THE CHARACTERISATION OF THE UPTAKE OF MICROPARTICULATES ACROSS INTESTINAL LYMPHOID TISSUE

a thesis submitted by

Kenneth Alan Howard BSc (Hons)

for the degree of Doctor of Philosophy
of the University of Nottingham

November 1994

CONTENTS

CHAPTER

CHAPTER

ABSTRACT

Poly(lactide-co-glycolide) (PLG) biodegradable microparticles were evaluated as an oral delivery system for immunisation against equine influenza virus. Model particulates (fluorescent polystyrene and gold labelled polystyrene) were used to characterise the route and mechanism of intestinal uptake. Peyer's patches were found to be the site of particulate intestinal absorption characterised by phagocytosis at the M cell surface. Microparticles were found within intercellular compartments indicating a paracellular route for the transit of microparticles from lumen to lymph. A quantitative method of counting the number of microparticles passing into the lymph in both the superior mesenteric and thoracic lymph ducts indicated levels of intestinal uptake high enough to deliver vaccines via this route.

PLG microparticles encapsulating equine influenza virus were prepared by the process of solvent evaporation. The immune responses were evaluated in mice after either systemic or oral immunisation of Balb\c mice using formalin treated equine influenza (Prague 56 H7N7) either encapsulated in PLG biodegradable microparticles or free in solution. Using the single-radial-immunodiffusion test, the haemagglutinin integrity of the microencapsulated influenza was found to be preserved during the microencapsulation process. When administered systemically the microencapsulated virus induced raised levels of anti-influenza IgG antibody in serum that were comparable with those obtained with the virus in solution. Oral administration of the microencapsulated virus induced raised levels of anti-influenza IgA antibody in saliva as well as levels of anti-influenza IgG comparable to those obtained after parenteral injection. Raised levels (compared to preimmune levels) of anti-influenza antibodies in both the systemic circulation and mucosal secretions indicates that orally administered microencapsulalated equine influenza virus represents a practical method of immunisation against influenza.

ACKNOWLEDGMENTS

First and foremost, I would like to thank Professor Bob Davis for giving me the opportunity to undertake a Ph.D in the Department of Pharmaceutical Sciences. I hope the findings presented in this Thesis justifies your faith in me. I would also like to express my gratitude to my other Supervisors; Dr Derek O'Hagan and Dr Norman Thomas, (Department of Human Morphology). I should like to extend special thank you to Norman for his unlimited patience and guidance throughout this work, and for always having the time to discuss matters both professional and personal.

I am grateful also to Drs Paul Jenkins, Paul M^{c}Gee and Allan Coombes for creative discussions and their willingness to provide advice throughout the years. I should like to thank Paul Jenkins especially, for performing flow cytometric analysis of lymph samples.

My thanks, also extend to Professor J Seifert, Dr Wolfgang Sass and their collegues for making my stay in Germany so interesting and enjoyable.

Finally, I would like to acknowledge all those people who have provided me with advice and assistance throughout the past four years: Mr Barry Shaw, Mr Alan Pyper, Mr I Jansen, Mr Mr T Self, I Harriman, Mr N Blackhall, Mrs Kate Parker, Ms Paula Barnes, Mr Lyndon Cochrane and Dr K Atkinson, Dr J Wood and Una to name just a few. A special thanks goes out to the secretaries, past and present.

To those who have inspired me throughout the years, especially G Nunn and F Gibson, many thanks.

This Thesis is dedicated to my mother and Sarah, two very special ladies.

CHAPTER 1

Introduction

Since the development of the first vaccine against smallpox in the 18th century (Jenner, 1796) the impact of vaccination on world health has been enormous. Smallpox is now a disease of historical interest only, following the declaration by the World Health Assembly on May 8th 1980 of its eradication (World Health 1980, Figure 1). In developed countries in particular, the widespread use of vaccines has led to a dramatic decrease in the incidence of such diseases as diphtheria, pertussis, tetanus (DPT), poliomyelitis, measles and tuberculosis (Hinman and Orenstein, 1990). In the third world over 60 million children are immunised each year against the common childhood diseases, preventing over 2.2 million infant deaths annually (Bloom, 1989). Despite this success, the majority of human diseases still requires a vaccine (Woodrow, 1990) and many of the presently available vaccines give inadequate protection. One of the reasons for this is that the traditional approach to vaccine development fail to address the initial invasive sites of the pathogen into the host. Most pathogens enter the host at the mucosal surfaces which are protected by a separate immune system termed the mucosal or local immune system. The traditional vaccines are ineffective at inducing local immunity and give little protection against pathogens whose initial portal of entry is the mucosa, for example the enteric pathogens that cause diarrhoeal diseases. Diarrhoeal diseases are thought to result in up to 15 million deaths each year (Woodrow, 1990). As a consequence there is a great need for new or improved vaccines that induce local immunity at mucosal surfaces.

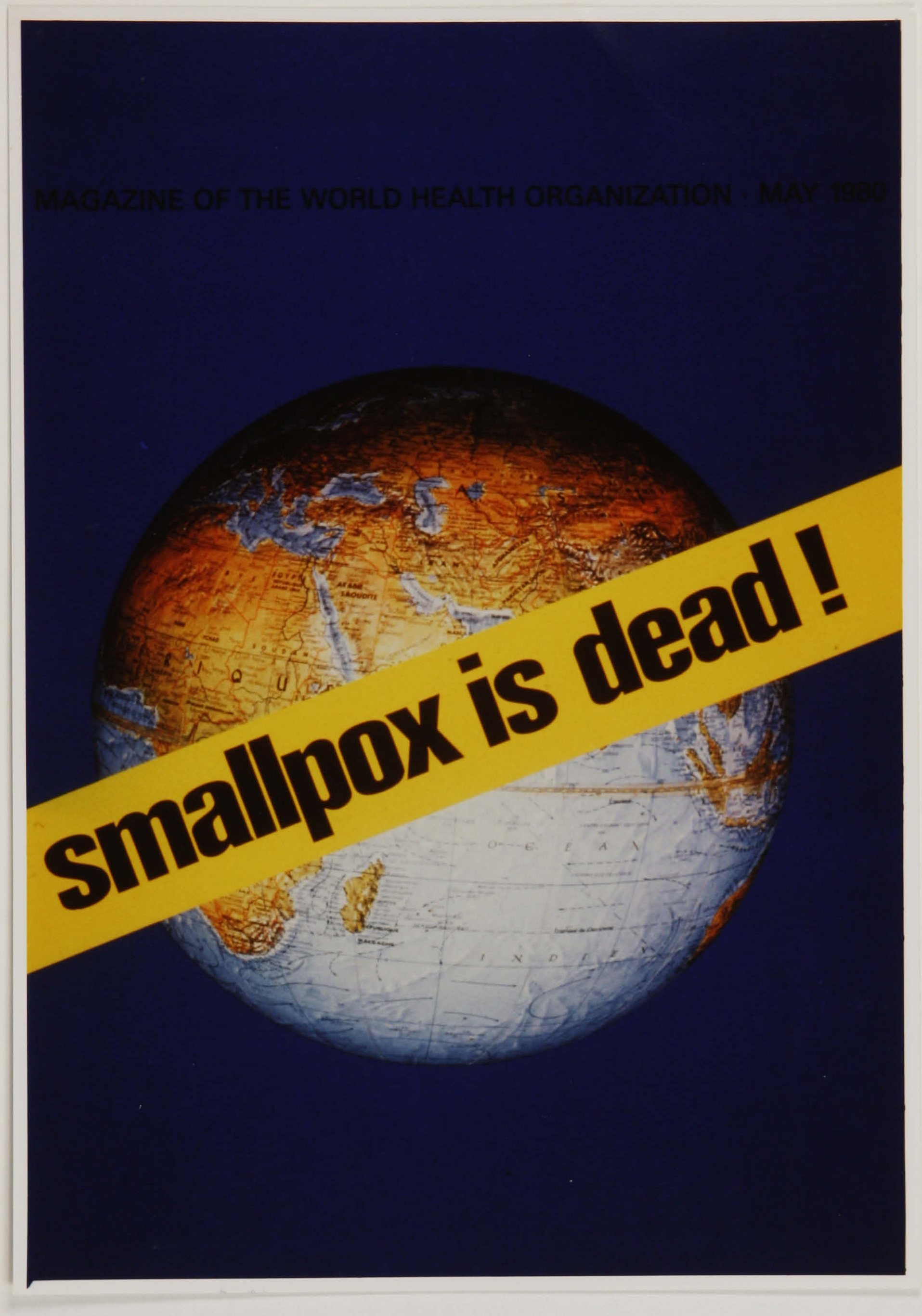


Figure 1

WHO poster proclaiming the eradication of smallpox

1.1 IMMUNOLOGICAL BASIS FOR VACCINES

It is the responsibility of the body's immune system to provide a defence against pathogenic microorganisms or substances. Exploitation of the natural protection provided by the cells and humoral factors of the immune system provides the immunological basis for vaccines. A vaccine can be defined as an artificial stimulus used to activate these defence mechanisms in providing protection (or immunity) against a invasive natural form of that stimulus. The immune system is divided into two categories the specific and non-specific; it is the specific which provides the memory and specificity required for vaccines (For a general text on the immune system refer to Riott, 1991).

Specific immunity is developed in response to previously encountered antigen and is specific to that antigen. The specific response can either provide humoral or cell mediated immunity. Humoral immunity is provided by antibodies originating from the antigenic stimulation of B-lymphocytes. On the surface of the B-lymphocyte are immunoglobulin (antibody) receptors. The receptor only recognise a specific sequence (epitope) on the surface of the antigen, therefore, each lymphocyte is specific to only one antigen. The B-lymphocyte clonally proliferates (under the control of helper T-lymphocytes) to form mature plasma cells which secrete antibody (primary response), whilst a proportion of the B-lymphocytes mature into memory cells. The antibody will only recognise the specific antigen which caused stimulation. Memory cells remain dormant in the body until that specific antigen is encountered again, at which time, they are activated to form antibody producing plasma cells. The secondary response is far greater and more rapid because the cells are already primed. Vaccines provide

a non-virulent form of the antigen to achieve the same responses. The boosters in an immunisation schedule provide the secondary stimulus for an enhanced response. Circulating antibodies provide protection such as neutralisation of the invasive pathogen by binding to epitopes important for pathogenesis, thus, preventing the onset of disease (Ada, 1990 and 1991). Depending on the pathogen, antibodies may not provide complete protection.

Viral pathogens which reside within cells can evade humoral immunity. Vaccines against such pathogens, must evoke cell-mediated immunity of which T-lymphocyte stimulation is required. T-lymphocytes only recognise antigen when it is presented on the surface of cells in association with a cellular marker, the major histocompatibility complex (MHC). Antigen presentation to the lymphocyte is provided by macrophages (non-specific cells which phagocytose and remove foreign material in the body). Macrophages present either peptides (in association with MHC class II), or infectious viral material (in association with MHC class I) on their surface. Each T-lymphocyte is specific to each antigen, but in contrast to B-lymphocytes, they respond to many epitopes from a microorganism. The macrophages secrete interleukins which provide chemical stimuli for the differentiation of the T-lymphocytes into effector helper or cytotoxic T-cells. Memory T-cells are also produced which provide a reservoir of cells mobilised to respond to a repeated attack from the antigen. Cytotoxic T-cells are able to destroy the invaded cell by mechanisms involving interferons. For this reason cell-mediated immunity is required to clear infection. Humoral and cell mediated immunity are intimately linked for example the stimulus for the differentiation of B-cells into antibody producing cells is provided by helper T-cells (formed in response to the same antigen). The specificity and memory are the essential components of the

specific immune system that can be exploited to produce vaccines, but antigen presentation (provided by non-specific antigen presenting cells) is equally important.

1.2 THE TRADITIONAL VACCINES

Before considering developing new or improved vaccines, it is important to outline the types of vaccine presently in use, in order to draw a baseline. Termed traditional because they have been developed using long-standing approaches, they can be divided into two catorgories: 1) live attenuated vaccines and 2) killed vaccines.

For a comprehensive text describing the scope of traditional vaccines the reader is referred to an overview by Ada (1990) and a general immunisation text such as that by Dudgeon and Cutting (1991).

The principle of attenuation is to reduce the virulence of the microorganism so that it is safe to the host but at the same time allowing replication. This can be achieved, for example, by changing the conditions in which the microorganism grows. A sustained dose of the antigen is presented to the immune system ensuring long lasting protection whilst the natural infection ensures cell-mediated as well as humoral immunity.

The approach to killed vaccines is to reduce the virulence of the microorganism by preventing replication (inactivation) without losing its immunogenicity. The microorganism can be inactivated by heat or chemical treatment. If the whole microorganism is used many antigens are available which means immunity is induced in most individuals. As a result of non-replication these vaccines need to be

administered repeatedly over time. Using the same approach, toxins (toxic products liberated from certain microorganisms) can be rendered harmless by chemical treatment to give toxoids which can induce protection.

Most of the vaccines approved for use in humans represent the above (refer to Table 1), there is another type of vaccine, the subunit vaccine which only consist of the important epitopes needed to confer immunity. Advances in genetic engineering and biotechnology have enabled these epitopes to be produced synthetically.

1.2.1 **Limitations of the traditional vaccines**

The types of vaccine described have advantages and inherent limitations. Killed vaccines and toxoids are considered safer because a non-replicating microorganism carries less virulence potential than a live vaccine containing a replicating microorganism. Live vaccines have been known to revert to a virulent form, for example the live poliomyelitis Sabin type 3 vaccine, this has resulted in some countries, such as Holland, replacing the OPV oral poliomyelitis vaccine with a new enhanced-potency killed parenteral IPV vaccine as a measure to overcome this problem (Hinman and Orenstein, 1990). The use of live vaccines in immunosuppressed individuals, the elderly and the young, is of special concern. Some killed vaccines, however, may also give adverse reactions such as the parenteral whole cell vaccines for typhoid (Levine et al. 1990) and pertussis (Ada, 1990). In the case of pertussis, the whole cell vaccine has been replaced in Japan by an acellular vaccine (Kimura and Kuno-Saki, 1988). Most vaccines in use today carry some risk to the host but the overall benefit to the whole community is the major objective in

vaccination programmes. Due to their safety, there is now a trend towards development of subunit vaccines, however, a problem remains, one of poor immunogenicity (Woodrow, 1990).

Live attenuated vaccines need to be stored in a cold chain to remain viable. Most of the presently available live and killed vaccines need to be stored between 2-8°C to remain active for any length of time (Dudgeon and Cutting, 1991). This is not only expensive, but poses several logistical problems for supply in isolated areas of the third world. The relative low cost of producing live attenuated and killed vaccines is counteracted by the need for cold storage and concomitant cost.

The major drawback in the use of killed and subunit vaccines is the necessity for multiple doses for adequate protection. Up to 5 injections of tetanus toxoid may be required for life long immunity. A typical immunisation schedule given to children in developed countries requires multiple contacts, four in the first year of life (Dungeon and Cutting, 1991). The BCG vaccine for tuberculosis and the measles vaccine (both attenuated and live) are the only presently available vaccines that require one dose. The dropout rate for boosters is high in the third world (Bektimirov *et al.* 1990). It is important to be immunised as soon as possible after birth, but because of the maternal antibodies circulating in the infant the Diptheria, Pertussis and Tetanus (DPT) vaccine cannot be given until 6 weeks of age. This is not such a major problem in the developed world because good sanitation ensures the mass population are protected from the onset of the disease. In the third world, however, poor sanitation results in a prevalence of disease in these areas and children are at

great risk as soon as they are born. Presently only two vaccines can be given at birth: the BCG vaccine for tuberculosis and the oral polio OPV vaccine (Bloom, 1990).

Most vaccines currently available are administered via the parenteral route; only the oral poliomyelitis vaccine is given orally (refer to Table 1). The oral route offers a number of advantages over parenteral administration: acceptability, convenience, and cost effectiveness (saving the need for trained personnel to administer injections and use of specialised equipment such as needles and syringes). The development of an oral route of administration as opposed to the parenteral is a major directive of the WHO transdisease vaccination programme initiated in 1987 (Bektimirov *et al*. 1990).

In order to develop improved vaccines the pathogenesis of the microorganism must be addressed. The route of invasion and subsequent dissemination of the pathogen within the host is important when devising protective strategies. Traditional vaccines, administered parenterally induce protective immunity in the serum of the host giving good protection against pathogens which directly invade the serum of the host such as *Clostridium tetani* (Tetanus) and viral hepatitis B, or have a stage during pathogenesis in which they infect systemic organs by passage through the bloodstream (viraemia and bacteraemic) such as poliomyelitis. Traditional vaccines provide poor protection at the mucosal surfaces which line the respiratory, gastrointestinal and genito-urinary tracts. For the majority of invasive pathogens these are the initial sites of entry into the host (refer to Table 2). The traditional vaccines were developed without consideration that a separate immunity exists to protect the mucosa - local immunity or the mucosal immune response. Vaccines that fail to induce immunity at the mucosa, fail to give adequate protection against pathogens that colonise or

TABLE 1

PRESENTLY AVAILABLE VACCINES FOR HUMAN USE

BACTERIAL VACCINES		ROUTE
Live attenuated	Killed inactivated	
BCG		Parenteral
	Diptheria	Parenteral
	Tetanus	Parenteral
	Anthrax	Parenteral
	Pertussis	Parenteral
	Typhoid	Parenteral
	Cholera	Parenteral
	Plague	Parenteral
	Pneumoccocal (subunit)	Parenteral
	Meningcoccal (subunit)	Parenteral
	H. influenza B (subunit)	Parenteral
VIRAL VACCINES		
Live attenuated	Killed vaccines	
Oral vaccine (OPV)		Oral
Yellow fever		Parenteral
Measles		Parenteral
Mumps		Parenteral
	Polio vaccine (IPV)	Parenteral
	Rabies	Parenteral
	Influenza A, B	Parenteral
	Japanese B encephalitis	Parenteral
	Influenza A, B (subunit)	Parenteral
	Hepatitis B (subunit)	Parenteral

TABLE 2
Infectious microorganisms whose initial portal of entry are the mucosal surfaces.

Invasive microrganisms initial site of infection		
Mucosal surface of the lower and upper respiratory tract	Mucosal surface of the Gastrointestinal tract	Mucosal surface of the Genito-rinary tract
Herpes simplex 1 *Bordetella pertussis* measles Influenza viruses Parainfluenza viruses Syncytial virus Rhinoviruses Adenoviruses Coronaviruses Coxsackieviruses Echoviruses *Neisseria meningitidis* *Streptococcus pneumoniae* *Haemophilus influenzae* *Group A Streptoccocus*	*Vibrio cholerae* *Escherichia coli* (ETEC) *Escherichia coli* (EPEC) *Shigella* species *Salmonella typhi* Rotavirus Reovirus Poliomyelitis *Mycobacteria*	HIV-1 Herpes simplex 2 *Eschericheria coli* *Neisseria gonorrhea*

penetrate via these surfaces. *Vibrio cholerae* for example, colonises the mucosal surface of the small intestine (Gillinghan and Po, 1991). The present parenteral killed vaccines give low and short lived protection (Holmgren *et al.* 1989). The fact that enteric diseases are estimated to account for up to a quarter of infant deaths in developing countries (Black, 1993) and the majority of the diseases included in Table 2 still require a vaccine, highlights the great need for new vaccines capable of inducing mucosal immunity. In order to evoke mucosal immunity the vaccine must first reach the mucosa. Substantial evidence suggests that a disseminated mucosal response can be initiated by orally administered antigens reaching the subepithelial lymphoid tissue of the gastrointestinal tract (refer to section 1.3). It is possible, therefore, to exploit for example the oral route to induce mucosal protection and overcome some of the inherent problems associated with parenteral delivery; inadequate protection at mucosal surfaces, inconvenience, poor patient acceptability and cost. However, other limitations associated with presently available vaccines also need to be considered, such as the need for cold storage and repeated dosage. In order to develop oral vaccines, the properties of the mucosal immune system must first be considered.

1.3 THE MUCOSAL IMMUNE SYSTEM

The mucosal membranes that line the respiratory, gastrointestinal and genito-urinary tracts cover a enormous surface area, over 400m^2 in the Human (M^{c}Gee and Kiyono, 1993). By inhalation, ingestion and sexual contact this vast area is a potential entry site for infectious microorganisms (refer to Table 2) and harmful environmental materials. A separate immune system, termed the mucosal immune system, comprising the mucosal tissue of these tracts as well as exocrine secretory glands (lacrimal, salivary and mammary) protects these surfaces against invasive pathogens and environmental materials.

The predominant antibody found in the external secretions that bathe the mucosa is secretory immunoglobulin A (sIgA) (Tomasi and Zigelbaum, 1963). Initially, it was thought that antibodies found in the mucosal secretions were a result of serum derived antibodies spilling over into the secretions and resulting in mucosal immunity- described as the spillover concept (Bergmann and Waldman, 1988). This was rejected in favour of local production at the mucosa, when Hanson (1961) demonstrated structural differences existed between serum and milk immunoglobulin A and later, Tomasi and Zegelbaum (1963), found that immunoglobulin A was the predominant antibody found in secretions.

Secretory IgA is composed of two or more IgA monomeric units as opposed to serum IgA which is monomeric (Hanson, 1961 and 1993). Secretory IgA also contains two additional components; the secretory component and the J chain. The secretory component is thought to facilitate the transport of polymeric IgA from plasma cells

within the submucosa to the mucosal surface where invasive pathogens are encountered (Russell and Mestecky, 1988), it is also thought to protect the heavy chains of the molecule from proteolysis by digestive enzymes (Rossen and Butler, 1973). Secretory IgA protects primarily by inhibiting the adherence of pathogens to the mucosal surface and stops invasion of the host. S-IgA can bind to bacterial toxins and viral surface epitopes important for cell attachment as well as specific and non-specific inhibitory effects on the adherence of bacteria to the mucosal surface (McGee and Mestecky, 1990).

1.3.1 **The Common Mucosal Immune Response**

The mucosal immune response is initiated when antigen is encountered by the subepithelial lymphoid tissue present throughout the mucosa - termed the mucosal associated lymphoid tissue (MALT). This can be divided into the bronchus associated lymphoid tissue (BALT) (Bienenstock and Johnson, 1973), found within the mucosa of the respiratory tract and the gut associated lymphoid tissue (GALT, O'Hagan, 1990), found within the mucosa of the gastrointestinal tract. These are thought to be strategically situated to respond to foreign antigens entering the host by inhalation and ingestion. Craig and Cebra (1971) were the first group to directly demonstrate that lymphoid tissue was the source of precursors for IgA producing plasma cells. When GALT (Peyer's patch) cells were transferred into irradiated allogenic rabbits there was a repopulation in the lamina propria of recipient small intestine by donor cells producing IgA. In contrast only small numbers of donor lymph node cells (peripheral lymphoid tissue) were found in the recipient intestine, the majority of which were producing IgG. They concluded that the GALT was the foci from which IgA producing plasma cells in the gut were derived. In a similar experiment, Rudzik *et*

al. (1975) demonstrated that BALT and GALT cells transferred to irradiated rabbits were able to repopulate both the lamina propria of lung and intestine with donor IgA containing cells. This study demonstrated that the production of precursor IgA secreting plasma cells was an inherent property of mucosal lymphoid tissue whether BALT or GALT. Rudzik *et al*. (1975) described the BALT and GALT as: "Lymphoid aggregates at sentinel sites guarding the portals of entry of potential pathogens". They also suggested the existence of a common mucosal immune system whereupon different mucosal sites are linked by migrating IgA committed lymphocytes. This hypothesis was supported by Roux *et al*. (1977) who demonstrated that mesenteric node (MLN) IgA lymphoblasts "home" to recipient mice mammary gland during late pregnancy and lactation. They proposed a system in which precursors of IgA derived from GALT enter mesenteric lymph nodes, differentiate, enter the general circulation via the thoracic duct and home to distant secretory sites. Jackson *et al*. (1981) confirmed these findings. Donor MLN cells (IgA lymphoblasts) were found to preferentially home to mammary glands (lactating) as well as the intestine, MLN and salivary glands in recipient mice. This was supported by M^{c}Dermott and Bienenstock (1979) who demonstrated donor mesenteric lymph node lymphoblast cells, the majority of which were of the IgA isotype, repopulated intestine, respiratory and genital tracts in recipient mice. However, they suggested a preferential homing of MLN to the gut and bronchial lymph cells to the lungs indicating an element of organ preference in the mucosal response.

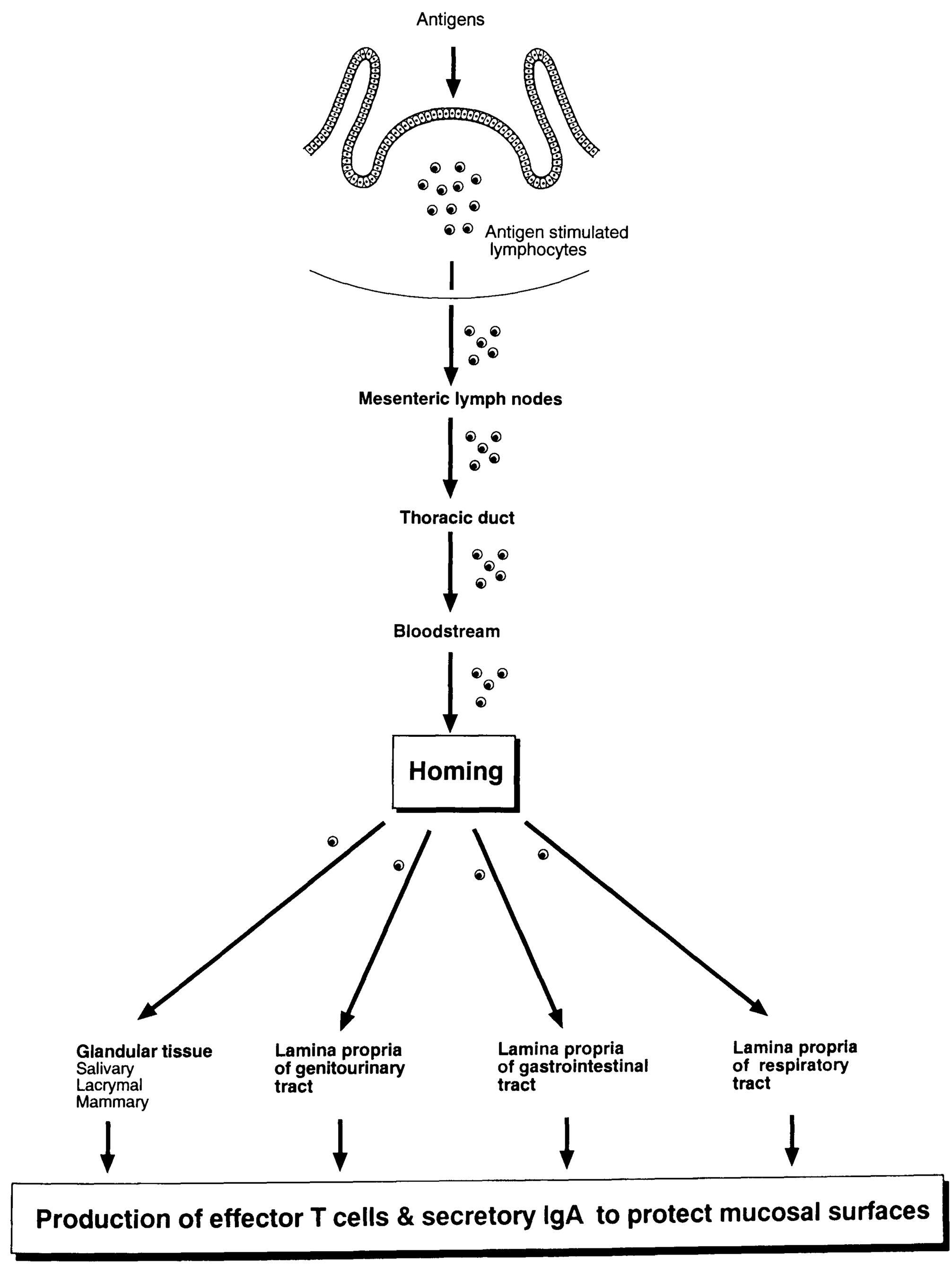
In humans, the evidence for a common mucosal system has been derived from clinical studies in which specific antibodies have found in mucosal secretions distant from the site of antigen encounter. In a study conducted by Waldman *et al*. (1982) an oral

administration of killed influenza vaccine (enterically coated) resulted in the presence of specific antibody in the nasal secretions. In a later study, IgA specific influenza antibodies were simultaneously found in tears, saliva and nasal secretions after oral administration of killed influenza (Bergmann *et al.* 1986).

Substantial evidence suggests that a common mucosal immune system does exist in which migratory IgA lymphoblasts derived from the MALT home to distant sites within the mucosal tissue, whereupon, secretory IgA is produced to protect mucosal surfaces (refer to Figure 2). Exploitation of the common mucosal immune response forms the basis for oral vaccine development. Orally delivered antigens encountered by subepithelial lymphoid tissue within the gastrointestinal tract evoke a disseminated mucosal response capable of evoking protection against pathogens invasive of distant mucosal sites, as well as the gastrointestinal tract for example an oral delivered vaccine against influenza virus that invades the mucosa of the respiratory tract.

Oral delivered antigens must first cross the intestinal epithelium and enter the subepithelial lymphoid tissue (GALT). The GALT is composed of organised lymphoid tissue and diffuse lymphoid tissue (O'Hagan, 1990). The organised tissue is composed of lymphoid follicles occurring as isolated follicles or in aggregates known as Peyer's patches. The diffuse tissue occurs as lymphocytes within the lamina propria (LPL) or between mucosal epithelial cells (IEL). Evidence suggests that the Peyer's patch are the source of the immune response (Craig and Cebra, 1971 and Rudzik *et al.* 1975) and numerous studies demonstrate that the main site of antigen uptake in the gut is the Peyer's patches (see following sections).

Figure 2 The common mucosal immune response



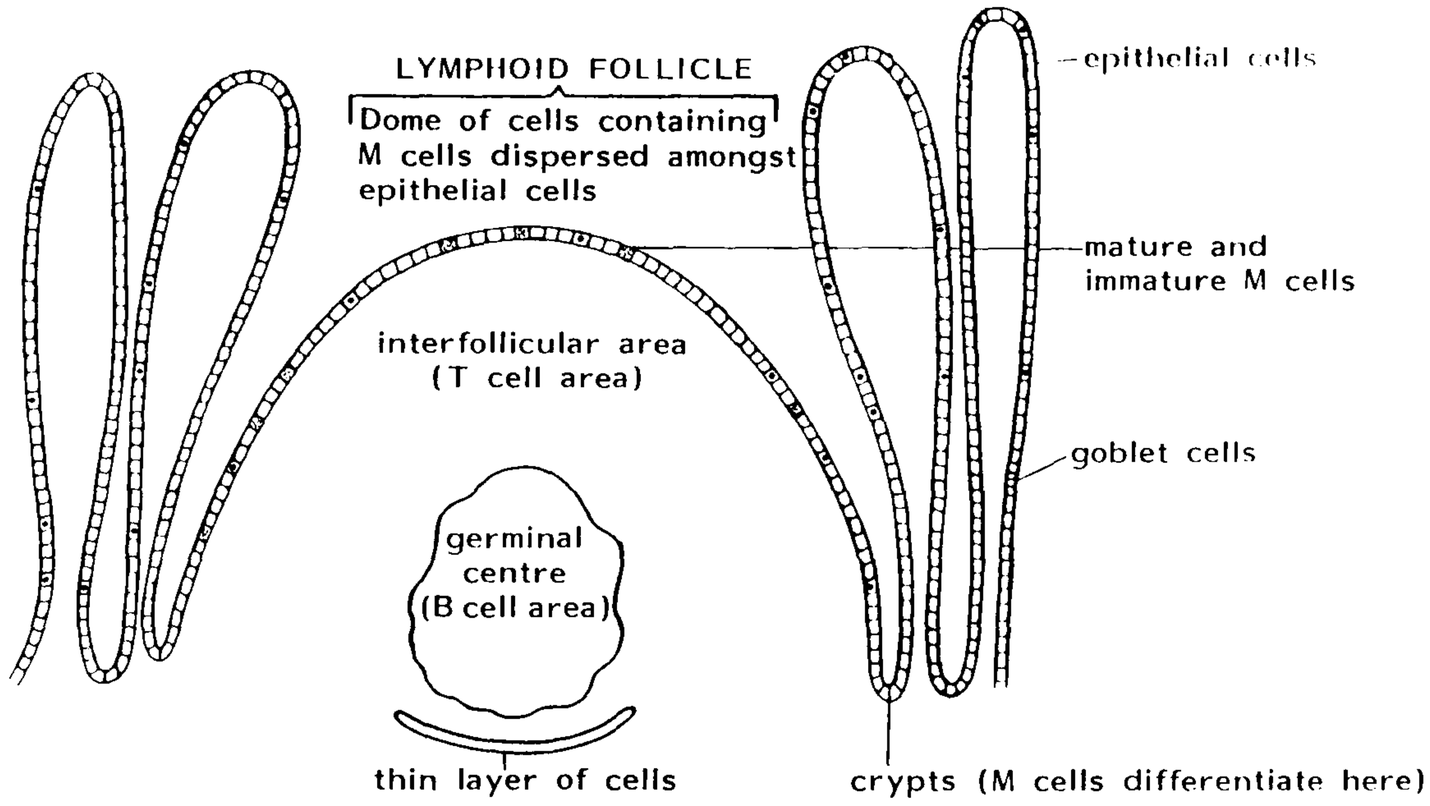
1.3.2 **Morphology of the Peyer's Patch**

Peyer's patches are collections of lymphoid follicles distributed throughout the small intestine, from the pylorus to the ileocecal valve, but they predominate in the ileum. They occur in a wide variety of species including man (Rubas and Grass, 1991). The primary difference between the species being the number follicles clustered together and the relationship of the folds and crypts to the lymphoid aggregate. The Peyer's patch contains a dome region consisting of one or two germinal centres (B-cell zone) situated towards the base of the follicle and parafollicular areas (T-cell zone) (refer to Figure 3). The adult Peyer's patch consists of 40-70% B lymphocytes found primarily within the germinal centre and 11-40% T lymphocytes predominantly found in the dome and interfollicular areas. Isotype switching of B cells bearing IgM isotype to IgA isotype is thought to occur in the dome as a result of switch T cells (Kawanishi *et al.* 1983). The dome region is also populated by macrophages and dendritic cells (LeFevre *et al.* 1985, Carlson and Owen 1987).

The epithelium overlying the dome of the follicles is termed the follicle associated epithelium (FAE) and allows the transport of luminal material into the dome. Specialised cells - M cells within the FAE are thought to facilitate this. This was demonstrated by Bockman and Cooper (1973), who compared the uptake of luminal carbon and ferritin into lymphoid follicle epithelial cells (in mouse Peyer's patch, rabbit appendix and chicken bursa of Fabricius) with that of neighbouring non-lymphoid epithelial cells. Ferritin and carbon were taken into the follicle associated epithelial cells but not the non-lymphoid epithelial cells. Bockman and Cooper (1973) demonstrated that the epithelium overlying lymphoid follicles contained specialised

Figure 3

Schematic diagram of a lymphoid follicle of a Peyer's patch (taken from CRC Critical Reviews in Therapeutic Drug Carrier Systems, vol. 4, issue 3, 1987).



cells capable of transporting luminal material to the domes and that this specialised cell was unique to lymphoid follicles, independent of species. Bienenstock (1976) described cells similar in appearance in the epithelium overlying the bronchus associated lymphoid tissue of rabbits, and reported the preferential uptake of luminal ferritin into the FAE - evidence that these specialised cells are found in all mucosa lymphoid tissue and share a common function. At this time the specialised cells were referred to as follicle associated epithelial cells which was confusing terminology as it implied all the cells in the FAE were specialised and did not take into account the normal columnar epithelial cells found in the FAE. These specialised cells were later named membranous cell or M cell due to the thin apical cytoplasm that separates the lymphocytes within the FAE from the lumen (Owen 1977).

1.3.3 **Morphology of the Membranous Cell (M Cell)**

The FAE consists of both M cells and absorptive columnar cells. The number of M cells vary between species but typically they occur as single cells scattered among the normal absorptive cells, separated by tight junctions. They possess a number of features which distinguish them from neighbouring absorptive cells (Owen and Jones 1974), refer to Figure 4. On their luminal surface they have a poorly defined glycocalyx which overlies stumpy microvilli. Unlike the microvilli of enterocytes, the microvilli of M cells are not aligned so as to form a brush border. The M cell organelles occur predominately just below the apical cytoplasm of the cells with an high occurrence of mitochondria but few lysozymes (Owen *et al.* 1986, Trier 1991). a basally located nucleus occurs within the cell. The lateral plasma membrane deeply invaginates its cytoplasm forming an intercellular compartment containing

Figure 4

Electron micrograph showing an M cell (M) of a rat. Characteristic features distinguishing these cells from normal enterocytes (E) are the shorter, fewer microvilli (Mv), an apical cytoplasm (AC) containing numerous mitochondria (Mc) and invading lymphocytes. Magnification x 9,620

lymphocytes, lymphoblasts and macrophages. This compartment has been referred to as the central hollow (Wolf and Bye 1984). These features suggest that if material is transported through the M cell, it will not be subject to lysosomal attack and so will be transported essentially unaltered, termed transcytosis (Rubas and Grass 1991) to an area rich in immunocompetent cells.

The immune response is initiated when antigen is presented to the B and T cells in the Peyer's patch dome by antigen presenting macrophage and dendritic cells. Antigen is presented in association with MHC II molecules to helper T cells. Antigen specific B cells are activated by antigen to form primed lymphoblasts. These primed lymphoblasts and antigen specific T blasts pass from the Peyer's patch via the lymphatics into the mesenteric lymph nodes and then the thoracic duct and into the blood. The primed B helper T cells "home" to mucosal sites at which differentiation into antibody producing plasma cells occurs under the influence of helper T cells. For viral pathogens it is important to induce cell mediated immunity as well as humoral immunity. CD8 phenotypic T lymphocytes, the precursors to cytotoxic T lymphocytes, are also present within Peyer's patches. Oral immunisation with viral antigens is known to induce specific cytotoxic T lymphocytes in distant mucosal and systemic sites (M^{c}Gee *et al.* 1992). For a comprehensive review of the mucosal immune response it is suggested the reader refers to ; Russell and Mestecky 1987, Mestecky 1987, M^{c}Gee *et al.* 1992 and M^{c}Gee and Kiyono 1993.

1.3.4 M Cell Uptake

The M cell has been described as an "antigen sampling" cell allowing the entry of luminal material into the Peyer's patch so an immune response can be mounted. Studies have been conducted to characterise the mechanism of M cell uptake and subsequent passage of material through the cell. Bockman and Cooper (1973) described a "pinocytotic" mechanism of entry of luminal ferritin and carbon into M cells of lymphoid tissue resulting in material filled vesicles and vacuoles within the apical cytoplasm. A pinocytotic entry of horse radish peroxidase into mouse M cells was also described by Owen (1977). Within 30 minutes HRP filled vesicles were observed within the M cell cytoplasm and after 60 minutes in the extracellular space being engulfed by lymphocytes. There was a retention of HRP enzymatic activity after uptake indicating no lysosomal attack.

The possible role of receptor mediated endocytosis in M cell uptake, uptake into the cell after initial binding to a specific marker on the cell surface (Rubas and Grass 1991), was investigated by Neutra *et al.* (1987). M cell apical surfaces contains anionic surfaces and membrane associated glycoconjugates shown by polycation and lectin binding sites (Owen and Bhalla 1983). Neutra *et al.* (1987) found *in vitro* that lectin-ferritin conjugates bound equally to M cells and absorptive cells. *In vivo*, however, the lectin-ferritin conjugates bound to M cells in greater numbers and were subsequently endocytosed and found in vesicles within the M cell. It was found that a simultaneous administration of an adherent conjugate (wheat germ agglutinin-ferritin) with that of a nonadherent BSA-colloidal gold conjugate resulted in a 50 times greater uptake of the adherent probe.

The unique features of M cells which allows entry of luminal material to pass unaltered to immunocompetent cells can also be exploited by pathogens as a means of escaping lysosomal breakdown. For example, intestinal lymphoid follicles are major sites for initial multiplication of *Salmonella typhi* before dissemination to distant systemic tissue (Gaines *et al*. 1967, Hohmann *et al*. 1978). Various studies investigating the involvement of M cells in the pathogenesis of microorganisms has further characterised the features of M cell uptake.

1.3.4.1 *The Uptake of Microorganisms into M Cells*

The uptake of *Vibrio cholerae* into rabbit Peyer's patches was documented by Owen et al. (1986). A mechanism in which pseudopodia extending from the M cell microvilli engulfing the bacteria was described. Similar to the transport of HRP (Owen, 1977) the bacteria was taken into vesicles within the M cell, transported through the cell and released into the extracellular space and taken up into macrophages. No invasion into the normal epithelial cells was seen. Only viable *cholerae* entered the M cell. This study demonstrated that a microorganism usually non invasive of the mucosa is actively taken into M cells indicating M cell sampling. It also indicates that components on the pathogen are necessary for uptake into M cells.

The role of specific ligands on microorganism adherence to M cells was investigated by Inman and Cantey (1984). The adherence of *Eschericheria coli* RDEC-1 strain to rabbit M cells was due to the possession of a pilus proteins. The transfer of the

plasmid that codes for the pili caused the specific adherence of a strain of *Shigella flexneri* to M cells. Controls lacking this plasmid did not adhere to the lymphoid follicles. The *Shigella* strain was seen deep within the cytoplasm of M cells and in association with dendritic cells resulted in an acute inflammatory response. In contrast, the RDEC-1 strain remained on the surface. After 14 days the RDEC-1 strain continued to colonise the lymphoid epithelium whilst the *Shigella* strain did not survive after 4 hours. Inman concluded the resistance to endocytosis of the RDEC-1 strain to the possession of "additional virulence factors" a possible escape mechanism from the induction of a mucosal immune response. This study showed that adherence alone does not ensure uptake into the M cell.

Wolf *et al*. (1981) investigated the entry of reovirus from the gastrointestinal tract to the systemic tissues. Using the murine model, reovirus type 1 was found preferentially to adhere and be taken up into M cells. No viruses were detected adhering to or inside normal absorptive cells. The virus was enveloped by vesicles within the cytoplasm of the cell and found in the extracellular space beneath the M cell close to lymphocytes. In a later study, reovirus type 3 was found adhering and inside lysosome bodies within the normal absorptive cells (Wolf, Kauffman, Finberg *et al*. 1983). No transport across these cells were observed. They were, however, transported across M cells in a similar manner described for type 1. The ability of the reovirus to adhere to the normal absorptive cells was attributed to the nature of the outer capsid protein haemagglutinin. Wolf *et al*. (1983), concluded that the surface characteristics of M cell and normal absorptive cells differ.

Sicinski *et al.* (1990) reported the specific adherence and endocytotic uptake of poliovirus type 1 into human epithelial M cells *in vitro*. The poliovirus was contained in vesicles within the apical cytoplasm of the M cell. No poliovirus was detected adhering or endocytosed by normal absorptive cells. It was concluded that the M cell route was being exploited by the virus to enter systemic tissue. A similar conclusion was made of Human Immunodeficiency Virus type 1 (HIV-1), in a study carried out by Amergen *et al.* (1991). HIV-1 selectively adhered and was endocytosed by mice and rabbit intestinal M cells *in vitro*. The virus was found within "endosomes" in the apical cytoplasm and next to lymphocytes in the extracellular space. The presence of M cells within human rectal mucosa suggests a possible invasive route in humans. Virus was not found adhering to or within the normal absorptive cells, within the lymphoid tissue or villous regions. They attributed this to specific surface markers on the apical surface of the M cell or to the lack of accessability to enterocytes due to their well defined glycocalyx.

A phagocytotic mechanism of uptake into rabbit M cells was observed for mycobacteria (Fujimura, 1986). At the apical surface pseudopodia extensions were close to the bacterium and subsequently contained in vesicles within the cytoplasm. The bacteria adhered specifically to the M cell with none detected inside normal absorptive cells. Bacteria were found within macrophages in the extracellular space indicating that an immune response to the pathogen was mounted.

In summary, luminal antigens are transported into M cells by a mechanism of endocytosis (pinocytosis or phagocytosis depending on the size of the material). This process is enhanced after prior adherence to the apical surface. Some pathogens can,

however, resist uptake by possessing additional virulence factors. Pathogen uptake into the M cell, in some cases, may be exploitation of the host to escape lysosomal breakdown but as these are the sites which initiate an immune response, this route is advantageous to the host.

1.4 APPROACHES TO ENHANCE MUCOSAL IMMUNITY

1.4.1. Antigen Degradation Within the Gastrointestinal Tract

The major drawback with oral immunisation is the problem of antigen breakdown within the gastrointestinal tract. The function of the gastrointestinal tract is to breakdown food and extract the nutrients needed to sustain life. To carry out this task, the tract has a system of mechanical, chemical and enzymatic components. These components are also capable of degrading orally administered antigens. For a general text on the digestive system refer to Sanford (1982).

In reaching the GALT (primarily the Peyer's patches within the small intestine) the antigen encounters numerous enzymes throughout the tract. Saliva in the oral cavity and oesophagus contains salivary amylase, lipase and lysozymes which breakdown polysaccharides, triglycerides and bacterial cell walls respectively. After transit through the oesophagus, the antigen must pass through the stomach. It is here that the food is processed to a semi-solid chyme and held until it passes into the small intestine. The environment within the stomach is extremely acidic with a pH in the range 1-2 resulting from the secretion of hydrochloric acid. This acidity can breakdown antigens. The stomach contains enzymes such as pepsin which breakdown

proteins to polypeptides by hydrolysis of the peptide bonds. The length of time the antigen would have to withstand these conditions before passing into the small intestine depends on a number of factors such as the fed state, but it could be up to 3 hours (Wilson and Washington, 1989). The small intestine contains enzymes potentially capable of destroying antigens, of particular concern are the enzyme components of the pancreatic secretions including amylase (which breakdowns polysaccharides), trypsin and chymotrypsin (which breakdowns polypeptides). ribonucleases and deoxyribonucleases are also contained in the pancreatic secretions which breakdown nucleic acids. A function of the small intestine is to facilitate nutrient absorption, therefore, food is held at the ileocecal junction until digestion is almost complete. The time varies but it may held here for 3-5 hours. This may be advantageous, in that antigen is retained in the area of maximum uptake (ileum) but it also means the antigen is subject to excessive enzymic attack. Possible ways to overcome the problem of antigen degradation in the gut are to (a) increase the amount of antigen administered or (b) enhancing (potentiating) the immunogenicity of the antigen by the use of mucosal adjuvants.

1.4.2 **Mucosal Adjuvants**

An adjuvant is defined as a substance, which when administered with an antigen enhances the immune response to the antigen (latin *adjuvare* - to help Riott, 1991). Adjuvants which work by acting directly on the cells involved in immunity are termed immunostimulants. Others increase antigen localisation in an area rich in immunocompetent cells and release antigen over a length of time to give continual stimulation of the immune system (this is termed the depot effect). The first adjuvants

were described by Ramon (1925), and since then a number of adjuvants have been developed, primarily for enhancing the immune responses to parenteral delivered antigens (reviews: Edelman, 1980, Warren *et al*. 1986 and Altman and Dixon, 1989). The problem with many of the currently available adjuvants is their toxicity; for instance, mineral oil adjuvants (Freund's adjuvants) are not licenced for human use because of the carcinogenesis associated with the mineral oils (Warren *et al*. 1986). The need for a new range of safer adjuvants is highlighted by the fact that Alum (aluminium salt) is the only presently available adjuvant licensed for use in humans (Edelman, 1980).

Mucosal adjuvants are adjuvants that enhance the immunogenicity of orally delivered antigens. Several approaches are currently being investigated (Lycke and Svennerholm 1990, O'Hagan, 1992) (refer to Table 3). Although some of the adjuvants listed in Table 3 act as immunostimulants, for example Muramyl dipeptidase (MDP) which acts directly on antigen presenting cells (Morisaki *et al*. 1983), most of the adjuvants act as antigen delivery vehicles with the aim of increasing the amount of antigen delivered to the mucosa. The live bacterial and viral vectors which express foreign antigens (review: O'Hagan, 1992) are able to replicate at the mucosa surface giving a prolonged delivery of antigen which is capable of acting as a secondary stimulus. The vector has to express non-virulence but at the same time retain the ability to colonise the mucosa, therefore, there is concern over safety (refer to section 1.2). Preexisting immunity to the carrier may also limit repeated doses. Ethical concerns over foreign gene insertion into humans seems limiting to live vector use in humans. Non-replicating delivery systems seem to offer a more likely option (O'Hagan, 1990). Adjuvants such as lectins (Pusztai *et al*. 1991) and cholera toxin and subunits

Table 3 MUCOSAL ADJUVANTS

1. REPLICATING (LIVE) ANTIGEN DELIVERY SYSTEMS

(A) LIVE BACTERIAL VECTORS

(i) *Salmonella*

(ii) *bacille Calmette-Guérin*

(B) LIVE VIRAL VECTORS

(i) Vaccina

(ii) Poliovirus

(iii) Adenovirus

2. NON-REPLICATING ANTIGEN DELIVERY SYSTEMS

(i) *Cholera* Toxin and subunits

(ii) Muramyl dipeptidase (MDP)

(iii) Lectins

(iv) ISCOMs

(v) Liposomes

(vi) Microparticles

(Holmgren *et al.* 1989) which bind to intestinal epithelium, have the drawback that conjugation to the antigen may effect the antigenic epitopes on the surface of the antigen. There also remains doubts over the toxicity of cholera toxin and some lectins (O'Hagan 1990).

A second type of non-replicating delivery system is those which aim to protect the antigen from the conditions encountered within the gut (refer to previous section). Liposomes are spheres of phospholipid bilayers containing an aqueous compartment in which antigen can be incorporated (Allison and Gregoriadis, 1976). Oral immunisation with liposomes containing purified *Streptococcus mutans* resulted in raised levels of salivary IgA in rats (Michalek *et al.* 1989). In a study conducted by Childers *et al.* (1990) liposomes were found to be taken into M cells of rats after administration via *in situ* intestinal loops. This indicated the adjuvant effect of liposomes may be due to delivery of antigen to the sites where a mucosal immune response is initiated. The drawback to the use of liposomes as mucosal adjuvants, however, is the possible instability of liposomes in the gut (O'Hagan, 1990). The use of ISCOMs (immunostimulating complexes) is another possible approach to enhance mucosal immunity. These are composed of Quil A, and membrane antigens of viruses incorporated into a micelles by hydrophobic interactions (Morein *et al.* 1984). Influenza virus ISCOMs (Sunquist *et al.* 1988) are licenced in Sweeden for parenteral use in horses. However, there is limited data at present on the stability of ISCOMs within the gastrointestinal tract.

There is, therefore, a need for more effective and safer delivery systems for orally administered antigens. The use of microparticulate antigen delivery systems composed of antigen incorporated into a polymer matrix is an approach gaining much interest.

1.4.3 **Microparticulate Antigen Delivery Systems**

The antigen is incorporated into a polymer matrix by the process of microencapsulation to form spherical microparticles (refer to section 5.1). The internalised antigen is, therefore, protected from the conditions encountered in the gastrointestinal tract. Microparticles can be monolithic in which the antigen is dispersed throughout the polymer matrix or a reservoir capsule in which the antigen lies in the centre surrounded by a core wall of polymer (Tice and Cowsar 1984).

The adjuvant effect of microparticulates was first recognised for parenterally delivered antigens. Kreuter *et al.* (1976) and Kreuter and Liehl (1978) demonstrated that split (1976) or whole influenza virus (1978) either adsorbed or encapsulated into polymethacylate particles was able to elicit an higher antibody response than soluble vaccines. The proposed mechanism of adjuvancy of the microparticulate was considered to be a result of a sustained release of the antigen from the microparticle resulting in antigen localisation in an area rich in immunocompetent cells (the depot theory first described by Glenny 1926). Kreuter *et al.* 1988, later found the immune response correlated with the hydrophobicity of the microparticulate (increase in hydrophobicity increased the response). This was supported by Tabata and Ikada (1987), who showed that an increase in hydophobicity increases macrophage phagocytosis. The adjuvant effect of microparticles administered parenterally seems

to be a combination of a depot effect and an increase in macrophage phagocytosis, both of which lead to greater antigen presentation and an increase in T cell dependent immune responses (Riott, 1991, refer to section 1.1).

Preis and Langer (1979) demonstrated that a pellet composed of ethylene-vinyl acetate copolymer implanted subcutaneously released antigen in a controlled manner into the surrounding tissues and evoked an enhanced antibody formation in serum over a period of six months comparable to the secondary response from antigen emulsified in Freund's complete adjuvant. This response was due to antigen, released over a prolonged period, acting as a secondary stimulus. The polymer used in this study was non-biodegradable and had to be removed surgically.

One of the major directives of the WHO programme for vaccine development (Bektimirov *et al.* 1990) is to develop methods to incorporate multiple vaccines into controlled release microparticles, made from biodegradable polymers, to release the vaccine at set times to mimic the booster immunisations by delivering a number of pulses of vaccine spaced over a number of months - the one shot vaccine.

The polymer used for microencapsulation needs to be both biodegradable (to avoid surgical removal) and bioresorbable (broken down into metabolites) to be considered safe for human use. The polyester, poly(lactide-co-glycolide) meets both these requirements and considerable research into their use as a polymer for sustained release antigen delivery vehicles has been carried out.

1.4.4 Poly(lactide-co-glycolide) (PLG) Microparticles as Controlled Release Vaccines

PLG is a polyester composed of lactic and glycolic acids (Tice and Cower 1984). PLG is non-toxic and has been used in humans as suture material (Wise *et al.* 1979), because of its biodegradability, biocompatability and low inflammatory reaction to tissue (Maulding 1987, Visscher *et al.* 1985 and Vissscher *et al.* 1987).

PLG has been used as microparticle drug delivery systems (Tice *et al.* 1989) and has been used to encapsulate peptides (Challacombe *et al.* 1992 and Eldridge *et al.* 1991), bacterial components (M^{c}Queen *et al.* 1993, Reid *et al.* 1993 and Edelman *et al.* 1993), whole viruses (Moldoveanu *et al.* 1993 and Ray *et al.* 1993) using various formulation techniques (refer to Chapter 5). The microparticle formulations can be lyophilised enabling storage at room temperature.

The release of antigen from PLG microparticles is dependent on the degradation of the polymer (caused by the hydrolytic cleavage of the ester bonds) and the diffusion of the antigen through the polymer matrix (Tice and Cowsar 1984). PLG polymers composed of different molecular weights and lactide to glycolide ratios can be produced which degrade at different rates (refer to section 5.6.1). By using different polymers the microparticulate delivery system can be tailored to release antigen at specific rates. In theory, by using a mixed population of microparticles composed of different PLG polymers one can deliver booster doses within a single immunisation.

PLG microparticles have been investigated as antigen delivery systems for parenteral immunisation. Eldridge *et al*. (1991) demonstrated that size was a crucial factor in the type of response elicited. Microparticles in the range 1-10μm were readily phagocytosed and transported to the draining lymph nodes. Therefore, a high concentration of antigen was available to lymphocytes, resulting in rapid and enhanced immune responses. The larger microparticles only elicited a response after sufficient degradation and subsequent phagocytosis. Using a combination of sizes they evoked a prolonged and enhanced immunity. Gander *et al*. (1993) investigated PLG degradation rates on the release of Tetanus toxoid in an attempt to mimic conventional immunisation schemes. A combination of fast and slow degrading polymers in a single administration gave enhanced responses at 4 weeks and 16 weeks and a persistent response after 8 months.

A series of experiments using particulate antigens has suggested that a particulate form of the antigen given orally elicits a greater mucosal immune response than a soluble form. Initial investigations conducted by Cox and Taubman (1984) reported that oral administration of a particulate form of dinitrophenylated bovine y-globulin (DNP-BCG) (by conjugating with *Streptococcus mutans*) was able to give greater secretory immune responses than the soluble form of the antigen. This effect was further investigated by O'Hagan *et al*. (1989). Ovalbumin adsorbed to microparticles (size 100nm) composed of a biodegradable polymer poly(butyl-2-cyanoacrylate) administered orally, elicited a greater IgA response in the saliva of rats than the soluble antigen. The same effect was obtained with ovalbumin encapsulated within microparticles composed of polyacrylamide (size 2.55μm) (O'Hagan *et al*., 1989). In all these studies the effect was attributed to the greater uptake of the particulates into

the gut associated lymphoid tissue, although there was no direct experimental demonstration of this effect. This outlines the need for detailed investigations into both the immune response to an oral microencapsulated antigen and the characterisation of the mechanism of uptake.

In summary, PLG microparticles have great potential as antigen delivery vehicles for oral vaccines. Microparticles have inherent adjuvant properties relating to phagocytosis by antigen presenting cells. PLG microparticles, which have already been used as parenteral antigen delivery systems, should give protection to the antigen in the environment found within the gastrointestinal tract. It has also been shown that a greater mucosal immune response can be evoked by a particulate form of the antigen. To elicit an oral immune response the microparticles must first cross the intestinal barrier and enter the GALT.

1.5 THE UPTAKE OF PARTICULATES ACROSS THE INTESTINAL EPITHELIUM

The intestinal epithelium is composed of normal absorptive enterocytes and the specialised cells of the follicle associated epithelium overlying lymphoid tissue. Particulate uptake across the intestinal epithelium has important implications for the possible toxic effects of ingested material, and the feasibility of the oral delivery of drugs and vaccines. To initiate a mucosal immune response the antigen delivery vehicles, discussed in the previous section, must breach the epithelium overlying the lymphoid tissue. Before discussing this, it is pertinent to review the evidence for particulate uptake across non-lymphoid tissue.

1.5.1 **Particulate Uptake Across Non-Lymphoid Tissue**

Sanders and Ashworth (1961) investigated the intestinal uptake of latex particles, 0.22μm in diameter, after intragastric administration in rats. Within 1 hour the particles were detected within cytoplasmic vesicles in the jejunal epithelial cells. The particles were later found in lymphatics. Uptake across normal absorptive cells was confirmed in a study conducted by Matsumo *et al*. (1983). Percoll particles, 20-30nm in diameter, orally administered to mice for 7 days were detected in vacuoles within enterocytes of the ileum. These particles were invariably associated with lysozymes. The low amount of uptake confirmed by the low level found within the subepithelial tissue of the villus mucosa was suggested by the authors as a consequence of discharge back into the lumen. Some particles, however, did reach the mesenteric lymph nodes and the liver.

Some authors have suggested a route in which particulates pass between enterocytes, rather than through them - the paracellular route. Polyalkylcyanoacrylate nanocapsules, 165nm in diameter, were administered to dogs using an *in situ* gut loop (Aprahamian *et al*. 1987). Scanning electron microscopy revealed the presence of the nanoparticles in the intercellular spaces between the enterocytes of the jejunum but not the cytoplasm of the enterocytes. The particles were especially prevalent in the intercellular spaces at the tips of villi where defects in the mucosa occurred as a result of cell dequamation. At these sites the particles reached the villus core and entered capillaries. The paracellular route of uptake has also been suggested for a series of controversial findings by Volkheimer (review, 1983) reporting the uptake of particulates in the size range 5 to 110μm. PVC particles and potato starch granules

with a diameter between 5-110μm were reported to cross the intestinal epithelium between enterocytes, especially at the tips of villi which showed mucosal defects. PVC particles were transported from the site of intestinal uptake either within portal blood or lymph and were later found in the cerebro-spinal fluid. These findings have been viewed with extreme scepticism: first, the entry of such large particles into the portal system seems unlikely considering the particles must cross the wall of the capillaries through fenestral channels of diameter 5-100nm (Aprahamian *et al.* 1987). Second, it is thought that only the smallest water soluble molecules are able to breach the blood/brain barrier, let alone particles in the micrometer range (Davis and Illum 1988).

In summary there are two routes by which luminal particulates cross non-lymphoid epithelium one, through the absorptive enterocytes and two, the paracellular route, between the absorptive enterocytes. Transport through the enterocytes takes place by a endocytotic mechanism and is restricted to small particulates, less than 200nm. After uptake, the particulate is prone to lysosomal breakdown within the enterocyte. The second route, the paracellular is proposed despite the tight junctions (occluding junctions) between the cells, although Phillips *et al.* (1987) reported the transport of horseradish peroxidase within occluding junctions but this macromolecule is only 5nm in size. It is therefore, not surprising that for large particulates the main site of reported paracellular transport is at the sites of defects in the mucosa such as the tips of villi.

1.5.2 **Particulate Uptake Across Lymphoid Tissue**

Evidence of particulates uptake into the GALT can divided into those studies which demonstrate 1) uptake into whole Peyer's patches and 2) uptake across the follicle associated epithelium (FAE) at the ultrastructural level.

In reviewing particulate uptake across the GALT, a number of points need to be considered:

1) The mechanism of uptake at the M cell surface.

2) The properties of particulates which determine uptake, for example, size and surface charge.

3) The mechanism and transit time for particulate transport through M cells.

4) The amount of particulates which cross the GALT and

5) The fate of particulates after uptake.

1.5.2.1 *Particulate Uptake into Peyer's Patches*

The first evidence of particulate uptake at the Peyer's patches was reported by groups investigating the potential toxic effects of ingested environmental particulates. An investigation into the uptake of carbon in mice was carried out by Joel *et al.* (1978). Carbon (20-50 nm) administered in drinking water was visible in the Peyer's patches after 2 days. The amount observed correlated with the length of feeding; after 2 months more carbon was found in the subepithelial dome regions of the patches the majority of which were within macrophages. No particles were present in the villi distant from the patch but a few particles were observed within villi adjacent to patches. After 6 months feeding black dots along the length of the small intestine

were visible. These were found to be isolated lymphoid follicles containing carbon. This indicated that the epithelium over lymphoid follicles was specialised for particulate uptake. LeFevre *et al.* (1978) extended these findings to investigated the intestinal uptake and migratory pathway of latex microparticles after chronic feeding. Latex microparticles were chosen for their uniform size and resistance to gastric breakdown. The microparticles (2μm) were given in drinking water for a period of 3 or 61 days. Sections of intestine, containing Peyer's patches, and mesenteric lymph nodes were taken and analysed using light microscopy for the presence of the microparticles. Microparticles were found within the domes of Peyer's patches in those mice which received high concentrations of microparticles, the amount observed correlating to the fed dose. Tissue taken 14 days after feeding had terminated contained more microparticles than tissue observed 74 days after feeding, indicating a migration of microparticles away from the patch over time. In the Peyer's patches from the mice fed high concentrations for 61 days, microparticles were present along the serosa, some of which were contained within macrophages. Some villi adjacent to the patch contained low numbers of microparticles whereas distant villi contained none. The presence of latex in the neighbouring villi was thought to result from the transfer through: "lymphatic channels". In the mice fed high concentrations over a 61 day period, microparticles were found in the mesenteric lymph nodes, some of which were contained within macrophages. LeFevre *et al.* (1978) suggested that migration of free and intracellular microparticles from the patch through lymphatics into the mesenteric lymph nodes had occurred.

LeFevre *et al.* (1980) investigated the influence of particulate size on the uptake into Peyer's patches. A comparison was made between the uptake and migratory pathway

of large (styrene divinylbenzene 15.8μm) and small (carbonised divinylbenzene 5.7μm) microparticles. The microparticles were administered as a single gavage or in drinking water for 30 or 60 days. Tissue sections and blood (heart drainage) were taken and analysed by light microscopy or a solubilised tissue technique for the presence of microparticles. For the single gavage and low particle concentration chronically fed (30 days) mice no microparticles were evident either in the blood or tissue sections, using both methods of detection. No accumulation of the 15.8μm microparticles were present in the Peyer's patches, blood, liver, spleen and mesenteric lymph nodes taken from mice chronically fed with high concentrations of microparticles. In contrast, Peyer's patches taken from the mice fed high concentrations of 5.7μm microparticles for 60 days contained microparticles, many within macrophages. Microparticles were not detected in intestinal non-lymphoid tissue, except as previously reported in the villi bordering the domes. Solubilised Peyer's patches, lungs and mesenteric lymph nodes contained microparticles, whereas the blood and spleen did not. Tissue taken longer periods after feeding showed more accumulation of microparticles in the mesenteric lymph nodes.

The significance of surface properties of microparticles to Peyer's patch uptake was investigated by LeFevre *et al.* (1985). Five crystallite particles, all less than 5μm, were orally administered to mice for 3 months. Carbon and iron oxide were found within the subepithelial and basal regions of Peyer's patches. In contrast, asbestos, quartz and carmine were not detected. This was attributed to the different hydrophobicities; carbon and iron oxide are hydrophobic whereas, the other crystallites are hydrophilic. LeFevre and Joel (1984) suggested that uptake into the Peyer's patch involved a phagocytotic event at the epithelial surface and, therefore,

would be influenced by: "factors known to influence phagocytosis". As previously discussed, macrophage phagocytosis of particulates is increased if the particulates are hydrophobic (Tabata *et al.* 1987 and Kreuter *et al.* 1988). The findings of LeFevre *et al.* (1985) were confirmed in studies conducted by Eldridge *et al.* (1990). This group investigated the uptake of fluorescent microparticles 1-10μm (showing various degrees of hydrophobicity) into mouse Peyer's patches. The amount of uptake into the dome region of the patch after a single gavage correlated with the degree of hydrophobicity; the more hydrophobic the greater the uptake. Also in agreement with LeFevre *et al.* (1980), was the finding that microparticles greater than 10μm were not taken into the patches. This study was the first to demonstrate the uptake and migratory pathway of microparticles composed of poly(DL-lactide-co-glycolide). After a single gavage of PLG microparticles the Peyer's patches, mesenteric lymph node (MLN) and spleen were taken at various times and analysed for the presence of microparticles. Microparticles were detected initially in the patches after time however, the maximum numbers decreased and more were found in the MLN and spleen. This migratory pattern was found to be size dependent, only microparticles less than 5μm were found in the MLN and spleen whilst those greater than 5μm remained in the patch. Migratory microparticles were reported to be within macrophages.

