

**THE BIOLOGICAL SIGNIFICANCE OF AChE
SECRETION BY *NIPPOSTRONGYLUS BRASILIENSIS*.**

W. S. Russell

**A thesis submitted for the degree of Doctor of Philosophy to
the University of London.**



Abstract

This study has demonstrated that the parasitic nematode *Nippostrongylus brasiliensis* secretes acetylcholinesterases (AChEs) *in vivo*. This was determined by recovering the contents of the jejunal lumen from rats at different time points post-infection and assaying AChE activity following separation by non-denaturing gel electrophoresis. High levels of butyrylcholinesterase (BuChE) were present in uninfected and infected animals, but AChE isoforms were only present in infected animals, and showed a similar profile of resolution to AChEs isolated from *in vitro* culture of parasites. Histochemical analysis of intestinal sections revealed focal AChE activity within the mucosa of infected but not uninfected animals, localised to the basement membrane of intestinal epithelial cells. Parasite AChEs were unable to traverse confluent epithelial cell monolayers *in vitro*, suggesting that the activity observed *in vivo* is either due to activation of endogenous AChEs, or that parasite enzymes gain access to the mucosa following an increase in epithelial cell permeability.

Localisation of muscarinic acetylcholine receptors (mAChRs) using a mAChR-specific antibody (M35) revealed positive staining in the external muscle of the small intestine and on the basement membrane of epithelial cells in both infected and uninfected animals, although an upregulation of receptor expression on enterocytes and cells in the lamina propria of infected animals was observed. Radioligand binding studies on isolated epithelial cells showed an increase in mAChR density and dissociation constant. Isolated epithelial and lamina propria cells expressed mRNA for the m3 mAChR subtype using reverse transcription polymerase chain reaction (RT-PCR).

Lymphocytes were isolated from *N. brasiliensis*-infected spleen and mesenteric lymph nodes (MLNs), and stimulated with a range of muscarinic agonists. These agonists had no effect on lymphocyte proliferation and did not augment proliferative responses to the T-cell mitogen concanavalin A (Con A). These data are discussed with reference to mucosal immunity to nematode parasites.

Acknowledgments

I would first like to thank my supervisors, Dr. Murray Selkirk and Dr. John Tippins for their help, advice and patience over the past few years. Murray (you pseudo-southern jessie), no matter how many times you describe your 'old drinking holes' in Salford...they've gone!

The past and present members of the lab have made the 'Selkirk experience' a thoroughly enjoyable one, with thanks:

to Ruth, for smuggling Welsh cakes out of Swansea (thanks Mr & Mrs T.!) and just being there,

to Matt, for the most amazing parties, BBQ's, dodgy geezer deals. One day soon you WILL have a cordless powertool!

to Clivey Clive and Nicola, for collaboration in the hectic world of fantasy league football, Siany Sian, Ayman, Gustavo (you animal) for their help and advice, and last but by no means least, Sero(sexy)phena, my lab mother/wife and general tormentor! to Luscious Lind, thanks for the advice and long-distance encouragement, I'm readily preparing for your 40th birthday!

to Chris and Helen, for feeding and watering me, and putting up with my absent mindedness over the summer.

to the footballing trio; Curly Kev, Stu, and Chopper Harris, for in-depth chats on all things football and those 'just one for the road' drinking binges.

to Dr. Graham Wilkin for the use of his lab, and to Suba and Cecilia for sharing the pain of writing-up, you'll soon be finished!.

to Brendan (Brenda-one times and a fish), a huge thanks for your help during the putting together of the thesis, I'll reserve you a blonde for when you come over.

to James (Ally M^cB), for living through my experiments in the kitchen, and keeping me sane (though this is debatable?!).

And finally, to my parents and brother Paul, whose love and support has helped me through the good and bad times-THANK YOU!

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Abbreviations

p.i.	Post infection
L3s	Third stage larvae
L4s	Forth stage larvae
GI tract	Gastro-intestinal tract
O-MALT	Organised Mucosa-Associated Lymphoid Tissue
D-MALT	Diffuse Mucosa-Associated Lymphoid Tissue
PP	Peyer's patches
Ig	Immunoglobulin
FAE	Follicle-associated-epithelium
M cells	Membranous epithelial cells
IEL	Intra-epithelial lymphocytes
LPL	Lamina propria lymphocytes
TDL	Thoracic duct lymphocytes
GALT	Gut associated lymphoid tissue
IEC	Intestinal epithelial cells
BMMC	Bone marrow derived mast cells
MLN	Mesenteric lymph nodes
TCR	T- cell receptor
ENS	Enteric nervous system
VIP	Vasoactive intestinal peptidergic
NA	Noradrenergic
NPY	Neuro peptide Y
WGA	Wheat germ agglutinin
RMCP	Rat mast cell protease
T _h	T helper
T _c	Cytotoxic T-cells
LPS	Lipopolysaccharide
EFS	Electrical field stimulation
GalNac	N-acetyl-D-galactosamide

ACh	Acetylcholine
mAChR	Muscarinic acetylcholine receptors
nAChR	Nicotinic acetylcholine receptors
AChE	Acetylcholine esterase
ChE	Choline esterase
VACht	Vesicular acetylcholine transporter
CGRP	Calcitonin gene related peptide
IFN	Interferon
IL	Interleukin
TNF	Tumour necrosis factor
5-HT	5- Hydroxytryptamine
PGE₂	Prostaglandin E₂
TTX	Tetrodotoxin
PAF	Platelet activating factor
GH	Growth hormone
NGF	Nerve growth factor
Oxo-M	Oxotremorine
PBS	Phosphate buffered saline
DAB	3, 3 Diaminobenzidine
BSA	Bovine serum albumin
ATCI	Acetylcholine iodide

CHAPTER 1

Introduction

1.1 The parasite

Nippostrongylus brasiliensis is a parasitic nematode of the small intestine. Its main natural hosts are *Rattus norvegicus* and *R. rattus*, but it has also been adapted for experimental use in the mouse (Ogilvie & Jones, 1971). Over the past 30 years the parasite has been widely used as a model system to study immunity against intestinal helminths and inflammatory responses within the intestinal tract. The main advantages of using this parasite is that it is easily maintained in the laboratory, has a relatively short life cycle and the host response^s resembles those^{made to} of[?] similar nematodes which are of medical or veterinary importance (Ogilvie & Jones, 1971).

The life cycle consists of two phases, with early development outside the host and the final stages of development occurring inside the host (Figure 1.1). Like most intestinal nematodes it has no intermediate hosts.

Following penetration of the host skin by the infective third-stage larvae (L3s), the L3s migrate through the lymphatic and vascular system to the lungs, which takes about 15 hours post-infection (p.i.). The L3s moult to the fourth-stage larvae (L4) and migrate up the trachea and down the oesophagus to the small intestine (the L4s are usually coughed up and swallowed), with most of the L4s reaching the intestine 3 days p.i. L4s undergo a final moult to the adult stage by day 4 p.i., and eggs are passed by the females which are expelled in the faeces by day 6 p.i. Eggs hatch after 24 hours, the rhabditiform larvae moult twice within 48 hours, finally moulting to the filariform L3-stage that exsheaths, stops feeding and migrates to the edge of the faeces to await a potential host.

In the laboratory the life cycle is generally maintained by injecting 5-6,000 infective L3s sub-cutaneously into the flank of the rat or 5-700 L3s into the flank of the mouse host.

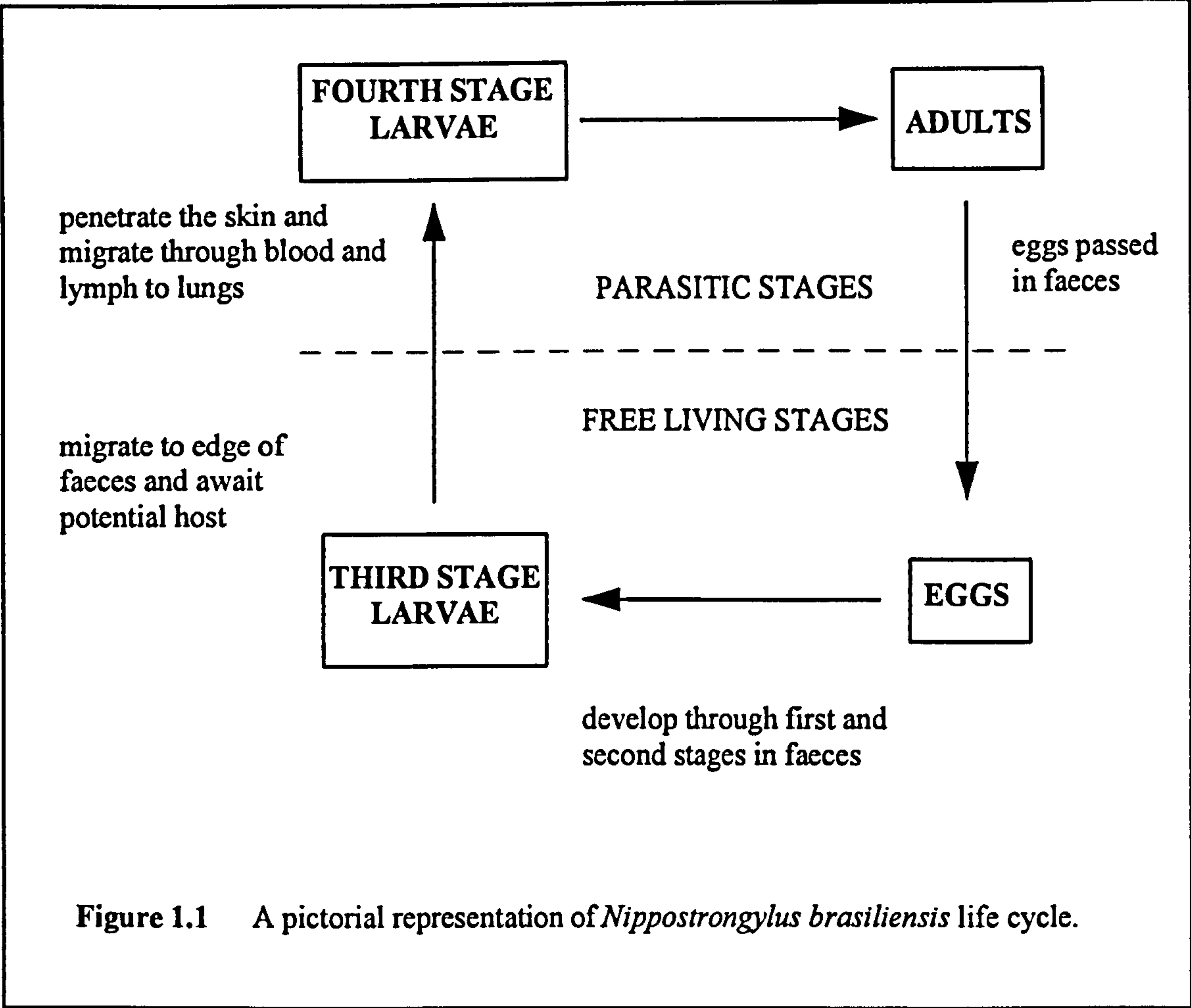


Figure 1.1 A pictorial representation of *Nippostrongylus brasiliensis* life cycle.

This challenge induces a strong inflammatory response resulting in expulsion of most worms, known as the ‘self-cure’ reaction, by the end of the second week (Mulligan *et al.*, 1965). Work by Kassai and colleagues (1987) demonstrated that worms undergoing rejection from the host were not killed or irreversibly damaged, but had decreased motility and impaired locomotory capacity. Host immunity therefore acts to remove the parasite rather than kill it. Experiments have been carried out using continual, low doses of L3s (five infective L3s, three times per week in rats) termed (2) ‘trickle infection’. This is thought to be more akin to natural infections. In these cases a chronic, residual infection is established whereby some worms appear to adapt and withstand host immunity (Jenkins & Phillipson, 1970, 1972). However, these effects could only be observed in young rats, and administration of continual low doses of L3s

to aged rats (11 weeks and older) resulted in parasite expulsion within two weeks (Jenkins, 1974). These findings suggested that parasite removal was dependent on the immune status of the host. In the past 30 years, researchers have tried to determine the processes by which the host immune system removes these parasites, and this work will be discussed later in this thesis.

1.2 The Gastro-intestinal tract

The primary role of the gastro-intestinal tract (GI tract) is the digestion and absorption of dietary molecules by the epithelium. Mucosal surfaces (GI tract, respiratory and urinogenital tracts) are unique, acting as a physical barrier between the external environment and the internal milieu and generally consisting of a single layer of cells. The intestinal epithelium represents a vast surface area (approximately 100-400 m²) that is constantly under assault from a diverse array of molecules, ranging from innocuous dietary products to noxious pathogens.

It is therefore not surprising that the intestinal mucosa is the largest immunological organ in the body in terms of lymphoid cells. The intestinal immune system can be divided into two functional compartments (Figure 1.2). The Organised Mucosa-Associated Lymphoid Tissue (O-MALT) which are highly specialised, local sites of inductive responses and Diffuse Mucosa-Associated Lymphoid Tissue: (D-MALT) consisting of effector sites diffusely distributed throughout the intestine.

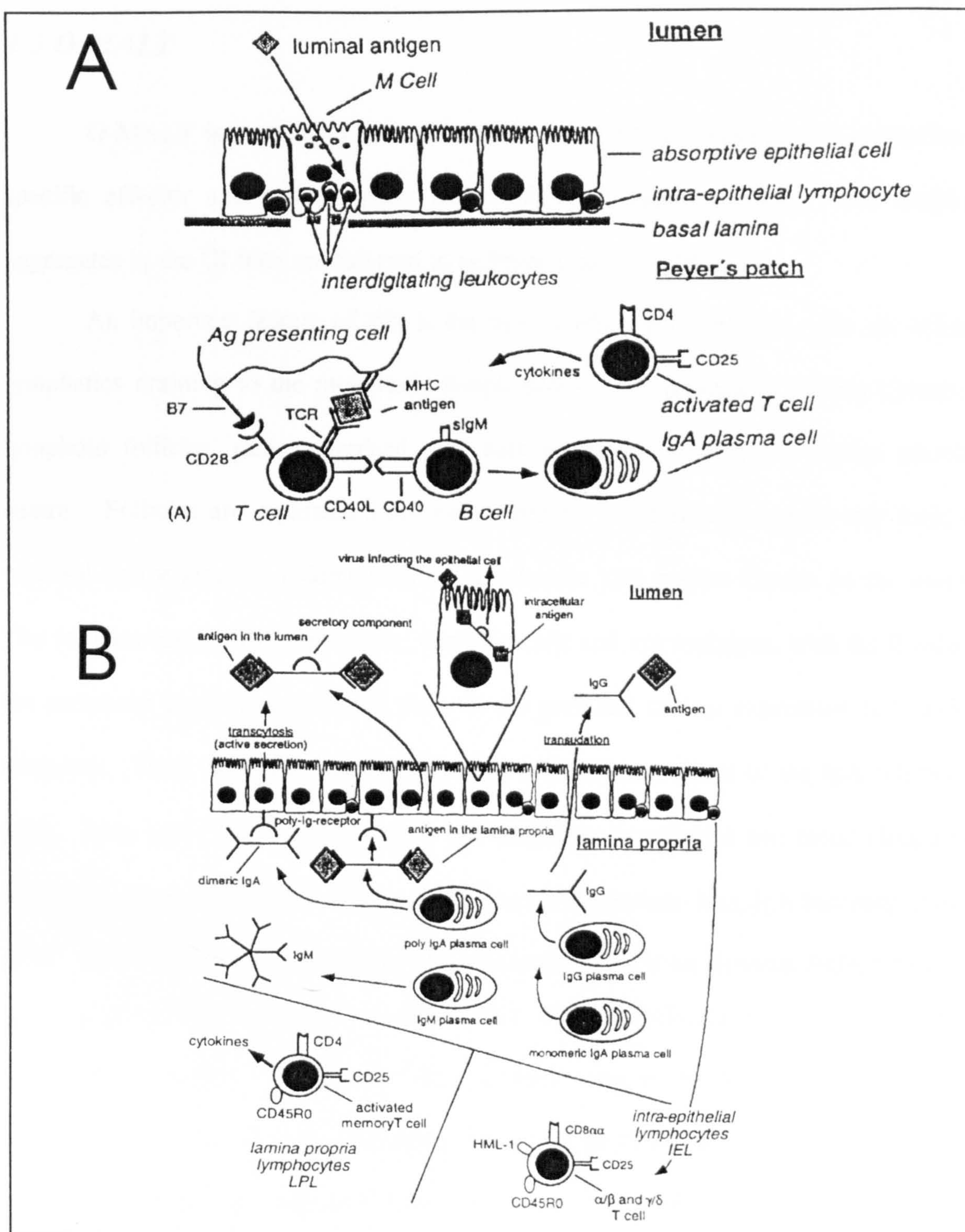


Figure 1.2. Schematic illustration of the two functional compartments of the intestinal immune system. A: O-MALT consists of Peyer's patches, which are the inductive sites for intestinal immune responses. Luminal antigens are transported across the epithelium by M cells, processed by antigen presenting cells and presented to T-cells. B: D-MALT is the effector component of the mucosal immune system, and is dominated by differentiated T-cells (IEL and LPL) and the secretory IgA system. (Adapted from Köhne *et al.*, 1996).

