the supernatants were collected at regular intervals post infection and frozen at –80°C.

A ten-fold dilution series of the supernatants was prepared in Leibovitz L-15 medium containing 2% FCS in duplicate and added to confluent AP61 mosquito cells as described in section 2.2.2.3. Two hours post infection, apical infection of AP61 cells was prevented by the addition of a mixture of carboxymethylcellulose and medium (1:1) to the monolayers and infection was restricted to that of neighbouring cells, which promoted the formation of foci of infection.

Cells were fixed with 3% paraformaldehyde in PBS and incubated with DEN 2-specific hyperimmune mouse ascitic fluid. Infected cells expressing DEN 2 viral antigens

Table 3.1. Detection of secreted DEN 2 virus in the supernatants of infected ECV304/T-24 cells. Supernatants were collected at regular intervals post infection and added to confluent C6/36 mosquito cell monolayers, on which the $TCID_{50}$ per mL was calculated.

Time (h.p.i.)	**DEN 2 infected cells** $TCID_{50}$ (virus/mL)	**'Mock' infected cells** $TCID_{50}$ (virus/mL)
8	2.5×10^1	<10
24	2.75×10^3	<10
46	5×10^4	<10
75	2.25×10^2	<10
119	2.25×10^2	<10
Positive control[a]	1×10^7	
Negative control[b]		<10

[a]represents supernatants collected from DEN 2 infected C6/36 cells 4 days post infection.

[b]represents Leibovitz L-15 medium containing 1% FCS.

were detected by the addition of HRP-conjugated anti-murine antibody. The number of focus forming units/mL was calculated for each time point and is presented in Table 3.2.

A curve of focus forming units in infected AP61 cells was obtained showing that infection of ECV304/T-24 cells was productive with the release of infected viral progeny from infected cells. The maximum number of infected cells was seen at 47.5 hours post infection. Free virus was detected at 1 and 4 hours post infection but was undetectable by 8 hours. This was due to the presence of residual virus and virus which had bound to the cell surface during infection and was later released into the supernatant.

The titres given as $TCID_{50}$/mL could be compared with the number of focus forming units/mL, by calculating the titre of the positive control or stock virus. It was found that 2 $TCID_{50}$/mL on C6/36 cells was equivalent to 1 ffu/mL on AP61 cells.

The results show that DEN 2 virus infection of ECV304/T-24 cells is a productive infection with the release of infectious viral progeny from the cells. Using either method, the time of maximum production of progeny virus from infected cells was found to be at approximately 48 hours post infection.

3.2.3 Cell Viability studies

Daily observation of cells under the microscope suggested that DEN 2 infection of ECV304/T-24 cells led to the detachment of some cells from the substratum into the supernatant, even though the remaining cells grew to confluence to form a monolayer. It was decided to investigate the effect of infection on the growth and viability of these cells using the trypan blue exclusion assay and the lactate dehydrogenase assay.

3.2.3.1 Trypan Blue exclusion assay

Exponentially growing ECV304/T-24 cells were seeded into tissue culture treated 48-well plates. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell, twenty-four hours post seeding and the number of viable cells was counted daily after infection. Corresponding 'mock' infected cells were counted as controls. All samples were prepared in duplicate. The results are the average of two independent experiments.

Table 3.2. Illustrates the detection of secreted DEN 2 virus from infected ECV304/T-24 cells. Supernatants were collected at regular intervals post infection and the titres were calculated on confluent AP61 mosquito cell monolayers as focus forming units per mL.

Time (h.p.i.)	DEN 2 infected ECV304/T-24 cells (ffu/mL)
1	7.5×10^2
4	2.5×10^1
8	<10
18	1.25×10^4
24	7.5×10^4
36	1×10^5
47.5	2×10^5
70	5×10^3
96	3×10^3
Positive control[a]	5×10^6
Positive control[b]	8.25×10^5
Negative control[c]	<10

[a] represents supernatants from DEN 2 infected C6/36 cells 4 days post infection.

[b] represents supernatants from DEN 2 infected C6/36 cells 5 days post infection.

[c] represents supernatant from 'mock' infected ECV304/T-24 cells, 47.5 hours post infection.

Trypsinized cells were diluted in an equal volume of trypan blue as described in section 2.2.3.1. Healthy cells excluded the dye and were translucent under a phase contrast microscope (Nikon) whilst dying cells had lost their membrane integrity, absorbed the dye and had a blue appearance.

'Mock' infected ECV304/T-24 cells grew exponentially to form a confluent monolayer at 6 days post seeding. DEN 2 infected ECV304/T-24 cells showed a reduction in the number of viable, adherent cells which was greatest at 5 days post infection, as shown in Fig. 3.5(a). Many detached cells were observed in the supernatants of infected cells. Figure 3.5(b) indicates the percentage of control cells adhering to the substratum following infection with DEN 2.

3.2.3.2 Lactate Dehdrogenase assay

The viability of infected ECV304/T-24 cells was measured for a longer time period using a more quantitative and less subjective method. The activity of the cytosolic enzyme lactate dehydrogenase is used to determine the viability of cells. Healthy intact cells retain the enzyme within the cytosol. Unhealthy cells release the enzyme from the cell into the medium and a fall in the level of enzymic activity, expressed in Units/L indicates a reduction in the viability of cells within the population (112).

The enzyme catalyzes the oxidation of lactate to pyruvate, with the simultaneous reduction of nicotinamide adenine dinucleotide (NAD) in the reaction shown below:

$$\text{Lactate} + \text{NAD} \longrightarrow \text{Pyruvate} + \text{NADH}$$

One unit of lactate dehydrogenase activity is defined as that amount of enzyme that will catalyze the formation of one micromole of NADH per minute. The formation of the reduced NADH results in an increase in absorbance of the solution at 340 nm. Lactate and NAD are added to the samples as substrates for the enzyme.

Exponentially growing ECV304/T-24 cells were infected with DEN 2 at a MOI of 10 $TCID_{50}$/cell. The supernatants from infected and 'mock' infected groups were collected and any cells present were pelleted by centrifugation. The adherent cells were

Fig. 3.5(a). Exponentially growing ECV304/T-24 cells were cultured in 48-well tissue culture-treated plates in medium containing 10% FCS and infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Duplicate samples were collected daily from infected and 'mock' infected samples, trypsinized and diluted in trypan blue. The cells were counted using a haemocytometer and Nikon inverted microscope. Fig. 3.5(b) presents the number of surviving adherent cells from infected samples as a percentage of control adherent 'mock' infected cells. The results are the average of two independent experiments.

Viability of exponentially growing ECV304/T-24 cells following infection with DEN 2, using the Trypan Blue exclusion assay

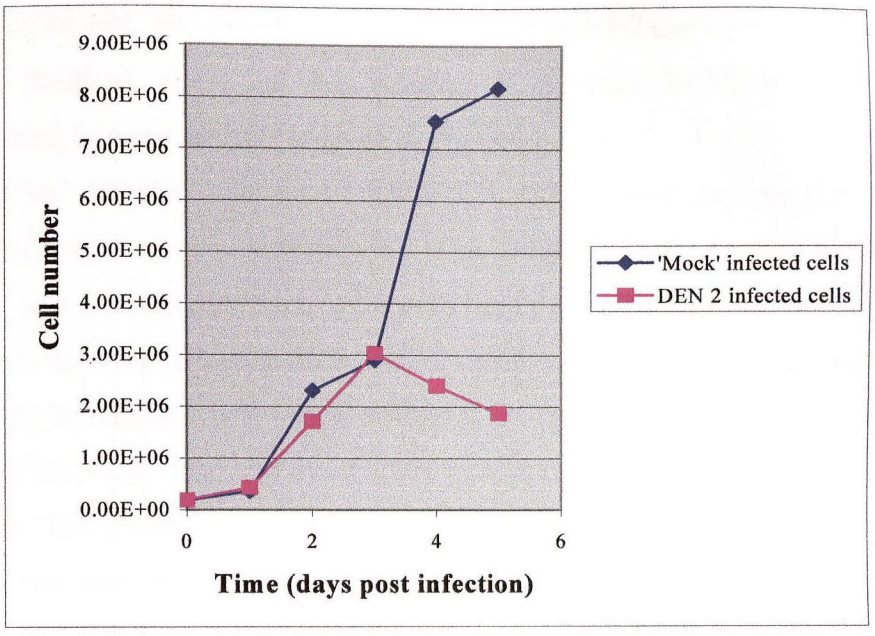

Fig. 3.5(a).

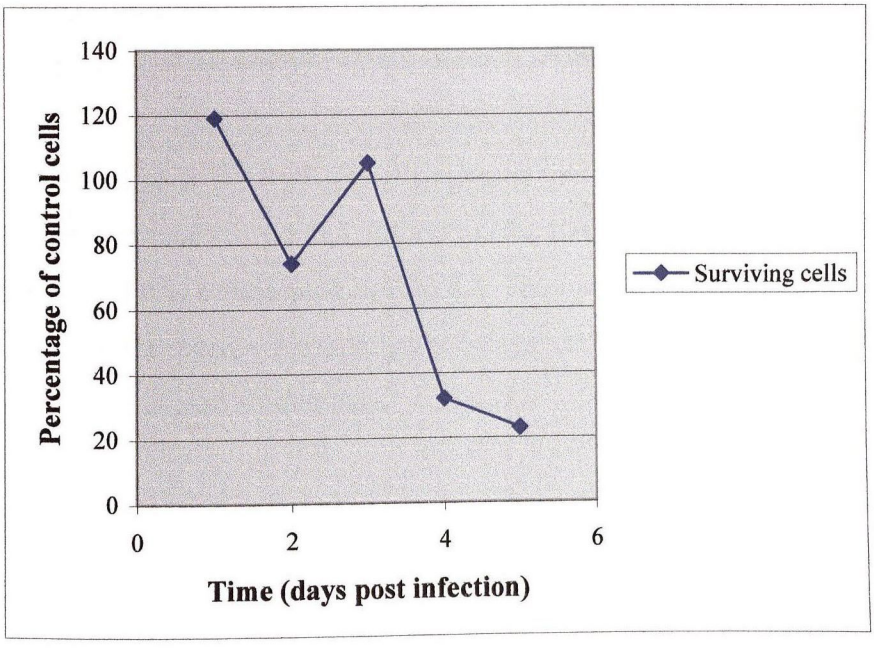

Fig. 3.5(b).

permeabilized and cytosolic extracts were prepared as described in section 2.2.3.2. The rate of enzymic activity was determined by calculating the absorbance of each sample at 2 min intervals for 10 min. The experiment was conducted at 30°C and in duplicate.

A standard curve of the number of healthy ECV304/T-24 cells against corresponding enzymic activity is given in Fig. 3.6.

'Mock' infected adherent ECV304/T-24 cells showed an increase in the level of LDH activity as the cells were growing. This level reached a maximum at 4 days post 'infection' and a plateau was seen as the cells became confluent. See Fig. 3.7(a).

Exponentially growing ECV304/T-24 cells which were infected with DEN 2 at a MOI of 10 $TCID_{50}$/cell, showed a difference in rate of growth and a reduction in the viability of the cells. The activity of LDH was much lower as fewer cells adhered to the substratum. The greatest difference between the viability of 'mock' infected and DEN 2 infected cells was evident at 5 days post infection, when there was a reduction of approximately 70% in the number of infected cells adhering.

In DEN 2 infected samples, the surviving cells grew to form a confluent monolayer as seen in Fig. 3.7(a). Seven days post infection, the viability of these cells was equivalent to that of their 'mock' infected counterparts. Figure 3.7(b) represents the percentage of control cell LDH activity present in the surviving adherent cells following infection with DEN 2.

Any detached ECV304/T-24 cells released into the supernatants of infected samples were collected and centrifuged at 1,300 r.p.m. for 10 min. The LDH activity of these cells was determined and is represented in Fig. 3.8. The number of cells which had detached in the infected samples reaches a maximum at 5 days post infection and was four times greater that that of it's uninfected counterpart.

Standard curve of ECV304/T-24 cell number versus corresponding Lactate Dehydrogenase activity

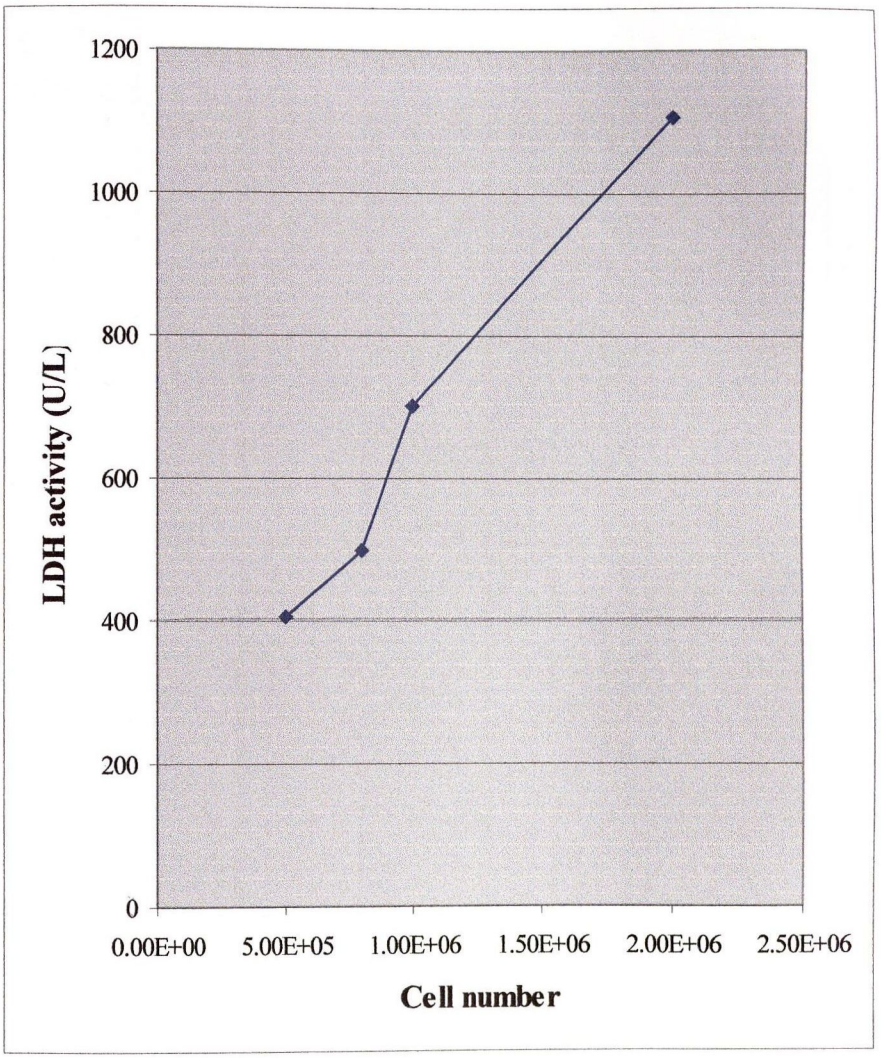

Fig. 3.6. Graphical representation of the lactate dehdrogenase activity of a dilution series of ECV304/T-24 cells, which was prepared in duplicate. Cells were trypsinized and counted using the trypan blue exclusion assay.

Fig. 3.7(a). Exponentially growing ECV304/T-24 cells were cultured in 48-well tissue culture-treated plates in medium containing 10% FCS and infected with DEN 2 at a MOI of 10 $TCID_{50}$/cell. Duplicate samples were collected daily from infected and 'mock' infected samples, permeabilized and the activity of LDH was determined at 30°C. Fig. 3.7(b) presents the LDH activity of surviving adherent cells from infected samples as a percentage of control adherent 'mock' infected cells. The results are the average of two independent experiments.

Viability of exponentially growing DEN 2 infected ECV304/T-24 cells using the Lactate Dehydrogenase assay

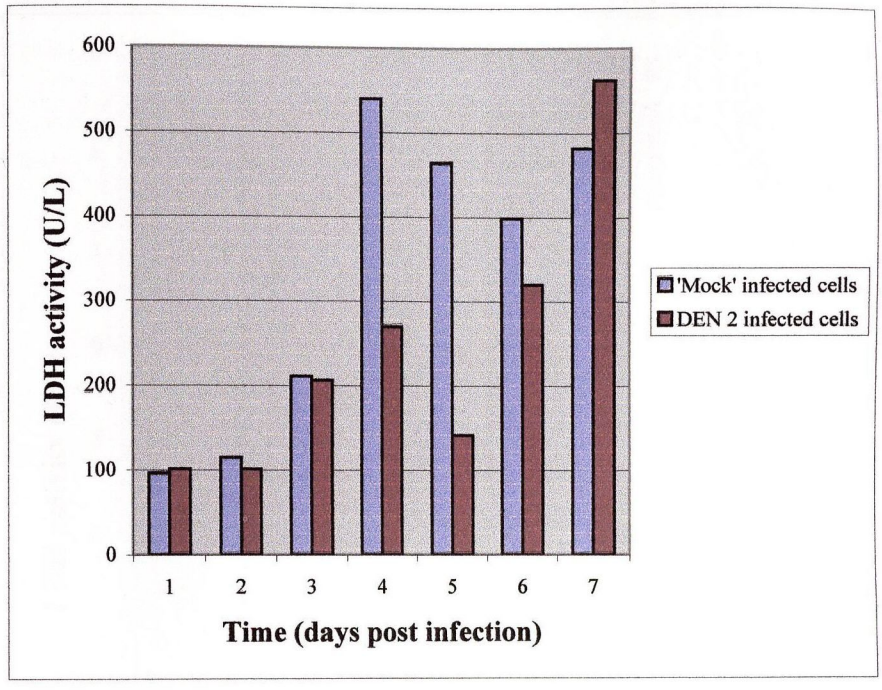

Fig.3.7(a)

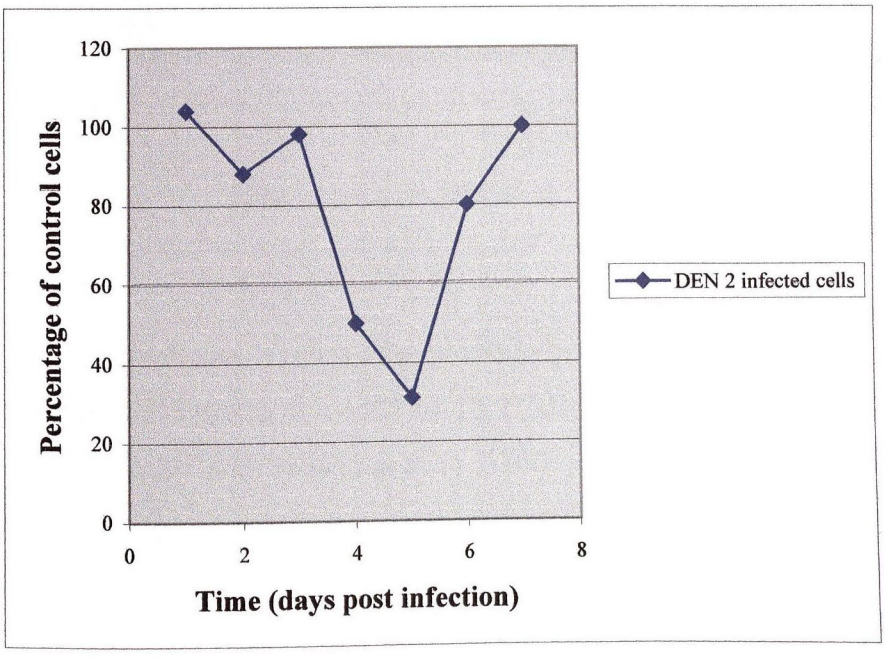

Fig. 3.7(b)

Lactate Dehydrogense activity of detached DEN 2 infected ECV304/T-24 cells

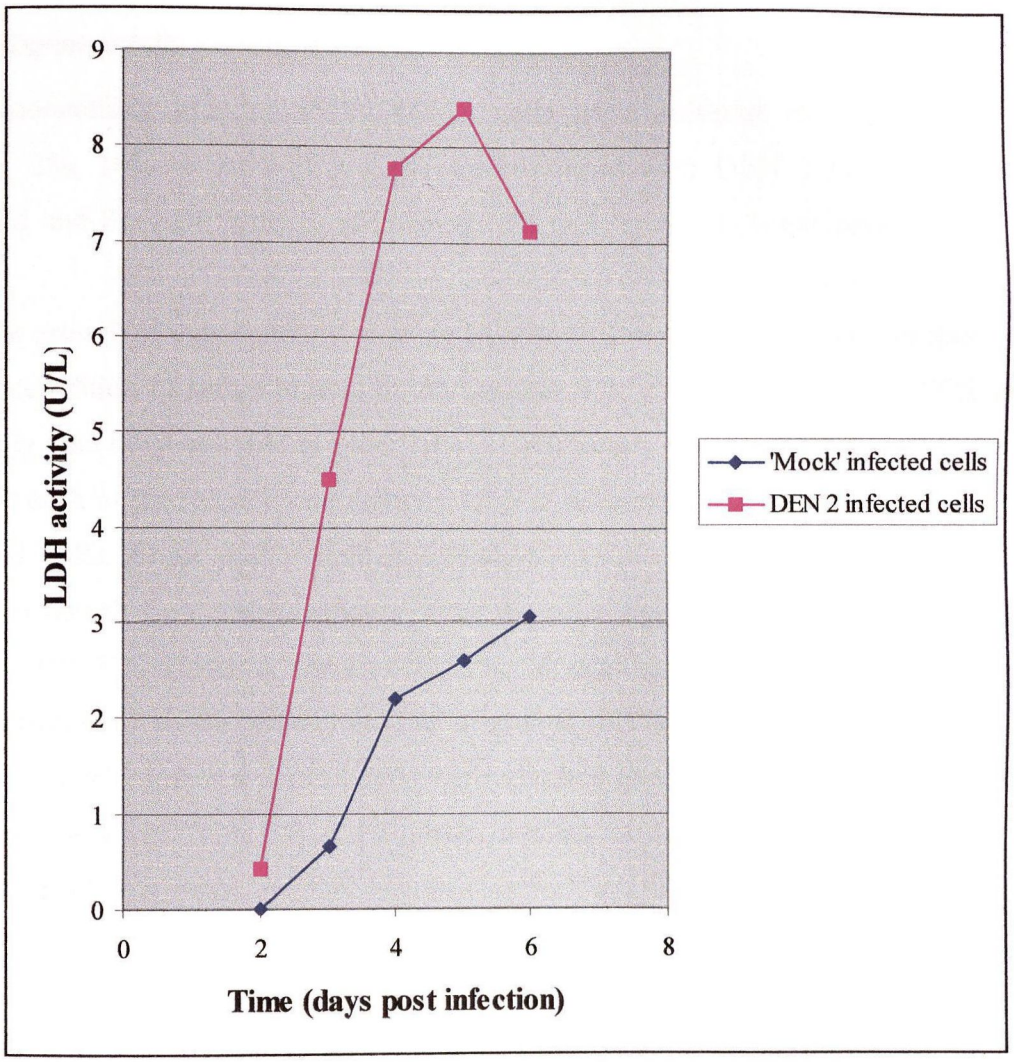

Fig. 3.8. Duplicate samples of exponentially growing ECV304/T-24 cells were infected with DEN 2 at a MOI of 10 $TCID_{50}$/mL and the detached cells were removed daily and centrifuged at 1,300 r.p.m. The activity of LDH was determined for infected and 'mock' infected counterparts at 30°C. The results are the average of two independent experiments.

3.2.3.3 The effect of serum concentration on DEN 2 infection of ECV304/T-24 cells

3.2.3.3.1 Exponentially growing ECV304/T-24 cells

Exponentially growing ECV304/T-24 cells were cultured in DMEM medium containing 2%, 10% or 20% FCS. Cells were infected with DEN 2 at a MOI of 10 $TCID_{50}$/cell and the LDH activity of infected cells and 'mock' infected counterparts was measured.

The growth of exponentially growing ECV304/T-24 cells was directly proportional to the concentration of serum present in the medium. The cells cultured in 2% FCS grew more slowly than those cultured in either 10% or 20% serum.

For each of the serum concentrations, DEN 2 infection resulted in a loss in viability of infected cells. From microscopic observations of infected monolayers, more cells appeared to detach from the substratum when the cells were growing faster, in medium containing 20% FCS. However, the growth curves for each group suggest that regardless of the concentration of serum present and rate of growth of the cells, approximately 70% of each of the 'mock' infected control cells were attached to the substratum at 6 days post infection as shown in Table 3.3. As a greater number of cells were present in the cells grown in 20% FCS, a greater number of cells detached, but the percentage of cells adhering at 6 days post infection was constant.

3.2.3.3.2 Polarised confluent ECV304/T-24 cells

Polarised confluent ECV304/T-24 cell monolayers were infected by DEN 2 at a MOI of 10 $TCID_{50}$/cell and the total LDH activity of the monolayer was compared to that of 'mock' infected counterparts. A subtle loss in the viability of infected monolayers was observed, as can be seen in Fig. 3.9. Infected cells detached from the monolayer and the level of LDH activity of the remaining cells decreased daily. The effects of infection were not adequate to cause massive cell loss with visibility of the underlying substratum. Polarised confluent ECV304/T-24 cells are not contact inhibited and continue to grow to form more than one layer of cells, which would render it difficult to determine whether

Table 3.3. The effect of serum concentration on the viability of DEN 2 infected, exponentially growing ECV304/T-24 cells. Cells were cultured in medium containing 2, 10 or 20% FCS and the LDH of the adherent cells was determined at 30°C.

	Lactate Dehydrogenase activity (U/L)					
Time (d.p.i.)	**2% FCS control**	**2% FCS +DEN 2**	**10% FCS control**	**10% FCS +DEN 2**	**20% FCS control**	**20% FCS +DEN 2**
1	25.96	33.37	25.96	27.25	32.14	32.14
2	105.51	112.53	118.17	116.88	132.65	88.86
3	140.69	182.89	225.10	158.28	296.73	243.83
6	258.18	184.16 (71%)[a]	491.09	337.62 (69%)[a]	825.30	556.08 (67%)[a]

[a]Values given in brackets are percentages of 'mock' infected control cells.

Viability of polarised confluent ECV304/T-24 cells infected with DEN 2 using the Lactate Dehydrogenase assay

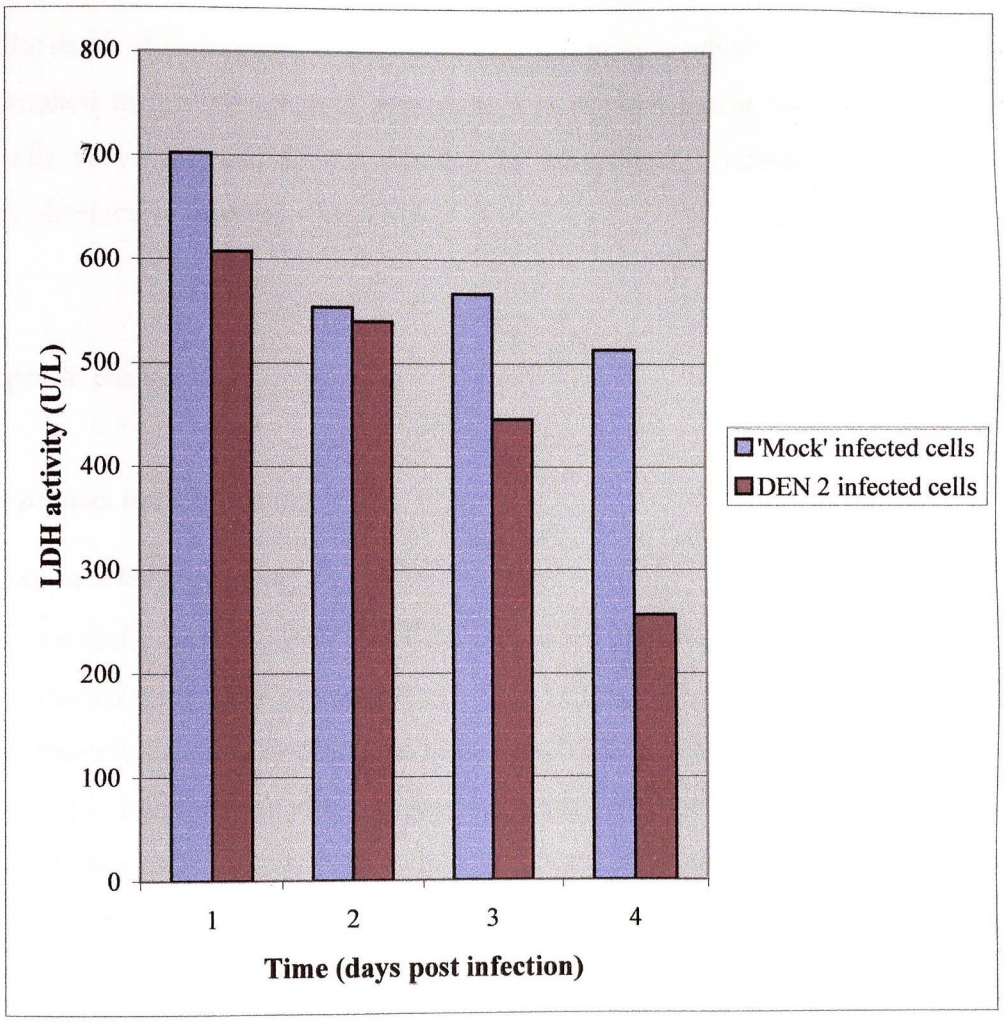

Fig. 3.9. Polarised confluent ECV304/T-24 cells were cultured in 48-well tissue culture-treated plates in medium containing 10% FCS and infected with DEN 2 at a MOI of 10 $TCID_{50}$/cell. Duplicate samples of 'mock' infected and infected adherent cells were permeabilized daily and the LDH activity was determined at 30°C.

infection of a contact inhibited monolayer would show the deleterious effects of infection more clearly.