The effect of size on uptake was further investigated by Ebel (1990). Mice were orally gavaged with two different sizes of fluorescent latex microparticle, 2.65μm and 9.13μm and the Peyer's patch and spleen removed. The tissues were solubilised and the number of microparticles counted using flow cytometry. Both types of microparticle were detected in the patches but none of the larger ones were found in

the spleen. This indicated that the large microparticles had remained in the patch and had not reached the general circulation.

The effect of size and physio-chemical properties of microparticles on uptake was reported by Jani, Halbert *et al.* (1989). Fluorescent carboxylated and non-carboxylated latex microparticles (100, 500, 1000, 3000nm) were administered orally to rats for 10 days. The 100, 500 and 1000nm microparticles were found in the serosa of the Peyer's patches, MLN, liver and spleen. Photomicrograph evidence showed 500nm non-carboxylated microparticles "transversing" a lymphatic vessel supporting the migratory path proposed by Eldridge *et al.* (1990). The smaller non-carboxylated 100 and 500nm microparticles were taken up to a greater extent than the 1000nm, whilst the 3μm microparticles could not be detected in the patches or MLN. Jani *et al.* (1989) also detected 100nm non-carboxylated microparticles in the villi of the small intestine suggesting an "endocytotic uptake" by normal enterocytes. Particulate uptake was further investigated by the same group using gel permeation chromatography to measure the amount of polystyrene in tissues after oral administration of microparticles (50, 100, 300, 500, 1000 and 3000nm) to rats for 10 days (Jani *et al.*, 1990). Jani *et al.* (1992) attempted to further investigate the intestinal uptake of microparticles using histological analysis. All the microparticles were present in the serosal layer of the Peyer's patch except the 3μm which were thought to adhere to the patch. Jani *et al.* (1992) conflicts with LeFevre *et al.* (1980), Eldridge *et al.* (1990) and Ebel (1990), in the claim that the upper limit for microparticle uptake across the intestine was 3μm. The localisation of the 50nm in the normal enterocytes led the group to suggest particulates 50-100nm may cross the

mucosa by endocytosis, which agrees with the findings of Sanders and Ashworth (1961) and Matsuno *et al.* (1983).

In the studies above, the mechanisms of uptake at the epithelial surface of the Peyer's patch or the specific cells in the FAE which facilitate uptake are not described. These studies, however, demonstrate that the primary sites of particulate uptake across the intestinal epithelium is the Peyer's patch. This phenomenon is influenced by a number of factors including the quantity administered and physio-chemical properties of the particulate such as hydrophobicity and charge and particulate size. In the studies discussed there is general agreement that the particulates leaving the Peyer's patches follow the lymphatic pathway (refer to chapter 4) although, hitherto, there is no direct evidence of microparticles in the lymph or whether they are transported in the lymph intracellularly or intercellularly. The significance of microparticles following the lymphatic pathway in respect to oral vaccines is that the antigen delivery vehicle will avoid the first pass effect of the liver and be disseminated to the systemic tissues.

1.5.2.2 *Particulate Uptake into M Cells*

The second type of study is where particulate uptake at the follicle epithelial surface at the ultrastructural level is investigated. A group of monoclonal antibodies specifically labelling M cells in the FAE of rabbits was raised by Pappo (1989); previously the M cell was identified by the morphological differences between M cell and enterocytes (Owen and Jones 1974). The specificity of these M cells to monoclonal antibodies was utilised in a study conducted by Pappo and Ermak (1989) investigating the uptake of fluorescent latex microparticles into the FAE overlying

rabbit Peyer's patches. Fluorescent latex microparticles (600-750nm) were administered into an *in situ* gut loop, containing Peyer's patches, and left for 90 minutes. The Peyer's patch was removed, sectioned, and observed using a fluorescent microscope for the presence of microparticles. The microparticles adhered to and were taken into the cells within the FAE of the patch after 30 minutes but not into the cells of the neighbouring villi. After 90 minutes the microparticles were present in the subepithelial domes. Monoclonal labelling of the FAE revealed that the actual site of microparticule entry into the FAE was at the M cells. The authors described uptake as occurring in: "synchronous waves", in which the microparticles are taken completely through the patch before more microparticles on the apical surface start as a second successive wave. The enhancement of M cell uptake by conjugating a monoclonal antibody directed against the M cell apical surface to polystyrene microparticles (1.0μm) was demonstrated by Pappo *et al.* (1991). Monoclonal antibody conjugated microparticles were taken up into M cells more than three times that of uncoated microparticles with no uptake observed over neighbouring villi. The interesting aspect of this study was that the monoclonal used had some specificity for enterocytes (Pappo 1989) but uptake of the conjugate was seen over M cells. This proved that, as well as the importance of binding to the cell surface, the M cell has intrinsic phagocytotic capabilities.

James *et al.* (1991) developed a technique to trace the passage of microparticles through the FAE of mouse Peyer's patches by optical sectioning using confocal microscopy. *In vitro* incubation of amidine-coated fluorescent latex (0.6μm) resulted in selective adherence and uptake into M cells (identified by the lack of alkaline phosphatase activity). The same technique for tracing the passage of luminal

microparticulates coated with antigens across mouse FAE was performed by Porta *et al*. (1992). Peyer's patches removed from *in-situ* gut loops injected with native latex beads (0.5μm) or coated with either immunoglobulin A (IgA) or bovine serum albumin were prepared for scanning electron microscopy or confocal microscopy. The IgA coated microparticles were observed selectively adhering to the surface of M cells and taken up into these cells. IgA coated microparticles adhered 4 times and were taken into the M cell 25 times greater than the BSA coated microparticles. In contrast, the native microparticles were not taken up across the FAE, which conflicts with previous reports by the other groups discussed.

In more recent studies using confocal microscopy and SEM, Jepson *et al*. (1993a) demonstrated that fluorescent latex microparticles (0.46μm), administered to rabbit *in situ* gut loops, bound selectively and were taken up by M cells across the FAE within 45 minutes of administration. SEM photomicrographs showed that the microparticles on the surface of the M cell were surrounded by protruding microvilli indicating a phagocytotic mechanism at the M cell surface. Microparticles taken across into the FAE were found in close association with lymphocytes occupying the central hollow. In agreement with the findings of Porta *et al*. (1991) binding and uptake of microparticles were greater over M cells at the periphery of the Peyer's patch dome, indicative of M cell distribution (Savidge *et al*. 1991). Studies by Jepson *et al*. (1993b), compared the extent of uptake across the follicle associated epithelium, between that of poly(lactide-co-glycolide) microparticles (0.5μm) and fluorescent latex into rabbit Peyer's patches. Whilst the latex microparticles were selectively adsorbed

to M cells, PLG microparticles also adhered to enterocytes within the FAE. The PLG microparticles were, however, taken across the M cells.

To visualise the mechanism of uptake at the M cell surface, the ultrastructure needs to be observed for example by the electron microscope. In agreement with the phagocytotic mechanism suggested by Jepson *et al.* (1993), Seifert *et al.* (1983) and Sass *et al.* (1990) showed the uptake of latex particles (0.5μm) into the M cells of rats. Using scanning electron microscopy (SEM), particles were identified on M cells surrounded by pseudopodia outpushings protruding from the M cell surface (Sass *et al.*, 1990).

In conclusion, the overwhelming evidence suggests the main sites for particulate uptake across the FAE is the M cell. Few studies have actually demonstrated the mechanism of uptake at the M cell surface but it is thought to occur as a phagocytotic event. The antigenic sites specific to the apical surfaces of the M cell, which would help to understand the specificity for uptake, are still largely unknown. Initial binding, however, to the apical surface seems to be a prelude to uptake indicative of receptor mediated endocytosis. Binding is not the total requirement for uptake. Both Neutra *et al.* (1987) and Pappo *et al.* (1991) found that conjugates that bind to both enterocytes and M cells are only taken into M cells *in vivo*. This suggests the M cell possesses an intrinsic capability for particulate uptake.

1.5.3 Quantitation of Microparticulate Uptake Across the Intestinal Epithelium

Fundamental to the use of microparticle delivery systems for oral vaccines, is the quantity which is taken across the intestinal epithelium. Several investigations have been conducted to elucidate this phenomenon. Ebel (1990) described a method for quantifying absorption of fluorescent particles from the mouse small intestine using the technique of flow cytometry. Less than 0.01% of the 10^6-10^8 administered oral dose of fluorescent particles (2.15μm) was taken across the intestine, and absorption was dependent on particle size, dose level and the fed state of the animal. This level of uptake is in agreement with the levels found by LeFevre *et al.* (1977) who reported less than 0.01% of a single administered dose of radioiodinated latex particles (170-227nm) was taken across the intestine of mice. Recent studies using confocal microscopy have indicated similar levels of intestine absorption: only $5x10^{-5}$% of a dose of polystyrene microparticles was taken across rabbit intestinal Peyer's patches (Jepson *et al.* 1993a) after administration via *in situ* gut loops. Pappo and Ermak (1989) investigated the amount of uptake across the FAE of rabbit Peyer's patches after an interluminal dose of fluorescent microparticles. Using fluorescent microscopy, total uptake was found to be 5% of the administered dose.

Other workers have reported levels of microparticle absorption several orders of magnitude higher. Alpar *et al.* (1989) claimed extremely high levels of microparticulate uptake across the intestine following oral administration; 39% of the administered oral dose of latex microparticles (1.1μm) was detected in the systemic circulation of rats 45 minutes after administration. Studies by Jani *et al.* (1990) also reported high levels of uptake following the oral administration of polystyrene

microparticles for 10 days to rats. The amount of polystyrene determined in systemic and gastrointestinal tissue, using gel permeation analysis, was 34% of the administered dose for 50nm microparticles and 26% for the 100nm microparticles.

Apart from there being a few claims of high uptake, the majority of evidence suggests that particulate uptake across the intestinal epithelium occurs at low levels. The possibility of this route for the delivery of therapeutic drugs is doubtful (unless extreme measures were taken! refer to Figure 5). The amount of antigen required to induce an immune response, however, requires smaller doses and therefore, this route may be exploited for delivering oral vaccines.

1.6 ORAL IMMUNISATION WITH PLG MICROENCAPSULATED ANTIGENS

Recently, for reasons discussed in previous sections (1.4.3), poly(lactide-co-glycolide) has been extensively used as a polymer for microparticulate antigen delivery systems. Consequently, a number of studies have investigated its use as oral vaccines. Early studies using PLG encapsulated antigens were conducted in collaborative work by groups at the Southern Research Institute and the University of Alabama U.S.A. Microparticles (size 1-5μm) (encapsulating a conjugated form of Hemocyanin) orally administered to mice (Tice *et al.* 1987) induced raised levels of antigen specific IgA antibodies in saliva and intestinal secretions. An increased serum response was also observed. The response was observed up to 28 days after antigen administration indicating that there was a sustained release of the encapsulated antigen as the polymer degraded. In studies conducted by the same group (Eldridge *et al.* 1990)

Figure 5 "Open wide"!

orally administered microparticles were found to selectively target Peyer's patches. giving direct evidence that the increased secretory immune responses with microparticulated antigens was a result of antigen delivery to the sites which initiate a disseminated mucosal response. In the same study (1990) it was demonstrated that PLG microparticles less than 5μm entered the general circulation through the lymphatic pathway, whereas, those greater than 5μm remained within the patch. This group attempted to exploit this phenomenon in order to evoke both a systemic and mucosal responses. Mice were orally dosed with either PLG microparticles (size 1-10μm) encapsulating *Staphylococcal* enterotoxin B (SEB) or soluble SEB. Raised levels of anti-toxin antibodies were obtained in the serum and external secretions (saliva and intestinal secretions) from the mice given microencapsulated antigen whereas, no secretory and very low serum responses were seen in the mice given soluble antigen. It was concluded that the small microparticles disseminated to the systemic lymphoid tissue to give a systemic response whilst the larger ones remained in the patch and, as a result of a sustained release of the antigen, induced a mucosal response.

In a study by Challacombe *et al.* (1992), PLG microparticles encapsulating ovalbumin were evaluated as oral delivery systems. Microparticles (size 3μm) containing antigen and soluble antigen were administered orally to mice on 3 consecutive days and boosted 4 weeks later. After the booster dose there was a significant increase of anti-OVA antibodies in the saliva (IgA isotype) and serum (IgG isotype) of mice administered microencapsulated antigen which persisted up to 8 weeks after the primary dose. In contrast, significantly lower responses were obtained with the soluble form.

PLG microparticles have also been used to encapsulate viruses and investigated for use as oral vaccines. Ray *et al.* (1993), encapsulated a purified human parainfluenza virus into PLG (50:50) microparticles (size 1-10μm). After oral administration in hamsters the serum and saliva antibody responses slightly increased but were significantly lower than the responses both in the saliva and serum of mice given microparticles intraperitoneally. Significant was the finding that the antigenic integrity of epitopes within the protein coat of the virus was retained after microencapsulation. It is against these epitopes that neutralising antibodies are formed to give immunity. The oral delivery in mice of PLG microencapsulated inactivated influenza virus was studied by Moldoveanu *et al.* (1993). Mice primed systemically with soluble antigen and boosted orally with microencapsulated antigen gave increased levels of anti-influenza antibodies in serum (IgG isotype) and saliva (IgA isotype) greater than those obtained with mice boosted orally with soluble virus. The responses were high enough to give protection in the nose, trachea and lungs when challenged with live influenza.

In a recent study by O'Hagan *et al.* (1993), cells secreting specific antibody to cholera toxin B subunit (CTB) were found in the spleen and mesenteric lymph node after oral administration to mice of PLG microencapsulated CTB. These responses were not detected when the mice were orally dosed with soluble CTB. Using SDS-PAGE and western blotting techniques, it was shown that the structural integrity of CTB was retained after the microencapsulation process. Of considerable significance to the development of oral vaccines against viruses, was the finding that OVA entrapped PLG microparticles elicited both T-cell and cytotoxic T cell responses after intraperitoneal immunisation.

Much work is currently being undertaken to develop oral vaccines against enteric diseases. One interesting strategy is to raise specific antibodies against the components on the pathogen essential for mucosal attachment. M^{c}Queen *et al.* (1993) encapsulated the pilus proteins of rabbit *E. coli* (RDEC-1), responsible for mucosal attachment, into PLG microparticles. Sections of ileum and caecum did not show *E. coli* attachment after challenge in the rabbits given microparticles intraduodenally. In contrast, sections from non-immunised rabbits showed bacterial attachment. Following this line of thought, oral vaccines against the pilus proteins of enterotoxigenic *E. coli* have been investigated by Edelman *et al.* (1993) and Reid *et al.* (1993).

1.7 INFLUENZA: A CANDIDATE FOR AN ORAL VACCINE

Influenza is a viral respiratory infection which accounts for an high rate of morbidity in both human and animal populations. Outbreaks may occur as epidemics and occasionally pandemics, such as the 1918 outbreak which killed 20 million people. Death usually occurs as a result of secondary bacterial infections and is particulary a risk to the infants, elderly and those with an history of cardiovascular, pulmonary or renal disorders (Kilbourne, 1988).

Influenza is a disease for which the development of an oral vaccine against would be particulary applicable. First, the initial route of entry into the host is the mucosal surface of the upper and lower respiratory tracts, primarily the ciliated columnar cells (Kilbourne, 1988). Therefore, local immunity at these surfaces would seem an attractive strategy for protection. As far back as 1940, Francis found that resistance

to influenza infections correlated better with specific antibodies found within nasopharyngeal secretions than in the sera. This was further supported by Fazekas de St Groth and Donnerly (1950) who found that antibodies in bronchial secretions, produced after local application, protected against influenza. The presently available vaccines are composed of killed inactivated virus administered parenterally. The killed vaccines induce specific circulating antibodies in the sera but fail to induce mucosal immunity or cytotoxic T-lymphocytes (Maasab *et al*. 1988) which as discussed earlier are important components for clearing a viral infection (refer to section 1.1). Influenza does infect systemic organs such as the lungs but there is no viremic stage (transit through the blood to other areas) and so circulating antibodies give limited protection. Present vaccines give approximately 70% protection and lasts approximately 1 year (Kilbourne, 1988).

Surface antigens on the influenza, to which neutralising antibody are formed, are susceptible to antigenic variation and so new variants arise which fail to be recognised by previously formed antibody, therefore, there is a need to change vaccines seasonally. If new strains are not recognised the vaccines in present use have limited effect. Secretory IgA has a broad range of protective functions (M^{c}Gee and Mestecky 1990) and is better suited to cope with antigenic variation: " Secretory IgA antibodies differ from antibodies in sera in that they have a wide spectrum of reactivity which increases their value in protecting against influenza when new antigenic variants of the virus appear" (Shvartsman and Zykov 1976).

In a series of fundamental studies conducted by Waldman and Bergman secretory immune responses have repeatedly been induced in the respiratory tracts of humans

and animals after oral immunisation with influenza virus (reviews: Bergman and Waldman, 1987 and 1988). This demonstrates that the common mucosal immune system can be successfully exploited by the oral route.

1.8 AIMS AND OBJECTIVES

Aims:

The aims of the work described in this thesis were to characterise the uptake of a model microparticulate across the intestinal lymphoid tissue and to exploit this route for the delivery of a microencapsulated oral vaccine against the equine influenza virus.

Objectives:

1. To demonstrate and characterise the uptake of microparticles across the intestinal lymphoid tissue of the rabbit and rat.
2. To identify the site and mechanism of uptake from the intestinal lumen.
3. To quantify microparticulate uptake across the intestinal epithelium.
4. To formulate PLG microparticles containing equine influenza virus and to investigate the structural and antigenic integrity after microencapsulation.
5. To measure the release of antigen from microparticle systems.
6. To assess the immunogenicity of the microencapsulated virus in mice.
7. To investigate the secretory immune response in mice after oral immunisation with PLG microparticles containing the equine influenza virus.

CHAPTER 2

The Uptake of Microparticles Across the Intestinal Lymphoid Tissue in the Rabbit and Rat

2.1 INTRODUCTION

Evidence suggests that Peyer's patches are the major sites for particulate uptake in the gastrointestinal tract (refer to section 1.5). The initial aim of this investigation was to provide definitive evidence for this phenomenon and to characterise it at the ultrastructural level. The experimental approach employed was to deliver a model microparticle to the Peyer's patches and trace the passage of particles across the follicle associated epithelium, using standard histological and electron microscopic techniques. In view of this approach, consideration had to be given to the nature of the particle and, depending on this, the histological techniques used for observation. Non-biodegradable latex microparticles were selected as the model particulate because they would resist both the digestive processes of the gastric environment (Sanders and Ashworth 1961, LeFevre *et al.* 1977.) and the chemicals used in histological analysis (Self *et al.* 1988).

Two approaches were employed (a) light and fluorescence microscopy to analyse whole Peyer's patch tissue and (b) transmission electron microscopy to observe tissue at the ultrastructural level. A critical problem in such studies is the identification of the particles within the tissue. Batches of latex microparticles can be made to various

sizes and exhibit monodispersity which helps in their identification. The fluorescence signal emitted by the fluorescent microparticles was very distinctive and markedly different from tissue autofluorescence. Particles were delivered either in a chronic feeding regime or by injection into *in situ* intestinal loops. While the latter is an artificial situation, it does ensure that microparticles reach the FAE and also the time of exposure is controlled. Chronic feeding limits the control of microparticle delivery to the intestine but is more in keeping with the aim to develop an oral vaccine. An important consideration was tissue preservation, the exact location of particles could only be determined if the structural integrity of the tissue was maintained.

2.2 MATERIALS AND METHODS

Animals

The rat and the rabbit were the animal models used in the uptake studies performed.

Adult male Wistar rats	200-400 gms
Adult female Netherland Dwarf rabbits	1000-1200 gms
Adult female New Zealand White rabbits	1000-1200 gms

The animals were obtained from the Department of Biomedical Services, University of Nottingham.

Anaesthesia

Adult rats were anaesthetised using an intraperitoneal injection of Nembutal (pentobarbitone sodium BP 60 mg/ml; 0.1 ml of Nembutal for every 100 gms of body weight).

Adult rabbits were anaesthetised by inhalation of halothane administered using a Boyles Apparatus (Flurotec II). Using an open circuit, an induction dose of 1% halothane was administered. The amount was then increased slowly to 2.5% for 10 minutes and then kept constant throughout the experiment at a maintenance dose of 1.5-2.5%. Oxygen and nitrous oxide were applied at 1 litre/minute each throughout the procedure. The level of consciousness, in both the rat and rabbit, was tested by the deep tendon reflex.

Microparticles

Fluoresbrite T_M non-carboxylated 0.94 μm YG (2.5% solids-latex) (Polyscience Laboratories, Warrington).

Fluoresbrite T_M non-carboxylated 0.11 μm YG (2.5% solids-latex) (Polyscience Laboratories).

Fluoresbrite carboxylated 0.093 μm YG (2.5% solids-latex) (Polyscience Laboratories).

Fluoresbrite carboxylated 1.1 μm BB (2.5% solids-latex) (Polyscience Laboratories).

Polybead-carboxylated 0.19 μm (2.5% solids-latex) (Polyscience Laboratories).

Polybead non-carboxylated 0.50 μm (2.5% solids-latex) (Polyscience Laboratories).

Polybead non-carboxylated 0.57 μm (2.5% solids-latex) (Polyscience Laboratories).

Latex microparticles 180 nm (Department of Pharmaceutical Sciences, University of Nottingham).

'OMP' ferromagnetic latex microparticles 3.7 μm (Nycomed, Denmark).

Buffers and Histological fixatives are described in the Appendix 2

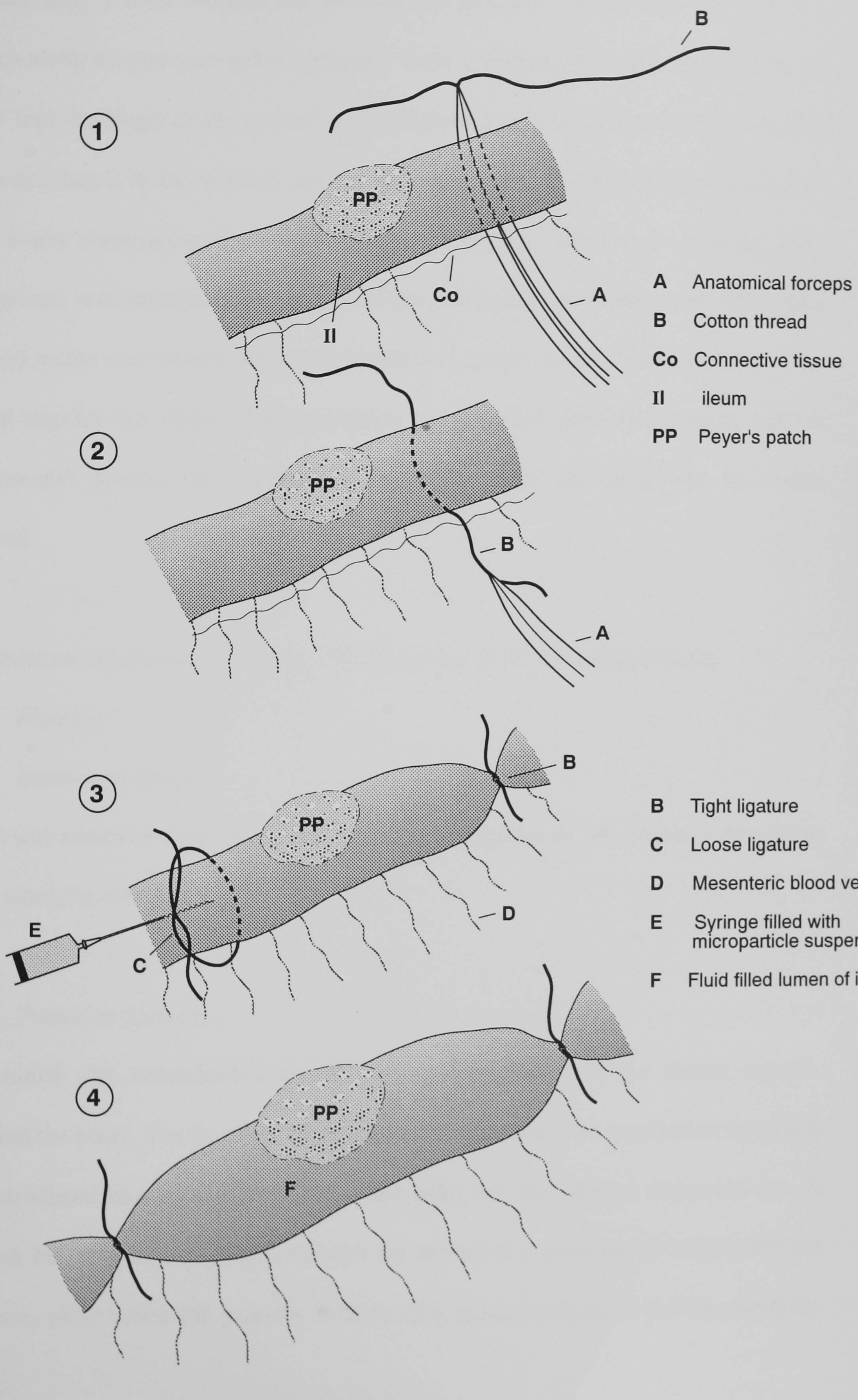
1) *In situ gut loops*

Each animal was fasted overnight to minimize bowel contents. It was anaesthetised and placed on a dissecting table heated to 37°C. The abdomen was opened in the midline to expose the small intestine. The length of the small intestine (from the duodenum to the ileocecal junction) was carefully searched for the presence of Peyer's patches. These are located on the antimesenteric surface of the ileum. Once located, the intestine was carefully ligated as follows: on either side of a patch the closed tips of watchmakers forceps were gently pushed through the mesentery close to the gut wall and opened to enlarge the perforation and a thread of damp cotton thread was drawn through the perforation. The two ends of the each thread were tied to form a ligature around the ileum. With one side loosely tied, the particulate suspension was introduced into the lumen using a G25 needle (refer to Figure 6). The suspensions were then left in the lumen for a time period. The tissue was then removed and processed for histological analysis.

2) *Chronic feeding via a stomach tube*

A piece of wood, free from sharp ridges and with a hole in the centre large enough to admit the passage of a rubber catheter, was placed under the incisors, raising the upper jaw. This results in the spread of the animal's jaw with the gag resting on the

Figure 6 Preparation of an *in-situ* intestinal loop



tongue with the centre of the gag in line with the centre of the pharynx. The catheter was carefully pushed through the hole of the gag and passed into the animal's stomach along the pharynx and oesophagus. Care is required to ensure that tube is not passed into the lungs of the animal and sufficient length of tubing must be used to ensure delivery is to the stomach and not the oesophagus. Oesophagus rupturing may occur if any suspensions are delivered here. A syringe, filled with the particulate suspensions, was attached to the tubing and the particulates were delivered. Phosphate buffered saline was passed after the particles to ensure all the microparticles were flushed through the tubing. This procedure was repeated daily (for dosage refer to experimental details) On completion, the animal was sacrificed and the tissue removed.

Transmission electron microscopy (TEM) analysis of Peyer's patch tissue:

1. Fixation.

A. Immersion fixation.

Tissue was removed from animal and immediately immersed into primary fixative to avoid autolytic changes.

B. Perfusion fixation.

The animal was anaesthetised (as previously described) and the thorax opened, exposing the heart. The tip of an 18-gauge cannula connected to a perfusion apparatus was introduced into the left ventricle of the heart and the inferior vena cava cut. A prewash buffer was then forced through the animal at a pressure of 100 mmHg for 1 minute, after which the primary fixative (3% glutaraldehyde in 0.1 M phosphate

buffer) was flushed through the animal for about 15 minutes or until the animal showed complete blanching. The tissue was then removed for further processing.

2. Microdissection of Peyer's patch material

Microdissection took place with the tissue still in primary fixative. Using a single edge razor blade and aided by a dissecting microscope (Vickers), the area around the dome of the follicle was trimmed away. This was achieved by removing the serosal muscle layer and the neighbouring villi to give a sample about 1 mm^2 (refer to Figure 7), which allowed better penetration of fixative and complete infiltration of embedding media later in the process.

3. Infiltration and embedding

A) The tissue was left in primary fixative for 48 hrs then washed three times with 5% sucrose buffer (1 hour each).

B) The tissue blocks were then post Fixed in a secondary fixative, 1% osmium tetroxide in Millonig's buffer, for 1 hour.

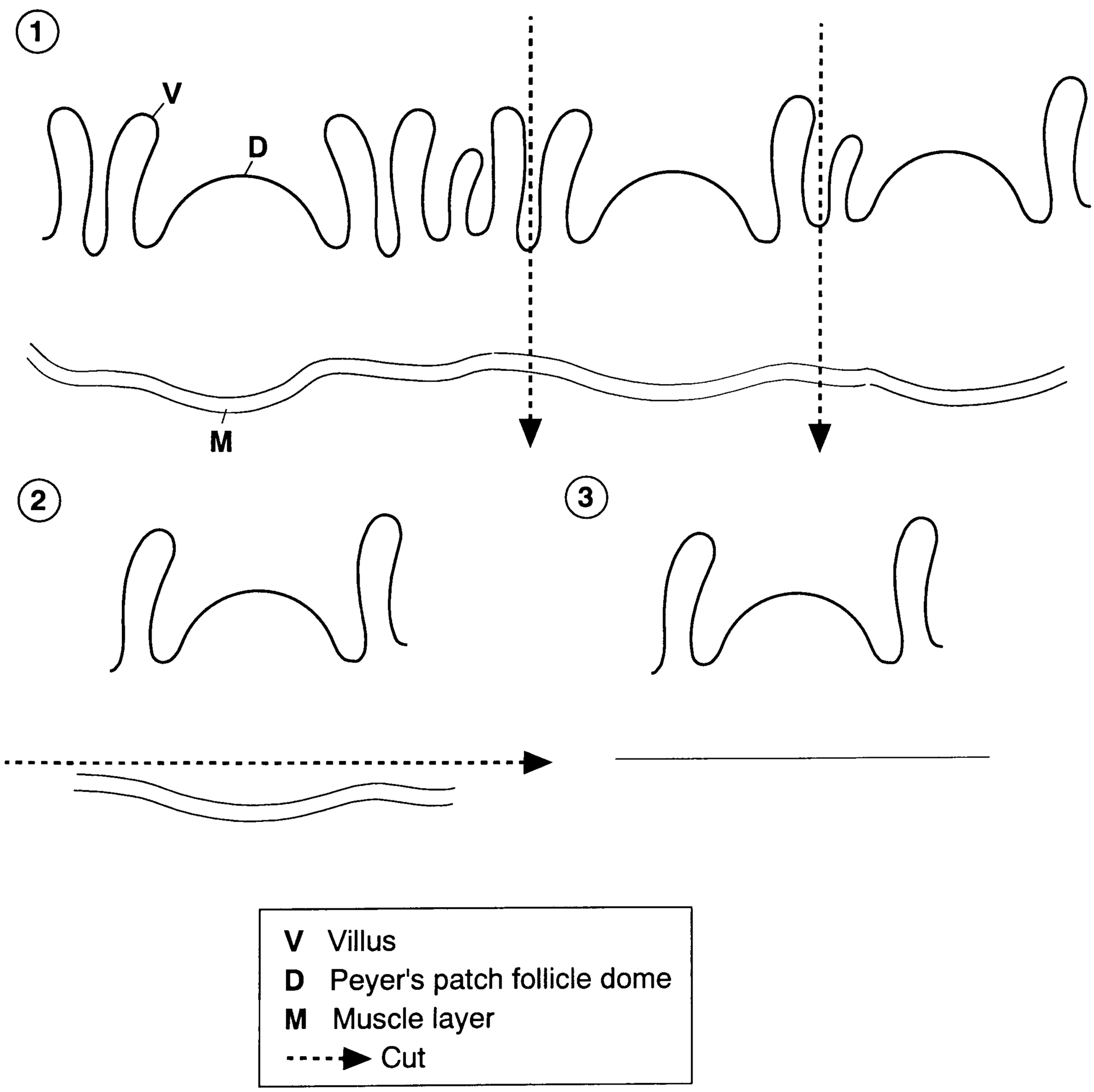
C) Tissue was dehydrated in graded alcohols (30, 50, 70, 100, 100%) for 15 minutes each.

D) Two washes in propylene oxide for 15 minutes each. The tissue was left in 50:50 propylene oxide:embedding medium overnight in an open vial.

E) Tissue was left for 24hrs in embedding medium (araldite) only in a stoppered vial.

F) The tissue was then blotted to remove excess resin and cast into moulds containing embedding medium, placed in a 60°C oven and left for 48 hours to polymerise.

Figure 7 Microdissection of a Peyer's patch



V Villus
D Peyer's patch follicle dome
M Muscle layer
----► Cut

4. Sectioning of polymerised tissue blocks.

One micron sections (semithins) were cut with a Reichert OMU3 ultra-microtome. A fine brush was used to pick the sections off the water in the knife reservoir, and placed on a glass slide. The sections were then stained for 2 minutes using toluidine blue, washed with absolute alcohol and viewed with a light microscope (Nikon) to identify the area being sectioned. When an area relating to the follicle associated epithelium (FAE) was found, ultra-thin sections were cut from that area. Gold sections with a thickness between 60-90 nm were prepared and mounted on 200 mesh copper grids. The grids were blotted dry.

5. Staining of tissue sections

The sections were double stained with filtered uranyl acetate (Bio-Rad) and filtered Renolds standard lead citrate (Bio-Rad). The copper grids, containing the sections, were placed on a drop of uranyl acetate for 15 minutes. The grid was rinsed using dropwise additions of double distilled water. The grids were placed on a drop of lead citrate and left for 6 minutes after which the grids were rinsed using dropwise additions of double distilled water. The grids were blotted dry and then observed using a Philips 300 or Philips 410 electron microscope.

Fluorescent microscopic analysis:

The fixed tissue samples were immersed in a cryoprotectant solution comprising 10% sucrose in 0.1 M phosphate buffer and stored at 4°C. Sections for fluorescent microscopic analysis were cut using either the freezing microtome or a cryostat (cold microtome).

1. Freezing microtome sections

Frozen sections were produced using a freezing microtome (MSE). A block of tissue was placed on the microtome stage with a drop of OCT freezing compound and the temperature reduced with a Percoll freezing apparatus. When the tissue was frozen, it was allowed to equilibrate prior to being sectioned at a microtome setting of 20 microns. Using a fine brush the sections were lifted from the knife and placed in a petri dish containing 0.2 M phosphate buffer. The sections were then mounted on glass slides using 0.2 M phosphate buffer as the mountant. During sectioning, the mounted sections were observed under a dissecting microscope until the follicle dome of a Peyer's patch was reached, at which time the sections were observed under the fluorescent microscope.

2. Cryostat (cold microtome) sections

Cryostat sections were produced using an open top cryostat chamber (Bright). The tissue sample was frozen to the object holder by adding OCT compound around the sample and then using forceps the holder was immersed into a beaker of chilled isopentane (brought to a temperature of -120°C by immersing in a dewer of liquid nitrogen) for about 20 seconds. The frozen sample was then placed in the chilled cryostat chamber (-17°C). The tissue block was left for at least 1 hour to equilibrate to the temperature of the chamber. The object holder was then clamped in position and the feed mechanism set to a thickness of 15 microns. The tissue block face was carefully lined up to the knife edge and trimmed until the sections being cut were representative of the tissue. The tissue sections were prevented from rolling or curling by using an antiroll bar which rests on the knife. A slide (at room temperature) was held a fraction over the frozen section on the knife causing the section to jump off on

to the slide. In between sections, the knife edge was scrupulously cleaned using a brush and kleenex tissues. The lid of the chamber was kept closed as much as possible to maintain the low temperature inside the cryostat. The slides were mounted using 0.2 M phosphate buffer and observed under the fluorescent microscope.

Fluorescent microscope

The fluorescent microparticles used in the uptake studies fluoresced either yellow/green (Excitation max 458nm, Emission max 540nm) or bright/blue (Excitation max 365nm, Emission max 468nm) when excitated with uv light, therefore, an excitation filter in the uv range was set on the microscope (Leitz Ortholux II) when the frozen and cryostat sections were viewed. Neither the OCT compound nor the mountant (0.2 M phosphate buffer, pH 7.2) showed fluorescence at these wavelengths.

Method for transmission electron microscopic processing of frozen sections taken from Peyer's patches

To investigate further what was observed in fluorescent microscopy, the frozen sections were processed for TEM analysis. Frozen sections (thickness of 20-40 microns) were carefully removed from the slide in which they have been mounted for previous fluorescent microscopic analysis and placed in a petri dish of 0.2 M phosphate buffer pH 7.2. The Peyer's patch sections were microdissected as previously described to give a single follicle dome. Due to the small size ($1mm^2$), the section was embedded in a block of agar to make processing easier. A small amount of 2% agar was poured on to a glass slide to give a film about 2 mm thick. Blocks of agar were cut and a small hole made in each for the Peyer's patch tissue to be

carefully placed. A small drop of warm 2% agar was then placed on top of the tissue to seal in the section. The blocks were then lifted from the slide and processed for TEM using the following procedure;

A. The agar block left in osmium tetroxide for 30 minutes.

B. The tissue was then dehydrated using graded alcohols (50, 70, 90, 100, 100%) for 10 minutes each.

C. The block was left in propylene oxide for 10 minutes and repeated.

D. It is then immersed in a 50:50 propylene oxide:embedding medium in an unstoppered vial overnight.

E. The block was immersed in embedding medium (araldite) for a further 24 hours.

F. The block was then cast into BEEM capsules containing embedding medium (araldite), ensuring the tissue lay flat, and placed in a 60°C oven for 48 hours.

For paraffin wax tissue-processing and staining methods, and Prussian blue iron test on TEM sections, refer to Appendix 3

2.3 UPTAKE STUDIES IN THE RAT

2.3.1 Acute Studies

Presentation of latex and fluorescent latex microparticles to rat Peyer's patches via closed intestinal loops

2.3.1.1 *Materials and Methods*

Male Wistar rats were fasted overnight. They were anaesthetised and microparticles injected into either an *in situ* intestinal loop performed around the Peyer's patches (refer to section 2.2.) or the whole length of the ileum (ligature made at the ileocecal junction). For the dosage and times refer to Table 4. At the end of this period Peyer's patch tissue was either perfusion fixed (study 1 and 3, fixative 3% glutaraldehyde in 0.1 M phosphate buffer and study 4, fixative 4% paraformaldehyde in 0.1 M phosphate buffer) or immersion fixed (study 2, fixative 3% glutaraldehyde in 0.1 M phosphate buffer) and the tissue processed for either TEM or fluorescent analysis. Control tissue (gut epithelium containing no Peyer's patch or microparticles) was also taken.

Table 4 Acute uptake studies in the rat

TRANSMISSION ELECTRON MICROSCOPY			
Animals	Microparticles		
Rats	Dose	Time in loop	
STUDY 1 1 2 3	 0.5 ml 0.8 ml 0.5 ml	 30 min 30 min 30 min	 0.19 μm carboxylated microparticles 0.50 μm non-carboxylated microparticles 0.57 μm non-carboxylated microparticles
FLUORESCENCE MICROSCOPY			
Animals	Microparticles		
Rats	Dose	Time in loop	
STUDY 2 4 5	 1.0 ml 1.0 ml	 15 min 15 min	 0.94 μm Fluoresbrite non-carboxylated 0.11 μm Fluoresbrite non-carboxylated
STUDY 3 6 7	 2.0 ml 2.0 ml Whole length of ileum	 30 min 30 min	 0.11 μm Fluoresbrite non-carboxylated 0.94 μm Fluoresbrite non-carboxylated
STUDY 4 8 9	 2.5 ml 3.0 ml Whole length of ileum	 15 min 15 min	 0.11 μm Fluoresbrite non-carboxylated 0.94 μm Fluoresbrite non-carboxylated

2.3.1.2 *Rat acute uptake study results*

(a) *Transmission electron microscopy analysis.*

In study 1, poor fixation of Peyer's patch material was evident in a number of tissue blocks, causing problems when cutting ultrathin sections for TEM analysis. The mass of lymphoid tissue lying below the epithelium of the follicle was difficult to fix leading to poor infiltration and poor cutting properties. Few M cells were detected. Tissue taken from rat 3, (0.57 μm latex microparticles administered) revealed the presence of microparticles lining the luminal surface of the intestinal epithelium (refer to Figure 8). Further observation of the tissue from rat 3 revealed what appeared to be a latex microparticle inside a epithelial cell (refer to Figures 9-10). The dense\tight prominent borders of the latex microparticle distinguished it from the cellular material of the cell. This cell seemed to be a normal enterocyte and not an M cell. Pseudopod formations were evident at the cell surface overlying the microparticle. Sections taken from rat 1, (0.19 μm latex microparticles administered) showed luminal microparticles; no microparticles however, were found intracellularly. No microparticles were found in the sections taken from rat 2.

(b) *Fluorescent microscopy analysis.*

In study 2, frozen sections (40μm) taken from rats 4 and 5 showed a distinctive dome arrangement of the lymphoid follicles of the Peyer's patch when viewed under the fluorescent microscope. Some sections from rat 4 and 5 showed a distinct bright yellow fluorescence relating to the microparticles within the dome regions and the tips of the neighbouring villi. It was difficult to determine, however, if the microparticles observed were intracellular. In an attempt to resolve this problem thinner sections

Figure 8

Electron micrograph showing latex microparticles (Mp, 0.57μm) in contact with the luminal surface of an enterocyte within the follicle associated epithelium of a rat Peyer's patch. Also labelled the microvilli (Mv) of the cell forming a brush border. Magnification x 12,850

Figure 9

Electron micrograph showing a enterocyte within the follicle associated epithelium of a rat Peyer's patch. A latex microparticle (Mp, 0.57μm) is shown within the cell, different from multivesicular bodies (Mvb) present. Pseudopodia formations (Pp) arising from the epithelium of the cell are apparent. Magnification x 22,680

Figure 10

High power electron micrograph of the latex microparticle in the above figure. Magnification x 39,310

Figure 9

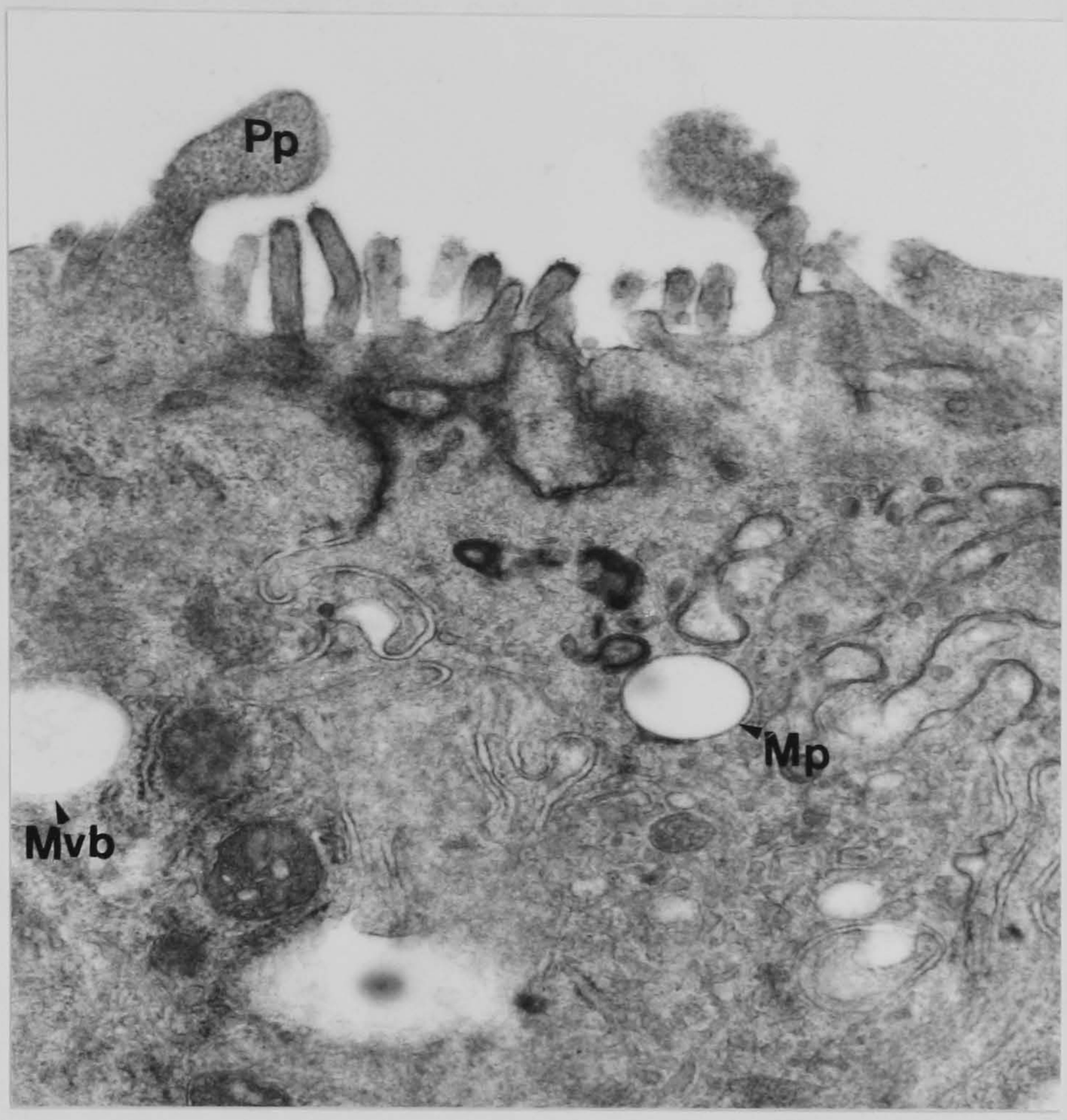
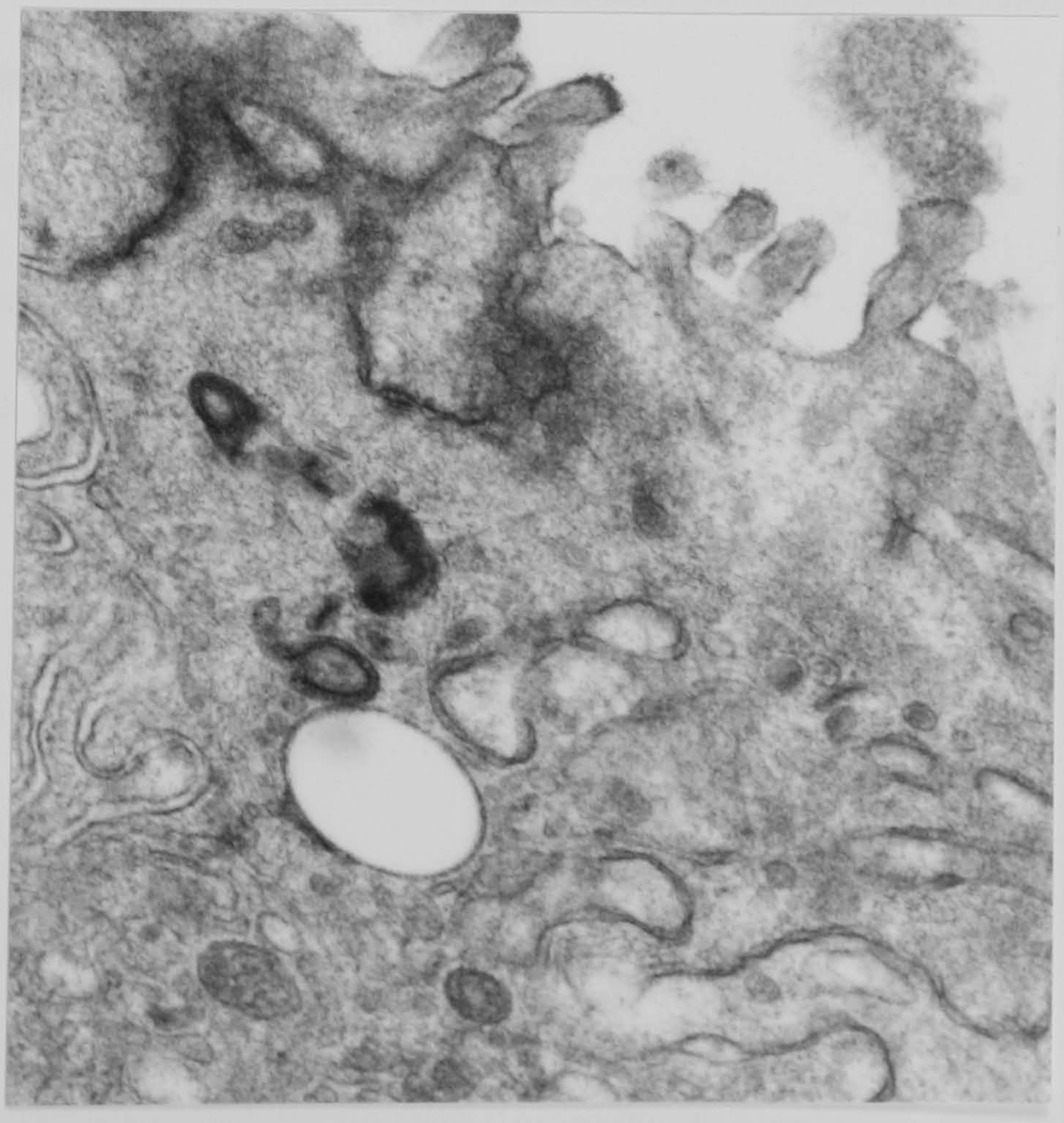


Figure 10



using the cryostat were obtained. Cryostat sections (15μm) showed poor dome preservation, leaving in most cases only intact villi. One section showing an intact follicle dome from rat 4 did reveal the presence of the 0.94μm microparticles within the dome. Clusters were abundant at the apex, centre and serosal surface of the follicle (refer to Figures 11 and 12). It was difficult, however, to determine if these microparticles were intracellular or on surface of the section. Too few intact structures were obtained to allow firm conclusions to be drawn. Similar results were obtained in study 3, cryostat sections showing poor preservation. In study 4, the use of paraformaldehyde as the fixative and buffered sucrose employed as a cryoprotectant used instead of glutaraldehyde resulted in better cutting properties giving more intact cryostat sections. Microparticles were evident in the dome regions of the patch but not to the same extent as the one section from rat 4. The problem remained as to whether these were intracellular or a luminal contaminant. Frozen sections that had already been viewed under the fluorescent microscope, were further processed for TEM analysis and viewed under the electron microscope. The semithins showed good preservation (considering the tissue had already been frozen and sectioned for fluorescent microscopy) when viewed under the electron microscope, however, no evidence of microparticles although no M cells were found.

Figure 11

Photomicrograph of a rat Peyer's patch showing the presence of fluorescent microparticles (0.94μm) within the apex of a follicle dome. Magnification x 100

Figure 12

Photomicrograph of a rat Peyer's patch showing the presence of fluorescent microparticles (0.94μm) within the serosa underlying a lymphoid follicle. Magnification x 100

Figure 11

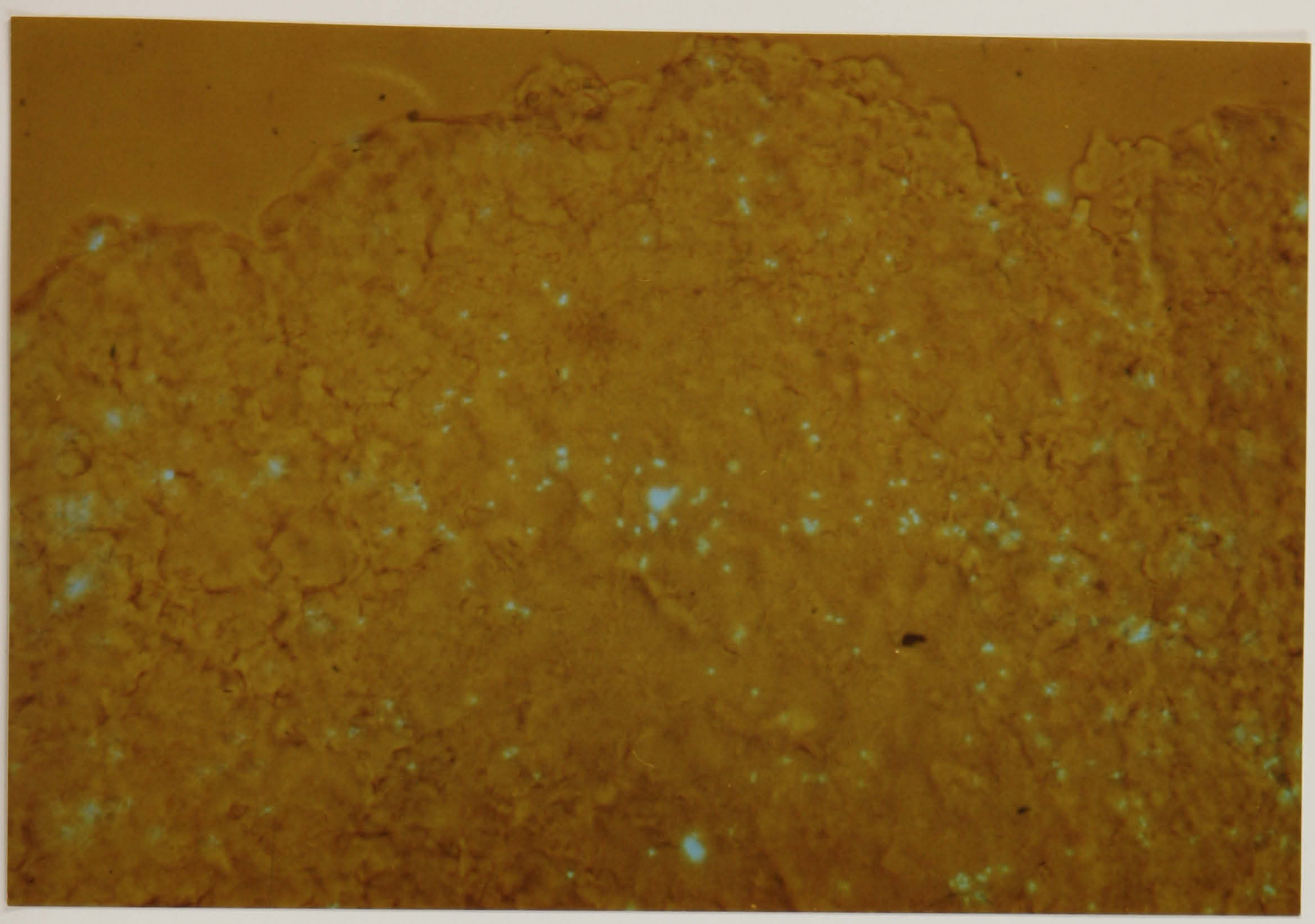
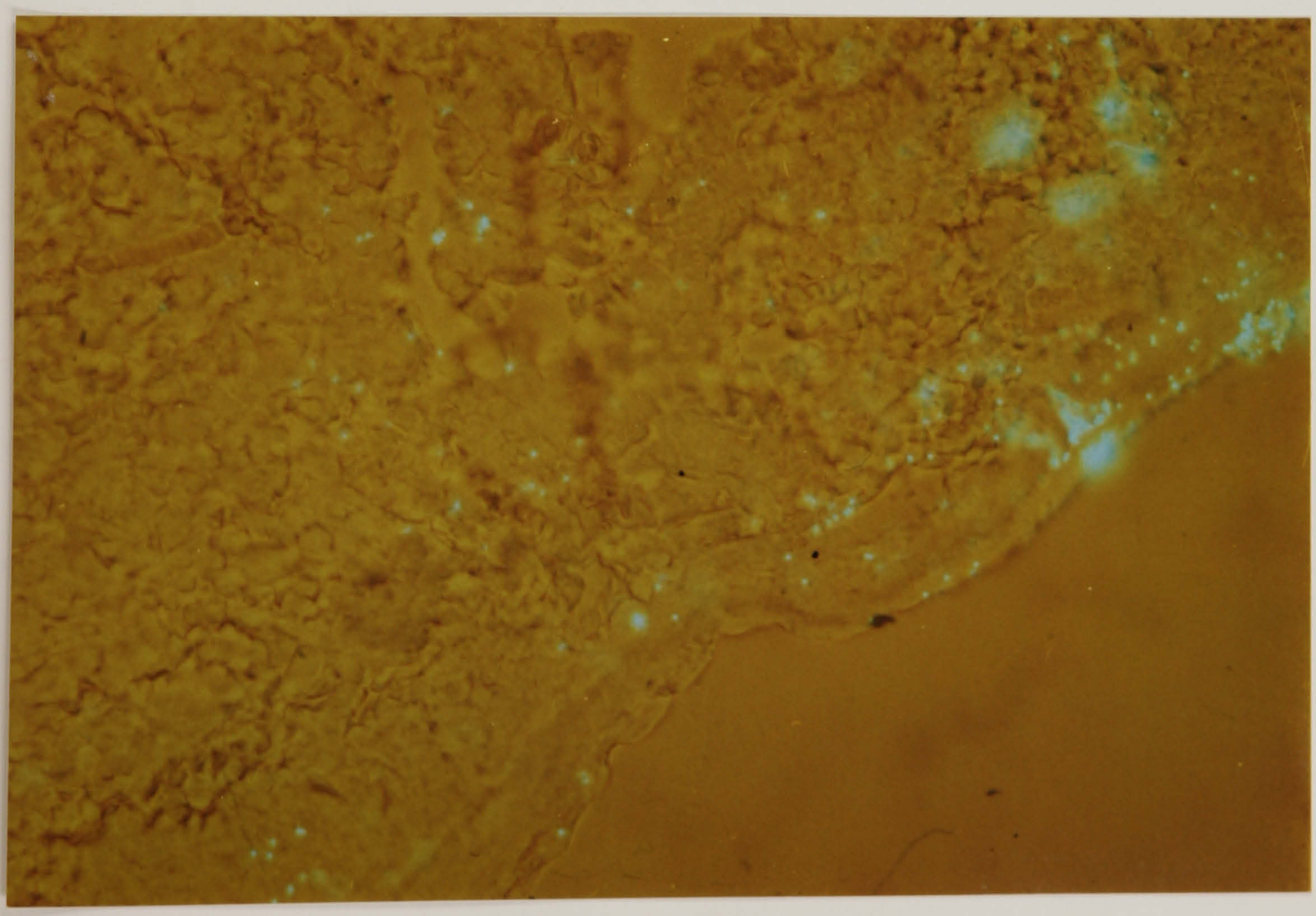


Figure 12



2.3.2 Chronic Feeding Studies

Presentation of microparticles to rat Peyer's patches using chronic feeding studies

2.3.2.1 *Materials and Methods*

Two chronic feeding studies were conducted in the rat. In the first, four male rats were dosed orally with 0.5 ml suspensions of microparticles for 7 weeks. At the end of this period, the animals were anaesthetised and the Peyer's patches removed. The tissue was immersion fixed in 3% glutaraldehyde in 0.1 M phosphate buffer and processed for TEM analysis.

In the second study, two groups of male Wistar rats (4 rats in each group) were dosed orally with a 0.2 ml suspension of fluorescent latex microparticles (diluted 1 in 18 with PBS) for 5 consecutive days. 0.2ml of a diluted suspension contained either approximately 6.1×10^8 (0.94μm) or 3.7×10^{11} (0.11μm). The same volume of PBS was administered to a group of controls (2 rats) simultaneously. On days 6 and 7 no microparticles were administered. The animals were fasted overnight on the evening of day 7. On day 8, they were anaesthetised and the Peyer's patches, mesenteric lymph nodes and liver tissue removed from each animal and immersion fixed (4% paraformaldehyde in 0.1 M phosphate buffer) overnight. The tissue was then transferred to 10% buffered sucrose solution. Frozen and cryostat sections were taken for fluorescent microscopic analysis.

Table 5 Chronic uptake studies in the rat

Animals	Microparticles	
Rats	Length of Feeding	
STUDY 1		
1,2	7 weeks	180 nm Latex microparticles
3,4	7 weeks	3.7 μm Ferro-magnetic microparticles
STUDY 2		
Group A	5 days	0.94 μm Fluoresbrite non-carboxylated
Group B	5 days	0.11 μm Fluoresbrite non-carboxylated
Group C	5 days	PBS
		Fluorescent microparticles diluted 1 in 18 with PBS

2.3.2.2 *Rat chronic uptake study results*

(a) *Transmission electron microscopy analysis.*

Samples taken from the animals dosed for 7 weeks with latex microparticles (study 1) showed good fixation which resulted in good cutting properties when sectioned for TEM analysis. Semithin sections (1μm) viewed under the light microscope, showed numerous follicles with an intact follicle associated epithelium containing M cells. These were characterised by a dip in the brush border, the presence of lymphocytes

and lighter staining with toluidine blue stain. Ultrathin sections (60-90nm) viewed under the electron microscope, revealed excellent preservation of tissue and the presence of M cells (refer to Figures 13-14). No latex microparticles were evident. either inside the cells or on the luminal surface. Tissue taken from rats 3 and 4 (study 1) was viewed under the light microscope for the presence of microparticles exhibiting the iron label. A microparticle smear on a glass slide gave a positive Prussian blue iron test. The Prussian blue iron test was then performed on wax (15 μm) and semithin (1 μm epoxy resin) tissue sections after the wax and resin had been removed, revealing a negative result for the presence of iron when viewed under the light microscope.

(b) *Fluorescent microscopy analysis.*

Only tissue from the group given 0.94 μm microparticles (group A) showed discrete microparticles in the Peyer's patches (refer to Figure 15), liver (refer to Figure 16) and mesenteric lymph nodes (refer to Figure 17). The numbers, however, were very low with the appearance of only 1 or 2 microparticles in any one slide. Out of 30 slides analysed under the fluorescent microscope, only 4/5 microparticles in total were observed. Group B tissue did not yield any microparticles from approximately 100 slides analysed.