1.3 O-MALT

O-MALT is thought to be the major site of antigen sampling, and generation of specific effector and memory cells (Kraehenbuhl & Neutra, 1992). The lymphoid aggregates in the GI tract are referred to as Peyer's patches (PP).

An important feature of PPs is the lack of afferent lymphatics, with the efferent lymphatics draining to the mesenteric lymph nodes. PP aggregates contain clusters of lymphoid follicles, each comprised of a dark peripheral zone and a lighter germinal centre. Follicles are separated from each other by intrafollicular spaces and from the mucosal epithelium by a dome-shaped, lymphocyte rich region known as the corona. The follicles contain B-lymphocytes, dendritic cells and macrophages, with the B-cells in the periphery expressing IgM and those in the germinal centres expressing IgA surface receptors. These cells are thought to be the immediate precursors of the IgA effector B-cells. They leave the O-MALT, enter the mesenteric lymphatics and blood circulation, migrating to remote mucosal sites where they differentiate into IgA-secreting plasma cells. The level of IgA in O-MALT is low because there is no terminal differentiation of IgA⁺ T_H lymphocytes predominate in the inter- and parafollicular regions. Both subsets of T-cells are present, with CD4⁺ T-cells predominating in the dome area and CD8⁺ T-cells abundant in the parafollicular zones (Moqbel & MacDonald, 1990).

The patches are separated from the lumen by a specialised epithelium (follicle-associated-epithelium: FAE) containing columnar epithelial cells interspersed with membranous epithelial cells (M cells) which represent 10% of the FAE, some lymphocytes and a few goblet cells.

M cells are highly adapted to promote the selective entry and transport of luminal antigens (Owen & Jones, 1974; Savidge, 1996), lacking the organised brush border and thick glycocalyx observed on the apical membrane of enterocytes. They possess a deeply invaginated basolateral membrane which acts as a pocket domain that accommodates lymphocytes and macrophages (Neutra *et al.*, 1987). Both B- and T-cells can be found in the pocket domain. The majority of T-cells are CD4⁺, are activated and express the memory cell marker CD45RO (Neutra *et al.*, 1996). In contrast the B-cell population expresses the naive cell marker CD45RA and express IgM rather than IgA or IgG.

The role of M cells in antigen trafficking and presentation has yet to be clearly understood. Molecules that adhere to the apical membrane of the M cell can be rapidly endocytosed, transported and released at the pocket domain within 10-15 minutes (Neutra *et al.*, 1996). It was generally thought that no degradation of the antigen occurred, resulting in intact antigen being released in close proximity to lymphocytes and macrophages. Here macrophages and naive B-cells could phagocytose the antigen and present it to T-cells via the MHC class II pathway. Owen and co-workers (1986) demonstrated that M cells possess acid phosphatase-containing pre-lysosomal and lysosomal compartments, but the presence of major histocompatibility complex class II (MHC class II) is required for conventional antigen presentation (Panja & Mayer, 1996). Very few M cells in the human intestinal tract express class II molecules (Bjerke & Brandtzaeg, 1988), but M cells from rodents possess MHC class II in the endosomal and lysosomal compartments (Allan *et al.*, 1993). This suggests that M cells from rodents may have a role in antigen processing and presentation.

1.4 D-MALT

Diffuse lymphoid tissue is distributed throughout the mucosal tissue, and consists of lymphocytes and plasma cells dispersed in the lamina propria and interstitial tissues (Kraehenbuhl & Neutra, 1990). D-MALT contains two populations of T-cells which are phenotypically distinct from each other, and from T-cells in other lymphocytic compartments. Those which reside between intestinal epithelial cells near the basement membrane are termed intra-epithelial lymphocytes (IEL) and those diffusely disseminated in the intestinal lamina propria are termed lamina propria lymphocytes (LPL).

T-cells can be divided into two classes based on the subunit composition of the T cell receptor (TCR). The alpha/beta ($\alpha\beta$) TCR is expressed by the majority of T-cells (approximately 90%), and these cells undergo development in the thymus before migrating to peripheral tissues. The second class of T-cell express the gamma/delta ($\gamma\delta$) heterodimer, constitute less than 5% of peripheral T-cells, and can develop extrathymically as well as in the thymus.

90% of IEL express the lymphocyte surface marker CD8 (Viney *et al.*, 1989), with equal numbers of cells expressing the $\gamma\delta$ or $\alpha\beta$ TCR (Goodman & Lefrancois, 1989). All the $\gamma\delta^+$ IEL and approximately a third of the $\alpha\beta^+$ IEL express the CD8 $\alpha\alpha$ homodimer rather than the CD8 heterodimer $\alpha\beta$ (Lefrancois, 1991; Lefrancois & Puddington, 1995).

$\gamma\delta$ T-cells have been implicated in the protective responses stimulated against intracellular bacteria (Mombaerts *et al.*, 1993), and work by Fujihashi and colleagues (1992, 1996) observed that mice deficient in $\gamma\delta$ T-cells (TCR $\delta^{-/-}$) have reduced numbers of IgA plasma cells and a decrease in levels of serum IgA in response to tetanus toxoid. It has been demonstrated in mouse and rat that intestinal epithelial cells (IEC) express MHC class II and

can present peptides to T-cells *in vitro* (Bland & Warren, 1986a, 1986b; Kaiserlian *et al.*, 1989; Hoyne *et al.*, 1993). Earlier work suggested that rat IEC preferentially activate CD8⁺ T-cells that display a suppressor phenotype (Bland & Warren, 1986b), whereas Kaiserlian and co-workers (1989) demonstrated that murine IEC present to, and activate a CD4⁺ T-cell hybridoma (CAK 1.22.3). However, recent work has shown that murine IEC downregulate IEL proliferation but not splenic lymphocyte proliferation *in vitro* (Yamamoto *et al.*, 1998). The inhibitory effect was apparent in both $\alpha\beta$ - and $\gamma\delta$ - IEL, involved a reduction of mRNA levels for T_h1 (IFN- γ and IL-2) and T_h2 (IL-4 and IL-5) cytokines in these cells and required cell-cell interaction. $\gamma\delta$ -IEL in turn have regulatory effects on IEC generation and proliferation (Komano *et al.*, 1995). IEL TCR δ ^{+/−} mice have reduced numbers of crypt cells, a down-regulation of crypt cell generation and a decrease in the expression of MHC class II (as crypt cells mature and migrate from crypt to villus they begin to express MHC class II antigens) in comparison to TCR δ ^{+/−} mice. These data suggest the cross-talk between IEL and IEC is complex, with IEC able to prevent potential inflammatory responses at the mucosal surface and IEL promoting epithelial growth and differentiation, which may play a role in healing damaged epithelia.

As discussed previously, lymphocytes that are diffusely disseminated in the intestinal lamina propria are termed LPL. This population consists of terminally differentiated B-cells (originating from O-MALT sites) mainly producing IgA, macrophages, neutrophils, MMC, with the majority of the T-cells expressing the CD4⁺ surface marker (Kraehenbuhl & Neutra, 1992). The CD4⁺ T-cells preferentially produce the cytokines IL-4, IL-5, IL-6 and IL-10 (T_h2 type cytokines), which induce the terminal differentiation of B-cells into IgA producing plasma cells, and promote IgA responses in the intestine (Beagley *et al.*, 1988; Xu-Amano *et al.*, 1993).

LPL T-cells appear to be in an activated state, determined by the surface markers they express. These T-cells express predominantly the CD45RO antigen and the IL-2 receptor (markers for memory T-cells), in contrast to peripheral blood lymphocytes (Köhne *et al.*, 1996). The increased state of activation of LPL T-cells may be a consequence of permanent exposure to antigens and mitogens in the intestinal lumen.

1.5 The enteric nervous system

The neural innervation of the intestine includes sympathetic, parasympathetic and enteric nerve fibres. The central nervous system (CNS) monitors sensory information from the intestine and sends commands via the sympathetic and parasympathetic neurons to the enteric ganglia, or directly to the mucosal cells to activate effector mechanisms (Cooke, 1986). Pre-ganglionic sympathetic fibres arise from neurons in the spinal column, between the fifth thoracic and the second or third lumbar segments. The cells have long post-ganglionic fibres that branch and innervate the intestine, terminating at enteric ganglia and blood vessels, with some fibres subjacent to epithelial cells. The pre-ganglionic parasympathetic fibres originate from the dorsal motor nucleus of the vagus and are carried by vagal efferent fibres to the intestine.

The enteric nervous system (ENS) is an independent, integrative system that consists of the myenteric plexus and the submucosal plexus (Figure 1.3). Both are ganglionated and interconnected into a single, functional system (Cooke, 1986). The texture, fluidity, volume, chemical composition and temperature of the lumen are monitored by the intramural and mucosal sensory receptors. Neurons from the submucosal ganglia project into the mucosa, possibly controlling and co-ordinating the

secretory and absorptive functions, blood flow and the contractility of the muscularis mucosae (Cooke, 1986).

The control mechanisms that regulate these functions involve extrinsic reflexes from the CNS and intrinsic reflexes within the enteric ganglia. Classic reflexes require the detection of stimuli by afferent sensory neurons, which are processed by the interneurons located in the ganglia and transmitted as signals to the effector/secretomotor neurons.

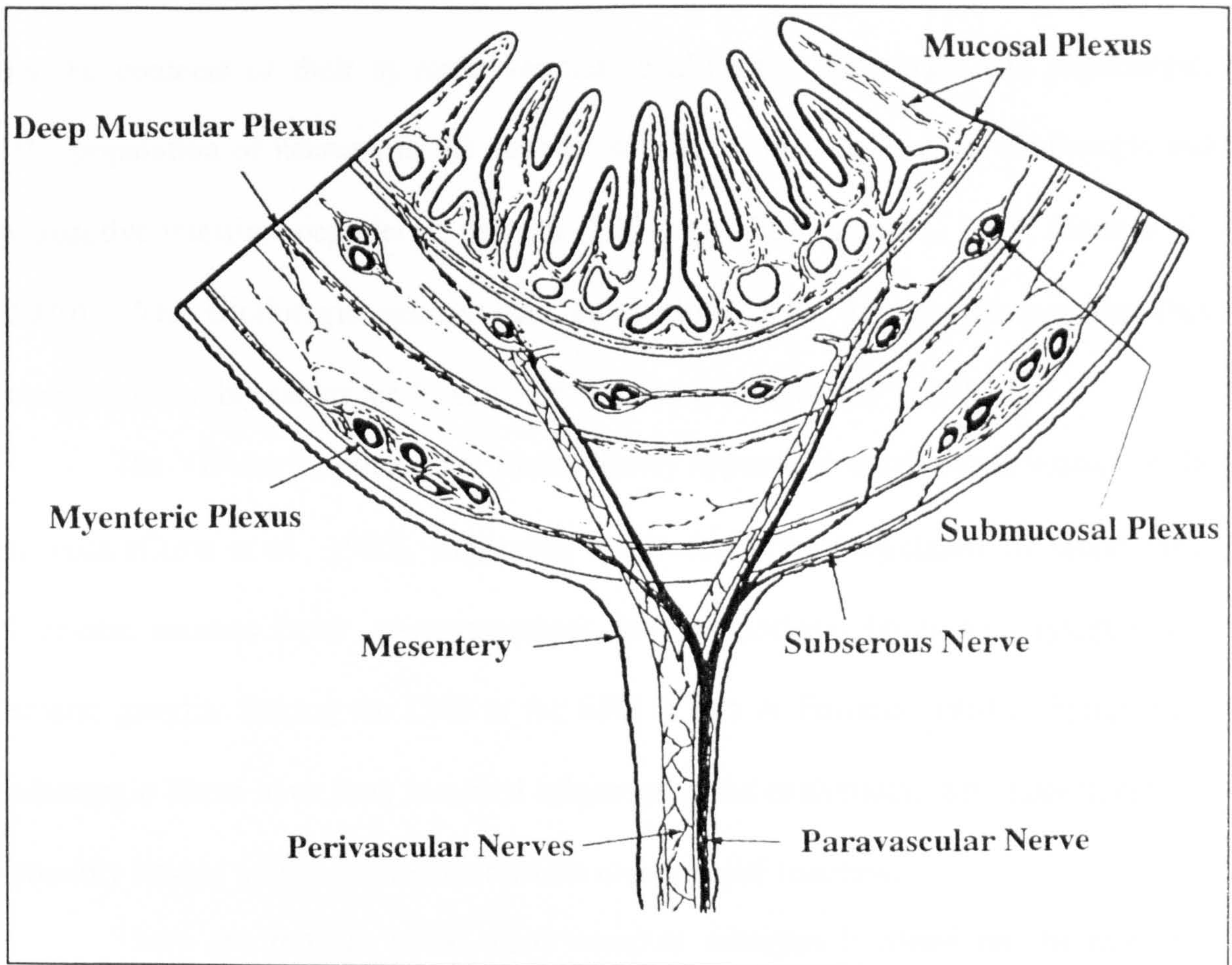


Figure 1.3. Diagram showing the arrangement of enteric nerves in a transverse section through the intestinal wall. (Adapted from Furness & Costa, 1980).

Reflexes may also occur when afferent sensory neurons cause the spread of action potentials into adjacent nerve processes and the release of neurotransmitters close to the mucosal effector sites (Cooke, 1994; Figure 1.4).

Histochemical studies have revealed three types of axons that are distinguished by the contents of their synaptic vesicles: cholinergic, adrenergic and peptidergic. The population of neurons in the submucosal plexus are made up of cholinergic and vasoactive intestinal peptidergic neurons (VIP-ergic: Furness *et al.*, 1983; Costa *et al.*, 1980). The cholinergic neurons can be subdivided by the specific peptides they contain, including substance P, somatostatin and Neuropeptide Y (NPY).