From these studies, it is clear that infection of ECV304/T-24 cells with DEN 2 results in the death of these cells. It was decided to investigate whether DEN 2 infection of the cells resulted in the induction of apoptosis. It is evident that a proportion of DEN 2 infected cells recovered and it was decided to investigate whether these cells were persistently/chronically infected with DEN 2.

3.2.4 Apoptotic studies

3.2.4.1 Propidium iodide staining

3.2.4.1.1 Exponentially growing ECV304/T-24 cells

Exponentially growing ECV304/T-24 cells were cultured in Falcon culture slides. Cells were infected with DEN 2 at a MOI of 1 or 4 $TCID_{50}$/cell, 24 hours post seeding and stained for intracellular virus as described in section 2.2.2.5. The cells were double-labeled with propidium iodide in order to investigate the integrity of the DNA within the nuclei of these cells. The presence of propidium iodide was detected using a fluorescent microscope (Leitz).

At time zero, there were no differences between the nuclei of infected and 'mock' infected cells. The nuclei were healthy and intact as seen in Fig. 3.10(a), (b), (c), (e) and (g). At forty-eight hours post infection, the cells were growing and there was evidence of mitosis within both groups of cells. A very small number of nuclei within the infected group appeared apoptotic. See Fig. 3.10(d).

Seventy-two hours post infection however, approximately 30% of the nuclei of infected cells had an apoptotic appearance with condensation of the chromatin and a complete loss of structure to the nucleus. Fig. 3.10(f) demonstrates how propidium iodide staining was far brighter in apoptotic nuclei, as the chromatin was more dense and occupied a smaller space. The plasma membrane of infected apoptotic cells was undergoing blebbing, which is characteristic of apoptosis and is visible in the figure. Some cells within the frame were undergoing mitosis and the separation of chromatids was visible within the

Propidium Iodide staining of nuclear DNA within DEN 2 infected, exponentially growing ECV304/T-24 cells

Fig. 3.10. Detection of apoptotic nuclei in DEN 2 infected ECV304/T-24 cells using propidium iodide staining. Exponentially growing cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. Cells were fixed with 3% PFA, at regular intervals post infection and observed under a fluorescent microscope. (a), (c), (e) and (g) are 'mock' infected control cells at 0, 48, 72 and 96 hours post infection. (b), (d), (f) and (h) are DEN 2 infected cells at corresponding time points. Examples of cells undergoing apoptosis are indicated by arrows (f).

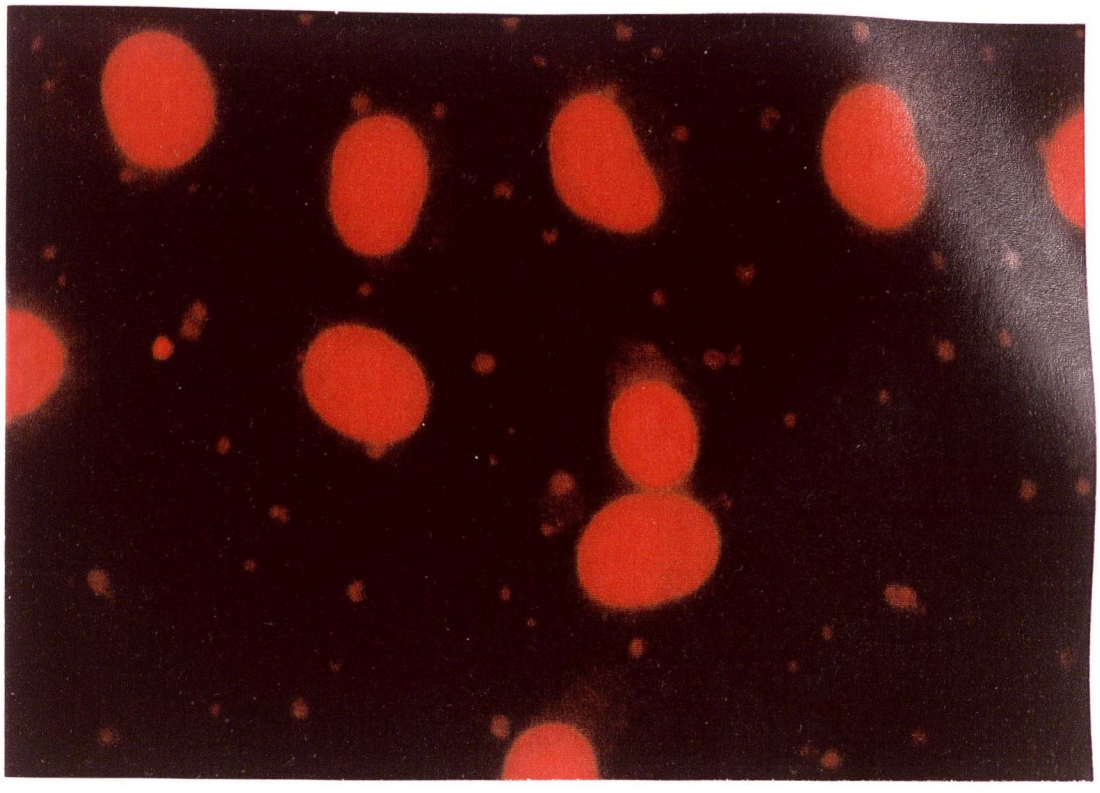

Fig. 3.10(a)

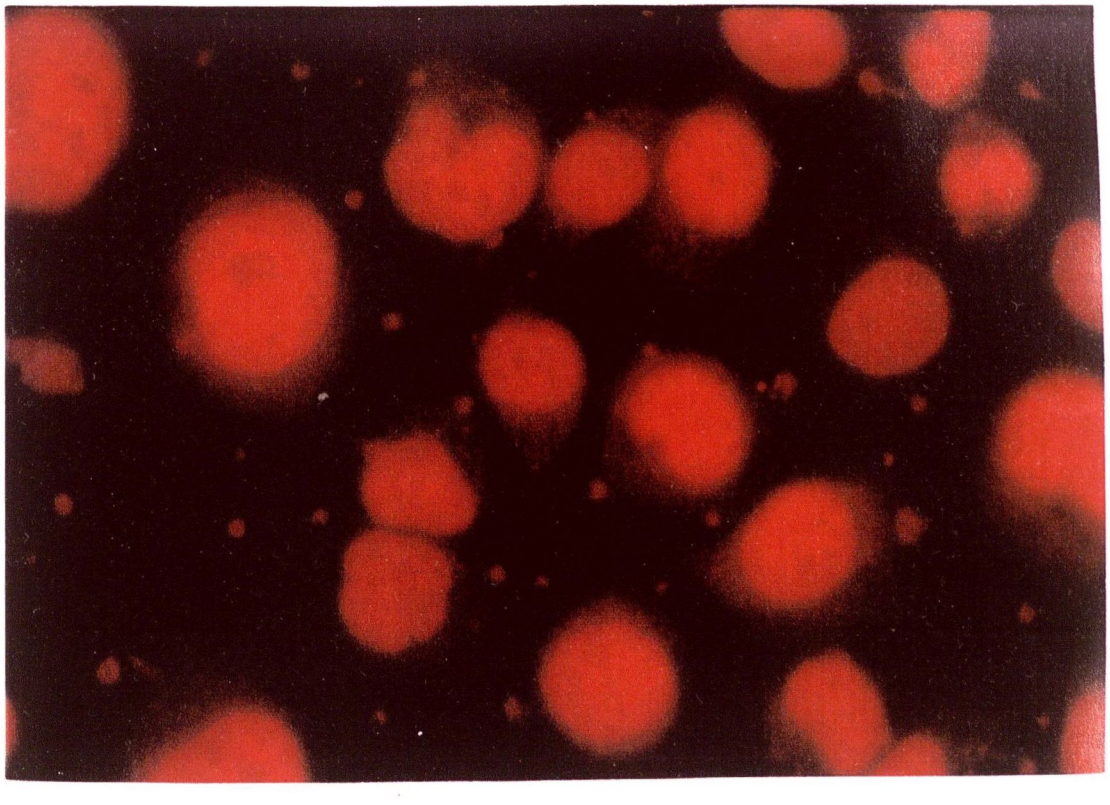

Fig.3.10(b)

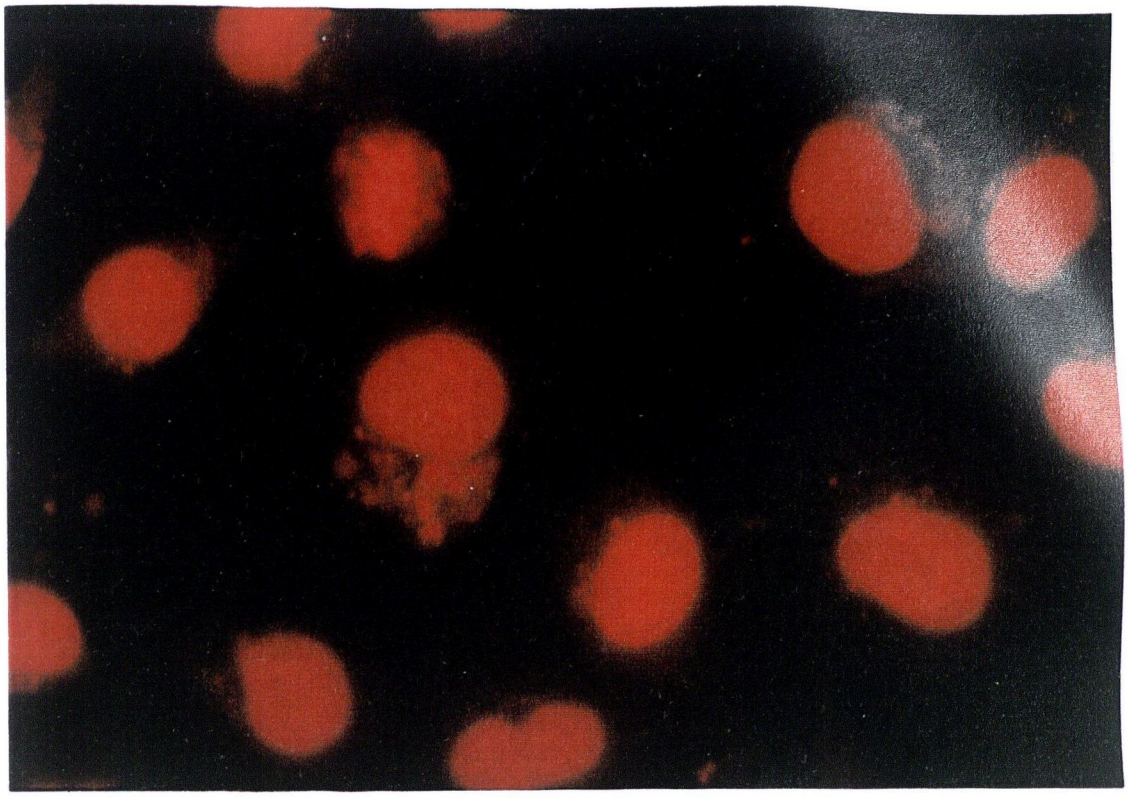

Fig. 3.10(c)

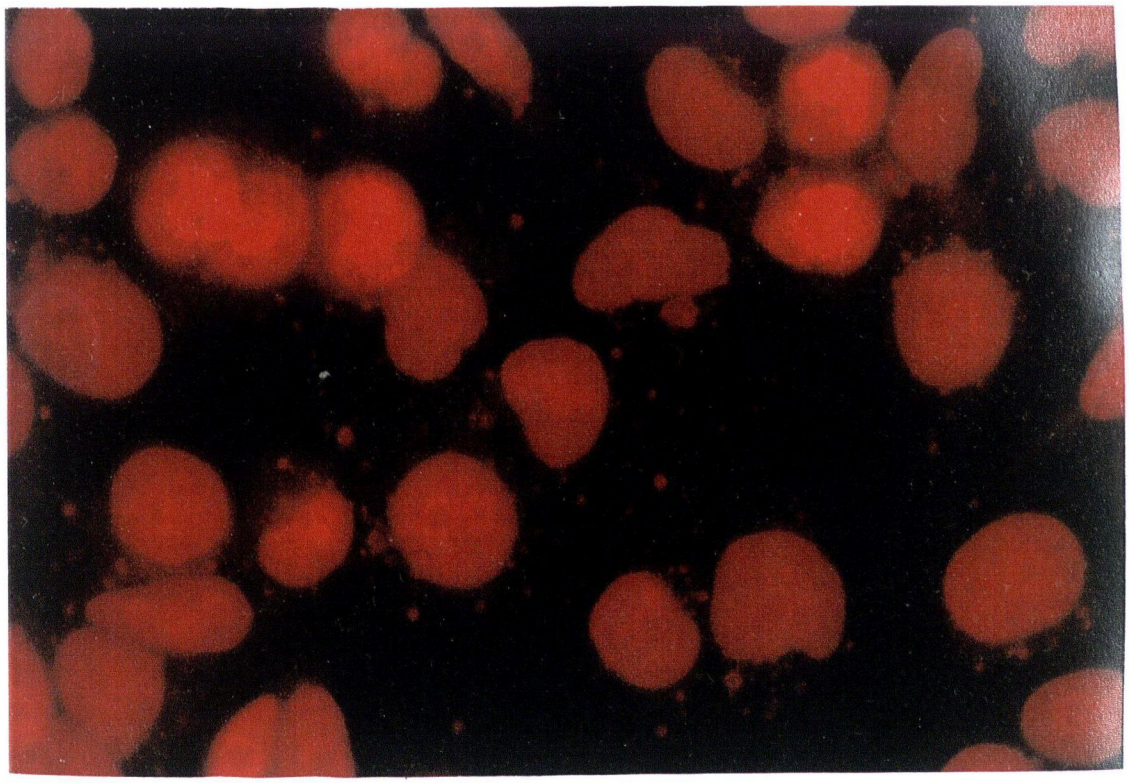

Fig.3.10(d)

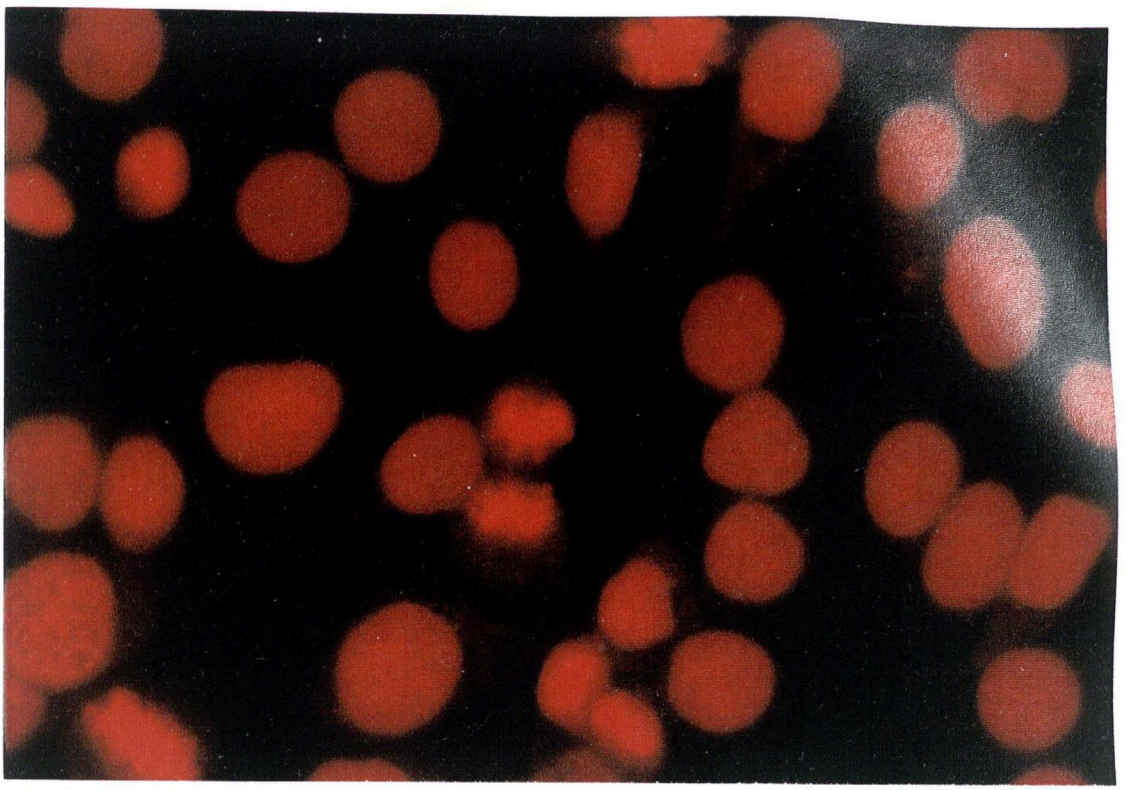

Fig. 3.10(e)

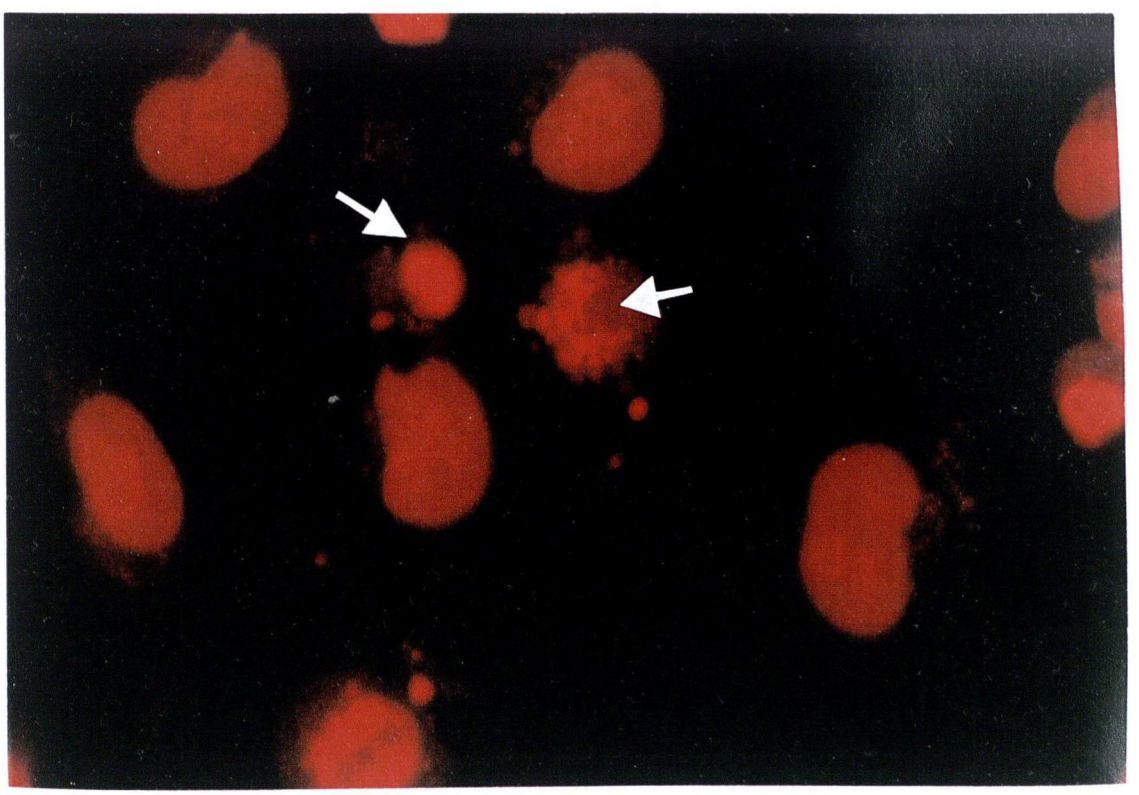

Fig.3.10(f)

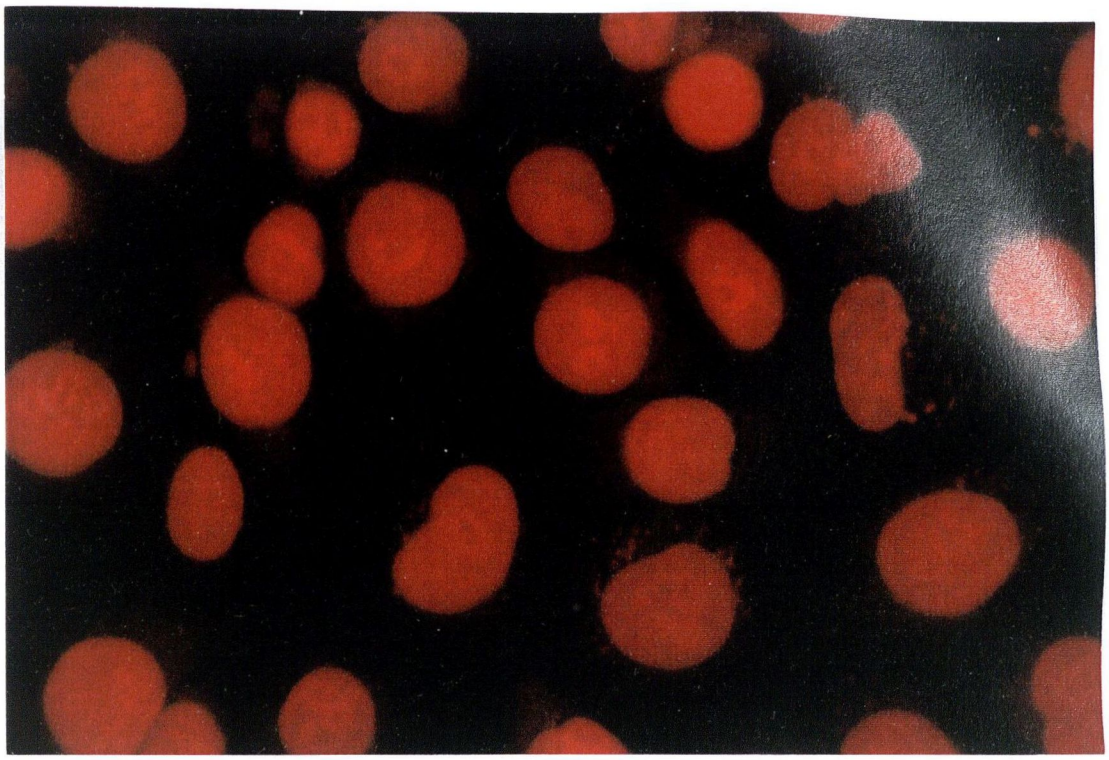

Fig. 3.10(g)

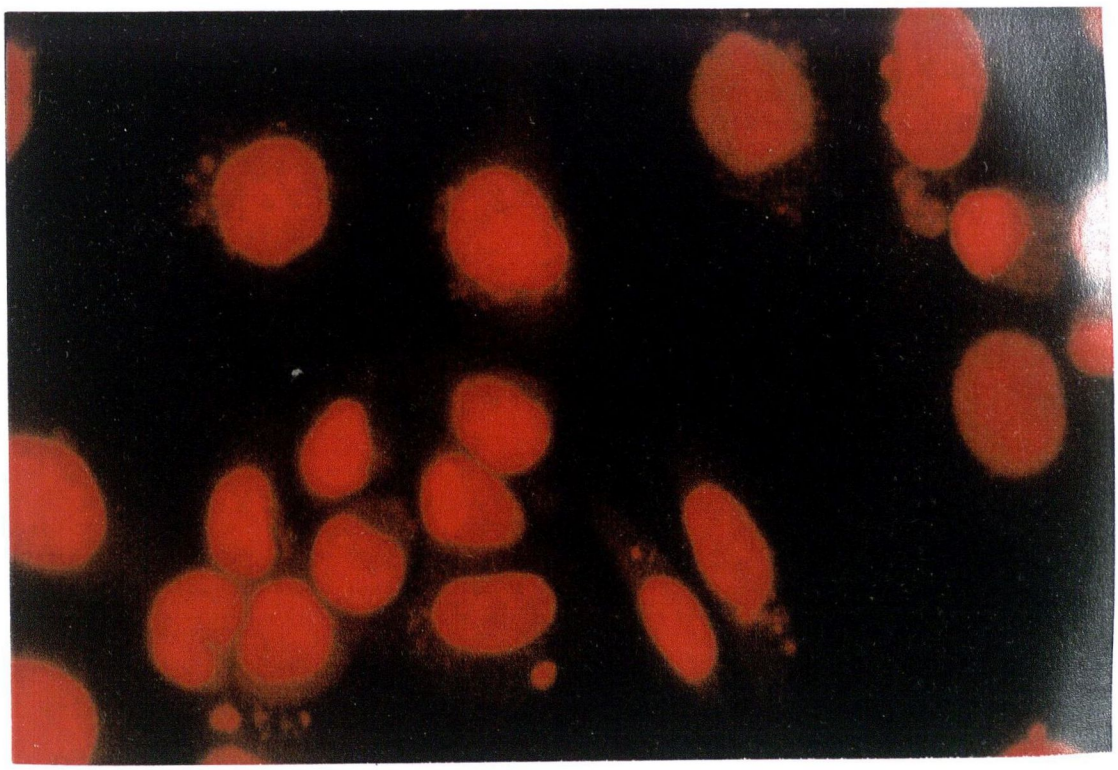

Fig.3.10(h)

picture. The nuclei had recovered at ninety-six hours post infection and there was virtually no evidence of apoptosis in infected cells, as shown in Fig. 3.10(h).

Apoptosis of DEN 2 infected ECV304/T-24 cells appeared to be directly due to viral replication and not a bystander effect as the images presented in figures 3.1 and 3.10 are of the same field of vision. Examples of cells which are positive for both apoptosis and DEN 2 antigen expression are indicated in both figures.

3.2.4.1.2 Polarised confluent ECV304/T-24 cells

Polarised confluent ECV304/T-24 cells were cultured on Falcon culture slides and were also double labeled with propidium iodide. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. There was no evidence of apoptosis within the 'mock' infected control group, as shown in figure 3.11(a). Apoptosis of the nuclei of infected monolayers was evident at 48 hours post infection. See Fig. 3.11(b). Seventy-two hours post infection, a greater number of infected cells had nuclei which were undergoing apoptosis (~15%) and can be seen in Fig. 3.11(c).

3.2.4.2 Differential staining of the cytoplasm and nucleus of DEN 2 infected ECV304/T-24 cells

Exponentially growing ECV304/T-24 cells were cultured in 9.5cm^2 tissue culture treated mini petri-dishes and 24 hours post seeding, were infected with DEN 2 at a MOI of 10 $TCID_{50}$/cell. 'Mock' infected control counterparts were also prepared. At seventy-two hours post infection, the supernatants were collected and any cells which had detached and were released into the supernatants were centrifuged at 1,300 r.p.m. for 10 min. The adherent cells were trypsinized and centrifuged as before.

Slides of DEN 2 infected and 'mock' infected adherent and detached ECV304/T-24 cells were prepared using a cytospin as described in section 2.2.4.3. Equal densities of monolayer cells were prepared for both groups. When observing the detached cells, the preparations contained all of the pelleted cells.

The cells were stained using Eosin Y and Methylene blue, mounted using DPX mountant and photographed using a light microscope. Nuclear material was stained pink

Propidium Iodide staining of nuclear DNA within DEN 2 infected, polarised confluent ECV304/T-24 cells

Fig. 3.11. Detection of apoptotic nuclei in polarised confluent DEN 2 infected, ECV304/T-24 cells using propidium iodide staining. Polarised confluent cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. Cells were fixed with 3% PFA, at regular intervals post infection and observed under a fluorescent microscope. 'Mock' infected cells are shown in (a) at 48 hours post infection. Figs. 3.11(b) and (c) represent DEN 2 infected cells at 48 and 72 hours post infection, respectively.