2.3.3 **Discussion of Microparticle Uptake in the Rat**

A limited amount of evidence for uptake was obtained from the acute study when analysed under TEM (study 1). Fixation was generally poor which led to difficulties when cutting ultrathin sections. Latex microparticles (0.57μm) were found lining the

Figure 13

Electron micrograph of the follicle associated epithelium of a rat Peyer's patch. M cells (M) are clearly identifiable from normal enterocytes (E), having shorter, stumpy microvilli (Mv) and invading lymphocytes (L). No microparticles are present inside the cells or on their luminal surface. Magnification x 5,390

Figure 14

Higher power of the M cell in the above micrograph showing a close up of the apical cytoplasm (Ac). Magnification x 11,930

Figure 13

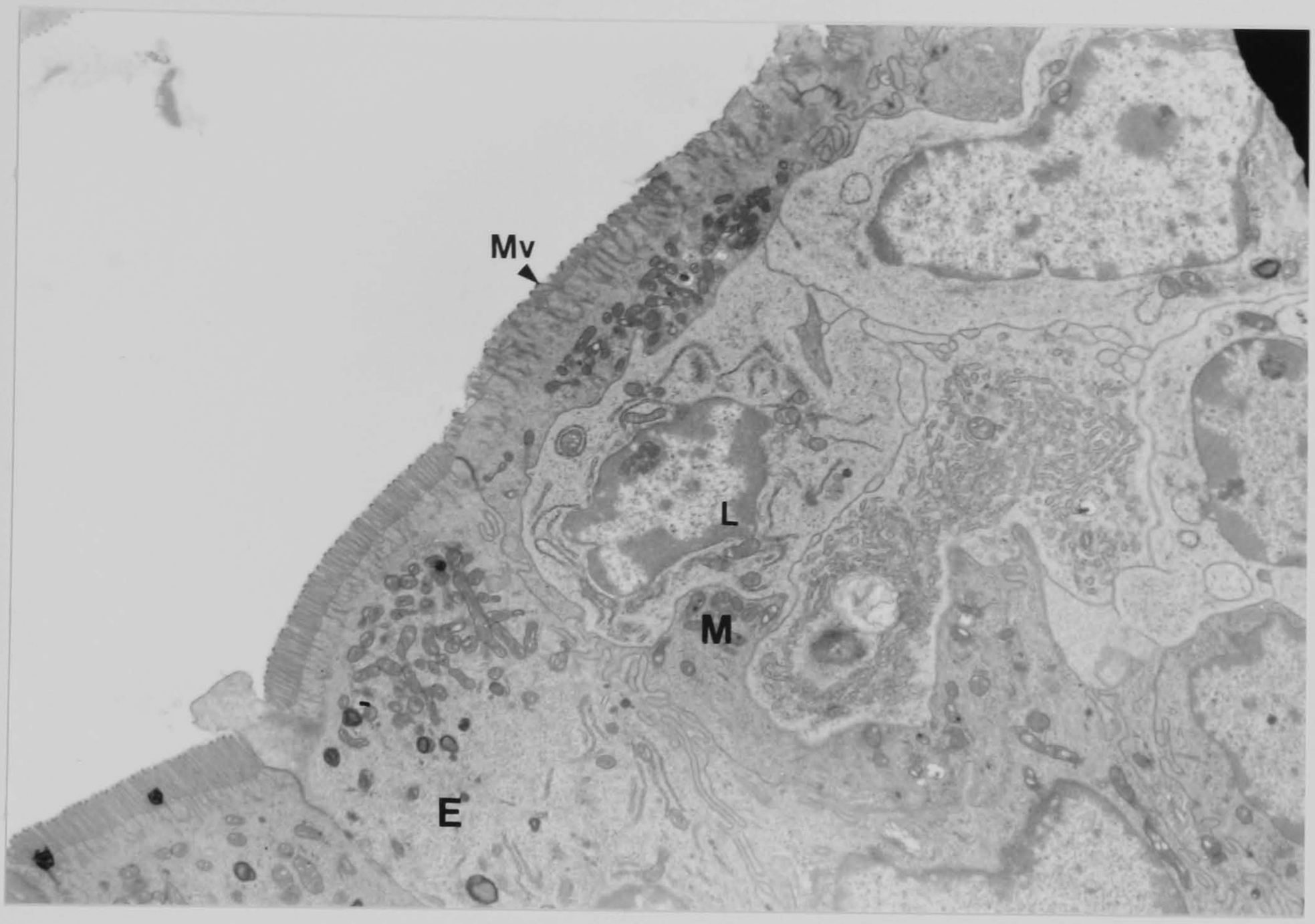


Figure 14

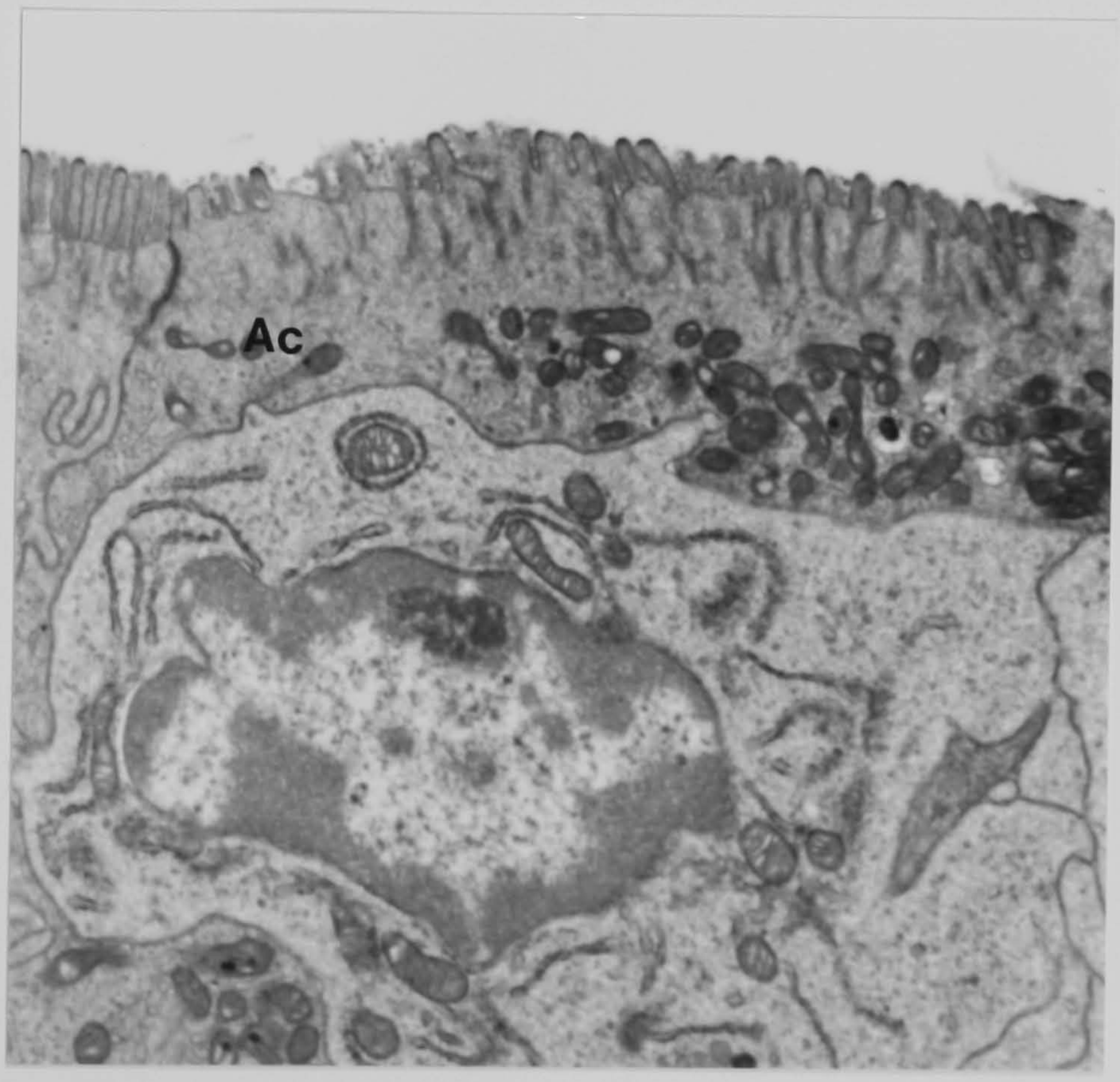


Figure 15

Photomicrograph of Peyer's patch tissue taken from a rat showing the presence of a fluorescent microparticle (0.94μm). Magnification x 250

Figure 16

Photomicrograph of liver tissue taken from a rat showing the presence of a fluorescent microparticle (0.94μm). Magnification x 250

Figure 15

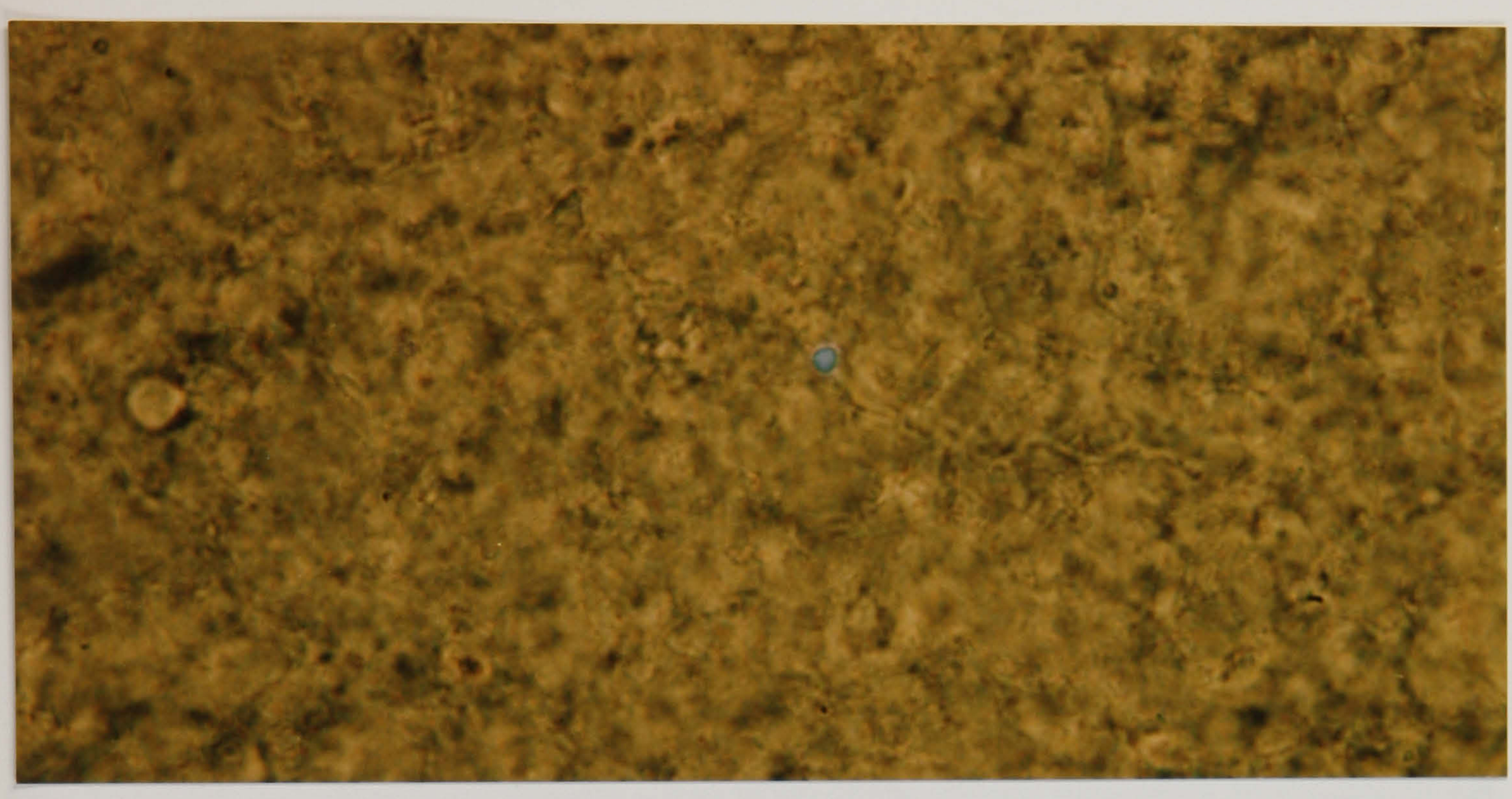
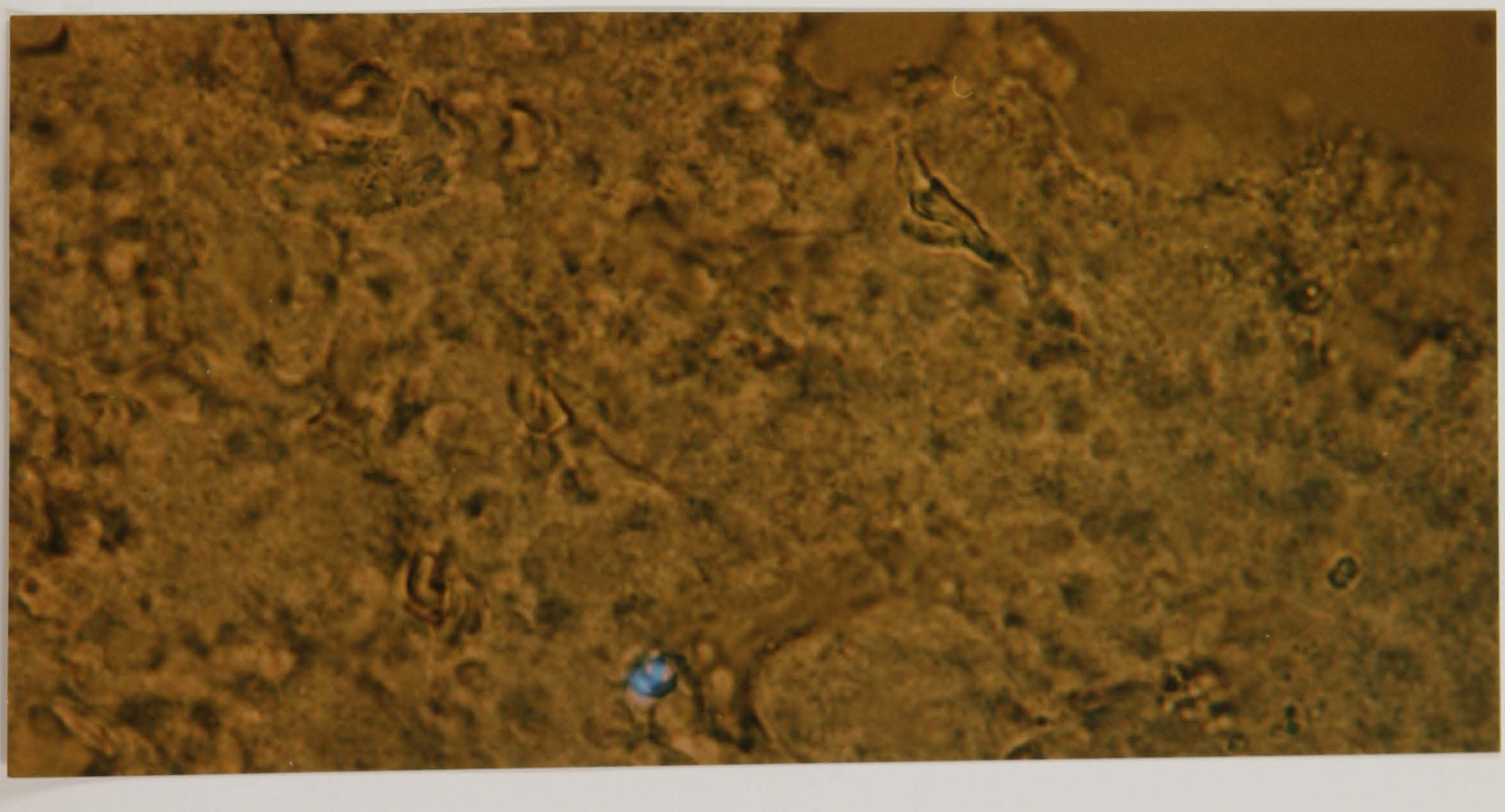


Figure 16



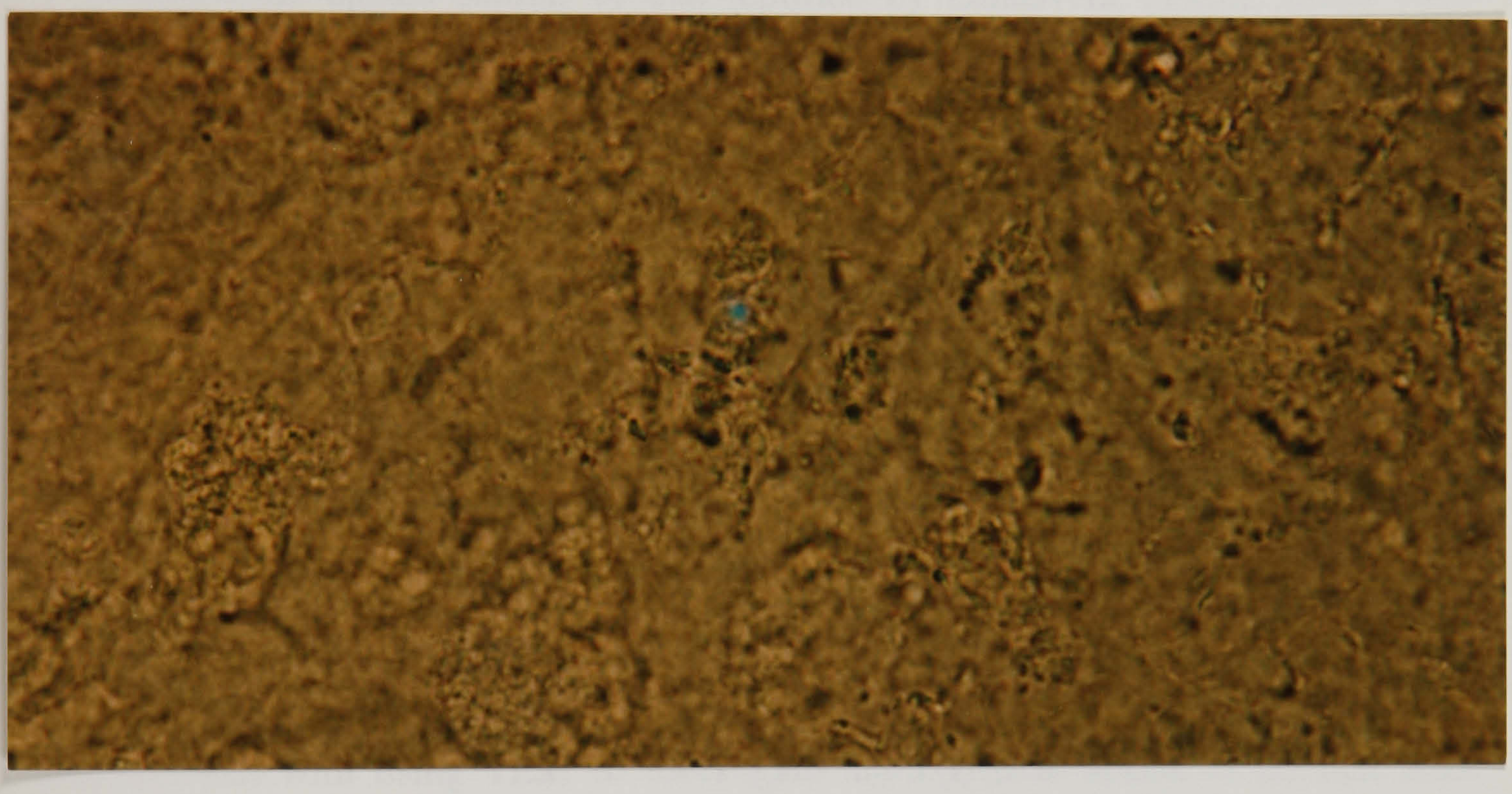


Figure 17

Photomicrograph of mesenteric lymph node tissue taken from a rat showing the presence of a fluorescent microparticle (0.94μm). Magnification x 250

luminal surface of the intestinal epithelium of Peyer's patches. This is a significant finding because it conflicts with various published accounts that the chemicals used in TEM processing destroyed latex (LeFevre *et al.* 1977, Lentzen 1984 and Jani *et al.* 1989). A microparticle was found inside an enterocyte within the FAE, just below pseudopodia formations arising from the epithelium. This result suggests a mechanism of phagocytotic uptake similar to that reported by Landsverk (1988); using TEM analysis, latex microparticles (610nm) were shown to be phagocytosed by pseudopodia before entry into the ileal Peyer's patches of calves. The cells involved in uptake were not M cells but epithelial cells. Calves have two types of M cells (Lansverk 1988). An interesting observation by Lansverk (1987) was that the M cells common to all mammalian species, found in calve jejunal Peyer's patches, did not show uptake.

The problem with using TEM to investigate microparticle uptake is (a) that latex microparticles are not electron dense, therefore, their identification in tissue is difficult and (b) semithin sections, viewed under the TEM, are less than 1 mm in diameter and 90 nm thick - a minute sample of the entire Peyer's patch. Due to the low number of intracellular microparticles observed and the difficulty in identifying latex under TEM, the whole patch was analysed at the light microscopic level, using fluorescence microscopy (studies 2-4). Fluorescent latex microparticles employed in these studies emitted a clear distinct single, easily recognisable within tissue. Frozen sections (40μm) and one cryostat section of tissue from study 2 revealed the presence microparticles in the areas relating to the follicle dome and the tips of the villi when viewed under the fluorescence microscope. The main problem, however, was determining if these microparticles were intracellular or surface contamination due to

the cutting action of the knife sweeping luminal fluorescence across the section. In an attempt to resolve this fluorescent sections were processed for TEM, when viewed under the electron microscope, however, no evidence of uptake was found, indicating that the fluorescence may have been due to surface microparticles. It was envisaged that cryostat sections less than 15μm in thickness would enable better identification of intracellular microparticles. Considerable problems were initially encountered in preserving the domes of the Peyer's patch when sections less than 15μm in thickness were taken. This was thought to be a result of using glutaraldehyde as the primary fixative. Subsequent studies (3 and 4) were conducted to try and improve tissue preservation and obtain intact cryostat sections of the follicle dome less than 15μm. In these studies luminal fluorescence was flushed out prior to sectioning to limit luminal contamination. It was found that a primary fixative of 4% paraformaldehyde in conjunction with a post wash of 10% sucrose in phosphate buffer, acting as a cryoprotectant, improved tissue preservation when cryostat section were taken. Although tissue preservation was improved, obtaining intact sections less than 15μm was still a problem. Microparticles were apparent at the tips of villi and the limited numbers found in the domes were restricted to the 0.94μm microparticles. The results were inconclusive as the problem remained as to whether these microparticles were intracellular or arose from surface contamination from luminal fluorescent microparticles.

A combination of techniques was employed to detect latex and ferromagnetic microparticles in the Peyer's patches after chronic feeding for 7 weeks. Tissue taken from chronic study 1, showed fine structural preservation when ultrathins were viewed under the TEM. Despite numerous M cells being found, no structures

resembling latex microparticles were detected. Structures were observed which seemed similar in appearance to microparticles but the lack of tight borders, such as that seen in Figures 9-10, indicated these were intracellular vesicles already present within the cell. Lefevre *et al.* (1978, 1980 and 1984), in a series of chronic feeding studies in mice, have described the uptake of particulates into Peyer's patches as a cumulative effect, uptake being dependent on the quantity and the length of feeding. with maximum uptake being shown after 60 days feeding. The lack of microparticles detected in study 1 may be a result of an insufficient length of feeding. It may also be a result of the difficulty in detecting latex microparticles under TEM. The difficulty in distinguishing latex microparticles from intracellular vesicles was the rationale behind using the ferro-magnetic microparticles in this study. Prussian blue iron tests were performed on wax sections (15μm) and epoxy semithins (1μm) in which the epoxy resin was removed. These sections gave a negative result for iron which could be possibly due to the sensitivity of the test rather than the absence of the ferromagnetic microparticles.

In the fluorescent microparticle feeding study (study 2) the mesenteric lymph nodes and liver were taken in addition to the Peyer's patches in order to follow the migratory pathway of the microparticles. A substantial amount of tissue was sampled and the signal emitted by the microparticles was very distinct, eliminating the any possibility that microparticles could be missed. Extremely low numbers of fluorescent microparticles were detected in the tissues and these were restricted to the 0.94μm microparticles. In similar experiments Jani *et al.* (1989, 1992) reported far greater numbers of fluorescent microparticles in the tissues of rats (Sprague Dawley) fed microparticles (50, 100, 500, 1000 and 3000nm) for 10 days. However, the total

numbers of microparticles administered in our study over the feeding period of 5 days was 5% of the total administered dose given over 10 days in the studies conducted by Jani *et al.* (1989, 1992). In our studies only discrete individual microparticles were recorded. Whilst, because of their difficulty in resolving microparticles of 50nm and 100nm, Jani *et al.* (1989 and 1992), relied on general tissue fluorescence as an indication of microparticle prescence. This may account for the different levels of microparticle uptake found.

In contrast to the results presented in this thesis, no evidence of microparticles found within the FAE of the Peyer's patches is given in the work of Jani *et al.* (1989 and 1990); all evidence is restricted to the serosal layer. After such a feeding regime you would have expected to have found some evidence of microparticles within the dome region of the patch (LeFevre *et al.* 1978).

In chronic feeding studies the presentation of the administered microparticles to the Peyer's patches cannot be guaranteed, therefore the possibility exists that the microparticles administered in our studies were not reaching the sites in the numbers required for identifiable evidence of uptake. Considering the limited evidence for uptake using *in situ* delivery of microparticles it is not surprising that little evidence was achieved using chronic delivery of microparticles.

2.4 UPTAKE STUDIES IN THE RABBIT

2.4.1 Acute Studies

Presentation of latex and fluorescent latex microparticles to rabbit Peyer's patches via intestinal closed loops

2.4.1.1 *Materials and Methods*

Two strains of rabbit, the Netherland Dwarf and New Zealand White were used. The rabbits were fasted overnight. They were anaesthetised and *in situ* gut loops formed around two different Peyer's patches in the same animal (refer to section 2.2.). The microparticles were injected into the loop (for dosage and time refer to Table 6). Fixative (study 1, 3% gluteraldehyde in 0.1 M phosphate buffer and study 2-5, 4% paraformaldehyde in 0.1 M phosphate buffer) was flushed through the lumen of the gut to remove residual particles in the lumen and then the Peyer's patches were removed, immersion fixed and processed for either TEM (study 1) or fluorescent analysis (study 2-5).

To compare the uptake of carboxylated microparticles of different size a mixed population of different coloured fluorescent microparticles were injected into the same loop (study 4).

Table 6 Acute uptake studies in the rabbit

TRANSMISSION ELECTRON MICROSCOPY			
Animals	Microparticles		
Rabbit Peyer's patch	Dose	Time in loop	
STUDY 1 ND♀ A B	 1.0 ml 1.0 ml	 20 min 20 min	 0.19 μm carboxylated microparticles 0.57 μm non-carboxylated microparticles
FLUORESCENCE MICROSCOPY			
Animals	Microparticles		
Rabbit Peyer's patch	Dose	Time in loop	
STUDY 2 ND♂ A B	 1.0 ml 1.0 ml	 40 min 40 min	 0.94 μm Fluoresbrite non-carboxylated YG 0.11 μm Fluoresbrite non-carboxylated YG
STUDY 3 NZW♀ A B	 1.0 ml 1.0 ml	 15 min 15 min	 0.94 μm Fluoresbrite non-carboxylated YG 0.11 μm Fluoresbrite non-carboxylated YG
STUDY 4 XNZW♂ A	 1.0 ml	 30 min	 0.093 μm Fluoresbrite carboxylated YG 1.1 μm Fluoresbrite carboxylated BB Particles dil. 1 in 5 then mixed *YG-yellow green fluorescence *BB-brilliant blue fluorescence

2.4.1.2 *Rabbit Acute Uptake Study Results*

(a) *Transmission electron microscopy analysis.*

When microdissecting the Peyer's patch tissue a multiple dome arrangement, surrounded by villi could be seen in the rabbit, as opposed to the characteristic single dome observed in the rat Peyer's patch. A well preserved follicle associated epithelium containing numerous intact M cells was observed in the semithin sections (1μm) taken from a Peyer's patch obtained in from study 1 when viewed under the light microscope. Ultrathins taken from these sections, viewed under the transmission electron microscope, revealed a remarkable feature. Pseudopod formations arising from the epithelium of an M cell were seemingly engulfing latex microparticles from the lumen. Structures relating to latex microparticles could be seen within the apical cytoplasm below the epithelium which were comparable in size to the administered microparticles (refer to Figures 18-20). However, without the presence of an electron dense marker on the microparticles it was impossible to conclude if these were actually microparticles or vesicles already present within the cell. No evidence of uptake was found within the patches from the tissue that received 0.57μm microparticles.

(b) *Fluorescent microscopy analysis*

Frozen sections taken of sample A from study 2, showed excellent tissue preservation. Multiple follicle arrangements were observed containing a well preserved follicle associated epithelium (FAE)(refer to Figure 21). Fluorescence relating to discrete 0.94μm microparticles was present in the domes of the follicles whilst absent in the neighbouring villi. Microparticles were present at the dome apices

Figure 18

Electron micrograph of an M cell lying within the follicle associated epithelium of a rabbit Peyer's patch. Latex microparticles (0.19μm, Mp) are in the process of being engulfed by pseudopodia (Pp) arising from the epithelium of the cell. Within the apical cytoplasm is a possible latex microparticle (Mp). Magnification x 26,160

Figure 19

High power of epithelial surface of the M cell in Figure 18. This shows pseudopodia (Pp) arising from the epithelium surrounding the latex microparticles. Magnification x 54,980

Figure 18

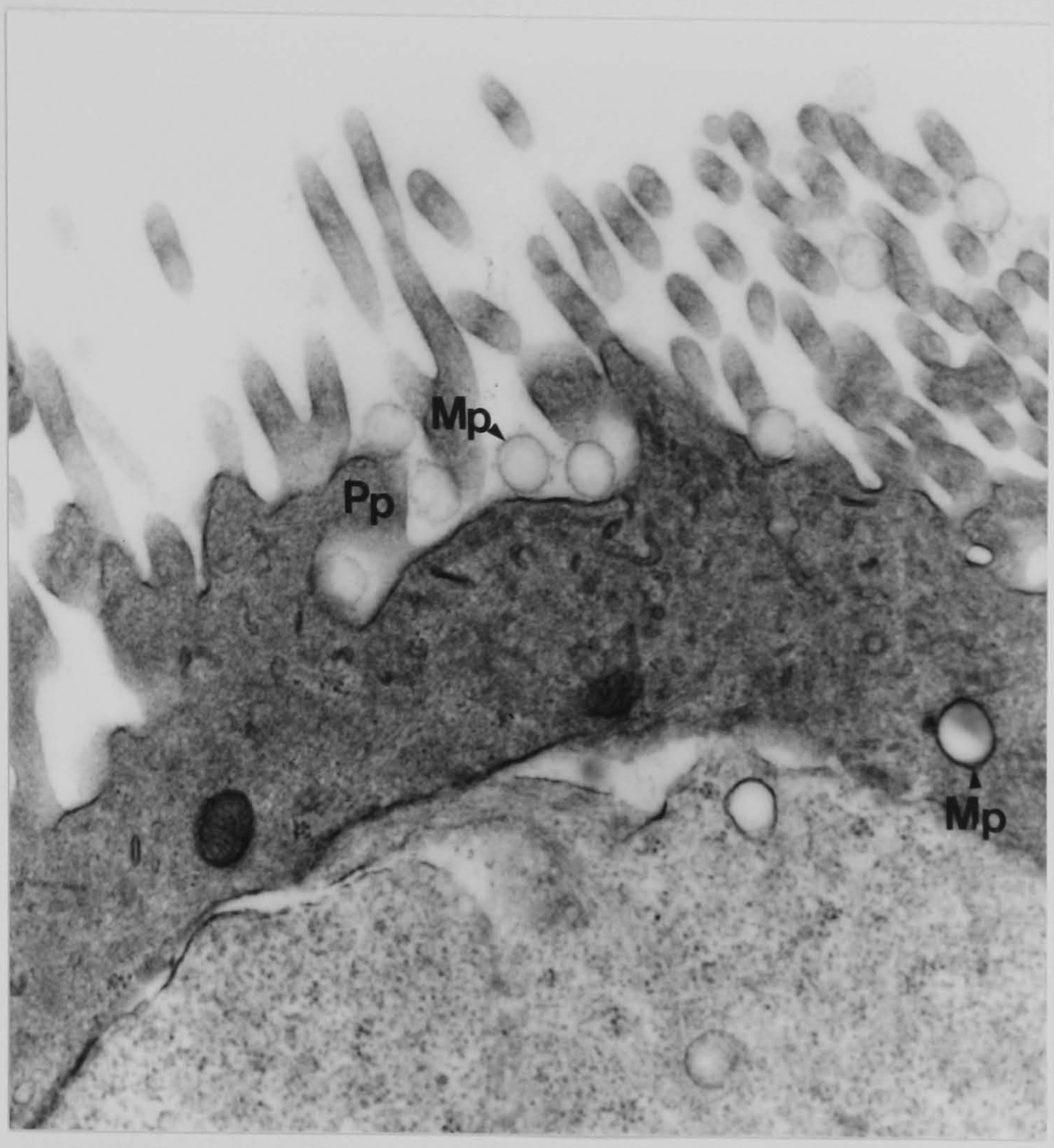
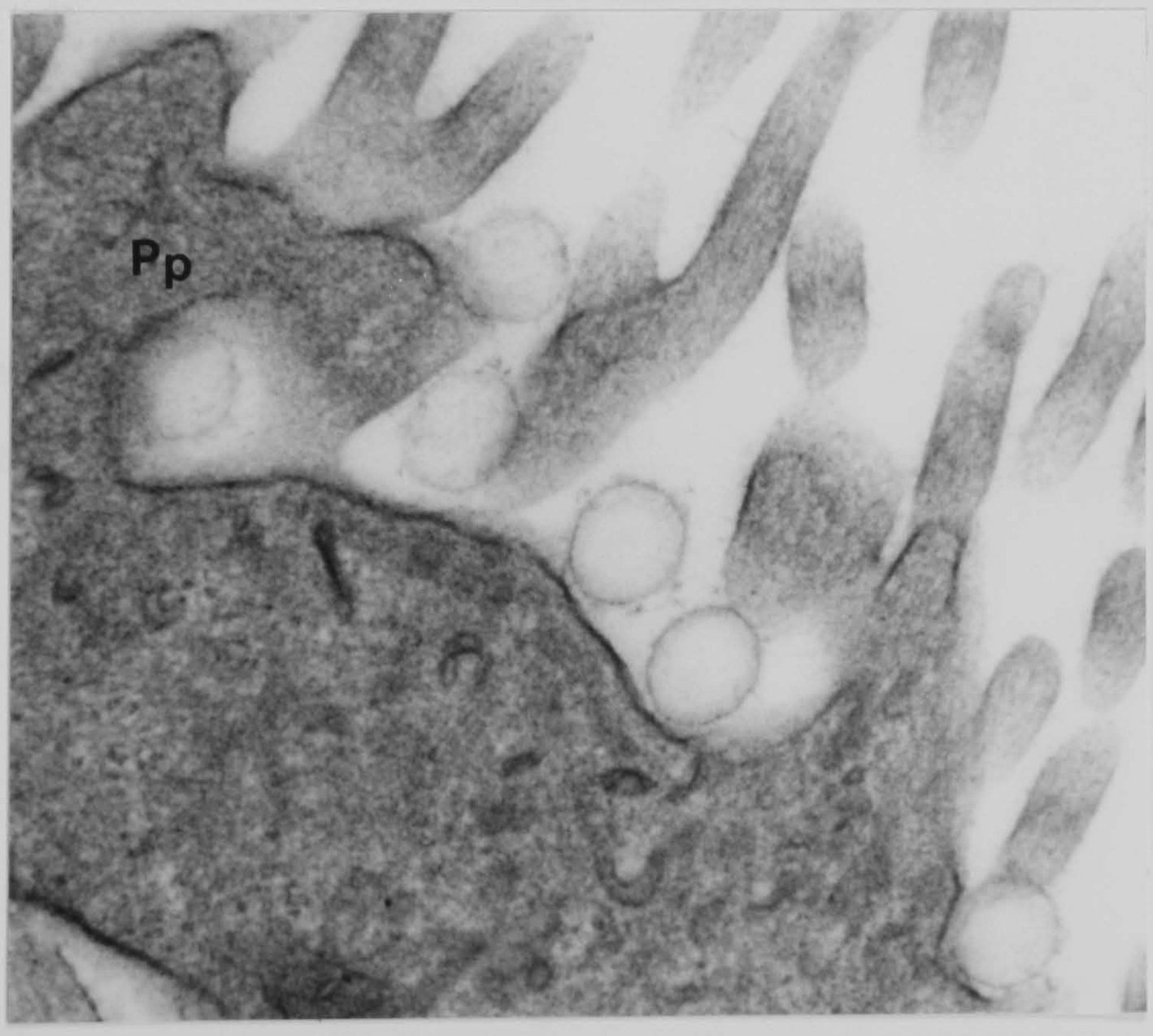


Figure 19



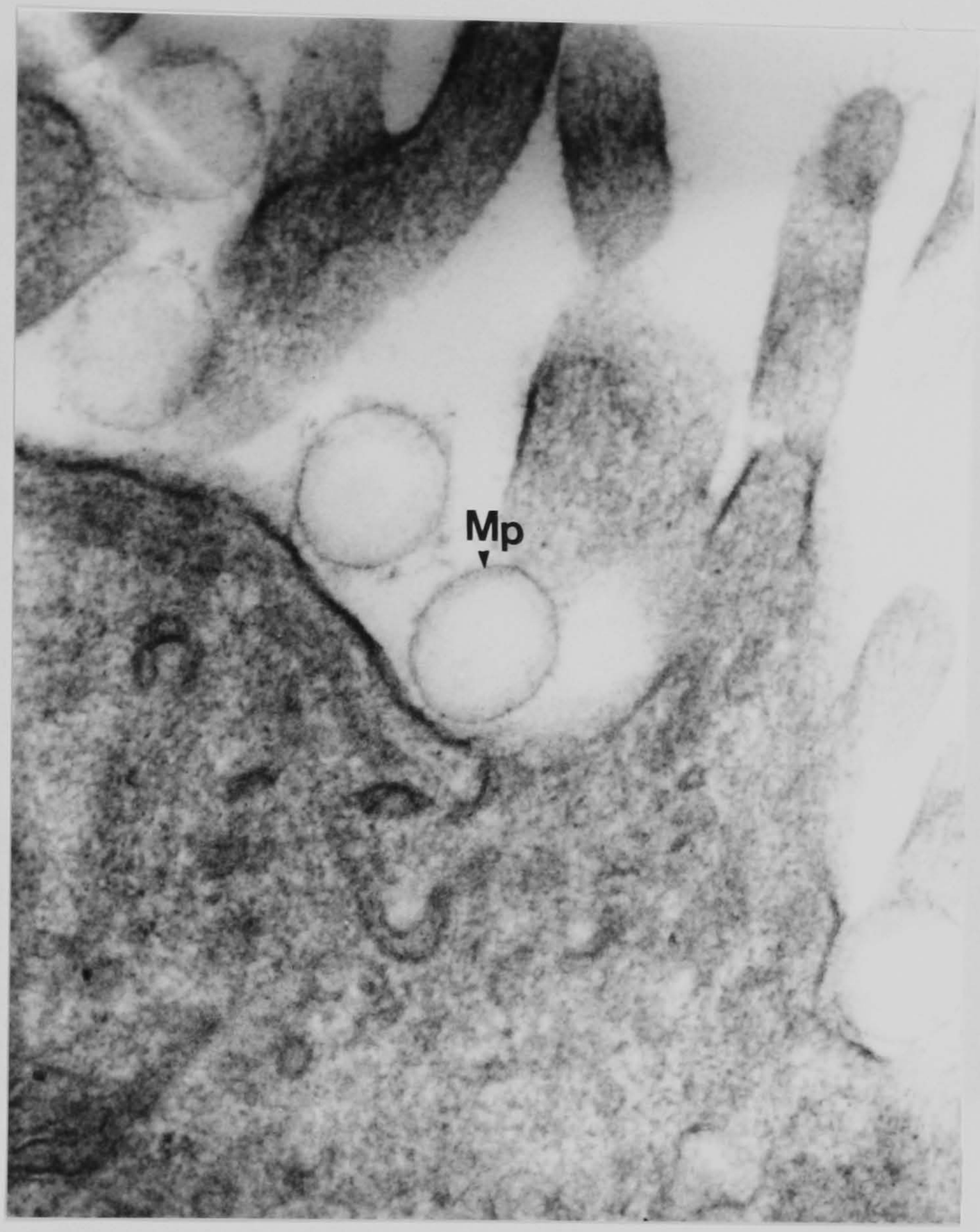


Figure 20

High power of the epithelium of the M cell in Figure 18. Latex microparticles (Mp) are clearly in contact with the epithelial surface of the cell. Magnification x 88,430

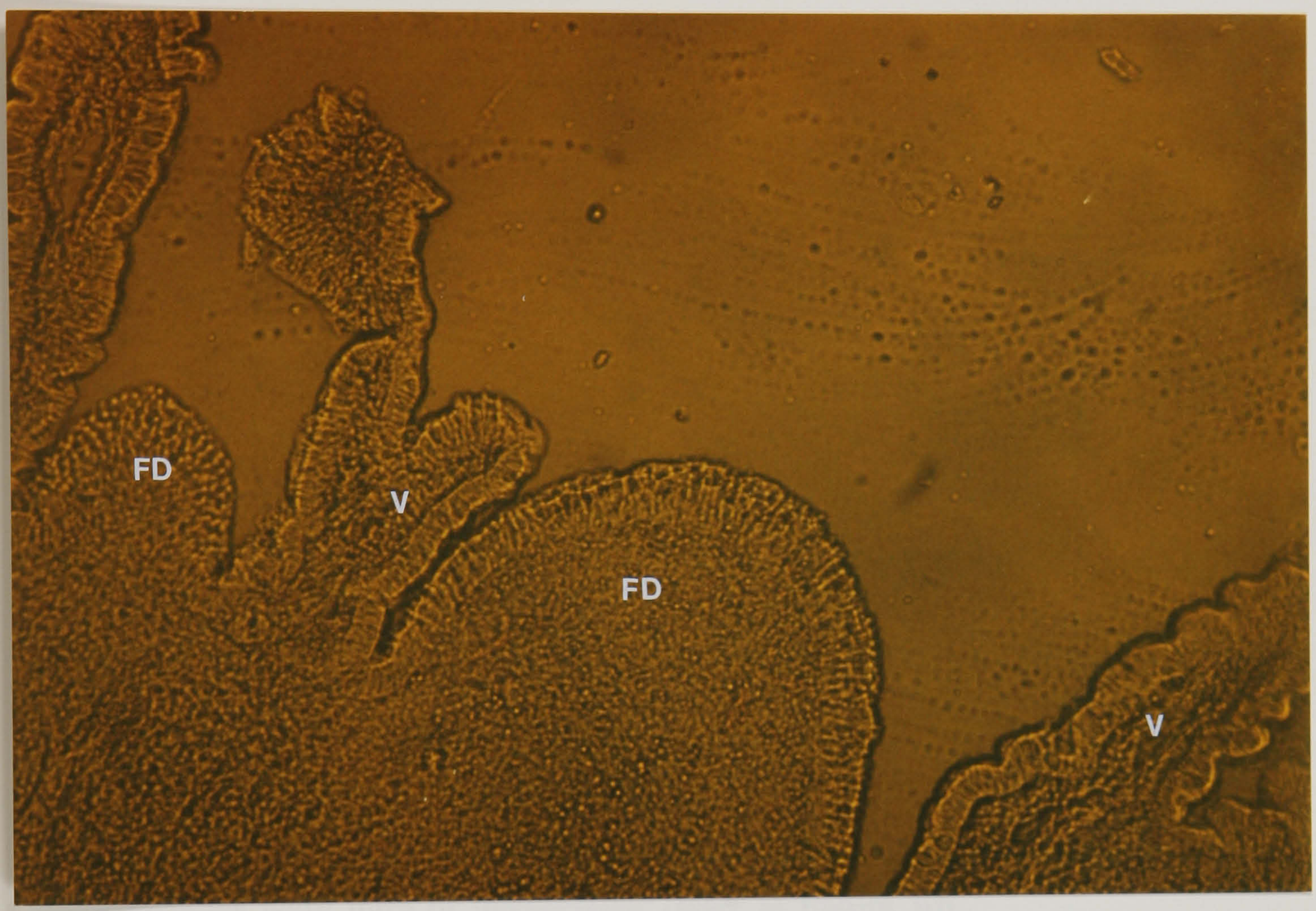


Figure 21

Photomicrograph of a rabbit Peyer's patch showing the follicle dome (FD) arrangement and the neighbouring villi (v). Magnification X 25

(refer to Figures 22-25) but absent at the serosal layer. This result was seen in every section observed. In frozen sections of sample B from study 2, 0.11μm microparticles were observed along the whole length of the serosa (refer to Figures 26-27), even within the serosa underlying villi (refer to Figures 28-29). The microparticles were present as discrete particles and in clusters. Cryostat sections taken from Peyer's patch B, study 2, showed less dome preservation than the frozen sections. The serosal layers were, however, intact and mimicked the results found in the frozen sections; the presence of 0.11μm fluorescent microparticles lining the whole length of the serosa. To investigate whether the 0.11μm microparticles observed were a result of adherence to the serosa rather than transport through the patch to the serosa, frozen sections from sample B were processed for TEM analysis and the ultrastructure of the serosal observed. Microparticles were present when viewed under the TEM, however, due to the poor preservation of tissue it was inconclusive as to whether these microparticles were intra or intercellular (refer to Figure 30).

Frozen sections of the sample A from a New Zealand white in study 3, showed similar results to the Netherland model. Discrete microparticles were present within the dome apices (refer to Figures 31-33). A few microparticles were seen transversing the dome but very few were present in the serosa. Frozen sections taken from Peyer's patch B, study 3 showed the presence of luminal microparticles trapped between the villi and domes. In agreement with the Netherland model, 0.11μm microparticles were present in the serosa with very little observed in the domes.

In study 4, cryostat sections were cut and viewed under the fluorescence microscope. The preservation of tissue was good enabling intact follicle domes to be viewed. Both

Figure 22

Photomicrograph of a follicle dome (FD) bordered by two villi (V) of a rabbit Peyer's patch. Fluorescent microparticles (FMp, 0.94μm) are present within the apex of the dome but absent from the villi. Magnification x 100

Figure 23

Photomicrograph of two follicle domes (FD) bordering a villus (V) of a rabbit Peyer's patch. Fluorescent microparticles (FMp, 0.94μm) are present within the domes but absent from the villus. Magnification x 100

Figure 22

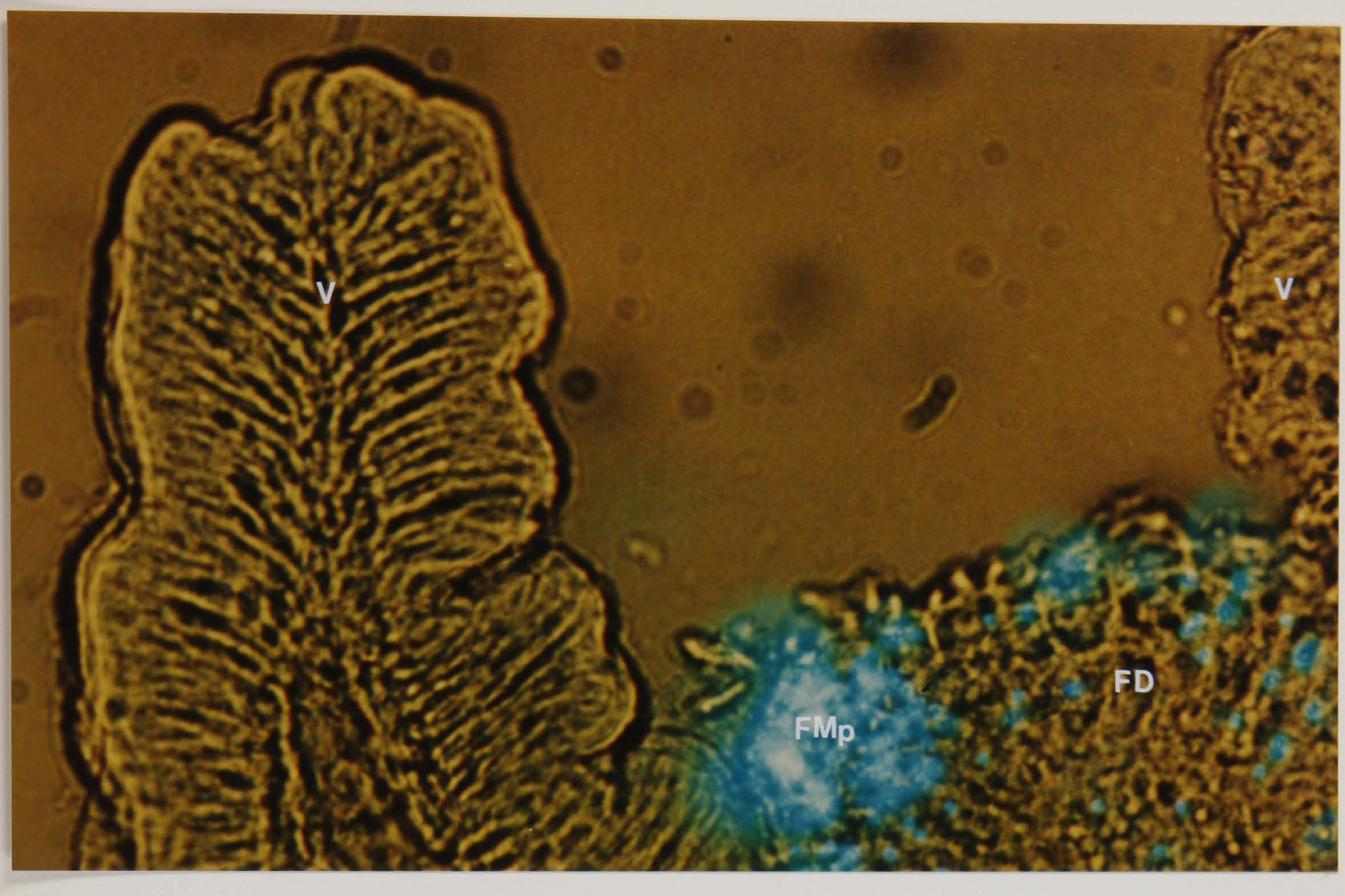


Figure 23

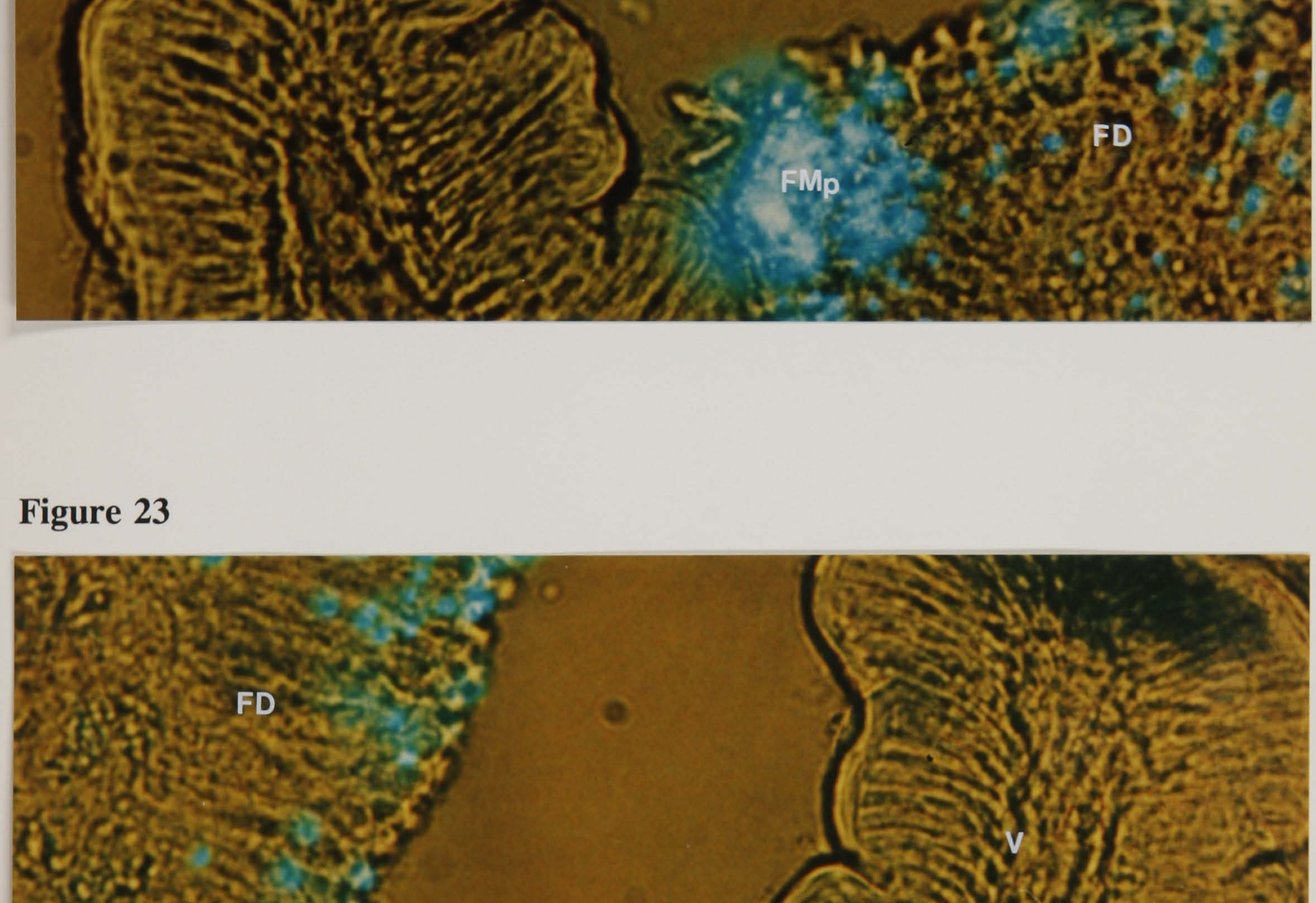


Figure 24

Photomicrograph of a follicle dome (FD) next to a villus (V) of a rabbit Peyer's patch. Fluorescent microparticles (0.94μm) are almost totally restricted to the follicle associated epithelium. Magnification x 100

Figure 25

Photomicrograph of a follicle dome (FD) of a rabbit Peyer's patch showing the presence of fluorescent microparticles (0.94μm). Magnification x 100

Figure 24

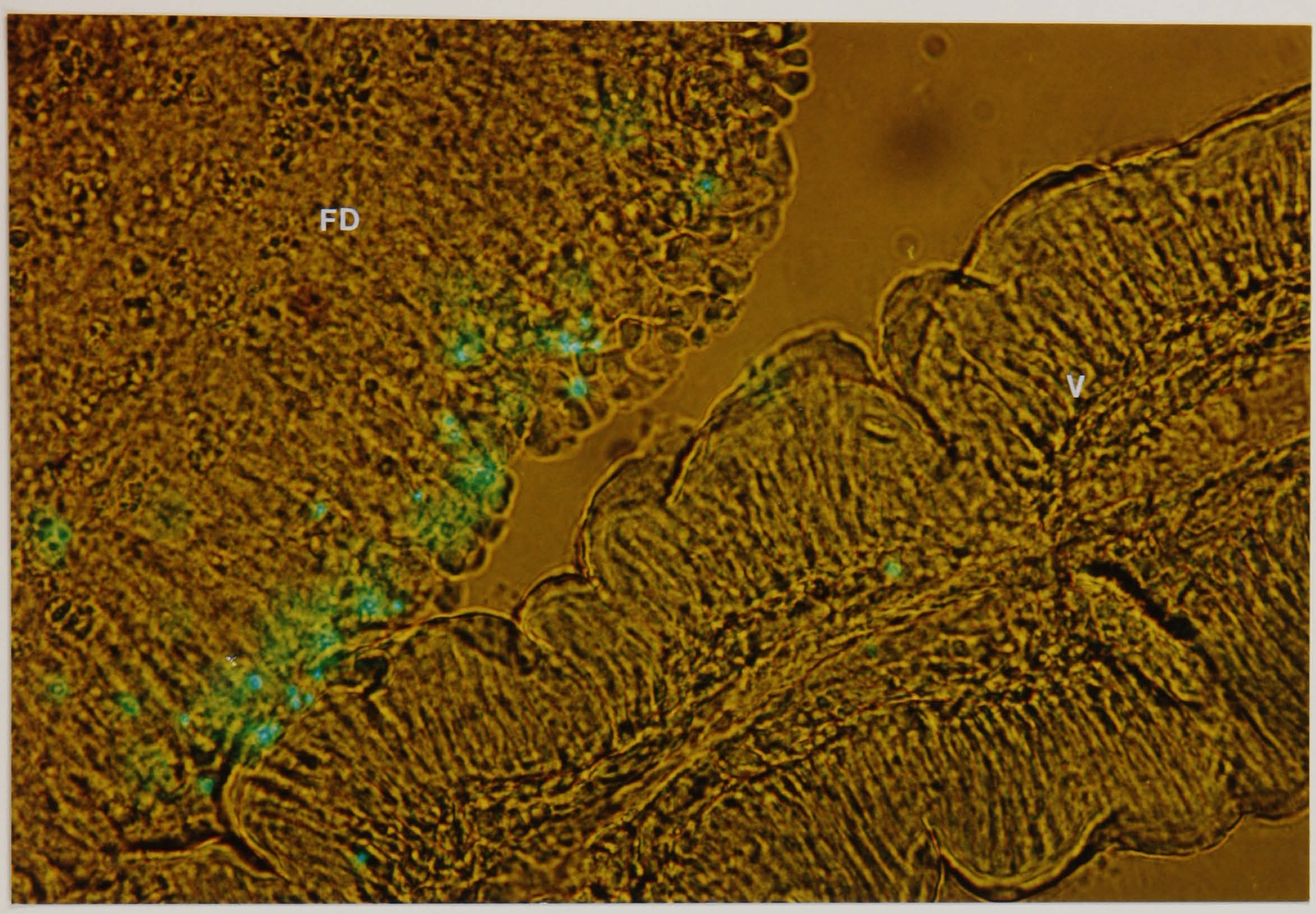


Figure 25

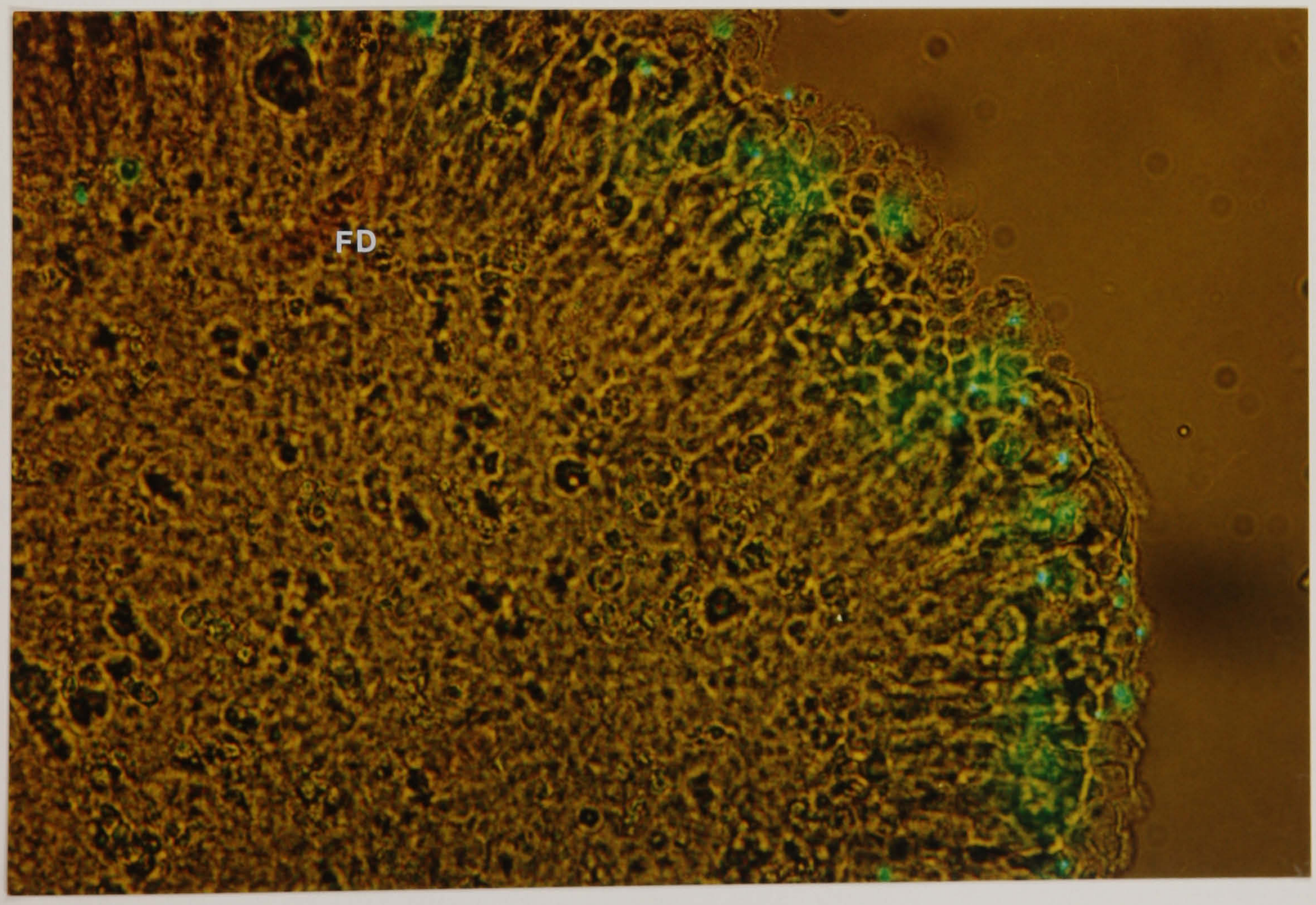


Figure 26

Photomicrograph of the serosal layer (S) of a follicle dome of a rabbit Peyer's patch showing the presence of fluorescent microparticles (FMp, 0.11μm). Magnification x 100

Figure 27

Photomimicrograph of the serosal layer of a follicle dome of a rabbit Peyer's patch showing the presence of fluorescent microparticles (FMp))(different area from shown in figure 26). Magnification x 250

Figure 26

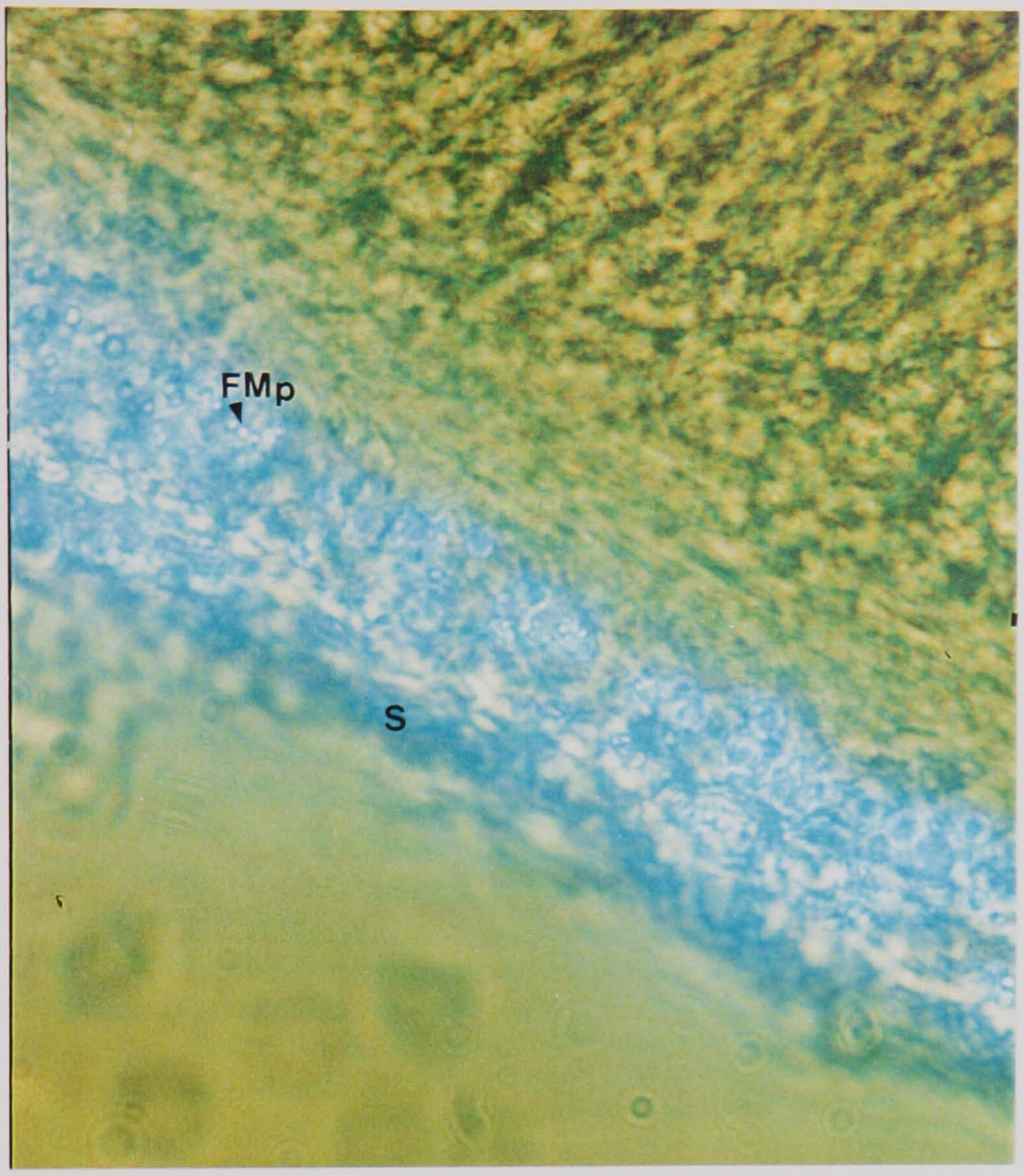


Figure 27

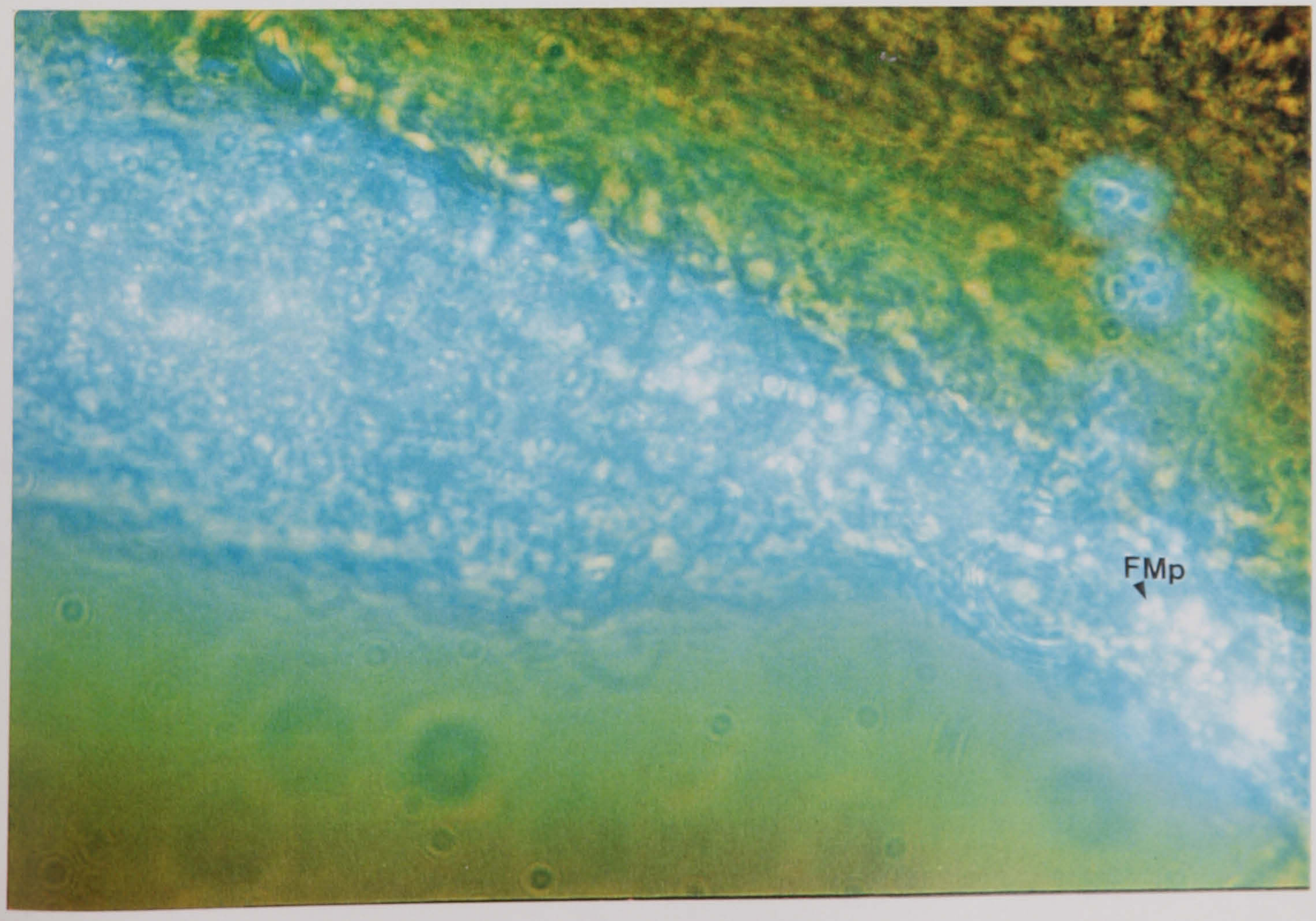


Figure 28

Photomicrograph showing villi (V) of a rabbit Peyer's patch. Also labelled: the serosal layer (S). Magnification x 250