The VIP-ergic neurons are found closely apposed to small blood vessels of the mucosa (Costa *et al.*, 1980), suggesting a role for VIP in regulation of blood flow. Extrinsic neurons from parasympathetic and sympathetic fibres also synapse with enteric ganglia, linking the CNS to the ENS (Costa & Furness, 1984). Sympathetic adrenergic fibres have been localised subjacent to the epithelium, with norepinephrine possibly having a direct or indirect action on epithelial function.

There are thought to be many receptor subtypes localised on the epithelial membrane. These include receptors for acetylcholine (ACh), substance P, VIP, NPY, somatostatin and adrenocorticotropin. Substance P, VIP and ACh are all involved in stimulating secretory processes and regulating intestinal fluid and electrolyte flow (Cooke *et al.*, 1995; Tapper *et al.*, 1978; Wang *et al.*, 1995). Fluid secretion is thought to be controlled by classic reflexes, but the role of extrinsic and intrinsic axon reflexes as a regulatory mechanism cannot be discounted (Cooke, 1994). The secretory reflexes limit the absorptive capacity of the intestine by stimulating the net flow of sodium and chloride ions, and the subsequent efflux of water into the lumen (Cooke, 1994).

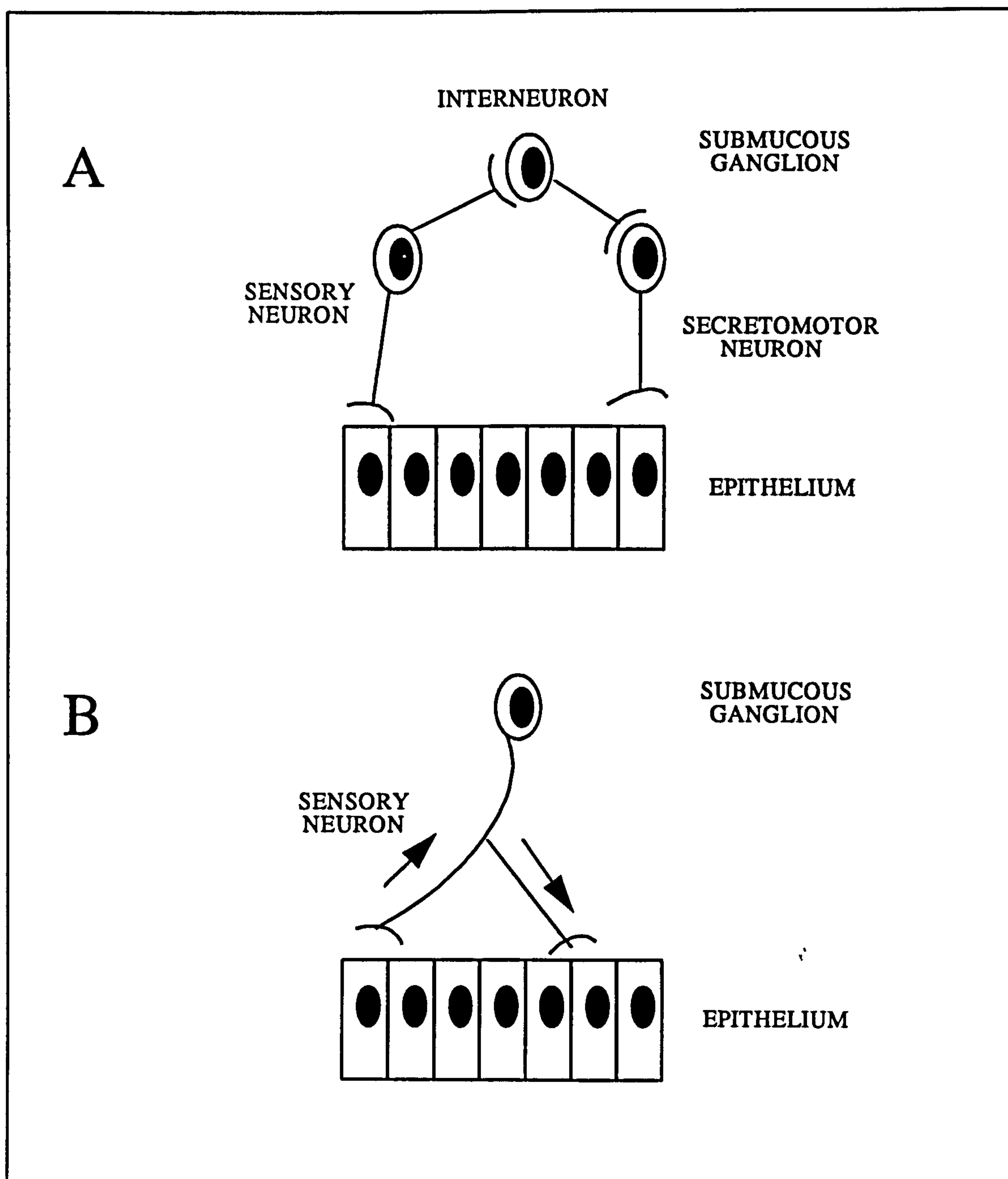


Figure 1.4. Diagrammatic representation of the types of reflex arc found within the intestine. A: the classic reflex, involving the detection of stimuli by sensory neurons, followed by processing within the inter-neurons and transmission of signals to effector neurons (motor neurons). B: axon reflex, where activation of afferent sensory neurons results in the antidromic spread of action potentials into adjacent axons near the epithelium. (Adapted from Cooke, 1994).

Sensory neurons are stimulated by changes in the lumen (including intestinal distension, mucosal stroking and luminal irritants), setting up reflex cascades with signals transmitted to cholinergic interneurons in the myenteric and submucous plexuses. These signals are passed to the epithelium through VIP or cholinergic secretomotor neurons, with nerve transmission to the epithelium via muscarinic cholinergic receptors or VIP receptors located on enterocytes (Figure 1.5; Cooke, 1986; 1994). The inhibition of fluid secretion is regulated by sympathetic input. Signals from sympathetic nerves shut down enteric reflexes, inhibiting the presynaptic transmission and postsynaptic suppression of neuronal excitability. This effect can be enhanced by the release of norepinephrine, which mobilises ions and water from the intestinal lumen into the circulation (Greenwood *et al.*, 1987; Hemlin *et al.*, 1987). Sympathetic innervation therefore, acts as a braking system for the secretory mechanism and prevents unnecessary secretion that could lead to physiological complications, such as debilitating diarrhoea (Perdue & McKay, 1994).

1.6 Immunopathology of *N. brasiliensis* infection

The immunological and pathological changes in the gastrointestinal tract on contact with *N. brasiliensis* antigen have been widely documented, but the precise protective events within the parasitised gut remain unclear (Moqbel, 1986). Local inflammation in the intestine is more pronounced in heavily infested areas of the small intestine, mainly the jejunum. This causes marked thickening of the muscularis externa and an increased muscle mass due to hypertrophy of the longitudinal smooth muscle (Castro, 1989).

During the inflammatory response it has been shown that the villi of the jejunum became shortened, atrophic and irregular in size. The microvilli are shortened by half and the brush border surface ^{becomes.} irregular and less densely packed ~
(Castro, 1989).

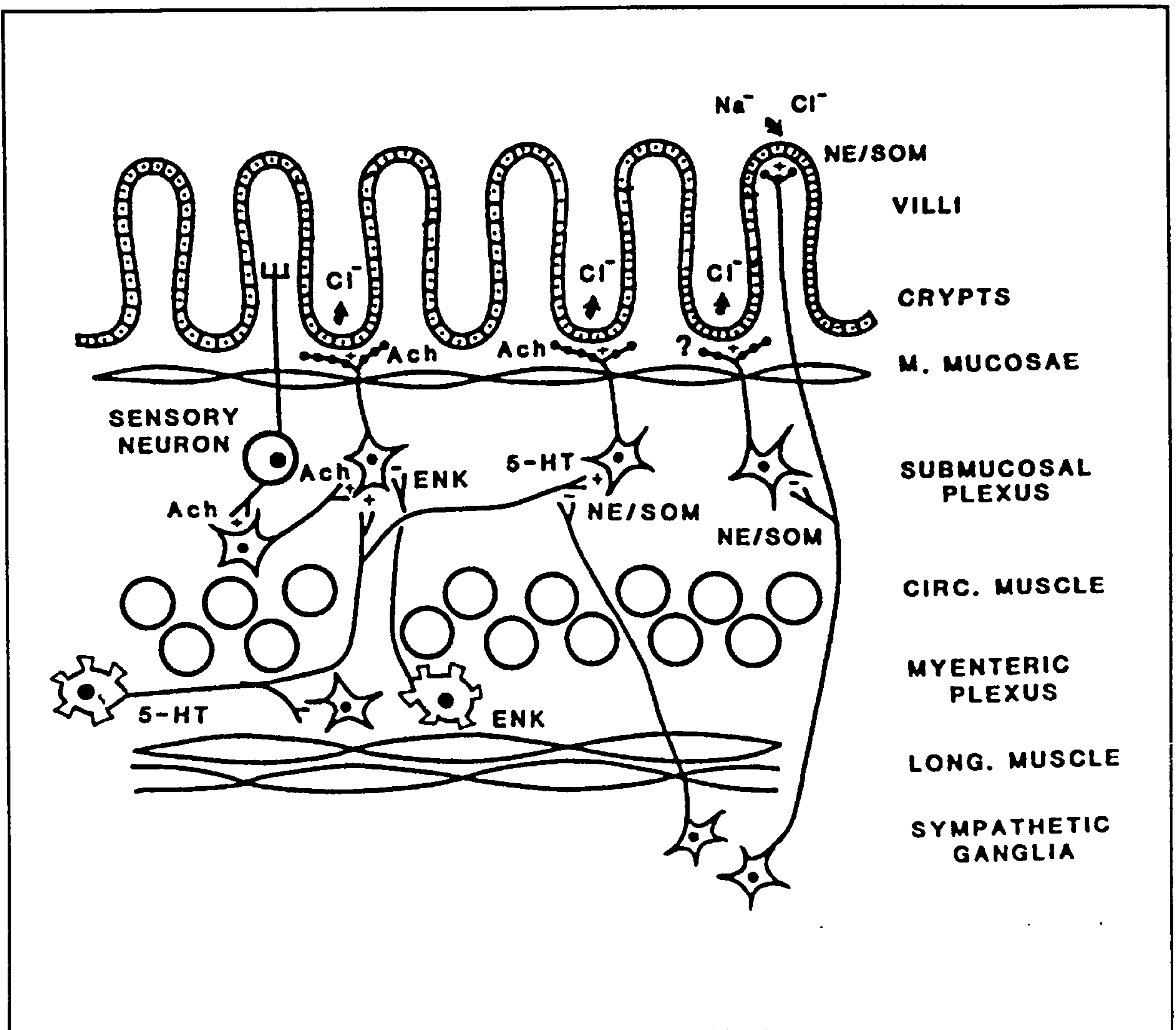


Figure 1.5. Model for control of mucosal transport by the nervous system. Sensory neurons within the mucosa are activated by chemical or mechanical stimuli, leading to activation of interneurons and/or cholinergic motor neurons innervating the intestinal crypts. Acetylcholine released from cholinergic motor neurons evokes chloride secretion by acting on muscarinic receptors found on the crypt enterocytes. This is modulated by intrinsic input from serotonergic and enkephalinergic neurons, and from extrinsic sympathetic release of norepinephrine (Adapted from Cooke, 1986).

It has been suggested that the loss of jejunal surface area may in some way contribute to malabsorption during infection (Symons, 1976). The malabsorption in the jejunum is not confined to the uptake of water and electrolytes. Glucose, methionine, histidine and palmitic acid absorption are also depressed during infection, with the degree of malabsorption directly related to the severity of infection (Symons *et al.*, 1971).

In addition, Castro and Harari (1986) showed that in both *Trichinella spiralis* and *N. brasiliensis*-infected intestine there was a reduced capacity of the brush border membrane (BBM) to bind the lectin wheat germ agglutinin (WGA), due to a decreased expression of terminal sialic acid residues on membrane glycoproteins.

The immune response to *N. brasiliensis* is complex and controversial. Ogilvie and Jones (1971) suggested a two stage response. The first stage was proposed to be mediated by humoral immunity, inducing what was termed 'metabolic damage'. This includes structural changes to the intestine and secretory glands of the parasite, as well as a decreased motility (Love *et al.*, 1975). The damage is reversible, demonstrated by the transfer of damaged worms to a naive second host, where the worms are able to establish successfully (Kassai *et al.*, 1987). The second stage of the immune response appeared to be mediated by T-cells, and it has been suggested that this phase brought about parasite elimination by the non-specific release of amines or T-cell lymphokines that interfered with parasite metabolism (Dineen *et al.*, 1973).

A different mechanism for expulsion was suggested by Larsh and Race (1975). Working with mice infected with *T. spiralis* (another parasitic nematode that has an intestinal phase in its life cycle), they showed that there was a close, direct pattern of acute intestinal inflammation correlated with parasite expulsion. The intensity and onset of inflammation were associated with the degree of sensitivity of the host challenged and

the size of challenge, and persistence of worms only occurred in the absence of acute inflammation. The first stage of expulsion was suggested to result from an immunologically specific, delayed-type hypersensitivity reaction between antigen-sensitive T-cells and antigen resulting in tissue injury. The second stage was proposed as an immunologically non-specific inflammatory response to the injury resulting in tissue changes that proved unfavourable for the parasite.

Infection with *N. brasiliensis* is generally self-limiting, with most of the adult worms eliminated from the intestine by a self-cure phenomenon by the end of the second week. Effector mechanisms are now known to be regulated by the CD4⁺, T helper (T_h) cells. T_h cells have been divided into several phenotypes, characterised by the cytokines they secrete (Mossmann & Coffman, 1989), with the cytokine environment and antigenic stimulus determining which subset proliferates (Figure 1.6). The T_h0 phenotype is indicative of naive cells, which may secrete many types of cytokines. T_h1 cells tend to secrete interferon gamma (IFN-γ), interleukin 2 (IL-2), IL-12 and tumour necrosis factor (TNF), and T_h2 cells IL-4, -5, -6, -9, -10 and -13. T_h1 and T_h2 phenotypes have opposing roles, with T_h1 eliciting a cytotoxic cellular response and T_h2 stimulating a humoral response. The T_h phenotypes have antagonistic effects. IFN-γ and IL-12 are known to downregulate T_h2 responses, pushing the immune response towards the T_h1 phenotype, while T_h2 cytokines IL-4 and IL-10 promote the T_h2 response (Urban *et al.*, 1992).