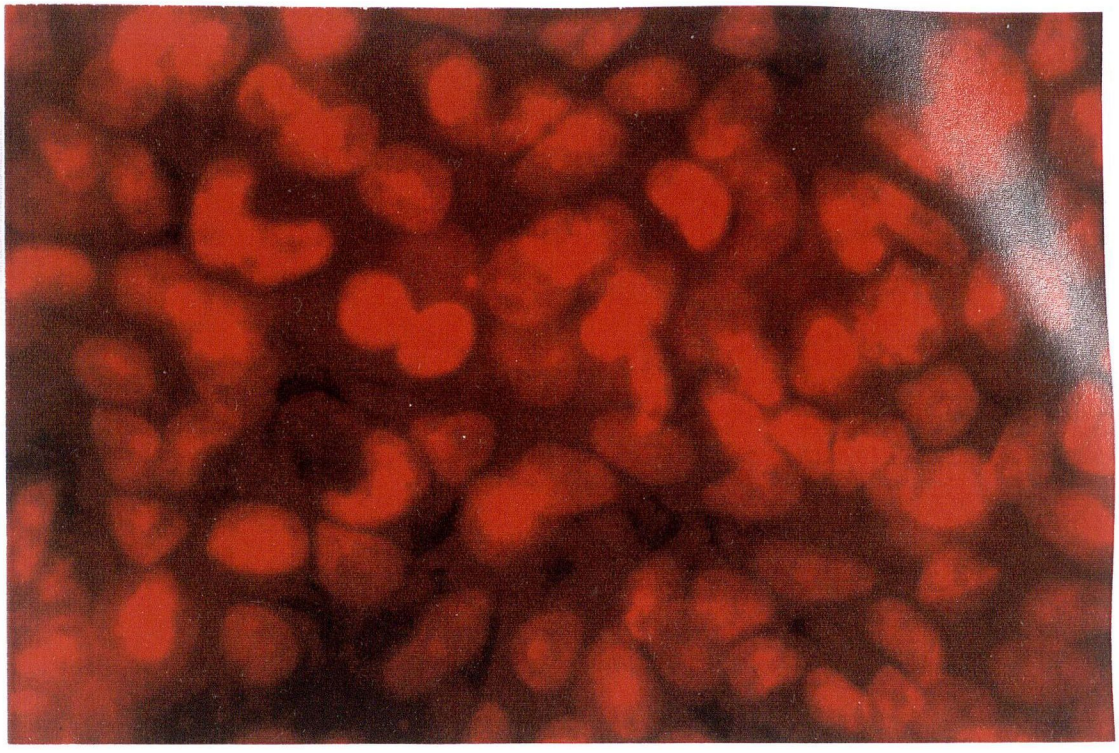

Fig. 3.11(a)

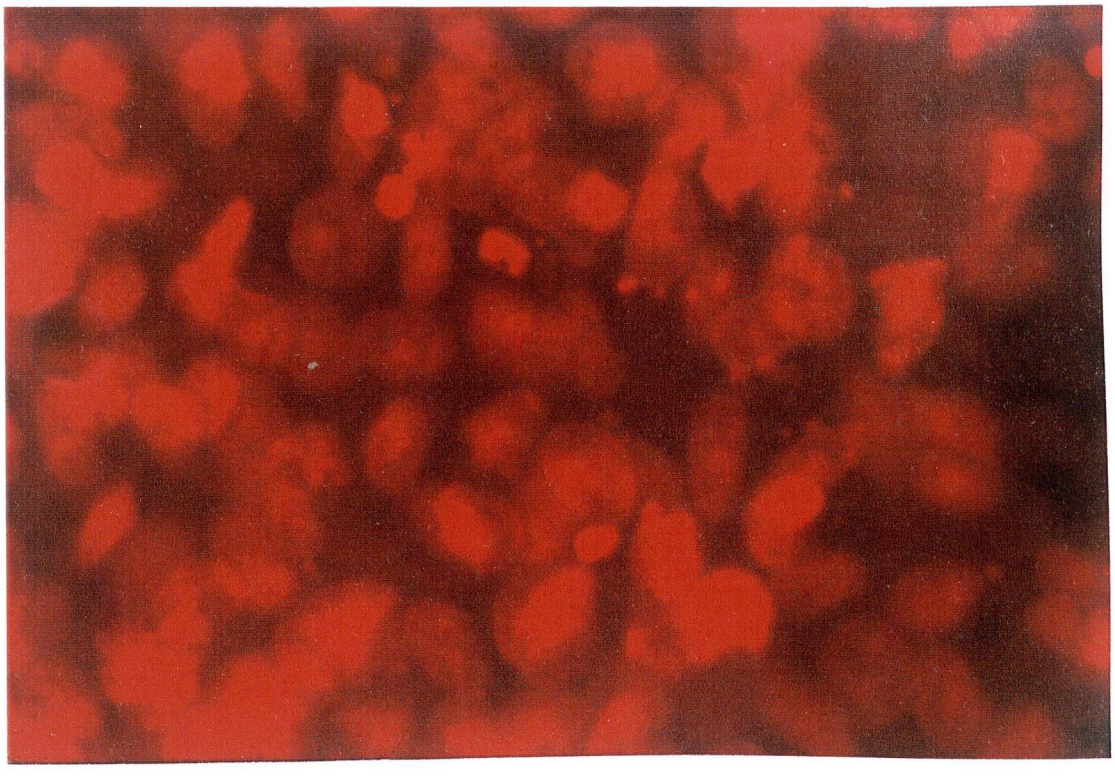

Fig.3.11(b)

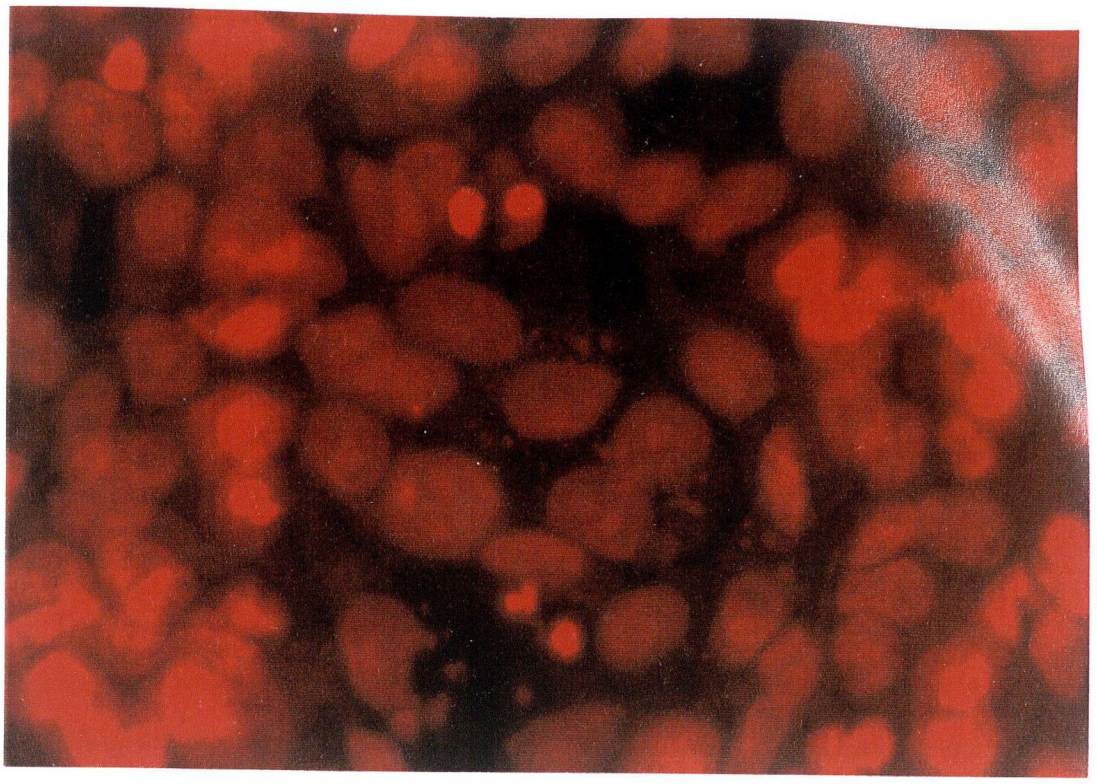

Fig. 3.11(c)

and the cytoplasm blue. Fig. 3.12(a) shows that 'mock' infected adherent cells were undergoing mitosis and had intact nuclei and plasma membranes. DEN 2 infected adherent cells were not as healthy and enlargement of the nuclear membrane and blebbing of the plasma membrane was visible. See Fig. 3.12(b).

Detached cells were very few in control groups one of which is shown in Fig. 3.12(c). In infected samples, the number of detached cells was much greater and the nuclei had lost their structure and condensation of the nuclear material had occurred as shown in figure 3.12(d).

3.2.4.3 TUNEL analysis of DEN 2 infected ECV304/T-24 cells

During apoptosis, breaks occur in the DNA strands. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay is used as a method of investigating cellular apoptosis. Fluorescein-conjugated dUTP and unlabeled dNTPs are incorporated into partially degraded DNA using the enzymes DNA polymerase and TdT in an *in situ* reaction. Apoptosis is detected using a fluorescein-conjugated label which binds the enzyme TdT. The affected cells are visualized using a fluorescent microscope and individual cells undergoing apoptosis can be detected, which is one of the advantages of this method over other apoptotic methods, such as DNA fragmentation analysis. The TUNEL method preferentially labels apoptotic cells over necrotic cells.

Exponentially growing ECV304/T-24 cells were cultured on Falcon chamber slides and infected with DEN 2 virus at a MOI of 4 $TCID_{50}$/cell. Seventy-two hours post infection, the cells were fixed in 3% paraformaldehyde in PBS and permeabilized. The cells are incubated with TUNEL enzyme and label as described in section 2.2.4.2. The cells were observed using a fluorescent microscope (Zeiss).

At seventy-two hours post infection, a number of nuclei were fluorescent, indicating that these nuclei are apoptotic, as can be seen in Fig. 3.13.

Differential staining of DEN 2 infected exponentially growing ECV304/T-24 cells

Fig. 3.12. Differential staining of nucleus and cytoplasm of DEN 2 infected ECV304/T-24 cells. Exponentially growing cells were infected with DEN 2 at a MOI of 10 $TCID_{50}$/mL and the adherent cells were trypsinized at 72 hours post infection. Any cells which had detached and were released into the supernatant were collected. Slides were prepared of adherent and detached samples using a cytospin and poly-L-lysine coated glass slides. 'Mock' infected adherent cells and detached cells are shown in Figs. 3.12(a) and (c) respectively. DEN 2 infected apoptotic, adherent cells are shown in Fig. 3.12(b) and infected detached cells are shown in Fig. 3.12(d).

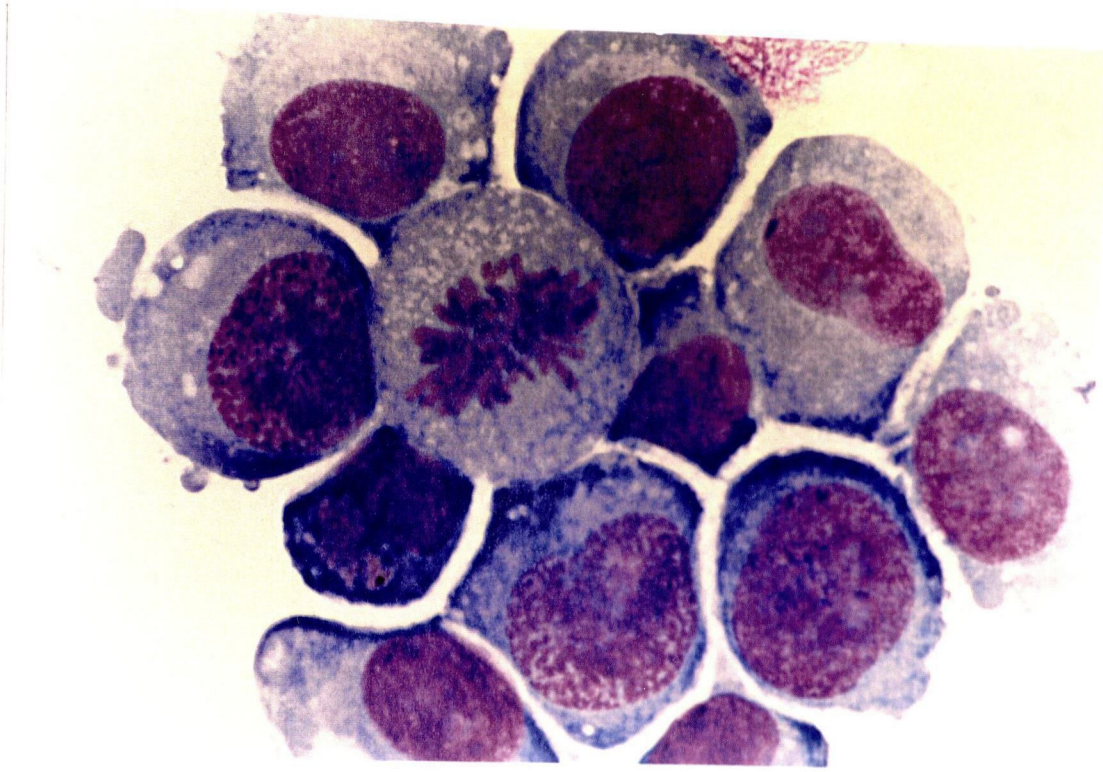

Fig. 3.12(a)

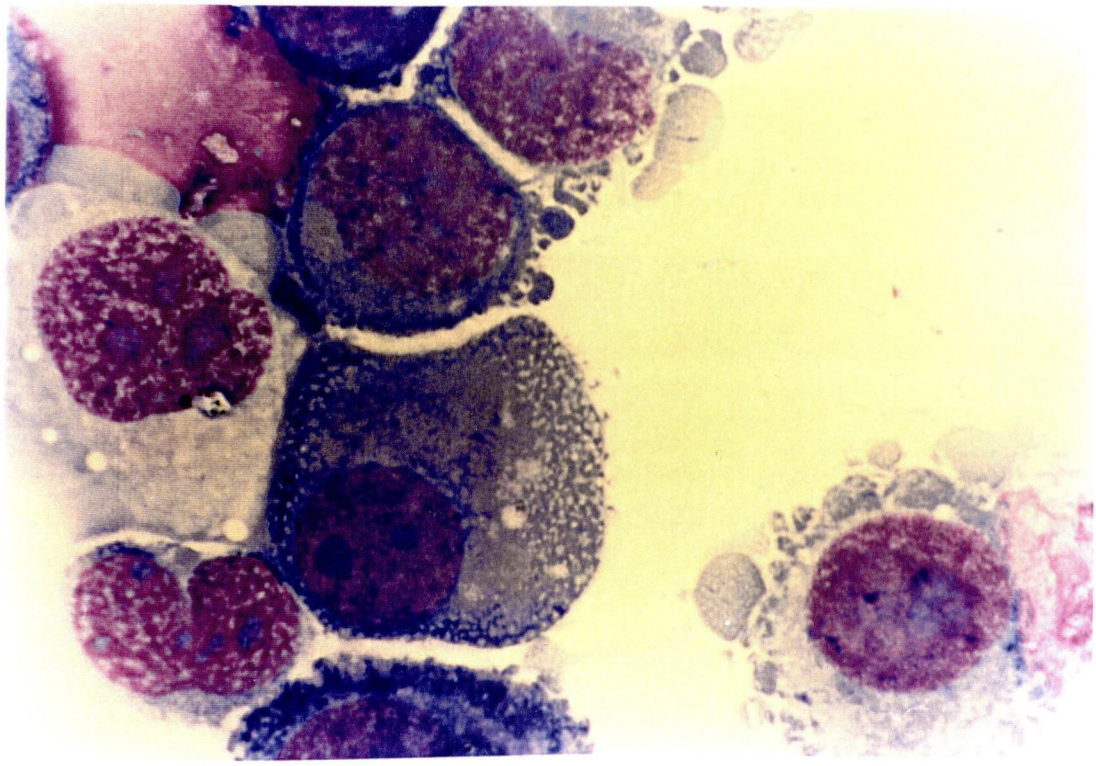

Fig.3.12(b)

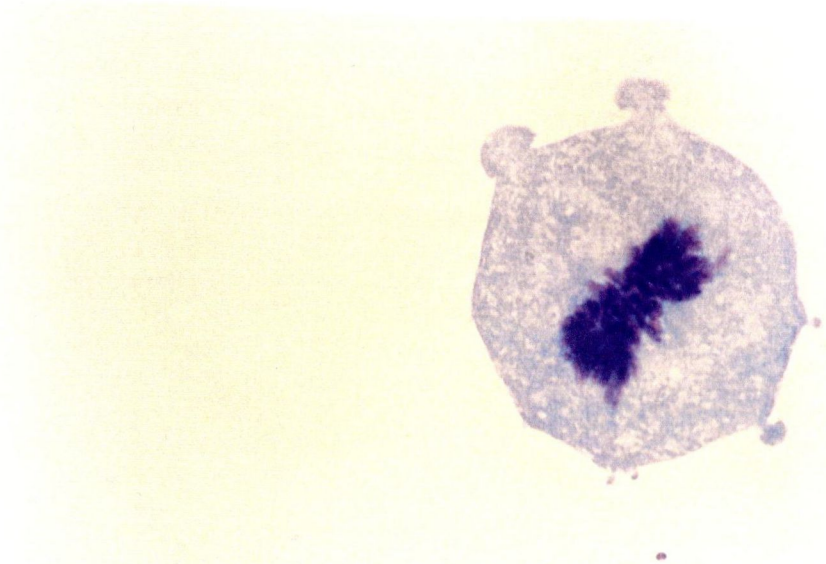

Fig. 3.12(c)

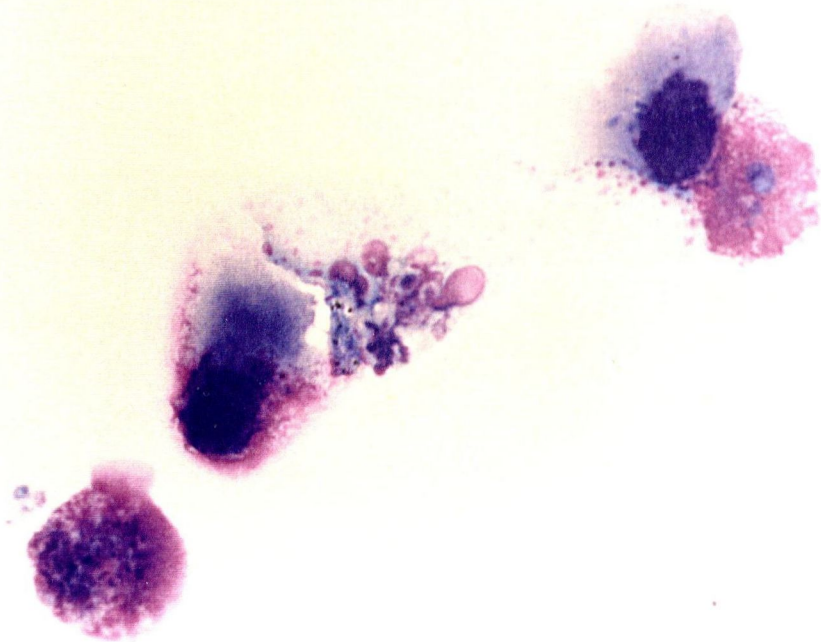

Fig.3.12(d)

TUNEL analysis of DEN 2 infected, exponentially growing ECV304/T-24 cells

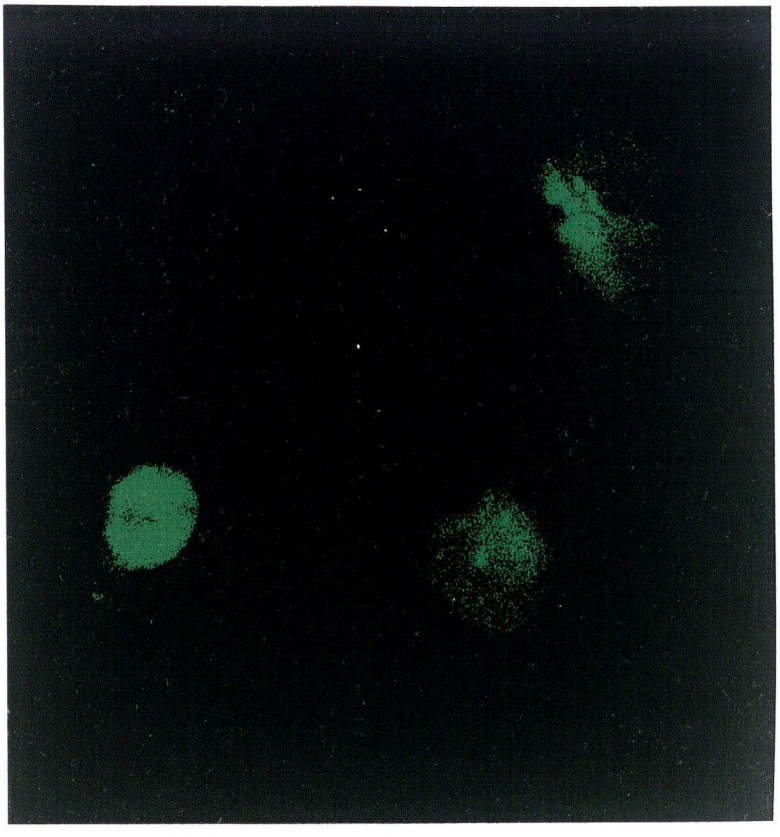

Fig. 3.13. Detection of apoptotic nuclei in polarised confluent DEN 2 infected, ECV304/T-24 cells using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis. Exponentially growing cells were cultured on Falcon chamber slides and infected with DEN 2 at a MOI of 4 infectious virus particles/cell. 72 hours post infection, cells were fixed with 3% PFA and observed under a fluorescent microscope. A fluorescein-conjugated label is used to detect apoptotic nuclei.

3.2.5 Persistence/chronicity of DEN 2 infection in ECV304/T-24 cells

3.2.5.1 Immunofluoresence detection of intracellular virus

Exponentially growing ECV304/T-24 cells were seeded into 25cm^2 tissue culture flasks and were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell, twenty-four hours post seeding. Infected and 'mock' infected cells were grown to confluence and passaged as described in section 2.2.1.1, at one-fifth the density of confluent cells. After five passages, the cells were seeded onto Falcon chamber slides and cultured for 24 hours. The cells were then fixed in 3% paraformaldehyde in PBS, labeled for intracellular DEN 2 viral antigens using indirect immunofluorescence analysis as described in section 2.2.2.5 and observed under a fluorescent microscope (Zeiss).

Figure 3.14 demonstrates the expression of intracellular viral antigens within DEN 2 infected ECV304/T-24 cells at five passages post infection. The fluoresence is displayed throughout the cytoplasm of infected cells, which are found in localized foci throughout the monolayer.

3.2.5.2 Intracellular and secreted viral presence in persistently infected ECV304/T-24 cells

ECV304/T-24 cells were cultured in 25cm^2 tissue culture treated flasks and infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. Cells were passaged up to five times and at 2, 3, and 5 passages supernatants and cells were collected. Supernatants were frozen at –80°C until use and cells were centrifuged at 1,300 r.p.m. for 10 min and 'snap' frozen in liquid nitrogen to ensure that lysis of the cells occurred.

Upon defrost, a doubling dilution series of each sample was prepared and the presence of infectious viral particles was assayed on C6/36 mosquito cell monolayers as previously described in section 2.2.2.2. The titres of infectious virus particles in both supernatant samples and intracellularly were calculated as $TCID_{50}$ and are described in Table 3.4.

The results show that infectious virus particles were located both intracellularly and secreted into the medium for up to five passages post infection.

3.2.5 Persistence/chronicity of DEN 2 infection in ECV304/T-24 cells

3.2.5.1 Immunofluoresence detection of intracellular virus

Exponentially growing ECV304/T-24 cells were seeded into 25cm^2 tissue culture flasks and were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell, twenty-four hours post seeding. Infected and 'mock' infected cells were grown to confluence and passaged as described in section 2.2.1.1, at one-fifth the density of confluent cells. After five passages, the cells were seeded onto Falcon chamber slides and cultured for 24 hours. The cells were then fixed in 3% paraformaldehyde in PBS, labeled for intracellular DEN 2 viral antigens using indirect immunofluorescence analysis as described in section 2.2.2.5 and observed under a fluorescent microscope (Zeiss).

Figure 3.14 demonstrates the expression of intracellular viral antigens within DEN 2 infected ECV304/T-24 cells at five passages post infection. The fluoresence is displayed throughout the cytoplasm of infected cells, which are found in localized foci throughout the monolayer.

3.2.5.2 Intracellular and secreted viral presence in persistently infected ECV304/T-24 cells

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The results show that infectious virus particles were located both intracellularly and secreted into the medium for up to five passages post infection.

Detection of intracellular DEN 2 viral antigens in persistently infected ECV304/T-24 cells

Fig. 3.14. Exponentially growing ECV304/T-24 cells were infected with DEN 2 at a MOI of 4 $TCID_{50}$/mL. Surviving cells were grown to confluence and passaged at a density of 1/5 the total number of cells. Following 5 passages, the cells were grown to confluence, fixed in 3% PFA and incubated with DEN 2-specific hyperimmune mouse ascitic fluid. DEN 2 antigens were detected using a fluorescein-conjugated anti-murine antibody and the samples were observed under a fluorescent microscope. 'Mock' infected counterparts are shown in (a) and foci of persistently infected cells are shown in (b).

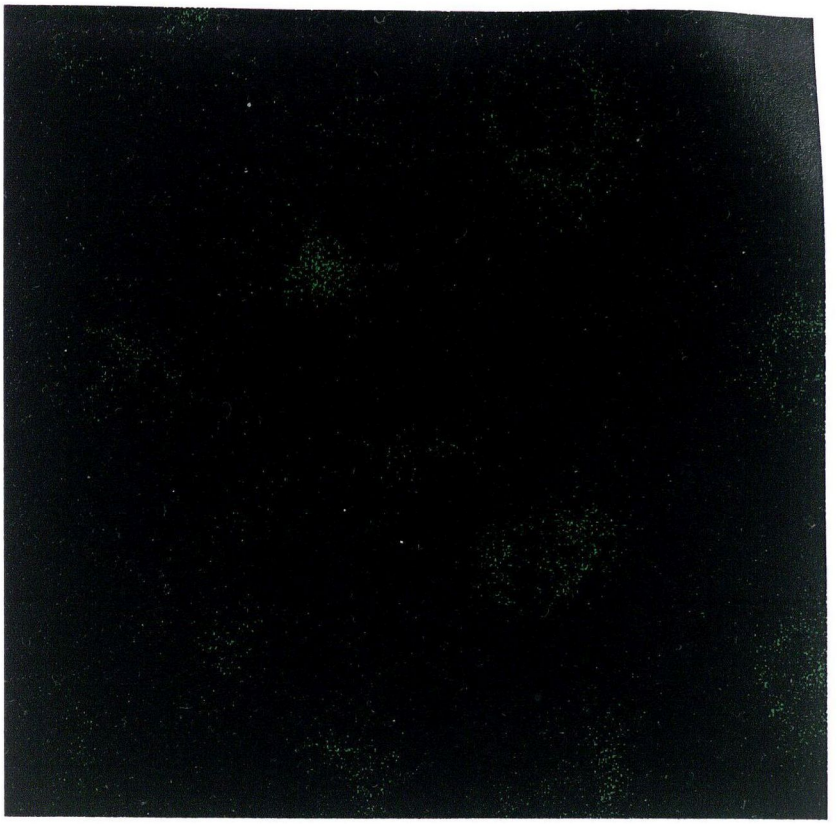


Fig. 3.14(a)

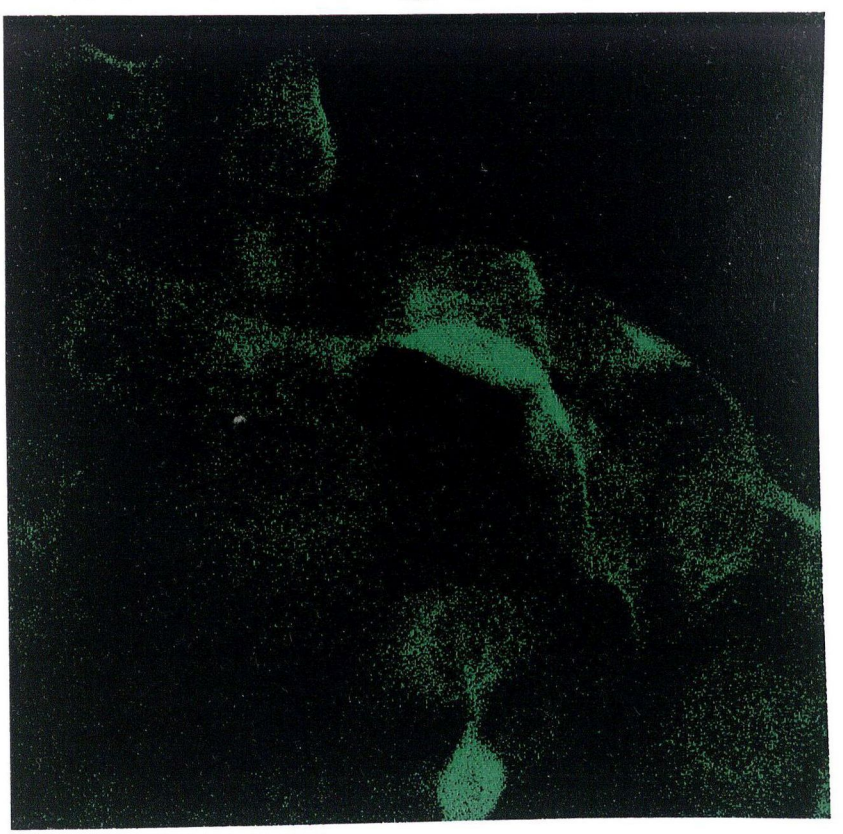


Fig.3.14(b)

Table 3.4. Persistence of DEN 2 infection of ECV304/T-24 cells. Exponentially growing cells were infected with DEN 2 at a MOI of 4 $TCID_{50}$/mL and grown until confluence. Cells were trypsinized and passaged for five passages. An aliquot of cells and supernatants were collected at each passage. Cells were 'snap' frozen in liquid nitrogen to release any intracellular virus. Titres were calculated as $TCID_{50}$/mL on confluent C6/36 mosquito cell monolayers.

Cell passage number	Supernatant $TCID_{50}$ (virus/mL)	Cell lysate $TCID_{50}$ (virus/mL)
2	N.D.	7.5×10^0
3	2×10^1	1×10^1
5	2×10^1	N.D.
Positive control[a]	1×10^7	N.D.
Negative controls[b]	0	0

[a]represents supernatants from DEN 2 infected C6/36 cells 4 days post infection.

[b]represents supernatants from 'mock' infected ECV304/T-24 cells corresponding to each of the above infected samples. No cytopathic effect was observed.

N.D. indicates not done.

3.2.5.3 Confirmation of secreted infectious virus from DEN 2 infected ECV304/T-24 cells

Exponentially growing ECV304/T-24 cells were cultured in $25cm^2$ tissue culture treated flasks and infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. 'Mock' infected cells were prepared as a negative control. At confluence, the supernatants were collected and frozen at −80°C. The cells were passaged and seeded at a one-twentieth the density of confluent cells and grown to confluence. The supernatants were collected and frozen at −80°C.

A ten-fold dilution series of the supernatants collected at both time points was prepared and titrated onto confluent AP61 mosquito cell monolayers as described in section 2.2.2.3. The titres of infectious DEN 2 particles present in the supernatants were calculated in focus forming units/mL. Supernatants from ECV304/T-24 cells collected at first confluence (7 days post infection) had a titre of 5×10^1 ffu/mL. Supernatants from cells which had been passaged once and subsequently grown to confluence had a titre of 1.25×10^2 ffu/mL.

3.2.6 Investigations into whether phenotypic changes of DEN 2 or adaptations of ECV304/T-24 cells have occurred in persistently infected cells

It was decided, using indirect immunofluorescence analysis, to test the ability of DEN 2 progeny virus secreted from infected ECV304/T-24 cells, to infect naïve ECV304/T-24 cells and result in apoptosis of these cells.