Figure 29

Photomicrograph of the serosal layer in Figure 26. The fluorescent microparticles (FMp, 0.11μm) within this area are clearly visible. Magnification x 250

Figure 28

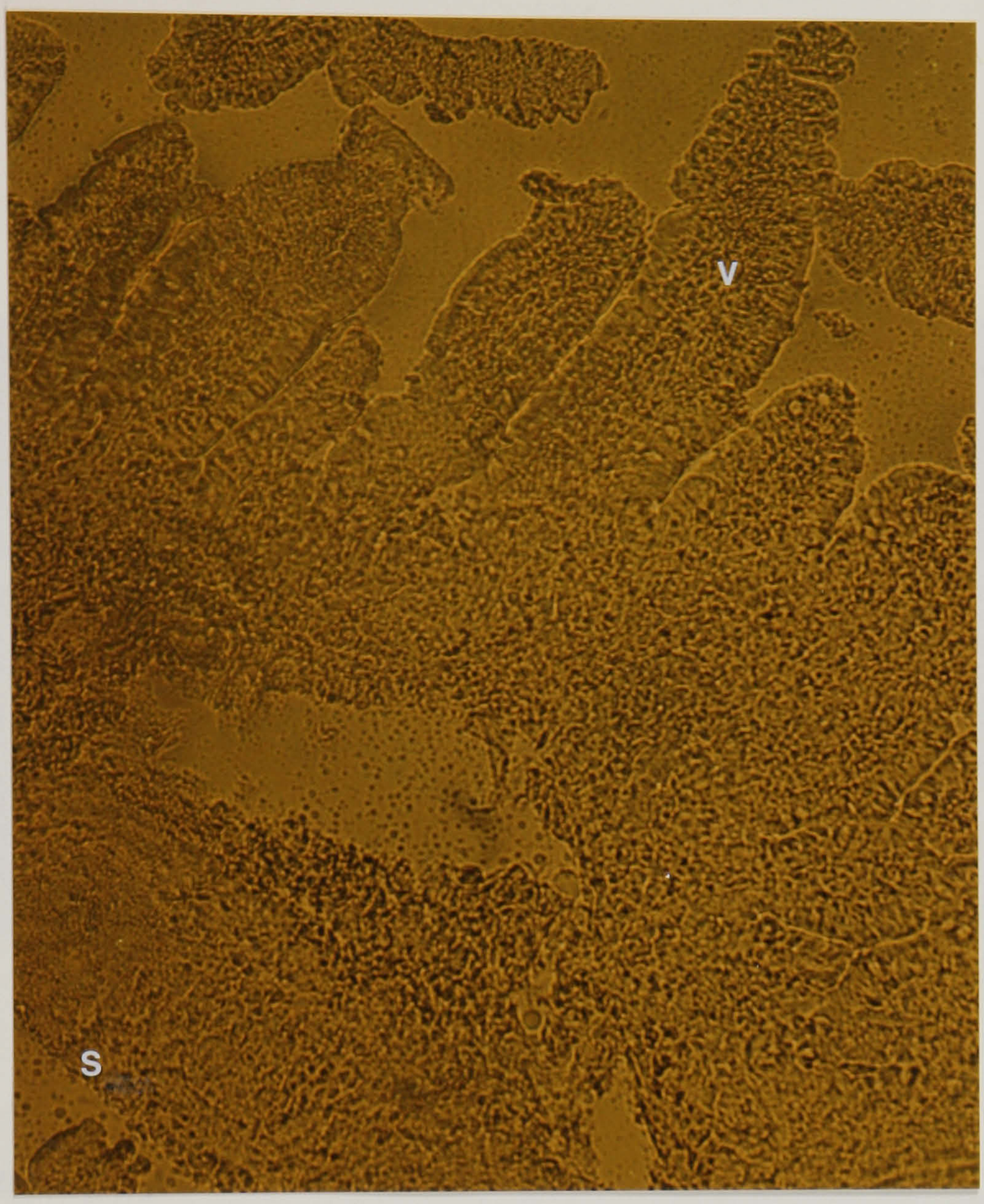
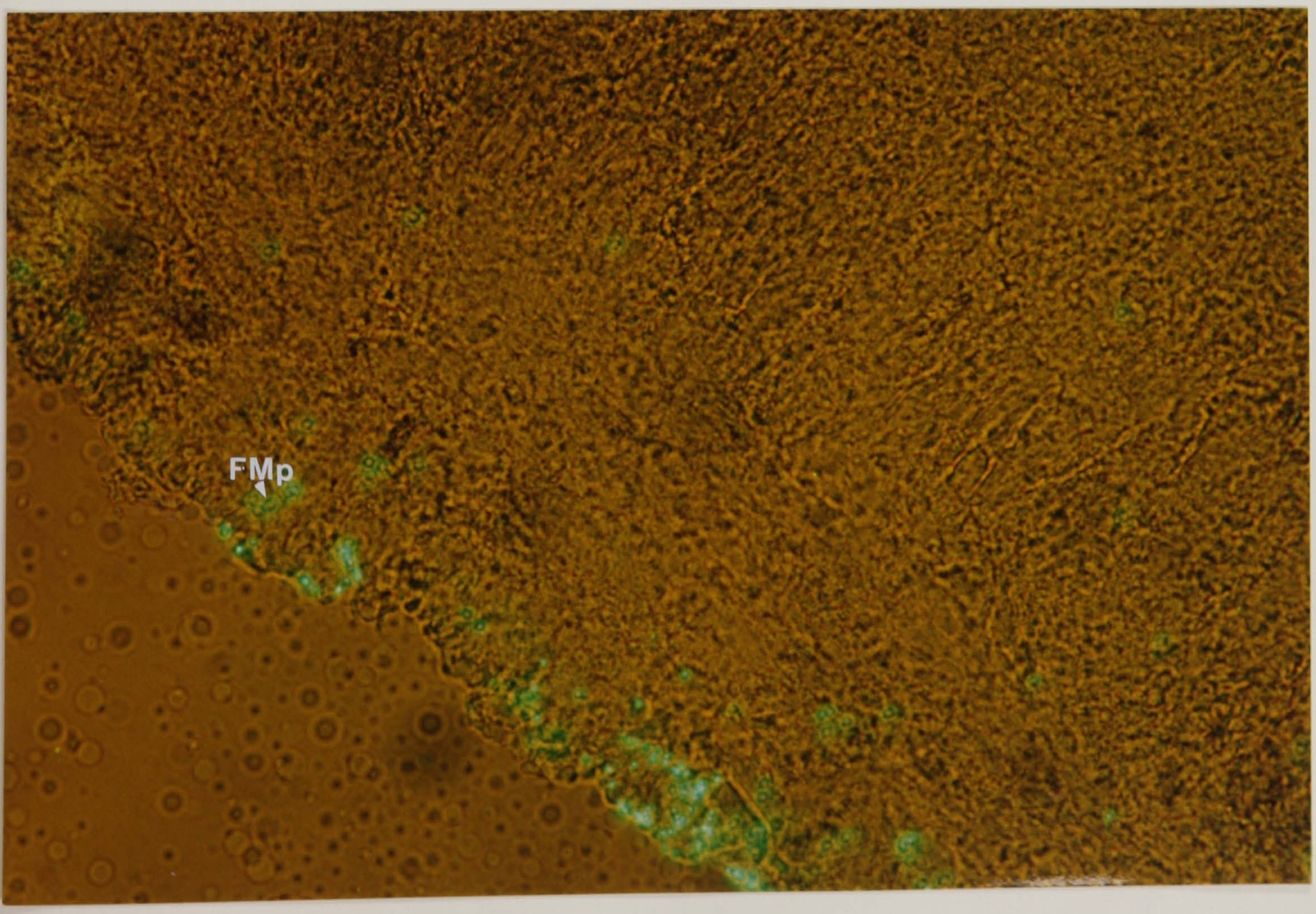


Figure 29



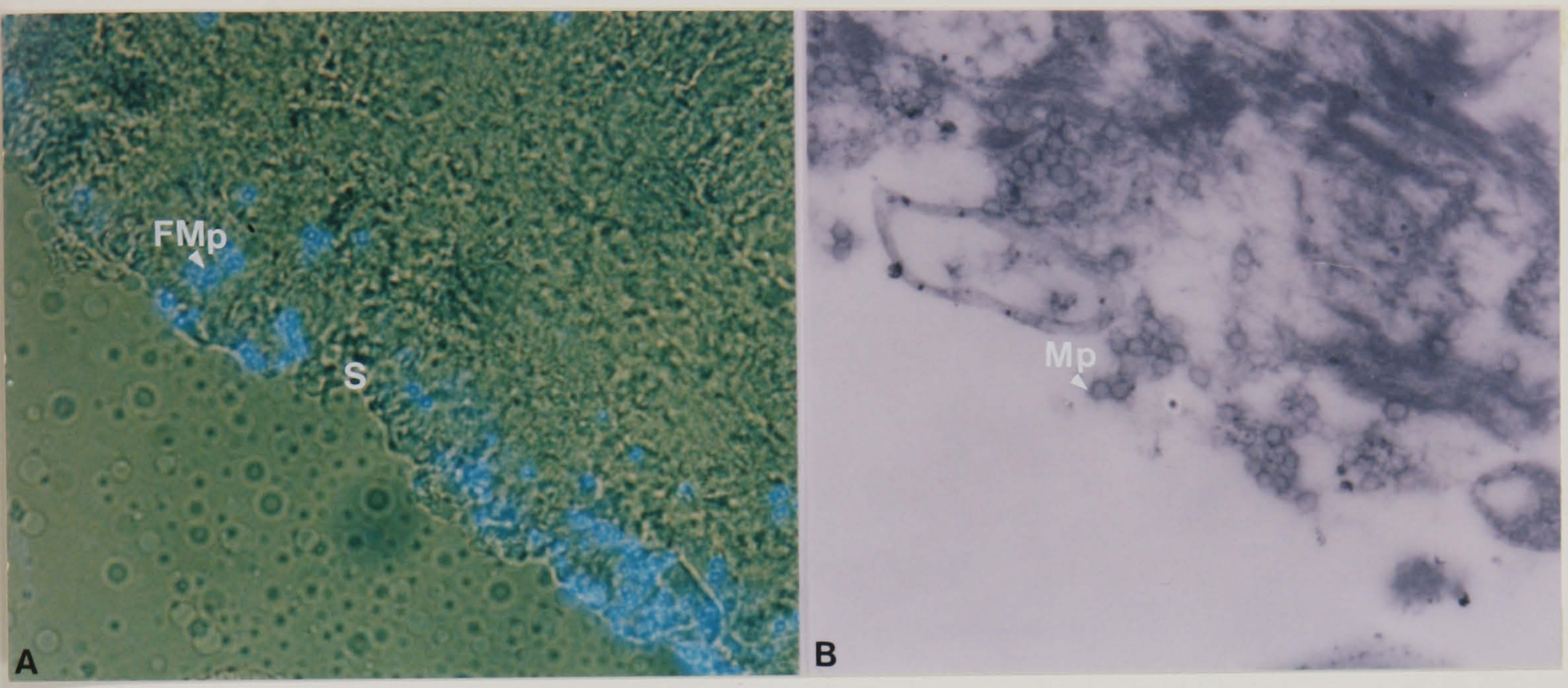


Figure 30

(A) Photomicrograph of the serosal layer (s) of a follicle dome of a rabbit Peyer's patch showing the presence of fluorescent microparticles (FMp, 0.11μm). Magnification x 100.(B) Electron micrograph of the serosal layer of the frozen tissue section shown in (A). Latex microparticles (Mp) are evident. Magnification x 14,000.

Figure 31

Photomicrograph of a rabbit Peyer's patch showing the follicle dome (FD) arrangement and the surrounding villi (V). Magnification x 25

Figure 32

Photomicrograph of the above structure in which the image has been turned down to emphasise the fluorescence emitted by the fluorescent microparticles (0.94μm). The fluorescence is almost totally restricted to the areas relating to the follicle domes. Magnification x 25

Figure 31

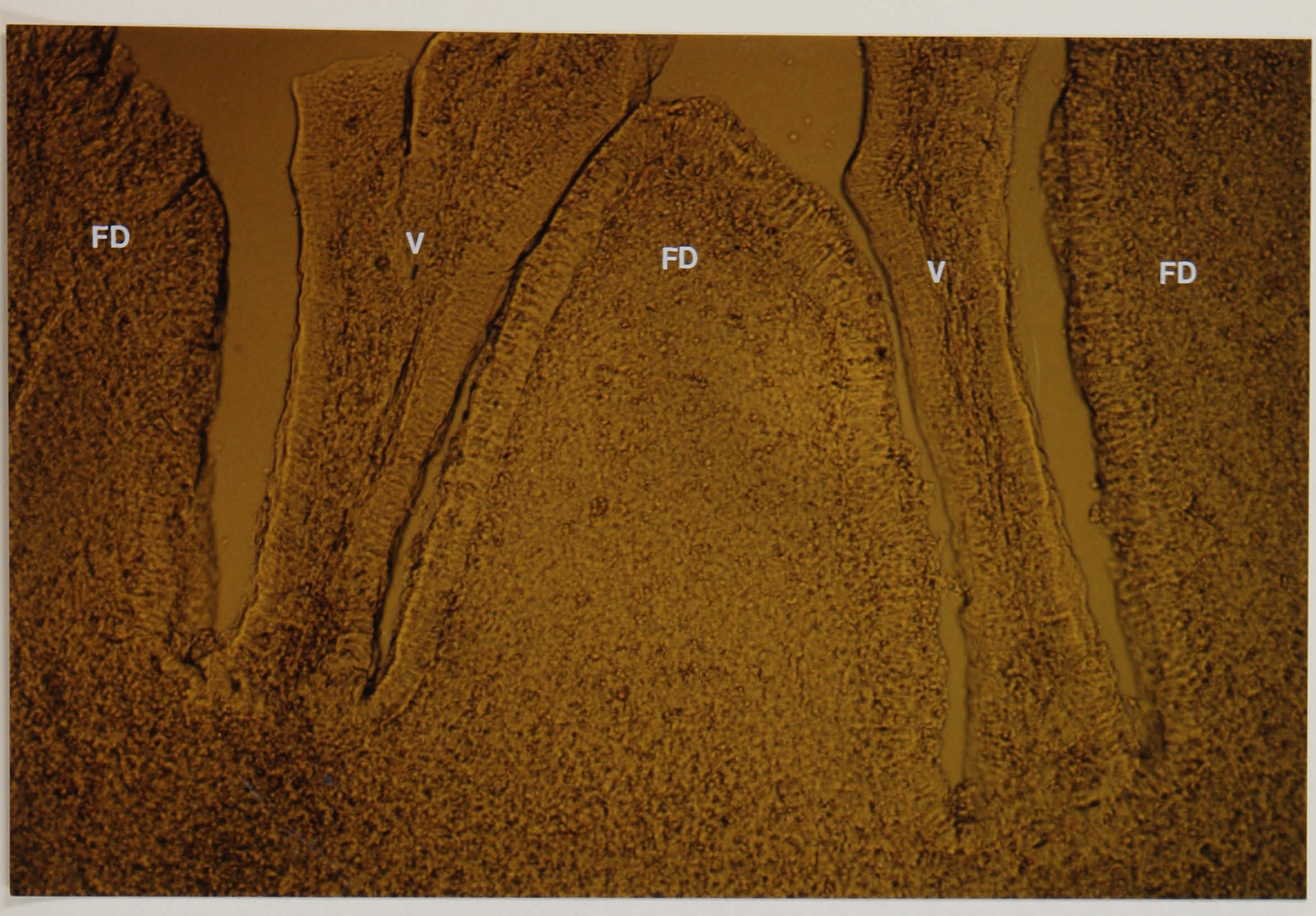
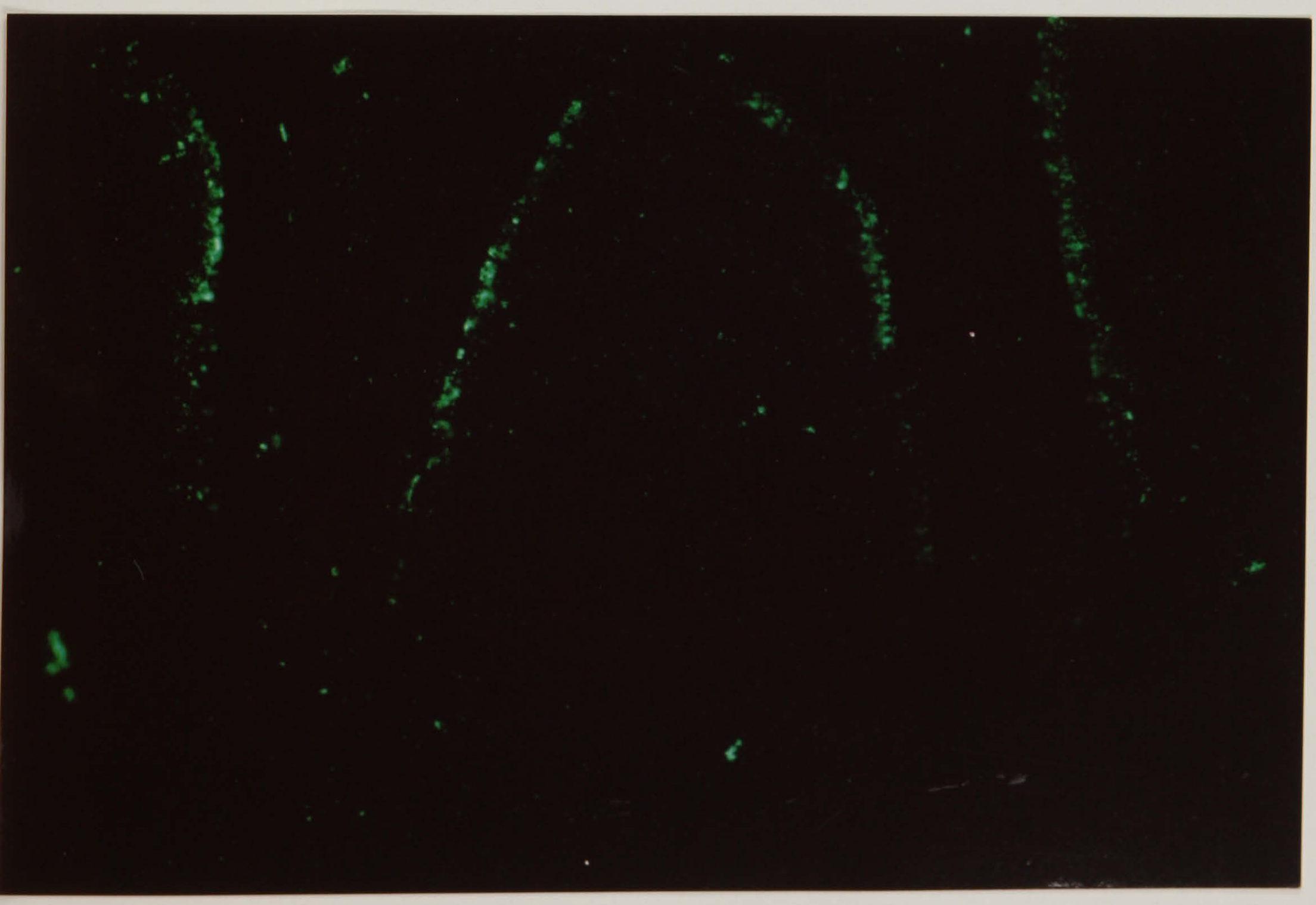


Figure 32



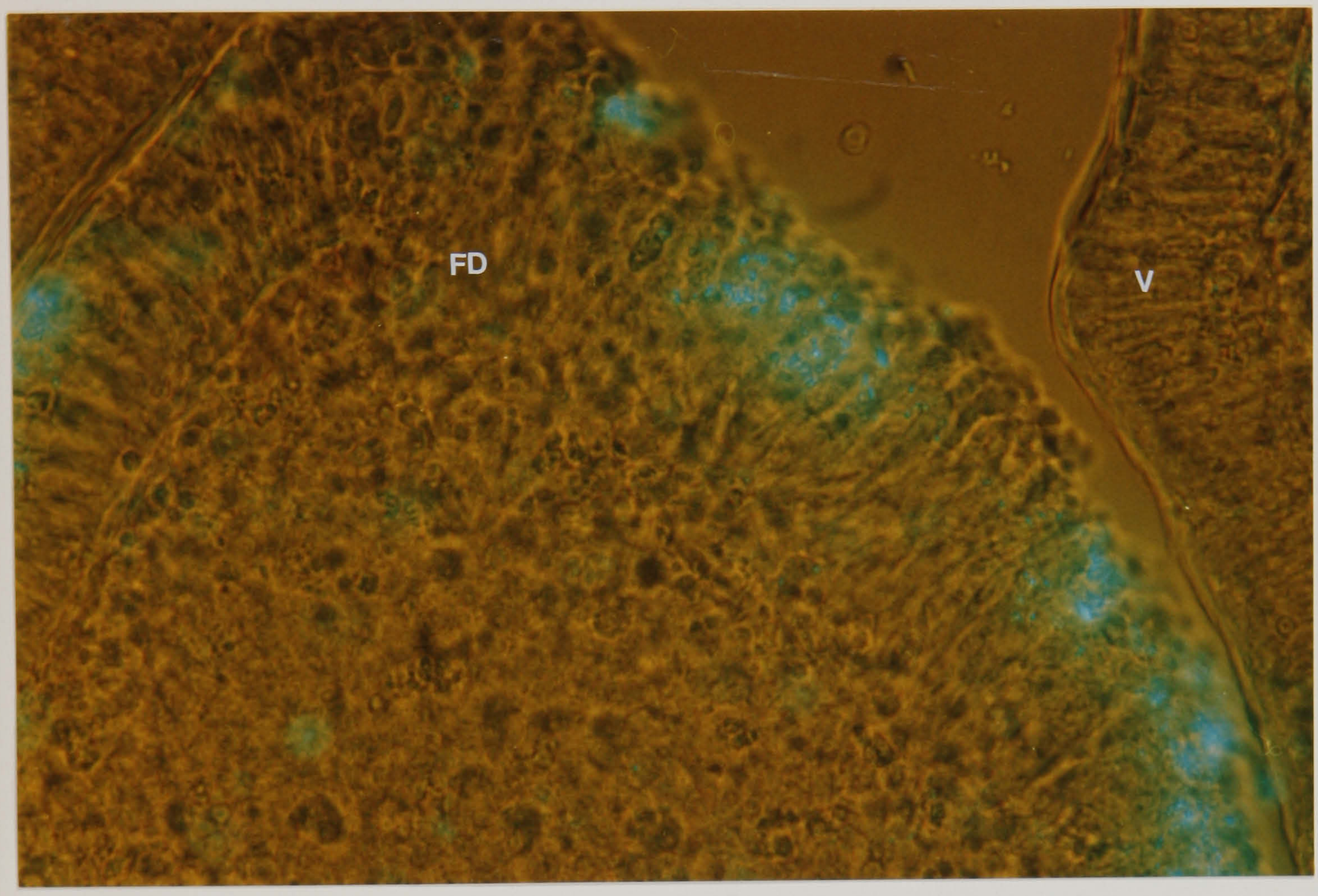


Figure 33

Photomicrograph of a follicle dome apex (FD) next to a villus (V) of a rabbit Peyer's patch. Fluorescent microparticles (0.94μm) are almost entirely present in the area relating to the follicle associated epithelium. Magnification x 250

the 0.093μm (yellow/green) and the 1.1μm (brilliant/blue) carboxylated microparticles were present at the serosa but were absent from the domes of the patch. Some luminal fluorescence was seen between the villi showing that luminal washing, prior to tissue harvest, was an inefficient method to ensure complete removal of all luminal contaminants. Frozen sections showed a mass of green and blue microparticles at the serosa but were absent from the domes. Some fluorescent sections were further processed for TEM (LR White resin) to investigate further if the microparticles were intracellular. Semithin sections (1μm) observed under the fluorescent microscope showed no evidence of microparticles.

2.4.2 **Chronic Feeding Studies**

Presentation of Fluorescent latex microparticles to rabbit Peyer's patches using a chronic feeding study

2.4.2.1 *Materials and Methods*

Two Netherland Dwarf rabbits were dosed orally with a 0.5 ml suspension of microparticles for 5 consecutive days. The same volume of PBS was administered to a control at the same time. On days 6 and 7, either no microparticles or PBS was administered. The animals were fasted overnight on the evening of day 7. On day 8, Peyer's patches, mesenteric lymph nodes and liver tissue was removed from each animal and immersion fixed (4% paraformaldehyde in 0.1 M phosphate buffer) overnight. The tissue was then transferred to 10% buffered sucrose solution. Frozen and cryostat sections were taken for fluorescence microscope analysis.

Table 7 Rabbit chronic feeding study

Animals	Microparticles	
Rabbit	Length of Feeding	
Group A	5 days	0.94 μm Fluoresbrite TM non-carboxylated
Group B	5 days	0.11 μm Fluoresbrite TM non-carboxylated
Group C	5 days	PBS
		Microparticles diluted 1 in 18 with PBS

2.4.2.2 *Rabbit Chronic Uptake Results*

Peyer's patches, mesenteric lymph nodes and liver from group A revealed no microparticles in any of the sections. A substantial amount of autofluorescence was present, especially in the liver samples but this was markedly different from the fluorescence signal emitted by the microparticles in previous experiments. Only 1 microparticle was observed in a liver section from a rabbit in group B, no microparticles were found in any of the Peyer's patch material nor within the mesenteric lymph node sections from this group.

2.4.3 Discussion of Microparticle Uptake in the Rabbit

In situ gut loops were performed on different Peyer's patches within the same animal to gain the maximum information from the least number of rabbits. In study 1, fine tissue preservation enabled intact M cells along the length of the FAE to be viewed under the TEM. Evidence of a phagocytotic mechanism of uptake was found at the site of an M cell; pseudopodia arising from the epithelium of an M cell were found surrounding microparticles at the epithelial surface. No such phenomenon was observed at the site of the normal enterocytes. This was a direct demonstration of the actual site and mechanism of microparticle uptake across the rabbit intestine. Structures thought to be microparticles were in the apical cytoplasm of the cell. In a comparable study using TEM, Fugimura (1986) reported a phagocytotic mechanism of particulate uptake in rabbits after in situ loop administration of mycobacteria (BCG). this study (Fugimura, 1986) highlights the problems encountered using TEM analysis; the electron micrographs clearly show extensions of the M cell epithelium surrounding the BCG outside the cell, but intracellular identification of the BCG is unconvincing. A phagocytotic mechanism of uptake of latex microparticles into rat Peyer's patches was described by Sass *et al.* (1990). Using scanning electron microscopy (SEM) microfolds on the surface of M cells were shown engulfing latex microparticles (0.5μm and 1.0μm) 10 minutes after administration into an *in situ* gut loop. This phenomenon was restricted to the M cells. A phagocytotic mechanism of uptake of latex microparticles (0.46μm) at the M cell surface was confirmed in rabbits by Jepson et al. (1993a) using SEM analysis. SEM enables a more rapid scan of the total Peyer's patch but has the disadvantage of not showing intracellular mechanisms.

In studies 2 and 3 two different strains of rabbit were used, the Netherland Dwarf (study 2) and the New Zealand White (study 3) to investigate fluorescent microparticle uptake after acute delivery. In both strains the 0.94μm microparticles were present along the apices of the follicle domes and the 0.11μm microparticles along the total length of the serosa from frozen sections. The localisation of the microparticles in the follicle domes and the absence in the villous regions indicates that this phenomenon was not due to luminal fluorescence swept over the tissue by the cutting action of the microtome knife. The frozen sections observed in these studies were 40μm, therefore, it was difficult to determine if the microparticles observed were intracellular or adhering to the surface of the follicle domes. Using the focus of the microscope, they did appear to lie in the plane of the section, indicating uptake.

Even if the microparticles observed at the apices of the follicle domes were due to adherence rather than uptake, it remains a significant finding. It suggests evidence for receptor mediated endocytosis into Peyer's patches where initial binding to the apical surface is a prelude to uptake. There were very few microparticles found transversing the dome; this may indicate that uptake occurs in synchronous waves, after the completion of which the process of uptake starts again. This process seems similar to the one described in rabbits by Pappo and Ermak (1989).

The presence of 0.11μm in the serosa underlying neighbouring villi seemed a curious finding, although, it agrees with those of LeFevre *et al.* (1978), who reported the transport of latex microparticles (2μm) to villi adjacent to the domes after uptake into the Peyer's patches of mice. TEM processing of frozen sections (0.11μm

microparticles administered) carried out to distinguish if the fluorescence observed at the serosa was intracellular or a string of luminal fluorescence adhering to the serosal layer was inconclusive due to the poor preservation of tissue after TEM, but microparticles were certainly present within the area relating to the serosa.

The results presented in this thesis indicate that uptake across the FAE and through the patch is an extremely rapid process, In study 2 and 3 the microparticles were left in the loop 40 minutes and 15 minutes respectively. Pappo and Ermak (1989) reported the uptake of fluorescent microparticles (600-750nm) across the FAE of rabbits within 10 minutes. Similar rates were reported by Jepson *et al.* (1993a) in rabbits and in a study by Sass et al. (1990) latex microparticles were found in the basal regions of the Peyer's patches and lymphatic capillaries within 10 minutes in rats.

Study 4 was conducted to investigate the effect of microparticulate charge on uptake. LeFevre *et al.* (1978), Herzog *et al.* (1983) and Jani *et al.* (1989) reported that chemical solvents (xylene, propylene oxide and ethanol respectively) may dissolve latex, therefore, to eliminate completely its possibility, half of the Peyer's patch material taken from study 4, was immediately placed in liquid nitrogen and cryostat sections were taken. The same extent of microparticle uptake was observed. Both the 0.093μm and 1.1μm carboxylated latex microparticles were found at the serosal layer of the Peyer's patches. The microparticles were virtually absent from the dome areas. This contrasts with the findings of study 2 and 3, where 0.94μm non-carboxylated microparticles were restricted to the dome. This indicates that charge is a factor in microparticle uptake. Jani *et al.* (1989) reported that carboxylated microparticles were taken into rat Peyer's patches to a lesser extent than non-carboxylated. In studies

presented in this thesis, it seems that carboxylated microparticles are taken into rabbit patches and transported to the serosal in greater numbers, although, the results are not unequivocal because of the difficulty in determining if the microparticles found at the serosa were the result of uptake through the patch or contamination by luminal overflow when the Peyer's patch was removed from the intestine. Some frozen sections were further processed for TEM. LR White embedding medium was used as this avoids the use of propylene oxide. Preservation in the LR White tissue was not as good as the epoxy resin as previously; the tissue easily fragmented when cutting ultrathins. Semithins viewed under the fluorescent microscope showed no presence of microparticles. This may be a limitation in the technique (poor preservation of tissue) rather than lack of uptake.

The lack of microparticles found in the rabbit chronic feeding study was surprising; only one, 0.11μm microparticle was found in a liver section. Peyer's patches, mesenteric lymph node and liver tissue were extensively analysed for the presence of microparticles. The lack of microparticles may have been a result of the short feeding regime, namely 5 days. The possibility also exists that the microparticles were not reaching the patches in the numbers required for uptake, the majority being flushed out with the luminal contents.

2.5 GENERAL DISCUSSION OF MICROPARTICLE UPTAKE IN THE RABBIT AND RAT

Considering the results gained from the two animal models, more evidence of microparticle uptake was achieved in the rabbit. This may be a result of the amount of Peyer's patch tissue in the two species. The size of the patch in rabbits may reach $10mm^2$ whilst that of the rat measures approximately $5mm^2$, therefore, considerably more follicle-associated epithelium is available in the rabbit. Under the light microscope, the arrangement of the follicles differ. In the rabbit the lymphoid follicles consist of a multiple array of domes whilst in the rat one dome is characteristic. The number of M cells in the FAE of rabbit is far greater than in the rat. Pappo (1988), reported over 50% of the FAE of the rabbit being made up of M cells; in the rat, only about 5 - 10% of the FAE consists of M cells (Smith *et al.* 1980). If M cells are the site for microparticulate uptake, and our results would suggest this, then a greater uptake will occur in rabbits. The appearance of pseudopodia above intracellular microparticles in both species indicates a phagocytotic mechanism of uptake common to both species. The main problem using TEM for analysis of latex microparticles is the similarity between the particles and structures already present within the cell. For this reason, the mechanism of microparticle transit through the cells could not be fully determined.

CHAPTER 3

ELECTRON DENSE MARKERS FOR THE IDENTIFICATION OF MICROPARTICLE UPTAKE USING TEM ANALYSIS

3.1 INTRODUCTION

When an electron beam is fired at a heavy metallic element in a transmission electron microscope, the electrons are deflected or stopped due to the electron dense cloud surrounding the metal. This phenomenon causes the heavy metal to appear dark when viewed under the TEM. This has been exploited for use TEM staining. Particulate gold is an electron dense material which has been used extensively in TEM as a cytochemical marker (Beesley 1985, Herzog and Farquhar 1983). Latex is electron lucid making its identification in tissue difficult under TEM, with an appearance similar to structures in the cell. This was the major difficulty when using latex microparticles to characterise uptake across lymphoid tissue (refer to Chapter 2). To trace clearly microparticle uptake through the cells of Peyer's patches an electron dense particulate or marker was required. For this reason particulate gold was used in uptake studies performed in rabbits.

Two strategies were employed; either (a) native colloidal gold particulates 100-150nm or (b) colloidal gold (10nm) labelled latex microparticles.

3.2 MATERIALS AND METHODS

Microparticles

Polybead amino microspheres 0.50μm (2.5% solids-latex) (Polyscience Laboratories).

Covospheres FX fluorescent microparticles 0.5μm (Duke Laboratories UK).

Biotinylation of latex microparticles (Hnatowich et al. method, 1987)

25 mg of amino latex microspheres was resuspended in 1 ml of bicarbonate buffer (50mM, p.H. 8.5). 0.4 mg NHS-LC-Biotin (Pierce, USA) was added and placed on ice for 2 hours. To remove unwanted biotin the mixture was centrifuged at 1000g for 15-30 minutes. After centrifugation the sample was diluted with 0.1M phosphate buffer p.H. 7.0. This was repeated two more times.

Biotinylation of latex microparticles (Sytowski method, 1990)

Briefly, 100 μl of Covosphere microparticles were washed twice with phosphate buffered saline (PBS pH 7.2), centrifuged at 10,000 g for 6 minutes and sonicated for 5 minutes. NHS-LC-biotin (Pierce USA) (750 μl in PBS) was added to the microparticles and the mixture was gently rotated for 60 minutes at room temperature. The biotinylated microparticles were then washed 3 times with PBS and dialysed against PBS overnight to remove excess NHS-LC-biotin.

Conjugation of streptavidin-gold (10nm) to biotinylated microparticles

30ul of biotinylated microparticles was added to 30ul of streptavidin-gold (diluted 1 in 100 with dilution buffer) and left 1 hour at room temperature. This was spun at

10,000 R.P.M. for 5 minutes. The pellet was resuspended in 30ul PBS. This was repeated 3 times. The pellet was finally resuspended in 30ul of PBS.

Anti-biotin-Colloidal gold labelling of biotinylated microparticles

To label the biotinylated microparticles with colloidal gold, the method of Sytowski (1990) was used. The biotinylated microparticles were sonicated for 10 minutes, 10 μl of anti-biotin-colloidal gold (Biorad)(10 nm) was then added to 100 μl of the microparticles and rotated gently for 25 minutes at room temperature. The preparation was centrifuged at 10,000 rpm for 6 minutes, the supernatant was removed and the microparticle pellet was resuspended in 200 μl of PBS (pH 7.2).

3.3 COLLOIDAL GOLD PARTICULATES

3.3.1 Preparation

Colloidal gold particulates were prepared using a method adapted from Frens (1973). The objective was to prepare gold particulates large enough (100-150nm) to use as an electron dense microparticulate in uptake studies. The method involved two stages (1) the formation of a gold sol by the chemical reduction of gold chloride by sodium citrate and (2) stabilisation of the gold sol by protein adsorption.

Protocol for the preparation of gold particles 100-150nm in size:

(a) 50 mls of gold chloride ($AuCl_4$, 0.01% by weight) was heated to 60°C (boiling point).

(b) 0.16 mls of sodium citrate (Nacitrate, 1% by weight) was added to the gold chloride, a reflux condenser applied and the mixture kept boiling for 30-40 minutes (the colour of the mixture at this stage was red/pink which indicated the completion of gold particulate formation)

(c) The gold sol is allowed to cool.

(d) 1 ml of PEG 20,000 was added to 40 mls of the gold sol (1% v/v) and the mixture centrifuged at 10,000rpm for 10 minutes (or until there is no colour in the supernatant).

(e) The supernant is discarded and the pellet resuspended in 1 ml of phosphate buffer (10mM p.H.7.2).

3.3.1.1 *Results*

The gold particulates were placed on a coated grid and observed under the transmission electron microscope (refer to Figure 34-35). In accordance with the method described by Frens (1973) large particulates up to 100nm were produced by reducing the volume of sodium citrate. The particulates were polydispersed with respect to size, ranging from 30-100nm, and very electron dense. The gold sol must be stabilised in order to avoid flocculation when electrolytes are added, this is maintained by electrostatic attractions between the gold and proteins adsorbed to their surface to give a gold-protein complex (Park 1989). In this experiment PEG (20,000) was used. If the gold is not stabilised the colour changes from a claret red to blue showing flocculation has taken place. These particulates were used to investigate particulate uptake into Peyer's patches.

Figure 34

Electron micrograph of a colloidal gold particulate. Magnification x 219,394

Figure 35

Electron micrograph of colloidal gold particulates. Magnification x 127,980

Figure 34

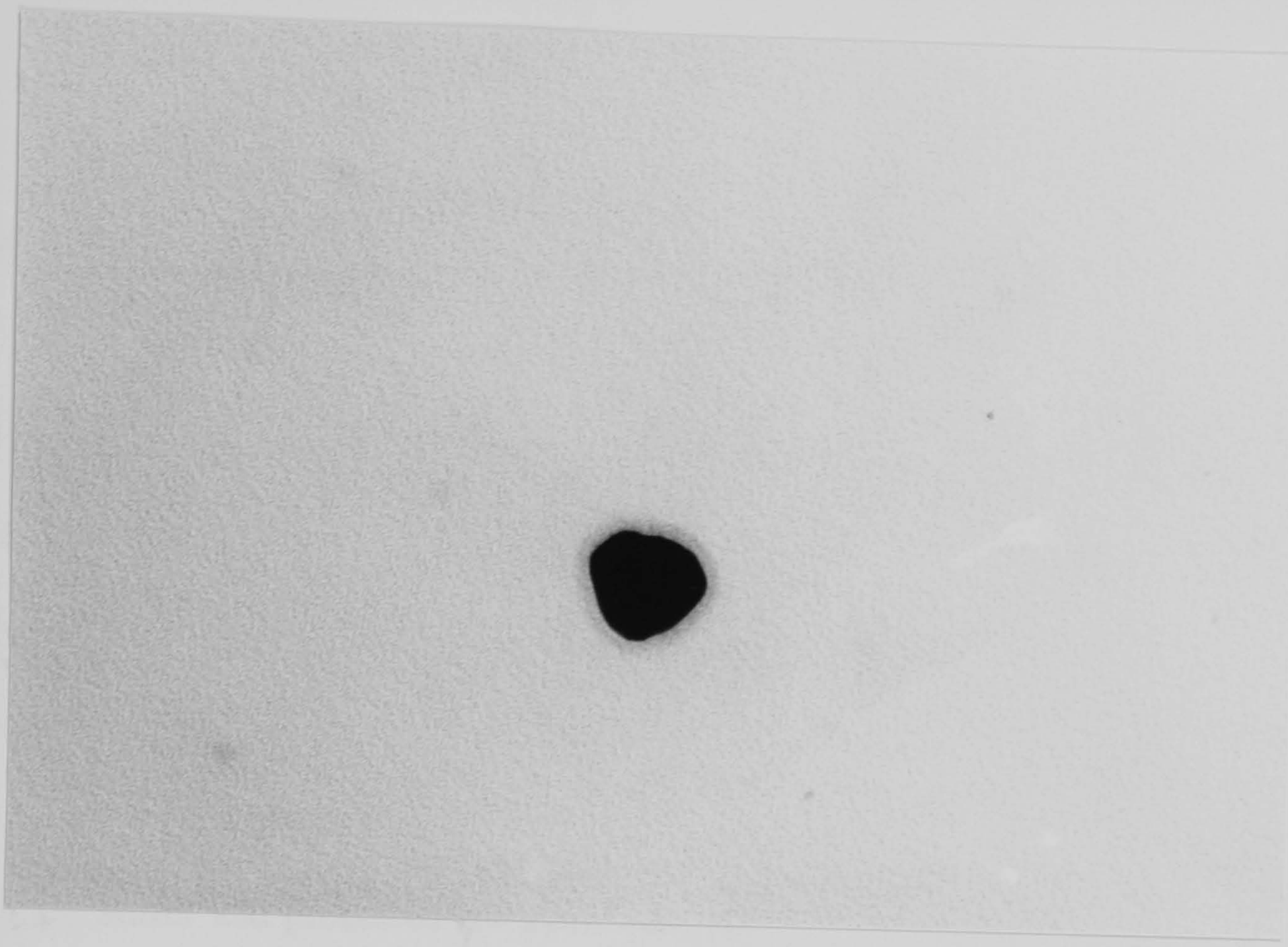
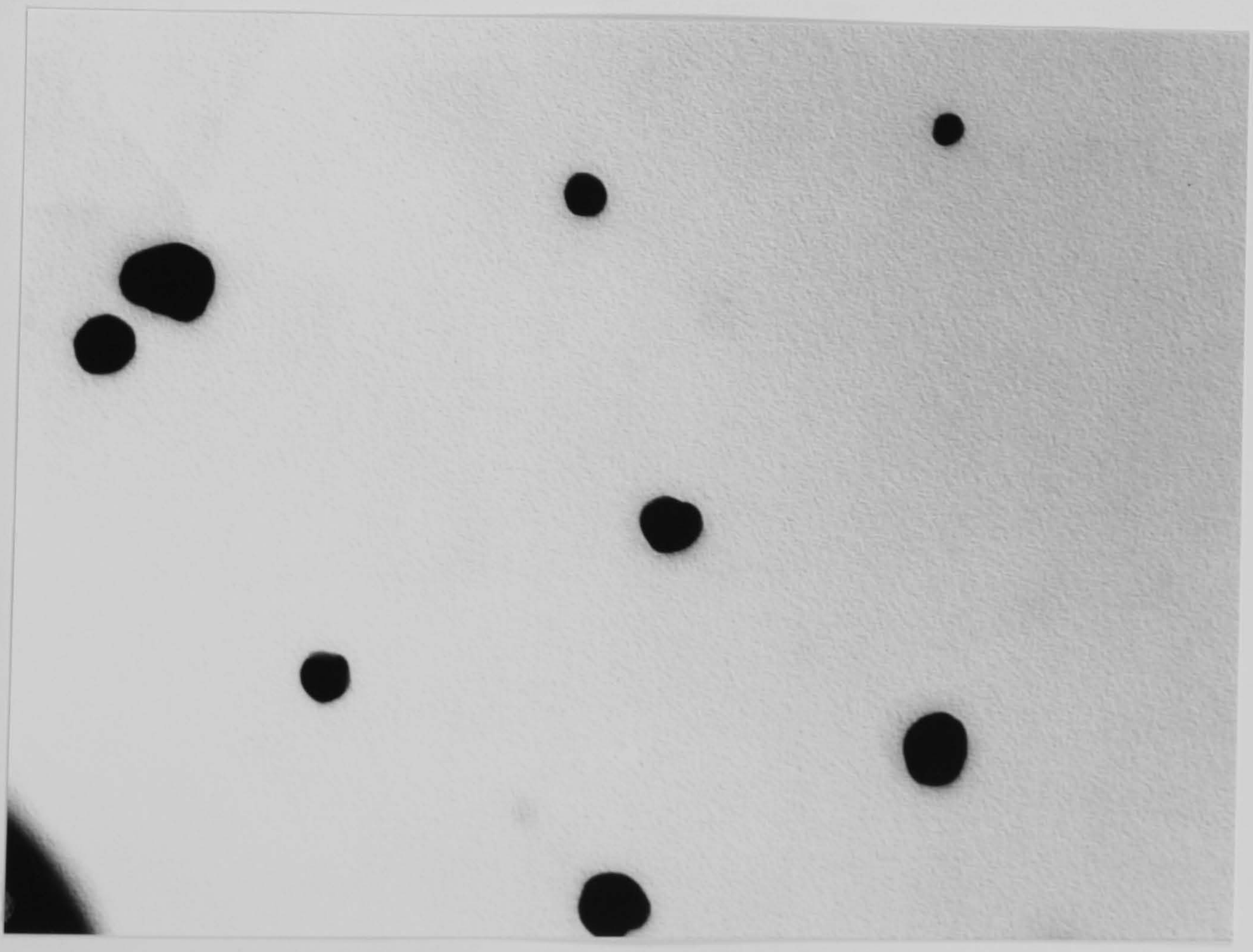


Figure 35



3.3.2 Presentation of Colloidal Gold Particulates to Rabbit Peyer's Patches via Closed Intestinal Loops

3.3.2.1 *Materials and Methods*

Male New Zealand White rabbits were fasted overnight. They were anaesthetised and *in situ* intestinal loops formed around the Peyer's patches. The gold particulates were injected into the loop and left for 30 minutes. In rabbit 2, the lumen of the gut was rinsed with 1% bovine serum albumin prior to the injection of the gold. The Peyer's patches were then removed and immersion fixed in 3% gluteraldehyde in 0.1 M phosphate buffer and processed for TEM analysis.

Table 8 Colloidal gold particulate uptake studies

Animals	Microparticulates		
Rabbit NZW	Dose	Time in loop	
STUDY 1	1.0 ml	30 min	Colloidal gold particulates stabilised with PEG 20 000 Resuspended in PBS
STUDY 2	1.0 ml	30 min	Colloidal gold particulates stabilised with 1% bovine serum albumin Resuspended in PBS

3.3.2.2 *Results of Colloidal Gold Uptake*

The structure of the Peyer's patches from both studies showed good preservation when viewed under the TEM. Ultrathin sections of M cells were isolated and viewed under the TEM. No evidence of colloidal gold particulates inside the cells was found. Luminal colloidal gold was, however, evident at the luminal surface (refer to Figure 36-37).

The problem with the colloidal gold particulate was that of polydispersity with respect to size. The range was 30-100nm, therefore, difficulties were encountered in clearly identifying the particulate even though they were electron dense under the TEM. A more monodispersed population would have been more advantageous for identification purposes.

In study 2, the rational behind stabilising the gold particles with bovine serum albumin was to enhance uptake by making the particulate more antigenic. Other workers have documented that antigenicity can enhance uptake. Rinsing the lumen with BSA before particulate administration was to prime the M cells antigenically, however, there was no difference between the evidence gained from study 1 and 2, although, the reason may be the small size and administered dose of the particulate. For exact identification, a monodispersed electron dense particulate was required. It was decided to combine the electron density exhibited by colloidal gold and the monodispersity shown by latex microparticles, by labelling the surface of latex microparticle with colloidal gold.

Figure 36

Electron micrograph showing a colloidal gold particulate (CGP) on the luminal surface of an enterocyte within the follicle associated epithelium of a rabbit Peyer's patch. Magnification x 12,852

Figure 37

High power of the enterocyte shown in figure 36. The colloidal gold particulate (CGP) is clearly visable. Magnification x 54,978

Figure 36

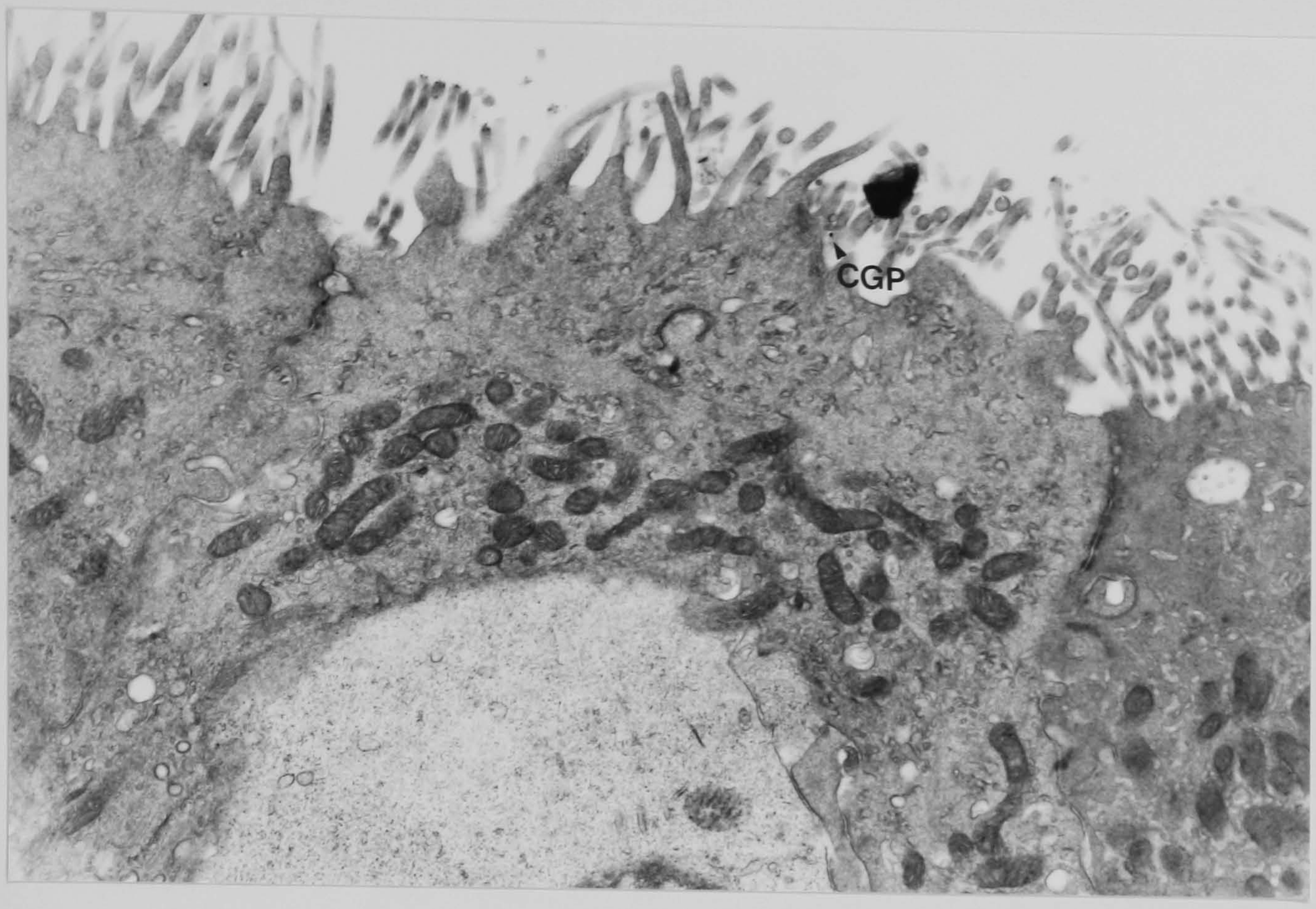


Figure 37

3.4 COLLOIDAL GOLD LABELLING OF LATEX MICROPARTICLES

3.4.1 Preparation

(a) Hnatowich *et al*. method.

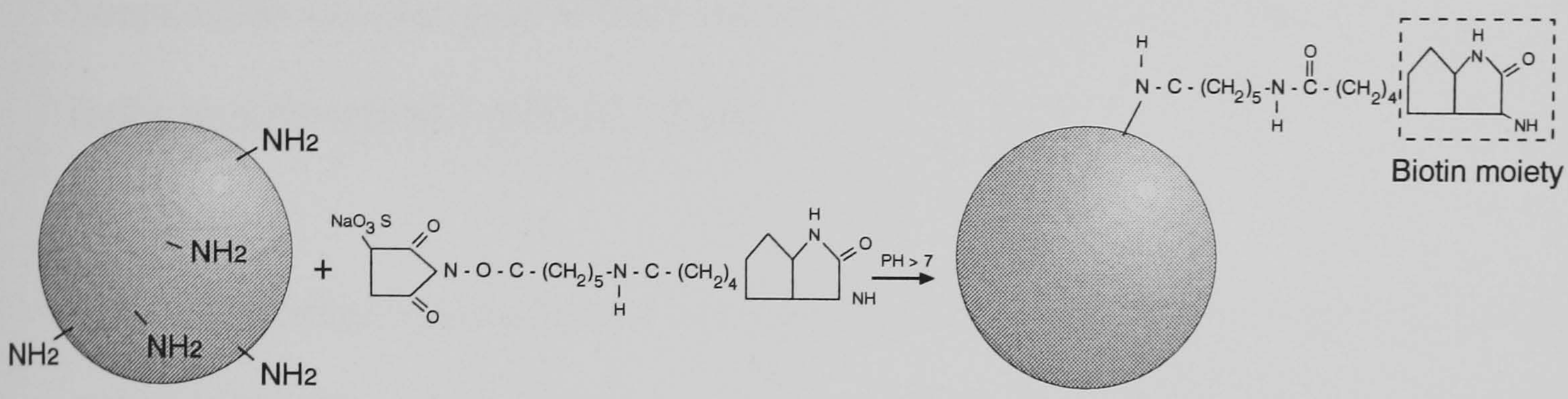
The initial approach used was a two step method using the avidin (streptavidin) - biotin system:

i) Conjugation of biotin to the surface of latex amino microparticles (0.50μm) (modified from that of Hnatowich et al. (1987) for biotinylating immunoglobulins). A substitution reaction occurs between the amine groups on the microparticles and the NHS-LC-biotin ester to link biotin through an amide bond to the surface of the microparticles and ii) formation of a complex between biotin (on the surface of the microparticles) and streptavidin (conjugated to a colloidal-gold probe, 10nm) to label the surface of the microparticles with colloidal gold. (refer to Figure 38).

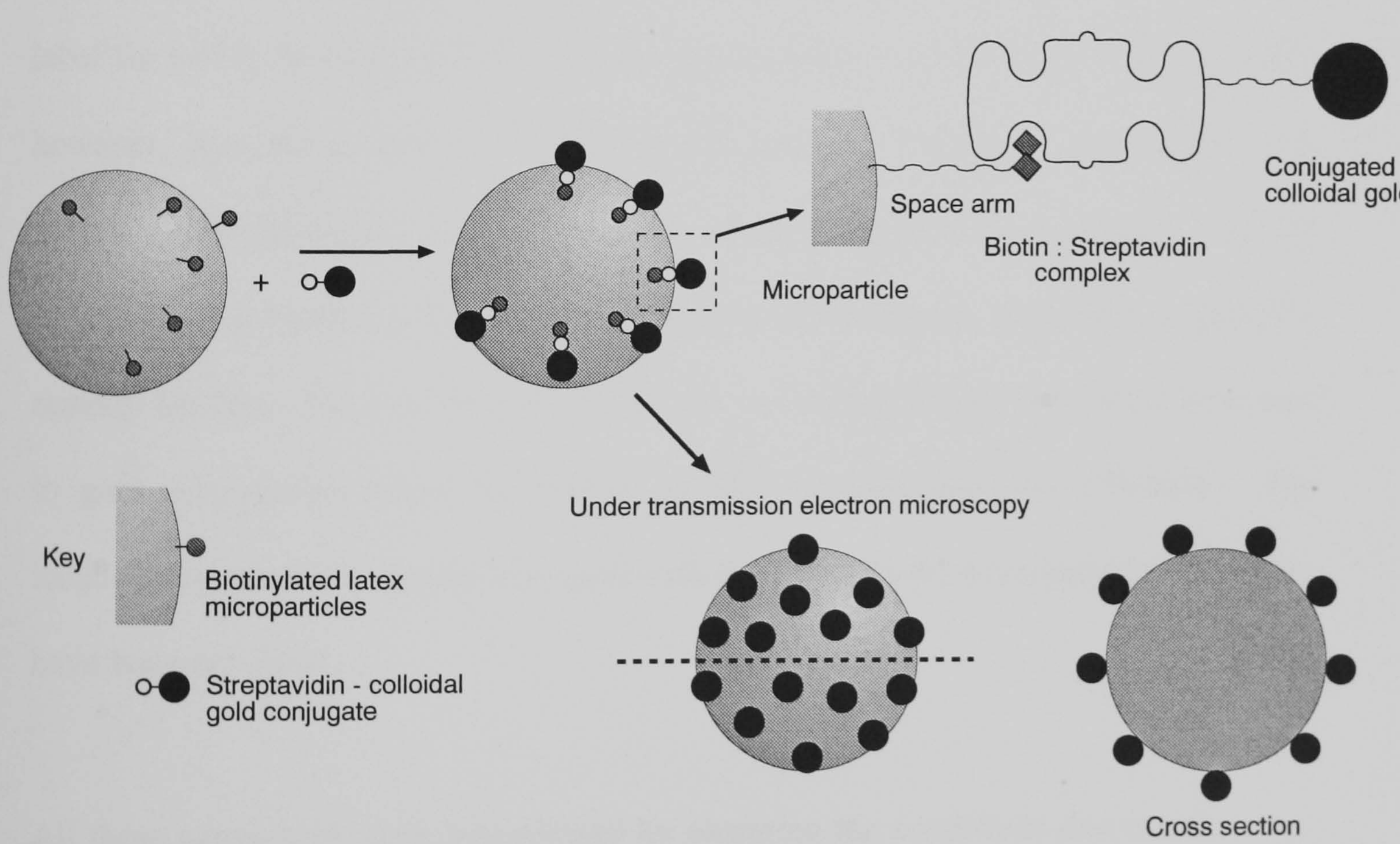
(for full details refer to materials and methods section)

Viewed under the TEM, there appeared to be no gold attached to the latex microparticles (refer to Figure 39). This may have been due to a low level of biotin conjugated to the microparticles. Little information concerning the amino groups on the microparticles were disclosed by Polyscience, therefore there may have been a lack of available amino groups on the microparticles for adequate biotinylation. For this reason a different method of biotinylating latex microparticles was used;

Figure 38 Colloidal gold labelling of latex microparticles using the Streptavidin/Biotin interaction



Step 1. Biotinylation of Latex microparticles by reaction with NHS - LC - Biotin



Step 2. Gold labelling of biotinylated microparticles via the streptavidin - biotin complex

(b) *Sytowski method.*

Adapting the method of Sytowski (1990), biotin was conjugated via amino groups on the surface of fluorescent latex microparticles (0.5μm, obtained from Duke Scientific) using NHS-LC-Biotin.

Streptavidin-colloidal gold (10nm) conjugation was carried out as before. (refer to materials and methods section).

The gold labelled microparticles were placed on a coated grid and viewed under the TEM. Colloidal gold (10nm) was clearly attached to the latex microparticles (refer to Figure 40). These microparticles also emitted a strong fluorescence when viewed under the fluorescence microscope, therefore, confirming the availability of a double label for use in fluorescence and electron microscopy. The amount of colloidal gold, however, was not as much as expected. The density of the gold was fainter than before; an indication that the reagents were losing their potency in storage. The use of antibiotin-colloidal gold instead of streptavidin-colloidal gold was a possible remedy for this. The low amount of gold on the microparticles may have been due to gold conjugation when suspending in PBS of high molarity (500mM). The incubation time of the streptavidin-gold with the biotinylated microparticles may also have been too short.

All these parameters were investigated by changing the conditions described;

(a) Fluorescent latex Covasphere + anti-biotin gold (10nm, washed and resuspended in 500mM PBS)

(b) Fluorescent latex Covasphere + anti-biotin gold (10nm, washed and resuspended in 10mM PBS)

Figure 39

Electron micrograph showing latex microparticles (unsectioned) with no colloidal gold label (Hnatowich method). Magnification x 18,650

Figure 40

Electron micrograph showing latex microparticles (unsectioned) with colloidal gold (CG, 10nm) associated with the surface (Sytowski method). Magnification x 88,430

Figure 39

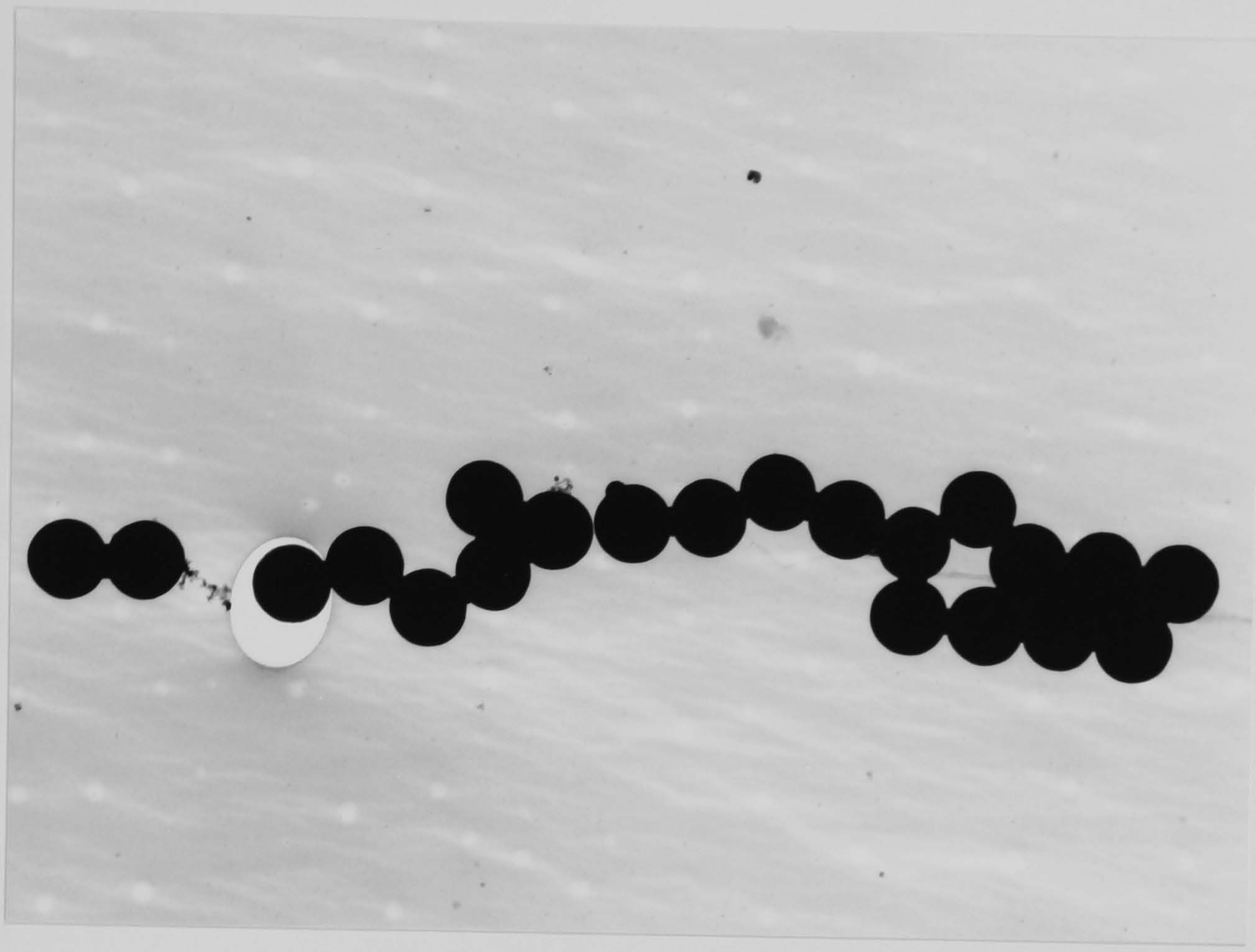
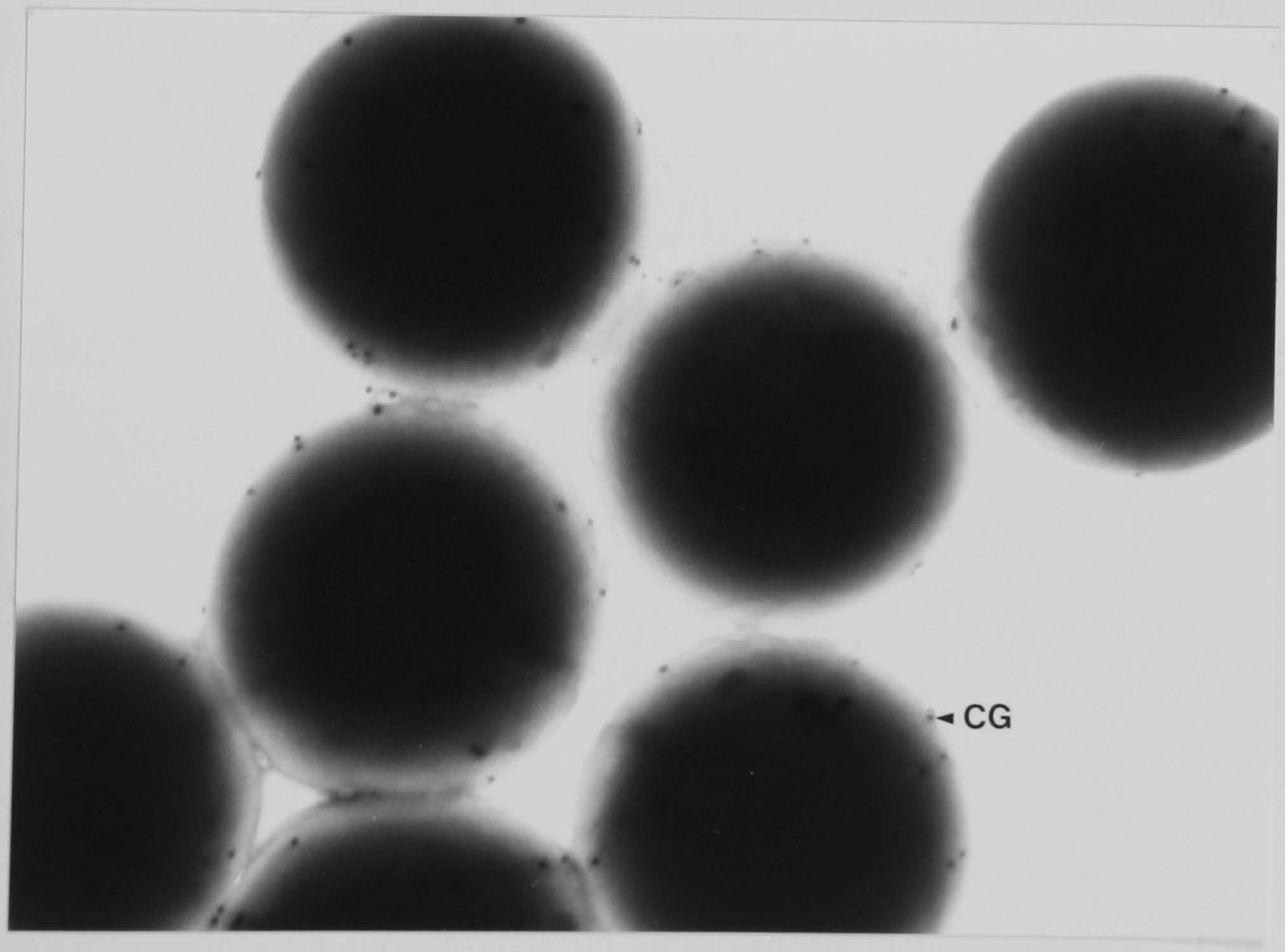


Figure 40



(c) Fluorescent latex Covasphere + streptavidin gold (10nm, washed and resuspended in 10mM PBS)

All samples were incubated for 1 hr 15 min.