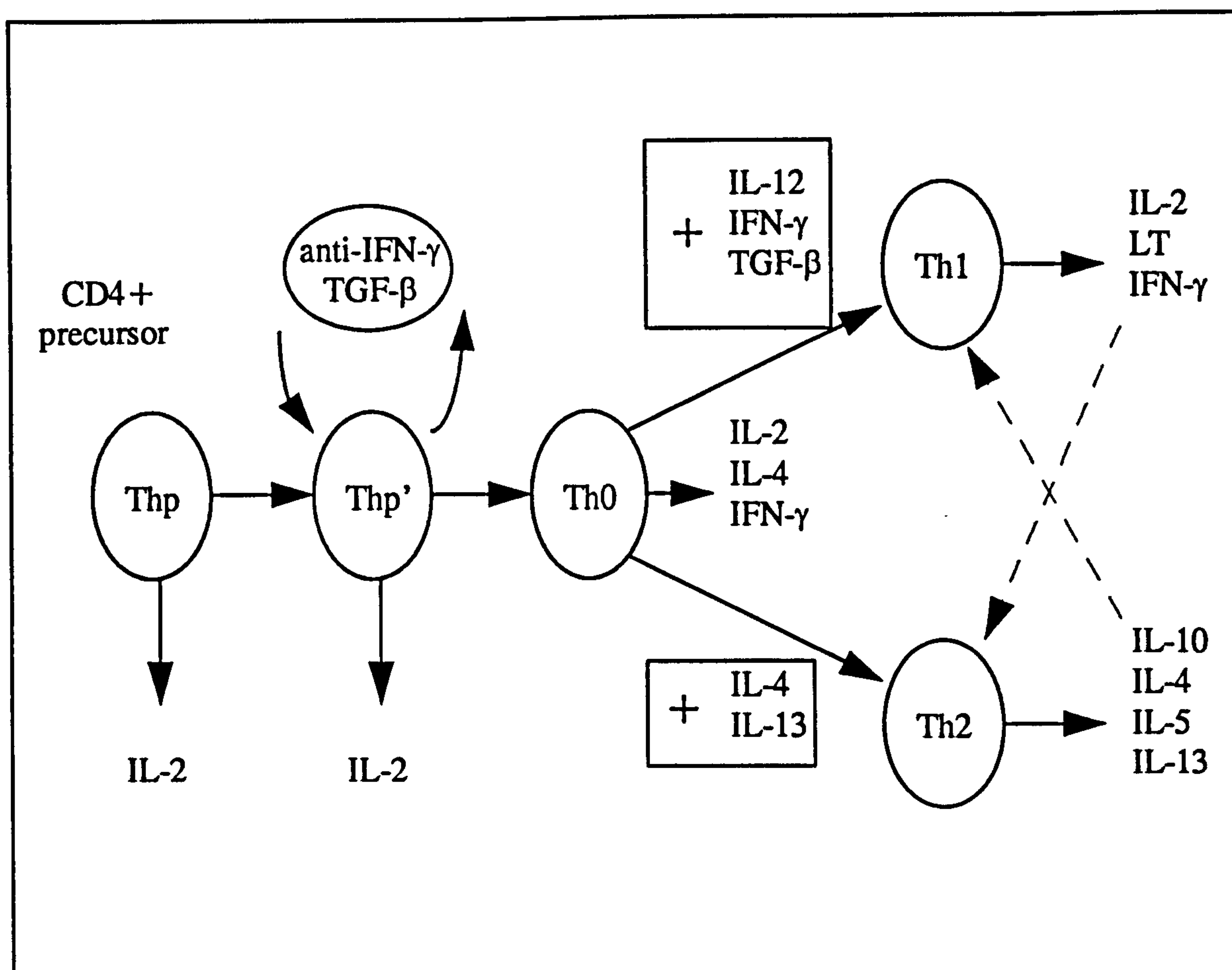


Figure 1.6.. Schematic representation of the development and differentiation of CD4+ T-helper cells (Th). Naive Th cells (Thp), on first contact with antigen (Thp') produce IL-2, then differentiate into either Th1 or Th2 cells secreting distinctive cytokines. These Th1/Th2 cytokines are cross-regulatory, with Th1 cytokines enhancing Th1 development (boxed) and inhibiting Th2 differentiation (broken line). Th cells can be prevented from developing into Th1/Th2 cells (maintained in the IL-2 secreting state) by administering TGF-β and anti-IFN-γ. Abbreviations: IFN-γ, interferon gamma; IL-2, interleukin 2; LT, lymphotoxin; TGF-β, transforming growth factor beta. (Adapted from Mossman & Sad, 1996).

The simplification of the T_h1/T_h2 system should however be treated with caution (Kelso, 1995; Allen & Maizels, 1997). The common notion is that these are two discrete cellular compartments derived from continued antigen exposure. Kelso (1995) suggested a heterogeneous cytokine secreting population of T-cell clones, with some secreting the whole array of T_h1- or T_h2-type cytokines, whilst other clones only secrete single cytokines.

The shift to a predominantly T_h1- or T_h2-phenotype occurs when the number of clones secreting the same cytokine profile within the cell population increases. To add to the confusion over the T_h1/T_h2 paradigm, CD8⁺ T-cells (cytotoxic T-cells; T_c) are now being subdivided into T_c1 and T_c2 subsets using the same principles of cytokine profiles and cross regulatory actions (Mosmann & Sad, 1996).

1.7 Cytokine regulation of host immunity

Typically, intestinal helminths induce a T_h2-type response, with production of IL-4, -5, -9, -10 and -13, favouring synthesis of IgG1 and IgE antibodies, with mast cell and eosinophil activation (Urban *et al.*, 1992; Urban *et al.*, 1998). IL-10 inhibits the production of IFN- γ , suppressing the activation of T_h1 cells, while IFN- γ has an antagonistic effect on T_h2 development. In strains of mice resistant to *Trichuris muris* the polarisation of the T_h response can be altered by reducing the infective dose and hence the antigen load. Under these conditions there is an increased expression of the T_h1 phenotype that leads to an inability to expel the parasite (Bancroft *et al.*, 1994).

As already discussed, the generation and classification of cells secreting cytokines are complex and yet to be fully understood. Many different species of gastrointestinal

nematode have been utilised in an attempt to dissect the protective responses that expel the parasite, including *T. spiralis*, *T. muris*, *Heligmosomoides polygyrus* and *N. brasiliensis*.

T. spiralis adult worms reside within intestinal epithelial cells, with the females releasing larvae that enter the vasculature and lymphatics and migrate throughout the body before they encapsulate in host striated muscle. The adult stages can remain in the intestines of large mammals for several weeks, but are generally removed from the rodent intestine within two weeks. *H. polygyrus* establishes chronic infections in mice, with the adult worms living in the gut lumen and feeding on the host mucosa. The whipworm *T. muris* has a spectrum of infection depending on the strain of mouse used, with some mouse strains maintaining chronic infections and others expelling the worms before they mature. The adult worms embed the filamentous anterior portion into the epithelium of the large intestine, with the posterior end extended out into the lumen. Finally, *N. brasiliensis* resides within the small intestine of rodents and produces an acute infection which is removed within two weeks.

Studies on the host protective effects of certain cytokines have highlighted differences in the responses generated to the above mentioned parasites. It has been demonstrated that IL-4 is important in the immunity to *T. muris* and *H. polygyrus*, but its role in *N. brasiliensis* and *T. spiralis* infections is less clear (Finkelman *et al.*, 1997). Administration of antibodies raised against IL-4 or IL-4 receptor (IL-4R) resulted in an increase in *H. polygyrus* adult survival and egg production (Urban *et al.*, 1991). In a strain of mice that is normally resistant to *T. muris* (BALB/k), the use of anti-IL-4R but not anti-IL-4 antibodies resulted in the development of a chronic infection (Else *et al.*, 1994). More recent work by Bancroft and colleagues (1998) demonstrated that both IL-

4- and IL-13-deficient mice were susceptible to infection with *T. muris*, in contrast to their wild-type littermates who expel the parasite. Experiments investigating the role of IL-4 in *T. spiralis* infection have been limited, due to the lack of antibody reagents available for use in the rat, and the suggestion that the rapid expulsion phenomenon does not exist in the mouse (Finkelman *et al.*, 1997). However, treatment of *T. spiralis* infected mice with anti-IL-4R antibodies increased the survival of adult worms (Finkelman *et al.*, 1997).

1.8 The immune response to N. brasiliensis

It has been shown in both the rat (Sinski & Holmes, 1977; Brown *et al.*, 1981) and the mouse models of *N. brasiliensis* infection (McElroy *et al.*, 1983; Katona *et al.*, 1988) that total serum immunoglobulin levels increase during infection. Specifically, levels of IgA and IgG are raised during the early stages of infection and parasite specific IgE is elevated as the parasite is eliminated, with the peak of specific IgE production occurring after parasite elimination (Yamada *et al.*, 1992). Sinski and Holmes (1977) showed that levels of parasite-specific IgG also reached maximal levels after parasite expulsion, suggesting that immunoglobulins (Ig) may not play an essential role in the elimination process.

The T-cell dependence of immunity to *N. brasiliensis* has been shown by *in vivo* treatment with neutralising antibodies to CD4 (Katona *et al.*, 1988) and the use of CD4-deficient mice (Fowell *et al.*, 1997). Animals depleted of CD4⁺ T-cells as well as congenitally athymic nude mice were unable to expel the parasite. Studies then performed to ascertain which cytokines were generated during infection determined that IL-4 and IL-5 levels increased rapidly from day 7 p.i. in the mesenteric lymph nodes,

whereas levels of IL-4 in the spleen were only detectable after the elimination phase (day 11 p.i.; Lawrence *et al.*, 1996). It has been demonstrated that treatment of infected mice with IFN- γ or alpha-interferon (IFN- α) increased worm egg production and prolonged worm survival by 48-72 hours in a primary infection (Urban *et al.*, 1993). Both IFN- γ and IFN- α almost totally inhibited the development of blood and tissue eosinophils, but only temporarily effected Ig isotype expression and mucosal mast cell hyperplasia. The T_h2 response to *N. brasiliensis* was completely suppressed by IL-12 treatment during the early stages of infection (Finkelman *et al.*, 1994). IgE and mast cell hyperplasia were no longer affected if IL-12 treatment was given 6 days after infection, due to the prior establishment of T_h2 cytokine production. Madden *et al.* (1991) treated *N. brasiliensis*-infected mice with neutralising monoclonal antibodies raised against IL-3 and IL-4. Depletion of both IL-3 and -4 had no effect on parasite expulsion, leading to the assumption that IL-4 is not required for the removal of *N. brasiliensis*. These findings were confirmed using mice lacking a functional IL-4 gene (Kopf *et al.*, 1993; Lawrence *et al.*, 1996).

Huels *et al.* (1995) devised a co-activation system using naive CD4⁺ cells and bone marrow-derived mast cells (BMMC). It was shown that IgE-activated BMDCs polarised the CD4⁺ cells to a T_h2 phenotype by secretion of IL-4. This was only partially inhibited by anti-IL-4 monoclonal antibodies, suggesting a role for other cytokines in CD4⁺ polarisation. IL-13 shares many common biological properties with IL-4, which includes a common receptor component, IL-4 receptor alpha chain (IL-4R α ; Obiri *et al.*, 1995; Zurawski *et al.*, 1995; Hilton *et al.*, 1996), IL-4R and IL-13R activation results in the induction of signal transducer and activator of transcription 6 (STAT6; Kohler *et al.*, 1994). Work by Urban and colleagues (1998) demonstrated that mice

deficient in the IL-4R α or STAT6 failed to expel *N. brasiliensis*. The role of IL-13 in parasite expulsion was tested using IL-4 deficient mice (IL-4^{-/-}), which expel the parasite normally (Madden *et al.*, 1991; Kopf *et al.*, 1993; Lawrence *et al.*, 1996). In these and wild-type animals, IL-13 was neutralised using soluble IL-13R α_2 -Fc fusion protein, and worm burdens and egg counts were determined. The neutralisation of IL-13 resulted in parasite survival in IL-4^{-/-} mice and more surprisingly in the wild type mice. These data suggest that IL-4R α and STAT6 are essential for parasite expulsion, and that IL-13 may have a more important role than IL-4 in host protection against *N. brasiliensis* (Urban *et al.*, 1998). These findings were substantiated by the work of Barner and colleagues (1998) using both IL-4R α - and IL-4-deficient mice, with parasite expulsion in mice lacking IL-4 but not IL-4R α . Recently, McKenzie *et al.* (1998) have developed an IL-13-deficient mouse (IL-13^{-/-}). These animals fail to clear *N. brasiliensis* infection within the first two weeks, despite the generation of a T_h2-type cytokine response. These results were also observed in IL-13^{-/-} mice infected with *T. muris*, which emphasizes the role of IL-13 in the protective immunity against intestinal nematodes.

1.9 Non-specific immune responses

It has been proposed that the second phase responses to parasite antigen are non-specific, taking place over several days during a primary infection, whereas in a secondary infection they are rapid, and detectable in minutes (Castro, 1989). These non-specific immunological responses to the parasite include goblet cell hyperplasia (Miller *et al.*, 1979), increased turnover of goblet cell mucins (Miller *et al.*, 1981), increased chloride secretion (Baird *et al.*, 1985; Castro, 1989), increased gastrointestinal motility (Farmer, 1981) and increased mast cell hyperplasia (Miller, 1981; Woodbury *et al.*,

1984). These responses are part of a local immediate hypersensitivity reaction that is thought to provide an unsuitable environment for parasite survival.

1.10 Goblet cell hyperplasia

Goblet cells are highly specialised exocrine cells that can be located along the entire length of the intestine. These cells are derived from stem cells at the base of the intestinal crypt, with development and differentiation occurring as the cells gradually migrate up from the crypts to the villus tip, where they are finally sloughed off and removed with excreta. There is a constant generation of cells, with the expansion from stem cell to death in mice taking 2-3 days (Merzel & Leblond, 1969). Goblet cells are characterised by the accumulation of secretory granules within the apical domain of the cell. The secretory granules contain mucins (which are large and heterogeneous glycopeptides), and a mixture of water, electrolytes and immunoglobulins that are slowly released by periodic exocytosis from individual granules (Specian & Oliver, 1991). As mucins are secreted they hydrate and gel, forming a protective mucus layer that overlays the intestinal epithelium. The mucus layer acts as a physical and chemical barrier, by preventing pathogens access to the mucosa, and providing a substrate for degradative enzymes released by the intestinal flora. Mucus also lubricates the intestine to avoid mucosal damage during the mechanical actions of digestion, and the transit of food debris.

The stimulatory signals that induce the slow, continual basal release of mucins have yet to be established. However, rapid discharge of stored mucin has been demonstrated by cholinergic stimulation (Specian & Neutra, 1980), intestinal anaphylaxis (Lake *et al.*, 1980), and intraluminal irritants (Neutra *et al.*, 1982). Secretion induced by

cholinergic agonists such as pilocarpine and carbachol is limited to goblet cells found within the crypts, with goblets cells derived from intestinal villi unresponsive to these agents (Specian & Neutra, 1980, 1982; Phillips *et al.*, 1984). The muscarinic^{receptor}/antagonist atropine inhibited goblet cell secretion (Specian & Neutra, 1982), and stimulated the rapid synthesis of new mucins (Specian & Oliver, 1991), implicating muscarinic cholinergic receptors in mucus secretion. Other neurotransmitters and hormones were tested as potential goblet cell secretagogues using intestinal explants from rabbits. Adrenergic agents and peptide hormones had no effect on goblet cell secretion, whereas histamine induced mucus secretion which was restricted to colonic crypt cells, and was insensitive to cholinergic antagonists (Neutra *et al.*, 1982). Work by Neutra and colleagues (Phillips *et al.*, 1984) examined the concept that both cholinergic and noncholinergic elements of the enteric nervous system play a role in goblet cell secretion. Intact epithelial sheets were isolated from rat ileum and colon, mounted in Ussing chambers, and the role of neurally mediated responses assessed by electrical field stimulation (EFS), which induces nonselective depolarisation of all nerve terminals. Both colonic and ileal crypt goblet cells secreted mucus in response to EFS, with secretion from the ileum totally abolished by treatment with atropine but only partially inhibited in the colon. This suggests that mucus secretion in the colon may be regulated by both cholinergic and non-cholinergic components of the enteric nervous system, whilst ileal secretion may be under the control of cholinergic elements (Phillips *et al.*, 1984).