The maximum production of progeny virus from ECV304/T-24 cells infected with stock virus had been found to be at 48 hours post infection as shown in Tables 3.1 and 3.2. Exponentially growing ECV304/T-24 cells were cultured on Falcon chamber slides and twenty-four hours later were infected with progeny DEN 2, at a MOI of 1 $TCID_{50}$/cell. At 72 hours post infection, when maximal apoptosis had been detected in cells infected with stock virus (see Fig. 3.10(f)), the cells were fixed in 3% paraformaldehyde in PBS. The cells were incubated with DEN 2-specific hyperimmune mouse ascitic fluids and fluorescein-conjugated anti-mouse IgG and double-labeled with propidium iodide as

described in section 2.2.4.1. The fluorescence was visualized using a confocal fluorescent microscope (Zeiss) and digital imaging system.

Very few of the cells which were infected with progeny virus displayed fluorescence. The fluorescence had a speckled appearance and was localized to the perinuclear region of the cell. No evidence of apoptosis was visible. See Fig. 3.15(a).

Infection of ECV304/T-24 cells with progeny virus does not elicit the same effect as infecting the cells with stock virus. Cells did not appear to die by apoptosis. This suggests, a phenotypic change may have occurred in the virus.

3.2.7 'Superinfection' of ECV304/T-24 cells with DEN 2

It was decided to look at the susceptibility of infected ECV304/T-24 cells to a sequential infection with original stock virus.

Exponentially growing cells were cultured on Falcon chamber slides and infected 24 hours post seeding with DEN 2 at a MOI of 1 $TCID_{50}$/cell. At 72 hours post infection, the cells were challenged with DEN 2 again at a MOI of 1 $TCID_{50}$/cell. 72 hours later (144 hours post primary infection), the cells were fixed in 3% paraformaldehyde in PBS, double-labeled for intracellular virus and apoptotic nuclei (see section 2.2.4.1) and observed under a fluorescent microscope.

Infected cells displayed fluorescence throughout the cytoplasm of the cell, with a pattern similar to that observed in ECV304/T-24 cells at 72 hours post infection with stock DEN 2 (see Fig. 3.1(f)). However, a much smaller number of cells were positive and these were located in foci of one, two or three fluorescent cells, within the monolayer. No evidence of apoptotic nuclei was found. See Fig. 3.15(b).

The cells which are positive for fluorescence are more than likely persistently infected cells similar to those found at 145 hours post infection with stock DEN 2 (Fig. 3.1(j)) and these cells are adapted to DEN 2 challenge and resistant to apoptosis.

Infection of ECV304/T-24 cells with progeny DEN 2 or re-infection of DEN 2 infected ECV304/T-24 cells with stock DEN 2

Fig. 3.15. Intracellular DEN 2 viral antigens were detected using indirect immunofluorescence analysis. (a) Exponentially growing ECV304/T-24 cells were cultured on Falcon chamber slides and 24 hours post seeding, were infected with DEN 2 progeny at a MOI of 1 $TCID_{50}$/cell. At 72 hours post infection, the cells were fixed with 3% PFA and incubated with DEN 2-specific hyperimmune mouse ascitic fluid. A fluorescein-conjugated antibody was used as a detection method and fluorescence was observed using a fluorescent microscope. (b) Exponentially growing ECV304/T-24 cells were cultured on Falcon chamber slides and 24 hours post seeding were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. At 72 hours post infection, the cells were again challenged with DEN 2 at a MOI of 1 $TCID_{50}$/cell and the presence of intracellular DEN 2 viral antigen was detected as above.

Fig. 3.15(a)

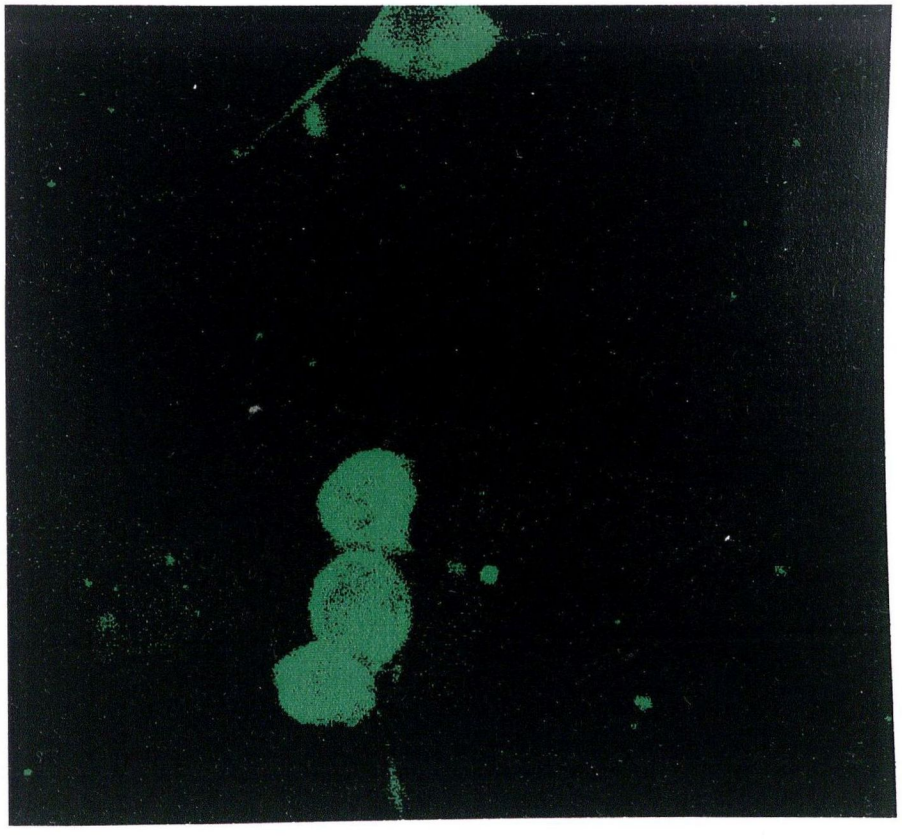


Fig.3.15(b)

3.3 DISCUSSION

From this study, DEN 2 has been shown to infect both ECV304/T-24 cells and primary HUVEC. The virus replicated within ECV304/T-24 cells and an increase in the number of cells expressing DEN viral antigens was observed with time, until a maximum number of antigen expressing cells was detected between 96 and 122 hours post infection. The localization of viral antigens within the cells altered with time, from an intense, continuous pattern of fluorescence being expressed throughout the cytoplasm in the earlier stages of infection, to a more speckled appearance, localized in foci, to the perinuclear area, possibly at the membrane of the endoplasmic reticulum.

The cells remained persistently infected for at least five passages, with virus being detected intracellularly and infectious viral particles being secreted from the cells. The virus did not seem to infect all of the cells of the monolayer. Immunofluorescence analysis revealed that only a limited number of cells, found in foci throughout the monolayer were positive for DEN 2 antigen synthesis. The titres of secreted virus released from the cells were very low, as can be expected in persistent infections. Re-infection of the cells with this progeny virus at a MOI equivalent to those used in primary infections with stock virus, did not result in the level of expression of viral antigens seen in cells experiencing a primary infection. The cells expressing DEN 2 antigens were found as single cells dispersed throughout the monolayer. Whether this difference in expression was due to alterations in the cells or the virus would have to be determined. One possible method would be the genome sequencing of both the parent and progeny virus and determining what, if any mutations may have occurred.

DEN 2 infection of ECV304/T-24 cells was found to be a productive infection, with the release of infectious viral progeny from the cells into the supernatant. Maximum production of progeny virus was found to occur at 48 hours post infection, with titres an order of 10^2 lower than those produced following infection of transformed mosquito cells.

DEN 2 infection of ECV304/T-24 cells and HUVEC resulted in the reduction of the viability of these cells and a visible detachment of these cells from the substratum into the supernatant. However, the level of pathogenesis detected was not sufficient to render the monolayers unable to recover and re-form intact, healthy monolayers. When the viability of ECV304/T-24 cells was investigated, the kinetics of cell death were constant using either

the Trypan Blue or Lactate Dehydrogenase assay. At the point of maximum destruction, which was found to be at five days post infection, using either method, the number of adherent surviving cells remained constant at approximately 30% that of control cells. A mechanism of survival has seemed to be 'switched' on by either the virus or cell, ensuring the recovery of the monolayer. The rate of growth of ECV304/T-24 cells did not seem to affect the percentage of cells which were dying. The control cells, which grew fastest in medium containing 20% FCS, had more than three times the number of cells of those grown in 2% FCS at 6 days post infection. However, the same proportion of cells from DEN 2 infected monolayers grown in 2% or 20% FCS survived. It is important to note however, that these transformed cells do not form contact-inhibited monolayers and that these cells do not grow to confluence, unlike primary endothelial cells or endothelial/epithelial cells found *in vivo*.

It was found that ECV304/T-24 cells which were infected with DEN 2, were dying by apoptosis. Maximum apoptosis was visible using propidium iodide staining at 72 hours post infection and remarkably, there was no visible sign of apoptotic nuclei by 96 hours. The programmed cell death seemed to be switched off, which allowed the recovery of the monolayer. The cells which survived at 5 days post infection and which became persistently infected, were found to be immune to subsequent challenge with stock DEN 2. No apoptosis of the cells was detected at 72 hours post challenge, the time point where maximum apoptosis occurred when the cells experienced a primary infection. Infection of healthy ECV304/T-24 cells with progeny virus at an equivalent MOI did not induce apoptosis in these cells, which suggests that the phenotype of the progeny virus is altered or the susceptibility of these cells which had been infected with DEN 2.

The confluence of ECV304/T-24 cells was not found to affect the ability of DEN 2 to infect and replicate within these cells and cause a loss in viability of the monolayers.

CHAPTER FOUR

Effects of DEN 2 Infection on the Pro-Inflammatory Immune Response

4.1 INTRODUCTION

The aim of this part of the study was to determine whether infection of primary HUVEC endothelial cells and transformed ECV304/T-24 bladder carcinoma cells by DEN 2 elicited a pro-inflammatory immune response in these cells. Elevated levels of the pro-inflammatory cytokines Tumour necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) have been detected in the serum of patients suffering from DHF (51). The pro-inflammatory cytokine Interleukin-1 (IL-1), has been shown to be induced in viral infections (113). Alterations in the levels of gene expression for these cytokines in DEN 2 infected and control cells was determined using a one-step, semi-quantitative RT-PCR method. The secretion of the cytokine from the cell was analysed using an ELISA method. As the functions of endothelial cells have been shown to alter with the state of confluence, it was decided to compare the effects of DEN 2 infection on exponentially growing cells with polarised confluent monolayers, where possible. It was decided to investigate if an immune response was elicited, whether it was due to a surface-stimulated signal transduction pathway or whether infection of the cell was necessary.

It has been postulated that monocytic cells are the major target cells for Dengue infection *in vivo*. Primary adherent monocytes and the human pro-monocytic cell line U937, were infected with DEN 2 and the expression of the cytokines TNF-α, IL-1 and IL-6 was investigated using the technique RT-PCR.

Most hypotheses for the pathogenesis of DHF, implicate a greater severity of disease following a secondary infection. ECV304/T-24 cells were pre-activated with the cytokines TNF-α and IFN-γ prior to infection with DEN 2, in order to simulate the environment late in infection, as monocytes secrete TNF-α during diapodesis, or during a second infection, when DEN-specific helper T lymphocytes secrete IFN-γ. The expression of pro-inflammatory cytokines was compared to that during a primary infection. Using immunofluorescence double-labelling, the presence of intracellular virus and the nuclei of pre-activated, infected cells was compared to cells which had not been stimulated with a cytokine prior to infection.

4.2 RESULTS

4.2.1 Pro-inflammatory cytokine gene expression studies using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

4.2.1.1 Standardization of template RNA

Equal numbers of cells were seeded into 9.5cm^2 mini petri dishes and divided into two groups: cells which were infected with DEN 2 and control cells which were 'mock' infected.

The number of cells between samples differed for a number of reasons. As cells grew, the number present increased (inter-assay variation). In some cases, infection with DEN 2 resulted in the death of cells (see chapter 3) and the number of cells in the infected group differed from the control cells (intra-assay variation). It was not possible to compare the level of gene expression in control and infected cells or the level of gene expression within samples at different time points without standardizing the input template RNA.

The total cellular RNA which was isolated from the cells as described in section 2.2.5.1 was analysed at 260 and 280 nm to determine the concentration and purity of the RNA. An A_{260} reading of unity was taken to be equivalent to 40μg/mL for denatured RNA. A 260/280 ratio between 1.8 and 2.0 was deemed pure and used in RT-PCR studies. It was necessary to subject the RNA to a number of cycles of freezing at –80°C and thawing in order to ensure that the RNA was completely in solution.

The total cellular RNA was then standardized by dilution with nuclease free H_2O and a nominal value of 4 ng/mL was used as template for each RT-PCR reaction.

The reverse transcription and polymerase chain reactions were performed in a single tube in a one-step reaction using the Access RT-PCR kit (see section 2.2.5.2). One primer from each pair created cDNA from template RNA using the enzyme Avian Myeloblastosis Virus (AMV) reverse transcriptase during the RT step and resulted in the production of RNA:cDNA hybrids. These were then amplified, using both primers and the enzyme *Thermus flavus* (*Tfl*) DNA polymerase, during a polymerase chain reaction. Primers were selected which crossed splice sites for each gene of interest and this ensured that any

possible genomic DNA contaminating the sample was not amplified and only spliced mRNA transcripts were used in both the RT and PCR steps (114).

All products were fractionated according to size and visualized using agarose gel electrophoresis as described in section 2.2.5.3. Gels were photographed and the density of each product was determined using a densitometer.

A number of negative controls were prepared. One consisted of reaction mixes without RNA template. This indicated whether possible contaminating DNA was present in the reaction components. A negative control without AMV reverse transcriptase was prepared which ensured that *Tfl* polymerase did not amplify genomic DNA. Another negative control consisted of the reaction mix without the enzyme *Tfl* polymerase, which indicated whether products of the correct size were prepared during the RT step and might influence the density of the final RT-PCR product.

4.2.1.2 Optimization of RT-PCR for each primer pair

Each set of primers required the optimization of the magnesium sulphate concentration within the mix, the annealing temperature (T_m) and the cycle number (n), to result in a single final product of the required size. The maximum level of stringency was necessary to ensure the specificity of primer binding to the template and the efficiency of the enzymes during the reaction. The optimal annealing temperatures were found to be either 55 or 60°C.

Magnesium concentration

For each primer pair, the optimal final concentration of magnesium sulphate was found to be 0.5 mM. A curve of magnesium concentration ranging from 0.5 to 3.0 mM in 0.5 mM increments was prepared for each primer pair and the concentration which yielded a single product was chosen.

Normalization

In order to ensure that an equal amount of RNA was used as template in each reaction, the amplification of a constitutively expressed 'house-keeping' gene was performed for each sample. The gene of choice originally selected had been the structural gene β–actin. A number of experiments included the activation of endothelial cells with the cytokines Tumour necrosis factor-α and Interferon-γ. However, reports have shown that exposure of endothelial cells to these cytokines can lead to the destabilization of β–actin (62, 115, 116). It was decided to substitute the gene glucose-6-phosphate dehydrogenase (G6PD) for β–actin as a constitutively expressed internal control in the reactions. G6PD is an integral enzyme in the glycolytic pathway of respiring human cells.

For each sample, the density of RT-PCR product for the cytokine genes was normalized with the level of G6PD amplified within that sample. It was then possible to determine if a difference in the level of cytokine gene mRNA expression occurred between infected and control samples and to compare the different levels of expression at different time intervals post infection. All data represented graphically in this chapter is comprised of normalized RT-PCR product densities.

Linearity

During non-competitive RT-PCR, theoretically the amount of product doubles during each cycle and there is a linear relationship between the quantity of input RNA and final product concentration. However, it has been shown that beyond a certain number of cycles, the efficiency of amplification decreases and a plateau effect occurs (108). To detect differences in gene expression, it was necessary to ensure that the reaction was stopped within the linear phase such that increasing the cycle number by one, or doubling the input RNA concentration, resulted in a twofold increase in output DNA. The minimum number of cycles which resulted in visible product formation were chosen and for each primer pair, the linearity of the reaction was confirmed, by preparing a doubling dilution series of input RNA at a fixed cycle number (see Fig. 4.1). The cycle numbers ranged from 29 to 34 cycles during the PCR step.

Specificity of RT-PCR

A 100 bp DNA ladder was run on each agarose gel to determine the size of each RT-PCR product formed. For each primer pair, a DNA probe specific to the sequence amplified between both primers was labeled with [γ-^{32}P]ATP and was hybridized to RT-PCR products which had been transferred to a nylon membrane. Bound probe was detected using autoradiography as described in section 2.2.5.5.

4.2.2 Pro-inflammatory cytokine mRNA expression in exponentially growing ECV304/T-24 cells following DEN 2 infection

It was decided to investigate the effect of DEN 2 infection on the expression of two cytokines TNF-α and IL-6, which are known to play a role in inflammation (62, 113), in the cell line ECV304/T-24. This cell line has many properties which are similar to those of endothelial cells (90). A difference between these cells and primary endothelial cells is that ECV304/T-24 cells do not produce factor VIII, nor do they produce the pro-inflammatory cytokine IL-1, which is known to have growth inhibitory properties. These cells are not contact inhibited at confluence (90).

4.2.2.1 Tumour necrosis factor-alpha

Exponentially growing ECV304/T-24 cells were cultured in 9.5cm^2 tissue culture treated petri-dishes and 24 hours post seeding, were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. At regular intervals post infection, total cellular RNA was isolated and the level of TNF-α mRNA expressed was determined for both infected and 'mock' infected cells using semi-quantitative RT-PCR, as described in section 2.2.5.2

The level of TNF-α expression within DEN 2 infected cells was compared to that of the 'mock' infected counterparts following densitometric analysis of both the G6PD and TNF-α RT-PCR products formed for each time point.

TNF-α mRNA expression was induced in DEN 2 infected ECV304/T-24 cells. At eight hours post infection, the level of expression was similar in both infected and 'mock' infected samples. Induction of TNF-α occurred at 24 hours post infection and reached a maximum by 46 hours, when levels were threefold higher in DEN 2 infected cells. At 51.5

hours, the level of TNF-α expression in infected cells was similar to levels detected at 24 h post infection and by seventy-two hours, levels of TNF-α gene expression in infected cells had fallen and were similar to those found in the 'mock' infected samples. Levels of expression had returned to basal levels by 119 hours post infection. See Fig. 4.1(a).

4.2.2.2 Interleukin-6

Total cellular RNA which had been isolated from exponentially growing ECV304/T-24 cells and used to determine TNF-α mRNA expression as described above, was also subjected to semi-quantitative RT-PCR using pimers specific for IL-6 transcripts.

Levels of expression were similar in both infected and 'mock' infected cells at 8 and 24 hours post infection. Induction of IL-6 mRNA was found in DEN 2 infected ECV304/T24 cells at forty-six hours post infection, which continued at 51.5 hours and reached a maximum at 75 hours post infection. The levels had returned to control levels by 119 hours post infection. See Fig. 4.1(b).

4.2.2.3 Comparison of TNF-α and IL-6 gene expression in DEN-2 infected exponentially growing ECV304/T-24 cells

The kinetics of the induction of TNF-α and IL-6 gene expression in DEN 2 infected, exponentially growing ECV304/T-24 cells differed. The induction of TNF-α gene expression occurred before that of IL-6. As TNF-α levels reached a maximum, the induction of IL-6 mRNA occurred. As IL-6 levels rose, a corresponding fall in the induced TNF-α expression was observed. At seventy-two hours post infection, TNF-α levels within DEN 2 infected cells were similar to those of the 'mock' infected counterparts and IL-6 induction was at it's maximum, as seen in Figure 4.2. The level of induction of expression of both TNF-α and IL-6 within infected cells was similar.

Fig. 4.1. Semi-quantitative RT-PCR of total cellular RNA extracts from DEN 2 infected exponentially growing ECV304/T-24 cells. Cells were infected with a MOI of 1 $TCID_{50}$/cell. (a) TNF-α RT-PCR products of 'mock' infected cells at 8, 24, 46, 51.5 and 75 hours post infection (lanes 1, 3, 5, 7 and 9). Even lanes from 2-10 represent DEN 2 infected counterparts. (b) IL-6 and (c) G6PD RT-PCR products of the same samples. Lane 6 in each sample represents DEN 2 infected cells at 46 h.p.i., the time of maximum TNF-α expression.

Linearity; To demonstrate the linearity of the TNF-α and IL-6 RT-PCR reactions, a two-fold dilution series was prepared of the RNA from DEN 2 infected cells at 46 h.p.i. (lane 6) and subjected to RT-PCR. Lanes 11, 12 and 13 of (a) and (b) show the RT-PCR products from this experiment. The linearity of the G6PD reaction is demonstrated in (c) in lanes 11-16. The RNA from growing U937 cells was isolated and subjected to RT-PCR, without standardisation. Lanes 11 and 12 represent RT-PCR products from 'mock' infected and DEN 2 infected cells respectively, at 0 h.p.i. Lanes 13 and 14 at 18 h.p.i. and lanes 15 and 16 represent RT-PCR products from 'mock' infected and DEN 2 infected cells at 46 h.p.i.

Induction of TNF-α and IL-6 mRNA expression in DEN 2 infected exponentially growing ECV304/T-24 cells

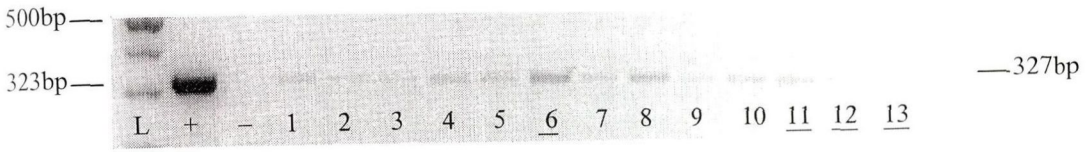


Fig. 4.1(a)

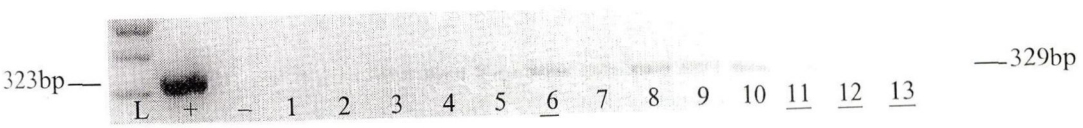


Fig. 4.1(b)

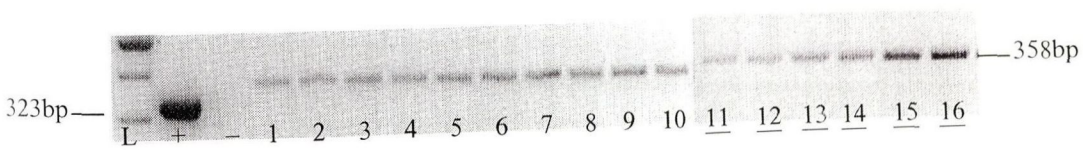


Fig. 4.1(c)

Kinetics of TNF-α and IL-6 mRNA expression in DEN 2 infected exponentially growing ECV340/T-24 cells

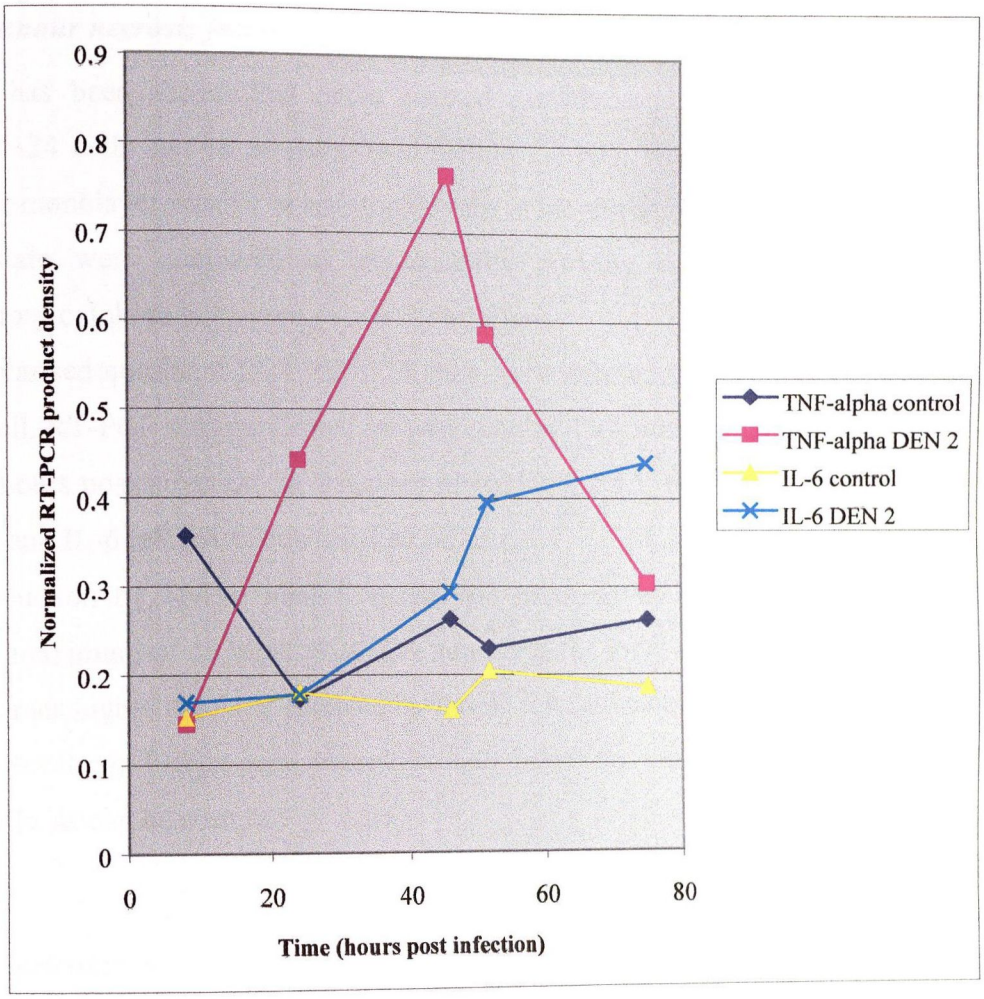


Fig. 4.2. Comparison of TNF-α and IL-6 RT-PCR products in exponentially growing ECV304/T-24 cells infected with DEN 2 virus at a MOI of 1 $TCID_{50}$/mL.

4.2.3 Pro-inflammatory cytokine mRNA expression in DEN 2 infected polarised confluent ECV304/T-24 cells

4.2.3.1 Tumour necrosis factor-alpha

It has been shown that under defined growth conditions, polarized confluent ECV304/T-24 cells can be prepared (see section 2.2.1.1), which can form a vasoactive responsive monolayer, similar to endothelial cells in blood vessels (46). ECV304/T-24 cells in this state were compared to exponentially growing cells with respect to pro-inflammatory cytokine inductions following infection.

Polarised confluent ECV304/T-24 cells were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. RT-PCR was performed on total cellular RNA which was isolated at 6, 24, 48 and 72.5 hours post infection, as described in section 2.2.5.1, to investigate the expression of TNF-α and IL-6 mRNA within infected cells.

Induction of TNF-α mRNA expression occurred in DEN 2 infected cells and reached a maximum at 48 hours post infection when the level of RT-PCR product formed was five times higher than that detected in 'mock' infected counterparts. At 72.5 hours post infection, levels of TNF-α gene expression had fallen, but were higher than those of the control cells, as can be seen in Fig. 4.3(a).

4.2.3.2 Interleukin-6

DEN 2 infection of polarised confluent ECV304/T-24 cells resulted in the induction of IL-6 mRNA expression. Total cellular RNA which had been isolated for use in TNF-α studies above, was subjected to RT-PCR. Induction of IL-6 mRNA expression was detected in DEN 2 infected cells.

Induction of IL-6 mRNA reached a maximum at forty-eight hours post infection, when levels detected within infected cells were nine times higher than those of 'mock' infected counterparts. At 72.5 h post infection, IL-6 mRNA was induced, but levels were approaching those of control cells. See Fig. 4.3(b).

Fig. 4.3. Semi-quantitative RT-PCR of total cellular RNA extracts from DEN 2 infected polarised confluent ECV304/T-24 cells. Cells were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. (a) Lanes 1-8 represent TNF-α RT-PCR products from 'mock' infected (odd lanes 1-7) and DEN 2 infected cells (even lanes 2-8) at 6, 24, 48 and 72.5 h.p.i. Lanes 9-16 represent IL-6 products of the same samples with lanes 9, 11, 13 and 15 representing 'mock' infected cells at 6, 24, 48 and 72.5 h.p.i. and even lanes 12-16 representing DEN 2 infected counterparts. (b) G6PD RT-PCR products of 'mock' infected (odd lanes 1-7) and DEN 2 infected (even lanes 2-8) cells at the same time intervals.

Induction of TNF-α and IL-6 mRNA expression in DEN 2 infected polarised confluent ECV304/T-24 cells

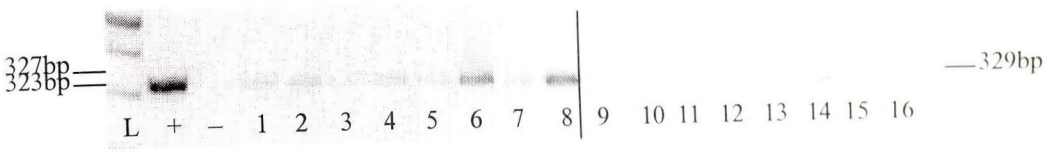


Fig. 4.3(a)

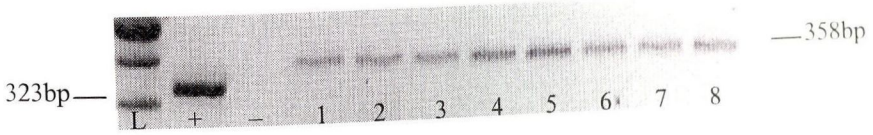


Fig. 4.3(b)

4.2.3.3 Comparison of TNF-α and IL-6 in DEN 2 infected polarised confluent ECV304/T-24 cells

The kinetics of TNF-α and IL-6 mRNA induction in DEN 2 infected ECV304/T-24 cells were similar. The induction of mRNA expression for both cytokines occurred simultaneously and reached a maximum for both at 48 hours post infection. The levels of expression of TNF-α and IL-6 had fallen by 72.5 hours post infection, but remained higher than those of control cells. See Fig. 4.4. The induction of IL-6 was almost twice as strong as that of TNF-α.