The greatest amount of labelled gold was found in (b), using anti-biotin-gold and 10mM PBS (refer to Figures 41). In (c) the amount of gold label increased by using 10mM PBS and 1 hr incubation but not as much as for the anti-biotin gold. It was decided to conduct an acute study in the rabbit using gold labelled microparticles prepared with the conditions described in (b).

3.4.2 **Presentation of Colloidal Gold Labelled Latex Microparticles to Rabbit Peyer's Patches via Closed Intestinal Loops**

3.4.2.1 *Materials and Methods*

A New Zealand White rabbit was fasted overnight. It was anaesthetised and *in situ* gut loops formed around two Peyer's patches. The lumen of the rabbit was rinsed extensively with PBS (10mM) prior to injecting the microparticles into the loop. The microparticles were left in the loop for 15 minutes. The Peyer's patches were then removed and immersion fixed either in 3% glutaraldehyde in 0.1 M phosphate buffer and processed for TEM analysis or 4% paraformaldehyde and processed for fluorescence analysis.

Table 9 Colloidal gold labelled latex microparticle uptake studies

Animals	Microparticles		
Rabbit Peyer's patch	Dose	Time in loop	
A	2.0 ml	15 min	gold-labelled Covaspheres 0.5μm diluted 1 in 30 with PBS
B	2.0 ml	15 min	Covaspheres 0.5μm diluted 1 in 30 with PBS

2.4.2.2 *Results of Gold Labelled Microparticle Uptake*

Frozen sections of sample A and B showed a fine follicle dome arrangement; microparticles were restricted to the domes of the patches but absent from the villi when viewed under the fluorescence microscope. Sample A tissue was processed for TEM. Ultrathin sections were taken from areas populated by M cells. Within one M cell was a microparticle which clearly showed the colloidal gold label (refer to Figure 42). The microparticle was intercellular, indicative of a paracellular pathway of transport through the cell. Other similar structures were also found (refer to Figure 43) but these were not as well defined as those found in Figure 42.

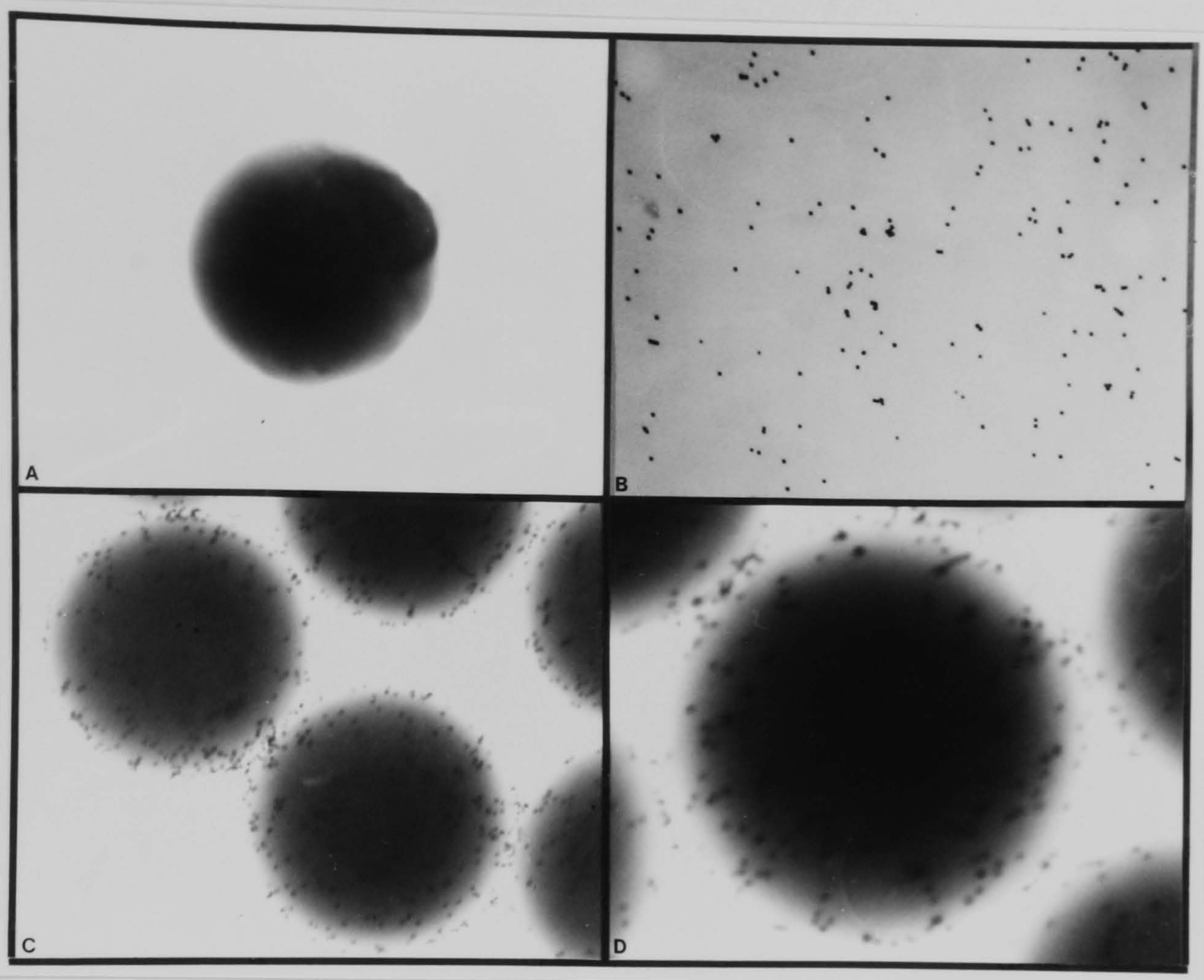


Figure 41

Electron micrograph showing colloidal gold labelling of latex microparticles (0.5μm Covaspheres) using the biotin/antibiotin bridge. (A) Unlabelled latex microparticles (0.5μm Covaspheres). (B) Colloidal gold particulates (10nm). (C) Latex microparticles (shown in A) labelled with colloidal gold, 10nm (shown in B), unsectioned. (Magnification A-C x 56,400). (D) Higher power of the labelled microparticle shown in C. (Magnification x 100,000).

Figure 42

Labelling of latex microparticles for visualisation in tissue under TEM. (A) Electron micrograph showing latex-biotinylated/antibiotin-colloidal gold microparticles (0.50μm) used in this study, unsectioned (Magnification x 30,300). (B) Electron micrograph showing an M cell (M) of a rabbit containing a latex-biotinylated/antibiotin-colloidal gold microparticle (Mp, 0.50μm) (Magnification x 5632). (C) Higher power of the latex microparticle shown in (B) (Magnification x 25,800).

Figure 43

Electron micrograph showing a cluster of latex microparticles (0.5μm) labelled with colloidal gold (Mp) within a paracellular compartment of an M cell. Magnification x 47,120

3.5 DISCUSSION

The rational behind using colloidal gold, either as a particulate itself or as a marker on the surface of latex microparticles, was to achieve unequivocal identification of particulates inside cells. Under the TEM, particulate gold 30-100nm was electron dense but was very polydisperse with respect to size. Little evidence was achieved in the uptake studies performed in the rabbit using these particulates. This may have been due to the dose administered rather than lack of identification. Colloidal gold markers on the surface of latex microparticles were more precise because of the monodispersity shown by the latex. The greatest amount of labelling was achieved with the Covaspheres using anti-biotin gold. A strong fluorescent signal was also emitted with these microparticles. In the uptake study performed with these microparticles only a few microparticles were apparent. These microparticles, however, were intercellular, indicating that the paracellular compartment is a component in the pathway for particulate transport from lumen to lymph. Childers *et al.* (1990) has suggested that liposomes are transported through the intercellular space of rat Peyer's patches, whilst Owen *et al.* (1986) demonstrated that *Vibrio cholerae* was transported through rabbit M cells in the intercellular spaces. Aprahamian *et al.* (1987) reported the presence of polyalkylcyanoacrylate nanoparticles (100-200nm) in the intercellular spaces of intestinal enterocytes. A more recent study by Damgé *et al.* (1991) reported a paracellular pathway of nanoparticles after uptake over rat Peyer's patches. The low numbers of microparticles observed in my study could be due a low dose and the small areas analysed under TEM.

CHAPTER 4

QUANTITATION OF MICROPARTICLE UPTAKE ACROSS THE INTESTINAL EPITHELIUM

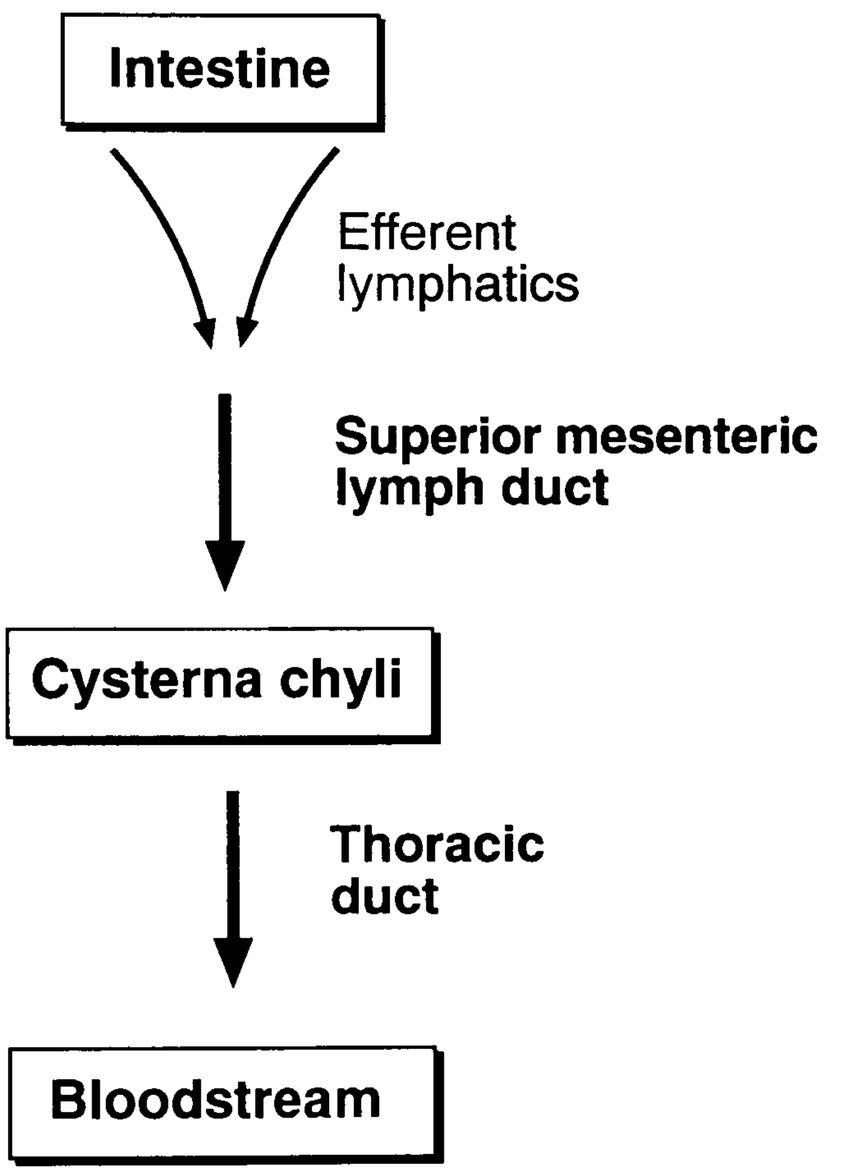
4.1 INTRODUCTION

To exploit the mechanisms of uptake described (refer to previous chapter) for the delivery of an oral vaccine in microparticles it was important to quantify the microparticulate uptake across the intestinal barrier. The most contentious issue surrounding this phenomenon is the degree of uptake. Many workers have investigated this, using analytical methods to count the numbers of microparticles in isolated tissue sections after the oral or intraluminal administration of microparticles (refer to section 1.5.3.).

Evidence suggests that absorbed microparticles are removed from the gut wall into draining lymph vessels (LeFevre *et al.* 1978, Eldridge *et al.* 1990, Jani *et al.* 1989, 1992, refer to section 1.5.2.). The mucosal efferent lymphatics merge to form the superior mesenteric lymph duct. This duct discharges into the cysterna chyli which in turn becomes the thoracic duct (refer to Figure 44).

The approach used, in this thesis, to quantify microparticle uptake across the intestine was to administer a dose of microparticles either orally or intraduodenally to rats, and cannulate the superior mesenteric and thoracic ducts and count the number of

Figure 44 Migratory pathway of lymph draining the intestine



microparticles within the draining lymph. Fluorescent latex microparticles of similar sizes to those used to characterise uptake in chapter 2 were employed in these studies. In addition, microparticles composed of poly(lactide-co-glycolide) (PLG) containing a fluorescent dye (rhodamine) were used to assess directly the feasibility of using an antigen delivery system (refer to section 1.4.4.) for oral vaccines.

Two methods of detection were used to count the numbers of fluorescent microparticles in lymph; (a) manual counting under the fluorescence microscope and (b) flow cytometry.

4.2 MATERIALS AND METHODS

Animals

The rat was the animal model used in the quantitative uptake studies that were performed.

Adult male Wistar rats (thoracic drainage)	300-400 grams
Adult male Wistar rats (mesenteric drainage)	140-180 grams

The animals were obtained from the Department of Biomedical Services, University of Nottingham.

Microparticles

Fluorescent polystyrene microparticles 0.15μm (Polyscience laboratories, Warrington, PA, U.S.A.)

Fluorescent polystyrene microparticles 1.0μm (Polyscience laboratories, Warrington, PA, U.S.A.)

Poly(DLlactide-co-glycolide) 50:50 rhodamine microparticles 1.10μm. (Produced in this work, refer to Appendix 3).

Surgical Procedures:

1) *Cannulation of the thoracic duct in the rat*

This procedure was undertaken in Germany at the University of Kiel, Department of Experimental Surgery under the direction of Dr. W. Sass.

The method of cannulating the thoracic duct was adapted from that described by Bollman *et al*. (1948) and Seifert and Sass (1991 personal communication). Adult rats 300-400 grams, which had been fasted for 24 hours, were anaesthetised. This was achieved by placing the animal in an ether filled bucket. When the animal was sedated an intraperitoneal injection of chlorohydrate (2 ml) was administered. The consciousness of the animal was tested by the deep tendon reflex and was monitored throughout the surgical procedure. The abdominal hair was shaved and an incision made approximately 2 cm long, exposing the abdominal cavity. Using cotton buds the intestine was carefully wrapped in moist cotton gauze (soaked in physiological saline maintained at 37°C) and laid to the right of the animal. The suprarenal abdominal aorta was localised and using a pair of curved anatomical forceps the connective tissue teased away by an opening and closing action parallel to the aorta. The thoracic duct (distinguished by milky white appearance of the contained lymph) was then teased away from the aorta and the connective tissue using the same instrument. A ligature was tied around the thoracic duct just posterior to the diaphragm (for preparation of ligatures refer to Figure 6), which resulted in the formation of a reservoir of lymph with the concomitant dilation of the thoracic duct. Two loose ligatures were prepared around the thoracic duct approximately 10 mm apart. Using

a pair of fine scissors an incision was carefully made 10 mm below the ligature. A cannula of perspex tubing (Portex, 0.5 mm internal diameter and 1.0 mm external diameter), was filled with heparin and inserted into the opening of the duct and pushed 5 mm into the duct using watchmakers forceps. The cannula was prepared with a U turn to allow a parallel lie. The cannula was then secured in place by tightening the two loose ligatures. The cannula was secured to the abdominal muscle using a suture and allowed to hang over the side of the dissecting bench. To allow gravitational flow, the length of the cannula was approximately 10 cm below the level of the rat. The abdominal cavity was closed using clips and the lymph was collected in Epindorf tubes attached to the end of the cannula.

2) *Cannulation of the superior mesenteric lymph duct in the rat.*

The method of cannulating the superior mesenteric lymph duct was adapted from that described by Bollman *et al.* (1948). Adult rats 170-180 grams were anaesthetised using an intraperitoneal injection of Nembutal (pentobarbitone sodium BP 60 mg/ml; 0.1 ml of Nembutal/100gms of body weight). The level of anaesthesia was tested by the deep tendon reflex and constantly monitored during the surgical procedure.

The animal was placed on an heated dissecting table (37°C) and the abdominal cavity was opened by making a cruciate incision along the midline. Using cotton buds, the stomach and intestine were laid to the left of the animal and wrapped in gauze soaked in phisiological saline (37°C). The superior mesenteric lymph duct was identified by the milky white appearance of the contained lymph to the left of the right kidney. The cannula consisting of a 20 cm length of perspex tubing (Portex 0.5 μm internal diameter, 1.0μm external diameter) was filled with heparin. Using anatomical forceps, a hole was pierced through the connective and fat tissue underlying the right

kidney, and the cannula taken within the tips of the forceps and drawn back through under the kidney so that the cannula was lying parallel to the mesenteric lymph duct. Using fine scissors, a small incision was made in the mesenteric lymph duct. The cannula was then fed into this hole and pushed 5 mm into the duct, a thin layer of connective tissue overlying the duct ensured the duct was rigid. When the lymph was flowing through the cannula it was held in place by the addition of a drop of cyanoacrylate adhesive at the site of insertion and the abdominal muscle. The abdominal cavity was then covered in moist gauze soaked in physiological saline (37°C). The cannula was extended below the level of the dissecting table and lymph collected in Eppindorf tubes.

Microparticle detection methods

The flow cytometric analysis was performed by Dr. P. J. Jenkins using an EPICS V flow cytometer (Coulter Electronics Ltd, Luton, U.K.).

The microscopic detection method is described in the Appendix 3

4.3 THE QUANTITATION OF THE ABSORPTION OF MICROPARTICLES INTO THE THORACIC LYMPH OF RATS

4.3.1 Materials and Methods

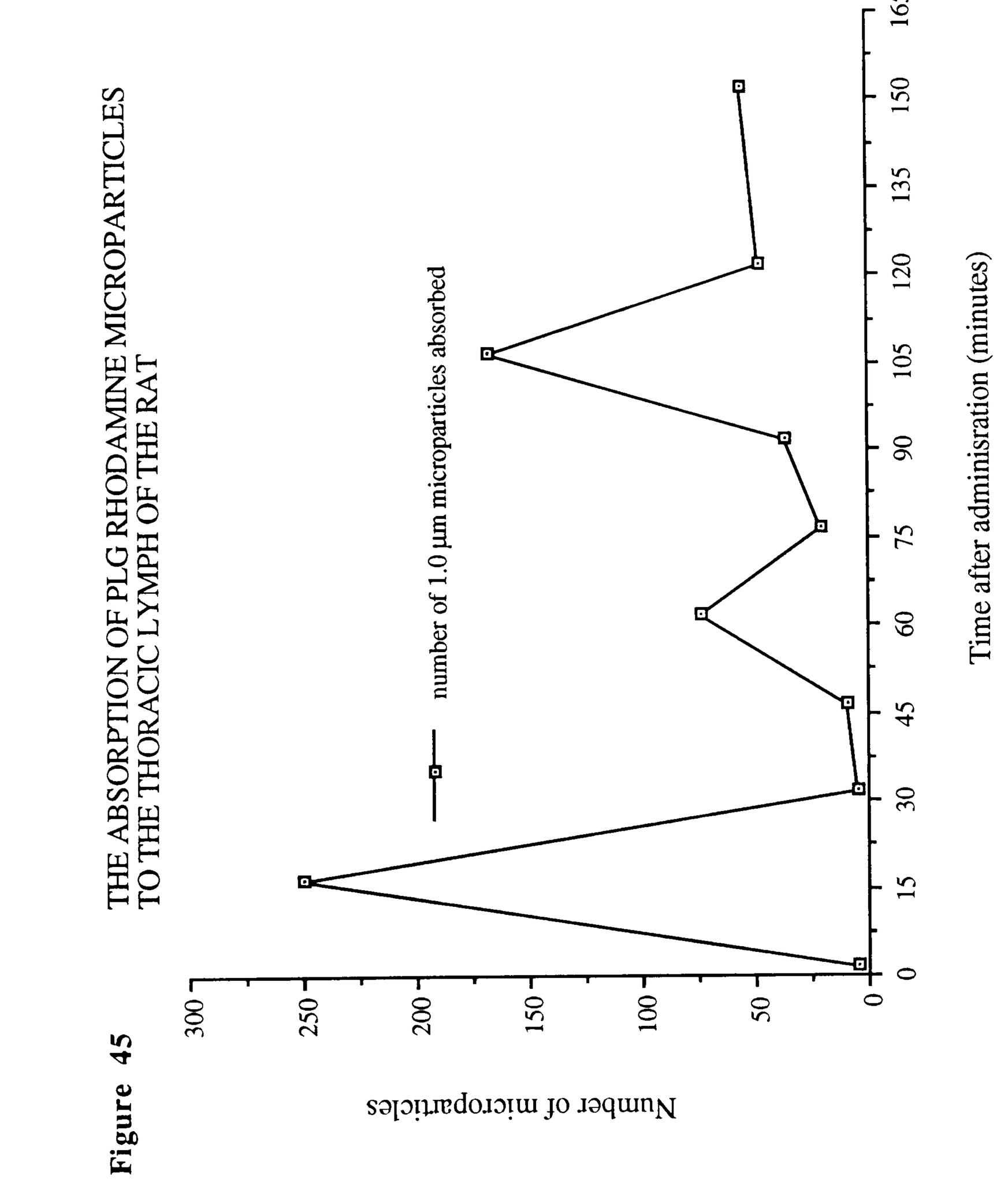
A male Wistar rat (320 gms.) was anaesthetised and the thoracic duct was cannulated according to the method described (section 4.2.).

When the lymph was flowing from the cannula, 1.7 mls. of a suspension (0.025 gms microparticles made upto 4 mls with distilled water) of PLG rhodamine microparticles (1.10μm) were administered orally using a gastric tube. The thoracic lymph was collected in 15 minutes aliquots over 3 hours and stored at 4°C. The number of microparticles in each lymph sample was calculated using the microscopic detection method described in Appendix 3.

4.3.2 Results

The numbers of microparticles found in each lymph sample was extremely low, reflected in the total levels found 2.9 x 10^{-6}% of the administered dose. The uptake of the microparticles into the thoracic lymph was a rapid event with maximal levels of microparticles found in the lymph only 15 minutes after microparticle administration. The temporal levels of microparticles found in the lymph can be seen in Figure 45. The microscopic analysis was time consuming and laborious but it enabled a direct observation of the microparticles. The microparticles were found to be intercellular with some closely associated with lymphocytes (refer to Figure 46).

Figure 45 THE ABSORPTION OF PLG RHODAMINE MICROPARTICLES TO THE THORACIC LYMPH OF THE RAT



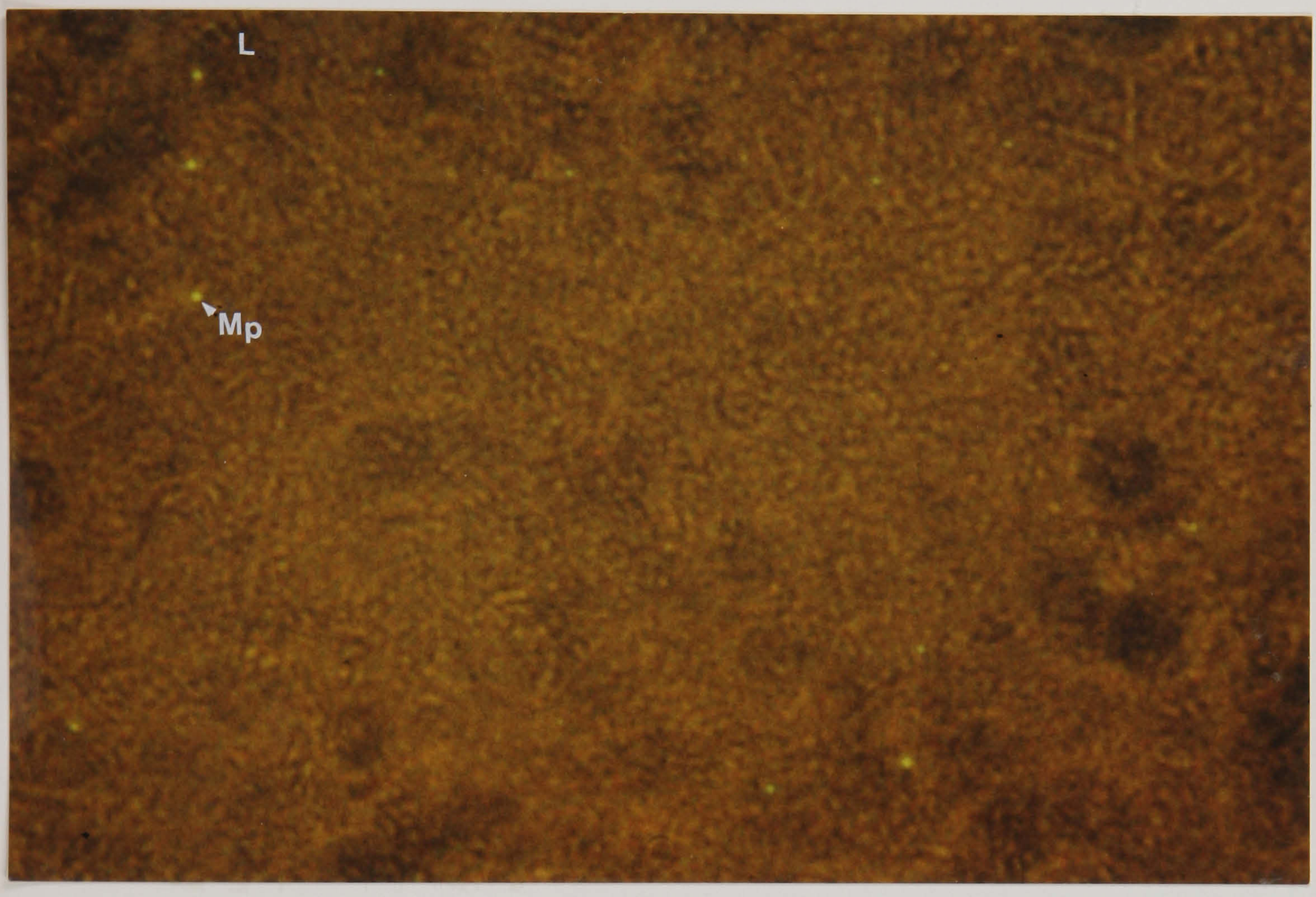


Figure 46

Photomicrograph showing rhodamine labelled PLG microparticles (Mp) in a sample of thoracic lymph. Some microparticles are closely associated with lymphocytes (L). Magnification x 400

4.4 THE QUANTITATION OF THE ABSORPTION OF MICROPARTICLES INTO THE MESENTERIC LYMPH OF RATS

4.4.1 Materials and Methods

Male Wistar rats (140-180 gms) were anaesthetised and the mesenteric lymph cannulated according to the method described (section 4.2.). 5 minutes after the lymph was flowing from the cannula, 1 ml of either 0.15μm (approximately $1x10^{13}$) or 1.0μm (approximately $1x10^{10}$) fluorescent latex microparticles were intraduodenally administered close to an observable Peyer's patch in groups of rats (n=5). Special care was taken to ensure that none of the microparticles contaminated the area where the cannula was inserted. A control group (n=2) were given 1 ml of saline intraduodenally. Lymph was collected from the cannula over a period of 90 minutes, in 5 minute aliquots and stored at 4°C. The number of microparticles in each sample was measured using the flow cytometric method.

4.4.2 Results

The flow cytometric analysis enabled a rapid and accurate calculation of the number of microparticles present in the mesenteric lymph. Microparticle absorption across the intestine, indicated by the numbers of microparticles found in the mesenteric lymph, occurred at a rapid rate but at low levels (refer to Figures 47-48 and Table 10). Both the 0.15μm and 1.0μm microparticles were found in the lymph 5 minutes after administration. Similar times for the maximal numbers were found for both sizes of microparticles; 35 minutes and 65 minutes for the 0.15μm and 25 minutes and 65

Figure 47 THE ABSORPTION OF 1.0 μm POLYSTYRENE MICROPARTICLES TO THE MESENTERIC LYMPH OF RATS

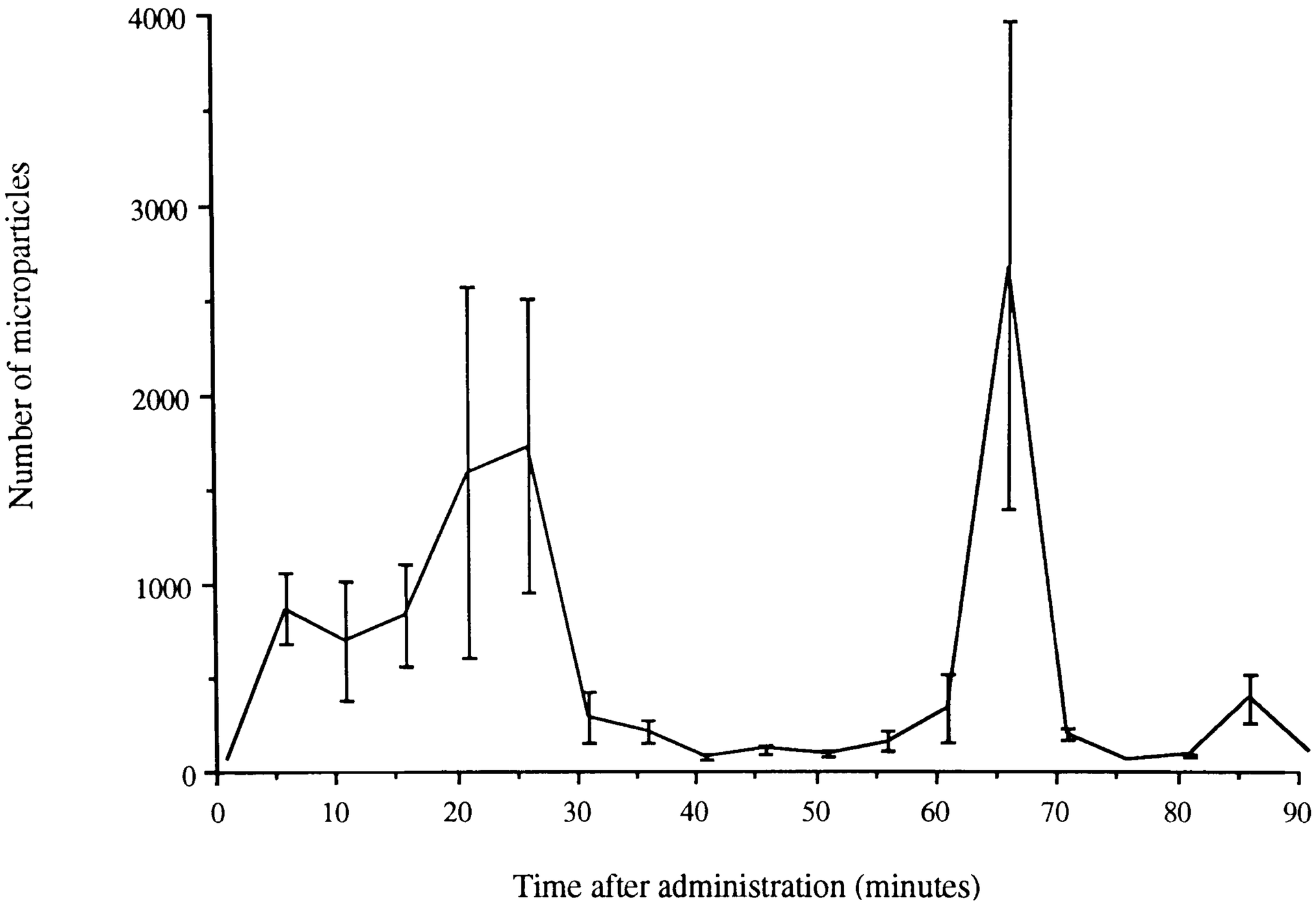


Figure 48

THE ABSORPTION OF 0.15μm POLYSTYRENE MICROPARTICLES TO THE MESENTERIC LYMPH OF RATS

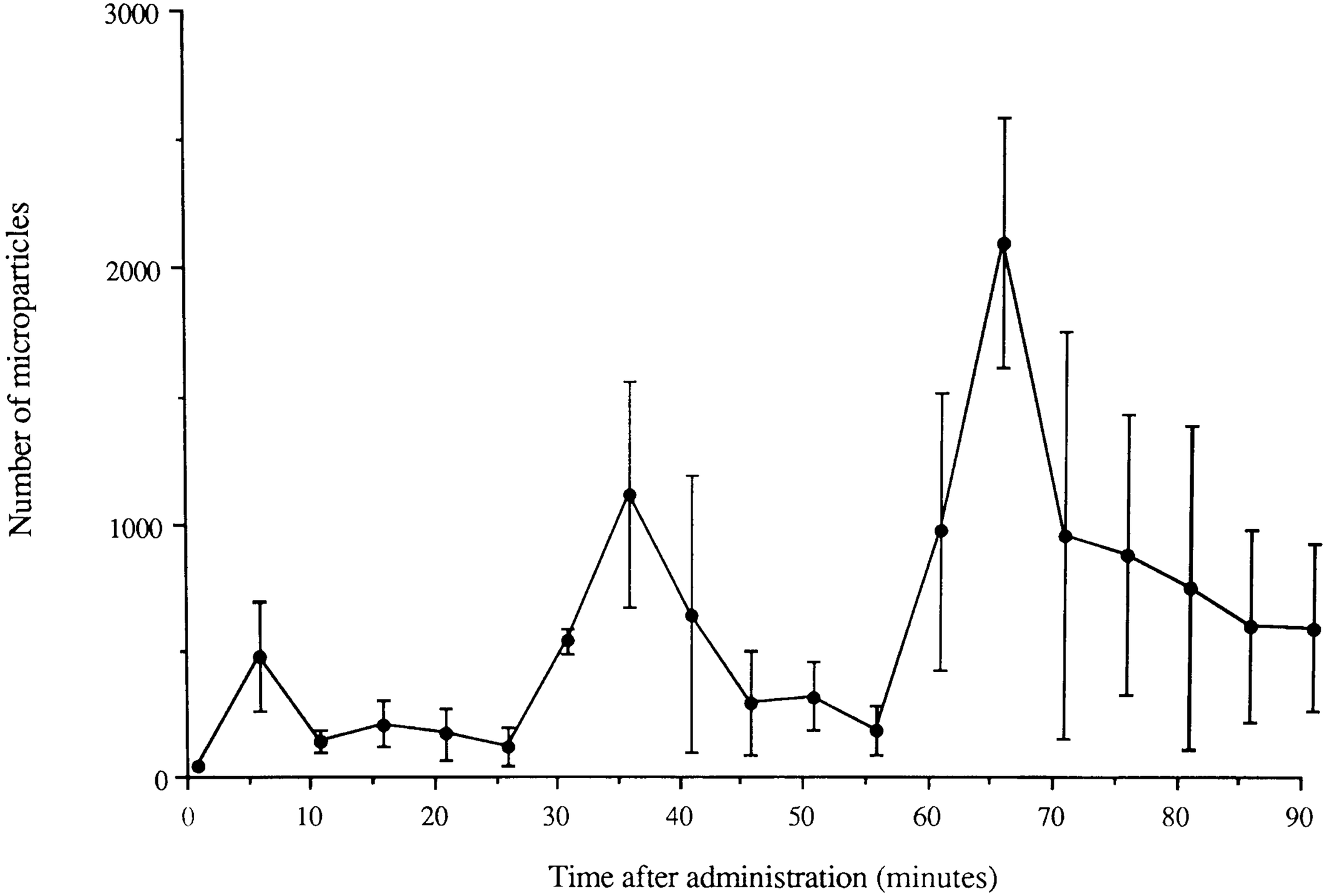


Table 10 **Times and levels of maximal absorption of 0.15μm and 1.0μm latex microparticles to the mesenteric lymph in rats.**

	Time of maximal absorption of microparticles	Maximal number of particles absorbed (% dose)	Total number of particles absorbed to the lymph (% determined by AUC).	Theoretical volumetric carrying capacity of total number of absorbed particles (μl).
0.15μm particles	65 minutes	2059 (2×10^{-8} %)	50006 (5×10^{-7} %)	0.88
1.0μm particles	65 minutes	2617 (2.6×10^{-5} %)	49964 (5×10^{-4} %)	36.9

minutes for the 1.0μm microparticles. The amount of microparticle absorption, expressed as a percentage of the dose was low; $5x10^{-4}$% for the 1.0μm and $5x10^{-7}$% for the 0.15μm microparticles.

4.5 DISCUSSION

The techniques presented here represent a valid approach to elucidate the numbers of microparticles crossing the intestine. Although it is generally accepted that, after intestinal uptake across lymphoid tissue, microparticles are carried by the lymphatic vessels, no direct evidence of microparticles in the lymph has been reported; previous evidence being based on microparticle detection in isolated mesenteric lymph node and systemic tissues (LeFevre *et al.* 1978, Eldridge *et al.* 1990, Jani *et al.* 1989, 1992). In contrast, the present method directly measures microparticles in flowing lymph derived from the intestine; the superior mesenteric duct receives lymph from most of the lymphatics draining the intestine while the thoracic duct receives lymph from peripheral tissues including the liver in addition to the intestine, 95% of cells in the thoracic lymph are derived from the nodes draining the intestine.

The results of the present studies clearly demonstrate lymphatic transport of absorbed microparticles. Additionally, the profiles for absorption can be interpreted as indicating a cyclic pattern. Microparticles were found in both the mesenteric and thoracic lymph 5 minutes after administration suggesting an extremely rapid uptake across the intestine. This agrees with the findings of Sass *et al.* (1990) who demonstrated the uptake of latex microparticles across rat M cells into the lymphatic capillaries within 10 minutes. Similarly, Pappo and Ermak (1989) reported that

microparticles crossed the follicle-associated epithelium in the rabbit within 10 minutes. The rapid rate of uptake of microparticles into the lymph also correlates with our findings of microparticle uptake across the follicle associated epithelium to the serosa within 15 minutes of intraluminal administration in the rabbit (refer to chapter 2). In contrast, Sanders and Ashworth (1961) reported that microparticles were found in the intestinal lymphatics 2-4 hours after oral administration in rats, although this may just have been a reflection of the times tissue was sampled.

The extent of intestinal uptake found in the present studies was low if expressed as a percentage of the administered dose; $2.9x10^{-6}$ (1.10μm) in the thoracic and $5x10^{-4}$ (1.0μm), $5x10^{-7}$ (0.15μm) in the mesenteric lymph. This does not reflect total uptake, however, because no attempt was made to quantify microparticles in the systemic tissues. These low levels correlate with the limited amount of microparticle uptake found in the TEM and fluorescent studies performed in chapter 2. The PLG microparticles were polydisperse with respect to size (refer to Figure 49) which presented a problem when counting under the microscope. The strong fluorescence signal emitted by the rhodamine, however, made identification relatively easy. The manual counting method was very time consuming. In contrast, the flow cytometric method represented a very efficient and accurate detection method. Interestingly, similar temporal profiles and low levels were found using the microscopic and flow cytometric detection methods. Although the data from the thoracic drainage represents only one animal, these levels are in agreement with the levels of latex microparticle in thoracic lymph measured by the manual counting method in experiments conducted by the group led by Professor J. Seifert, Kiel, Germany (personal communication). The levels of microparticle uptake in the present study agree with a number of studies

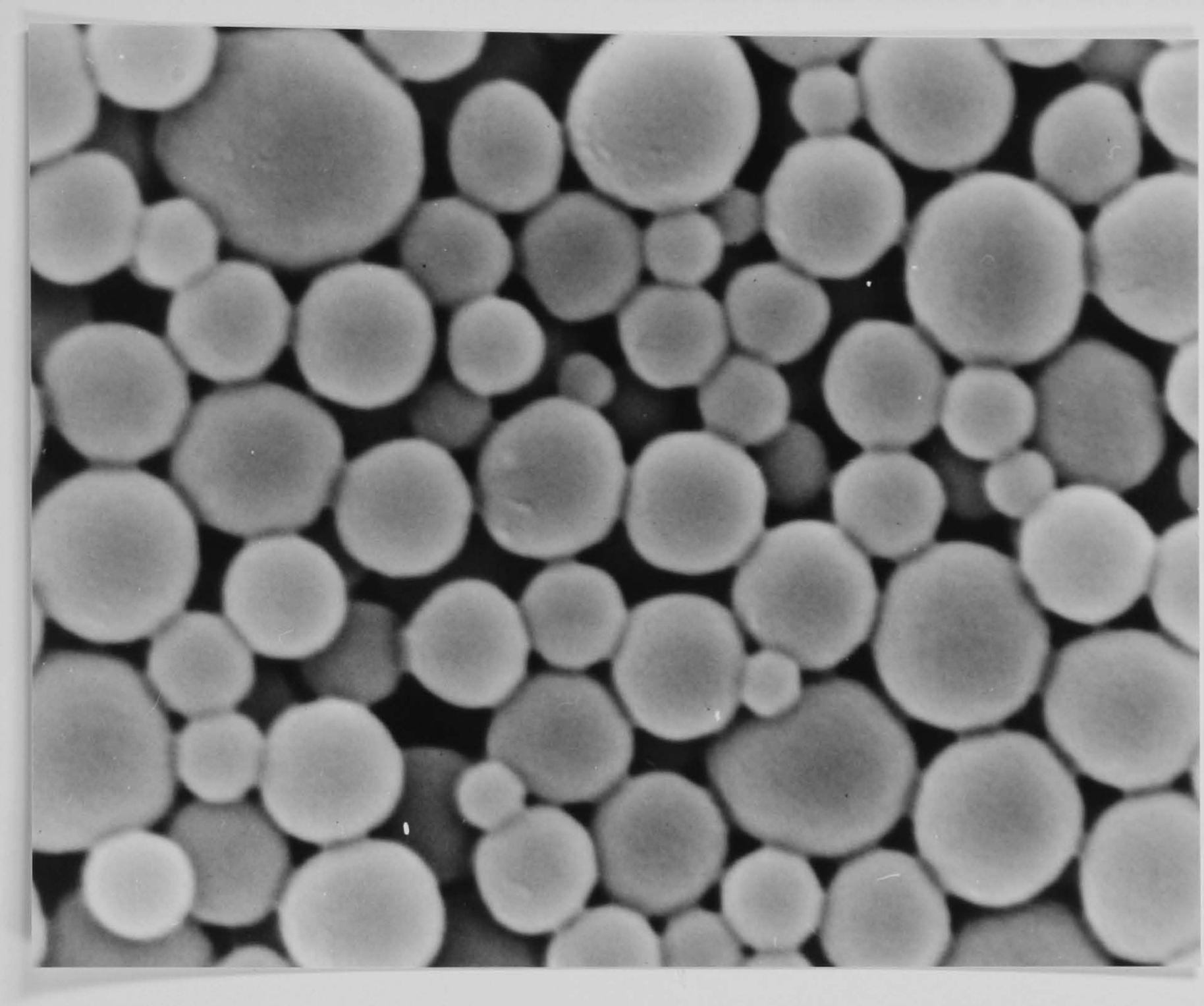


Figure 49

Scanning electron micrograph of PLG (50:50), microparticles containing rhodamine. Magnification x 52,000

investigating uptake; LeFevre *et al.* (1977) reported that 0.01% of radioiodinated latex microparticles (170-227nm) were taken across the intestine in mice after a single gavage. This estimate was calculated by measuring the radioiodinated label in the body tissue. The levels in the study conducted by LeFevre *et al.* (1977) may not be totally accurate, however, due to the instability of the label on the microparticles. Using a flow cytometric detection method, similar to the one used in our mesenteric studies, Ebel (1990), also found less than 0.01% of an oral dose of fluorescent latex microparticles (2.65μm) in mice was taken across the intestine, estimated by the numbers in Peyer's patch, mesenteric lymph node and spleenic tissue. Interestingly the levels were found to be greater in fed mice as opposed to fasted animals, Ebel (1990) attributed this to a possible increase in M cell activity due to increased luminal contents.

Jepson *et al.* (1993a) used confocal microscopy to measure the uptake of latex microparticles across rabbit lymphoid tissue. They found that $5x10^{-5}$% of a dose of fluorescent polystyrene microparticles (0.46μm) was taken across the follicle associated epithelium. This figure was calculated by counting the amount of microparticles within the FAE of each Peyer's patch dome (1×10^5 microparticles per dome). Also in the rabbit, Pappo and Ermak (1989) reported that 5% of an intraluminal dose of fluorescent microparticles (600-750nm) was taken across the follicle associated epithelium as detected by fluorescent microscopy analysis of Peyer's patch tissue. This was an order of magnitude greater than the amount of microparticle uptake found in the murine model (Pappo and Ermak 1989). Pappo and Ermak reasoned that this may be a result of the greater number of M cells found in

the rabbit; 50% of the follicle associated epithelium in rabbits is composed of M cells (Pappo *et al.* 1988) as opposed to 5-10% in rats (Smith 1980).

In contrast to the low levels described, much higher levels of intestinal uptake of microparticles has been reported; Alpar *et al.* (1989) claimed that 39% of an oral dose of latex microparticles (1.1μm) given to rats was found in blood 45 minutes after administration. This seems an extraordinary finding especially when no mechanism or route across the intestine was demonstrated. High levels of intestinal uptake was also described by Jani *et al.* (1990); in this study latex microparticles (50nm and 100nm) were fed to rats for 10 days, isolated tissue was then digested and an assay was used to measure the amount of latex present. Levels of microparticulate uptake of 34% for the 50nm and 26% for the 100nm were reported. The problem with this analytical method was that it did not disassociate those microparticles adhering to the luminal surface of the intestine from those taken across the intestine. Jani *et al.* (1990) suggested that uptake into isolated tissue would reflect a more accurate assessment; giving levels of 7% (50nm) and 4% (100nm) of the dose.

The various uptake levels reported seem to fall into one of two catorgories: (a) less than 0.01% and (b) 5% and over. The differing reported levels of microparticle uptake are probably a result of the different experimental and analytical detection methods employed as well as the species used in the study.

In the present studies a greater level of uptake was found for the 1.0μm latex microparticles ($5x10^{-4}$% of the dose) than the 1.0μm PLG microparticles ($2.9x10^{-6}$% of the dose). This is an important finding for the development of PLG microparticles as an antigen delivery system. However, it is unclear whether this is due to the

different techniques and detection methods used. Jepson *et al*. (1993b), reported that PLG microparticles were taken across rabbit follicle associated epithelium at similar levels to those found in the present studies. Using the technique of confocal microscopy to count the numbers in Peyer's patches Jepson *et al*. (1993) found the levels of PLG to be an order of magnitude less than they found for latex. Jepson *et al*. (1993) suggested that this reflected the greater binding capacity of the polystyrene to the M cells.

Under the fluorescence microscope, samples taken from both the thoracic and mesenteric lymph showed intercellular microparticles. In the thoracic lymph, however, a proportion of the microparticles seemed to be associated with lymphocytes. For pathogenic microorganisms entering the host through M cells, phagocytosis by macrophages in the Peyer's patch represents an important defence. Owen *et al*. (1986) demonstrated the association of *Vibrio cholera* with macrophages in the central hollow of rabbit M cells whilst, Fujimura (1986) reported that mycobacteria were taken up by macrophages in the follicle associated epithelium of rabbits.

LeFevre *et al*. (1978), detected latex microparticles (2μm) in macrophages within Peyer's patches and mesenteric lymph nodes suggesting intracellular migration. LeFevre *et al*. suggested that a proportion may migrate as: "free microparticles". Eldridge *et al*. (1990) also suggested a migratory macrophage population containing microparticles originating in the dome of the Peyer's patch and later found in the mesenteric lymph nodes and spleen. Although all microparticles were found within

macrophages in the patch only those less than 5μm migrated. The association of microparticles with macrophages within Peyer's patch follicle domes has also been reported by Pappo and Ermak (1989), Sass *et al.* (1990) and Jepson *et al.* (1993). The results from the present studies suggest that the majority of microparticles found in the draining lymph are transported intercellularly but there is evidence of microparticles closely associated with lymphocytes. The low levels of microparticles found in the lymph suggest that oral delivery of therapeutic drugs in microparticles appears improbable but the low levels of antigen required to elicit an immune response indicates that the delivery of an oral vaccine by this route is indeed possible.

For an illustrated summary of the microparticle uptake findings contained in this thesis refer to Figures 50 - 51.

Figure 50

Schematic representation of microparticle uptake findings at the ultrastructural level

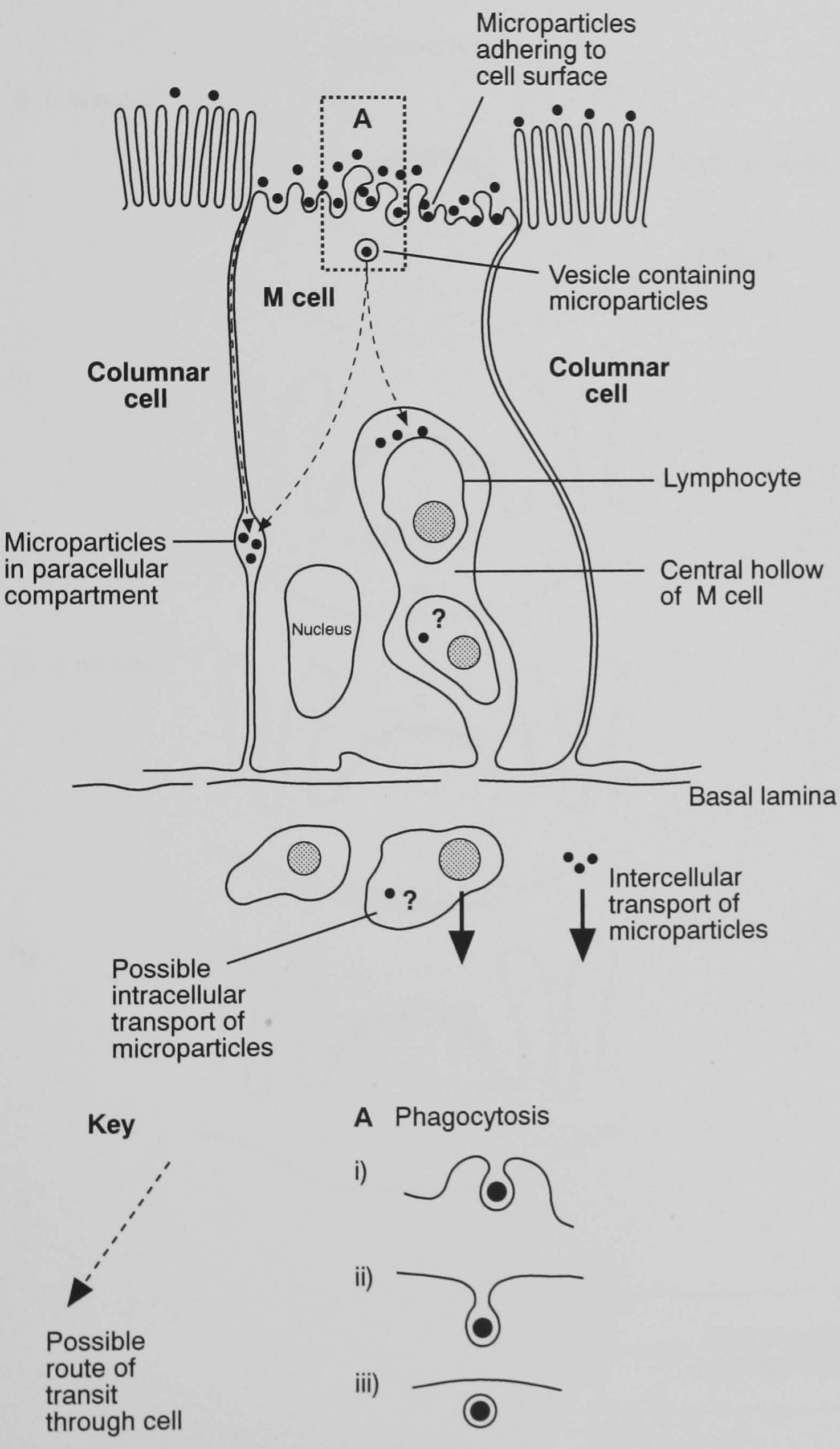
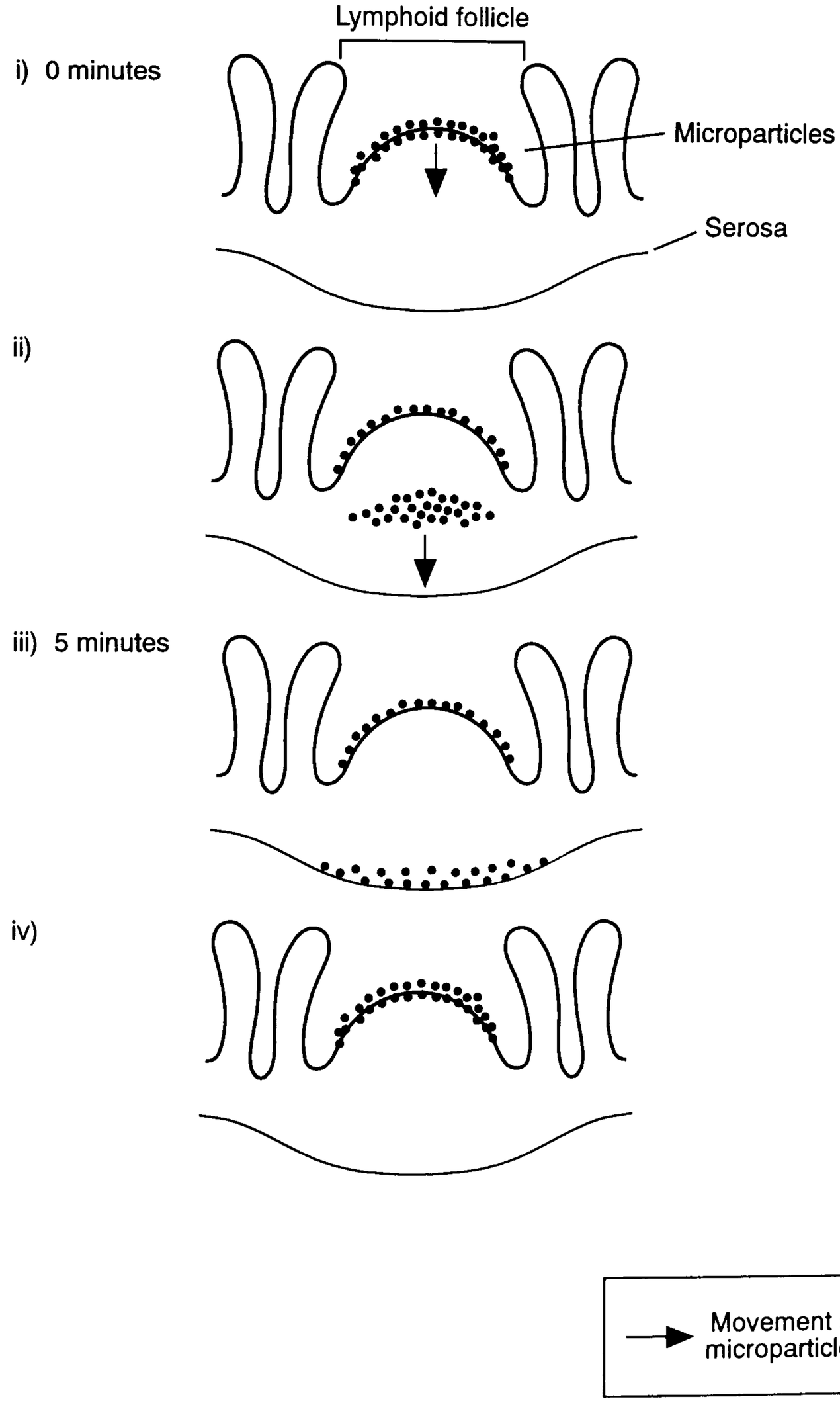


Figure 51

Schematic representation of the temporal movement of microparticles through Peyer's patches



CHAPTER 5

THE FORMULATION AND CHARACTERISATION OF POLY(LACTIDE-CO-GLYCOLIDE) MICROPARTICLES CONTAINING INFLUENZA VIRUS

5.1 INTRODUCTION

The work conducted in chapters 2-4 has given unequivocal evidence that the primary route for microparticle uptake across the intestine is the Peyer's patches (refer to Chapters 2-4). Evidence suggests that a disseminated mucosal immune response is initiated at these sites (refer to section 1.3). In order to investigate the feasibility of this route for the delivery of a microparticulate oral vaccine, the antigen must ideally be incorporated into microparticles.

The process of incorporating an antigen into a polymer matrix is termed microencapsulation. Various microencapsulation techniques are currently in use (Morris *et al.* 1994). Recently, the process of solvent evaporation has been employed extensively to microencapsulate drugs and antigens using the poly(lactide-co-glycolide) (PLG) copolymers (Tice and Gilley 1985, Jeffery *et al.* 1991).

As previously discussed, poly(lactide-co-glycolide) microparticles have been used as antigen delivery systems for both parenteral (refer to section 1.4.6) and oral

immunisation (refer to section 1.4.6). Poly(lactide-co-glycolide) (PLG) is non-toxic and has been approved by the FDA for human use. The biodegradability of PLG can potentially be exploited to give sustained or pulse release of antigen.

The antigen selected for microencapsulation was influenza virus. This is an ideal candidate for an oral vaccine for the reasons described in section 1.7. Equine influenza was the type of influenza used in these studies. It is a orthomyovirus (A group) of which there are two subtypes A/equi-1 and A/equi-2 (Paccaud, 1970). The pathogenesis of equine influenza mimics that in humans with initial invasion at the respiratory mucosa (Gerber, 1970). Outbreaks can be rapid and severe (Wood, 1988), leading to considerable economic cost especially in racehorse populations. The common vaccine in present use in Britain is composed of inactivated whole viruses (variants of the two subtypes) adsorbed to alum which is administered intramuscularly (licensed name-PREVAC*). These induce short term responses (Wood, Mumford, Folkers *et al.* 1983), requiring boosters at least every 9 months, and low protection (Mumford, Wood, Scott *et al.* 1983).

This chapter describes the production and characterisation (size, morphology and antigen content) of PLG microparticles containing influenza virus, the release of antigen from the microparticle system and the antigenic integrity of the encapsulated virus.

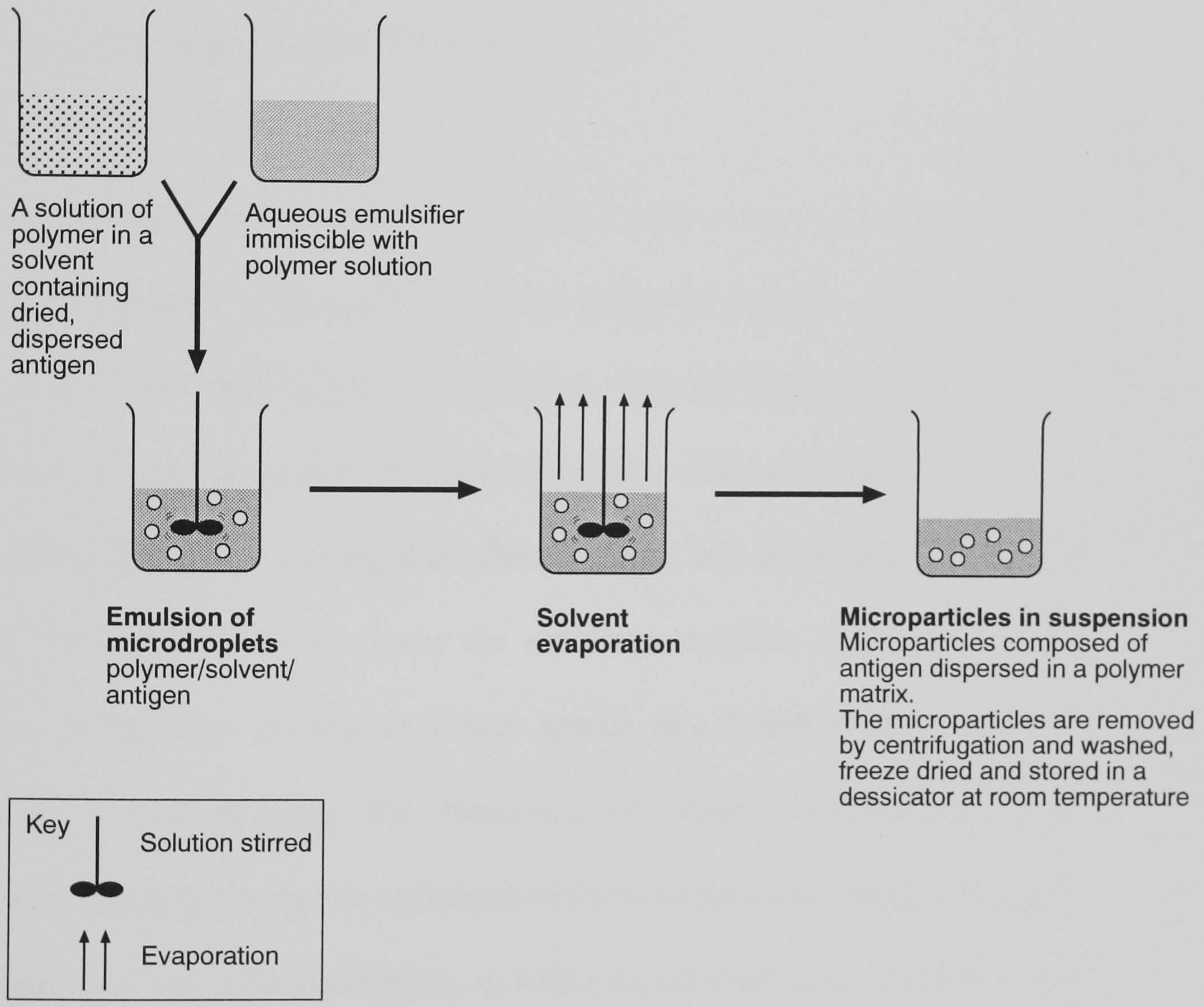
5.2 PRINCIPLES OF THE SOLVENT EVAPORATION MICROENCAPSULATION TECHNIQUE

The basic principle is the formation of an emulsion in which microdroplets (comprising of polymer, drug or antigen and solvent) referred to as the discontinuous phase are dispersed in a continuous phase. The solvent is then removed by evaporation leaving microparticles consisting of the drug or antigen dispersed within the polymer.