Not only do mucins act as a physical and chemical barrier, goblet cell hyperplasia and mucus trapping of parasites have been observed concurrently with expulsion of *N. brasiliensis* (Miller *et al.*, 1981; Miller & Nawa, 1979). In the first week of infection goblet cell numbers are depressed, increasing to a maximum around day 10 p.i. (Miller &

Nawa, 1979). Goblet cell numbers increase prior to and during the expulsion phase (Miller *et al.*, 1979; Miller & Nawa, 1979), implicating a role in parasite expulsion.

It has been demonstrated that implanting 'normal worms' (worms taken from a non-immune host that are able to re-establish on transfer to a naive rat are termed normal) into the intestine depresses goblet cell differentiation, suggesting these worms are able to actively suppress this response (Miller & Nawa, 1979). 'Damaged worms' (these are worms that have been affected by the host immune response and are rapidly expelled on transfer to a naive animal) do not possess this inhibitory factor and are rapidly expelled (Ishikawa *et al.*, 1993), but if mixed populations of 'normal' and 'damaged' worms are implanted they are able to survive (Ishikawa *et al.*, 1994a). Interestingly, the goblet cell response to *N. brasiliensis* includes an alteration in the mucin sugar residues, with an increase in *N*-acetyl-D-galactosamine (GalNAc) containing mucins observed during the expulsion phase, using lectin histochemistry (Ishikawa *et al.*, 1993). The changes in mucin composition were suppressed in mixed populations of 'normal' and 'damaged' worms. Priming animals with 'damaged' worms followed by re-challenge with 'normal' worms resulted in almost complete expulsion of the parasite within 4 hours, and an increase in GalNAc positive mucins (Ishikawa *et al.*, 1994a, b). The alteration in the composition of mucins produced during expulsion of *N. brasiliensis* was also demonstrated by enzyme histochemistry, with a shift from neutral sialomucins to acid sulphated mucins in both crypt and villus goblet cells at the time of parasite removal from the intestine (Koninkx *et al.*, 1988).

Miller and Nawa (1979) showed that thoracic duct lymphocytes (TDLs) from immune rats, when transferred to naive animals, brought about an earlier onset of goblet cell differentiation and hyperplasia. In contrast to infected rats, no initial depression in

goblet cell numbers were seen. The T-cell dependence of the immune response to *N. brasiliensis* was described earlier, where administration of anti-CD4 antibodies resulted in prolonged worm survival (Katona *et al.*, 1988). Work by Khan and colleagues (1995), using a mouse strain that rejected *N. brasiliensis* within seven days, demonstrated that parasite expulsion was delayed by treatment with anti-CD4 antibodies, and a significant decrease in mucus release was observed. Hypothymic and euthymic rats were used to examine the effects of the immune system on the kinetics of parasite expulsion and goblet cell proliferation. Implantation of 'normal' worms into the intestine of euthymic rats results in the establishment of parasites for up to 7 days before the expulsion process is initiated, whereas hypothymic rats maintain parasites for up to 3 weeks (Ishikawa *et al.*, 1994b). In contrast, the majority of 'damaged' worms were expelled from the intestine of both strains of rat 5 days after implantation, with euthymic rats showing an increase in the number of goblet cells and an alteration in mucin composition (an increase in GalNAc positive mucin). Alteration of goblet cell mucins were also observed in hypothymic rats, but there was no proliferation of goblet cells. Treatment with the immunosuppressant drug dexamethasone resulted in a failure to expel parasites, with a lack of goblet cell proliferation and change in mucin composition (Ishikawa *et al.*, 1994b). On the basis of these results Nawa and colleagues (1994) supported a two-step hypothesis for the expulsion of *N. brasiliensis*. The first step involves T-cell dependent worm damage and goblet cell proliferation, followed by T-cell independent ~~induced~~ changes in mucin composition and parasite expulsion. The effects of cytokines on the differentiation and proliferation of goblet cells have yet to be established. However, addition of purified IL-1 or lipopolysaccharide (LPS)-induced macrophage culture supernatants resulted in increased mucus secretion from mouse

duodenal explants in a dose- and time-dependent manner (Han *et al.*, 1987). In contrast, it has been reported that IFN- γ inhibits cholera toxin and VIP induced mucus secretion *in vitro* (Jarry *et al.*, 1992). Most studies investigating cytokine effects on parasite expulsion fail to assess goblet cell changes, although recent work by McKenzie *et al.* (1998) demonstrated that IL-13^{-/-} mice were unable to expel *N. brasiliensis*, which coincided with a lack of goblet cell hyperplasia.

It was generally considered that mucins act as a non-specific, protective barrier (Specian & Oliver 1991), which prevents parasitic worms access to their preferred niche. However, there is increasing evidence the mucins act selectively. Nawa and colleagues (reviewed in Nawa *et al.*, 1994) have attempted to dissect the effector mechanisms for the expulsion of intestinal nematodes, using *in vivo* studies and concurrent infections of *N. brasiliensis* and *Strongyloides* species. Mucosal mast cells appear to be involved in the removal of *Strongyloides* spp., with expulsion of *S. ratti* and *S. venezuelensis* delayed in mast cell deficient mice (W/W^v; Nawa *et al.*, 1985; Khan *et al.*, 1993). Repeated administration of IL-3, a mast cell growth factor, to nude mice infected with *S. ratti* resulted in parasite expulsion in conjunction with mast cell hyperplasia (Abe & Nawa, 1988). The role of mucosal mast cells in *N. brasiliensis* expulsion is still somewhat controversial. It has been reported that expulsion of *N. brasiliensis* in W/W^v mice is at a slower rate than normal littermates (Crowle & Reed, 1981; Ishikawa *et al.*, 1994c), or at the same rate as normal littermates (Über *et al.*, 1980). In contrast, work by Miller and Jarrett (1971) suggested a temporal relationship between parasite expulsion and mastocytosis, with mast cell hyperplasia and the levels of the mast cell product, rat mast cell protease II (RMCP II) peaking during parasite rejection (Woodbury *et al.*, 1984). The possibility of selective effector mechanisms in the removal of different species of

parasite was first predicted by Nawa and Korenga (1983). Rats infected with *N. brasiliensis* and *S. ratti* undertake two separate expulsion events, with goblet cell hyperplasia removing the former, and intestinal mastocytosis the latter parasite (Figure 1.7). As discussed earlier, immunising rats with ‘damaged’ *N. brasiliensis*, followed by re-challenge with ‘normal’ worms results in rapid expulsion. In contrast, re-challenge with *S. venezuelensis* induces the characteristic goblet cell hyperplasia but the parasite is able to persist (Ishikawa *et al.*, 1994b).

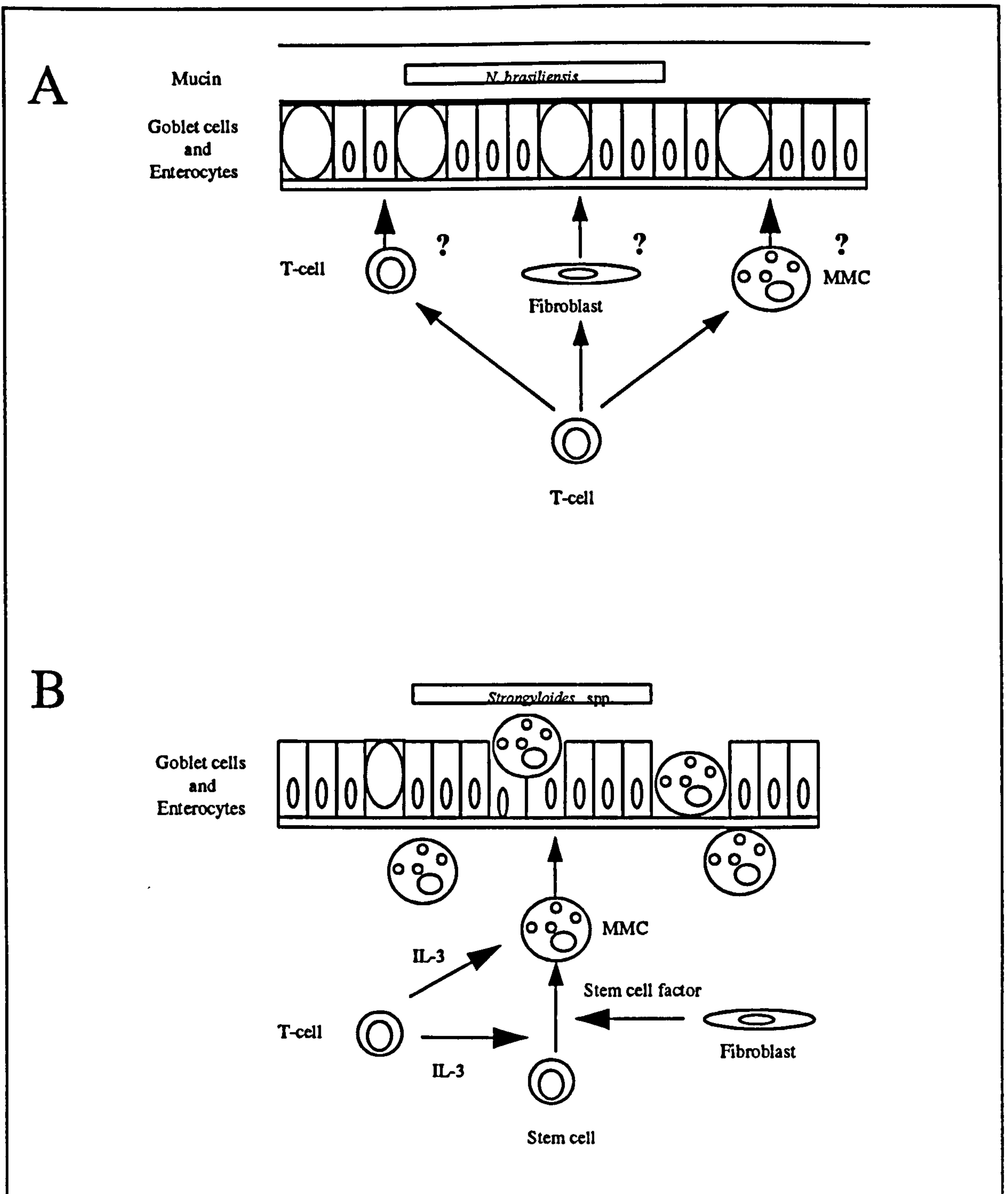


Figure 1.7. Proposed mechanisms for the selective expulsion of different parasitic nematodes. A: the nematode *N. brasiliensis* evokes goblet cell proliferation and mucus secretion, which leads to the removal of the parasite but not concurrently infected *Strongyloides* spp. B: *Strongyloides* spp. induce mast cell differentiation and hyperplasia which causes parasite expulsion. Abbreviations: IL-3, interleukin 3; MMC, mucosal mast cell. (Adapted from Nawa *et al.*, 1994).

1.11 Intestinal anaphylaxis

As mentioned earlier, immediate type hypersensitivity reactions (anaphylaxis) in the gut have been shown to coincide with antigenic challenge of previously-sensitised animals (Castro, 1989; Castro & Arntzen, 1993). Mucosal mast cells (MMC's) play a vital role in intestinal anaphylaxis, and their preformed and lipid mediators are thought to have a stimulatory role in fluid secretion (Bern *et al.*, 1989). The majority of the work to date regarding anaphylaxis induced fluid secretion has utilised the rapid expulsion phenomenon associated with the removal of *T. spiralis*. Changes in fluid secretion from tissue segments can be measured in Ussing chambers, which are modified organ baths.

Application of an electrical current maintains the trans-epithelial potential difference at zero, with transient increases in the short circuit current (Isc) indicative of a net flow of ions across the tissue. An increase in Isc is due to the luminal transport of electrogenic chloride ions (Cl⁻), creating an electrochemical gradient which results in the secretion of water (Baird & O'Malley, 1993).

An increase in fluid secretion into the lumen of the small intestine of *T. spiralis*-sensitized guinea-pigs and rats has been demonstrated on further challenge with *T. spiralis* antigen. The fluid secretion was observed to be bi-phasic. The first phase occurred within two minutes following antigen challenge and appeared to be caused by histamine and 5-hydroxytryptamine (5-HT) derived from mast cells (Zhang *et al.*, 1991). These mediators could be detected in samples taken from everted sacs and intestinal loops re-stimulated with antigen. The first phase peak could be selectively inhibited using histamine (diphenhydramine and cimetidine) and 5-HT (cinancerin and metogoline) antagonists (Castro, 1987). The second phase lasted for twenty minutes, peaked after

eight minutes and appeared to be generated by prostaglandin E₂ (PGE₂) synthesized from mast cells (Castro, 1989). The transfer of serum from *T. spiralis* immunised animals to naive recipients resulted in a reduction in worm burden when naive animals were challenged with the parasite. As well as having a reduced worm burden these animals were able to respond electrophysiologically to antigen challenge, demonstrated by a rapid monophasic increase in fluid secretion. These responses were not observed in animals which received heat-treated immune serum or non-immune serum, suggesting that anti-parasite antibodies have a role in parasite rejection and concurrent increase in fluid secretion (Harari *et al.*, 1987).

Baird and co-workers (1985) found that mepyramine (a histamine H1 receptor antagonist) and indomethacin (a cyclo-oxygenase inhibitor) had no effect on fluid secretion in the *N. brasiliensis*-sensitized rat colon challenged with parasite antigen. More recent work by Masson and colleagues (1996) observed that during *N. brasiliensis* infection, nerve function appeared to be suppressed. Jejunal segments from rats infected ten days previously with *N. brasiliensis* displayed a reduced secretory response to nerve stimulation, which was insensitive to the cholinergic antagonist atropine. Exposure to the cholinergic agonist, bethanechol, resulted in secretory responses comparable to those of control animals. This suggests that the epithelium was able to respond to exogenous cholinergic stimulation, but that the cholinergic nerve function was defective (Masson *et al.*, 1996). It was also observed that exogenous substance P (the peptidergic neurotransmitter which has pro-inflammatory and secretagogue actions) mediated fluid secretion was reduced during infection. The epithelial insensitivity to substance P was concurrent with an increase in the levels of immunoreactive substance P within the mucosa and an upregulation of substance P-reactive nerve profiles (Masson *et al.*, 1996).

It has been suggested that these contradictory findings could imply a downregulation of secretory responses to substance P by receptor tachyphylaxis (McKay & Fairweather, 1997).

Tetrodotoxin (TTX), a neurotoxin that inhibits action potentials by blocking sodium channels, decreased fluid secretion in animals immunised against *T. spiralis* and re-challenged with parasite-derived antigen, further implicating the enteric nervous system in this process, but secretory responses to PGE₂ were not altered in the presence of TTX. PGE₂ is thought to mediate secretion by acting directly on secretory cells in the intestinal crypts (Castro, 1989). Work by MacNaughton and Gall (1991) showed that another inflammatory mediator, platelet activating factor (PAF), also induced fluid secretion in rat intestine. PAF might act by stimulating the synthesis of PGE₂ which enhances Ca²⁺ entry into enterocytes. Bern *et al.* (1989) concluded that the immune system and the enteric nerves of the intestine must both have an interactive role in fluid secretion. They found that immune cell stimulants (anti-rat IgE and N-formyl-methionine-leucine-phenylalanine; FMLP) generated brief fluid secretion, while immune cell products (PAF, PGs and hydrogen peroxide) caused bi-phasic responses. McKay and co-workers (1995) investigated the role of the immune system in fluid secretion further using athymic rats infected with *N. brasiliensis*. As described earlier, athymic rats are unable to expel the parasite. This study demonstrated a delayed onset of goblet cell hyperplasia and mucosal mastocytosis. There was no significant alteration in timing of fluid secretion, but infected tissue showed a reduced ability to respond to electrical stimulation. Addition of exogenous histamine to these tissues resulted in fluid secretion, which demonstrated the tissue was viable. It was concluded that fluid secretion in

response to antigen occurred independently of T-cell help and was ineffective at removing parasites in the absence of goblet cell hyperplasia (McKay *et al.*, 1995).