4.2.4 Pro-inflammatory cytokine mRNA expression in DEN 2 infected human umbilical vein endothelial cells (HUVEC)

In this study, it was decided to investigate the effects of DEN 2 infection on the inflammatory aspect of the immune response in primary endothelial cells and compare it with that of the cell line ECV304/T-24. The mRNA expression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1 was studied in exponentially growing HUVEC following infection with DEN 2, using the technique semi-quantitative RT-PCR.

Commercially obtained HUVEC were used in this study. The preparation of polarised confluent monolayers was not satisfactory using these cells, once the cells had undergone more than one passage.

4.2.4.1 Tumour necrosis factor-alpha

Exponentially growing HUVEC were seeded into 9.5cm^2 mini petri dishes at a density of 3 x 10^5 cells/dish. Cells were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell and the total cellular RNA was isolated from infected cells and their 'mock' infected counterparts at regular intervals post infection. The RNA was standardized and primers specific for TNF-α cDNA were used to amplify mRNA specific to that gene using the technique of semi-quantitative RT-PCR. Template RNA concentration and RT-PCR conditions used for HUVEC were identical to those used for ECV304/T-24 cells.

Following agarose gel electrophoresis, no TNF-α products were detected in either infected or 'mock' infected cells at 2, 4, 23 or 73 hours post infection, as shown in Fig.

Kinetics of TNF-α and IL-6 mRNA expression in DEN 2 infected polarised confluent ECV340/T-24 cells

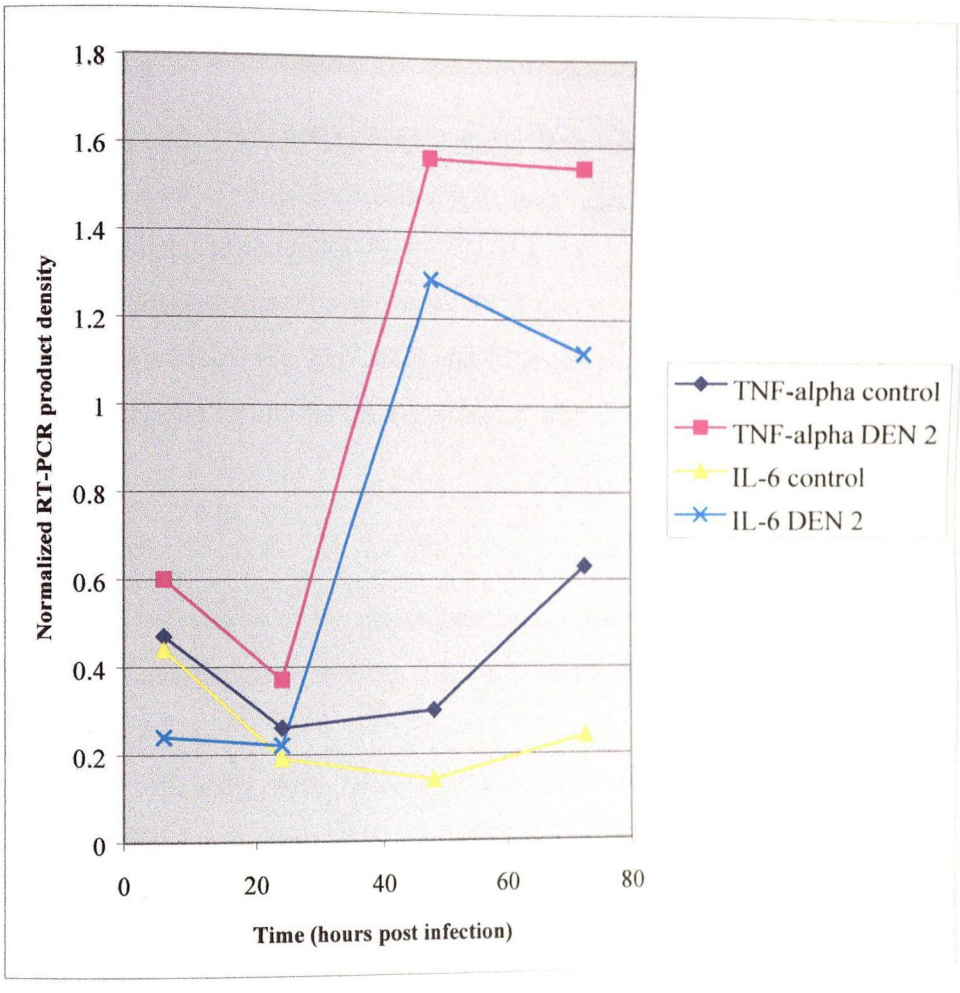


Fig. 4.4. Comparison of TNF-α and IL-6 RT-PCR products of polarised confluent ECV304/T-24 cells which were infected with DEN 2 at a MOI of 4 $TCID_{50}$/mL.

4.5(a). The cycle number was increased to 40 and the process was repeated. No products were obtained.

4.2.4.2 Interkeukin-6

Total cellular RNA which had been isolated from exponentially growing HUVEC and used to investigate TNF-α expression was then used to investigate IL-6 mRNA expression using semi-quantitative RT-PCR.

Following densitometric analysis of RT-PCR products, a higher level of expression was found in DEN 2 infected HUVEC at 23 and 73 hours post infection than in the 'mock' infected control cells. The results are shown in Fig. 4.5(b).

4.2.4.3 Interleukin-1

The mRNA expression of the pro-inflammatory cytokine IL-1, was investigated in DEN 2 infected exponentially growing HUVEC using semi-quantitative RT-PCR as described in section 2.2.5.2.

Infection of HUVEC with DEN 2 at a MOI of 4 $TCID_{50}$/cell resulted in the induction of IL-1 mRNA expression. Higher levels of IL-1 RT-PCR products were found in infected cells at 23 and 73 hours post infection than in 'mock' infected control cells. Photographs of RT-PCR products are shown in Fig. 4.5(c).

From these studies, it was evident that DEN 2 infection of ECV304/T-24 cells and primary HUVEC resulted in the induction of a pro-inflammatory immune response *in vitro*. Infected ECV304/T-24 cells transcribed mRNA for the cytokines TNF-α and IL-6. The effect was observed in both exponentially growing and polarised confluent cells, with differing kinetics. DEN 2 infection of primary HUVEC resulted in the induction of mRNA expression for the pro-inflammatory cytokines IL-1 and IL-6. These cells did not express TNF-α mRNA in either infected or 'mock' infected cells.

Fig. 4.5. Semi-quantitative RT-PCR of total cellular RNA extracts from DEN 2 infected primary HUVEC. Cells were infected with DEN 2 at a MOI of 4 $TCID_{50}$/cell. (a) Lanes 1-4 represent TNF-α RT-PCR products from 'mock' infected (lanes 1 and 3) and DEN 2 infected cells (lanes 2 and 4) at 23.5 and 48 h.p.i. Lanes 5-8 represent RT-PCR products of U937 cells at 24 and 48 hours post infection (odd lanes = 'mock' infected, even lanes = DEN 2 infected). Lane 9 = DEN 2 infected ECV304/T-24 cells at 48 h.p.i.
(b) IL-6 RT-PCR products of DEN 2 infected HUVEC. Lanes 1, 3, 5 and 7 represent 'mock' infected samples at 2, 4, 23 and 73 hours post infection. Lanes 2, 4, 6 and 8 represent DEN 2 infected counterparts. (c) IL-1 RT-PCR products of DEN 2 infected HUVEC. Odd lanes 1-7 represent 'mock' infected samples at 2, 4, 23 and 73 h.p.i. Even lanes 2-8 represent DEN 2 infected counterparts. (d) G6PD RT-PCR products of the samples presented in (b) and (c).

Induction of IL-6 and IL-1 mRNA expression in DEN 2 infected HUVEC

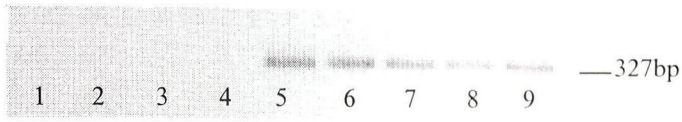


Fig. 4.5(a)

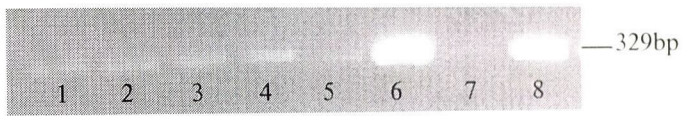


Fig. 4.5(b)

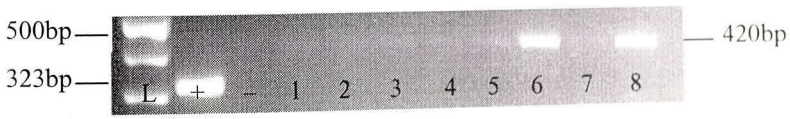


Fig. 4.5(c)

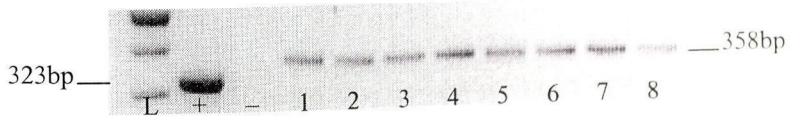


Fig. 4.5(d)

4.2.5 Pro-inflammatory cytokine mRNA expression in the human promonocytic cell line U937, following infection with DEN 2

4.2.5.1 Tumour necrosis factor-alpha, Interleukin-6 and Interleukin-1

U937 cells were seeded at a density of $3x10^5$ cells/mL and infected with DEN 2 viral-immune complexes (section 2.2.2.1,) at a MOI of 1 $TCID_{50}$/cell. Control cells were exposed to medium containing antiserum only. Reports have shown that infection of U937 cells by DEN 2 virus occurs solely via the Fcγ receptor (42).

Total cellular RNA was isolated at 6, 23, 49 and 77 hours post infection and TNF-α mRNA expression investigated using semi-quantitative RT-PCR. TNF-α was constitutively expressed in these cells. No difference in the level of TNF-α mRNA expression was observed for U937 cells infected with DEN 2-immune complexes when compared with control counterparts. U937 cells did not express IL-6 mRNA in either DEN 2-immune complex infected cells or control cells. U937 cells failed to express IL-1 mRNA using the technique RT-PCR (as shown in Fig. 4.8(b)). No product was detected in either DEN 2-immune complex infected or uninfected cells. See Fig. 4.6.

4.2.6 Pro-inflammatory cytokine mRNA expression in DEN 2 infected primary adherent monocytes

4.2.6.1 Susceptibility of primary monocytes to DEN 2 infection

Primary adherent monocytes were isolated from human buffy coats using the method of preparation described in section 2.2.1.3. This method differs from other methods, in that under new regulations, the buffy coats were held at the Blood Transfusion Service Board for two days (at room temperature) until the absence of pathogens was confirmed.

To ensure that the adherent monocytes obtained using this preparation were a pure culture of monocytic cells, the cells were incubated with antibodies to human CD11b and CD14, which were conjugated to phytoerythrin and the presence of these ligands on the surface of the cells was detected using FACS analysis as described in section 2.2.1.4. The

TNF-α and IL-6 mRNA expression in DEN 2 viral-immune complex infected U937 cells

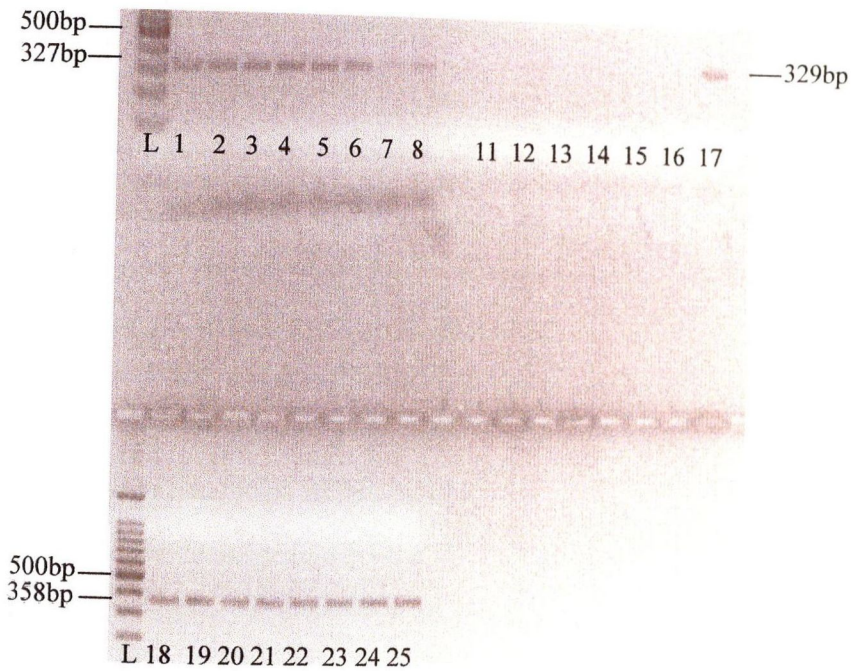


Fig. 4.6. Semi-quantitative RT-PCR of total cellular RNA extracts from U937 cells infected with DEN 2 viral-immune complexes. Even lanes from 18-20 represent G6PD products for 'mock' infected cells at 6, 23, 49 and 77.35 hours post infection. Odd lanes = infected counterparts. Lanes 1-8 = TNF-α products of 'mock' infected (odd lanes) and infected (even lanes) samples at the above time points. Lanes 11-16 = IL-6 products of U937 cells and lane 17 = IL-6 product of DEN 2 infected ECV304/T-24 cells 48 hours post infection.

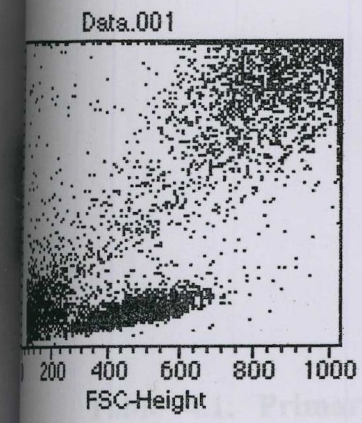
results of this experiment are presented in Fig. 4.7. Negative control cells were exposed to antibodies to murine IgG1.

The monocytes were infected with DEN 2-immune complexes at a MOI of 4 viruses/cell and the susceptibility of these cells to infection was investigated. Supernatants from infected cells were collected at 1, 2, 7 and 8 days post infection. A doubling dilution series was prepared and the presence of secreted virus from the cells was determined by calculating the $TCID_{50}$ on C6/36 monolayers as described in section 2.2.2.2. The cells were found to be susceptible to infection via the Fcγ receptor and the results are given in Table 4.1.

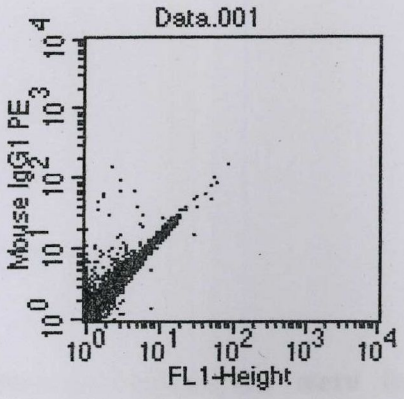
4.2.6.2 Tumour necrosis factor-alpha, Interleukin-6 and Interleukin-1

Total cellular RNA was isolated from DEN 2-immune complex infected cells and the expression of TNF-α, IL-6 and IL-1 mRNA was investigated using semi-quantitative RT-PCR. The time course was extended for monocytes as reports have shown that monocyte response to alphavirus infection may occur as late as one week post infection (117). Following densitometric analysis of normalized RT-PCR products, TNF-α mRNA was found to be induced in DEN 2 infected cells above 'mock' infected control levels, at 6 hours post infection. See Fig. 4.8(a). IL-1 induction was observed 24 hours post infection. See Fig. 4.8(b). IL-6 induction occurred at 7 days post infection, as shown in Fig. 4.8(c).

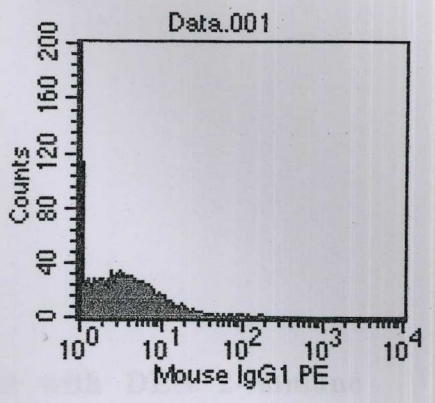
From these studies it is evident that primary adherent human monocytes and the transformed promonocytic cell line U937 respond very differently to infection by DEN 2 virus via the Fcγ receptor. DEN 2 infection of primary cells did result in an inflammatory response whereas, U937 cells did not express the cytokines IL-6 and IL-1 and failed to show an induction in transcription of the pro-inflammatory cytokine TNF-α.



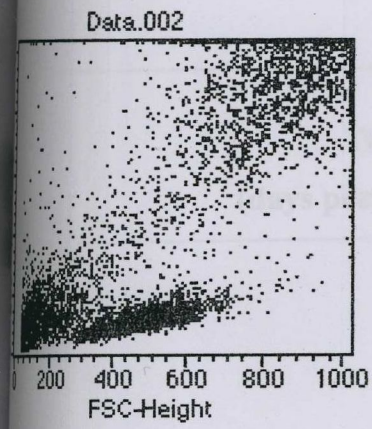
FSC/SSC



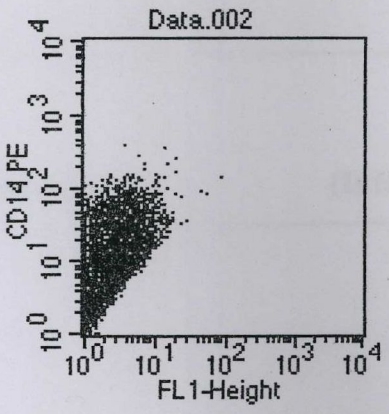
FL1/FL2 Igg1PE



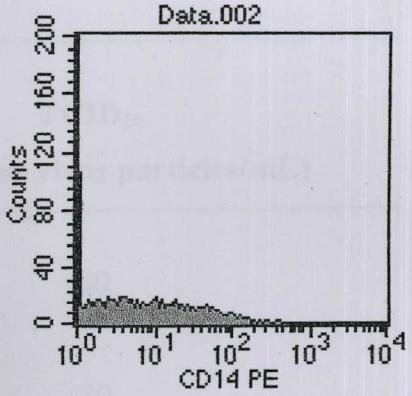
Histogram Igg1 PE



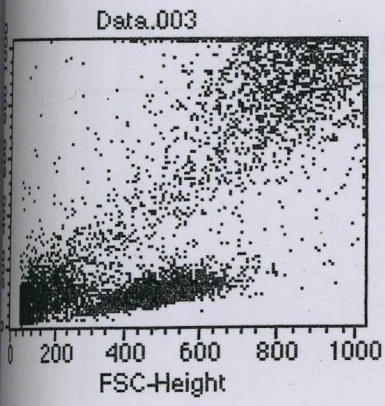
FSC/SSC



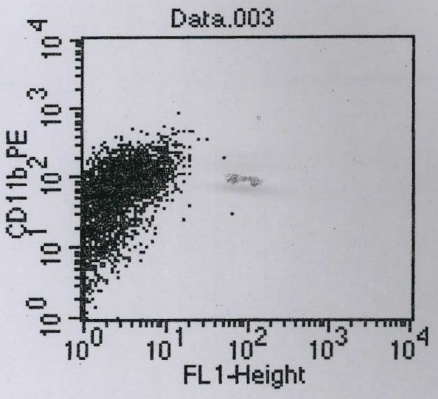
FL1/FL2 CD14 PE



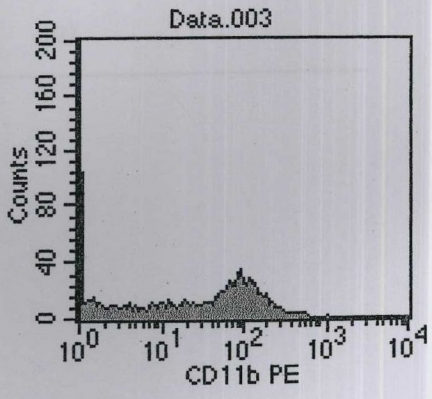
Histogram CD14 PE



FSC/SSC



FL1/FL2 CD 11b PE



Histogram CD11b PE

Fig. 4.7. FACS analysis of primary adherent monocytes incubated with antibodies to murine IgG1 (as a negative control), CD14 and CD11b.

Table 4.1. Primary adherent monocytes were infected with DEN 2-immune complex and the titre of virus secreted from the cells was determined by calculating the $TCID_{50}$ on C6/36 monolayers.

Time (days post infection)	$TCID_{50}$ (Infectious virus particles/mL)
1	40
2	40
7	40
8	80

Fig. 4.8. Semi-quantitative RT-PCR of total cellular RNA extracts from DEN 2 infected primary adherent monocytes. Cells were infected with DEN 2 or DEN 2 viral-immune complexes at a MOI of 4 $TCID_{50}$/cell.

(a) TNF-α RT=PCR products. Lanes 1 and 2 = positive and negative controls. Lanes 5 and 9 = unstimulated control cells at 25 and 48 h.p.i. Lanes 3, 7 and 11 = antiserum stimulated negative controls at 6, 25 and 48 h.p.i. Lanes 6 and 10 = DEN 2 infected cells at 25 and 48 h.p.i. Lanes 4, 8 and 12 = DEN 2 viral-immune complex infected cells at 6, 25 and 48 h.p.i. (Lanes 13-15 = PMA stimulated cells at 48 h.p.i. Lane 13 = PMA treated only, 14 = PMA treated and 'mock' infected, lane 15 = PMA treated and DEN 2 viral-immune complex infected).

(d) Lanes 1-13 = G6PD products of the same samples (with different lane numbering). Lanes 3 and 7 = unstimulated control cells at 25 and 48 h.p.i. Lanes 1, 5 and 9 = antiserum stimulated negative controls at 6, 25 and 48 h.p.i. Lanes 4 and 8 = DEN 2 infected cells at 25 and 48 h.p.i. Lanes 2, 6 and 10 = DEN 2 viral-immune complex infected cells at 6, 25 and 48 h.p.i. (Lanes 13-15 = PMA stimulated cells at 48 h.p.i. Lane 13 = PMA treated only, 14 = PMA treated and 'mock' infected, lane 15 = PMA treated and DEN 2 viral-immune complex infected).

(b) IL-1 products of 'mock' infected and DEN 2 viral-immune complex infected U937 cells (lanes 1 and 2) and primary monocytes (lanes 3 and 4) at 24 hours post infection.

(c) IL-6 RT-PCR products of 'mock' infected (odd lanes 1-7) and DEN 2 viral-immune complex infected (even lanes 2-8) cells at 1, 2, 7 and 8 days post infection. (d) Lanes 14-21 = G6PD RT-PCR products of these samples.

TNF-α, IL-6 and IL-1 mRNA expression in DEN 2 viral-immune complex infected primary adherent monocytes

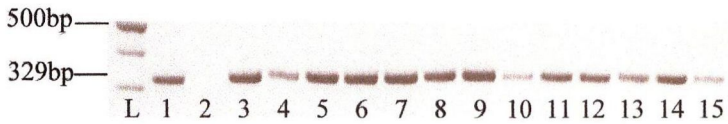


Fig. 4.8(a)

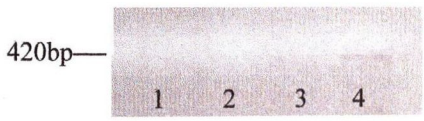


Fig. 4.8(b)

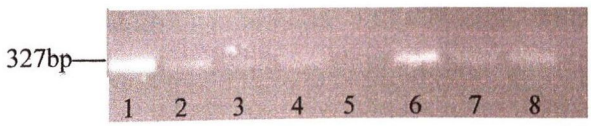


Fig. 4.8(c)

Fig. 4.8(d)

4.2.7 Effects of exposure to inactivated DEN 2 on the host cell cytokine response

To determine whether the effects on the host cell immune response were due to the binding of DEN 2 to the cell surface, or entry of the virus into the cell and replication of DEN 2 within the cell, the virus was inactivated with the compound β–propiolactione (BPL). BPL has been shown to inactivate viruses (106), including DEN virus (105) without a loss in the antigenicity of the virus.

4.2.7.1 β–propiolactone and Borate buffer

A number of different conditions have been described for the inactivation of viruses with BPL. A series of experiments was designed to determine the best conditions for inactivation of stock DEN 2 collected from C6/36 cells with titres of 10^7 $TCID_{50}$/mL or 5 x 10^6 ffu/mL, without resulting in toxicity to mosquito or human cells.

DEN 2 was incubated at 37°C for 15 min in a solution containing 0.05%(v/v) BPL in borate buffer, pH 9.0, as described previously (105). A doubling dilution series of the solution was prepared and the infectivity of the virus was tested by calculating the $TCID_{50}$ on C6/36 mosquito cell monolayers, as described in section 2.2.2.2. A titre of 40 $TCID_{50}$/mL was detected and this was deemed insufficient for the inactivation of stock DEN 2. A range of concentrations of BPL were prepared and tested.

4.2.7.2 Preparation of a titration curve of β–propiolactione concentrations

A titration curve of BPL concentrations, ranging from 0.015 – 0.1%(v/v) BPL in borate buffer, pH 9.0, was then prepared and incubated with stock DEN 2 at 37°C for 15 min. Controls were prepared for each concentration which did not include DEN 2. All samples were tested in duplicate. $TCID_{50}$s were calculated and are represented in Table 4.2.

At BPL concentrations of 0.05%(v/v) and lower, a viral cytopathic effect was observed on C6/36 mosquito cells, which indicated that DEN 2 remained infective and active. Each of the control samples were tested on C6/36 cells to determine whether the concentration of BPL present was toxic to cells. A massive loss of C6/36 cells was found

Table 4.2. Effect of exposure to DEN 2 (titre of 10^6 $TCID_{50}$/mL) incubated in a range of β–propiolactone concentrations on the viability of C6/36 mosquito cell monolayers.

BPL concentration % (v/v)	+DEN 2 $TCID_{50}$	–DEN 2 $TCID_{50}$
0.015	≥160*	<10
0.025	80*	10[§]
0.05	40*	20[§]
0.06	30[§]	40[§]
0.07	30[§]	40[§]
0.08	40[§]	40[§]
0.10	60[§]	40[§]

* indicates a cytopathic effect typical of DEN 2 infection of C6/36 cells with syncytial invagination.

[§] indicates a complete loss of C6/36 cells from the surface of the well.

with BPL concentrations of 0.025% (v/v) and above. The cytopathic effect was unlike that caused by DEN 2 infection of C6/36 cells.

4.2.7.3 β–propiolactione and Tris buffer

Another report (106) suggested virus inactivation by incubation with a preparation of 0.015%(v/v) BPL in a Tris-HCl buffer, pH 7.5 at room temperature for 24 hours, shielded from light, to allow the hydrolysis of β–propiolactone to it's product β–hydroxypropionic acid, an agent which is not toxic to cells.

It was decided to incubate DEN 2 with 0.015% and 0.06%(v/v) BPL in Tris buffer, pH 7.5, at room temperature, shielded from light for 48 hours and to determine whether the solutions were toxic to C6/36 cells and if inactivation of DEN 2 had occurred. Each concentration of BPL used was compared with a solution of BPL in borate buffer, pH 9.0.

A positive control was prepared which consisted of DEN 2 incubated at room temperature and shielded from light for 48 hours, to test if virus remained infective under these conditions. The results are given in Table 4.3.

The positive control sample which contained DEN 2 following incubation at room temperature for 48 hours, remained infective. This indicated that BPL was necessary for inactivation.

When the negative controls, which comprised of inactivation solution without DEN 2 were added to the cells, it was clear that the presence of borate buffer, pH 9.0, was toxic to the mosquito cells. The higher concentration of BPL (0.06%(v/v)) in borate resulted in a greater $TCID_{50}$. The virus did not become inactivated when incubated with 0.015 or 0.06%(v/v) BPL in borate buffer.

When either 0.015 or 0.06%(v/v) BPL with Tris buffer was added to the mosquito cells, the solutions were not toxic to the mosquito cells. When DEN 2 was incubated in these solutions, the virus became inactivated and exposure of C6/36 cells to the solutions did not result in a cytopathic effect. It was decided to inactivate DEN 2 in the presence of 0.06%(v/v) BPL in Tris pH 7.5, at room temperature for 48 hours shielded from light before use in immune response studies.

Table 4.3. The effects of exposure of C6/36 mosquito cells to DEN 2 (titre of 10^7 $TCID_{50}$/mL) incubated in a range of BPL concentrations, prepared in either borate or Tris buffer. Solutions were incubated at room temperature for 48 hours and shielded from light.

BPL concentration % (v/v)	+DEN 2 $TCID_{50}$	–DEN 2 $TCID_{50}$
0.015 Borate buffer, pH 9.0	≥160*	15[§]
0.015 Tris buffer, pH 7.5	<10	<10
0.06 Borate buffer, pH 9.0	≥160*	40[§]
0.06 Tris buffer, pH 7.5	<10	<10
Positive control[a]	≥160*	<10

[a] indicates DEN 2 incubated at room temperature for 48 h, shielded from light.