The first step involves dissolution of the polymer in an organic solvent. The antigen is added in lyophilised form to the polymer solution. This dispersion is then added to an immiscible aqueous phase containing a surfactant such as Polyvinyl alcohol and mixed by stirring to form an oil-in-water emulsion. The microdroplets, (a term described by Tice and Gilley 1985) are stabilised by molecules of the emulsifier in the continuous phase forming a coating around the microparticles. The organic solvent is then slowly removed by evaporation necessitating the use of a solvent whose boiling point is lower than water. The microdroplets harden as the solvent is removed to form stable microparticles comprising of antigen dispersed throughout the polymer matrix. The microparticles are harvested by a process of centrifugation and washing to remove excess surfactant (refer to Figure 52).

One problem associated with using the solvent evaporation technique is the possible adverse effects on the antigen due to contact with the organic solvent. The solvent may destroy important antigenic epitopes and affect the subsequent antigenicity of the antigen. This may be avoided by first dissolving the antigen in an aqueous solution

Figure 52 **Production of microparticles using oil-in-water (o/w) solvent evaporation technique**

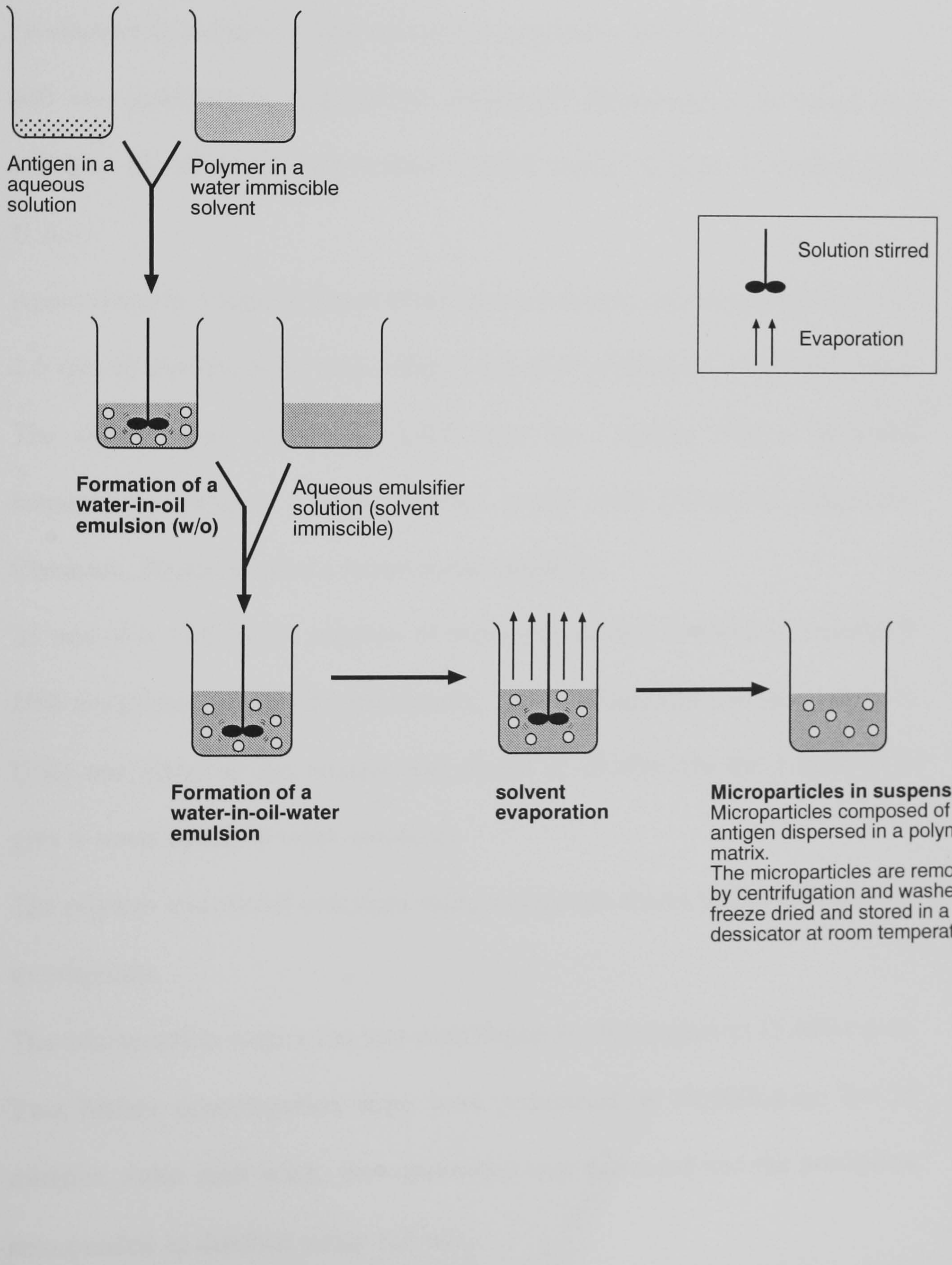


which is then added to the polymer solution, thereby avoiding direct contact between the antigen and the solvent. This results in a water-in-oil (w/o) emulsion which subsequently forms a water-in-oil-in-water (w/o/w) emulsion when mixed with an aqueous phase containing the emulsion stabiliser (refer to Figure 53).

Microparticles of submicron size can be made by solvent evaporation. From the data presented in Chapters 2-4, it is evident that microparticles of approximately 1μm are required for optimum uptake across the Peyer's patches.

Changing process parameters such as the size and speed of the stirrer, size of the mixing vessel and polymer concentration can all result in changes in microparticle size (Watts *et al.* 1990, and Jeffery *et al.* 1991). One of the principle factors in producing small or large microparticles is the magnitude of the shearing forces acting on the microparticle droplets; stirring at maximum speeds in a small volume increases the shearing forces which break down the emulsion droplets resulting in small microparticles. In contrast, stirring at slower speeds in a larger volume results in lower shearing forces allowing the formation of large microparticles. PLG microparticles containing the equine influenza virus were prepared using a water-in-oil-in-water solvent evaporation technique, in which selected process conditions were varied to produce microparticles of submicron and 10 micron respectively.

Figure 53 **Production of microparticles using water-in-oil-in-water (w/o/w) solvent evaporation technique**



5.3 MICROENCAPSULATION OF EQUINE INFLUENZA VIRUS INTO BIODEGRADABLE PLG MICROPARTICLES

The whole inactivated influenza virus, prague strain (A/Equine/Prague/56) (supplied by Behringwerke, Germany) was used for encapsulation studies.

1) *Production of submicron microparticles containing influenza*:

(a) 400 mgs poly(lactide-co-glycolide) copolymer (Boehringer, Germany) was added to 10 mls of Dichloromethane (HPLC grade, Fissons, Loughborough, U.K.).

(b) Approximately 5 mgs of freeze dried equine influenza were resuspended in 2-2.5 mls of distilled water and added to the PLG solution in a 100 ml beaker.

(c) The mixture was stirred at 12,400 rpm for 1 minute with a Silverson homogeniser using a 12mm diameter stirrer head (Silverson Machines, Chesham, Bucks) to give a water-in-oil emulsion.

(d) 25 mls of a 10% (w/v) solution of polyvinyl alcohol in distilled water)(87-89% hydrolysed 13,000-23,000 daltons, Aldrich Chemical Company, Dorset, U.K) was added to the mixture and stirred at 12,400 rpm for 5 minutes to give a water-in-oil-in-water emulsion.

(e) The mixture was stirred overnight using a magnetic stirrer to allow the solvent to evaporate.

(f) The microparticle suspension was centrifuged for 30 minutes at 15,000 r.p.m. Two further centrifugation steps were performed at 10,000r.p.m. for 15 minutes. After each wash, the supernatant was discarded and the precipitate resuspended in distilled water (15 mls).

(g) The microparticles were freeze dried and stored in a desiccator at 25°C.

For the appearance of a typical batch of submicron microparticles refer to Figure 54.

2) *Production of microparticles 5 micron and over containing influenza virus*:

(a) 900 mgs polylactide-co-glycolide polymer was added to 10 mls of Dichloromethane.

(b) Approximately 5 mgs of freeze dried equine influenza were resuspended in 2-2.5 mls of distilled water and added to the poly(lactide-co-glycolide) solution in a 500 ml glass beaker.

(c) The solution was stirred at 8,100 rpm for 1 minute with a Silverson homogeniser using a 16mm diameter stirrer to give a water-in-oil emulsion.

(d) 500 mls of a 0.5% solution of polyvinyl alcohol was added to the mixture and stirred at 8,100 rpm for 5 minutes to give a water-in-oil-in-water emulsion.

(e) The mixture was stirred overnight using a magnetic stirrer to allow the solvent to evaporate.

(f) The microparticle suspension was centrifuged for 15 minutes at 14,000 r.p.m. Two further centrifugation steps were performed at 14,000 r.p.m. for 15 minutes. After each wash, the supernatant was discarded and the precipitate resuspended in distilled water (15 mls).

(g) The microparticles were then freeze dried and stored in a desiccator at 25°C.

For the appearance of a typical batch of microparticles greater than 5μm refer to Figure 55.

Figure 54

Scanning electron micrograph of a typical batch of PLG submicron microparticles containing equine influenza virus.

Figure 55

Scanning electron micrograph of a typical batch of PLG microparticles over 5μm containing equine influenza virus.

Figure 54

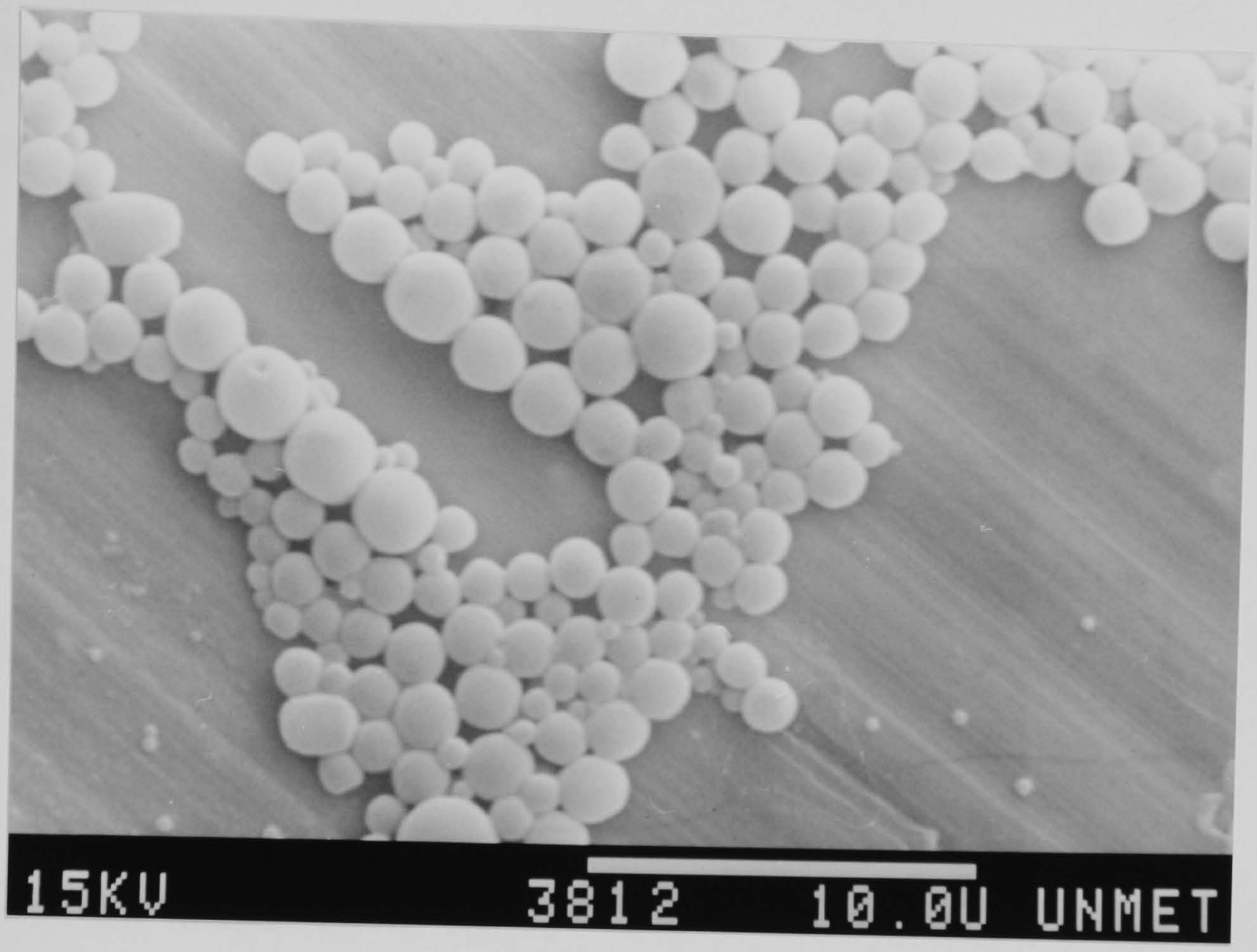
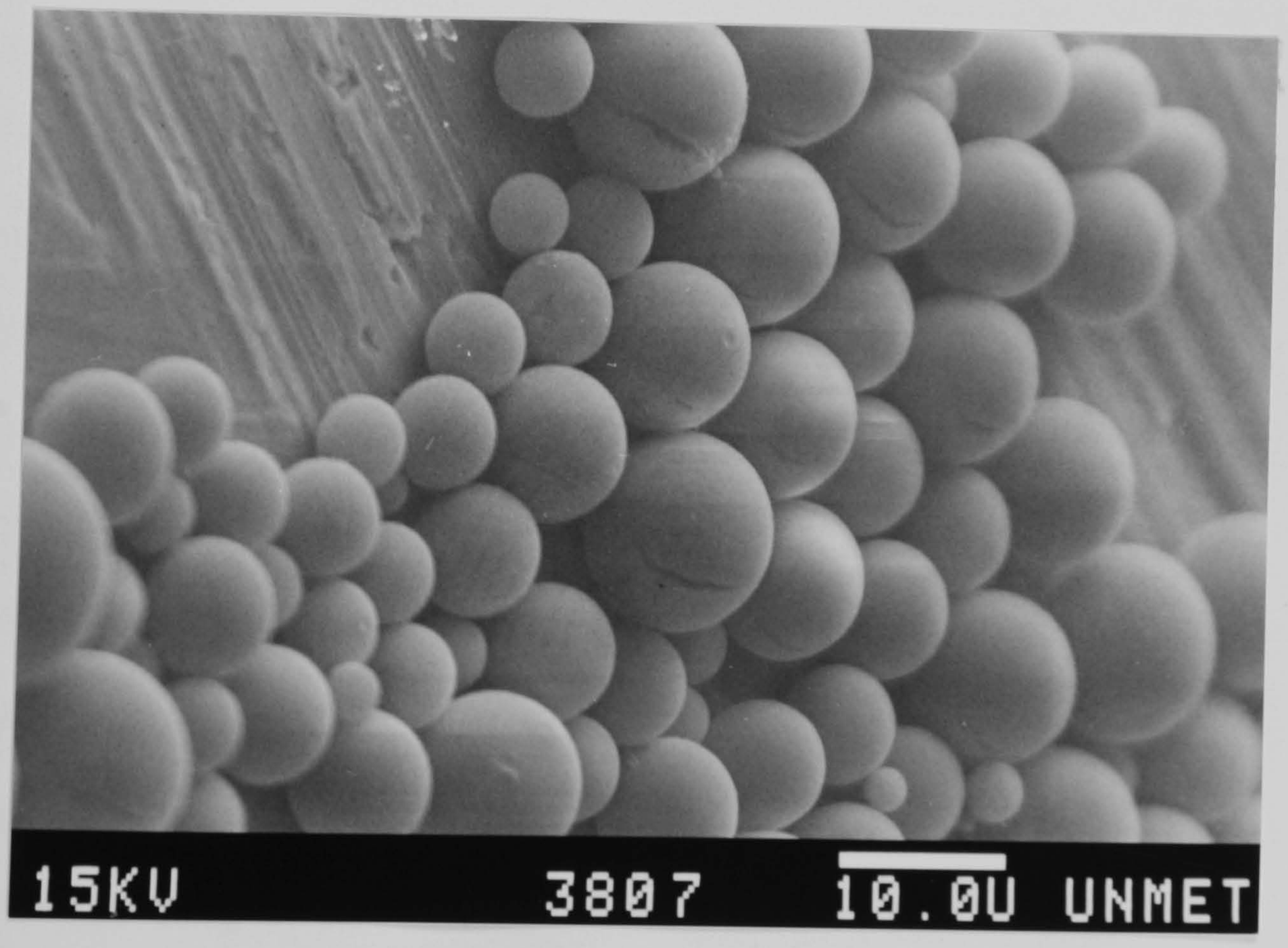


Figure 55



The procedures described above was used for the production of blank PLG microparticles (dependent on size of microparticles required) with omission of the antigen, thus, the blank microparticles were formed from an oil-in-water emulsion.

5.4 CHARACTERISATION OF PLG MICROPARTICLES CONTAINING THE EQUINE INFLUENZA VIRUS

5.4.1 Morphological Analysis Using Scanning and Transmission Electron Microscopy

The external morphology of the microparticles was investigated using scanning electron microscopy (SEM, JOEL 6400 Winsem). Both the small and large microparticles prepared in section 5.3. were spherical in shape and showed a smooth surface, with an absence of surface pores (this is important for the controlled release of entrapped antigen). If the current of the SEM was high, however, the PLG microparticles distorted and formed visible pores. For this reason the current was kept at 0.2×10^{-10} amps. Pores also formed if the volume of the internal aqueous phase (virus in water) was high (over 4 ml in production of small microparticles). This is possibly the result of an unstable primary emulsion (w/o) (Cohen *et al.* 1991). The use of the formulation approaches described in section 5.3 resulted in microparticles of either submicron or 5 micron and over (refer to Figure 54-55). The microparticles, however, did show a degree of heterogeneity with respect to size within batches (confirmed using Photon correlation spectroscopy, Malvern 2600). One possible reason is non-uniform mixing as the mixing vessel is manually moved with respect

to the stirrer head, thus, some regions of the emulsion are stirred more than others resulting in a heterogenous size population.

Transmission electron microscopy was used (TEM, Phillips 300 and 410) to determine the interior morphology of the microparticles. This work was performed using a preparation of large PLG microparticles (approximately 10μm) containing equine influenza virus. The procedure for resin embedding of PLG microparticles is listed in the Appendix 3. The polymerised resin blocks containing microparticles were sectioned using a Reichert OMU3 ultra-microtome (refer to section 2.2.).

Initially semithins (1μm sections) were placed on a carbon disc overlying aluminium stubs, sputter coated, and viewed under the SEM. Poor contrast between the topography of the microparticles and surrounding resin resulted in unsatisfactory images when viewed under SEM. The image did, however, indicate an homogenous matrix (refer to Figure 56). Ultrathin sections (90nm) of the PLG microparticles were viewed under the TEM revealed grey spherical structures relating to the microparticles (refer to Figure 57). The internal matrix appeared homogenous and was surrounded by a dense border. This indicates that the influenza is dispersed throughout the whole matrix (referred to as monolithic by Tice and Cowsar 1984). Influenza virus could be seen on the outside of the microparticles (refer to Figure 58). In the absence of specific labelling of the virus, however, it was difficult to observe microencapsulated material.

Figure 56

Scanning electron micrograph of a TEM section of PLG microparticles containing equine influenza virus.

Figure 57

Electron micrograph of sectioned PLG microparticles containing equine influenza virus, showing a homogenous internal matrix. Magnification x 26,150

Figure 56

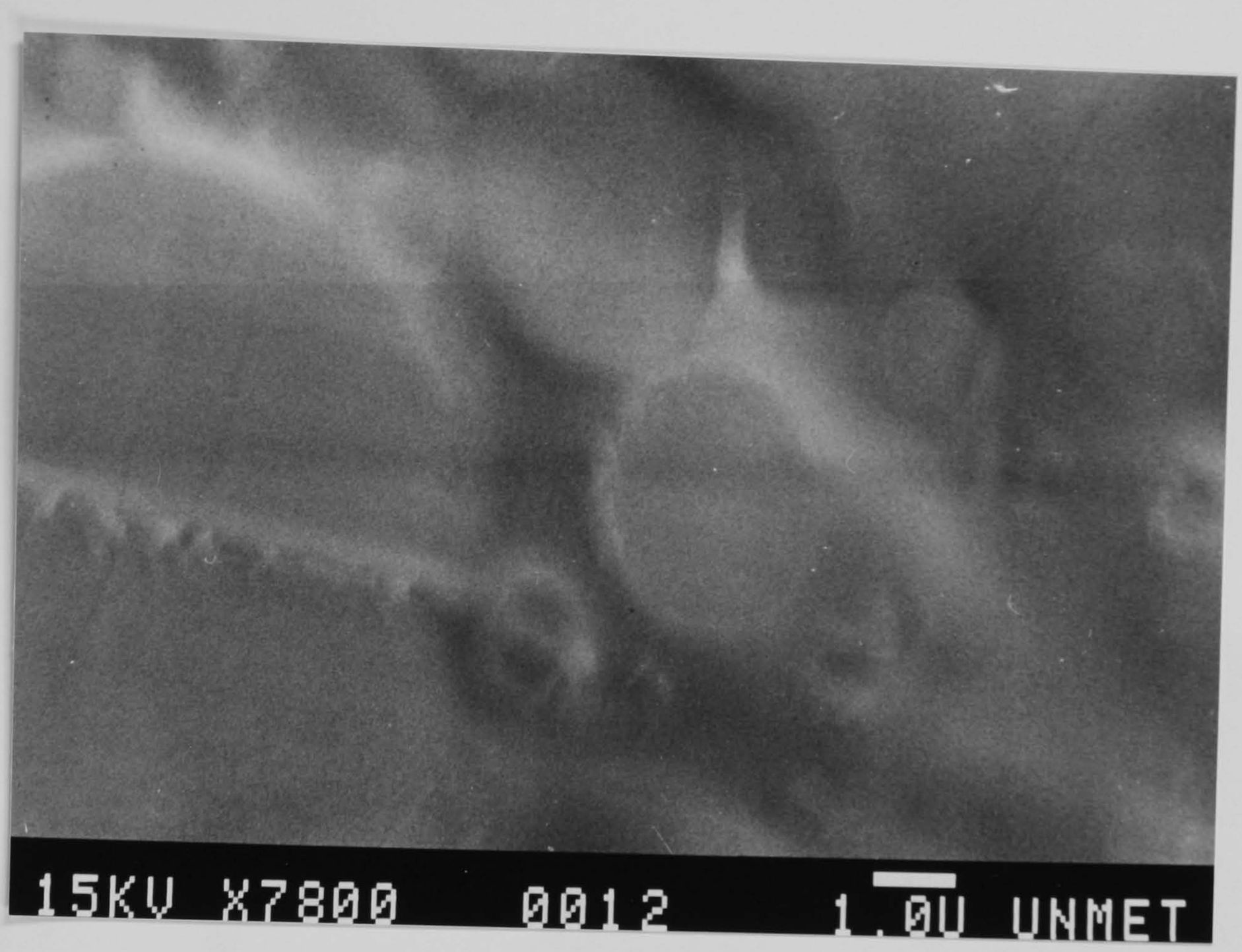


Figure 57

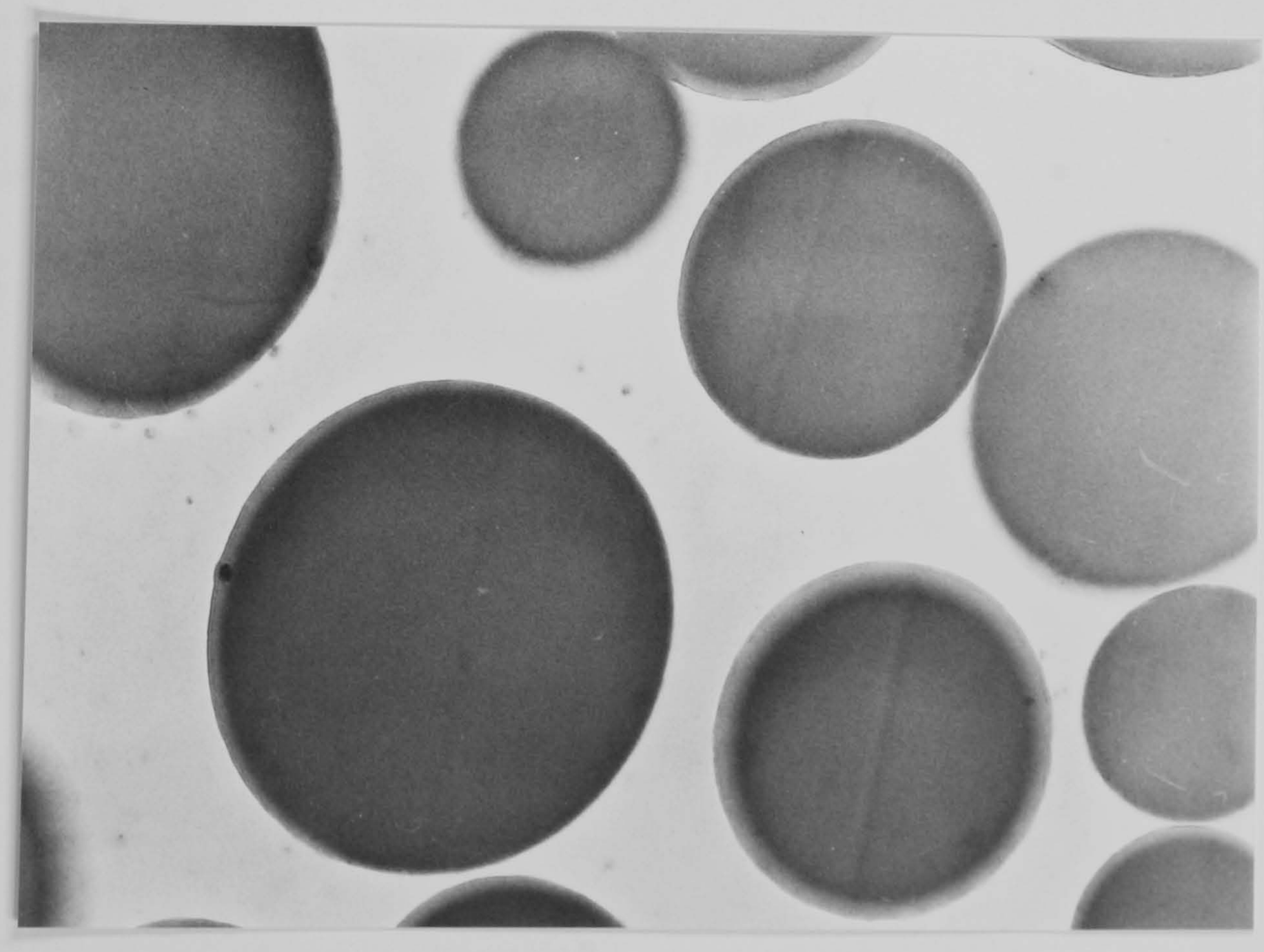


Figure 58

Electron micrograph of PLG microparticles (containing equine influenza virus) showing the equine influenza (V) on the outside. Magnification x 54,980

5.4.2 Determination of the Amount of Entrapped Virus in PLG Microparticles

The amount of influenza virus entrapped in the PLG microparticles was determined using a bicinchoninic acid protein assay (BCA, Smith *et al*. 1985). The basis of the assay is a two step reaction (a) the reduction of Cu^{2+} to Cu^{1+} ions by protein in an alkaline environment and (b) the formation of a purple complex between the resulting Cu^{1+} ions and bicinchoninic acid which has an absorbance maximum at 562nm. The intensity of this purple complex can be related to protein concentration using a standard calibration curve.

In order to measure the amount of viral material in PLG microparticles, the encapsulated virus was released by digesting the PLG microparticles. This was achieved by incubating the PLG microparticles in a solution of 0.1M sodium hydroxide containing 5% SDS (p.H. 7.4) on a shaker system overnight.

BCA assay procedure for determining the amount of influenza virus in PLG microparticles:

(a) 15-25mgs of freeze dried microparticles were added to 1 ml of a solution of 0.1M NaOH/5% SDS (performed in triplicate) and shaken at room temperature overnight. The assay was also performed on blank (protein-free) PLG microparticle.

(b) A dilution series of influenza virus was prepared as a standard using the same virus used to prepare the microparticles (prague) in 0.1M/5% SDS (100μg/ml - 800μg/ml).

(c) 2 ml of BCA solution (50ml BCA + 1 ml copper sulphate) was added to 0.1 ml of microparticle suspension and standards (all tests were performed in duplicate). 0.1 ml samples of 0.1M NaOH/5% SDS digestion medium were also tested.

(d) The samples were incubated in a water bath set at 60°C for 25 minutes and allowed to cool.

(e) The samples were centrifuged at 4,000 r.p.m for 5 minutes to sediment the digested microparticles.

(f) The supernatant was collected and the absorbance measured in a spectrophotometer set at wavelength 562nm.

A standard curve was plotted from the dilution series of influenza virus and the amount of virus in the assay sample was determined. This was then used to calculate the amount of virus associated with the total microparticle sample.

Example:

Weight of PLG microparticle containing virus used for assay = 13.7 mg.

Amount of virus (calculated from standard curve) = 49.05μg in 0.1 ml (volume used for assay).

1 ml of digestion medium was used, therefore, 490.5μg of virus are contained in 13.7mg of microparticles.

$$\frac{\textit{total protein}}{\textit{weight of sample}} \times 100 = \%\ w/w.$$

$$e.g. \quad \frac{490.5}{13700} \times 100 = 3.58\% \text{ weight of virus/weight of PLG.}$$

The same calculation was performed for the blank PLG microparticles and this was subtracted from the protein content figure to allow for any absorbance reading due to PLG.

Initially the Lowry-Hess (1977) protein assay was used to measure the amount of virus contained in PLG microparticles. However, it was found that protein free PLG blanks were giving comparable absorbance readings to the virus containing PLG microparticles. It was apparent that some constituent used in the microparticle formulation process was interfering with the assay. Polyvinyl alcohol was subsequently found to give a strong absorbance reading indicating that PVA was present in the PLG microparticle preparation. Polyvinyl alcohol did not give a significant absorbance reading when the BCA assay was used to estimate protein content.

Considerable problems were encountered when using the BCA assay to measure the viral content. Initially the PLG remnant was removed following digestion by centrifuging and the BCA assay was performed on the supernatant. However, no absorbance reading was obtained on analysis of the supernatant. It was later found that the virus was associated with the PLG sediment (refer to section 5.6). The protocol was subsequently adapted; removing the PLG sediment only after the BCA colour reaction had taken place. The level of viral entrapment varied between 0.25%

- 3.6% (weight of virus/weight of polymer) and was found to be dependent on the size of the microparticles (refer to section 5.6).

5.5 INVESTIGATION OF THE ANTIGENIC INTEGRITY OF THE EQUINE INFLUENZA VIRUS AFTER MICROENCAPSULATION USING THE SINGLE-RADIAL-IMMUNODIFFUSION ASSAY

5.5.1 Introduction

Influenza is an orthomyxovirus, existing of 3 types A, B and C. They are spherical in shape composed of a central capsid containing ribonucleoproteins and an outer capsid composed of protruding spikes of either haemagglutinin or neuraminidase. The haemagglutinin antigen on the surface of influenza, is involved in cell attachment and is the main antigen to which neutralising antibodies are formed to confer immunity. The success of a microencapsulated vaccine against influenza depends on the antigenic integrity of these epitopes remaining intact during the microencapsulation process. During the solvent evaporation process the virus may be subjected to high shearing forces as well as solvent exposure (Dichloromethane).

The single-radial-diffusion (SRD) assay was used to investigate antigen integrity. This assay measures the amount of haemagglutinin in influenza preparations expressed as micrograms of antigen activity (Wood *et al.* 1983). The assay is based on an antigen (haemagglutinin epitopes) /antibody (specific to the HA) interaction. The SRD system comprises of an agarose gel containing the specific antiserum to which a influenza virus, disrupted by treatment with detergent, is added in wells cut in the agarose. As

the virus diffuses out of the wells a radially produced precipitation zone is formed, as a result of the HA of the virus reacting with the anti-HA, the size of which correlates to the amount of haemagglutinin in the influenza virus preparation. The assay, therefore, works for influenza retaining haemagglutinin activity. Using this principle, an SRD test performed on influenza virus before and after microencapsulation would, therefore, indicate loss of haemagglutinin antigenicity.

Experiments were performed to investigate the antigenic integrity of PLG microencapsulated influenza virus after production by solvent evaporation. To investigate the effect of time of microencapsulation, various microparticle preparations were incubated in PBS at 37°C and SRD tests were performed at various time points. The aim of the investigation was to determine whether antigen integrity was maintained after microencapsulation. No quantification of virus content was attempted.

5.5.2 **Materials and Methods**

Preparation of PLG microparticles containing equine influenza virus

Two PLG polymers, resomer 858 (85:15 lactide-glycolide ratio,53kD) and resomer 503 (50:50,9kD) (Boehringer, Germany) were used to microencapsulate equine influenza, prague strain (A/Equine/Prague/56) (the virus was kindly supplied by NIBSC, Potters Bar, England). The microparticles were produced using the method described in section 5.3. for the preparation of submicron microparticles.

Incubation of PLG microparticles containing equine influenza virus

Test-tubes containing 10-20 mg of freeze dried microparticles were dispersed in 3 mls of phosphate buffered saline (p.H. 7.4) and kept at 37°C. Tubes were removed at 0, 1 week, 6 weeks, 11 weeks and 12 weeks and stored at -20°C. SRD tests were then performed on each sample. A substantial amount of virus was surface associated as revealed by *in vitro* release studies (refer to section 5.6). Therefore, in order to have sufficient virus to perform the SRD test both the microparticle suspension and the supernatant was used for the SRD assay.

The single-radial-immunodiffusion procedure (adapted from the method used at NIBSC, Potters Bar, England) was conducted at NIBSC under direction of Dr. J. Wood.

Antigenic reagents:

Influenza virus used for microencapsulation (A/Equine/Prague/56 (H7N7) 2707μgHA/ml NIBSC ref. H10).

Standard influenza virus (A/Equine/Prague/56 (H7N7) 110μgHA/ml NIBSC ref. 85/553).

Anti-influenza virus serum: Goat anti-A/Equine/Prague/56 HA. NIBSC ref. 84/673.

For preparation of the reagents used in the SRD test refer to Appendix 2.

(a) Two glass plate (12 x 12cm) was cleaned using a detergent (Decon) and dried.

(b) Agarose was melted in a boiling water bath and dispensed into prewarmed glass universals in a 50°C water bath (14 ml agarose per plate).

(c) Hot agarose was smeared on the glass plates using a tissue and allowed to dry.

(d) A perspex mould (internal diameter 9 cm) was placed on each glass plate and the edges sealed with hot agarose. This was left to dry for 5 mins.

(e) The 84/673 antiserum was added to the agarose (7.5ul per ml agarose), gently rotated by hand, and poured into the mould on a level surface (14 mls per plate). Bubbles in the agarose were removed using a Pasteur pipette. The agarose was left for 10 minutes before removing the mould.

(f) 5 ul of detergent (Zwittergent) was added to 45 ul of microparticle suspension in a multiwell plate and shaken for 30 minutes at room temperature. A standard prague sample (85/553) and a sample of the prague virus used for microencapsulation were used as reference samples and diluted to the same concentration with phosphate buffered saline (these were also disrupted with detergent).

(g) A metal hole punch (5 mm diameter) was used to punch a series of wells. The gel in each hole was removed using a Pasteur pipette attached to a vacuum.

(f) 20 uls of sample was introduced into the wells.

(g) The plates were covered by a perspex lid and placed in a plastic container lined with moist blue roll tissue. Diffusion was allowed to take place for a minimum of 18 hours at room temperature.

(h) After 18 hours the plates were washed under tap water. The plate was then placed on paper towels and the gel covered with filter paper (Whatman 1, 15 cm.). Lint was then placed on top and finally a metal weight (approx. 650 gms) for 15 minutes to tightly compress the gel to the glass plate.

(i) The weight and lint were removed and the gel (with filter paper attached) dried under warm air at 37°C. When dried (approximately 2 hours), the filter paper was removed and the gel stained in Coomassie Blue stain for 15 minutes. The gel was then destained in methanol-acetic acid until the blue precipitation zones were evident. The plates were allowed to dry and viewed with a magnifying lens.

5.5.3 **Results and Discussion**

Only the standard influenza virus sample (85/553) and the week 12, 503 microparticle sample gave a positive SRD reaction (refer to Figure 59). Of the two polymers used to encapsulate the virus for this study, 503 is the faster degrading. At 12 weeks the 503 microparticle sample was clear indicating that the PLG had totally degraded whereas all the other microparticulate samples showed a cloudy appearance due to non-degraded PLG. It would appear that the presence of a PLG suspension interferes with the SRD reaction possibly restricting the diffusion of the influenza virus through the agarose gel. The colour reaction inside the wells indicates a non-specific reaction between the PLG suspension and the Coomassie stain. On close examination, using an eye lens, the ring evident in the week 12, 503 sample was fainter than the reference standard and was slightly crenated which may indicate slight disruption of the HA during microencapsulation, although a level of antigenicity remains. Factors accounting for the faint ring could be either (a) trace remains of PLG interfering with the reaction or (b) the shift of pH found in the closed *in vitro* system in which the microparticles were kept; over the incubation period the pH of the external medium changed from 7.4 (0 weeks) to approximately 3.0 (12 weeks)(see section 5.6). The haemagglutonin spikes can be disrupted at this pH (Dr John Wood, NIBSC, Personal communication). Earlier a series of SRD tests were performed on unencapsulated equine influenza virus supplied by Behringwerke, Germany. A negative result was achieved, however, due to the high level of formalin used to inactivate the virus (which cross-linked the haemagglutinin spikes). For this reason virus inactivated with lower formalin concentrations was used in the studies described.

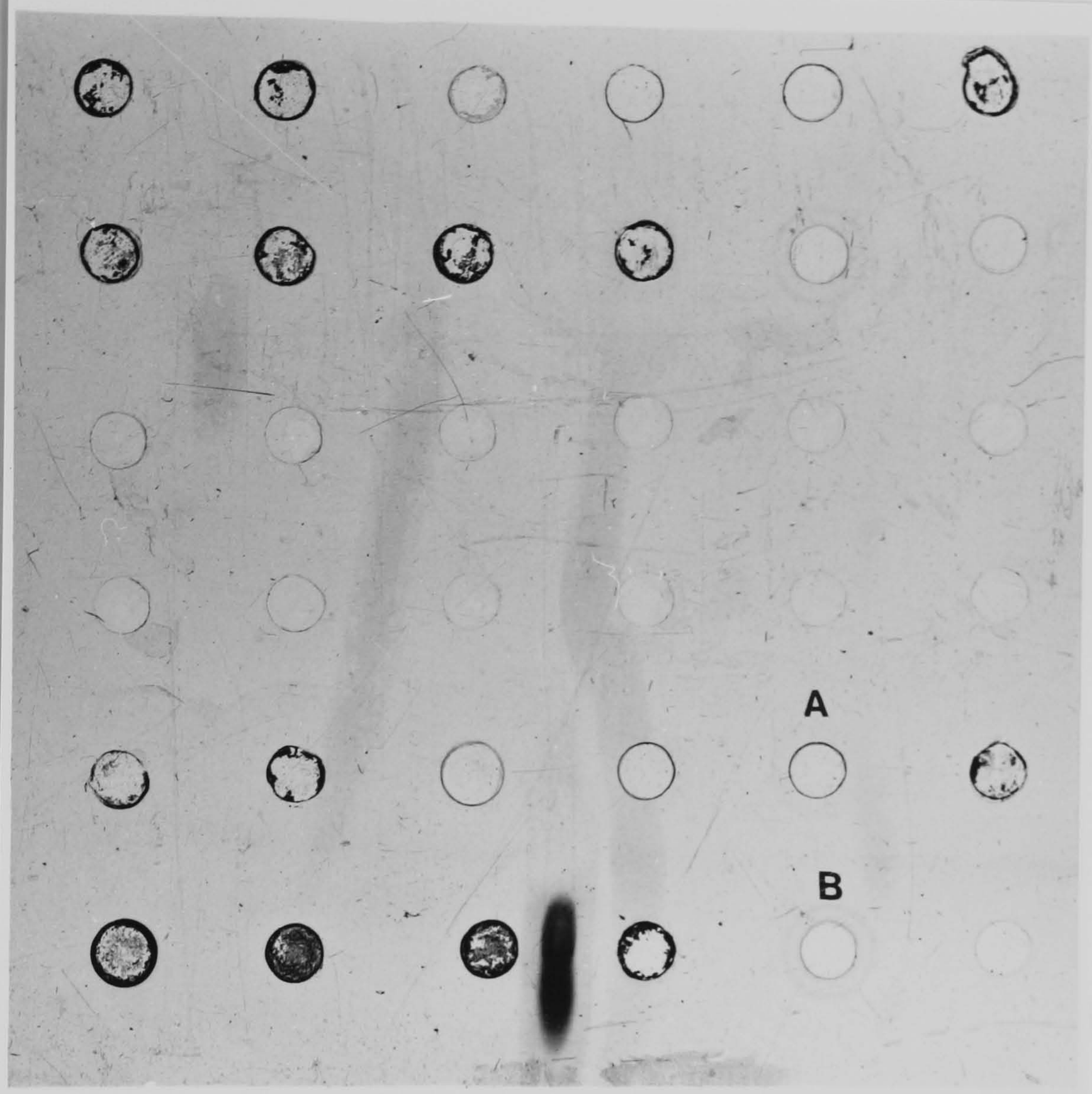


Figure 59

Single-radial-immunodiffusion plate showing precipitation zones (positive reaction) for A, PLG (resomer 503) microencapsulated 12 week sample and B, control aqueous equine influenza sample.

The results suggest that haemagglutinin activity remains after microencapsulation but there may be a slight loss which may be attributed to remaining PLG or the limitations in the *in vitro* system used. To routinely use the SRD test, however, methods to completely remove residual PLG must be sought.

In summary, PLG microparticles of various sizes, containing equine influenza virus were prepared. These microparticles were both spherical and smooth, showing an homogenous internal matrix. The antigen integrity of the haemagglutinin spikes on the virus was found to remain after microencapsulation. The levels of entrapment indicated that these microparticles could be useful as an antigen delivery system for influenza virus. First, however, it was important to investigate the *in vitro* release of influenza from the PLG microparticles to determine the virus release pattern.

5.6 INVESTIGATION OF THE *IN-VITRO* RELEASE OF EQUINE INFLUENZA FROM PLG MICROPARTICLES

5.6.1 Introduction

The release of antigen from PLG microparticles depends on the degradation of the polymer and the diffusion of the antigen through the matrix. The importance of diffusion on release depends on the permeability of the entrapped material within the polymer matrix. Peptides and proteins have low permeability through the PLG polymer and are the principle factor in their release is the degradation of the polymer matrix (Tice and Cowsar 1984, and Cohen *et al*. 1991).

The rate of degradation of PLG depends on a number of factors, principally of which are the molecular weight of the polymer and the lactide/glycolide ratio. PLG is broken down by a hydrolytic cleavage of the ester bonds linking the polymer to give lactic and glycolic acid (Tice and Gilley 1984). High molecular weight polymers require more hydrolysis to break down the long chain molecules resulting in a lower rate of degradation compared with low molecular weight polymers (Wang *et al.* 1990). The lactide to glycolide ratio in the polymer also determines the rate of hydrolysis and subsequently the rate of degradation since the methyl groups on the lactide segments in the cytoplasm tend to shield the ester groups from hydrolysis. Miller *et al.* 1977 found that a polymer composed of 100% lactide degraded in 6 months *in vivo*. If the glycolide content was increased the degradation rate increased; a 50:50 ratio polymer degraded in 1 week. The glycolide units in the copolymer are more hydrophillic than the lactide unit and, therefore, increase the amount of water uptake (Gilding and Reed 1979, Hollinger and Battistone 1986). Microparticles composed of a polymer with a higher lactide content will result in a slower rate of degradation (Wang *et al.* 1990 and Splenlehauer *et al.* 1989). Size factors must also be taken into account in that smaller microparticles present a larger surface area for hydrolytic attack and water uptake which will influence microparticle degradation. PLG microparticles of different composition (lactide/glycolide ratio) and molecular weight should release antigen at different rates and over varying time periods. The significance of this is that microparticles composed of different polymer compositions may be formulated to release antigen at predetermined times, giving a pulse release profile or continuous release.

To investigate the release profiles of equine influenza virus from PLG microparticles, an *in-vitro* release study was conducted using polymers of different compositions (lactide/glycolide ratio) and molecular weight.

5.6.2 **Materials and Methods**

Several batches of PLG microparticles containing the equine influenza virus (praque strain supplied by Behringwerke, Germany) were produced using the solvent evaporation method (refer to Table 11). Blank PLG microparticle containing no virus were also produced from each of the PLG polymers under investigation (Boehringer, Germany).

Test-tubes containing 10-20 mg of freeze dried microparticles dispersed in 3 mls of phosphate buffered saline (PBS, p.H 7.4) were placed in a water bath at 37°C. The test-tubes were shaken periodically to keep the microparticles in suspension. At regular time intervals test-tubes were removed and assayed for protein content using a bicinchoninic acid protein assay as described in section 5.4.2. From these results the percentage of equine influenza released at each sample time (percentage of the original protein content) was calculated and plotted against time to give the cumulative release profiles. To investigate the morphology changes of the microparticles over time, samples taken from the *in-vitro* test-tubes were washed in distilled water (to remove the PBS) and viewed under a scanning electron microscope (JOEL 6400 Winsem).

Table 11 Microparticles encapsulating equine influenza virus using different Poly(lactide-co-glycolide) polymers.

Batch	Lactide/Glycolide	%Influenza encapsulated %w/w	Particle size(μm)
6	50/50(503,9kD)	1.13	0.5
4	50/50(505,18kD)	0.8	1.0
8	85/15(858,53kD)	1.02	1.0
9	50/50(858,53kD)	0.54	0.2-1.0
10*	85/15(858,53kD)	1.0	0.2-1.0

*NIBSC inactivated virus

5.6.3 Results of the *In-Vitro* Release Studies

Polymer - PLG 50:50 (lactide/glycolide)(9kD) Resomer 503

At time zero 65% of the virus content was detected by the BCA method (refer to Figure 60), which indicated the presence of virus adsorbed on the surface of the microparticles. The amount of virus then decreased over the next 8 hours. The 50:50 (lactide/glycolide), low molecular weight polymer was expected to degrade rapidly and release encapsulated virus over a short time scale. Figure 61, shows a temporal release of the virus but even after 12 weeks only 70% of the original virus content was released. Under the SEM (refer to Figure 62), pores were apparent on the surface of microparticles after 2 weeks and by 12 weeks total degradation had taken place. Thus, 100% release of the virus was anticipated by 12 weeks.

Polymer - 50:50 (18kD) Resomer 505

The profile in Figure 63 shows that 55% of the virus was released at time zero which indicated the disassociation of adsorbed surface virus from the microparticles. Being a higher molecular weight polymer the degradation rate was expected to be slower than the 503 resomer. Under the SEM (refer to Figure 64) surface degradation was not apparent until after 3 weeks. 100% of virus was detected by the BCA assay at 4 weeks and then the amount declined thereafter. However, a controlled release of virus appears to be taking place over 4 weeks in vitro.

Polymer - 85:15 (53kD) Resomer 858

The 85:15 copolymer was expected to degrade at a lower rate than the other two copolymers due to the higher lactide content and high molecular weight. SEM

examination showed that the microparticles morphology appeared unaltered even after 24 weeks, indicative of a very slow degradation rate (refer to Figure 65). The initial 28-50% detected by the BCA assay of virus at time zero (refer to Figure 66-67) was attributed to surface associated virus. The level of release did not reach 100%. The difference in the profiles obtained with batch 8 and 10 could be due to the origin of the virus Batch 10 virus was supplied from NIBSC using a low level of formalin for inactivation.

5.6.4 **Discussion**

An initial "burst" release of virus was apparent for all the microparticle systems investigated *in vitro*. A similar phenomenon has been recorded by others investigating the in vitro release of antigen from PLG microparticles; Cohn *et al*. 1991 found an initial burst of FITC-BSA (20-60% cumulative release dependent on the polymer molecular weight. Wang *et al*. (1990) found 70-80% of BSA was released within 1 day from microparticles composed of 50:50 PLG copolymer. Both groups attributed this initial burst of release to loosely bound surface protein. The results from the present studies indicate the presence of surface protein which is supported by SEM evidence. No detectable morphological deterioration or pore formation (which could account for virus release) was observed in any of the microparticles within the first week. The SEM evidence supported the theory that degradation decreases in PLG polymers with increased lactide content and higher molecular weight (degradation 50:50,9kD greater than 50:50,18kD which is greater than 85:15,53kD).

Only the 505 resomer *in vitro* system released 100% of the entrapped virus, where a controlled release of virus took place upto 4 weeks. The 858 microparticles did not show any morphological changes after 24 weeks indicating that complete release of virus would occur only after this period. The fact that total release was not detected in the 503 resomer, although SEM evidence showed complete degradation of the microparticles after 12 weeks, may indicate that a decrease in sensitivity of the virus to the BCA assay. One factor encountered in the *in vitro* release studies was a marked pH shift in the external medium (PBS) over time (refer to Table 12). This was due to lactic acid formation as the polymer degrades. As expected a marked pH shift was seen in the 503 system compared to the other polymers, indicative of greater degradation to monomers. In the *in vitro* system, each test-tube was sampled rather than aliquots taken and the external medium (PBS) replenished, therefore, the released virus was not removed from the system and may be degraded by the increasingly acid release medium; as previously discussed the influenza can be disrupted at pH 3-4 (Dr John Wood, NIBSC, Personal communication). The optimum pH for the BCA assay is 11.25 (Smith *et. al.* 1985), with an acidic sample the pH of the assay may be altered and diminish the sensitivity of the assay.

The use of an *in vitro* system in which the external medium was changed over time would remove released virus and overcome the problem of pH shift. This condition is a result of the limitation of the *in vitro* system used in these experiments and would not necessarily occur *in vivo*.

Figure 60 Influenza release from 1μm PLG (503,batch 6) microparticles

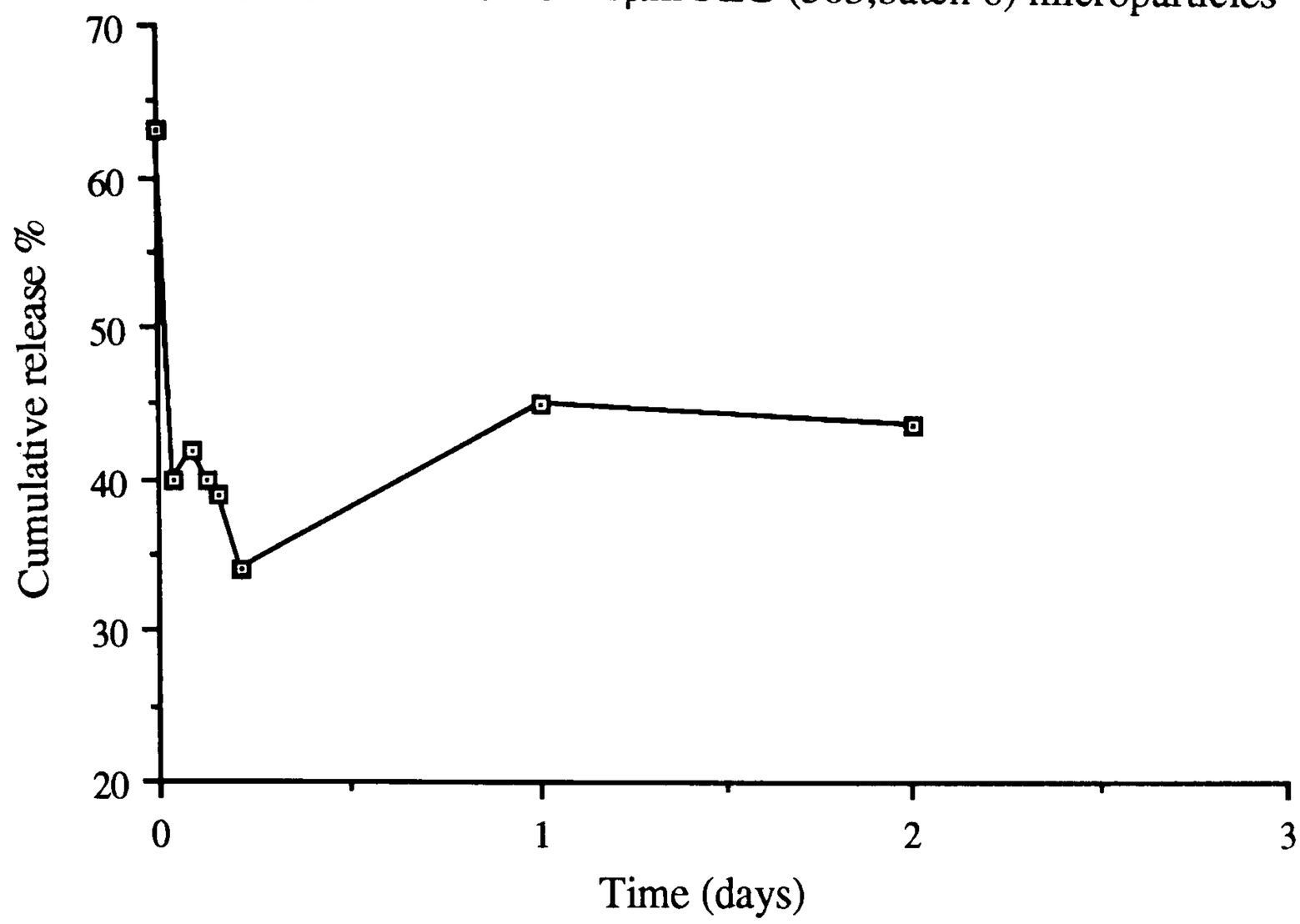


Figure 61 Influenza release from 1μm PLG (503,batch 9, NIBSC) microparticles

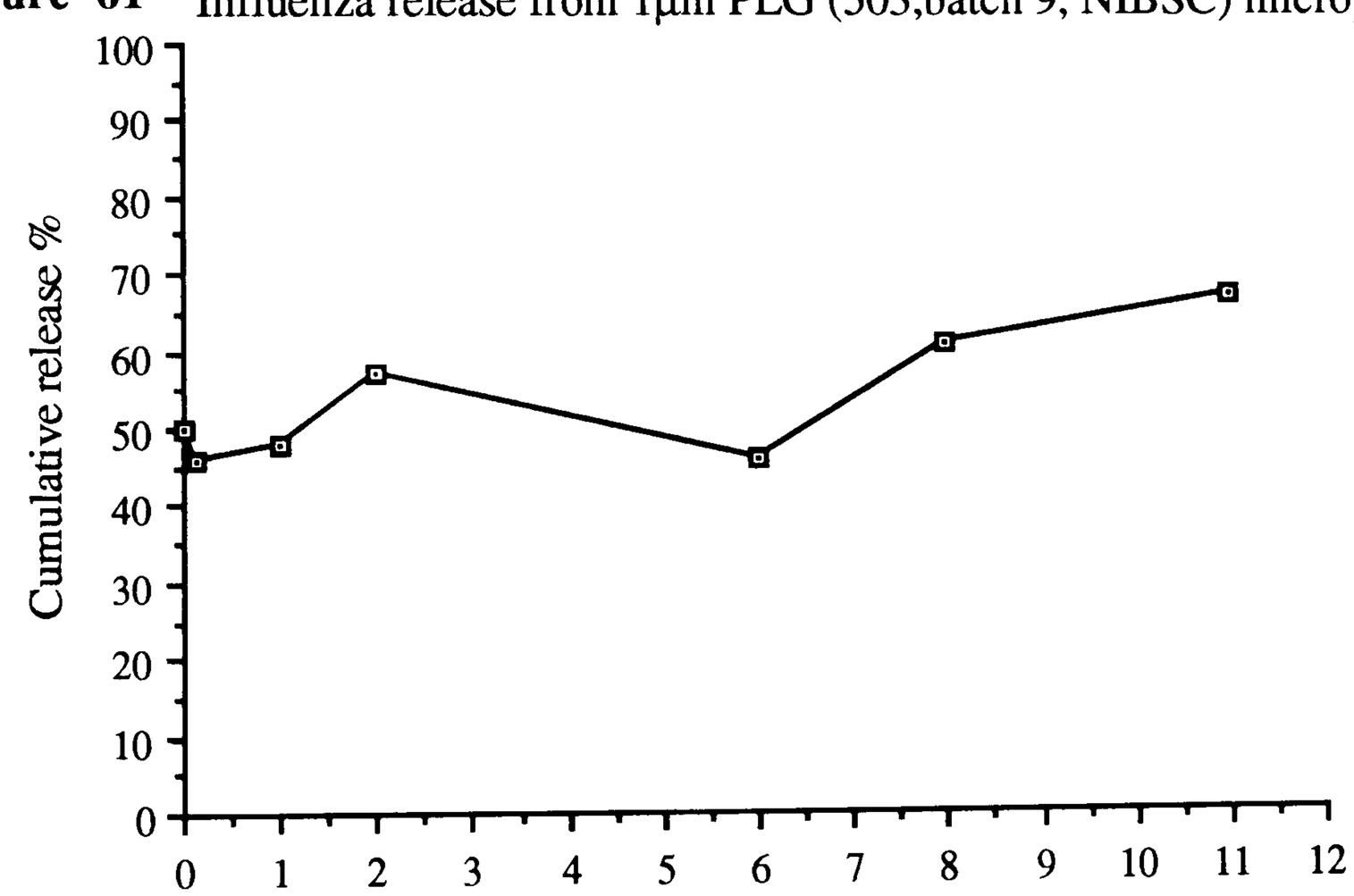
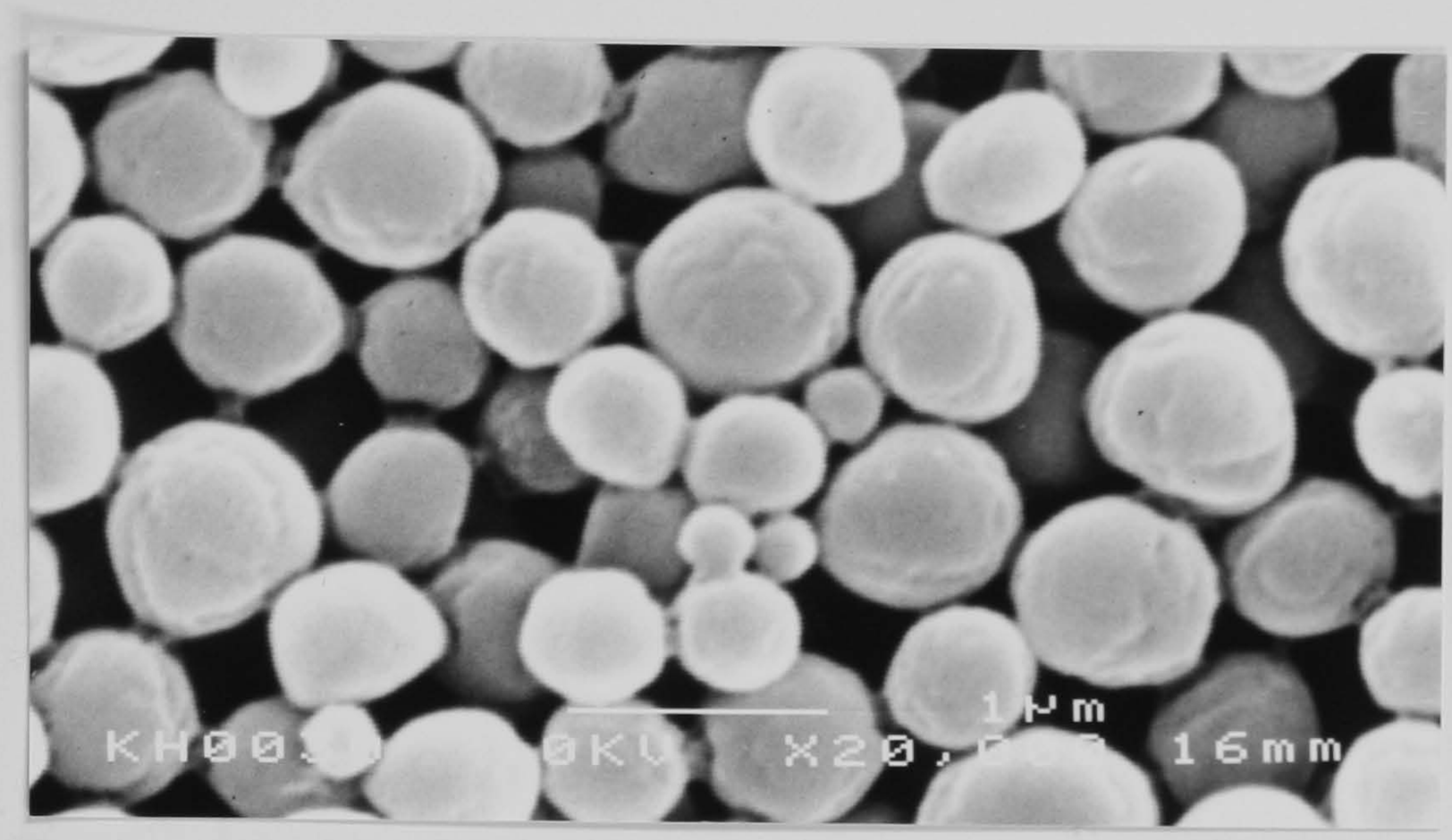
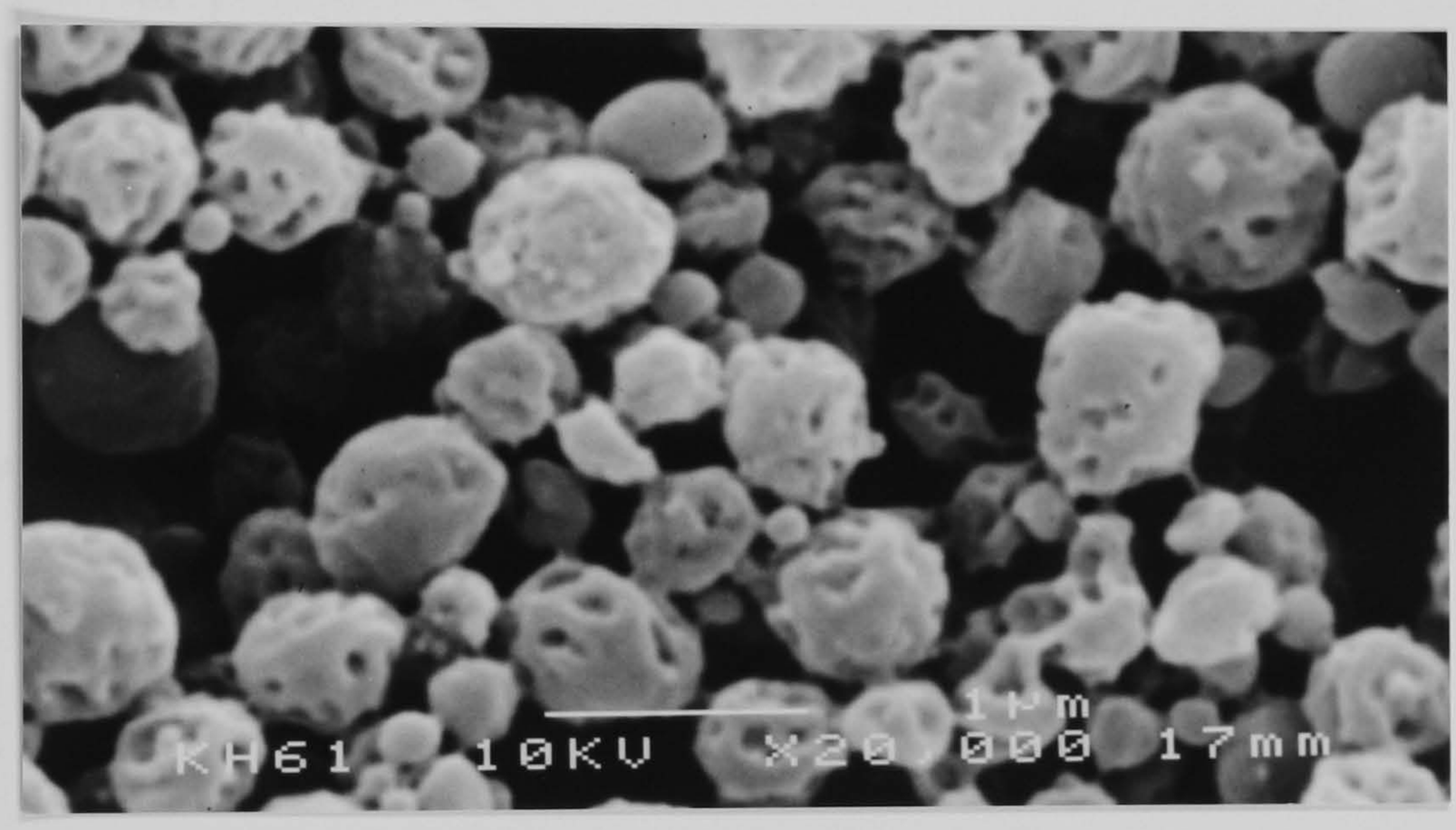


Figure 62

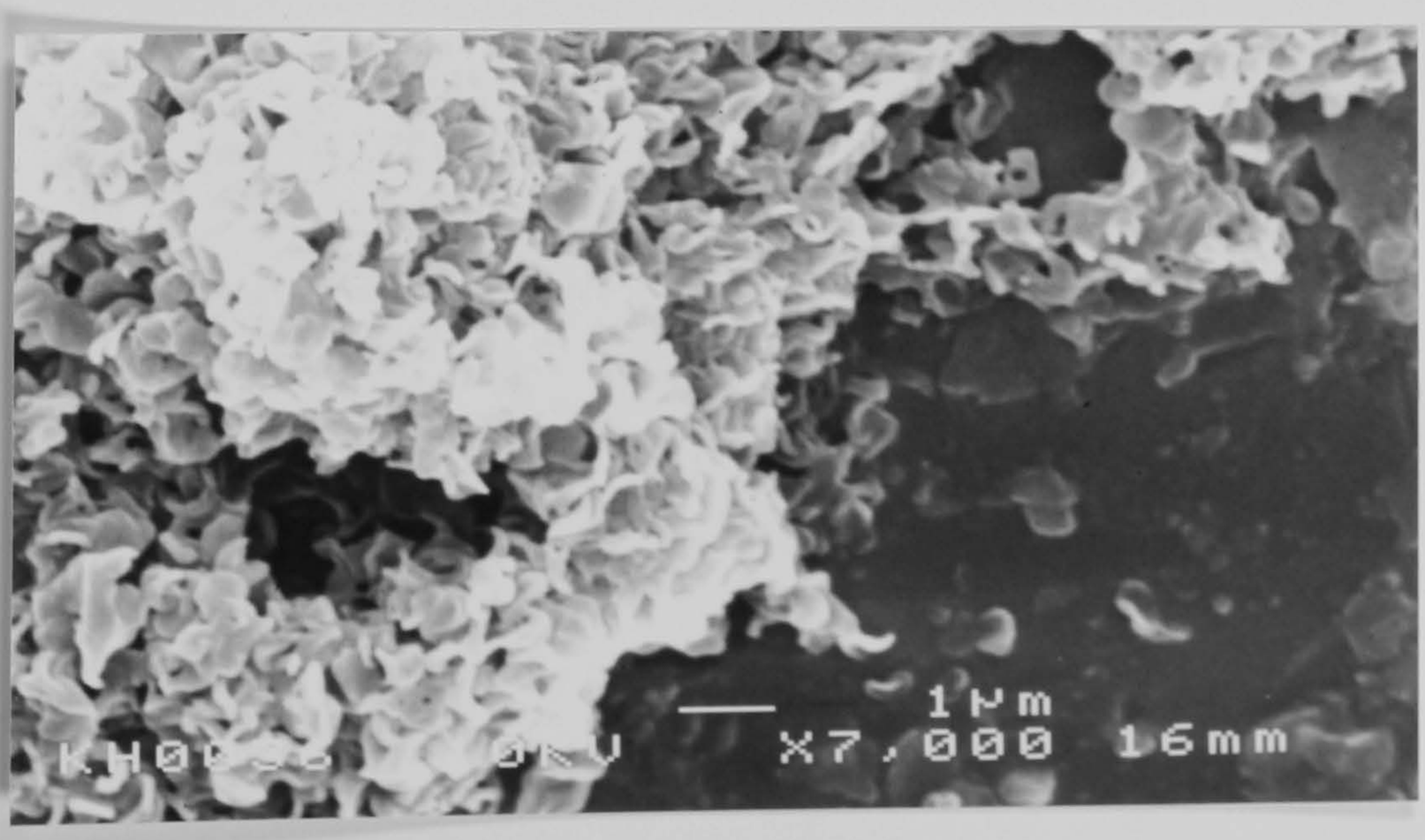
Scanning electron micrographs of PLG microparticles (50:50, resomer 503) containing equine influenza virus following incubation in PBS.