1.12 Neuro-immune interactions

The nervous and immune systems were once thought of as autonomous, working independently of each other. But, in the past two decades advances have been made and new concepts developed. It is now generally accepted that there is bi-directional communication between the immune, nervous and endocrine systems, and this field has been reviewed extensively (Figure 1.8; Stead *et al.*, 1987; Mathison *et al.*, 1994; Madden & Felten, 1995; Savino & Dardenne, 1995).

To determine if neurotransmission occurs within lymphoid organs, several criteria need to be fulfilled. There needs to be innervation and localisation of nerve fibers, neurotransmitter release, the presence of receptors for the neurotransmitter on the target cells and finally, a functional role for innervation (Felten *et al.*, 1987).

1.13 Noradrenergic innervation of lymphoid tissue

Using immunohistochemistry, it has been shown that both the primary and secondary lymphoid organs are innervated by sympathetic noradrenergic (NA) and peptidergic nerve fibres. NA fibres enter the bone marrow with the vasculature, are distributed throughout the tissue, and are thought to influence haemopoiesis and cell migration. The subcapsular, cortico- and cortico-medullary regions and the blood vessels of the thymus are also in contact with NA fibres (Felten *et al.*, 1987). It was proposed that the fibres exert an inhibitory effect on thymocyte proliferation and migration (Scheid *et al.*, 1973).

The NA fibres enter the spleen with the splenic artery and then branch throughout the spleen. Here T-cells, B-cells and macrophages are all in contact with NA fibres and are exposed to neurotransmitters.

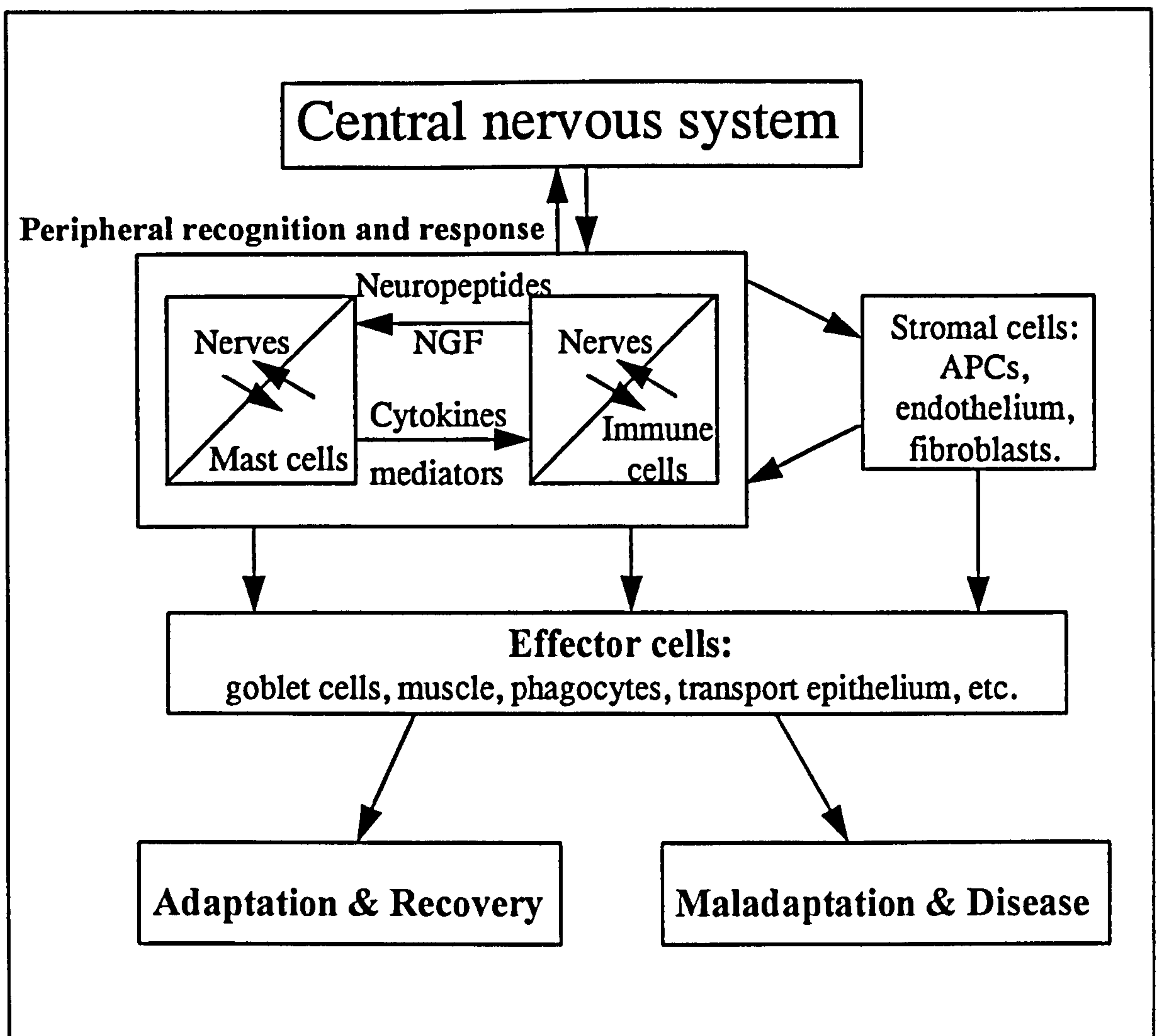


Figure 1.8. Schematic representation of the interactions between the nervous and immune systems in response to antigenic challenge. Arrows depict the cellular responses to antigen. An appropriate response leads to antigen elimination whereas an exaggerated or inappropriate response leads to chronic inflammation. Abbreviations: APCs, antigen presenting cells; NGF, nerve growth factor (Adapted from Savino & Dardenne, 1995).

The lymph nodes and Peyer's patches exhibit the same pattern of innervation. There are NA fibres within the T-dependent zones of the gut-associated lymphoid tissue (GALT), located in the lamina propria, and in contact with immunoglobulin-secreting plasma cells at the luminal surface (Madden & Felten, 1995).

1.14 Peptidergic innervation of lymphoid tissue

Several neuropeptides have been implicated in having a direct influence on immune reactivity. Neuropeptide Y (NPY) is co-localised in NA fibres in the rat spleen and thymus where it is in direct contact with immunocytes. Other neuropeptides such as vasoactive intestinal peptide (VIP) and substance P are associated with non-NA nerve fibres.

Substance P is found in the CNS, peripheral sensory neurons, and the nerve plexi of the gut, spleen, lymph nodes and thymus. It is thought to have a role in the regulation of immune responses during inflammation, where levels of substance P are elevated. Vasodilation, vascular permeability, increased phagocytosis by neutrophils and a potentiation of the effects of vasoactive amines such as histamine have all been linked to substance P (McGillis *et al.*, 1987). VIP is found within neurons of the CNS, the thymus, and is especially abundant in nerve fibres within mucosal tissues such as the intestinal tract and Peyer's patches (Ottaway *et al.*, 1984).

1.15 Lymphocyte expression of neuropeptide receptors

T-cells, B-cells, macrophages and neutrophils have all been shown to possess β -adrenoreceptors (Ottaway, 1984; Pochet *et al.*, 1979). The receptors are upregulated in the presence of β -blockers and downregulated in the presence of β -agonists. The density



of receptor expression on cells changes with activation or differentiation, with mature T-cells expressing more receptors than naive T-cells. Ablation of sympathetic nerve terminals in recipient adult rats, resulted in reduced migration into the GALT of adoptively transferred mesenteric lymph node cells from normal donors. The mucosal immune response in these animals was also reduced. Immunisation with ovalbumin (OVA) resulted in fewer anti-OVA antibody containing cells in the lamina propria of sympathectomised animals in comparison to normal animals (González-Ariki & Husband, 1998).

Both T- and B-cells are thought to express substance P receptors. This was shown by the blockade of substance P-mediated T-cell proliferation using a non-functional analogue (Payan & Goetzl, 1985). Substance P enhances polyclonal IgA production by Peyer's patch and mesenteric lymph node (MLN) cells *in vitro*, whereas IgM is less affected and IgG synthesis unchanged (Stanisz *et al.*, 1986).

High affinity VIP receptors have been demonstrated on T-cells and B-cells (Ottaway & Greenberg, 1984; O'Dorisio *et al.*, 1989). VIP causes an inhibition of T-cell proliferation to mitogens *in vitro*, inhibits IL-2 production (Ganea & Sun, 1993) and impairs T-cell migration to the MLNs and Peyer's patches (Ottaway, 1984).

Lymphocytes of mice, rats and humans have been shown to possess muscarinic acetylcholine receptors (mAChR; Atweh *et al.*, 1984; Maslinski *et al.*, 1987; Eva *et al.*, 1989). However, the majority of studies to date have used radioligand binding techniques, which provide inconsistent results (Bronzetti *et al.*, 1996) and the observation that antagonists display lower affinity for the receptors expressed on lymphocytes than in other tissues (Costa *et al.*, 1988; Eva *et al.*, 1989). Acetylcholine (ACh) has been reported to stimulate chemotaxis, phagocytosis, lysosomal enzyme

secretion and antibody-dependent cellular cytotoxicity of polymorphonuclear leukocytes (Rhoads, 1984). The muscarinic agonist, pilocarpine, has been demonstrated to inhibit proliferation and IFN- γ synthesis in human peripheral blood mononuclear cells, with an increase in the CD8:CD4 T-cell ratio interpreted as enhancing suppressor cell function (Arzt *et al.*, 1989; Sterin Prynec *et al.*, 1992).

1.16 The Hypothalamo-Pituitary-Adrenal axis (HPA)

The HPA axis has a role in the modulation of immune function and host resistance. Lymphocytes have the ability to secrete peptide hormones and express peptide receptors that are classically described as HPA hormones (Savino & Dardenne, 1995). The hormones of the HPA axis have many effects on the immune system. Growth hormone (GH) deficiency leads to a reduction in size of both primary and secondary lymphoid organs that can be restored by GH treatment (Fabris *et al.*, 1971). GH has also been shown to prime macrophages and neutrophils to produce superoxide anions (Edwards *et al.*, 1988).

Cytokines, which are generally accepted as mediators of activation and inflammatory responses within the immune system, are also expressed in the nervous system (Hopkins & Rothwell, 1995). They can perform growth and differentiation functions, in addition to acting as trophic factors in nerve regeneration. IL-1 β has been demonstrated to induce sympathetic nerve growth and raise levels of substance P by the production of a soluble intermediate, which is functionally similar to leukaemia inhibitory factor (LIF, a cholinergic differentiation factor; Freidin & Kessler, 1991; Shadiack *et al.*, 1993). Both IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to enhance neurite outgrowth and elevate choline acetyltransferase

activity in rat and mouse central cholinergic neurons (Kamegai *et al.*, 1990a; 1990b). This raised the possibility that the immune system may participate in shaping neuronal connectivity. Stead and colleagues (1987b) investigated the relationship between mast cells and the enteric nerves in normal and nematode-infected rats. It was found that 67% of the intestinal MMCs were in intimate contact with the subepithelial nerves. The size of the vesicles within the axons were consistent with peptidergic neurotransmitters, and immunohistochemical staining demonstrated both substance P and Calcitonin gene-related protein (CGRP) containing nerves adjacent to the MMCs. *N. brasiliensis*-infected animals undergo mucosal nerve remodeling that has been divided into two phases (Stead *et al.*, 1991). The first phase is characterised by a degeneration of a proportion of mucosal nerves during the acute phase of inflammation. This is followed by a period of re-innervation, which peaks 18 days p.i. and then gradually declines.

A co-culture system, using superior cervical ganglia explants (SCG) and various lymphoid tissues, was used to determine the extent and efficacy of mediators derived from the immune system on neurite outgrowth (Kannan *et al.*, 1994; 1996). Nerve outgrowth stimulated by adult thymus was similar to that of positive controls, with normal mesenteric lymph node explants (MLN) having minimal effect. However, MLN explants from animals infected with *N. brasiliensis* (Nb-MLN) showed enhancement of neurite outgrowth, which was maximal three weeks post infection in rats (Kannan *et al.*, 1994). The use of neutralising antibodies revealed that nerve growth factor (NGF) accounted for the majority of neurite outgrowth towards thymus, but only 50% of outgrowth towards Nb-MLN. Antibodies raised against IL-1 β , IL-3, IL-6 and GM-CSF significantly inhibited nerve outgrowth stimulated by Nb-MLN, with IL-1 β but not IL-3 outgrowth blocked by anti-NGF antibodies (Kannan *et al.*, 1996). These data suggest

that the nervous system shows a degree of plasticity during inflammatory reactions. With damage and degeneration occurring during the immune response and nerve remodelling and innervation aided by the immune system.

1.17 Acetylcholinesterases

As described earlier, ACh is a neurotransmitter that is localised both in the CNS and the peripheral nerves. Receptors for ACh can be divided into two groups, nicotinic receptors (nAChRs) and muscarinic receptors (mAChRs). Nicotinic AChRs are coupled to cation channels and mediate excitatory transmission at neuromuscular junctions, autonomic ganglia and sites within the CNS. Muscarinic AChRs consist of seven transmembrane segments which are G-protein coupled, they are expressed in the CNS and periphery, and have been split into 5 subtypes (termed m1-m5). The enzyme acetylcholinesterase (AChE) hydrolyses ACh to acetate and choline, thus terminating nerve transmission at AChRs. Two different enzymes that hydrolyse ACh have been described in mammals, AChE (EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8). Both enzymes are closely related in structure, but differ in tissue distribution, substrate specificity, inhibitor profile and function (Figure 1.9; Chatonnet & Lockridge, 1989).

AChEs can exist in globular (G) or asymmetric (A) forms (Taylor, 1991). The three G forms (G₁, G₂ and G₄) correspond to a monomeric, dimeric or tetrameric composition of catalytic subunits. The G forms can be further subdivided into hydrophilic and amphiphilic forms, the latter formed by the association of G₁ and G₂ enzymes with glycolipid and amphiphilic non-catalytic subunits. The A forms (A₄, A₈ and A₁₂) are associations of one, two or three tetramer subunits attached to a collagen *regulator*

tail. Invertebrates appear to possess only G forms of AChE, while vertebrates are known to possess all forms (Massoulié *et al.*, 1993).