* indicates a cytopathic effect typical of DEN 2 infection of C6/36 cells with syncytial invagination.

[§] indicates a complete loss of C6/36 cells from the wells.

4.2.7.4 Effects of ECV304/T-24 cell exposure to inactivated DEN 2 on TNF-α and IL-6 mRNA expression

Polarised confluent ECV304/T-24 cells were infected with either DEN 2, or inactivated DEN 2 (as described above) at a MOI of 1 $TCID_{50}$/cell. Total cellular RNA was isolated from the cells at 23.5, 48 and 72 hours post infection and the expression of the genes TNF-α and IL-6 was investigated using semi-quantitative RT-PCR as described in section 4.2.1. Following agarose gel electrophoresis, RT-PCR products were photographed and densitometric analysis of the products was performed.

Levels of TNF-α and IL-6 in ECV304/T-24 cells which were exposed to inactivated DEN 2, were found to be similar to those of the 'mock' infected untreated counterparts as seen in Figure 4.9. To ensure that the chemicals present in the inactivation solution did not affect the cytokine response, ECV304/T-24 cells were exposed to the solution without the presence of DEN 2. No difference in the level of TNF-α or IL-6 mRNA expression was detected, when compared to that of negative control cells. A positive control sample consisting of mRNA isolated from DEN 2 infected ECV304/T-24 cells for 48 hours, was also included in the study.

From these results, it is evident that infection of ECV304/T-24 cells by active DEN 2 is necessary for the induction of gene expression of the pro-inflammatory cytokines TNF-α and IL-6. Exposure of ECV304/T-24 cells to inactivated DEN did not induce an inflammatory response. The effects of DEN 2 on the induction of TNF-α and IL-6 in ECV304/T-24 cells are not as a result of a cell-surface stimulated signal transduction pathway.

4.2.8 Secretion of tumour necrosis factor-α from DEN 2 infected ECV 304/T-24 cells

The secretion of the pro-inflammatory cytokine TNF-α from DEN 2 infected ECV304/T-24 cells was investigated using a sandwich ELISA method as described in section 2.2.5.6. A murine anti-human TNF-α antibody was used as the capture antibody and biotinylated rabbit anti-human TNF-α antibody was used as the secondary antibody. Bound antibody was detected using HRP-conjugated streptavidin and TMB, a substrate for HRP.

Infection of ECV304/T-24 cells with DEN 2 is necessary for the induction of TNF-α and IL-6 mRNA expression

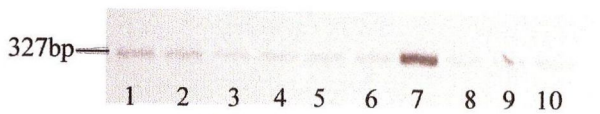


Fig. 4.9(a)

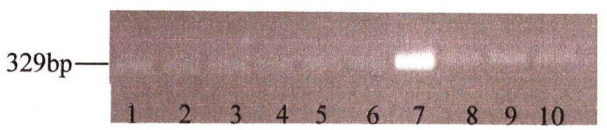


Fig. 4.9(b)

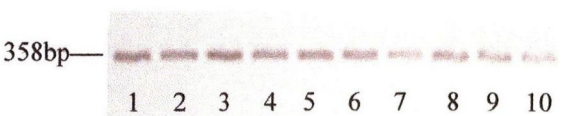


Fig. 4.9(c)

Fig. 4.9. Semi-quantitative RT-PCR of total cellular RNA isolated from polarised confluent ECV304/T-24 cells exposed to inactivated DEN 2. (a) Lanes 1, 4 and 8 = TNF-α products of 'mock' infected cells at 23.5, 48 and 72 hours post infection. Lanes 2, 5 and 9 = cells stimulated with BPL only. Lanes 3, 6 and 10 = cells exposed to inactivated DEN 2 for 23.5, 48 and 72 hours. Lane 7 = TNF-α RT-PCR products of DEN 2 infected cells, 48 h.p.i. (b) IL-6 and (c) G6PD RT-PCR products of the same samples.

Sulphuric acid was used to stop the reaction. The resulting solution was assayed colorimetrically at an absorbance of 450 nm.

A standard curve was prepared of a doubling dilution series of human recombinant TNF-α, ranging from 1000 pg/mL to 15.625 pg/mL with the corresponding absorbance of TMB for each concentration and is given in Fig. 4.10.

Polarised confluent ECV304/T-24 cells were infected by DEN 2 and the supernatants of these cells and of 'mock' infected counterparts were collected at regular intervals post infection and frozen at –80°C until assayed. The presence of secreted human TNF-α was detected in the supernatants of DEN 2 infected ECV304/T-24 cells and is shown in Figure 4.11.

The results are an average of two independent experiments and show that at 18 hours post infection, the level of TNF-α detected in the supernatant was equivalent to that of the 'mock' infected control group. This had increased to an average of 35.23 pg/mL by 42 hours and 70.91 pg/mL by 66.5 hours post infection. 'Mock' infected control levels ranged from 7.85 to 13.55 pg/mL.

DEN 2 infection of ECV304/T-24 cells resulted in the release of the pro-inflammatory cytokine TNF-α from the cells and this occurs at some time point between 18 and 42 hours post infection.

4.2.9 Effects of TNF-α pre-activation on pro-inflammatory cytokine induction by DEN 2 infection of ECV304/T-24 cells

In this study, it was decided to activate polarised confluent ECV304/T-24 cells with cytokines which have been shown to be present during a 'second infection' or secreted late in infection. The shock associated with Dengue Shock Syndrome/Dengue Haemorrhagic Fever occurs quite late in infection (32).

Dengue virus activates T_H-1 and T_H-2 memory T lymphocytes upon a sequential infection and induces the secretion of cytokines specific to these cells, such as Interferon-γ (37). Monocytes are known to secrete Tumour necrosis factor-α during diapodesis, as they pass through the capillary wall. It was decided to simulate the *in vitro* model of polarised confluent ECV304/T-24 cells with the cytokines TNF-α and IFN-γ for 24 hours and subsequently challenge these cells with DEN 2. The effect of infection following pre-

Standard curve of secreted TNF-α and corresponding absorbance, using an ELISA

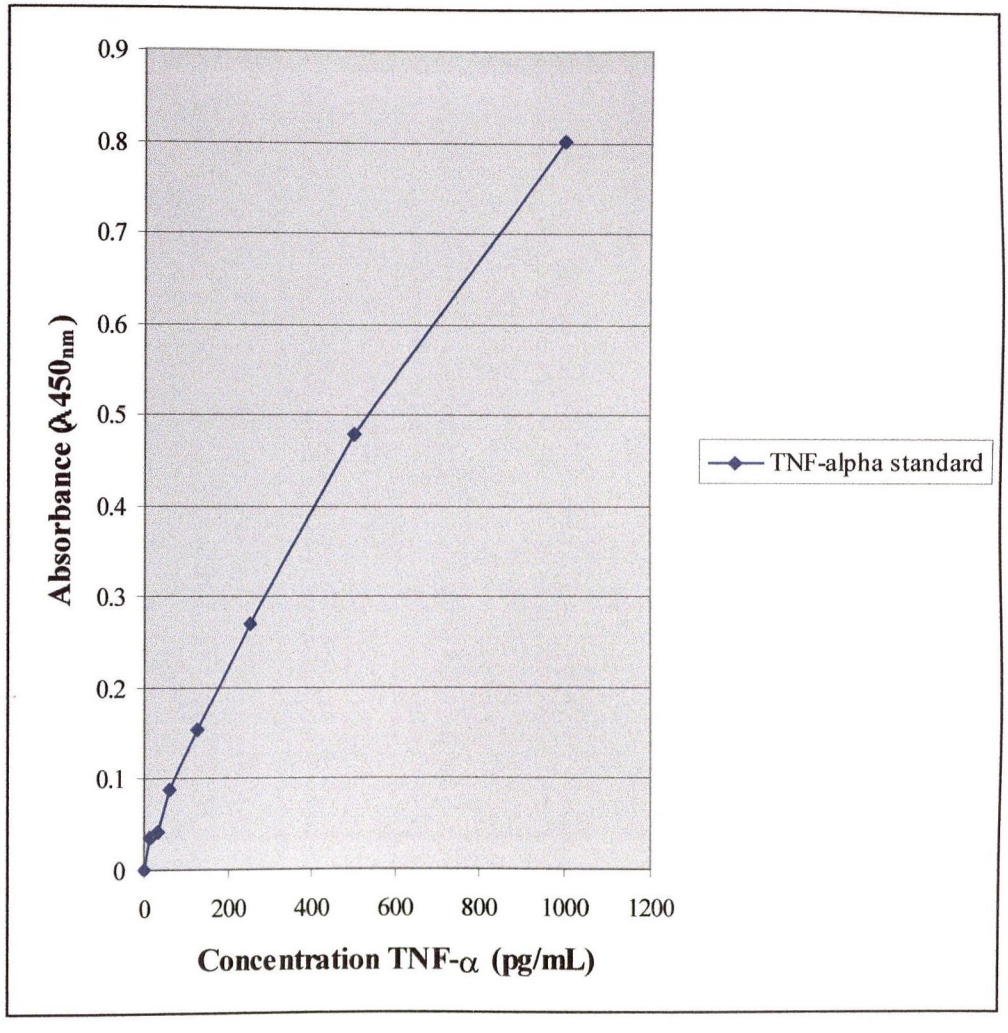


Fig.4.10. Standard curve of TNF-α concentrations ranging from 15.625pg/mL to 1000pg/mL and the corresponding absorbance of the solution at 450nm, using an ELISA.

Secretion of TNF-α from DEN 2 infected polarised confluent ECV304/T-24 cells

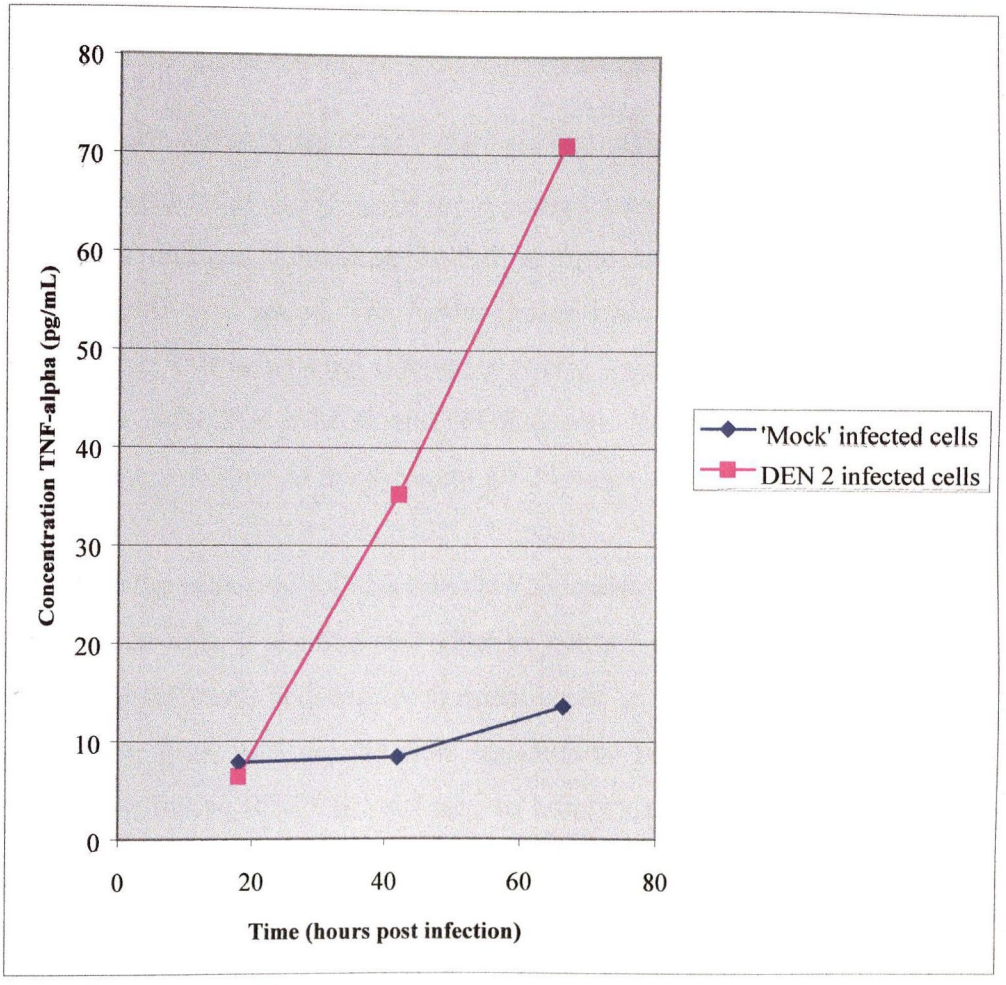


Fig. 4.11. Secreted TNF-α from DEN 2 infected polarised confluent ECV304/T-24 cells was detected using an enzyme-linked immunosorbant assay (ELISA). Supernatants from DEN 2 infected and 'mock' infected cells were collected at 18, 42 and 66.5 hours post infection.

activation on the ability of these cells to induce TNF-α and IL-6 mRNA (as seen in section 4.2.3) was investigated using RT-PCR.

4.2.9.1 Tumour necrosis factor-α mRNA expression

Polarised confluent ECV304/T-24 cells were stimulated for 24 hours with TNF-α at levels within a clinical range (118). Cells were seeded into 24-well tissue culture treated plates and 2mL of medium containing TNF-α at concentrations of 10ng/mL, 1ng/mL 100pg/mL or 10pg/mL was added. The levels chosen have been found to lie within the clinical range *in vivo,* following viral infections (118). Twenty-four hours later, the cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Negative control samples were treated with each concentration of the cytokine for 24 hours and were then 'mock' infected for 48 hours.

To compare the effects of DEN 2 infection following pre-activation with TNF-α, to an infection of naïve cells, a positive and negative control group was included, in which cells were not stimulated with TNF-α prior to infection or 'mock' infection.

Total cellular RNA was isolated from the cells at 48 hours post infection and the technique semi-quantitative RT-PCR was used to amplify mRNA specific to the TNF-α gene (as described in section 2.2.5.2). RT-PCR products were fractionated and visualized on an agarose gel and densitometric analysis was used to determine whether differences in the levels of gene expression were detected.

In the positive control group which comprised cells which were infected with DEN 2 for 48 hours, but not pre-activated with TNF-α, an induction of TNF-α mRNA expression occurred. Previous results indicated that maximum levels of induction of TNF-α in DEN 2 infected ECV304/T-24 cells occurred at 48 hours post infection (see sections 4.2.2.1 and 4.2.3.1).

When ECV304/T-24 cells were pre-activated with TNF-α and 'mock' infected, levels of TNF-α were very similar to those of unstimulated 'mock' infected negative control cells, regardless of the concentration of cytokine used.

However, when ECV304/T-24 cells were activated with TNF-α prior to infection with DEN 2, an induction of TNF-α was observed for each concentration of cytokine used. This level was lower than that of the positive control cells. The suppressive effect of TNF-α

pre-treatment on the ability of DEN 2 to induce TNF-α mRNA was dose dependent, with the greatest inhibitory effect found at a concentration of 1ng/mL. See Fig. 4.12.

As a control experiment, a trypan blue exclusion assay was prepared to determine whether the addition of TNF-α to ECV304/T-24 cells affected the viability of these cells. Exponentially growing cells were seeded at a density of 1.28 x 10^4 cells/9.5cm^2 mini petri dish and grown to confluence. Five days post seeding, the cells were stimulated with TNF-α at concentrations of 25, 5, 2.5, 1.25, and 0.5ng/mL. Twenty-four hours post addition of TNF-α to the cells, a trypan blue assay was performed (see section 2.2.3.1). No reduction in the number of viable cells was found at any of the concentrations of cytokine added.

4.2.9.2 Interleukin-6 mRNA expression

The effect of TNF-α pre-activation on the induction of IL-6 mRNA following DEN 2 infection of polarised confluent ECV304/T-24 cells was investigated using semi-quantitative RT-PCR. Confluent ECV304/T-24 cells were stimulated for 24 hours with TNF-α at 10ng/mL, 1ng/mL, 100pg/mL or 10pg/mL and subsequently infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 48 hours. Total cellular RNA was isolated and the expression of IL-6 mRNA was investigated.

Control cells which had been treated with TNF-α and 'mock' infected showed low levels of IL-6 mRNA expression as did the unstimulated 'mock' infected cells (negative control). A dose responsive minor induction in IL-6 mRNA may be evident as the concentration of TNF-α increased.

DEN 2 infected cells were affected by TNF-α pre-activation. IL-6 mRNA was induced in these cells, above levels of the corresponding control cells and the negative control. Induction of IL-6 was however, lower than that observed in DEN 2 infected cells which had not been pre-activated with TNF-α. This effect was dose dependent and as with TNF-α mRNA expression, the greatest suppressive effect was evident when cells were exposed to 1ng/mL. See Fig. 4.13.

These results indicate that TNF-α acts as an anti-viral agent. The induction of TNF-α and IL-6 mRNA which occurs following infection with DEN 2, is suppressed in cells which are stimulated with the cytokine TNF-α, prior to infection.

The effects of TNF-α pretreatment on the induction of TNF-α mRNA expression following DEN 2 infection of ECV304/T-24 cells

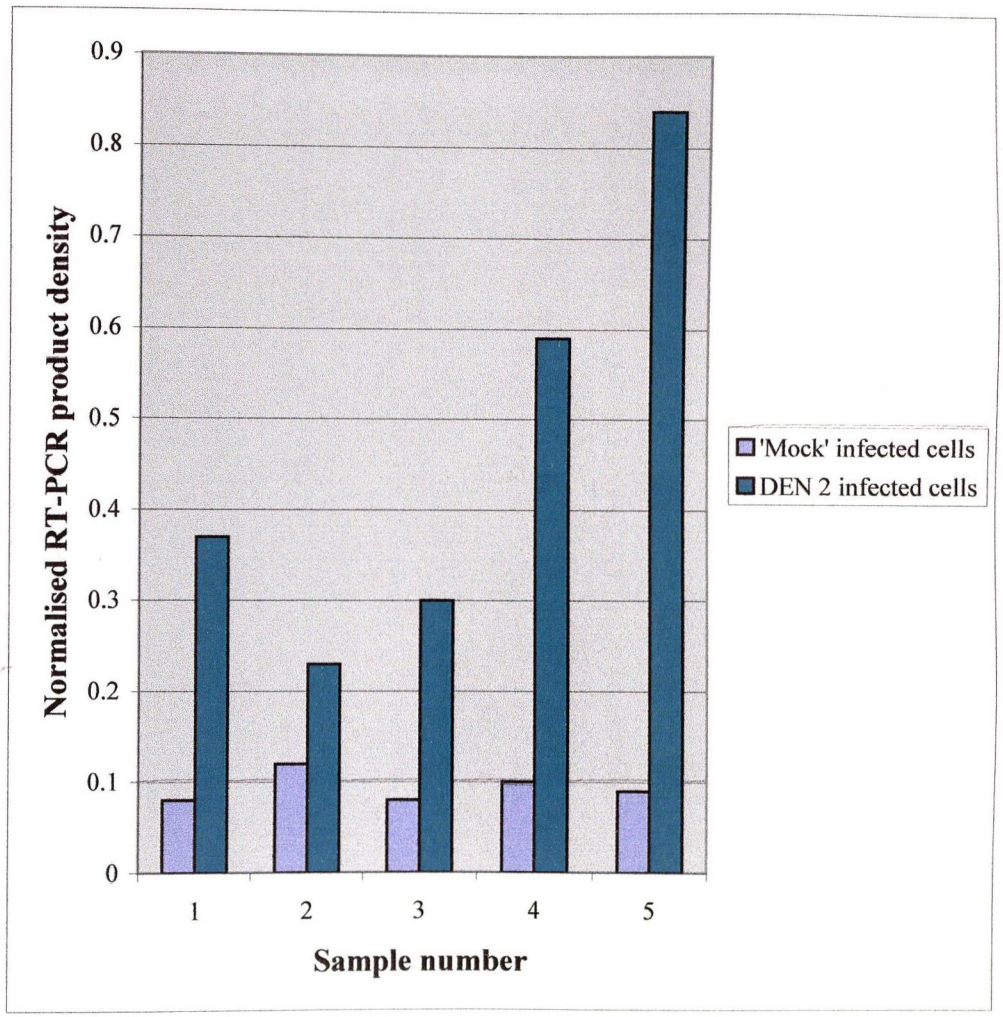


Fig. 4.12. Effects of TNF-α pre-activation on TNF-α mRNA expression following DEN 2 infection of polarised confluent ECV304/T-24 cells. Cells were stimulated with TNF-α at concentrations of 10ng/mL(lane 1), 1ng/mL(lane 2), 100pg/mL(lane 3) and 10pg/mL(lane 4) for 24 hours prior to infection. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 48 hours. Total cellular RNA was isolated and TNF-α mRNA expression was detected using semi-quantitative RT-PCR. Lane 5 represents control cells which were not exposed to TNF-α.

The effects of TNF-α pretreatment on the induction of IL-6 mRNA expression following DEN 2 infection of ECV304/T-24 cells

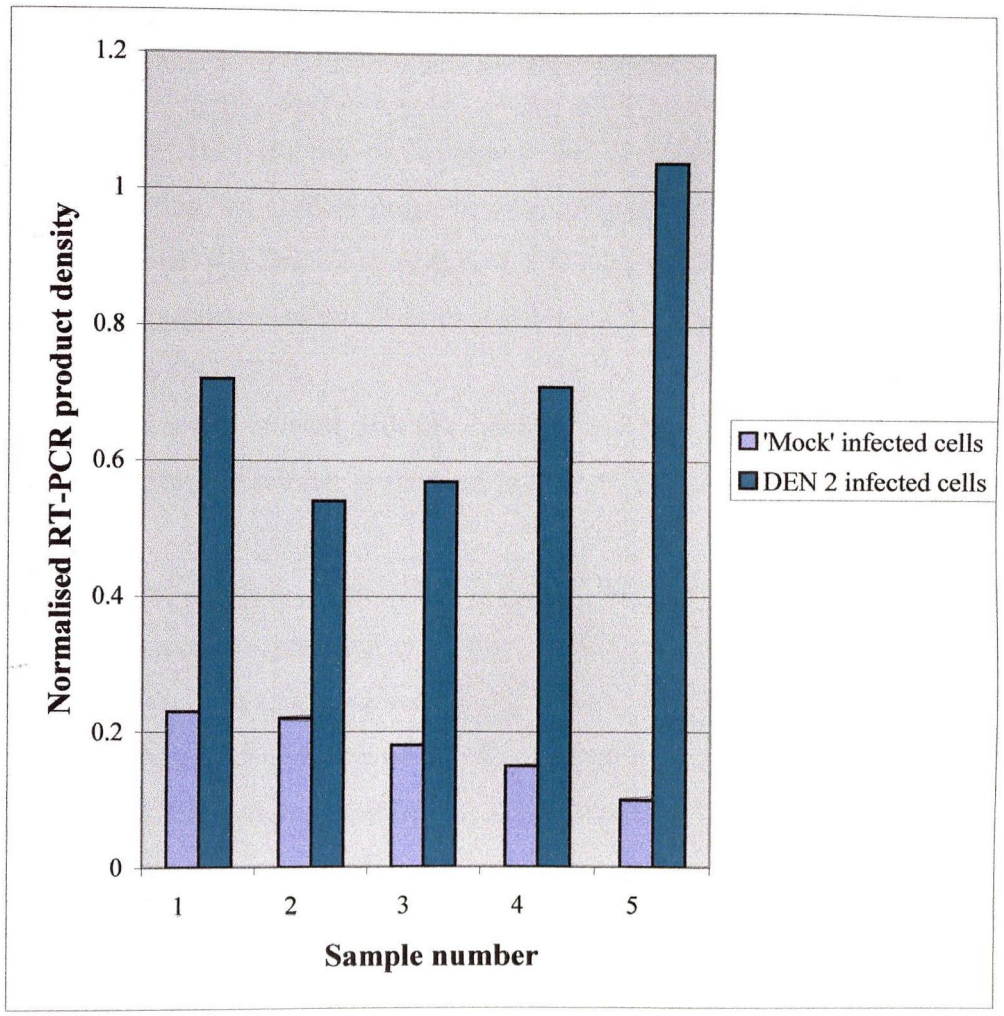


Fig. 4.13. Effects of TNF-α pre-activation on IL-6 mRNA expression of polarised confluent ECV304/T-24 cells following DEN 2 infection. Cells were stimulated with TNF-α at concentrations of 10ng/mL(lane 1), 1ng/mL(lane 2), 100pg/mL(lane 3) and 10pg/mL(lane 4) for 24 hours prior to infection. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 48 hours. Total cellular RNA was isolated and IL-6 mRNA expression was detected using semi-quantitative RT-PCR. Lane 5 represents control cells which were not exposed to TNF-α.

4.2.10 Effects of IFN-γ pre-activation on pro-inflammatory cytokine induction by DEN 2 infection of ECV304/T-24 cells

4.2.10.1 Tumour necrosis factor-α mRNA expression

Polarised confluent ECV304/T-24 cells were stimulated with IFN-γ at concentrations of 10ng/mL, 1ng/mL or 100pg/mL for 24 hours. The levels chosen have been found to lie within the clinical range *in vivo,* following viral infections (118). The cells were then infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell and the total cellular RNA was isolated 48 hours post infection. The expression of TNF-α mRNA was investigated using semi-quantitative RT-PCR.

Cells which were stimulated with the cytokine and 'mock' infected, showed levels of TNF-α mRNA expression similar to those of untreated 'mock' infected negative control cells.

Stimulation of the cells with IFN-γ prior to DEN 2 infection, did affect the expression of TNF-α mRNA. Induction of TNF-α mRNA levels was observed but the level of induction was lower than that observed in cells which had been infected with DEN 2 only. The suppressive effect of IFN-γ on DEN 2 infection was dose dependent with the greatest effect seen at the highest concentration of cytokine used (10ng/mL). See Fig. 4.14.

As a control experiment, the effects of Interferon-γ on the viability of ECV304/T-24 cells was also investigated using the Lactate Dehydrogenase assay, as previously described (section 2.2.3.2). IFN-γ was added to the cells at concentrations of 100, 10, 1.0, 0.1 and 0ng/mL. No reduction in the viability of treated cells was observed, when compared to control cells.

4.2.10.2 Interleukin-6 mRNA expression

The expression of IL-6 mRNA in polarised confluent ECV304/T-24 cells which had been stimulated with IFN-γ prior to infection with DEN 2 was then investigated using semi-quantitative RT-PCR. Cells were stimulated with IFN-γ at concentrations of 10ng/mL, 1ng/mL or 100pg/mL for 24 hours and subsequently infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 48 hours.

The effects of IFN-γ pretreatment on the induction of TNF-α mRNA expression following DEN 2 infection of ECV304/T-24 cells

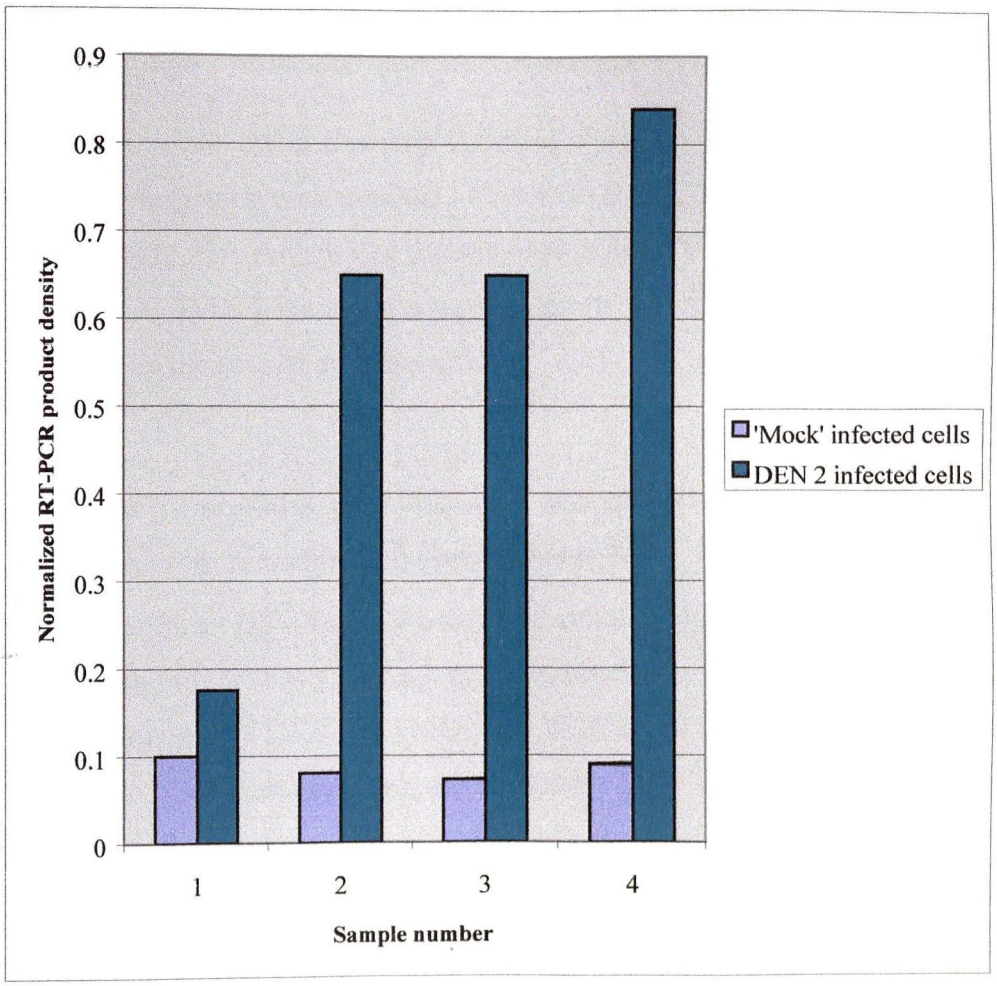


Fig. 4.14. Effects of IFN-γ pre-activation on the induction of TNF-α mRNA expression following DEN 2 infection of polarised confluent ECV304/T-24 cells. Cells were stimulated with 10ng/mL(lane 1), 1ng/mL(lane 2) and 100pg/mL(lane 3) for 24 hours prior to infection. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 48 hours. Total cellular RNA was isolated and TNF-α mRNA expression was detected using semi-quantitative RT-PCR. Lane 4 represents control cells which were not exposed to IFN-γ.