0 Weeks



2 Weeks



8 Weeks

Figure 63 Influenza release from 1μm PLG (505, batch 4) microparticles

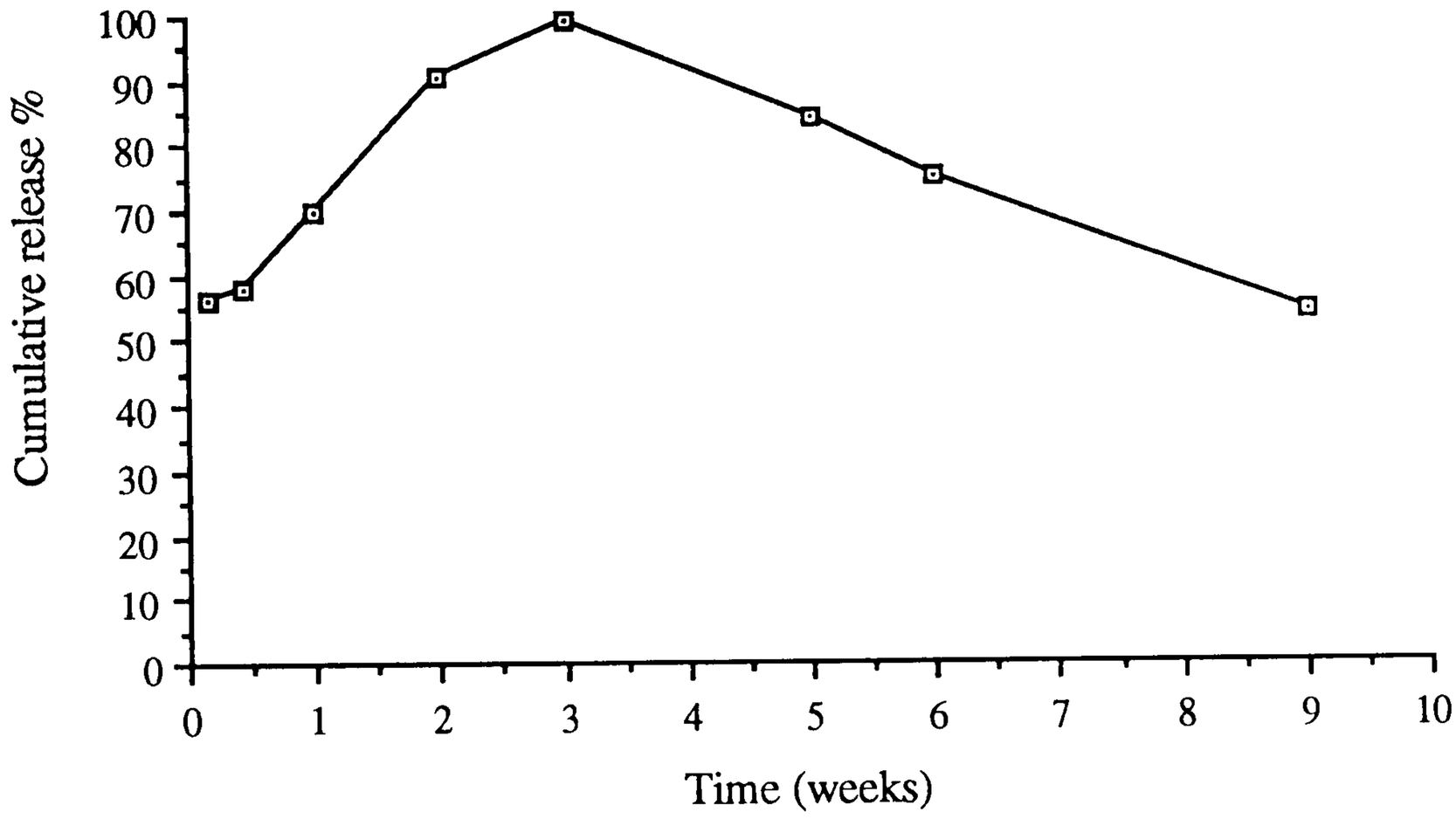
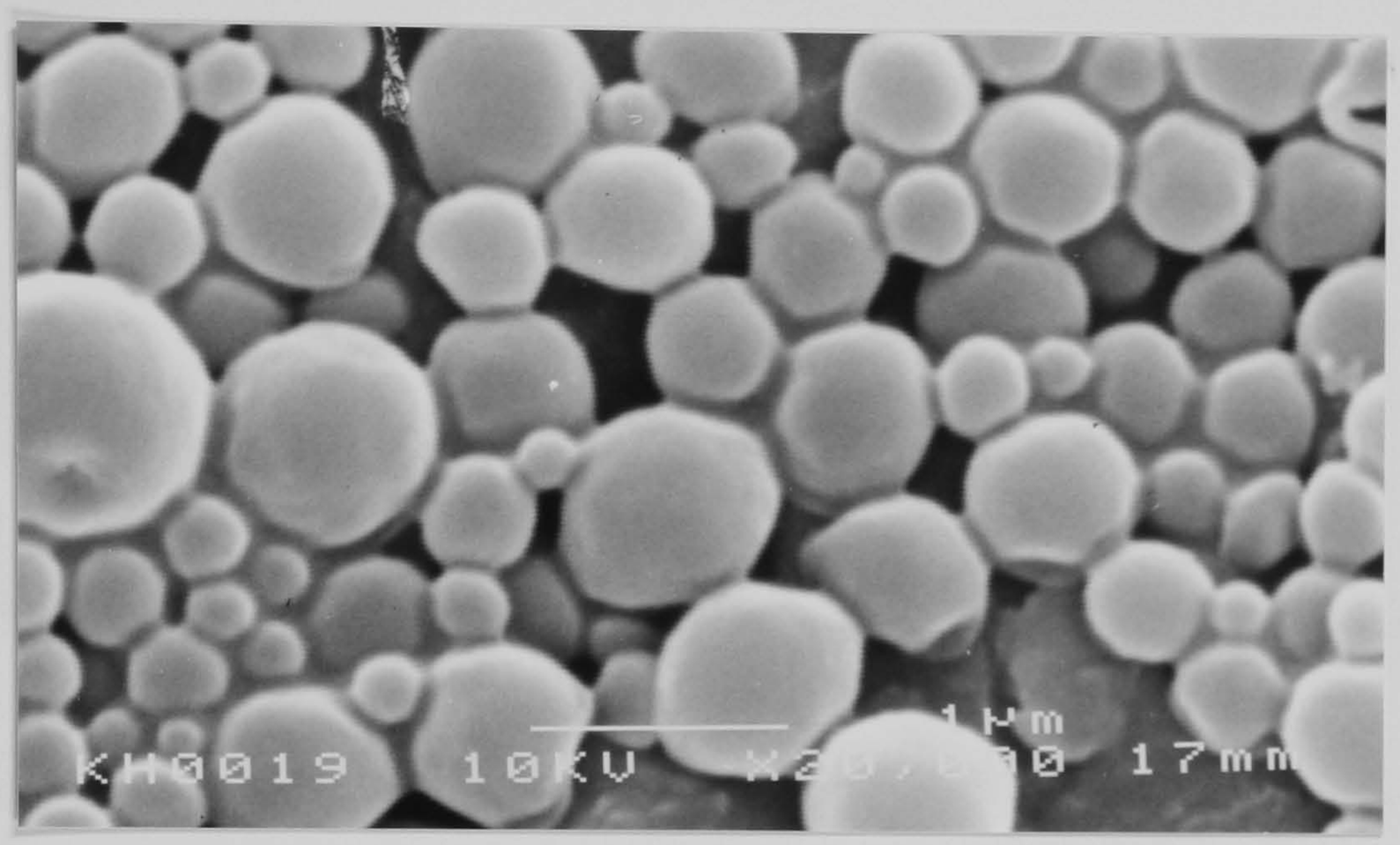
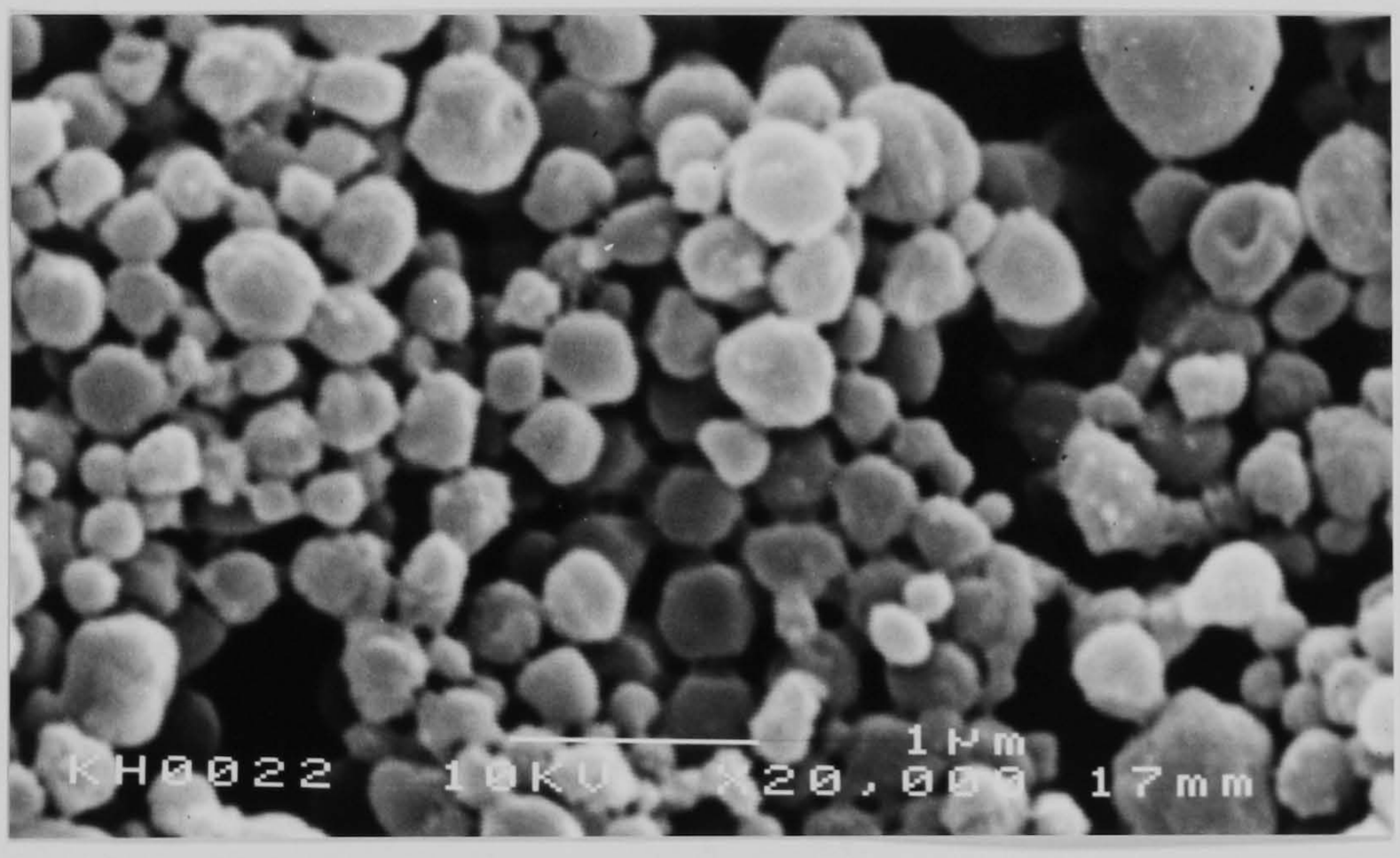


Figure 64

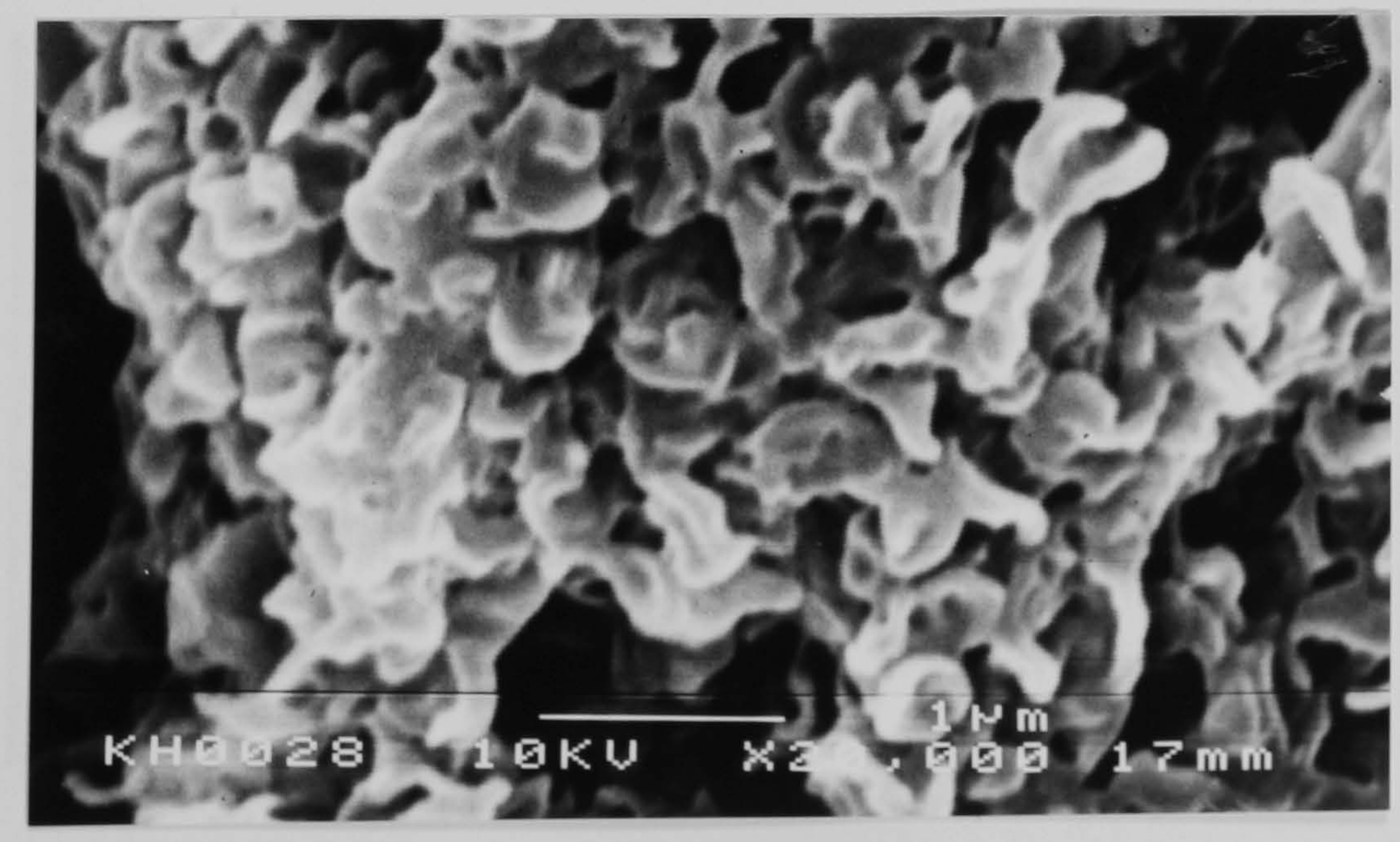
Scanning electron micrographs of PLG microparticles (50:50, resomer 505) containing equine influenza virus following incubation in PBS.



0 Weeks



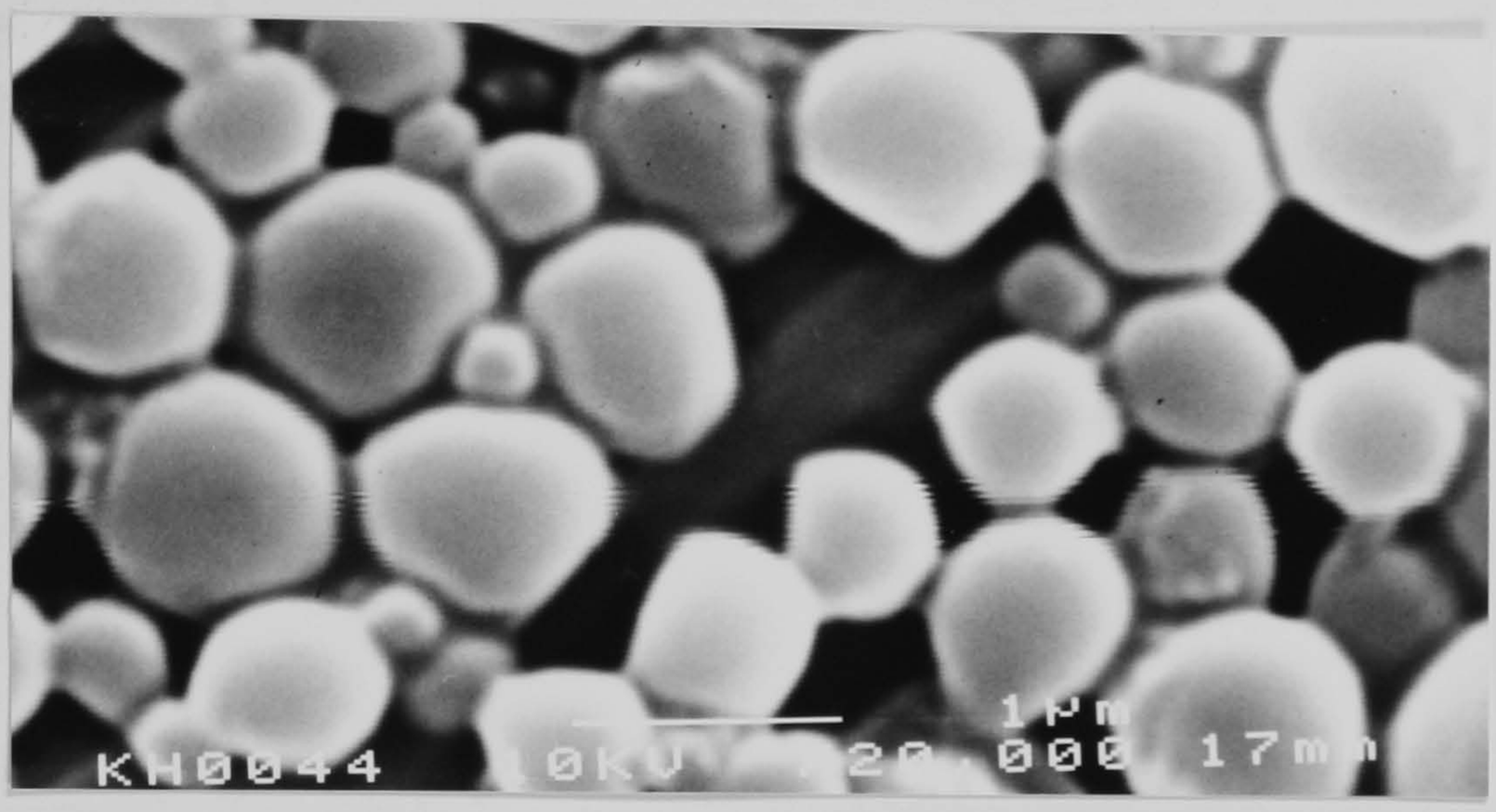
3 Weeks



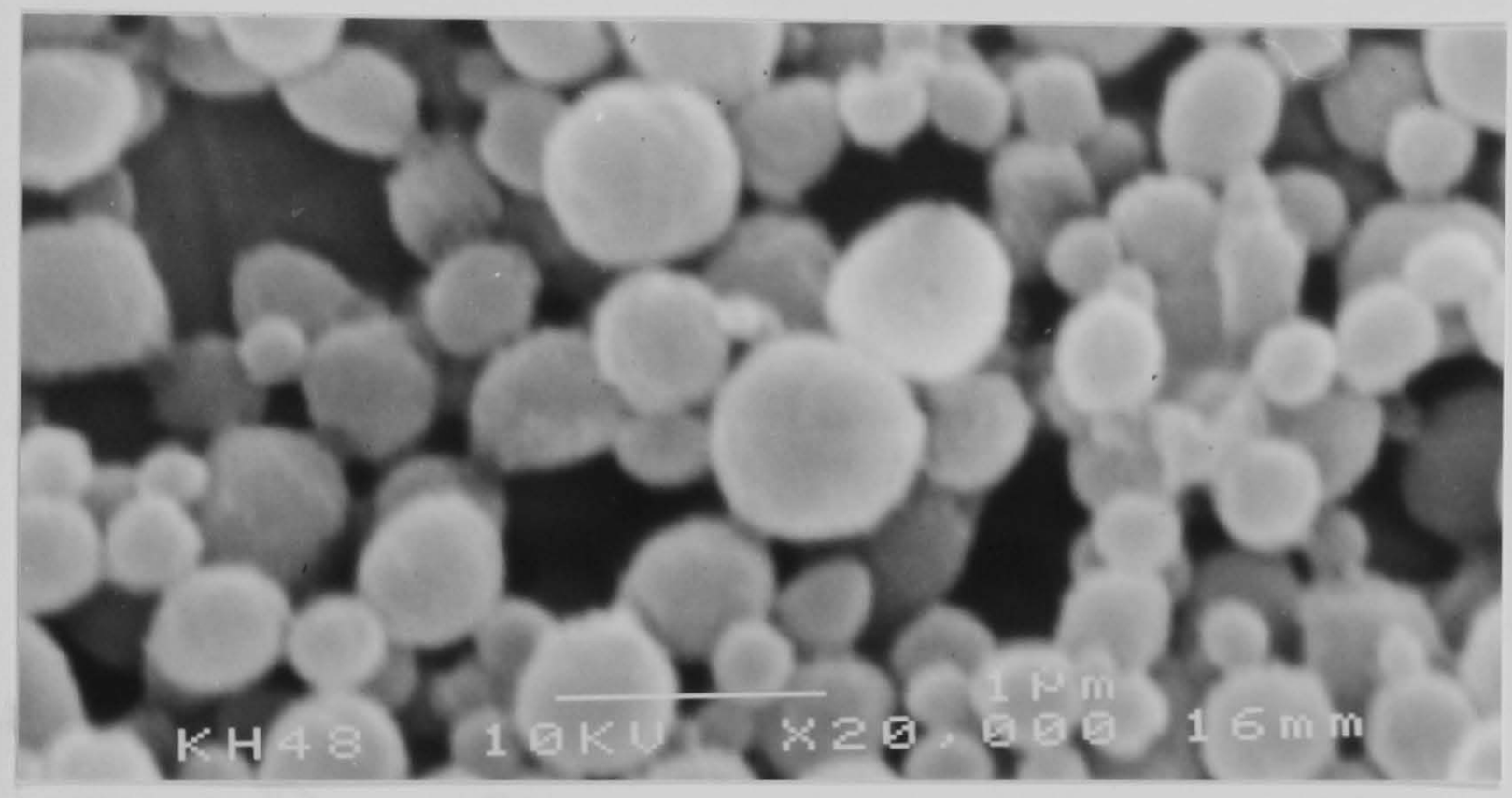
9 Weeks

Figure 65

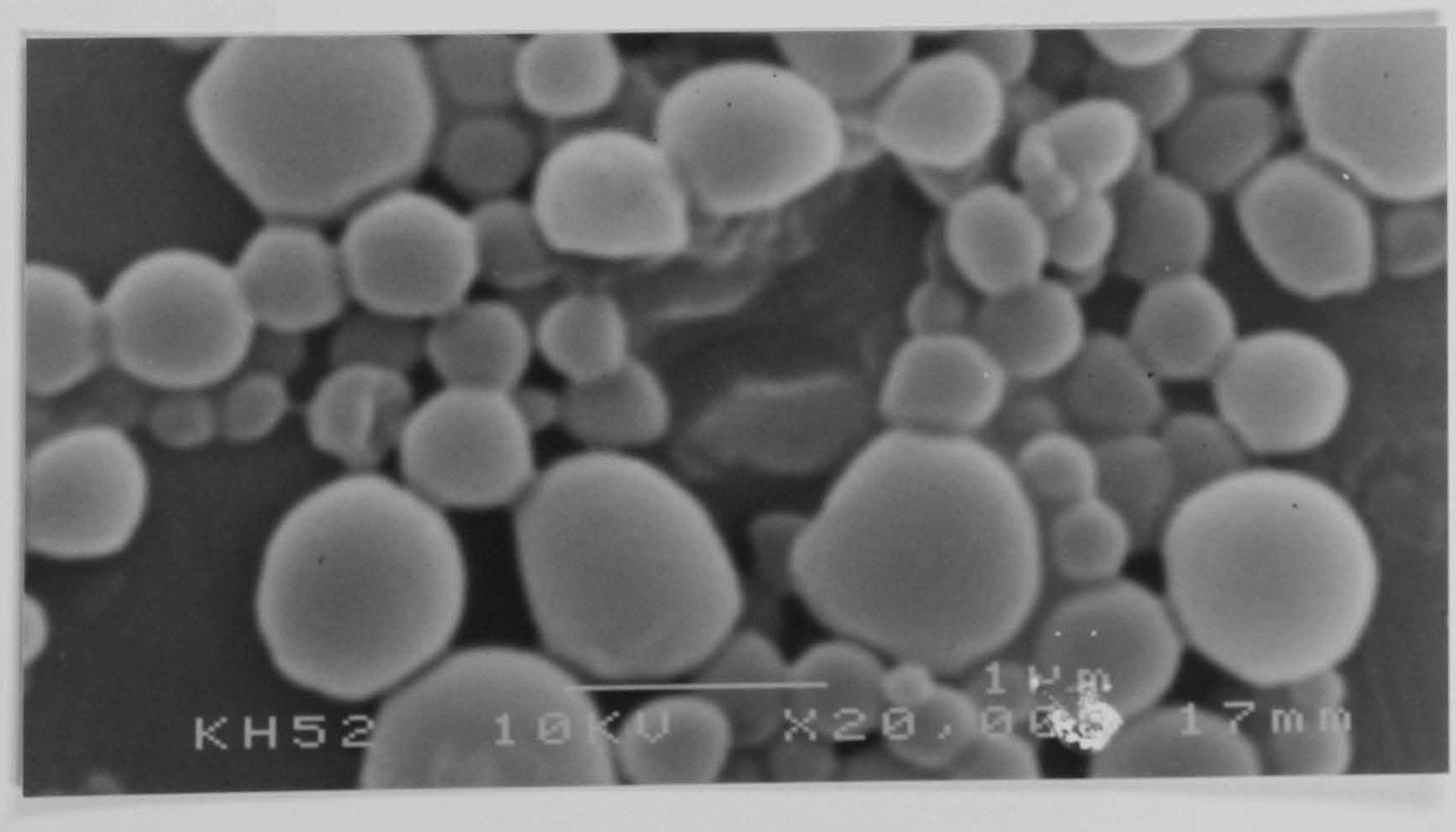
Scanning electron micrographs of PLG microparticles (85:15, resomer 858) containing equine influenza virus following incubation in PBS.



0 Weeks



4 Weeks



24 Weeks

Figure 66 Influenza release from 1μm PLG (858, batch 8) microparticles

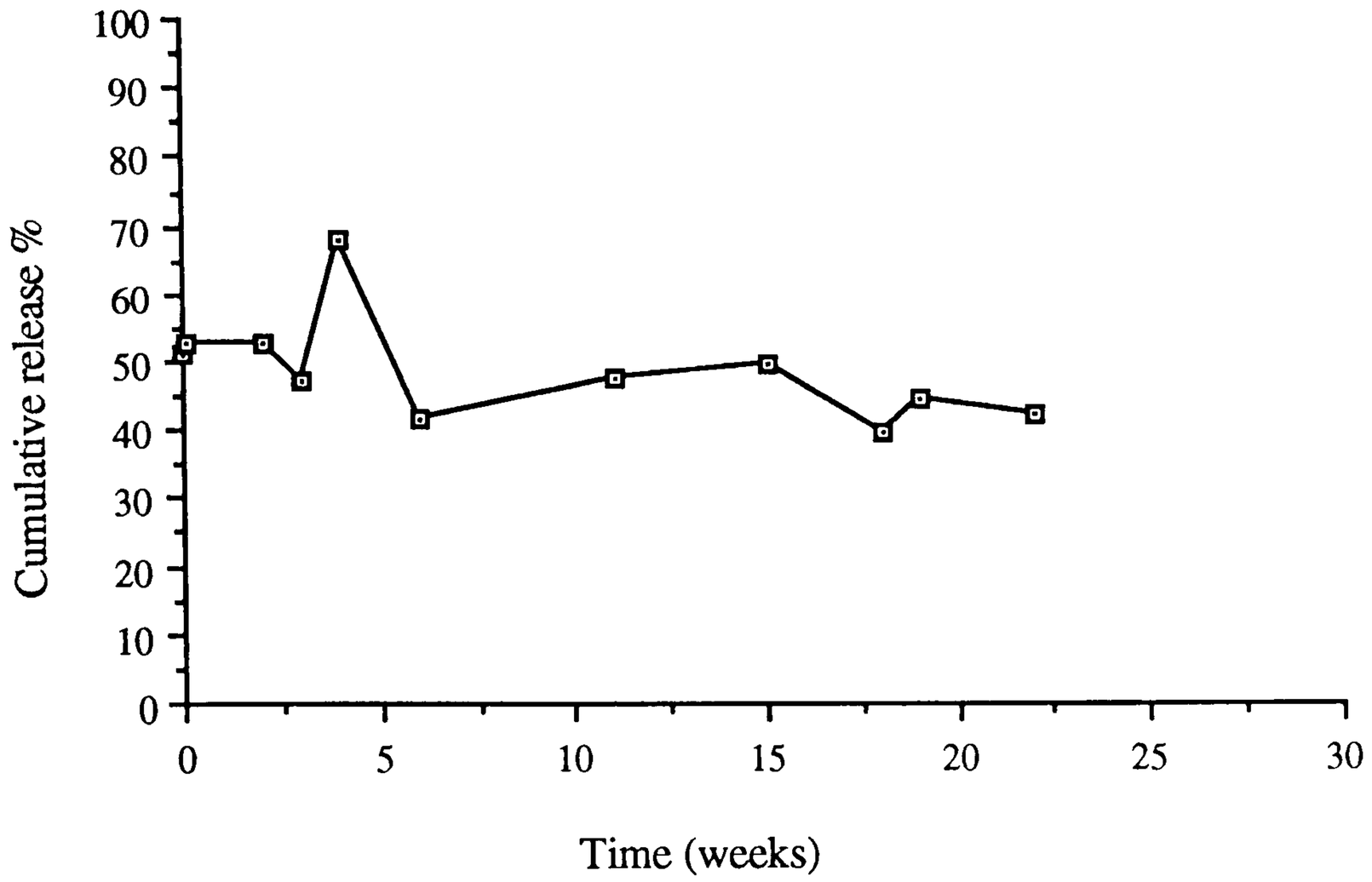


Figure 67

Influenza release from 1μm PLG (858,batch 10,NIBSC) microparticles

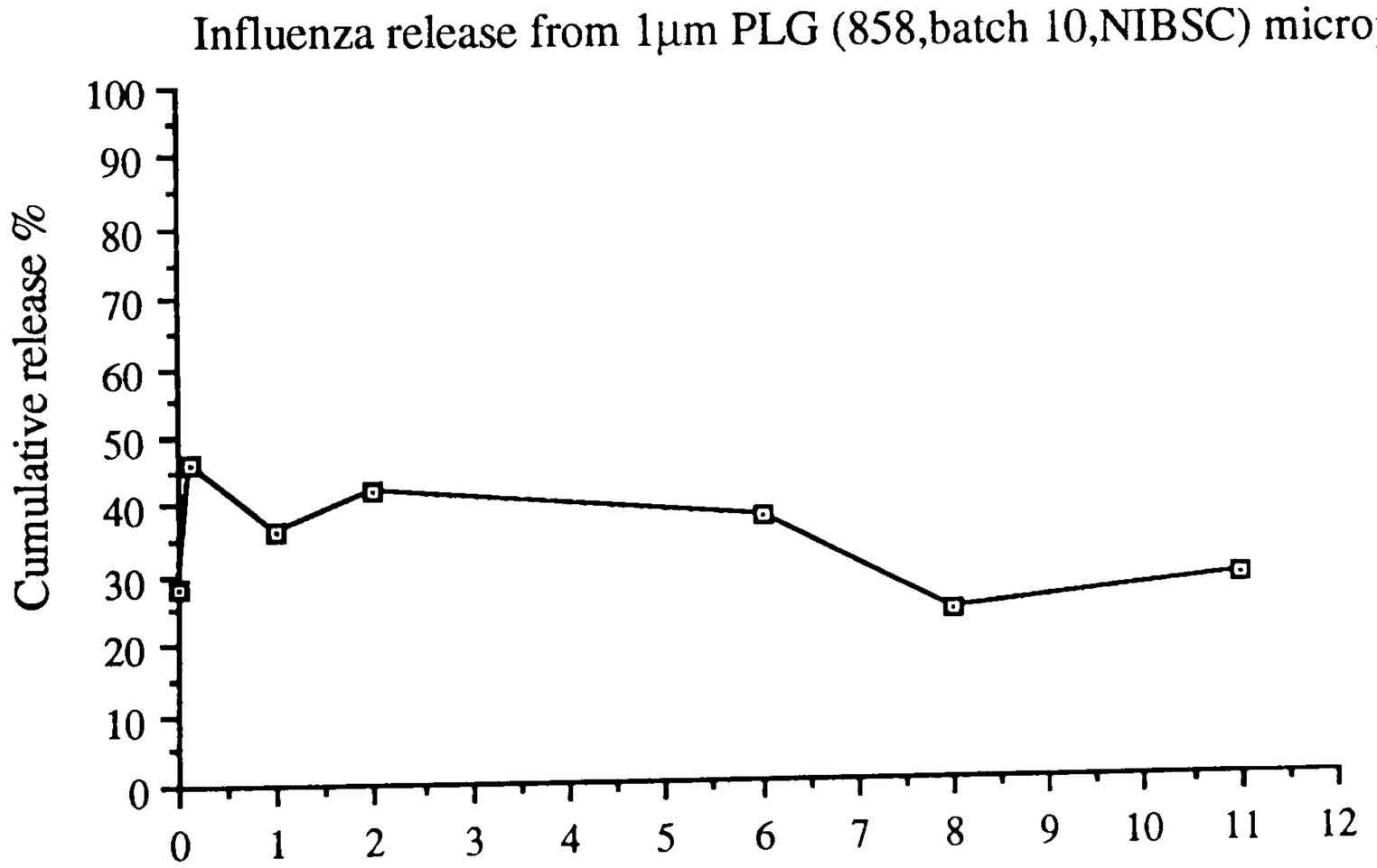


Table 12 **pH shift of the external medium of Poly(lactide-co-glycolide) microparticles during *in-vitro* studies.**

Batch	Time (weeks)	pH of external medium
3 (resomer 505)	0	7.4
	6	4.5-5.0
8 (resomer 858)	0	7.4
	26	6.5
9 (resomer 503)	0	7.4
	10	3.0-4.0

CHAPTER 6

IMMUNISATION STUDIES USING PLG MICROPARTICLES CONTAINING INFLUENZA VIRUS

6.1 INTRODUCTION

PLG microparticles containing equine influenza virus had been formulated and characterised (refer to Chapter 5). The next step in the development of an oral vaccine against influenza was to assess the immunological responses to these microparticles *in vivo*. Immunisation studies were conducted in mice and the immunological responses were assessed by measuring the levels of anti-influenza antibodies produced utilising a specific enzyme linked immunosorbant assay (ELISA).

Two studies were conducted (a) a subcutaneous study (s.c) to evaluate the immunogenicity of the microencapsulated influenza virus in mice and (b) an oral study to investigate the mucosal immune response in mice after immunisation with equine influenza virus encapsulated within PLG microparticles.

6.2 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

For a comprehensive review of the principle of the ELISA the reader is referred to Kemeny, A practical guide to ELISA (1990).

The basis of this assay is the antibody:antigen interaction (immunoassay) in which an antibody or antigen linked to an enzyme is added to either an antibody or antigen immobilised on a solid phase. The type of solid supports vary but plastic microtitre plates are routinely used which are composed of a series of wells in which the reaction takes place. The subsequent antibody:antigen reaction results in a bound fraction with enzymatic activity. Addition of the enzyme substrate gives a colour reaction, the intensity of which is related to the amount of antibody:antigen complex. The plates are washed after each step to remove unbound reagents. ELISA`s can be divided into non-competitive such as described above, and competitive where a mixture of enzyme labelled antigen and unlabelled antigen compete for the binding sites on an immobilised antibody.

There are many formats and types of ELISA dependent on the component is being measured, but all work by the basic principles described above. To measure antibody levels in biological fluids the non-competitive sandwich ELISA is commonly employed. The plates are coated with a dilution of the antigen, the biological fluid is added and the specific antibodies bind to the plate via an antigen/antibody complex. A specific secondary enzyme conjugated antibody is added which forms a antigen\antibody\enzyme conjugated antibody. Enzyme substrate is subsequently added to give a colour reaction.

Each step of the ELISA protocol requires optimisation to ensure an efficient and accurate assay. This is achieved by running test ELISA using different concentrations of reagents (changing only one parameter each time) and finding the optimal conditions. Interpretation of ELISA data can be a simple "yes" or "no" for the presence of the specific antibody signified by colour development. For a more

quantitative assessment the colour intensity (optical density) of the reaction is measured and compared to a reference curve. The reference curve is obtained by performing an ELISA on a series of dilutions of antisera (or other biological fluid). containing an high content of the antibody being investigated, and plotting the optical densities against dilution to give a linear curve.

6.3 PARENTERAL IMMUNISATION STUDIES IN MICE USING PLG MICROPARTICLES ENCAPSULATING EQUINE INFLUENZA VIRUS

The objectives of this study were twofold; (a) To evoke the production of antibodies to the equine influenza virus entrapped within a biodegradable carrier system administered parenterally and (b) To compare the effect of different size carrier systems to the immunological response.

6.3.1 Materials and Methods

Animals:

Male Balb/c mice (25g) were halothane anaesthetised and immunised subcutaneously with a 25G (5/8 inch) needle.

PLG microparticles with entrapped virus:

The microparticles were formulated and characterised according to the methods described in Chapter 5.

Virus - Inactivated Influenza A/equine/prague/56 (H7N7)(supplied by Behringwerke, Germany)

PLG Polymer - PLG 50:50 (9kD) resomer 503 (Boehringer, Germany)

Microparticles - (1μm) 2.1% w/w entrapped virus

(30μm) 3.6% w/w entrapped virus

Immunisation schedule:

The mice were divided into four groups of 10, and were given the following by s.c. injection:

Group A. PLG microparticles (1μm)(1000 HA, 17μg protein) in saline.

Group B. Inactivated influenza (1000 HA, 17μg protein) in saline.

Group C. Inactivated influenza (1000 HA, 17μg protein) adsorbed to Alum (2%) in saline.

Group D. PLG microparticles (30μm)(1000 HA, 17μg protein) in saline.

Immunisations were repeated after 6 weeks.

Blood samples were collected from the tail vein of halothane anaesthetised mice at 14 day intervals. The specific anti-influenza IgG antibody content of each serum sample was determined in an established ELISA and standardised against the positive control antiserum obtained in an hyperimmunisation study with whole influenza A/equine/Prague/56 (H7N7).

Hyperimmune study

Two mice male Balb/C 6-8 weeks old (25g) were immunised IP with inactivated equine influenza (A/equine/Prague/56,H7N7) in Freund's complete adjuvant (FCA) the administered dose contained 1,000 HA units. Two weeks after the initial immunisation the mice were given an IP injection of equine influenza virus (Prague strain H7N7) in Freund's incomplete adjuvant (FIA) containing 1,000HA units of protein. The mice were given a third IP immunisation of equine influenza in Freund's incomplete adjuvant containing 1,000HA units of protein four weeks after the initial immunisation.

Blood was removed 1 week after the third immunisation and the serum separated.

ELISA for measurement of anti-influenza IgG antibodies in serum

(a) 40μl inactivated influenza (A/Equine/Prague/56,H7N7, supplied by Behringwerke, Germany) was added to 20 mls of coating buffer, gently mixed and 200ul dispensed into all the wells of a microtitre multiwell plate (NUNC-immuno plate Maxisorp F96)(0.48μg influenza per well). The plate was left overnight at 4°C.

(b) The plate was emptied by flicking and washed 3 times with wash buffer using a microplate washer (Titertek). The plate was then tapped on sheets of blue towel to remove residue wash buffer.

(c) 200 uls of blocking buffer was then added to all the wells except a blank column and left for 2 hours at room temperature.

(d) Step (b) was repeated.

(e) The serum samples and raised antisera (positive control) was diluted in wash buffer and 150 ul of diluted sample dispensed into the wells, and left for 2 hours at room temperature.

(f) Step (b) was repeated.

(g) The secondary enzyme conjugated antibody (Mouse anti-IgG conjugated to horseradish peroxidase) was diluted with wash buffer (1 in 2000 dilution) and 150ul added to each well except the blank column. This was left 2 hours at room temperature.

(h) Step (b) was repeated.

(i) The chromogen solution (substrate) was prepared by adding 40ul of hydrogen peroxide to 40mg of o-Phenylenediamine (OPD), mixed, and added to 50mls of a 1:1 citrate:phosphate solution (25mls citrate, 25mls phosphate). When fully dissolved, 100ul was added to all wells (including blanks). The glassware used for the preparing the chromogen solution was scrupulously cleaned with dilute sulphuric acid then citrate/phosphate buffer before hand.

(j) After 15 minutes in the dark the reaction was stopped by adding 50ul of dilute sulphuric acid to each well and the optical density of the colour reaction measured using a plate reader set at 492nm.

For buffers refer to Appendix 2.

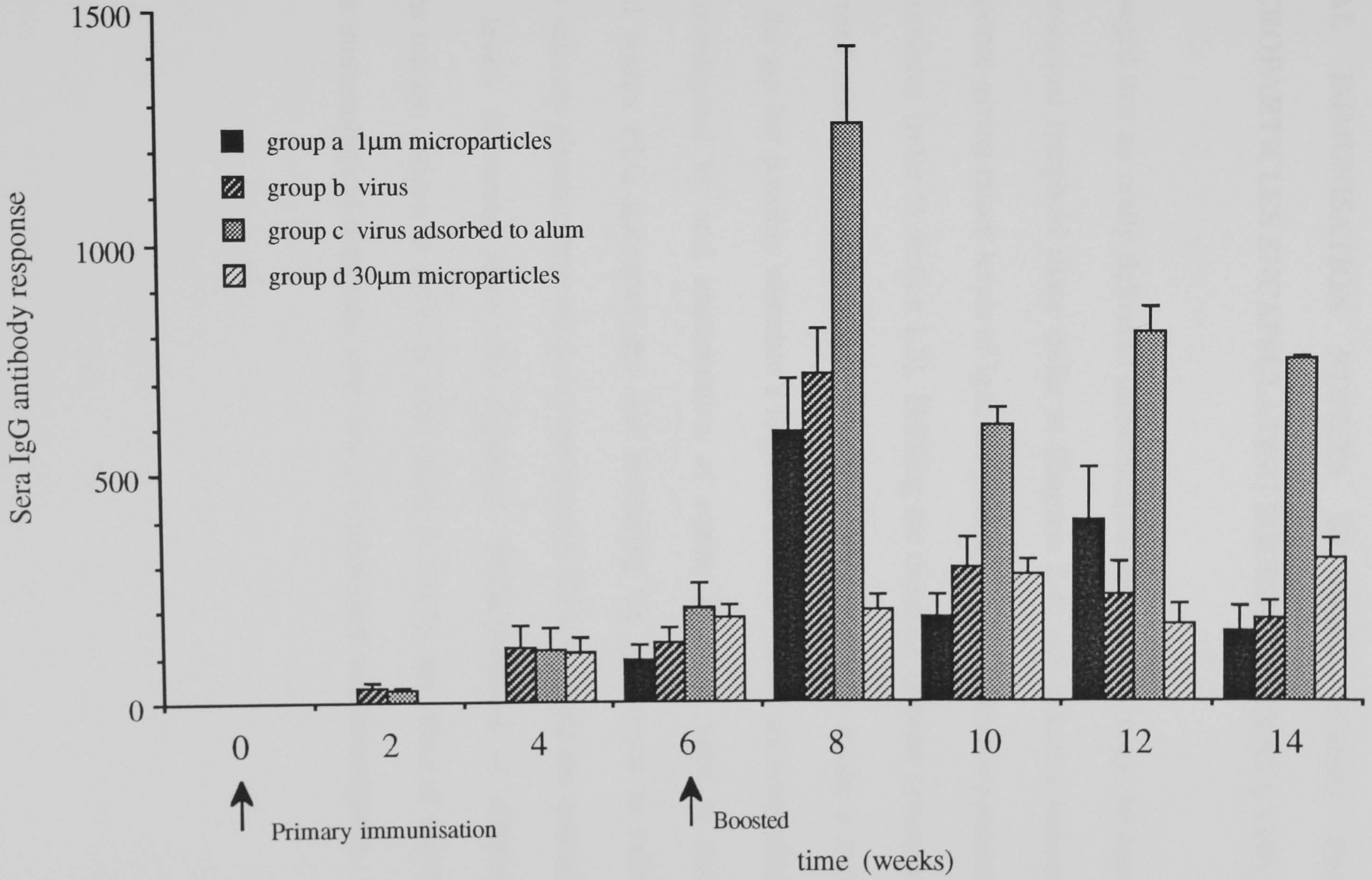
6.3.2 **Results and Discussion of Parenteral Study**

For the serum IgG antibody responses obtained in the parenteral study refer to Figure 68. For statistical evaluation a two-way analysis of variance was performed on the data using a Unistat statisical package (version 4.50).

The animals were given 1,000HA of equine influenza, Prague strain which is the concentration used in the commercial vaccine for horses (PREVAC*- Hoechst Animal Health). An IgG antibody response was seen in all 4 groups. The greatest response was seen in the group administered with virus adsorbed to Alum. This is due to the adjuvant nature of Alum. Although, a raised humoral response was observed with the Alum formulation it is worth bearing in mind that Alum is not thought to evoke cell mediated responses (Warren *et al.* 1986). The serum IgG response in the microparticle groups was not greater than the virus only group. This may be explained by the size of the virus, being 80-100nm it may itself be perceived as a particulate by the macrophage system and be treated in a similar manner to small microparticles. In a recent study conducted by Moldoveanu *et al.* (1993), inactivated influenza virus, A/H3N2, either encapsulated in PLG microparticles or as an aqueous solution of virus were administered subcutaneously to mice. As in our studies the microencapsulated virus induced levels of virus-specific antibodies in sera comparable but not greater to those obtained after immunisation with the aqueous virus. The polymer used in our study is known to degrade after approximately one week (Miller *et al.* 1977) therefore, both the large and small microparticles would release the entrapped virus soon after administration. This may account for the similar responses observed in the two microparticulate groups. The results of the present study,

Figure 68

Subcutaneous study with 1μm and 30μm PLG microparticles containing Equine Influenza virus (Prague strain)



however, demonstrate an evoked immunological response *in vivo* to equine influenza entrapped within a biodegradable carrier system administered parenterally.

6.4 ORAL IMMUNISATION STUDIES IN MICE USING PLG MICROPARTICLES ENCAPSULATING EQUINE INFLUENZA VIRUS

It was envisaged that an orally delivered microencapsulated antigen would be taken across the intestinal lymphoid tissue (refer to Chapters 2-4) and induce a mucosal immune response giving raised levels of IgA, the primary immunoglobulin produced at mucosal surfaces (refer to section 1.3). Utilising the common immune system an orally delivered microparticulate containing antigen might not only evoke a local response in the gut but possibly stimulate a response at more distant secretory sites. This was investigated by oral immunisation of equine influenza virus to mice encapsulated within PLG microparticles and measuring the IgA levels in saliva (secreted by salivary glands connected to the oral cavity). To investigate the systemic effect IgG levels in serum were also measured. PLG polymers of different compositions release antigen at different rates (refer to 5.6.1), the effect of this on the resultant immunological responses after oral administration was investigated.

6.4.1 **Materials and Methods**

Animals:

Male Balb/c mice (25g) were gastrically intubated with a curved blunt-ended oral dosing needle.

PLG microparticles with entrapped virus:

The microparticles were formulated and characterised according to the methods described in Chapter 5.

Virus - Inactivated Influenza A/equine/prague/56 (H7N7)(supplied by Behringwerke, Germany).

Polymer - PLG 50:50 (9kD) and PLG 85:15 (53kD) resomer 858 (Boehringer)

Microparticles - (1μm) 9kD 1.2% w/w entrapped virus

(1μm) 53kD 1.1% w/w entrapped virus

Oral immunisation schedule:

The mice were divided into three groups of 10, they were fasted 4 hours prior to receiving the following by gastric intubation on three consecutive days:

Group E. Virus (10,000 HA, 53μg protein) in saline.

Group F. PLG microparticles 1μm (9kD)(10,000 HA, 53μg) in saline.

Group G. PLG microparticles 1μm (53kD)(10,000 HA, 53μg) in saline.

Immunisations were repeated 4 weeks later.

Blood samples were collected from the tail vein of halothane anaesthetised mice at 7 day intervals. Salivary samples were collected from halothane anaesthetised mice at 7 day intervals after an intraperitoneal injection of pilocarpine. The specific anti-influenza IgG antibody content of each serum sample and the specific anti-influenza IgA antibody response of each saliva sample was determined in an established ELISA and standardised against a positive antiserum obtained in an hyperimmunisation study (refer to section 6.3.1) with whole equine influenza (Prague) virus.

ELISA for measurement of anti-influenza IgA antibodies in saliva

(a) 55μl influenza (A/Equine/Prague/56,H7N7, supplied by Behringwerke. Germany) was added to 20 mls of coating buffer, gently mixed and 190ul dispensed into all the wells of a microtitre multiwell plate (NUNC-immuno plate Maxisorp F96)(0.47μg influenza per well). The plate was left overnight at 4°C.

(b) The plate was emptied by flicking and washed 3 times with wash buffer using a microplate washer (Titertek). The plate was then tapped on sheets of blue towel to remove any residue wash buffer.

(c) 200uls of blocking buffer was then added to all the wells except column 1 (blank column) and left for 2 hours at room temperature.

(d) Step (b) was repeated.

(e) The saliva samples and raised antisera (positive control) was diluted in wash buffer and 150ul of diluted sample dispensed into the wells and left for 2 hours at room temperature.

(f) Step (b) was repeated.

(g) Sheep anti-mouse IgA was diluted with wash buffer (1 in 1000 dilution) and 110ul added to each well except the blank column. This was left 2 hours at room temperature.

(h) Step (b) was repeated.

(g) Donkey anti-sheep IgG alkaline phosphatase conjugate (Sigma) was diluted with wash buffer (1 in 300 dilution) and added to each well except the blank column. This was left for 2 hours at room temperature.

(h) Step (b) was repeated with an additional wash in distilled water.

(i) The chromogen solution was prepared by dissolving 4 tablets of p-nitrophenyl phosphate (pNPP, Sigma) to 20 mls of diethanolamine buffer. 100uls of this was added to each well.

(j) The colour reaction was allowed to develop in the dark for 15 minutes. The reaction was stopped by adding 50ul of NaOH (3M) to each well.

(k) The optical density of the colour reaction was measured at 405nm in a plate reader.

For buffers refer to Appendix 2.

6.4.2 **Results and Discussion of the Oral Study**

For the serum IgG responses obtained in the oral study refer to Figure 69. For statistical evaluation a two-way analysis of variance was performed on the data using a Unistat statisical package (version 4.50).

The IgG antibody responses were similar in all three groups. The levels of IgG antibody in the serum after oral microparticulate delivery were comparable to levels produced after s.c. immunisation (refer to 6.3.2). This is a good result because, although, we are specifically trying to evoke a mucosal immune response with the oral administration of antigen it is advantageous to induce anti-influenza antibodies in the systemic circulation. These results are in contrast to those of Waldman *et al.* (1986) and Bergmann *et al.* (1986), who found no serum antibody (IgG) increase in humans to orally administered inactivated influenza virus.

For the salivary IgA responses obtained in the oral study refer to Figure 70. Raised levels of IgA was seen in all the groups indicating that oral administration of inactivated influenza can induce a disseminated mucosal response in areas distant from the gut. The greater response was elicited from the group containing soluble virus. A number of reasons may account for this. As described earlier, the virus size may enable the virus to act as a particulate and itself and be taken up by the Peyer's patches in a similar manner to the microparticle formulations. There has been numerous reports of the uptake of viruses through M cells of Peyer's patches: reovirus (Wolf *et al.* 1981, 1983), Poliovirus (Sicinski *et al.* 1990) and HIV (Amergen *et al.* 1991). Various workers have reported increased IgA levels in

Figure 69

Sera IgG antibody responses in mice following oral administration of Equine Influenza virus (Prague strain) as virus or as microparticles

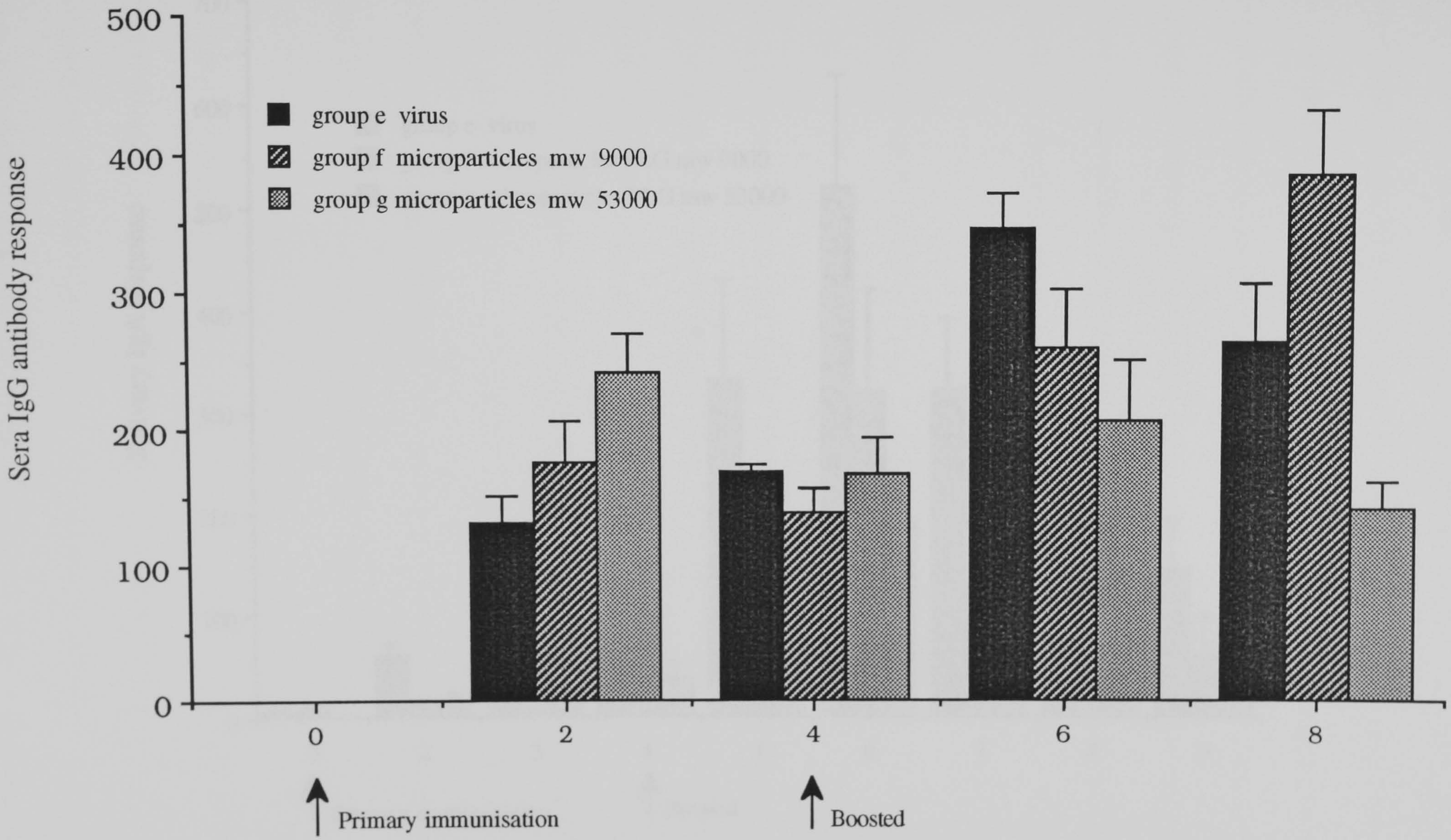
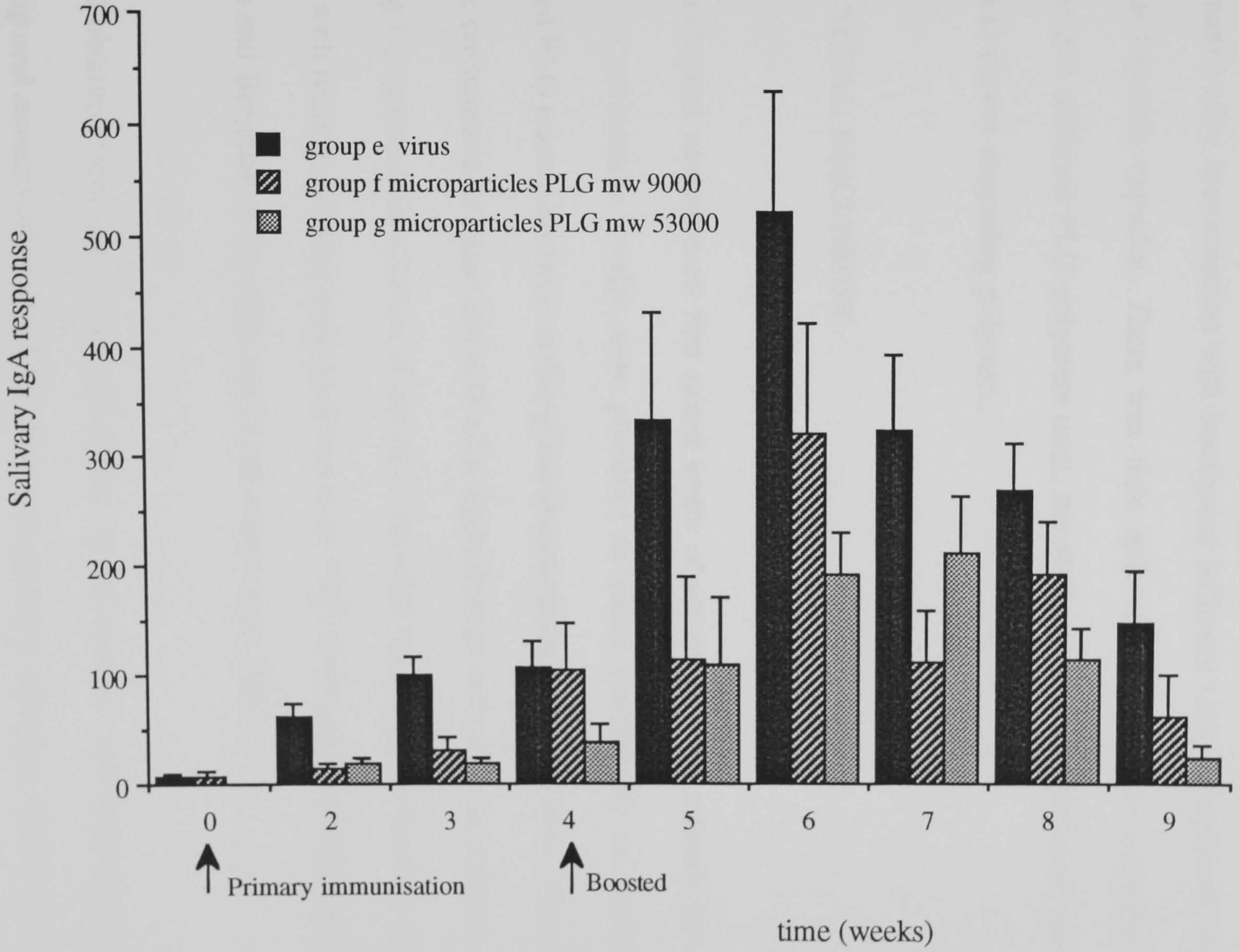


Figure 70

Salivary IgA responses in mice following oral administration of Equine Influenza virus (Prague strain) as virus or in microparticles



mucosal secretions after oral administration of an aqueous solution of inactivated influenza virus in humans and animals; Chen *et al.* (1987), Chen and Quinnan (1988) and Chen and Quinnan (1989) found raised levels of specific secretory IgA in pulmonary secretions in mice whilst in humans, Waldman *et al.* (1986) and Bergmann *et al.* (1986), also demonstrated increased levels of IgA in external secretions (tears, saliva and nasal) after immunisation with inactivated influenza virus administered in enteric-coated gelatin capsules. There was little difference in the IgA responses between the two different PLG polymers used, the 9kD rapidly degrading polymer and the 53kD slower degrading polymer.

6.5 GENERAL DISCUSSION

The immunological studies show that raised levels of serum IgG and salivary IgA (compared to preimmune levels) were produced to either parenterally or orally administered PLG microparticles containing inactivated equine influenza virus. These levels were comparable to those obtained after immunisation with virus in solution. Our finding that an aqueous solution of the virus can elicit an immune response is in agreement with findings of other workers (Chen *et al.* 1987,1988 and 1989, Waldman *et al.* 1986 and Bergman *et al.* 1986 and Moldoveanu *et al.* 1993).

The results obtained from our studies are similar by comparison with other groups investigating oral immunisation with PLG microencapsulated influenza. Moldoveanu *et al.* (1993) evaluated PLG microparticles as an oral antigen delivery system with human influenza virus type A/H3N2. Two systemic immunisations with

microencapsulated virus induced levels of antibodies in sera that were comparable but not greater than those obtained after immunisation with virus in solution. Only by priming systemically with virus in solution were the levels of IgA and IgG induced by an oral boost of microencapsulated virus slightly higher than those induced by an oral boost of the virus in solution. This group however, did not investigate the effect of priming and boosting orally with microencapsulated virus. Importantly, using the oral route of immunisation in the studies presented in this thesis, raised levels of anti-influenza antibodies in mucosal secretions and the systemic circulation were induced. The IgG antibody levels in sera obtained in the oral study were comparable with those obtained with the parenteral study. This contrasts with the findings of Bergmann *et al.* (1986) and Waldman *et al.* (1986) where no serum antibodies were raised after oral immunisation with inactivated virus.