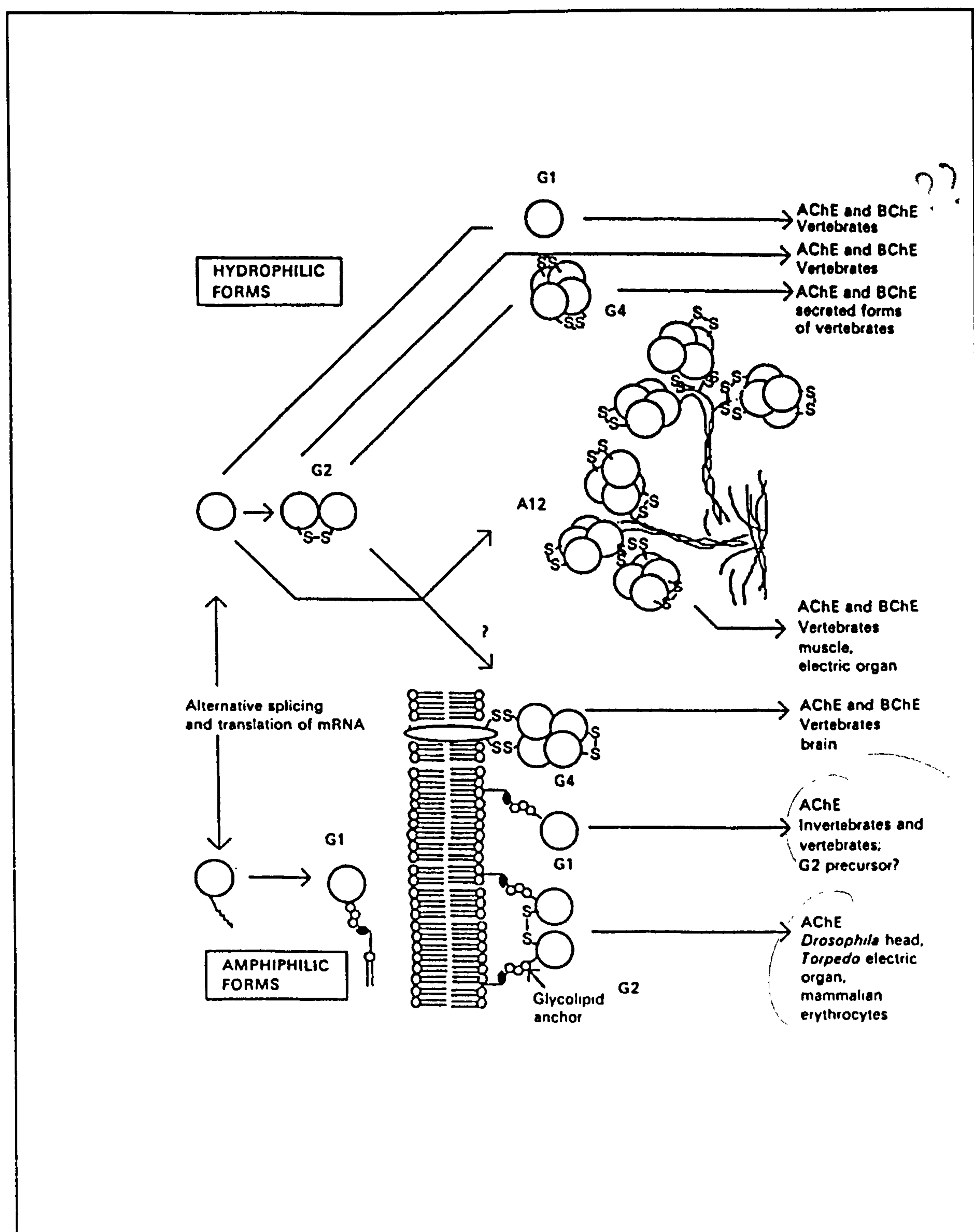


Figure 1.9. Schematic model to demonstrate the molecular polymorphism of AChE and BuChE. The catalytic subunits are represented by open circles and the disulphide bonds by S-S. Globular (G) forms of ChE are found as monomers (G1), dimers (G2), and tetramers (G4) of the catalytic subunit which can be subdivided into hydrophilic and amphiphilic forms. The asymmetric (A) forms consist of G4 subunits attached to a collagen tail. (Adapted from Chatonnet & Lockridge, 1989).

1.18 Nematode secretory acetylcholinesterases

N. brasiliensis and other nematodes of the gastro-intestinal tract have been observed to secrete AChEs during *in vitro* culture (Figure 1.10; Burt & Ogilvie, 1975; Jones & Knox, 1990; Pritchard, 1994; Sanderson & Ogilvie, 1971; Edwards *et al.*, 1971). *N. brasiliensis* possesses at least 5 isoforms of AChE, determined by non-denaturing PAGE gels (Edwards *et al.*, 1971). Total enzyme activity rapidly increases as the parasite develops from an egg to the L4 larval stage, and the adult worms express fifteen times greater enzyme activity per wet weight than that of the egg. Enzyme activity is further doubled in the last seven days of infection as the parasites are damaged and expelled (Sanderson & Ogilvie, 1971).

The three major enzyme isoforms have been termed A, B and C, and display apparent masses of 74, 69 and 71 kDa respectively when resolved by SDS-PAGE under non-reducing conditions. These isoforms were confirmed as true AChEs, determined by substrate and inhibitor specificities, existing as hydrophilic G₁ forms (Grigg *et al.*, 1997). Their pattern of expression alters dramatically during infection (Edwards *et al.*, 1971, Blackburn & Selkirk, 1992). The amounts of B and C forms increase while the A form decreases and is totally lost as the expulsion phase begins. Transfer of day 12 or day 14 worms into an immunologically naive host results in a decrease in the levels of AChE synthesised, suggesting that the enzyme is produced in response to host immunity (Sanderson *et al.*, 1976a). Interestingly, the A isoform of AChE is significantly increased in worms that have adapted to the host response and can resist expulsion for a prolonged period (Edwards *et al.*, 1971).

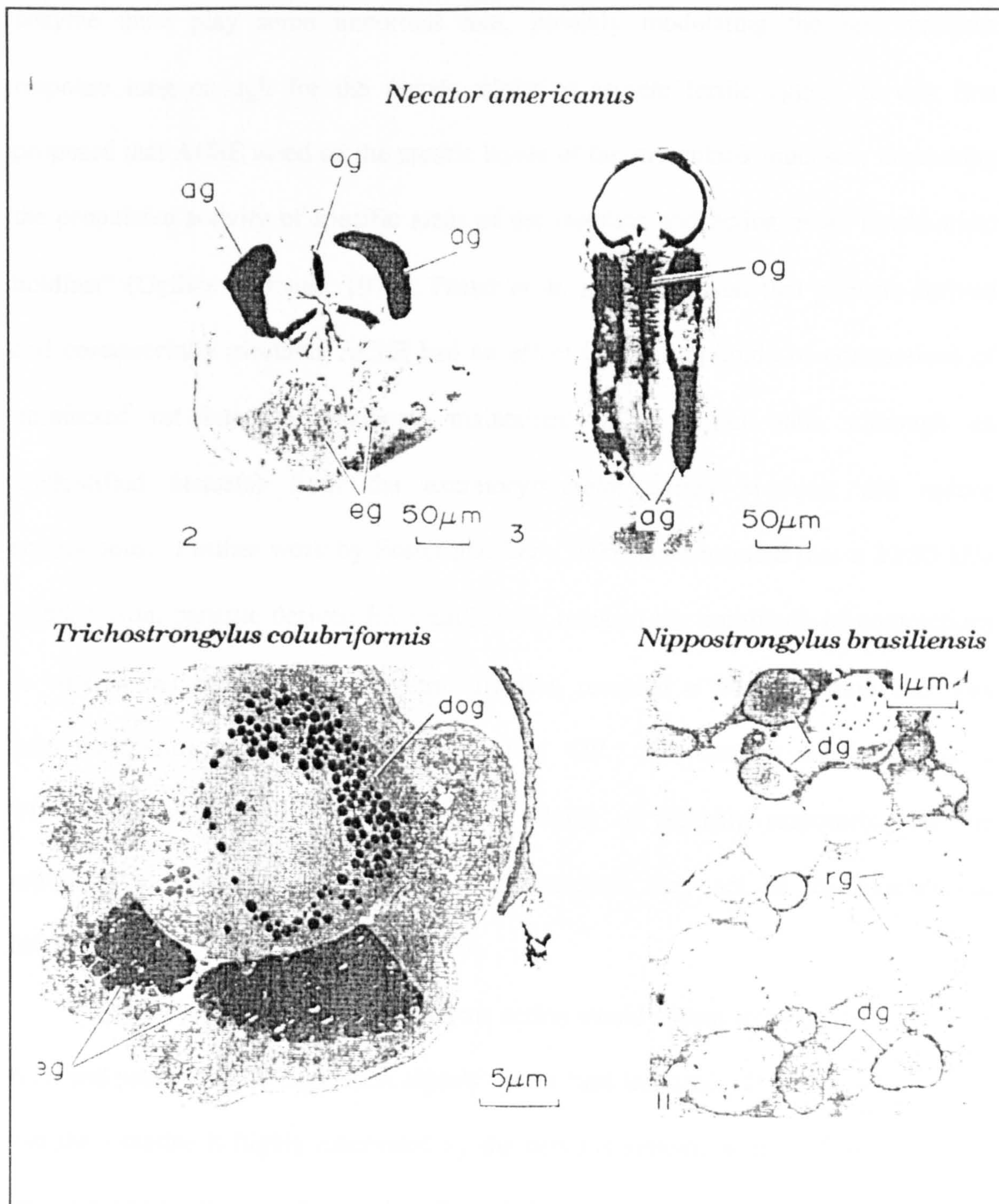


Figure 1.10. The localisation of AChE to the secretory glands of parasitic nematodes. Transverse and longitudinal sections of adult *N. americanus*, *T. colubriformis* L4s, and adult *N. brasiliensis*. Abbreviations: ag, amphidial gland; dg, dense granules; dog, dorsal oesophageal gland; eg, excretory gland; og, oesophageal gland; rg, reticulate granules (Adapted from McLaren *et al.*, 1974).

As the parasite secretes such large quantities of AChE, it suggests that this enzyme must play some important role, possibly modulating the host immune response long enough for the female adults to secrete fertile eggs. It was first proposed that AChE acted on the muscle layers of the muscularis mucosae, depressing the propulsive activity of specific areas of the intestine and acting as a "biochemical holdfast" (Ogilvie & Jones, 1971). Foster *et al.* (1994) showed that parasite-derived and commercially available AChE had no effect on the amplitude of contractions of uninfected rat intestinal segments maintained in an organ bath, although an unidentified factor(s) from the excretory/secretory (ES) products did reduce contractions. Further work by Foster and Lee (1996) demonstrated that a 30-50 kDa fraction from parasite derived ES significantly reduced the amplitude of contractions *in vitro*. Gel electrophoresis of this fraction revealed a 30 kDa band that was immunologically cross-reactive to mammalian VIP. The authors concluded that a VIP-like protein, rather than AChE, was wholly or partially responsible for the reduction in gastrointestinal motility, and therefore may act as the biochemical holdfast (Foster & Lee, 1996; Lee, 1996).

The most plausible role for enzyme action would appear to be the hydrolysis of ACh and prevention of 'activation signals' in the host intestine. It is well documented that the intestine is highly innervated by the nervous system, with mAChRs localised on enteric neurons, smooth muscle cells and the basement membrane of epithelial and enteroendocrine cells (Goyal, 1988; Isaacs *et al.*, 1982; Tein *et al.*, 1985; Rimele *et al.*, 1981). Addition of ACh or AChR agonists to intestinal epithelial cells and goblet cells evokes fluid and mucus secretion respectively (Cooke, 1984; Bradbury *et al.*, 1980). These effects can be blocked by the muscarinic antagonist, atropine, indicating

the involvement of muscarinic cholinergic receptors (Cooke, 1984; Phillips *et al.*, 1984). It has also been documented that lymphocytes express mAChRs (described previously), but the functional significance of these receptors remains unclear. In order to inactivate ACh which might otherwise stimulate epithelial cell or mucosal lymphocyte function, the parasite AChE would have to cross the intestinal epithelium. Work by Jones & Knox (1990), using sheep infected with *Trichostrongylus colubriformis* suggests that nematode secretory AChEs may cross the epithelial barrier. They detected an increase in the levels of AChE in the mucosa of infected animals compared to controls, but could not definitively determine the origin of the enzyme. It should be noted that parasite secreted AChEs have only been observed during *in vitro* culture, and *in vivo* secretion has been inferred by the production of antibodies to these enzymes (Lee, 1996). Therefore, it is essential to determine secretion of these enzymes *in vivo* before possible functional roles are investigated.

CHAPTER 2

Materials and Methods

2.1 PARASITES

2.1.1 Maintenance and recovery

N. brasiliensis were maintained by serial passage of 5-6,000 infective third-stage larvae (L3s) through Sprague-Dawley rats. Infective L3s were obtained by collecting the faeces of infected rats from days 5 to 9 post-infection. Collected faeces were moistened with distilled water (dH₂O) for one hour and mixed with activated charcoal to form a paste. The paste was plated onto 90 mm plastic petri dishes and incubated in the dark for up to seven days at 26°C. Third-stage larvae were recovered in a Baermann apparatus, consisting of a funnel leading down to a collecting tube, with the funnel covered by muslin gauze. The funnel was filled with dH₂O at 37°C, the infective larvae-faecal mixture placed on the the gauze, and the larvae recovered from the collecting tube and washed three times in phosphate buffered saline (PBS).

Adult worms were harvested from rats on day 7 p.i. The rats were killed by cervical dislocation, the small intestine rapidly dissected, opened up longitudinally and then placed on Baermann apparatus filled with, 0.9% Sodium chloride (NaCl) at 37°C. Parasites were collected after 2 hours incubation and washed extensively in PBS.

2.1.2 In vitro culture

Recovered adult worms were cultured in serum-free RPMI-1640 (Gibco) supplemented with 2 mM L-glutamine, 2% glucose, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 20 mg/ml Gentamycin. Before culture the parasites were repeatedly washed with sterile medium. They were then incubated in sterile medium at 37°C, 5% CO₂, and the medium was changed daily.

2.1.3 Concentration of Excretory/Secretory products

Culture medium containing ES products was concentrated using Centricon 50 microconcentrator filters (Amicon), according to manufacturers' instructions. Once concentrated the protein was washed into sterile phosphate buffer (pH 7.0). Protein concentration was determined using the Bradford assay (Bradford, 1976), and acetylcholinesterase (AChE) activity was measured by the method of Ellman *et al.* (1961). Concentrated ES samples were stored in aliquots at 4°C, or at -70°C.

2.1.4 Collection of E/S products secreted in vivo

Rats were starved 24 hours prior to sacrifice to reduce amounts of faecal material retained in the intestine. Rats were killed by cervical dislocation and the small intestine rapidly removed. Intestines were repeatedly flushed out with ice-cold, 50 mM sodium phosphate (pH 7.4) containing: 1 µM Leupeptin, 1 mM EDTA, 1.4 µM Pepstatin, 0.3 µM Aprotinin, 0.1 mg/ml Bacitracin, and 1 mM Benzamidine. Supernatants were centrifuged at 1,000 xg to remove residual faecal matter, and then passed through a 0.2 µm filter. The samples were concentrated, and protein concentration and AChE activity determined.

2.1.5 Preparation of parasite adult antigen extract

Adult worms were washed three times in PBS, homogenised and then sonicated for three, thirty second bursts on ice before centrifugation for thirty minutes at 15,000xg at 4°C. The supernatant was recovered and then passed through a 0.2 µm filter, and the protein concentration determined using the Bradford assay (Bradford, 1976). Aliquots were stored at -20°C.

2.2 HISTOCHEMISTRY

2.2.1 Tissue Sections

Segments of jejunum (1 cm) were rapidly dissected from sacrificed animals, carefully flushed through with ice cold 4% paraformaldehyde and then ice cold PBS. Segments were embedded with Tissue-Tek® O.C.T. compound (Miles Inc., U.S.A), mounted onto cork boards and snap frozen in isopentane (BDH) on dry ice. 10-12 µm thick sections were cut on a cryostat and stored at -20°C until required.