'Mock' infected cells which were stimulated with IFN-γ did not show any significant difference in the level of IL-6 expression when compared with untreated 'mock' infected negative controls.

Stimulation of the cells with IFN-γ prior to infection resulted in a lower level of IL-6 induction than that observed in cells which had been infected without pre-treatment with the cytokine. However, the effect was only observed when the cells were stimulated with 10ng/mL of IFN-γ, the highest concentration of cytokine added. See Fig. 4.15.

From this study, it is evident that the stimulation of ECV304/T-24 cells with IFN-γ prior to infection with DEN 2 results in a suppression of the inflammatory response. This effect is dependent on the dose of cytokine added.

4.2.11 Detection of intracellular virus following pre-activation with Tumour necrosis factor-α, Interferon-γ or Transforming growth factor-β

Polarised confluent ECV304/T-24 cells were cultured on Falcon chamber slides and stimulated with 10ng/mL of IFN-γ, 1ng/mL of TNF-α or 10ng/mL of TGF-β_2 for 24 hours. Cells were then infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell.

Seventy-two hours post infection, the cells were fixed in 3% paraformaldehyde in PBS. The presence of intracellular virus was detected using DEN 2-specific hyperimmune mouse ascitic fluids and a fluorescein-conjugated anti-mouse IgG, as described in section 2.2.2.5. The integrity of the nuclei was investigated by double-labeling the cells with propidium iodide. Fluorescence was detected using a confocal fluorescent microscope (Zeiss) and BioRad digital imaging system.

Cells which were infected with DEN 2 without pre-activation, showed a speckled pattern of fluorescence predominantly in the perinuclear region of the cell and a large proportion of the nuclei were apoptotic, see Figs. 4.16(a), Fig. 3.1(f) and Fig. 3.10(f). In this experiment, the majority of the cells forming the monolayer were positive for intracellular virus.

When the cells were activated with TNF-α prior to infection with DEN 2 however, a difference in the number of infected cells and in the pattern of fluorescence was observed. A smaller number of cells were positive for intracellular virus and the fluorescence was

The effects of IFN-γ pretreatment on the induction of IL-6 mRNA expression following DEN 2 infection of ECV304/T-24 cells

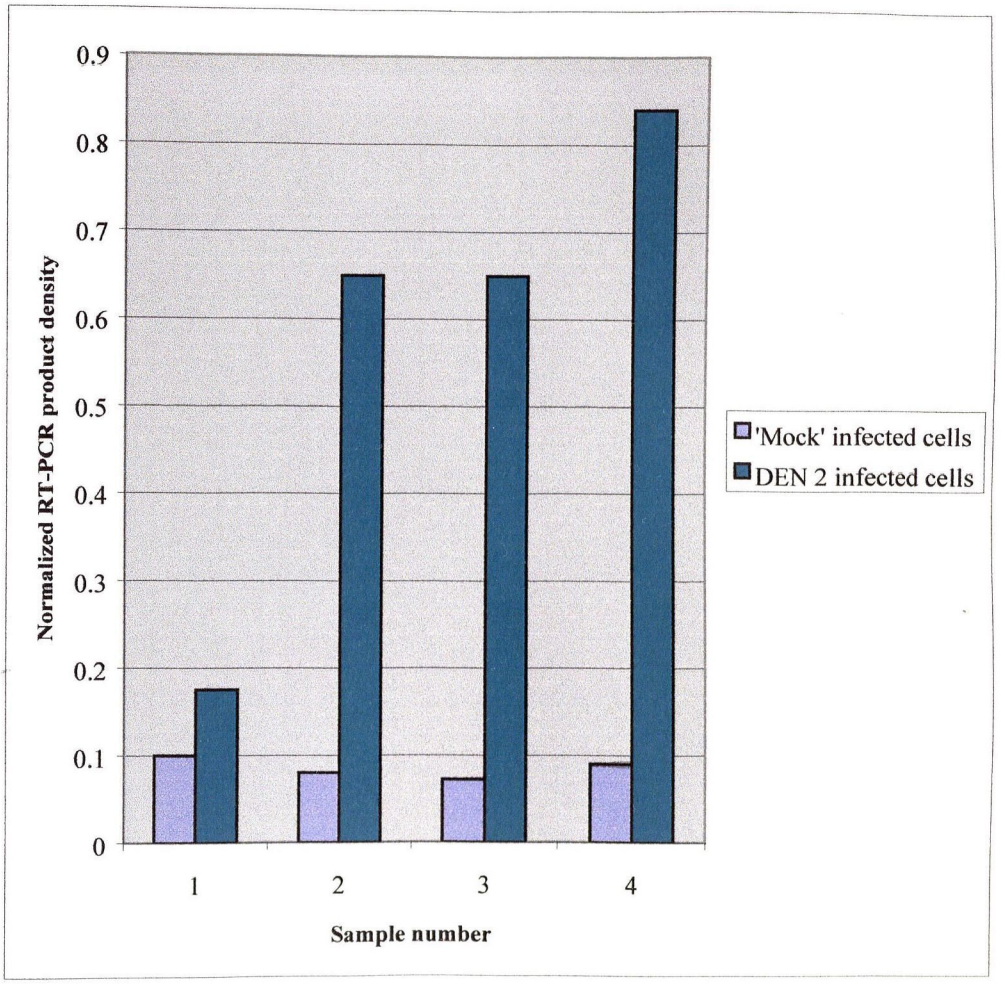


Fig. 4.15. Effects of IFN-γ pre-activation on the induction of IL-6 mRNA expression following DEN 2 infection of polarised confluent ECV304/T-24 cells. Cells were stimulated with 10ng/mL(lane 1), 1ng/mL(lane 2) and 100pg/mL(lane 3) for 24 hours prior to infection. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 48 hours. Total cellular RNA was isolated and IL-6 mRNA expression was detected using semi-quantitative RT-PCR. Lane 4 represents control cells which were not exposed to IFN-γ.

Detection of intracellular DEN 2 viral antigens and apoptotic nuclei in polarised confluent ECV304/T-24 cells using double-labeling

Fig. 4.16. Detection of intracellular DEN 2 antigens using indirect immunofluorescence and apoptotic nuclei using propidium iodide staining in polarised confluent ECV304/T-24 cells. (a) Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 72 hours. (b) Cells were treated with 1ng/mL of TNF-α for 24 hours and infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell for 72 hours. (c) Cells were treated with 10ng/mL of IFN-γ prior to infection and (d) cells were treated with 10ng/mL of TGF-β_2 for 24 hours prior to infection. 72 hours post infection cells were fixed in 3% PFA in PBS and double-labeled. Fluorescence was observed under a confocal microscope.

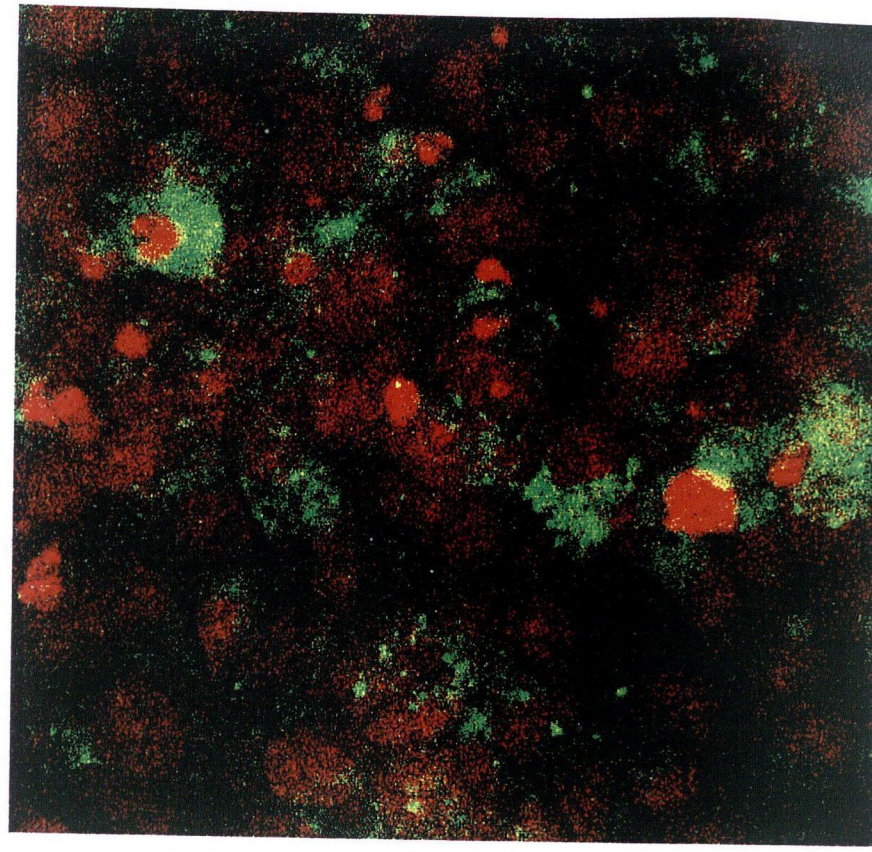


Fig. 4.16(a)

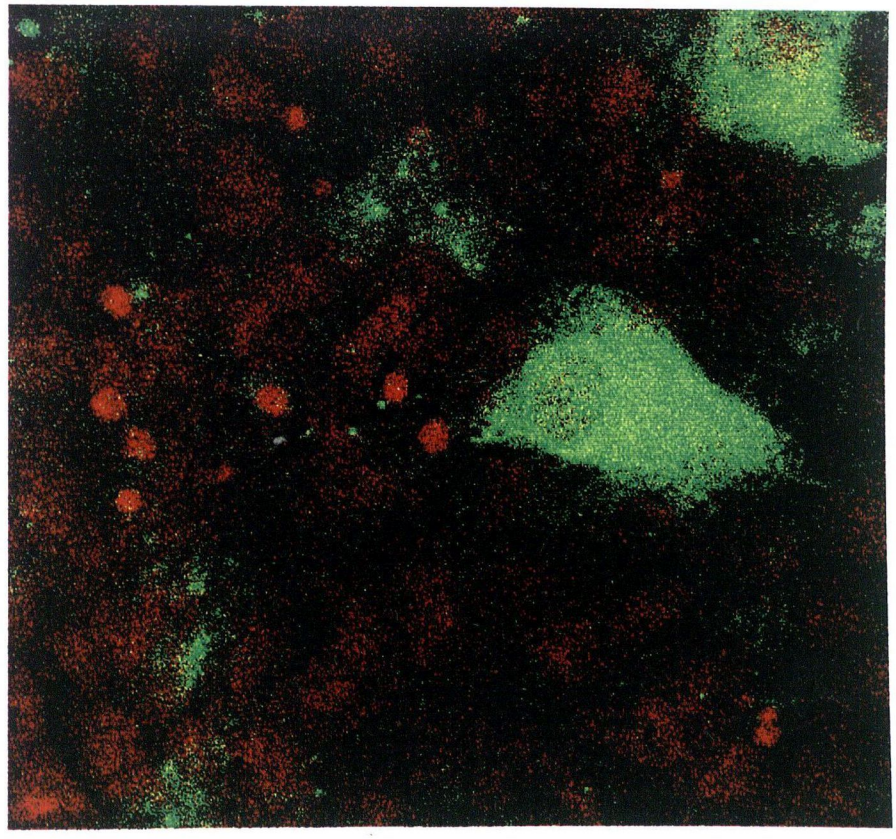


Fig. 4.16(b)

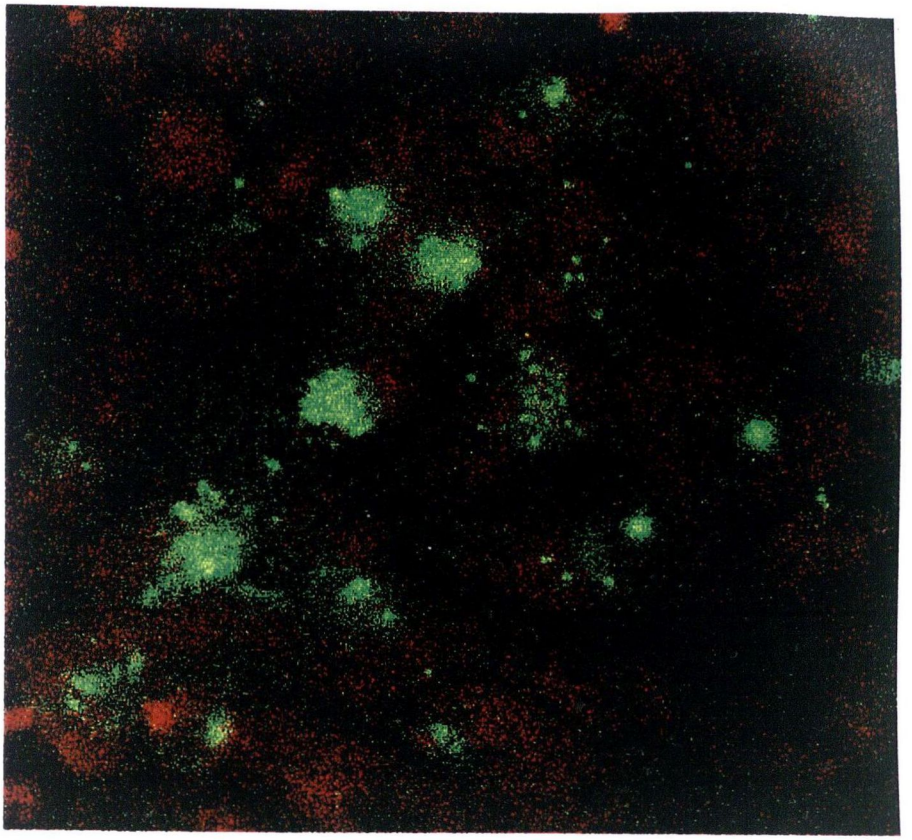


Fig. 4.16(c)

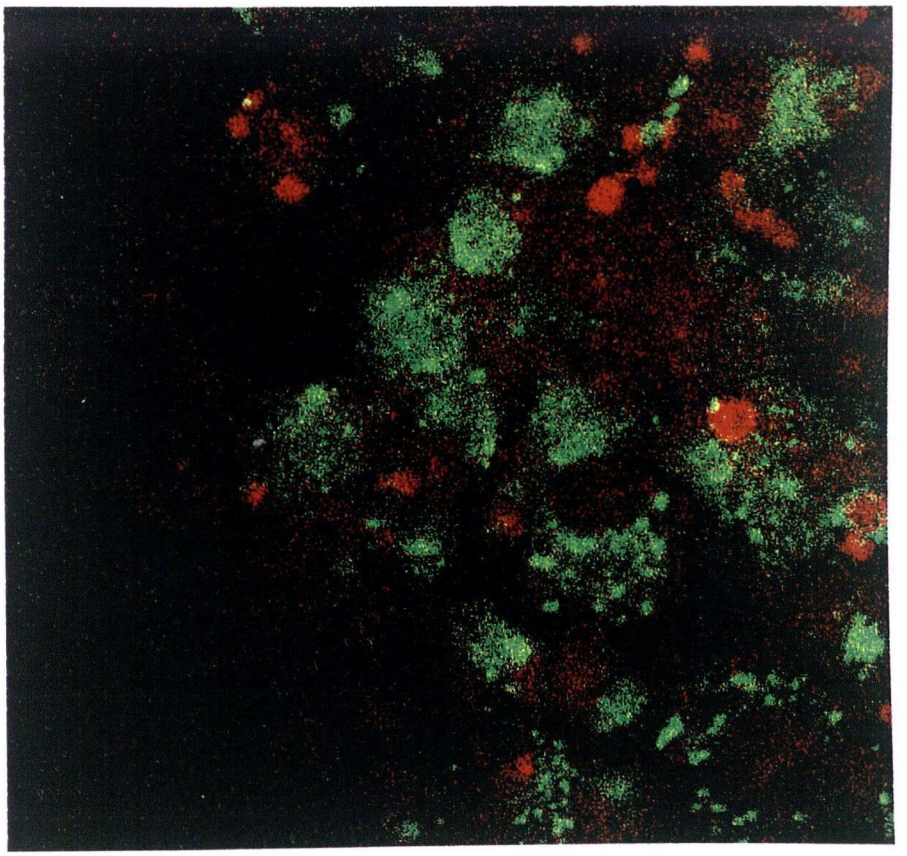


Fig. 4.16(d)

displayed throughout the cytoplasm of the cell. Apoptosis was evident in the infected monolayer. See Fig. 4.16(b).

Figure 4.16(c) shows how treatment of monolayers with IFN-γ prior to infection resulted in a reduction of the number of cells that were positive for intracellular virus. The fluorescence was localized to the nuclear region of the cell. There was a reduction in the percentage of cells displaying apoptotic nuclei.

TGF-β_2 treatment of the monolayers prior to infection with DEN 2 appeared to have no effect on the cells. A similar number of cells were positive for intracellular virus and demonstrated a speckled pattern of fluorescence, as was evident in untreated infected cells (see Fig. 4.16(a)). A similar percentage of apoptotic nuclei were visible. See Fig. 4.16(d).

4.3 DISCUSSION

DEN 2 infection of ECV304/T-24 cells, HUVEC and primary adherent monocytes resulted in the induction of one aspect of the pro-inflammatory immune response. The mRNA expression of the cytokines TNF-α, IL-1 and IL-6 were studied using the technique semi-quantitative RT-PCR. This method, once optimized, proved very reliable and reproducible following a series of internal and external controls.

TNF-α and IL-6 were induced in ECV304/T-24 cells and these cells were unable to produce IL-1. The kinetics of induction of each of these cytokines suggested that IL-6 may activate a negative feedback mechanism for TNF-α. It has been postulated that IL-6 induction leads to the protection of cells (75) and rescues cells from apoptosis. Interestingly, the maximum level of TNF-α gene expression was detected at 48 hours post infection and maximum secretion of the cytokine from the cells was detected at 72 hours, which corresponded with the maximum level of apoptosis within infected monolayers. No signs of apoptosis were visible at 96 hours, as shown in chapter 3. IL-6 gene expression reached a maximum at 72 hours post infection, which corresponded with a suppression of TNF-α expression. The induction of these cytokines was not as a result of a cell surface-stimulated event, as exposure of the cells to inactivated DEN 2 did not result in an alteration in the level of either TNF-α or IL-6 expression. Induction of TNF-α at 48 hours following stress to the cells is late, indicating the action of DEN 2 on the immune response is later than other pro-inflammatory stimuli. The confluence of ECV304/T-24 cells did not affect the induction of these cytokines, but did affect the kinetics of expression with the maximum levels of expression of both TNF-α and IL-6 occurring simultaneously.

DEN 2 infection of primary HUVEC resulted in the induction of the pro-inflammatory cytokines IL-1 and IL-6. These cells failed to express TNF-α and there has been no evidence to suggest that these cells do express this cytokine from the literature. In this study, it is difficult to know whether the cell line ECV304/T-24 or primary HUVEC are an indicator of the situation *in vivo*.

It was thought that simulating the conditions present late in infection or during a secondary infection would enhance the pathogenesis of the infection and result in a more pronounced immune response, but this proved not to be the case. Pre-treatment of ECV304/T-24 cells with the cytokines TNF-α and IFN-γ, not only failed to induce TNF-α

or IL-6 mRNA expression in these cells, but suppressed the induction of these cytokines in DEN 2 infected cells. Previous reports involving the stimulation of cells *in vitro* with these cytokines demonstrated the use of arbitrary concentrations, whereas in this study, the concentrations chosen to stimulate the cells were found to lie within the clinical range (118). These cytokines acted in an anti-viral manner, with a reduction in the proportion of cells expressing DEN 2 antigens within infected monolayers for both and also a reduction in the number of cells dying by apoptosis. It has been shown previously that Interferon has a role in controlling viral infections (119). The action of these cytokines is a complex process, as the effects of pre-activation differed with different concentrations and the maximum level of suppression for TNF-α was at an intermediate level, not simply at the highest concentration, as was the case for IFN-γ.

The immune response elicited by the infection of primary human monocytes by DEN 2 was unexpected. The primary monocytes used in this study were analysed using FACS analysis to ensure that these cells were homogeneous in population and were indeed monocytic cells. TNF-α, IL-1 and IL-6 expression was induced in these cells, following DEN 2 infection via the Fc receptor, but the effects were short lived and never lasted for greater than 24 hours. TNF-α and IL-1 were induced early in infection, at 6 and 24 hours respectively, whereas IL-6 induction occurred at 7 days post infection. Alphaviruses have been shown to induce pro-inflammatory cytokines in monocytes as late as 7 days post infection (117). This suggests that these events are indeed independent and short-lived. ECV304/T-24 cells and primary monocytes were found to be susceptible to DEN 2 infection, but the levels of virus secreted from these two cell types differed greatly. Infection of ECV304/T-24 cells yielded maximum titres with an order of magnitude 10^4 times higher than those detected for monocytes. The immune response was much weaker for these cells than for ECV304/T-24 cells or primary HUVEC. U937 cells, once infected by DEN 2 via the Fc receptor also, did not induce an inflammatory response. No alterations in the level of TNF-α mRNA was detected and these cells failed to express mRNA for the cytokines IL-1 and IL-6 following infection with DEN 2.

If a pro-inflammatory immune response plays a role in the induction of vascular permeability during DHF, from this *in vitro* study, the endothelial cells HUVEC and the

epithelial/endothelial cell line ECV304/T-24 are of far greater significance than the previously implicated monocytic cells.

CHAPTER FIVE

Mechanism of Action of DEN 2 on the Host Cell Immune Response

5.1 INTRODUCTION

From the data presented in the previous chapter, it was found that infection of ECV304/T-24 cells and HUVEC by DEN 2 resulted in the induction of the pro-inflammatory cytokines Tumour necrosis factor-alpha, Interleukin-6 and Interleukin-1. Chapter three illustrated how DEN 2 infection of ECV304/T-24 cells resulted in the death of approximately 70% of those cells by apoptosis at five days post infection.

The transcription factor NF-κB is known to activate a wide array of genes in response to a diversity of stimuli including those that promote inflammation and apoptosis (77, 120, 121). In unstimulated cells, this dimeric transcription factor exists in a latent form sequestered in the cytosol, complexed to an inhibitor protein of the IκB family. A number of IκB proteins exist, all of which contain multiple copies of an ankyrin repeat motif which binds the Rel homology domain (RHD) of NF-κB (120, 122). There are a number of potential mechanisms for regulating NFκB activity, including the availability of IκB proteins. Upon degradation of IκB, NF-κB is rapidly released and is free to translocate to the nucleus and activate target genes such as TNF-α, by binding with high affinity to the κB elements in their promoters. The degradation of the IκB protein IκB-α, occurs following phosphorylation at the serine residues Ser 32 and Ser 36 (123).

The cytokine IL-1 is known to activate NF-κB by IκB-α phosphorylation and degradation and was used in this study as a control. The effects of DEN 2 infection on the phosphorylation of IκB-α at Ser 32 and it's subsequent degradation were investigated. Whole cell extracts were prepared and the presence of IκB-α and of phospho-IκB-α (Ser 32) were detected using Western immunoblotting. The ability of DEN 2 to activate NF-κB and result in it's translocation to the nucleus of infected cells following infection of ECV304/T-24 cells was also investigated. Nuclear extracts were isolated from DEN 2 infected cells and the binding of the protein to it's κB consensus sequence was examined using an electrophoretic mobility shift assay.

5.2 RESULTS

5.2.1 Effects of stimulation of ECV304/T-24 cells with Interleukin-1α on IκB-α

5.2.1.1 IκB-α degradation

ECV304/T-24 cells were seeded at a density of $2.25x10^5$ cells/mini petri dish (9.5 cm^2), cultured for 3 days and subsequently stimulated with the cytokine IL-1α, at a concentration of 10ng/mL. Untreated cells (time 0) were used as the negative control group. Stimulation with the cytokine was stopped by the addition of ice cold PBS to the monolayers. Whole cell extracts were prepared from both groups at 0, 3, 8, 15 and 30 min post stimulation and equivalent concentrations of protein were subjected to SDS-PAGE through a 5% stacking gel and a 10% resolving gel. The proteins were electrotransferred onto a nitrocellulose membrane and the membrane was then incubated with a monoclonal antibody to human IκB-α and detected by autoradiography using diagnostic film (see section 2.2.6).

Levels of IκB-α expression in IL-1α stimulated cells were similar to control cells (0 min) at 3 and 8 min post addition of the cytokine. Degradation of the protein was observed at 15 and 30 min post addition of IL-1α to the cells, as shown in Fig. 5.1.

5.2.1.2 Phosphorylation of IκB-α

A duplicate gel was run using the above whole cell extracts prepared following IL-1α stimulation of ECV304/T-24 cells and the membrane was then incubated with a polyclonal antibody to human phospho-IκB-α (Ser 32). Phosphorylated IκB-α was detected at 8 min post addition of the cytokine to the cells and can be seen in Fig. 5.2. No product was detected at 0, 3, 15 or 30 min post stimulation. As a positive control for DEN 2 studies, the whole cell extract prepared from ECV304/T-24 cells at 8 min post stimulation with IL-1α was used. This sample was positive for the presence of both IκB-α and phospho-IκB-α (Ser 32).

IκB-α degradation in ECV304/T-24 cells following IL-1α stimulation

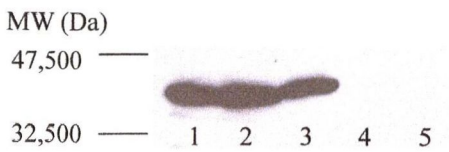


Fig. 5.1. Western blot analysis of whole cell extracts prepared from IL-1α stimulated confluent ECV304/T-24 cells. Cells were stimulated with IL-1α at a concentration of 10ng/mL. Whole cell extracts were prepared at 0 (lane 1), 3 (lane 2), 8 (lane 3), 15 (lane 4) and 30 (lane 5) min post stimulation. The membrane was incubated with anti-human IκB-α antibody.

IκB-α phosphorylation in ECV304/T-24 cells following IL-1α stimulation

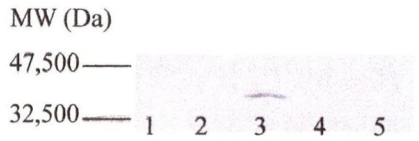


Fig. 5.2. Western blot analysis of whole cell extracts prepared from IL-1α stimulated confluent ECV304/T-24 cells. Cells were stimulated with IL-1α at a concentration of 10ng/mL. Whole cell extracts were prepared at 0 (lane 1), 3 (lane 2), 8 (lane 3), 15 (lane 4) and 30 (lane 5) min post stimulation. The membrane was incubated with anti-human phospho-IκB-α (Ser 32) antibody.

5.2.2 Effects of infection of ECV304/T-24 cells by DEN 2 on IκB-α

5.2.2.1 IκB-α degradation

ECV304/T-24 cells were seeded at a density of $2.25x10^5$ cells per mini petri dish (9.5 cm^2) and three days post seeding were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Whole cell extracts were prepared from DEN 2 infected cells at 5 min intervals post addition of the virus to the cells. Control cells consisted of 'mock' infected counterparts.

In the first experiment, samples were collected from 5 to 65 min post addition of DEN 2 to confluent ECV304/T-24 cells and degradation of IκB-α was observed at 50 min after addition of the virus to the cells. The protein was completely degraded by 55, 60 and 65 min post infection, as can be seen in Fig. 5.3.

Following these results, a longer time course was prepared. Whole cell extracts were prepared from DEN 2 infected ECV304/T-24 cells at 5 min intervals from 45 to 105 min post addition of virus to the cells and at regular intervals for up to 96 'hours' post infection (97.5 hours post addition of virus to the cells). As described in previous chapters and in section 2.2.2.1, the number of' 'hours' post infection refers to the number of hours following the 90 min incubation of virus with the cells (time zero).

In this second experiment, degradation of IκB-α began at 80 and 85 min post addition of DEN 2 to confluent ECV304/T-24 cells. Complete degradation had occurred at 90 and 105 min post addition. However, by one hour post infection (2.5 hours post addition of the virus to the cells), IκB-α was again detected in the cytosol of infected cells. The protein was detected at 4, 8, 18, 24, 36, 47.5, 70 and 96 hours post infection. See Fig. 5.4. Negative controls comprised of cells exposed to DEN 2 virus inactivated with β-propiolactone, the results of which are described below.

5.2.2.2 Infection is necessary for the degradation of IκB-α

To ensure that infection of ECV304/T-24 cells by DEN 2 was necessary to cause the degradation of IκB-α and that the effect was not as a result of a cell surface stimulated signal transduction pathway, the cells were exposed to inactivated virus. DEN 2 was

IκB-α degradation in ECV304/T-24 cells following infection with DEN 2

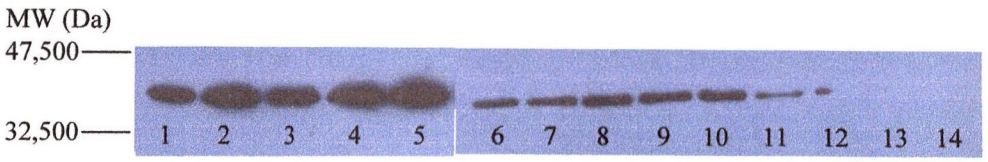


Fig. 5.3. Western blot analysis of whole cell extracts prepared from DEN 2 infected confluent ECV304/T-24 cells. Cells were infected with DEN 2 at a MOI of 1 $TCID_{50}$/cell. Whole cell extracts were prepared at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 min post addition of virus to the cells (lanes 2-14). Whole cell extract of 'Mock' infected cells at 60 min post addition (lane 1). The membrane was incubated with anti-human IκB-α antibody. Differences between the intensities of the two panels were due to intra-experimental differences.