The fact that the microencapsulated virus did not give greater antibody responses in comparison to the aqueous virus may have been due to a loss of antigen integrity during microencapsulation (refer to 5.5). As mentioned earlier, in the studies carried out by Moldoveanu *et al.* (1993), the greatest levels of IgA antibody and protection were produced after first priming with aqueous influenza virus systemically and then boosting orally with PLG encapsulated virus. Cox and Taubman (1984), also found the greatest level of IgA response to a particulate form of antigen, given orally, was achieved after first priming systemically with the aqueous form of the antigen. Our levels may be increased if an oral boost after priming systemically is performed, indicating this route of vaccination may provide a simple and inexpensive means of providing "booster" immunisations.

In the present study measurements were made of the IgA levels in saliva which protects the pharynx, we did not however, measure other mucosal secretions important in protecting against influenza invasion such as bronchial and pulmonary secretions. It would be important in any future studies to determine antibody levels in these fluids.

CHAPTER 7

FINAL DISCUSSION

The aim of the work contained in this thesis was to investigate the feasibility of developmening an oral vaccine against influenza virus, using a microparticulate antigen delivery system. The work was conducted on the hypothesis that an oral microparticulate containing antigen would be taken into intestinal lymphoid tissue and induce a disseminated mucosal response. To test this hypothesis, a series of experiments were conducted. The objective of this section is not to reiterate the results and discussions presented earlier in the thesis, but to highlight the major findings and relate them to published work. Conclusions will be drawn as whether, these studies further the development of an oral vaccine for influenza and how future investigatory work might be directed.

Initial work was carried out to investigate the uptake of a model microparticulate (latex) across the intestine. Peyer's patches were found to be the primary site of microparticle (size, 0.94μm and 0.11μm) uptake. This confirmed the findings of numerous workers using latex microparticles of a similar size range (LeFevre *et al.* 1978, Pappo and Ermak 1989, Jani *et al.* 1989 and 1992 and Eldridge *et al.* 1990). Most evidence of uptake was achieved when microparticles were administered using an *in situ* intestinal loop. The drawback to this technique, however, is the possible contamination by microparticles still present in the gut lumen. This problem does not occur if a clearing out period is allowed after chronic feeding. Low levels of

microparticles were found in the chronic feeding studies conducted in this thesis. however, in comparison to other studies investigating microparticle uptake after chronic feeding (LeFevre *et al*. 1978, Jani *et al*. 1989 and 1992). This may have been due to the lower dose (5 % of the dose used in the study by Jani *et al*. 1989) and shorter period of feeding used in my studies. To eliminate the problems associated with histological sectioning (frozen and cryostat sections) the technique of confocal microscopy could be used for the visualization of microparticles within tissue. This method does not have the inherent problem of section contamination because it optical sections tissue. This method has already been used to follow the disposition of microparticles through intestinal lymphoid tissue (James *et al*. 1992 and Jepson *et al*. 1993a and 1993b). The implication of preferential microparticle uptake across Peyer's patches is that antigen contained within microparticles will be delivered to the intestinal lymphoid tissue, which is thought to be a site for the induction of a mucosal immune response (Russell and Mestecky 1988, Eldridge *et al*. 1990, and M^{c}Gee and Kiyono 1993).

Transmission electron microscopy (TEM) was used to investigate microparticle uptake at the ultrastructural level. In the rabbit a phagocytotic mechanism of uptake was found at the M cell surface. This result is confirmation of the finding by Sass *et al*. (1990) and Jepson *et al*. (1993a) using scanning electron microscopy (SEM), proposed a similar mechanism of phagocytosis over the M cells of rats and rabbits, respectively. The limitation of SEM, however, is it does not allow the identification of intracellular microparticles. In contrast, the work presented in this thesis is more convincing because possible microparticles within vesicles in the apical cytoplasm of

the M cell were found giving more evidence to the presented mechanism (refer to Figure 50). The only other account using TEM evidence to demonstrate a phagocytotic mechanism of microparticle uptake over follicle associated epithelium, is that proposed by Landsverk (1988), however, there was no evidence of uptake into M cells. Other workers have reported M cell uptake of microparticles (Pappo and Ermak 1989, James *et al*. 1992, Porta *et al*. 1992) but have not directly demonstrated the mechanism at the M cell surface using electron microscopic analysis. The importance of microparticle uptake into M cells is the entrapped antigen will escape lysosomal breakdown due to the reduced number of lysosomes found in M cells (Owen *et al*. 1986). It is concluded that data presented in this thesis supports the hypothesis that M cells are antigen sampling cells allowing the entry of luminal material to immunocompetent cells where a mucosal immune response can be initiated (Owen 1977).

Gold labelled microparticles were used to clearly identify intracellular microparticles. This was the first study in which TEM had been used to detect microparticles labelled with colloidal gold. Microparticles were found in paracellular compartment of M cells; it is unlikely that the microparticles enter the cell through the tight junctions because, a more likely situation is that microparticles enter the paracellular compartment after uptake at the luminal surface and transport through the M cell cytoplasm and are discharged at the lateral plasma membrane (refer to Figure 50). This finding was found in a study conducted by Damagé *et al*. (1991) who described a paracellular pathway of nanocapsules transport through cells. The consequence of a paracellular route is the microparticles (hence entrapped antigen) may escape further lysosomal breakdown. The advantage of using TEM is the position of the

microparticle in relation to cell organelles can be detected. The limitation is the small area that is viewed and the time required to analyse the whole follicle associated epithelium. This may be one reason for the low levels of microparticles observed in the TEM morphological studies. A more rapid means of investigating microparticle uptake over the FAE would be to use confocal microscopy (Jepson *et al.* 1993a and 1993b).

The work presented in this thesis investigated microparticle uptake in the rabbit and the rat and represents a direct comparison between two species. More uptake was found in the rabbit, which may be a reflection of the greater number of M cells found in the rabbit (Pappo 1988) compared to the rat (Smith *et al.* 1980). The feasibility of oral vaccines may depend on the species for which the vaccine is to be used or more specifically the number of M cells within the lymphoid tissue.

Previous evidence used indirect methods to quantify microparticle uptake across the intestine by counting numbers in isolated tissue samples (Pappo and Ermak 1989, Jani *et al.* 1990, Ebel 1990 and Jepson *et al.* 1993). The advantage of the methodology presented in this thesis is that each and every microparticle within lymph draining the intestine is counted. A disadvantage with this method is it does not measure any microparticles transported in the portal blood and does not allow visualisation of microparticles within tissue. The presence of microparticles in the mesenteric and thoracic lymph confirmed the migratory pathway taken by microparticles crossing the intestine. This was the first evidence of microparticles actually within lymph. Previous evidence of microparticles within the lymph system were provided by histological analysis of lymphatic vessels (Jani *et al.* 1989 and 1992) and lymph nodes

(Eldridge *et al.* 1990). The presence of microparticles in the thoracic lymph 5 minutes after oral delivery suggests a rapid rate of intestinal uptake suggesting that a greater amount of morphological evidence would have been achieved if tissue was taken shorter periods after microparticle administration; future investigatory studies into microparticle uptake across the intestine should bear this in mind.

Qualitative assessment of microparticle uptake of histological samples whether *in situ* loop or chronic feeding indicated a low level of uptake, and this was confirmed by the quantitative studies of thoracic and mesenteric samples; if assessed as a percentage of the administered dose approximately 10^{-4} - 10^{-8}% were detected, although this does not include microparticles in tissues or blood. These levels are in agreement with a number of workers (LeFevre *et al.* 1977, Ebel 1990, Jepson *et al.* 1993) but contrasts with the level of uptake described by Jani *et al.* (1990). The latter authors (Jani *et al.* 1990) did not distinguish between microparticle absorption and microparticle adhesion to the gut wall which may have led to an overestimation. In a recent study by Jenkins *et al.* (1993) the flow cytometric detection method has been used to measure microparticles in digested tissue (adapted from a method by Ebel 1990). The level of microparticle uptake across the intestine did not significantly increase by including the numbers found in Peyer's patch and mesenteric lymph node tissues. The method of lymph drainage provides an efficient method to investigate intestinal uptake of microparticulates, where uptake parameters such as size of microparticulate can easily be investigated (Jenkins *et al.* 1993) without the inherent problems described for histological sectioning.

In contrast to the numerous studies that describe the role of macrophages in the translocation of microparticles within the Peyer's patch and their subsequent migration away from the patch (Lefevre *et al*. 1978, Pappo and Ermak 1989, Sass *et al*. 1990 and Jepson *et al*. 1993) the majority of microparticles detected microscopically in this study were found to be intercellular, although some microparticles were found closely associated with lymphocytes. This finding is supported by previous studies at Nottingham (McKenzie, 1993). The possibility also exists that microparticles were taken up by macrophages in areas not examined in the present studies such as mesenteric lymph nodes. Macrophages present antigen to helper T-cells to induce T-cell responses (refer to section 1.1), the significance of macrophage evasion from a point of view of using microparticles as antigen delivery systems is reduced cell mediated immunity. This would be a disadvantage in the development of oral vaccines against viral pathogens where cell mediated immunity is important (McGee and Kiyono 1993, section 1.1), in particular, to viruses such as influenza (Wood, 1988) whose epitopes are prone to antigenic drift because of the broader specificity of T-cells (Ada, 1990). However, the adjuvant effect of microparticles as a result of increased delivery to antigen presenting cells (macrophages) is well documented (Tabata *et al*. 1987, Kreuter *et al*. 1988, O'Hagan *et al*. 1991 and Eldridge *et al*. 1992). In addition, a recent study by O'Hagan *et al*. (1993) showed raised T-cell responses to PLG microparticles entrapping ovalbumin. It is difficult to draw conclusions on T-cell presentation from the work contained in this thesis because cell mediated responses were not investigated but future studies would need to address this important point.

PLG microparticles were found in thoracic lymph but no morphological studies using PLG were conducted to investigate the site of intestinal uptake. However, their presence in thoracic lymph and previous reports showing PLG microparticle uptake into mice Peyer's patches (Eldridge *et al.* 1990), and more specifically M cells in the FAE of rabbits (Jepson *et al.* 1993b) suggest that the route of uptake across the intestine is the lymphoid tissue. An essential extension to the morphological evidence of latex microparticle uptake is to investigate this phenomenon using PLG microparticles.

To exploit microparticle uptake into intestinal lymphoid tissue and test the hypothesis that antigen taken into intestinal lymphoid tissue can induce a disseminated immune response (Russell and Mestecky 1987, Mestecky 1987, M^{c}Gee *et al.* 1992) equine influenza was incorporated into PLG microparticles and immunological responses investigated.

Equine influenza belongs to the same group of orthomyovirus that infects humans, group A (although the subtypes are species specific, Paccaud 1970, Wood, 1988) and mimics the pathogenesis in humans (Gerber, 1970). Therefore, equine influenza can be used as a model for the development of an oral vaccine against influenza in humans.

Other workers have encapsulated Influenza (Moldoveanu *et al.* 1993) and Parainfluenza virus (Ray *et al.* 1993) into PLG microparticles using solvent evaporation although technical details were not described. The work in this thesis presents an extensive protocol for encapsulating influenza into microparticles of various sizes and is the first account of encapsulating equine influenza into PLG. Moldoveanu *et al.* (1993) and Ray *et al.* (1993) assessed the antigenicity of the

microencapsuled antigen by measuring antibody responses in sera of mice. The single-radial-immunodiffusion method presented in this thesis represents a unique, more convenient and rapid method to assess antigenicity of encapsulated influenza. The importance of this test was highlighted when it indicated the haemagglutinin epitopes were affected by the production processes employed by Behringwerke for their commercial influenza vaccine; in view of this finding Behringwerke may wish to assess there manufacturing procedures.

An important finding was the change in pH as the PLG polymer degrades. The significance of this in relation to PLG as antigen delivery vehicles is the possible effect on the encapsulated antigen as the pH of the internal matrix of the microparticles change. This point has not been previously addressed and may limit the use of PLG containing high levels of lactide for encapsulating antigens affected by low pH. Future work will have to investigate the effect of pH on the encapsulated antigen.

When PLG microparticles containing equine influenza were administered parenterally to mice comparable levels of serum IgG was found between the microencapsulated form and inactivated virus in solution. This was a similar finding to parenteral study conducted by Moldoveanu *et al*. (1993). Presently available vaccines for influenza in man and horses in Britain are killed inactivated virus administered parenterally which need to be stored at refrigeration temperatures between 2°C and 8°C (Dudgeon and Cutting 1991). Prior to the parenteral study the PLG microparticle preparation was lyophilised and stored at room temperatures. From a commercial point of view,

microparticle vaccines omitting the necessity for refrigeration offer an obvious advantage (refer to 1.2.1).

The finding that raised levels of IgA was produced in a site distant to the gut (salivary glands) after oral administration of PLG microencapsulated influenza and influenza in solution confirmed the presence of a common mucosal immune response (refer to section 1.3). The finding that oral administration of influenza induces a disseminated mucosal immune response has been reported previously in humans (Bergmann *et al.* 1986 and Waldman *et al.* 1986) but these studies did not use microencapsulated virus. In my studies raised IgG serum levels were found indicating a concurrent mucosal and systemic immunity. This result contrasts with those of Waldman *et al.* (1986) and Bergmann *et al.* (1986), who found no serum antibody (IgG) increase in humans to orally administered inactivated influenza virus.

The only other study investigating oral immunisation with microencapsulated influenza was the study conducted by Moldoveanu *et al.* (1993) who found raised levels of serum IgG and salivary IgA. However, mice were systemically primed with a solution of influenza before oral administration. Other workers have reported that systemically priming with the soluble form of the antigen prior to the administration of the microencapsulted form increases IgA levels in external secretions (Cox and Taubman 1984, and Eldridge *et al.* 1991). Consequently future studies should investigate if systemic priming is a prerequisite to oral immunisation with microencapsulated antigen. The results presented in this thesis, however, indicates that an immunisation regime using microencapsulated antigen as boosters might be

a possibility. The current influenza vaccines in humans (Kilbourne, 1988) and horses (Hoechst Animal Health) requires repeated boosters every 9-12 months by parenteral injection. Oral microencapsulated boosters offer an advance to the present vaccines by being more convenient and more cost effective (omitting the need for refrigeration, specialised equipment and personel to administer, refer to 1.2.1).

An argument against the use of sustained release vaccines is the scenario where administration results in to an adverse reaction in an individual. During normal immunisation regimes the treatment can be stopped this cannot be done in a single controlled release delivery system. This highlights the need for allergy testing in each individual before one shot vaccines can be administered. Although IgA levels in mucosal secretions have been found previously to correlate with protection against influenza (Liew *et al.* 1984, Moldoveanu *et al.* 1993), challenge experiments will need to be performed in order to assess the full value of the PLG influenza delivery system presented in this thesis.

Future work should investigate methods of increasing the delivery of antigen across intestinal lymphoid tissue as means of enhancing immune responses. One possible way to increase the levels of microparticle uptake is to increase lymphatic absorption by increasing the lipophilicity of the microparticle preparation. Compounds with increased lipophilicity produce an increase in lymphatic transport (Charman and Stella 1991). Targeting the apical cytoplasm of M cells is another possible method to increase uptake: monoclonal antibodies have been produced which are specific to M cells (Pappo 1989), which when conjugated to microparticles have been shown to enhance uptake (Pappo 1991). An interesting extension to would be to exploit the

avidin/biotin interaction to pretarget the M cell using a monoclonal conjugated to avidin (or streptavidin) and deliver a biotinylated microparticle. Formation of the avidin\biotin complex would result in targetting to the M cell.

Neutra *et al*. (1987) found microparticles conjugated to lectins were specifically taken up by M cells in rabbit Peyer's patches. However, preliminary work carried out during this thesis investigating the uptake of latex microparticles conjugated to PHA (reported to bind to epithelial glycoconjugates in the intestine, Pusztai *et al*. 1991). did not show any preferential adherence or uptake across the follicle associated epithelium in the rat. This may have been due to structural alterations to the lectin binding sites during the conjugation process. Weltzin *et al*. (1989) found that secretory IgA bound specifically to M cells, Porta *et al*. (1992) extended this finding to increase M cell uptake of microparticles by coating the microparticles with IgA.

In conclusion the work conducted in this thesis supports the contention that oral vaccines using biodegradable microparticulate antigen delivery systems are possible. An oral vaccine against influenza was investigated but due to the nature of the mucosal immune response the findings in this thesis can be used for the development of an oral vaccine against an array of pathogens. The findings included in this thesis have futhered the development of an oral vaccine against influenza, however, before this goal can be realised further investigatory studies outlined need to be conducted. The work included in this thesis justifies further research and I hope, will encourage others to join this field of study.

REFERENCES

Ada, G.L (1990): Modern vaccines. The immunological principles of vaccination. The Lancet, 335, 523 - 526.

Ada, G (1991): Strategies for exploiting the immune system in the design of vaccines. Mol. Immunology, 28, 225 - 230.

Allen, P.M., Unanue, E.R. (1984). Antigen processing and presentation by macrophages. Am. J. Anatomy, 170, 483 - 490.

Allison, A. C. and Gregoriadis, G (1990). Vaccines: recent trends and progress. Immunology Today, 11, No. 2.

Alpar, H.O., Field, W.N., Hyde, R. and Lewis, D.A (1989). The transport of microspheres from the gastrointestinal tract to inflammatory air pouches in the rat. J. Pharm. Pharmacol., 41, 194 - 196.

Altman, A. and Dixon, F. J (1989). Immunomodifiers in vaccines. Advan. Vet. Sci. Comparative Med., 33, 301 - 343.

Amergen, H. M., Weltzin, R., Farnet, C. M., Michetti, P., Haseltine, W. A. and Neutra, M. R (1991). Transepithelial transport of HIV-1 by intestinal M cells: A mechanism for transmission of AIDS. J. Acquired Immune Deficiency Syndromes, 4, 760 - 765.

Amoss, H. L. and Taylor, E (1917). Neutralization of the virus of poliomyelitis by nasal washings. J. Exp. Med., 25, 507 - 523.

Aprahamian, M., Michel, C., Humbert, W., Devissaguet, J-P. and Damage, C (1987). Transmucosal passage of polyalkylcyanoacrylate nanoparticles as a new drug carrier in the small intestine. Biol. Cell., 61, 69 - 76.

Beesley, J. E (1985). Colliodal gold: A new revolution in marking cytochemistry. Proc. RMS, 20, 187 - 196.

Bektimirov, T., Lambert, P. H. and Torrigiani, G (1990). Vaccine development perspectives of the World Health Organisation. J. Med. Virology, 31, 62 - 64.

Bergmann, K-ch., Waldman, R.H., Tischner, H. and Pohl W.D (1986). Antibody in Tears, Saliva and Nasal Secretions following Oral Immunisation of Humans with Inactivated Influenza Virus Vaccine. Int. Archs. Allergy appl. Immun., 80, 107 - 109.

Bergmann, K-Ch., Waldman, R. H., Tischner, H. and Pohl, W-D (1986). Antibody in tears, saliva and nasal secretions following oral immunization of humans with inactivated influenza virus vaccine. Int. Archs Allergy appl. Immun., 80, 107 - 109.

Bergmann, K-C. and Waldman, R. H (1987). Oral immunization with influenza viruses: Experimental and clinical studies. 83 - 89.

Bergmann, K-C. and Waldman, R. H (1988). Stimulation of secretory antibody following oral administration of antigens. Rev. Infect. Dis., 10, 939 - 950.

Bienenstock, J. and Johnston, N (1976). A morphologic study of rabbit bronchial lymphoid aggregates and lymhoepithelium. Lab. Invest., 35, 343 - 348.

Black, R. E (1993). Epidemiology of diarrhoeal disease: implications for control by vaccines. Vaccine, 11, 100 - 106.

Bloom, B. R (1989). Vaccines for the Third World. Nature, 342, 115 - 120.

Bockman, D. E. and Cooper, M. D (1973). Pinocytosis by epithelium associated with lymphoid follicles in the bursa of fabricius, appendix, and Peyer's patches. An electron microscopic study. Am. J. Anat., 136, 455 -478.

Bodian, D (1955). Emerging concept of poliomyelitis infection. Science, 122, 105 - 108.

Bollman, J. L., Cain, J. C. and Grindlay, J. H (1948). Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J. Lab. Clin. Med., 33, 1349 - 1352.

Carlson, J. R. and Owen, R. L (1987). Structure and functional role of Peyer's patches. In: Immunopathology of the small intestine. Ed: Marsh, M. N. Wiley, J. and Sons Ltd.

Challacombe, S. J., Rahman, D., Jeffery, H., Davis, S.S. and O'Hagan, D. T (1992). Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen. Immunology, 76, 164 - 168.

Charman, W. N. and Stella, V. J (1991). Transport of lipophilic molecules by the intestinal lymphatic system. Advanced Drug Delivery Reviews, 7, 1 - 14.

Chen, K.S. and Quinnan, G.V (1988). Induction, persistence and strain specificity of haemagglutonin-specific secretory antibodies in lungs of mice after intragastric administration of inactivated influenza virus vaccine. Journal of General Virology, 69, 2779-2784.

Chen, K.S., Burlington, D.B. and Quinnan, G.V (1987). Active Synthesis of haemagglutonin-specific immunoglobulin A by lung cells of mice that were immunised intra-gastrically with inactivated Influenza virus vaccine. Journal of Virology, 61, 2150 -2154.

Chen, K-S. and Quinnan, G. V (1989). Secretory immunoglobulin A antibody responses is conserved in aged mice following oral immunisation with influenza virus vaccine. J., gen., Virol., 70, 3291 - 3296.

Childers, N. K., Denys, F. R., M^{c}Gee, N. F. and Michaelek, S. M (1990). Ultrastructural study of liposome uptake by M cells of rat Peyer's patch. An oral vaccine system for delivery of purified antigen. Reg. Immunol., 3, 8 - 16.

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L. H. and Langer, R (1991). Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm., Res., 8, 713 - 720.

Cowsar, D. R., Tice, T. R., Gilley, R. M. and English, J. P (1985). Poly(lactide-co-glycolide) microcapsules for controlled release of steroids. Methods Enzy., 112, 101 - 116.

Cox, D. S. and Taubman, M. A (1984). Oral induction of the secretory antibody response by soluble and particulate antigens. Int. Archs Allergy Appl. Immun., 75, 126 - 131.

Craig, S. W. and Cebra, J. J (1971). Peyer's patches: An enriched source of precursors for IgA-producing immunocytes in the rabbit. J. Exp. Med., 134, 188 - 200.

Davis, S. S. and Illum, L (1988). Targetting using physical approaches and particulate drug carriers - interaction with the biological milieu. In: Targetting of drugs, anatomical and physiological considerations, Plenum press, New York, pp 177 - 187. Eds: Gregoriadis G and Poste G.

Damagé, C, Detantaine, L., Aprahamian, M., Michel, C., Humbert, W. and Devissaguet, J (1991). Preferential uptake of nanocapsules through Peyer's patches in the rat. Proc. Int. Symp. Control Rel. Bioact. Mater., 18, 349 - 350.

Dudgeon, J. A. and Cutting, W. A. M (1991). Immunization: Principles and practice. Chapman and Hall Medical. London.

Ebel, J.D (1990). A method for quantifying particle absorption from the mouse small intestine. Pharm. Res., 7, 848 - 851.

Edelman, R., Russell, R. G., Losonsky, G., Tall, B. D., Tacket, C. O., Levine, M. M. and Lewis, D. H (1993). Immunization of rabbits with entertoxigenic E. coli colonization factor antigen (CFA/I) encapsulated in biodegradable microspheres of poly (lactide-co-glycolide). Vaccine, 11, 155 - 158.

Edelman, R (1980). Vaccine adjuvants. Rev. Infect. Dis., 2, 370 - 383.

Eldridge, J. H., Staas, J. K., Meulbroek, J. A., Tice, T. R. and Gilley, R. M (1991). Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for Staphyloccal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. Infec. Immunity, 59, 2978 - 2986.

Eldridge, J. H., Hammond, C. J., Meulbroek, J. A., Staas, J. K., Gilley, R. M. and Tice, T. R (1990). Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches. J. Controlled Release, 11, 205 - 214.

Eldridge, J.H., Staas, J.K., Tice, T.R., Gilley, R.M. (1992). Biodegradable poly(DL-lactide-co-glycolide) microspheres.
Res. Immunol., 143, 557 - 563.

Fazekas de St. Groth, S. and Donnerly, M (1950). Studies in experimental immunology of influenza. iv. The protective value of active immunization. Aust. J. Exp. Biol. Med. Sci., 28, 61 - 75.

Francis, T. Jr (1941). Factors conditioning resistance to epidemic influenza. The Harvey lectures.

Frens, G (1973). Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature (Phys. Sci.), 241, 20 - 22.

Fujimura, Y (1986). Functional morphology of microfold cells (M cells) in Peyer's patches - phagocytosis and transport of BCG by M cells into rabbit Peyer's patches. Gastroenterologia Japonica, 21, 325 - 335.

Gaines, S., Sprinz, H., Tulley, J. G. and Tigertt, W. D (1967). Studies on infection and immunity in experimental typhoid fever.

Gander, B., Thomasin, C., Merkle, H. P., Men, Y. and Corradin, G (1993). Pulsed tetanus toxoid release from PLGA-microspheres and its relevance for immunogenicity in mice. Proceed. Intern. Symp. Control. Rel. Bioact. Mater., 20, 65 - 66.

Gilding, D. K. and Reed, A. M. (1979). Biodegradable polymers for use in surgery - polyglycolic/poly(lactic acid) homo and copolymers. Polymer, 20, 1459-1464.

Gilligan, C. A. and Li Wan Po, A (1991). Oral enteric vaccines-clinical trials. J. Clin. Pharmacy and Therap., 16, 309 - 335.

Glenny, A. T., Buttle, G. A. H., Stevens, M. F (1931). Rate of disappearance of diphtheria toxoid injected into rabbits and guinea-pigs: toxoid precipitated with alum. J. Pathol. Bacteriol. 34, 267 - 275.

Hanson, L. A (1961). Comparative immunological studies of the immune globulins of human milk and blood serum. Int. Arch. Allergy, 18, 241 - 267.

Hanson, L.A. (1993). Early work on secretory immunoglobulin A. Mucosal immunology update. 1, Number 2, 10 - 11.

Herzog, V. and Farquhar, M. G (1983). Use of electron-opaque tracers for studies on endocytosis and membrane recycling. Meths. Enzymol., 98, 203 - 225.

Hinman, A. R. and Orenstein (1990). Modern vaccines: Immunisation practice in developed countries. Lancet, 335, 707 - 710.

Hnatowich, D. J., Virzi, F. and Rusckowski, M (1987). Investigations of avidin and biotin for immaging applications. J. Nuclear. Med., 28, 1294 - 1302.

Hohmann, A. W., Schmidt, G. and Rowley, D (1978). Intestinal colonization and virulence of salmonella in mice. Infec. Immunity, 763 - 770.

Hollinger, J. O. and Battistone, G. C. (1986). Biodegradable bone repair materials. Synthetic polymers and ceramics. Clin. Orthop. Rel. Res., 207, 290-305.

Holmgren, J., Clemens, J., Sack, D. A. and Svennerholm, A-M (1989). New cholera vaccines. Vaccine, 7, 94 - 96.

Inman, L. R. and Cantey, J. R (1984). Peyer's patch lymphoid follicle epithelial adherence of a rabbit enteropathogenic Escherichia coli (strain RDEC-1): Role of plasmid-mediated pili in initial adherence. J. Clin. Invest., 74, 90 - 95.

Jackson, D. E., Lally, E. T., Nakamura, M. C. and Montgomery, P. C (1981). Migration of IgA-bearing lymphocytes into salivary glands. Cell. Immun., 63, 203 - 209.

James, P. S., Savidge, T. C. and Smith, M. W (1992). A new method to identify and study surface interactions taking place on mouse Peyer's patch M cells. J. physiol., 446, 60.

Jani, P.U., Florence, A.T. and M^{c}Carthy, D.E (1992). Further histological evidence of the gastrointestinal absorption of polystyrene nanospheres in the rat. Int. J. Pharmaceut., 84, 245 - 252.

Jani, P., Halbert, G. W., Langridge, J. and Florence, A.T (1989). The uptake and translocation of latex nanospheres and microspheres after oral administration to rats. J. Pharm. Pharmacol., 41, 809 - 812.

Jani, P., Halbert, G. W., Langridge, J. and Florence, A.T (1990). Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J. Pharm. Pharmocol., 42, 821 - 826.

Jeffery, H., Davis, S. S. and O'Hagan, D. T (1991). The preparation and characterisation of poly(lactide-co-glycolide) microparticles. I. Oil-in-water emulsion evaporation. Int. J. Pharmaceutics, 77, 169 - 175.

Jenkins, P. G, Howard, K. A., Blackhall, N.W., Thomas, N.W., Davis S. S. and O'Hagan, D. T (1993) Microparticulate absorption from the rat intestine. J. Controlled release, 29, 339 - 350.

Jenner, E (1801). The origin of the vaccine inoculation. Shury, D. N., London.

Jepson, M. A., Simmons, N. L., O'Hagan, D. T. and Hirst, B. H (1993b). Comparison of poly(DL-lactide-co-glycolide) and polystyrene microsphere targeting to intestinal M cells. J. Drug Targ, in press.

Jepson, M. A., Simmons, N. L., Savidge, T. C., James, P. S. and Hirst, B. A (1993a). Selective binding and Transcytosis of latex microspheres by rabbit intestinal M cells. Cell Tiss. Res., 271, 399 - 405.

Joel, D. D., Laissue, J. A. and LeFevre, M. E (1978). Distribution and fate of ingested carbon particles in mice. J. Reticuloendothel. Soc., 24, 477 - 487.

Kawanishi, H., Saltzman, L. E. and Strober, W (1983). Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. I. T cells derived from Peyer's patches that switch sIgM B cells to sIgA B cells *in vitro*. J., Exp., Med., 157, 433 - 450.

Kemeny, D. M (1990). A practical guide to ELISA. Ed: Kemeny, D. M. Pergamon publishers, Oxford.

Kilbourne, E. D (1988). Inactivated influenza vaccines. In: Vaccines. Ed: Plotkin, S. A. and Mortimer, E. A. W. B. Saunders company, Philadelphia.

Kimura, M., Kuno-Saki, H (1988). Pertussis in Japan. Acta. Paediatr. Jap., 30, 143 - 153.

Kreuter, J., Berg, U., Liehl, E., Soliva, M. and Speiser, P. P (1986). Influence of the particle size on the adjuvant effect of particulate polymeric adjuvants. Vaccine, 4, 125 - 129.

Kreuter, J., Mauler, R., Gruschkau, H. and Speiser, P. P (1976). The use of new polymethylmethacrylate adjuvants foe split influenza vaccines. Expl. Cell Biol., 44, 12 - 19.

Kreuter, J. and Liehl, E (1978). Protection induced by inactivated influenza virus vaccines with polymethylmethacrylate adjuvants. Med. Microbiol. Immunol., 165, 111 - 117.

Kreuter, J., Liehl, E., Berg, U., Soliva, M. and Speiser, P. P (1988). Influence of hydrophobicity on the adjuvant effect of particulate polymeric adjuvants. Vaccine, 253 - 256.

Lansverk, T (1987). The Follicle-associated epithelium of the ileal Peyer's patches in ruminants is distinquished by its shedding of 50nm particles. Immunol. Cell, Biol.. 65, 251 - 261.

Landsverk, T (1988). Phagocytosis and transcytosis by the follicle-associated epithelium of the ileal Peyer's patch in calves. Immunol. cell Biol., 66, 261 - 268.

LeFevre, M. E. and Joel, D. D (1984). Peyer's patch epithelium: An imperfect barrier. In: Schiller, C. M. (ed.), Intestinal toxicology, Raven Press, New York, pp. 45 - 56.

LeFevre, M. E., Warren, J. B. and Joel, D. D (1985). Particles and macrophages in murine Peyer's patches. Expl. Cell Biol., 53, 121 - 129.

LeFevre, M. E., Joel, D. D., Laissue, J. A., EL-Aasser, M. S. and Vanderhoff, J. W (1977). Stability of ^{125}I label after intragastric or intravenous administration of radioiodinated latexes to mice. J. Reticuloendothelial Soc., 22, 189 - 197.

LeFevre, M. E., Hancock, D. C. and Joel, D.D (1980). Intestinal barriers to large particulates in mice. J. Toxicol. Environ. Health, 6, 691 - 704.

LeFevre, M. E., Olivo, R., Vanderhoff, J. W. and Joel, D. D (1978). Accumulation of latex in Peyer's patches and its subsequent appearance in villi and mesenteric lymph nodes. Proc. Soc. Exp. Biol. Med., 159, 298 - 302.

Lentzin, H., Agrawal, B., Noske, W. and Herken, H (1984). Isolation and Characterisation of internalised glioma cell membranes. Cell Tiss. Res., 236, 147 - 151.

Liew, F. Y., Russell, S. M., Appleyard, G., Brand, C. M., Beale, J. (1984). Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum or cytotoxic T cell reactivity. Eur. J. Immunol., 14, 350 - 356.

Lycke, N. Y., Svennerholm, A-M. (1990). Presentation of immunogens at the gut and other mucosal surfaces. In: New generation vaccines, ed: Woodrow, G. C. and Levine, M. M. Publisher Marcel Dekker.

Maassab, H. F., LaMontagne, J. R. and DeBorde, D. C (1988). Live influenza virus vaccines. In: Vaccines. Ed: Plotkin, S. A. and Mortimer, E. A. W. B. Saunders company, Philadelphia.

Madara, J. L., Bye, W. A. and Trier, J. S (1984). Structural features of and cholesterol distribution in M cell membranes in guinea pig, rat and mouse Peyer's patches. Gastroenterology, 87, 1091.

Matsuno, K., Schaffner, T., Gerber, H. A., Ruchti, C., Hess, M. W. and Cottier. H (1983). Uptake by enterocytes and subsequent translocation to internal organs. eg, the thymus, of percoll microspheres administered per Os to suckling mice. J. Reticuloendothelial Soc., 33, 263 - 273.Maulding, H. V (1987). Prolonged delivery of peptides by microcapsules. J. Controlled release, 6, 167 - 176.

M^{c}Dermott, M. R. and Bienenstock, J (1979). Evidence for a common mucosal immunologic system: I. Migration of B immunoblasts into intestinal, respiratory. and genital tissues. J. Immunology, 122, 1892 - 1898.

M^{c}Gee, J. R., Mestecky, J., Dertzbaugh, M. T., Eldridge, J. H., Hirasawa, M. and Kiyono, H (1992). The mucosal immune system: from fundamental concepts to vaccine development. Vaccine, 10, 75 - 88.

M^{c}Gee, J. R. and Mestecky, J (1990). In defence of mucosal surfaces: Development of novel vaccines for IgA responses protective at the portals of entry of microbial pathogens. Infect. Dis. Clinics of N. Am., 4, 315 - 331.

M^{c}Gee, J. R. and Kiyono, H (1993). New perspectives in vaccine development: Mucosal immunity to infections. Infectious Agents and disease, 2, 55 - 73.

M^{c}Queen, C. E., Boedeker, E. C., Jarboe, D., Wolf, M., Le, M. and Brown, W. R (1993). Pili in microspheres protect rabbits from diarrhoea induced by E. coli strain RDEC-1. Vaccine, 201 - 206.

Mestecky, J (1987). The common mucosal immune system and current strategies for induction of immune responses in external secretions. J. Clin. Immun., 7, 265 - 276.

Michalek, S. M., Childers, N. K., Katz, J., Denys, F. R., Berry, A. K., Eldridge, J. H., M^{c}Gee, J. R. and Curtiss, R. (1989). Liposomes as oral adjuvants. Curr., Top., Microbiol., Immunol., 146, 51 - 58.

Miller, R. A., Brady, J. M., Cutright, D. E (1977). Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. J., Biomed., Mater., Res., 11, 711 - 719.

Moldoveanu, Z., Novak, M., Huang, W-Q., Gilley, R.*^T Staas, J. K., Schafer, D., Compans, R. W. and Mestecky, J (1993). Oral immunisation with influenza virus in biodegradable microspheres. J. Infect. Dis., 167, 84 - 90.

Morein, B., Sunquist, B., Hoglund, S., Dalsgaard, K. and Osterhaus, A (1984). Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature, 308, 457 - 460.

Morisaki, I., Michalek, S. M., Harmon, C. C., Toril, M., Hamada, S. and M^{c}Gee, J. R (1983). Effective immunity to dental caries: enhancement of salivary anti-streptococcus mutans antibody responses with oral adjuvants. Infect., Immun., 40, 577.

Morris, W., Steinhoff, M. C. and Russell, P. K (1994). Potential of polymer microencapsulation technology for vaccine innovation. Vaccine, 12, 5 - 11.

Mumford, J., Wood, J. M., Scott, A. M., Folkers, C. and Schild, G. C (1983). Studies with inactivated equine influenza vaccine: 2. Protection against experimental infection with influenza virus A/equine/Newmarket/79(H3N8). J. Hyg., Camb..90, 385 - 395.

Neutra, M. R., Phillips, T. L., Mayer, E. L. and Fishkind, D. J (1987). Transport of membrane-bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patche. Cell tissue Res., 247, 537 - 546.

O'Hagan, D. T., Palin, K., Davis, S.S., Artursson, P. and Sjoholm, I (1989). Microparticles as potentially orally active immunological adjuvants. Vaccine, 421 - 424.

O'Hagan, D. T., Palin, K. J. and Davis, S.S (1989). Poly(buty-2-cyanoacrylate) particles as adjuvants for oral immunization. Vaccine, 7, 213 - 216.

O'Hagan, D. T (1990). Intestinal translocation of particulates - implications for drug and antigen delivery. Advanced Drug Delivery Reviews, 5, 265 - 285.

O'Hagan, D. T (1990). Novel nonreplicating antigen delivery systems. Curr., Opin., Infect., Dis., 3, 393 - 401.

O'Hagan, D.T., Rahman, D., McGee, J.P., Jeffrey, H., Davies, M.C., Williams. P., Davis, S.S., Challacombe, S.J. (1991). Biodegradable microparticles as controlled release antigen delivery systems. Immunology, 73, 239 - 242.

O'Hagan, D. T (1992). Oral delivery of vaccines. Formulations and clinical pharmacokinetic considerations. Clin. Pharmacokinet., 22 (1), 1 - 10.

O'Hagan, D. T., M^{c}Gee, J. P., Holmgren, J., Mowat, M. C., Donachie, A. M., Mills, K. H. G., Gaisford, W., Rahman, D. and Challacombe, S. J (1993). Biodegradable microparticles for oral immunization. Vaccine, 11, 149 - 154.

Ohtani, O. and Murokami, T (1990). Organisation of the lymphatic vessels and their relationships to blood vessels in the rabbit Peyer's patch. Arch. Histol. Cytol., 53 (suppli.), 155 - 164.

Owen, R. L., Pierce, N. F., Apple, R. T. and Cray, W. C (1986). M cell transport of Vibrio cholerae from the intestinal lumen into Peyer's patches: A mechanism for antigen sampling and for microbial transepithelial migration. J. Infec. Dis., 153, 1108 - 1118.

Owen, R. L. and Jones, A. L (1974). Epithelial cell specialization within human Peyer's patches: An ultrastructural study of intestinal lymphoid follicles. Gastroenterology, 66, 189 - 203.

Owen, R. L (1977). Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: An ultrastructural study. Gastroenterology, 72, 440 - 451.

Owen, R. L. and Bhalla D. K (1983). Cytochemical analysis of alkaline phosphatase and esterase activities, and of lectin-binding and anionic sites in rat and mouse Peyer's patch cells. Am. J. Anat., 168, 199 - 212.

Paccaud, M. F (1970). The virology of equine influenza. Proc. 2nd int. Conf. Equine infectious Diseases, Paris 1969, pp 81 -93. Karger, Basel.

Pappo, J., Ermak, T. H. and Steger, H, J (1991). Monoclonal antibody-directed targeting of fluorescent polystyrene microspheres to Peyer's patch M cells. Immunology, 73, 277 - 280.

Pappo, J.and Ermak, T.H (1989). Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake. Clin. Exp. Immunol., 76, 144 - 148.

Pappo, J (1989). Generation and characterization of monoclonal antibodies recognizing follicle epithelial M cells in rabbit gut-associated lymphoid tissue. Cellular Immunology, 120, 31 - 41.

Park, K (1989). A new approach to study mucoadhesion: colloidal gold staining. Inter., J., Pharmaceutics, 53, 209 - 217.

Phillips, T. E., Phillips, T. L. and Neutra, M. R (1987). Macromolecules can pass through occluding junctions of rat ileal epithelium during cholinergic stimulation. Cell Tissue Res., 247, 547 - 554.

Porta, C., James, P. S., Phillips, A. D., Savidge, T. C., Smith, M. W. and Cremaschi, D (1992). Confocal analysis of fluorescent bead uptake by mouse Peyer's patch follicle-associated M cells. Exp. Physiology, 77, 929 - 932.

Preis, I. and Langer, R. S (1979). A single-step immunization by sustained antigen release. J. Immunological methods, 28, 193 - 197.

Pusztai, A., Ewen, S. W. B., Grant, G., Peumans, W. J., Van Damme, E. J. M., Rubio, L. A. and Bardoez, S (1991). Plant (food) lectins as signal molecules: effect on the morphological and bacterial ecology of the small intestine. Lectin Reviews, 1, 1 - 15.

Ramon, G (1925). Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de serum antidiphterique. Bull., Soc., Centr., Med., Vet., 101, 227 - 234.

Ray, R., Novak, M., Duncan, J. D., Matsuoka, Y. and Compans, R. W (1993). Microencapsulated human parainfluenza virus induces a protective immune response. J. Infect. Dis., 167, 752 - 755.

Reid, R. H., Boedeker, E. C., M^{c}Queen, C. E., Davis, D., Tseng, L-Y., Kodak, J., Sau, K., Wilhelmsen, C. L., Nellore, R., Dalal, P. and Bhagat, H. R (1993). Preclinical evaluation of microencapsulated CFA/II oral vaccine against enterotoxigenic E. coli. Vaccine, 11, 159 - 167.

Riott, I (1991). Essential Immunology. Blackwell Scientific Publications. Oxford.

Rossen, R. D. and Butler, W. T (1973). Immunologic responses to infection at mucosal surfaces. In: Viral and mycoplasmal infections of the respiratory tract. Ed: Knight, V. Lea and Febiger, Philadelphia.

Roux, M. E., M^{c}Williams, M., Phillips-Quagliata, J. M., Weisz-carrington, P., and Lamm, M. E. (1977). Origin of IgA-secreting plasma cells in the mammary gland. J. Exp. Med., 146, 1311 - 1322.

Rubas, W. and Grass, G. M (1991). Gastrointestinal lymphatic absorption of peptides and proteins. Advanced Drug Delivery Reviews, 7, 15 - 69.

Rudzik, R., Clancy, R. L., Perey, D. Y. E., Day, R. P. and Bienenstock, J (1975). Repopulation with IgA-containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patch and bronchial lymphocytes. J. Immunology, 114, 1599 - 1604.

Russell, M. W. and Mestecky, J (1988). Induction of the mucosal immune response. Rev. Infect. Dis., 10, 440 - 446.

Sanders, E. and Ashworth, C. T (1961). A study of particulate intestinal absorption and hepatocellular uptake: Use of polystyrene latex particles. Exp. Cell Research, 22, 137 -145.

Sanford, P. A (1982). Digestive system physiology. Edward Arnold Publishers.

Sass, W., Dreyer, H-P. and Seifert, J (1990). Rapid insorption of small particles in the gut. Am. J. Gastro., 85, 255 -260.

Seifert, J., Sass, W. & Dreyer, H.P. (1983). Mucosal permeation of macromolecules and particles in: E. Skadhauge and K. Heintze (Eds), Intestinal Absorption and Secretion, Falk Symposium 36, Kluwer Academic Publishers, Dordrecht, pp. 505-513.

Self, T.J., Johnson, S.A., Thomas, N.W., Warren, M., Fry, J. and Wilson, C. G (1988). Uptake of microspheres by rat hepatocytes in tissue culture. J. Anat., 158, 221 - 223.

Shvartsman, Y. S. and Zykov, M. P (1976). Secretory anti-influenza immunity. Adv. Immunol., 22, 291 - 330.

Sicinski, P., Rowinski, J., Warchol, J. B., Jarzabek, Z., Gut, W., Szczygiel, B., Bielecki, K. and Kock, G (1990). Poliovirus type 1 enters the human host through intestinal M cells. Gastroenterology, 98, 56 - 58.

Smith, P. K., Krohn, R. I., Hermanson, A. K., Mallia, A. K., Gartner. F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J. and Klenk, D. C (1985). Measurement of protein using bicinchoninic acid. Analyt., Biochem., 150. 76 - 85.

Smith, M. W. and Peacock, M. A (1980). M cell distribution in follicle associated epithelium of mouse Peyer's patch. Am. J. Anat., 159, 167 - 175.

Splenlehauer, G., Vert, M., Benoit, J. P. and Boddaert, A (1989). In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials, 10, 557 - 563.

Sunquist, B., Lovgren, K. and Morein, B (1988). Influenza virus ISCOMs: antibody response in animals. Vaccine, 6, 49 - 53.

Sytkowski, A. J (1990). Detection of cell surface components using fluorescent microspheres. Meth. Enzymol., 184, 353 - 356.

Tabata, Y. and Ikada, Y (1988). Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophages. Biomaterials, 9, 356 - 362.

Tabata, Y. and Ikada, Y (1990). Drug delivery systems for antitumor activation of macrophages. Crit. Rev. Therapeutic Drug Carrier Systems, 7, 121.

Tice, T. R., Staas, J. K., Gilley, R. M. and Eldridge, J. H (1987). Method and formulation for orally administering bioactive agents to and through the Peyer's patch. European patent application, No. 87309286.0.

Tice, T. R. and Gilley, R. M (1985). Preparation of injectable controlled-release microcapsules by a solvent-evaporation process. J., Controlled release, 2, 343 - 352.

Tice, T. R., Mason, D. W. and Gilley, R. M (1989). Clinical use and future of parenteral microsphere delivery systems. In: Novel drug delivery and its therapeutic application. Ed. Prescott, L. F. and Nimmo, W. S. John Wiley and Sons Ltd.

Tice, T. R. and Cowsar, D. R (1984). Biodegradable controlled-release parenteral systems. Pharmaceutical Tech., Nov., 26 - 35.

Tomasi, T. B. and Zigelbaum, S. D (1963). The selective occurrence of y1A-globulinin certain body fluids. J., Clin., Invest., 42, 1552.

Trier, J. S (1991). Structure and function of intestinal M cells. Gastro. Clinics of N. Amer., 20. 531 - 547.

Van Dongen, J. J., Remie, R., Rensema, J. W. and Van Wunnik, G. H. G (Eds) (1990). Manual of microsurgery on the laboratory rat. Part 1: General information and experimental techniques. Elsevier Science Publishers B. V. (Biomedical division).

Visscher, G. E., Robinson, R. L., Maulding, H. V., Fong, J. W., Pearson, J. E. and Argentieri, G. J (1985). Biodegradation of and tissue reaction to 50:50 poly(DL-lactide-co-glycolide) microcapsules. J. Biomedical Materials Res., 19, 349 - 365.

Visscher, G. E., Robinson, R. L. and Argentieri, G. J (1987). Tissue response to biodegradable injectable microcapsules. J. Biomaterials Appli., 2, 118 - 131.

Volkheimer, G (1983). Haematogenous dissemination of ingested polyvinyl chloride particles. Annals N.Y. Acad. Sci., 409, 164 - 171.

Waldman, R. H., Lazzell, V. A., Bergmann, C., Khakoo, R., Jacknowitz, A. I., Howard, S. A. and Rose, C (1982). Oral immunization against influenza. Trans. Am. Clin. Climatol Assoc., 93, 133 - 140.

Waldman, R.H., Stone, J., Bergmann, K-ch., Khakoo, R., Lazzell, V., Jacknowitz, A., Waldman, E.R. and Howard S (1986). Secretory Antibody following Oral Influenza Immunisation. Am. J. Med. Sci., 292, 367 - 371.

Wang, H. T., Palmer, H., Linhardt, R. J., Flanagan, D. R. and Schmitt, E (199). Degradation of poly(ester) microspheres. Biomaterials, 11, 679 - 685.

Warren, H. S., Vogel, F. R. and Chedid, L. A (1986). Current status of immunological adjuvants. Ann. Rev. Immunol., 4, 369 - 388.

Wilson, C. G. and Washington, N (1989). Physiological pharmaceutics. Biological barriers to drug absorption. John Wiley and Sons. New York.

Wise, D. L., Fellman, T. D., Sanderson, J. E. and Wentworth, R. 1 (1979). Lactic/glycolic acid polymers, in Drug carriers in biological medicine. Ed: Gregoriadis, 237 - 270. Academic press publishers.

Watts, P. J., Davies, M. C. and Melia, C. D (1990). Microencapsulation using emulsification/solvent evaporation: An overview of techniques and applications. Crit., Rev., Therapeutic Drug Carrier Systems, 7, 235 - 259.

Weltzin, R., Lucia-Jandris, P., Michetti, P., Fields, B. N., Kraehenbuhl, J. P. and Neutra, M. R (1989). Binding and transepithelial transport of immunoglobulins by intestinal M cells: Demonstration using monoclonal IgA antibodies against enteric viral proteins. J. Cell Biology, 108, 1673 - 1685.

Wolf, J. L., Rubin, D. H., Finberg, R., Kauffman, R. S., Sharpe, A. H., Trier, J. S. and Fields, B. N (1981). Intestinal M cells: A pathway for entry of reovirus into the host. Science, 212, 471 - 472.

Wolf, J. L., Kauffman, R. S., Finberg, R., Dambrauskas, R., Fields. B. N. and Trier, J. S (1983). Determinants of reovirus interaction with the intestinal M cells and absorptive cells of murine intestine. Gastroenterology, 85, 291 - 300.

Wolf, J. L. and Bye, W. A (1984). The membranous epithelial (M) cell and the mucosal immune system. Ann. Rev. Med., 35, 95 - 112.

Wood, J. M., Mumford, J., Folkers, C., Scott, A.M. and Schild, G. C (1983). Studies with inactivated equine influenza vaccine: 1. Serological responses of ponies to graded doses of vaccine. J. Hyg., Camb., 90, 371 - 384.

Wood, J. M., Schild, G. C., Folkers, C., Mumford, J. and Newman (1983). The standardisation of inactivated equine influenza vaccines by single-radial immunodiffusion. J., Biological Standardisation, 11, 133 - 136.

Wood, J. M (1988). Antigenic variation of equine influenza: A stable virus. Equine Vet. J.,20, 316 - 318.

Woodrow, G. C (1990). An overview of biotechnology as applied to vaccine development. In: New generation vaccines, Ed: Woodrow, G. C. and Levine, M. M. Publisher Marcel Dekker.

World Health Magazine, May 1980.

APPENDICES

APPENDIX 1: PUBLICATIONS DURING Ph.D STUDIES

K.A. Howard, N.W. Thomas, R. Minhas, S.S. Davis and D.T. O'Hagan (1993). The uptake of microparticles across the intestinal epithelium in the rabbit and rat. Poster presentation to the Anatomical Society of Great Britain and Ireland, University of Nottingham, Nottingham Meeting, 1992.
Journal of Anatomy, 182, part 1, February 1993.

K.A. Howard, P.G. Jenkins, N.W. Thomas, S.S. Davis and D.T. O'Hagan (1993). Microparticulate uptake into intestinal lymphoid tissue - as a route for the delivery of oral vaccines.
Poster presentation at the 20th International Symposium on Controlled Release of Bioactive Materials, Washington D.C., U.S.A., 25-28 July 1993. Proceed. Intern. Symp. Control. Rel. Bioact. Mater., 20 (1993). Controlled Release Society, Inc.

K.A. Howard, N.W. Thomas, P.G. Jenkins, S.S. Davis and D.T. O'Hagan (1994). The Absorption of Microparticles into Peyer's patches in the rabbit and rat. Accepted (January 1994) for publication in Pharmaceutical Science Communications.

P.G. Jenkins, K.A. Howard, N.W. Blackhall, N.W. Thomas, S.S. Davis and D.T. O'Hagan (1993). Aspects of microparticulate absorption across the intestine: a prelude to effective oral vaccination.
Poster presentation at novel vaccine strategies: Mucosal immunisation, adjuvants and genetic approaches, Bethesda, Maryland, U.S.A., 6-9 October 1993. Published in the Proceedings of conference.

P.G. Jenkins, K.A. Howard, N.W. Blackhall, N.W. Thomas, S.S. Davis and D.T. O'Hagan (1994). The absorption of polystyrene microparticles across the intestine of Wistar rats.

Poster presentation to the Anatomical Society of Great Britain and Ireland, University of Wales College of Cardiff Meeting, 13-15 July 1993.

Journal of Anatomy, 184, part 1, Febuary 1994.

P.G. Jenkins, K.A. Howard, N.W. Blackhall, N.W. Thomas, S.S. Davis and D.T. O'Hagan (1994). Microparticulate absorption from the rat intestine.

J. Controlled release, 29, 339 - 350.

P.G. Jenkins, K.A. Howard, N.W. Blackhall, N.W. Thomas, S.S. Davis and D.T. O'Hagan (1994). The quantitation of the absorption of microparticles into the intestinal lymph of Wistar rats. Int., J., Pharmaceutics, 102, 261 - 266.

APPENDIX 2: BUFFERS AND HISTOLOGICAL FIXATIVES

Phosphate buffer 0.2 M (pH 7.2)

Solution A: 15.6 gms sodium dihydrogen phosphate dissolved in distilled water up to 500 mls.

Solution B: 35.8 gms disodium hydrogen phosphate dissolved in distilled water up to 500 mls.

Add solution A to solution B until a pH of 7.2 is reached at 20°C.

Phosphate buffered saline

Disodium hydrogen phosphate 2.13 gms.
Sodium dihydrogen phosphate 0.23 gms.
Sodium chloride 0.36 gms.
Made up to 1 litre with distilled water.

5% sucrose buffer

0.2 M phosphate buffer (pH 7.2) 500 mls.
Sucrose 50 gms.
Made up to 1 litre with distilled water.

10% sucrose buffer

0.2 M phosphate buffer (pH 7.2) 500 mls.
Sucrose 100 gms.
Made up to 1 litre with distilled water.

Prewash

Sodium barbital	5.88 gms
Sodium acetate.$3H_2O$	2.88 gms (or 2.33 gms ofanhydrous)
Sodium chloride	5.5 gms
Potassium chloride	0.3 gms
Calcium chloride.$2H_2O$	0.2 gms
Magnesium chloride.$6H_2O$	0.05 gms

Dissolve into approximately 400 mls of distilled water. Then measure out 220-230 cm^3 of 0.1 M hydrochloric acid and use this to reduce the pH of the prewash to 7.2. When at 7.2 make up to 1 litre with distilled water and store at 4°C. Just before use, to 500 mls of the prewash solution add 15 g ficoll and 0.5 gm procaine hydrochloride. When dissolved filter the solution.

Embedding medium (full strength araldite)

Araldite CY212 (epoxy resin) 100 ml.
Dodecenyl succinic anhydride (DDSA) 100 ml.
The solutions are mixed thoroughly together.
One drop of accelerator (Benzyldimethylamine (BDMA)) per ml of araldite is added.

1% toluidine blue

1 g of toluidine blue is added to 100 ml of 0.05 M phosphate buffer (pH 7.2).
Epoxy resin removal stock solutions

A Potassium hydroxide 0.5% in absolute methyl alcohol.
B Pioric acid 10% in acetone.
C Potassium hydroxide (30%) 1.5 mls.
Alcohol (80%) 40 mls.

Primary fixative (TEM) 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2)

0.2 M phosphate buffer (pH 7.2) 500 mls.
Glutaraldehyde (25% EM grade) 120 mls.
Made up to 1 litre with distilled water.

Secondary fixative (TEM) 1% osmium tetroxide in Millonig's buffer

1 g of O_2O_4 is added to Millonig's buffer made up of:

Sodium dihydrogen phosphate	75 ml (2.26%)
Sodium hydroxide	15 ml (2.52%)
Glucose	10 ml (5.4%)

Divided into aliquots and stored at 0°C.

4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2)

0.2 M phosphate buffer (pH 7.2) 500 mls.
10% paraformaldehyde 400 mls.
Made up to 1 litre with distilled water.

ELISA buffers

Coating buffer: 0.1M Bicarbonate p.H 9.6 500 mls.

Sodium carbonate	2.12 gms
Sodium Bicarbonate	2.52 gms

Made upto 500 mls with double distilled water.

Wash buffer:
0.15M PBS + 0.05% Tween 20 v/v. p.H 7.4.

Blocking buffer:
0.15M PBS + 0.3% Tween 20 v/v + 1% dried milk.

Developing buffer for IgA ELISA: Diethanolamine p.H 9.8.

101 mg of Magnessium chloride $6H_20$ dissolved in 800 mls double distilled water.
Add 97 mls of Diethanolamine to the magnessium chloride solution. Make upto 1 Ltr. with double distilled water.

Reagents for the single-radial-immunodiffusion (SRD) assay

Dulbeccos PBSA

NaCl	100 gms
KCl	2.5 gms
Na_2HPO_4	14.4 gms
Dist Water	10 litere
10% sodium azide solution	50 mls
	p.H 7.4

Agarose

Seakem MEM	10 gms
(Marine Colloids Inc	
Rockland	
USA)	

PBSA	1 litre
10% sodium azide solution	10 mls

a) Add agarose to PBSA at 4° and mix well
b) Dissolve and melt agarose in PBSA over boiling water bath while stirring
c) If necessary, adjust volume to 1 litre with warm (60°C) water
d) Add NaN_3 solution
e) Decant into 20-25 mls amounts

Zwittergent 3-14 detergent 1gm
(Calbiochem-Behring Corp
La Jolla, CA 92037
USA)
Distilled water 9 mls

Destain solution

Acetic acid	200 mls
Methanol	1 litre
Distilled water	1 litre

Stain

Coomassie Brilliant Blue G 250 (Serva Feinbiochemica Heidelberg)	1.5 gms
Destain solution	500 gms

Dissolve stain and filter through Whatmans Grade 113V filter paper.

APPENDIX 3: METHODS

Paraffin wax tissue-processing

Tissues for paraffin wax embedding were fixed in 4% glutaraldehyde for 24 hours. The water was removed from the tissue using graded alcohols (70, 90, 100, 100%) 1 hour in each. The tissue was then placed in a 50:50 chloroform:alcohol solution for 3 hours then replaced by chloroform and left overnight. With the tissue in a glass jar, melted paraffin wax was added from a paraffin wax dispenser (Electrothermal). The unstoppered glass jar was then placed under vacuum (Hearson) for 5 minutes. The paraffin wax was replaced with fresh and the tissue placed under vacuum for a further 30 minutes. This was repeated again with the tissue left under vacuum for 1 hour. This process eliminates the chloroform while the paraffin wax infiltrates the tissue. Prolonged treatment in molten paraffin wax causes shrinkage and hardening of the tissue, therefore the tissue should be left no longer than is necessary. The tissue was then put into a plastic mould containing warm paraffin wax. Using warm forceps the tissue was orientated in the bottom of the mould in the correct plane for sectioning. For Peyer's patch material the epithelium must lie flat to the bottom of the mould so all the FAE strata will be seen in the finished slide. The paraffin wax was allowed to set at room temperature for 24 hours. The paraffin wax blocks are removed from the moulds and trimmed using a single edge razor blade to remove excess paraffin wax around the tissue. A warm knife was used to melt the paraffin wax block onto a wooden base. The block is sectioned on a rotary microtome (Spenser '820'). The section's thickness was set using a feed mechanism (7 μm). It is important to ensure the upper and lower edges of the paraffin wax blocks are parallel to the knife

edge if ribbon sections are to be cut. Using a camel hair brush to support the leading edge of the ribbon section, they were removed from the microtome and placed in a paraffin wax section mounting bath (Electrothermal) set at 55-50°C. When the sections flatten out they were carefully picked up using glass slides.

The sections were then flattened by exerting pressure using damp filter paper (Whatman No.1). The slides were then put on a slide dryer (Spenser) set at 60°C for 1-2 minutes or until the wax melts. They were left overnight on a slide dryer set at 34°C to remove moisture from the slides.

Staining of paraffin wax sections

The sections were stained using haematoxylin and eosin dyes. The haematoxylin (Harris formula) stain nuclei blue whilst eosin counterstains cytoplasm red. Before staining all the paraffin wax was removed and the section brought down to water. With the sections in a metal slide rack, they were placed in xylene for 3 minutes then graded alcohols (100, 90, 70, 50%) for 1 minute each. The slides were then washed under tap water. The sections were then placed in a solution of Harris haematoxylin for 2 minutes and then washed in tap water. This was regressive stain in that overstaining of the nuclei can be discolourised to remove excess colour with dilute acid alcohol. A solution of 0.5% hydrochloric acid in 70% alcohol was employed for this reason for 10-20 seconds. The sections were then washed in tap water and the sections viewed under the light microscope for nuclei staining. If adequately stained the sections were then placed in a 1% solution of eosin for 30 seconds. Excess dye stain was removed using 50% alcohol. The sections were then dehydrated in graded alcohol (50, 70, 90, 100%) for 1 minute each and a final incubation with xylene for 3 minutes. The sections were then mounted using DPX mounts and are viewed using a light microscope.

Iron test on TEM sections

(i) Epoxy resin removal from tissue sections

Semi-thin sections (1 μm) on glass slides were placed in a coplin jar containing a solution of 1:2:2 potassium hydroxide/acetone/benzene for 5 minutes. The slides were then rinsed in a solution of equal parts acetone/benzene for 1 minute and repeated. A further rinse in a solution of equal parts picric acid/acetone/benzene for 2 minutes

was followed by a solution of equal parts picric acid/acetone for 2 minutes. Further incubation with graded alcohols (100, 100, 95, 95, 80, 80%) for 1 minute each was carried out. The tissue sections were bleached for 3-5 minutes in a solution of potassium hydroxide/alcohol (80%). A rinse in 80% alcohol was followed by a final wash in distilled water.

(ii) Perls' Prussian Blue reaction for ferric ion (Perls, 1867)

The ferric ion, which is released from proteins by treatment with dilute hydrochloric acid, reacts with dilute potassium ferrocyanide to precipitate insoluble blue compound ferric ferrocyanide (Prussian blue). A solution containing 2% potassium ferrocyanide, 2% hydrochloric acid (prepared fresh before use) was added to the tissue sections for 10-20 minutes, after which the solution was washed off with water. The tissue sections were then observed under the light microscope.

Preparation of submicron PLG microparticles containing rhodamine

(a) 300mg of poly(lactide-co-glycolide) polymer was added to 5mls Dichloromethane (HPLC grade).

(b) 5 mgs of rhodamine dye was added to 2ml of distilled water.

(c) The polymer solution was added dropwise to the rhodamine solution, and the mixture stirred at 12,400 rpm with a Silverson homogeniser using a 12mm diameter stirrer head for 2 minutes, to give a water-in-oil (w/o) emulsion.

(d) 20 mls 10% w/v polyvinylalcohol in distilled water (PVA) was added to the mixture and stirred at 12,400 rpm for 5 minutes.

(e) The mixture was stirred overnight using a magnetic stirrer to allow the solvent to evaporate.

(f) The microparticle suspension was centrifuged for 45 minutes at 10,000 r.p.m. The supernatant was discarded and the microparticle suspension was redispersed in distilled water (15mls). This step was repeated two times for 20 minutes.

(f) The microparticles were freeze dried and stored in a desiccator at 25°C.

Microscopic counting method to count the number of rhodamine fluorescent microparticles in lymph

A 5 ul aliquot of lymph was placed on a glass slide. A coverslip was placed over the sample and sealed using nail varnish gloss to prevent drying. Each slide was viewed under a fluorescent microscope set for rhodamine fluorescence (excitation maximum 553nm, emission maximum 619nm). A grid on the microscope stage was used so the total area of the slide was viewed and the total numbers of microparticles in the sample was counted. The number of microparticles in a microparticle suspension was calculated using a Neubauer chamber viewed under the microscope.

PLGA microparticles embedding for TEM analysis

(a) Approximately 5 mg of a freeze dried preparation was resuspended in 2 mls of distilled water and spun in a bench centrifuge at 10,000 r.p.m. for 30 minutes to form a pellet.

(b) The pellet was fixed in 1 ml of 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer for 1 hour.

(c) The pellet was fixed for 1 hour in 1% osmium tetraxide for 1 hour.

(d) The pellet was washed three times in 0.1 M sodium cocodylate in 6% sucrose for 45 minutes.

(e) The pellet was washed three times in distilled water for 45 mins.

(f) The pellet was placed in 2% uranyl acetate for 30 minutes.

(g) Dehydration was performed by placing the pellet in graded alcohols (40%, 60%, 80%, 100%, 100%, 100%) for 20 minutes in each.

(h) The pellet was left o/n in a 3:1 ethanol:resin (Epon resin) mixture.

(g) The pellet was left in a 1:1 ethanol:resin mixture for 8 hours.

(h) The pellet was left in resin for 1 day.

The pellet was resuspended during each step and then pelleted by being spun for 2 minutes at 10,000 r.p.m using a bench centrifuge.

(g) The pellets were then cast into Epindorfs containing epon resin and placed in a 60°C oven for 48 hours.

PLG microparticle preparation for SEM analysis

(a) Freeze dried preparations were suspended in distilled water and a drop of the solution pipetted onto an aluminium stub and air dried overnight.

(b) The samples were then coated with gold using a sputter coater (EMSCOPE 500) at a coating current of 15mA for 2 minutes.