Infection of ECV304/T-24 cells with DEN 2 is necessary for the degradation of IκB-α

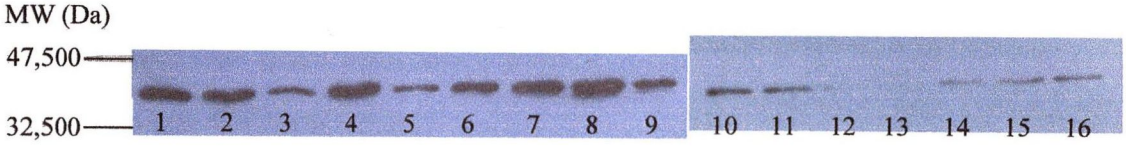


Fig. 5.4(a)

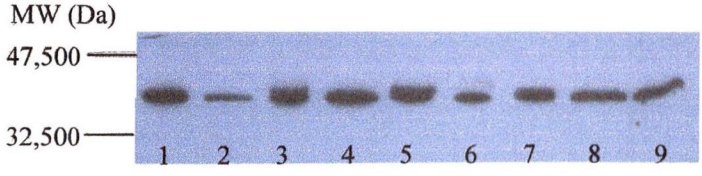


Fig. 5.4(b)

Fig. 5.4. Western blot analysis of whole cell extracts prepared from DEN 2 infected confluent ECV304/T-24 cells. (a) Lane 9 = 'mock' infected cells at 75 min post addition. Lanes 1, 5, 15 and 16 = extracts of cells exposed to BPL-inactivated DEN 2 at 45, 60, 75 and 90 min post addition of the virus respectively. Lanes 2-4, 6-8 and 10-14 represent extracts of DEN 2 infected cells at 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 105 min post addition. The membrane was incubated with anti-human IκB-α antibody.
(b) IκB-α immunoblot of whole cell extracts of cells infected with DEN 2 for 1, 4, 8, 18, 24, 36, 47.5, 70 and 96 hours (lanes 1-9).

incubated with 0.06%(v/v) β–propiolactone in Tris-HCl buffer, pH 7.5 at room temperature for 48 hours and shielded from light as described in section 4.2.7.

Whole cell extracts were prepared at 45, 60, 75 and 90 min post addition of inactivated DEN 2 virus to the cells and were run alongside infected samples. Fig. 5.4 shows that no degradation of the cytosolic protein IκB-α was detected in ECV304/T-24 cells exposed to inactivated DEN 2 virus, whereas by 90 min post addition of stock DEN 2 virus to the cells, IκB-α had been completely degraded.

5.2.2.3 Phosphorylation of IκB-α

Duplicate gels were run of the whole cell extracts of DEN 2 infected ECV304/T-24 cells prepared for the IκB-α study. The proteins were electrotransferred onto nitrocellulose membranes as described in section 2.2.6 and the membranes were incubated with a polyclonal antibody to human phospho-IκB-α (Ser 32).

Phosphorylation of the protein IκB-α was observed at 85, 90 and 105 min post addition of DEN 2 to the cells as shown in Fig. 5.5(a). The experiment was repeated and the time of exposure of the membrane to the diagnostic film was increased. At 70, 75 and 80 min post addition of virus to the cells the presence of phospho-IκB-α (Ser 32) was detected but the intensity of binding was much lower than that of IL-1α stimulated ECV304/T-24 cells. See Fig. 5.5(b). No products were detected in the negative control groups. A final exposure of 3 days of the membrane to the film was prepared and a product was detected for each of the time points. The level of intensity was much lower than the level of phosphorylated IκB-α detected following stimulation of the cells with IL-1α for 8 min. See Fig. 5.5(c).

From these results, it is evident that DEN 2 infection of ECV304/T-24 cells resulted in the phosphorylation and subsequent degradation of IκB-α. This effect was not permanent and the protein was sequestered into the cytosol again within one hour of degradation. Infection of the cells was necessary, as cells which were exposed to DEN 2 inactivated with the compound β-propiolactone, showed neither phosphorylation nor degradation of IκB-α.

IκB-α phosphorylation in ECV304/T-24 cells following infection with DEN 2

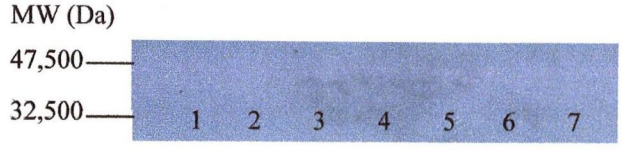


Fig. 5.5(a)

Fig. 5.5(b)

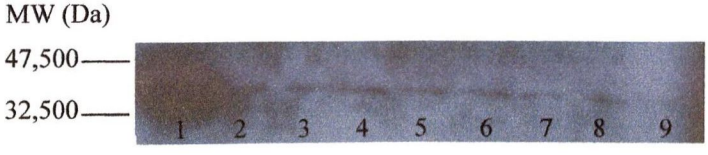


Fig. 5.5(c)

Fig. 5.5. Western blot analysis of whole cell extracts prepared from DEN 2 infected confluent ECV304/T-24 cells. (a) Extracts were prepared at 75, 80, 85, 90 and 105 min post addition of virus (lanes 1-5). Extracts of 'mock' infected cells at 75 min post addition (lane 6) and of cells exposed to inactivated DEN 2 at 75 post addition (lane 7). (b) IL-1α stimulated cells (lane 1) and DEN 2 infected cells at 60, 65, 70, 75 and 80 min post addition (lanes 2-6). (c) IL-1α stimulated cells (lane 1) and DEN 2 infected cells at 55, 60, 65, 70, 75, 80, 85 and 90 min post addition of DEN 2 to the cells (lanes 2-9). The membrane was incubated with anti-human phospho-IκB-α (Ser 32) antibody.

5.2.3 Activation of the transcription factor NF-κB in DEN 2 infected ECV304/T-24 cells

ECV304/T-24 cells were seeded at a density of $2.25x10^5$ cells/9.5 cm^2 mini petri dish. Three days post seeding, the cells were infected with DEN 2 virus at a MOI of 1 $TCID_{50}$/cell. Control cells consisted of 'mock' infected counterparts. At regular intervals post infection, nuclear extracts were prepared from both infected and control cells, as described in section 2.2.7.

The nuclear extracts were incubated with a 22-base pair oligonucleotide probe 5'-AGTTGA**GGGGACTTTCCC**AGGC-3', which included the κB consensus sequence (shown in bold). When nuclear extracts from DEN 2 infected cells were analysed using an electrophoretic mobility shift assay, the migration of the DNA probe through a 5% polyacrylamide gel was retarded, as a result of it's binding to NF-κB and the formation of DNA-protein complexes. The bound oligonucleotides were visualized as the upper band in Fig. 5.6. Very low levels of NF-κB activation were detected in 'mock' infected control cells.

The activation NF-κB and it's subsequent translocation to the nucleus of DEN 2 infected ECV304/T-24 cells was detected six hours post infection. The most intense binding of the oligonucleotides to NF-κB was observed at 24 hours post infection and levels remained more intense than those of their control counterparts at 46 and 75 hours post infection. By 93.5 hours post infection, levels of expression were similar to those of 'mock' infected cells. Unbound oligonucleotides migrated more easily through the gel and are visible as the lower band in Fig. 5.6.

From this study, it is evident that infection of ECV304/T-24 cells with DEN 2 resulted in the translocation to the nucleus and binding of the transcription factor NF-κB to the κB elements in it's target DNA. The greatest activation of NF-κB was detected at 24 hours post infection.

NF-κB activation in ECV304/T-24 cells following infection with DEN 2

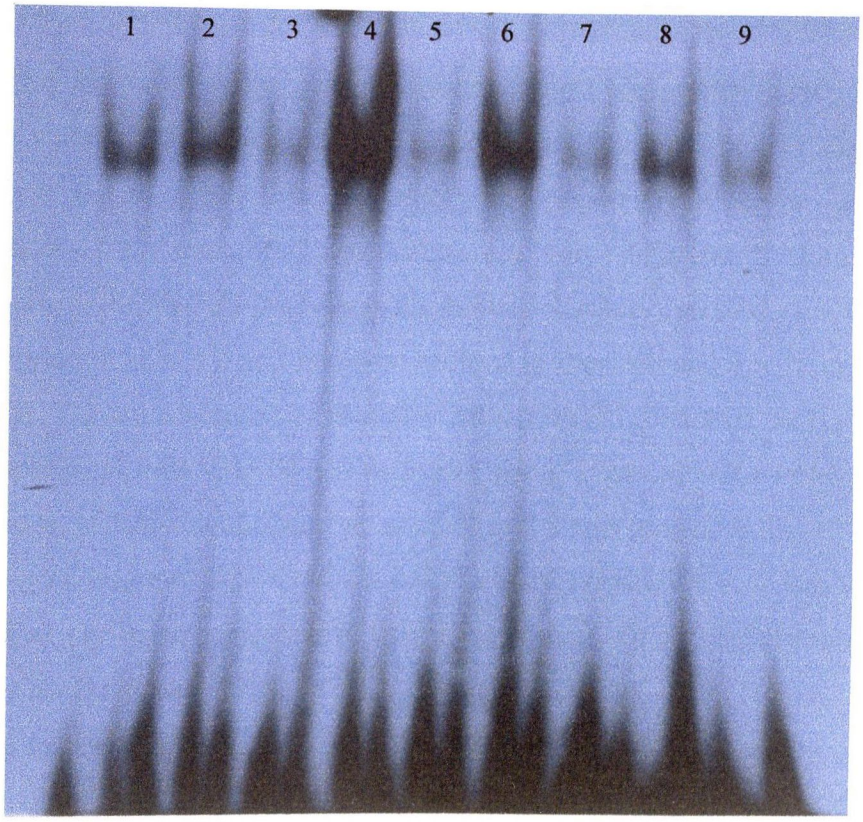


Fig. 5.6. Electrophoretic mobility shift assay of nuclear extracts from DEN 2 infected ECV304/T-24 cells. Nuclear extracts were incubated with a radio-labeled oligonucleotide probe which included the κB consensus sequence. NFκB binding resulted in retardation of the probe and formation of the upper band. Lanes 1, 3, 5, and 7 represent 'mock' infected samples at 6, 24, 46 and 75 hours post infection. Lanes 2, 4, 6, 8 and 9 represent DEN 2 infected samples at 6, 24, 46, 75 and 93.5 hours post infection.

5.3 DISCUSSION

DEN 2 infection of ECV304/T-24 cells resulted in the phosphorylation of the cytosolic protein IκB-α at Ser 32. In two independent experiments, the protein was subsequently degraded, however the kinetics differed slightly for each experiment, with degradation occurring at 55 min post addition of virus in one experiment and at 80 min post addition in another. IκB-α was not permanently degraded. Forty-five minutes after the last observed time of absence, the protein was once again sequestered in the cytosol and was present for up to 96 hours post infection at the time points tested. Interestingly the phosphorylation of the protein was not a discrete process occurring for only 5 min, but was continuous and at a low level, occurring simultaneously with the ubiquitination and degradation of the protein. These events are very different to those observed after stimulation of ECV304/T-24 cells with the cell surface ligand IL-1α. IL-1 induced the phosphorylation of IκB-α at 8 min post addition to the cells and this form was not detected at 15 min. The concentration of phospho-IκB-α (Ser32) generated by IL-1 was far greater than that elicited following DEN 2 infection, using identical cell numbers and conditions. Degradation of IκB-α was observed as early as 15 min post addition of IL-1 to the cells. Following exposure of ECV304/T-24 cells to inactivated DEN 2, it was found that neither degradation nor phosphorylation of IκB-α was found. DEN 2 infection of ECV304/T-24 cells was necessary to elicit these effects.

Infection of ECV304/T-24 cells by DEN 2 resulted in the activation of the transcription factor NF-κB. The kinetics of this activation reflected the kinetics of TNF-α induction, as would be expected and occurred prior to the detection of apoptosis within these cells. The binding of the transcription factor NF-κB to the consensus sequence was maximum at 24 hours post infection, indicating that the maximum concentration of the protein within the nucleus was present at this time. The time points investigated in this study were chosen in order to investigate the possible implications of the role of NF-κB in the induction of the pro-inflammatory cytokines and apoptosis observed in DEN 2 infected ECV304/T24 cells.

The kinetics of IκB-α degradation and of NF-κB activation did not coincide. This suggests that, during the late events induced by DEN 2 infection, IκB-α did not play a role. This can be expected as IκB-α is known to function in early intracellular stimulatory events

and the proteins IκB-β and ε have been implicated in later events within cells. IκB-β has also been shown to mediate the persistent activation of NF-κB (124).

CHAPTER SIX

Final Perspectives

6.1 FINAL PERSPECTIVES

The elucidation of the pathogenesis of DHF and the identification of the target cells of Dengue virus infection, still elude researchers. There is as yet, no *in vivo* model system for DHF and as a result the tools available for investigating the underlying mechanisms of DEN action and of the pathogenesis of the virus on its target cell(s) are limited. This study has attempted to understand some aspects of the action of DEN 2 on endothelial cells and even in this capacity, is limited. The models of cells used, ECV304/T-24 cells which were originally believed to be of endothelial origin have, since the commencement of this study, been discovered to be derived from an epithelial carcinoma. It is quite clear however, that these cells do possess many of the characteristics of endothelial cells (90) and form monolayers which are responsive to the action of vasoactive cytokines (46). The cells along with many transformed cells, lack the ability to produce the pro-inflammatory, growth inhibiting cytokine IL-1, whose activities include the induction of vascular permeability and apoptosis. However, there is no readily available commercial substitute. This study attempted to investigate whether transformed endothelial cells and primary endothelial cells responded in an identical manner to DEN 2 infection. The results have shown that with respect to the immune response, both cell types respond in a pro-inflammatory way, with ECV304/T-24 cells producing TNF-α and HUVEC producing IL-1. The failure of HUVEC to produce TNF-α has implied that these cells may be very different to the human microvascular endothelial cell *in vivo* and this would be understandable due to the foetal nature of these cells. It is logical to infer that no one cell type is an exact representation of the clinical situation, but by using two or more, a more complete comparison may be made. The immune response generated by these cells using this system is of course not a true clinical picture and is more than likely influenced by endothleial cell contact with all of the other possible cells and chemical mediators within the body, at the endothelial/blood interface. In an *in vivo* situation, there would be a greater number of cytokines and chemokines present and interplay between these mediators. Chemokines and cytokines which have been shown to be induced in ECV304/T-24 cells infected with DEN 2 since the commencement of this work include RANTES and IL-8 (111).

To date, most of the research into the pathogenesis of DHF has implicated the monocytic cell as the primary target of Dengue virus and has presented that the most severe

disease is observed in patients suffering from a secondary infection with a heterologous serotype. The data available to date however, have not offered compelling evidence that monocytic cells are the target cells, capable of initiating the cascade of events, which would eventually lead to DHF. In this study, it was decided to investigate a different aspect of the pathogenesis of DHF. In this study, the effects of DEN 2 infection on both transformed and primary endothelial and monocytic cells, was compared. It has been possible to show that in this *in vitro* system, which involved the primary infection of these cells by DEN 2, the effects of infection on the viability and immune response of endothelial cells are far more pronounced than observed for monocytic cells. It is possible to envisage the endothelium as one site of the pathogenesis of DHF. The possible influence this subset of cells could have in most areas of the body and on the immune system is immense.

This study has attempted to decipher two aspects of the infection of endothelial cells by DEN 2; the viability of the cell following infection and one subset of the pro-inflammatory immune response of these cells to DEN 2. In summary, if the data obtained in this study is presented chronologically, that is, in the order of appearance of each effect elicited in DEN 2 infected exponentially growing ECV304/T-24 cells, infection resulted in the sequence of events described below. Initially, the cytosolic inhibitory protein IκB-α was phosphorylated and subsequently degraded and this occurred at approximately 1 hour post addition of the virus to the cell. This was followed by the release and activation of the transcription factor NF-κB, which was located in the nucleus from 6 hours post infection and was at it's highest concentration 24 hours post infection. The induction of the transcription of the cytokine TNF-α followed at 24 hours, which reached a maximum at 46 hours post infection. This was found to be secreted from the cell at 48 hours post infection and the highest concentration was detected approximately 72 hours post infection. IL-6 transcription was induced at 46 hours and the maximum levels of mRNA were detected at 75 hours post infection. It is possible that TNF-α secretion acted in an autocrine manner and in turn activated the transcription of the cytokine IL-6. See Figure 6.1.

Simultaneous with these responses, the virus had penetrated the cell and was replicating within the cell and producing DEN 2 antigens. The most intense production of viral antigens was detected at 72 hours post infection and by 96 hours, the antigens seemed located at perinuclear region, perhaps incorporated into the ECV304/T-24 endoplasmic

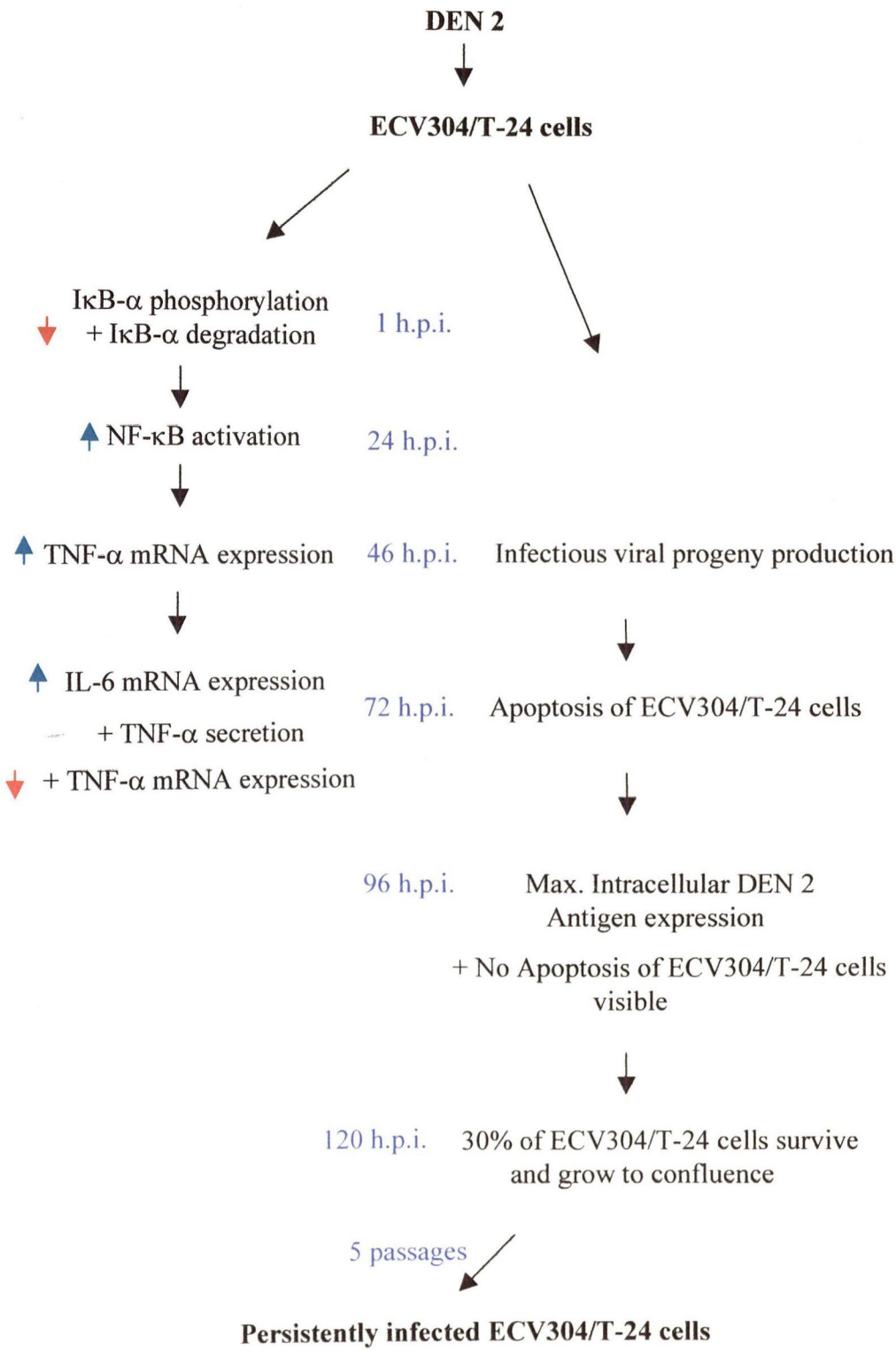


Fig. 6.1 Pathogenesis of DEN 2 infection of exponentially growing ECV304/T-24 cells

reticulum. During replication, the virus was producing infectious progeny which were released from the cells possibly in a non-lytic manner. The maximum concentration of secreted virus was detected from the cells at 48 hours post infection, using different methods of detection. At this time, either the cells were responding to these events or the virus was inducing a response in the form of programmed cell-death. Apoptosis was highest within the monolayers at 72 hours post infection. The aspects measured in this determination included the condensation of the nuclei (propidium iodide staining) and the cleavage of nuclear DNA (TUNEL analysis). This continued induction of apoptosis was not detected in the cells after this time point and the nuclei present seemed healthy by 96 hours post infection. The aftermath of these results culminated in the gradual loss of cells from the monolayer, commencing at 96 hours post infection and reaching a maximum at 120 hours post infection, with the loss of 70% of the cells present. The survivors recovered by 144 hours following infection and these cells grew to confluence. This suggests that if this occurred *in vivo,* and other immune cellular responses were neither synergistic nor protective, then the delay between the destruction of the endothelial cells and their recovery would take no longer than 48 hours. It also led us to investigate in a limited manner, whether these cells which had recovered, were persistently infected with a variant of DEN 2 which did not result in apoptosis to the cells. After five passages, the results do suggest this to be the case. The cells were found to be persistently infected, secreting low levels of infectious progeny, which elicited the same type of cytopathic effect on mosquito cells, but did not result in the induction of apoptosis in ECV304/T-24 cells. It would be interesting if this progeny virus were used to infected naïve HUVEC or other endothelial cells and ascertain if this phenotypic change is specific for the parental cells only. When surviving ECV304/T-24 monolayers were investigated for viral antigen expression, foci of cells were visible which were producing the viral antigens, rather than the entire monolayer, at a low level. The implications of this on the pathogenesis of DHF during a primary infection with DEN 2 are not obvious. However, it is feasible to assume that during a secondary infection with DEN 1, 3, or 4, these infected endothelial cells may indeed become targets of a fortified immune response, which may have recruited heterologous antibody and 'memory' cytokines. A schematic representation based on this study, of a hypothesis of one possible aspect of the pathogenesis of DHF, is presented in Figure 6.2.

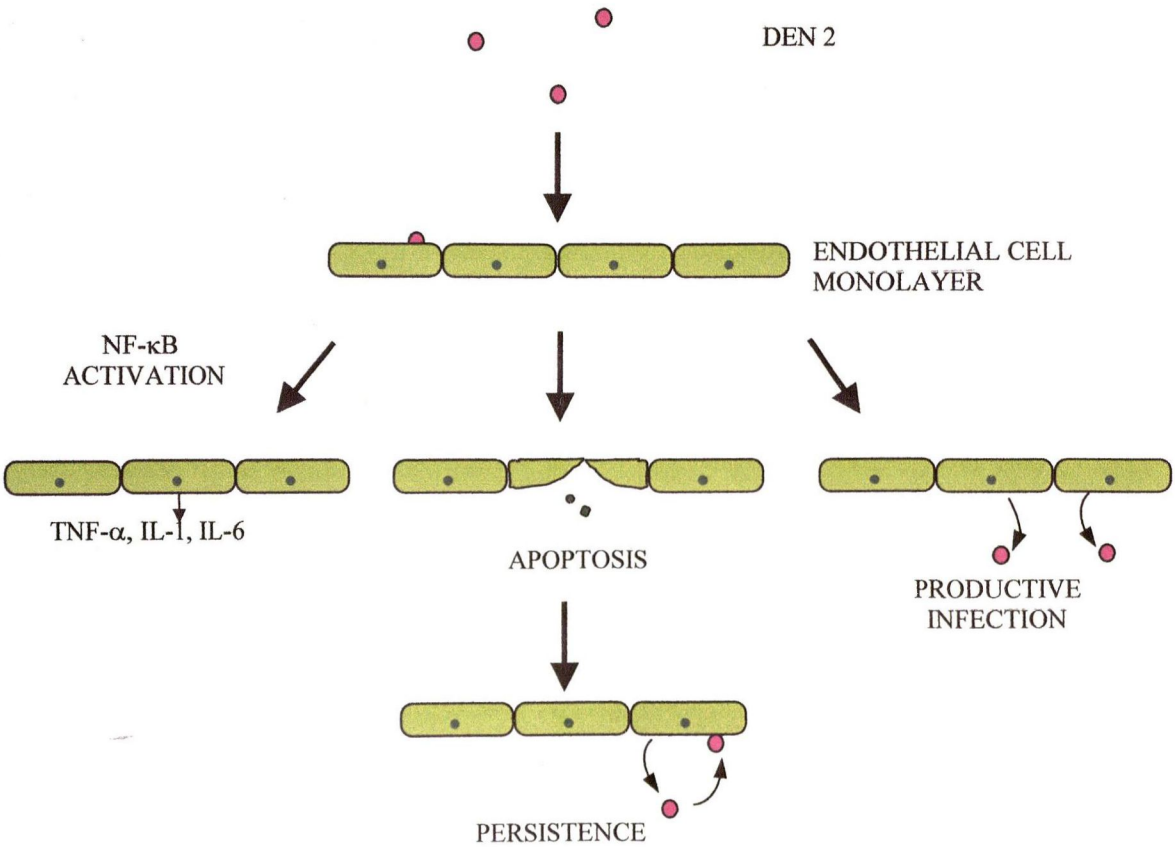


FIG 6.2 Schematic representation of one possible aspect of the pathogenesis of DHF.

All of these results suggest that each of the effects detected in DEN 2 infected ECV304/T-24 cells are regulated in a very tight manner. It would be very interesting to elucidate the mechanism(s) involved in the induction and suppression of inflammation and of apoptosis and infectious viral progeny production within these cells. The cellular protein Bcl-2 has been shown to be involved in the conversion of a lytic infection to a persistent infection by alphaviruses (96, 103) and may indeed be involved in the promotion of a persistent infection in this situation. It is possible that following TNF-α induction of IL-6, this protein may have blocked TNF-α production and activated the *bcl-2* gene which would result in a pro-survival and anti-apoptotic state in the cell.

When DEN 2 was inactivated with β–propiolactone, which retains the antigenicity of the virus, the cells did not produce these cytokines nor was phosphorylation/degradation of IκB-α detected. This infers that infection of the cells is integral in the elucidation of these effects. We have attempted to keep the parameters of this system as close to the clinical picture as possible, using cytokines within the clinical range and comparing exponentially growing cells (which may represent the growing endothelium of children) with polarised confluent cells. The responses were very similar for both systems.

HUVEC were more difficult to grow and where possible, were included in this study as a comparison with ECV304/T-24 cells. Infection of these cells resulted in the detachment of the cells from the substratum as with ECV304/T-24 cells and elicited a pro-inflammatory cytokine response. This was very similar to that of ECV304/T-24 cells, with two differences, TNF-α was not induced in these cells, even when conditions used during RT-PCR were at the minimum stringency and IL-1 was induced in these cells.

An unexpected result was obtained quite early in this study. Both human primary monocytes (which were analysed to ensure their lineage) and transformed U937 cells did not demonstrate a strong immune response or loss of cell viability when infected with DEN 2. The cells were found to be susceptible to infection by the virus which acted in a very subtle manner in these cells. The cells may harbour the virus and act as reservoirs of DEN 2 in the body. The cells, when differentiated may also play significant roles in a phagocytic or complement-activated response within the tissues, but they do not seem to contribute to a possible inflammatory response.

As a result of these findings, it is possible to envisage DEN 2 infecting the endothelium *in vivo*. The cells may act as a reservoir or play an active role in hypovolaemic shock induced by increased vascular permeability.

A number of future possibilities exist using this system. It would be interesting to establish a persistently infected ECV304/T-24 cell model and introduce the other serotypes of DEN to it. Whether endothelial cells do play a role in a secondary infection with a heterologous serotype would then be answered. The use of micro-arrays, would reveal exactly which genes were induced and suppressed at very close time intervals within infected cells. A full map of the cytokines expressed in this cell would then be available to researchers and allow speculation as to other external influences responsible for the induction of these genes would become apparent. The mechanism of action of the virus on intracellular signal transduction pathways might be elucidated. Recent studies in our laboratory by Dr. S. Zhou have included the addition of subsets of human primary lymphocytes and mononuclear cells to polarised confluent ECV304/T-24 monolayers. The aim of these investigations included the determination of the affects on of other infected cells on the permeability of endothelial cells and the immune response of endothelial cells following such exposure.

The problem of DHF is a growing one and new areas of the world are experiencing the introduction of the vector of the Dengue viruses, *Aedes aegypti*. The disease is the most important arthropod-borne disease today. Epidemics have been reported in Southern Vietnam in 1998 with 342 deaths (125); in Bangladesh in July/August this year killing at least 28 people (Reuters Medical News); in French Polynesia in 1996-1997 (126); in Brazil in 1996-1997 (127). Recent advances in the development of a safe vaccine include the preparation of an attenuated tetravalent vaccine which is presently in clinical trials.

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