2.2.2 Immunocytochemistry

First and second layer antibodies were diluted in PBS. Sections were fixed in acetone (-20°C) for staining of lymphocyte surface markers or paraformaldehyde (4% in PBS) for staining of muscarinic acetylcholine receptors (ten minutes at room temperature). Between each sequential step all sections were washed three times in PBS, with five minutes per wash. Endogenous peroxidase was blocked by incubating sections in hydrogen peroxide (3% in methanol) for two ten minute incubations and endogenous biotin blocked using an avidin/biotin blocking kit (Vector Labs., U.K). Sections were incubated in avidin D solution for fifteen minutes, briefly washed in PBS, incubated in biotin solution for fifteen minutes and then carefully blotted dry. Sections were overlaid with primary antibodies overnight at 4°C. Biotinylated monoclonal antibodies W3/25 (anti-CD4: Serotec), MRC OX-33 (anti-CD45RA: Serotec) and MRC OX-8 (anti-CD8: Serotec) were diluted in PBS at 1:60, 1:90 and 1:60 respectively. All sections were incubated in avidin-conjugated peroxidase (Vector Labs., U.K) diluted 1:1000, for thirty minutes at room temperature. Sections were also stained with a mouse

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monoclonal antibody M35 (Argene-Biosoft, France), which was raised against muscarinic acetylcholine receptors isolated from calf forebrain and has been shown to be pan-specific for all muscarinic receptor subtypes (André *et al.*, 1983, 1984). Slides were first pre-incubated in 5% normal goat serum (NGS; Vector Labs., U.K) for one hour at room temperature and then overlaid with M35 (diluted 1:1000 in PBS with 1% NGS) overnight at 4°C. The sections were incubated in horseradish peroxidase conjugated anti-mouse IgM (μ -chain specific; Serotec) diluted 1:200 for one hour at room temperature. The bound peroxidase was visualised in 0.05% 3, 3' diaminobenzidine (DAB)/0.01% H₂O₂ in PBS and the reaction monitored by microscopy. The reaction was stopped by several washes in dH₂O, before sections were dehydrated and mounted in DPX.

2.2.3 Histochemical staining of intestine for cholinesterase activity

Cholinesterase activity was detected using the method of Karnovsky and Roots (1964). Sections were fixed for ten minutes in 4% paraformaldehyde at room temperature, then washed three times in PBS. The sections were then incubated in substrate buffer for 1-2 hours at room temperature, in the dark. The buffer consisted of: 5 mg acetylthiocholine iodide dissolved in 6.5 ml 0.1 M sodium phosphate buffer (pH 6.5), to which the following were added sequentially: 0.5 ml 0.1 M sodium citrate; 1 ml 30 mM copper sulphate; 1 ml water and 1 ml 5 mM potassium ferricyanide. The specificity of the enzyme was checked by pre-incubating the samples for 30 minutes in 10⁻⁴ M 1,5,-bis(4 allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51) or 10⁻⁴ M tetramonoisopropylpyrophosphotetramide (iso-OMPA), specific inhibitors of AChE and BuChE respectively (Austin & Berry, 1953), substituting water for the

relevant inhibitor in the substrate buffer. After incubation sections were washed several times in water, dehydrated and mounted.

2.3 PROTEINS

2.3.1 Protein determination

Total protein concentration was determined by the method of Bradford (1976). The Bradford Microassay was performed using the Coomassie Plus Protein Assay Reagent (Pierce, Illinois, USA). Sample was mixed with the Coomassie reagent in a ratio of 1:30 respectively and the optical density read at 600 nm. Bovine serum albumin (Sigma, USA) was used as the protein standard at a range of 0.1-1.5 mg/ml. The protein concentration was calculated from the BSA calibration curve.

2.3.2 Acetylcholinesterase activity

Samples were assayed for AChE activity using the method of Ellman *et al.* (1961). Aliquots (25 µl) were added to 96 well microtitre plates containing 1 mM 5,5' dithiobis (2-nitrobenzoic acid) (DTNB; Sigma) and 1 mM acetylthiocholine iodide (ATCI) in 100 mM sodium phosphate buffer, pH 7.0 in a final volume of 200 µl. The change in absorbance was measured at 414 nm, with AChE activity calculated from the extinction coefficient of DTNB (Ellman *et al.*, 1961). One unit of AChE activity is defined as that which hydrolyses 1 µmol of substrate per minute at 20°C.

2.3.3 Polyacrylamide gel electrophoresis.

Proteins were generally resolved by 15% SDS-PAGE. The constituents of the main gel were:

15% acrylamide / 0.4% bisacrylamide (Protogel, National Diagnostics, Atlanta, USA)

0.4 M Tris base pH 8.9

0.1% SDS

0.04% TEMED (Tetraethylmethylenediamine, Sigma Chemicals Co., St. Louis, USA)

0.1% APS (Ammonium persulphate)

The stacking gels were composed of:

5% acrylamide / 0.13% bisacrylamide

0.125 M Tris base pH 6.8

0.1% SDS

0.15% TEMED

0.625% APS

After addition of the sample to gels pre-cooled to 4°C, samples were electrophoresed at 20 mA in running buffer (0.25 M glycine, 0.025 M Tris base, 0.1% SDS, pH 8.3) until the tracking dye had completely run off the base of the gel. Gels were either fixed and stained with Coomassie brilliant blue or samples transferred by Western blotting.

Non-denaturing conditions were required for the separation and determination of acetylcholinesterase activity. All the buffers and solutions were made as described above, with the omission of SDS. Samples were resolved by electrophoresis in 7.5% polyacrylamide gels and enzyme activity was detected by an adaptation of the Karnovsky and Roots (1961) method (Blackburn & Selkirk, 1992). Briefly, after electrophoresis

gels were washed in distilled water for 30 minutes, and then incubated in substrate buffer (described in 'Histochemical staining of intestine for cholinesterase activity'). Enzyme activity resulted in the appearance of brown bands in the gel following incubation at room temperature for 30 minutes. The species of enzyme was determined by pre-incubation with either the acetylcholinesterase inhibitor BW284C51 (10^{-4} M) or the butyrylcholinesterase inhibitor iso-OMPA (10^{-4} M).

2.3.4 Staining with Coomassie Brilliant blue

Gels were fixed and stained for 30 min in stain 1 (25% isopropanol, 10% acetic acid and 0.01% Coomassie Blue R-250), 30 min in stain 2 (10% isopropanol, 10% acetic acid and 0.025% Coomassie Blue R-250) then destained for 1 hr in 15% methanol, 7.7% acetic acid.

2.3.5 Western blotting

Blotting of proteins from polyacrylamide gels onto nitrocellulose membrane was carried out using a Biorad Trans-blot Electrophoretic Transfer Cell. Blotting was performed for 3 hr at a constant current of 250 mA in transfer buffer.

Transfer buffer was made up as follows:

192 mM Glycine

25 mM Tris-base

20% Methanol and adjusted to pH 8.3.

2.4 CELL CULTURE

2.4.1 Epithelial Cell culture

The epithelial cell lines, Madin-Darby canine kidney (MDCK) and colonic carcinoma (Caco-2) cells were kindly donated by Dr. C. Isaacs (Dept. of Biology, Imperial College). MDCK cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 0.37% (w/v) sodium bicarbonate and 15 µg/ml phenol red. Caco-2 cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were grown to confluency in tissue culture flasks held at 37°C, 5% CO₂, before being transferred onto permeable tissue culture inserts (Costar) at a cell density of 1×10^6 cells/ml. The cells were grown to confluency, and the permeability of the monolayer assessed by the inulin assay.

2.4.2 Inulin assay

Monolayers grown on permeable inserts were washed in fresh medium before being tested for tight junction formation. [³H]-inulin was added to the apical chamber at a concentration of 1 µCi/ml and the plates were incubated at 37°C. Aliquots of medium (200 µl) were taken from both apical and basolateral chambers after 30 minutes and 1 hour, and the presence of [³H]-inulin determined by scintillation counting. Monolayers were determined to be tightly sealed when less than 0.5% of the total [³H]-Inulin was detected in the basolateral chamber after 1 hour. Permeable inserts without cells were used as positive controls.

2.4.3 Transport studies

All experiments were carried out at 37°C, 5% CO₂, using cells that had formed tightly sealed monolayers.

1) Aliquots of unfractionated ES from adult parasites (100 µl; 600-800 µg) were added to the apical chambers of Transwell inserts with confluent MDCK cells, and the distribution of AChE examined by removing 100 µl samples of medium from each chamber at intervals over 24 hours. Cultures were carried out in DMEM supplemented with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 0.37% (w/v) sodium bicarbonate and 15 µg/ml phenol red. The effect of rat mast cell protease II (RMCP II) on the permeability of MDCK cells with respect to passage of AChE was assessed by addition of 10 µg/ml to the basolateral chamber. RMCP II was kindly provided by Prof. Hugh Miller, University of Edinburgh.

2) Aliquots of unfractionated ES from adult parasites (100 µl; 600-800 µg) were added to the apical or basolateral chambers of Transwell inserts with confluent Caco-2 cells, and the distribution of AChE examined by removing 100 µl samples of medium from each chamber at intervals over 24 hours. Cultures were carried out in Caco-2 cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The effects of RMCP II (10 µg/ml) and sheep mast cell protease (SMCP; 50 µg/ml) on the permeability of Caco-2 cells with respect to passage of AChE was assessed by addition of proteases to basolateral chambers. RMCP II and SMCP were kind gifts from Prof. Hugh Miller, University of Edinburgh.

3) Approximately 100 live adult worms or 100 heat killed worms (worms maintained at 100°C for 10 minutes, and washed twice in sterile PBS) were added to the apical chambers of Transwell inserts containing confluent Caco-2 cells. [³H]-inulin was added to the apical chamber (1 µCi/ml) to assess changes in monolayer permeability, and the distribution of AChE and proteins were examined by removing 100 µl samples of medium from each chamber at intervals over 24 hours.

2.5 LYMPHOCYTE ASSAYS

2.5.1 Mononuclear cell isolation

The spleens and mesenteric lymph nodes of control and infected animals were rapidly dissected and maintained in ice cold RPMI 1640 medium (Gibco BRL). The tissue was mashed using sterile scalpel blades and filtered through sterile cheesecloth. The cells were centrifuged at 500 x g for 5 minutes, resuspended in RPMI 1640 medium, then centrifuged and the supernatant removed. The cells were then resuspended in RPMI and mononuclear cells isolated by centrifugation (400 x g) in Histopaque-1077 (Sigma Diagnostics). Mononuclear cells were removed and washed twice and resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum (Gibco BRL), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin and counted using a haemocytometer.

2.5.2 T cell proliferation assay

Mononuclear cells were isolated from the spleen and mesenteric lymph nodes of uninfected and *N. brasiliensis*-infected (d7 p.i) rats as described above. Cells (2×10^5

/well) were incubated in 96 well plates in the presence of Concanavalin A (5 ug/ml), a range of muscarinic receptor agonists alone for 24 hours (oxotremorine-M 10 μ M, carbachol 10 μ M), or were pre-treated with mAChR agonists for 12 hours prior to Con A stimulation. Proliferation was assessed by the addition of 1 μ Ci of [3 H]-thymidine/well for the last 8 hours of a 72 hour incubation. Cells were harvested (Skatron Instruments cell harvester) and [3 H]-thymidine incorporation determined in a scintillation counter

2.5.3 Isolation of intestinal cells

Intestinal epithelial and lamina propria cells were isolated by an adaptation of the procedure described by Poussier (1996). Rat small intestine was removed and placed into a siliconized beaker containing sterile PBS held at 37°C. The intestine was carefully flushed through several times with PBS, using a 10 ml syringe, before the Peyer's patches were removed and the intestine opened up longitudinally onto a Kimwipe^(?). The gut lumen was gently blotted with Kimwipe to remove faecal material and mucus. Intestinal tissue was cut into 1 cm pieces, incubated in Hanks Balanced Salt Solution without calcium or magnesium (HBSS-CMF; Gibco BRL), containing 2 mM dithiothreitol (DTT), 5% FCS for 15 minutes at 37°C with continuous stirring. The supernatant was removed and discarded. Tissue pieces were incubated in HBSS-CMF containing 2 mM DTT, 5% FCS, 1 mM EDTA, held at 37°C with continuous stirring. After 20 minutes the supernatant was removed, filtered through four layers of sterile cheesecloth into 50 ml falcon tubes and centrifuged at 500 x g, for 5 minutes at room temperature. Fresh medium was added to the tissue and the procedure repeated three times. The supernatants were discarded and the cell pellets resuspended in HBSS-CMF containing 5% FCS, pooled, and centrifuged (500 x g for 5 minutes at room

temperature). Cell pellets were resuspended in HBSS-CMF/5% FCS and counted. This fraction contains epithelial enterocytes and IELs.

To obtain lamina propria cells, tissue pieces (after epithelial cell isolation) were washed vigorously in HBSS-CMF containing 1 mM EDTA three times to remove residual epithelium. The intestinal tissue was then incubated with HBSS containing 10% FCS, and 10U/ml collagenase (Sigma) for three x 20 minute incubations at 37°C with vigorous stirring. The supernatants were removed, passed through sterile cheesecloth and centrifuged at 500 x g for 5 minutes at room temperature. Pellets were resuspended, pooled and again centrifuged to remove any collagenase activity. The final pellet was resuspended in HBSS containing 10% FCS and the cells counted.

2.6 RADIOLIGAND BINDING

2.6.1 Membrane preparation

Freshly isolated or frozen cell preparations were homogenised in membrane buffer (10 mM Tris, 2 mM EDTA, pH 7.4), containing a cocktail of protease inhibitors (1 µM Leupeptin, 1 mM EDTA, 1.4 µM Pepstatin, 0.3 µM Aprotinin, 0.1 mg/ml Bacitracin, and 1 mM Benzamidine), at 4°C using a Jencons glass homogeniser. Cell homogenates were centrifuged for 15 minutes at 27, 000 x g in an ultracentrifuge maintained at 4°C. The supernatants were discarded, the pellet resuspended in membrane buffer and re-centrifuged. Finally, the supernatants were discarded, the pellet resuspended in membrane buffer and the protein content determined (Bradford, 1976). Samples were stored at -70°C in 1 ml aliquots.

2.6.2 Membrane preparation

Binding assays were performed in a final volume of 250 µl of incubation buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, pH 7.5), in 96 well microtitre plates. The incubation was started by the addition of membrane preparation to wells containing various concentrations of [³H]-Quinuclidinyl Benzilate ([³H]-QNB; NEN). Specificity of binding was assessed by the addition of the muscarinic inhibitor, atropine (10⁻⁴ M). Incubations were performed at 37°C for 1 hour. The membrane preparations were collected on Whatman GF/C filters using a Skatron cell harvester. Filters were allowed to dry, then added to scintillant and counted.

2.7 MOLECULAR BIOLOGY

2.7.1 RNA extraction

Whole tissue segments were frozen in liquid nitrogen and ground with a mortar and pestle (which were kept cold by intermittent addition of liquid nitrogen). The resulting powder was transferred to an eppendorf tube and the RNA extracted using Triazol reagent (Gibco) according to the manufacturers' instructions. Isolated cells were resuspended in Triazol reagent and RNA extracted as for whole tissue segments. The RNA pellets were resuspended in 25 µl DEPC-treated water and 1 µl of each sample was quantified by optical density. RNA was resolved on a 2% agarose gel in TAE (0.04 M Tris-acetate 0.001 M EDTA).

2.7.2 DNase treatment and Reverse Transcription

Before reverse transcription, contaminating DNA was removed from the RNA samples by treatment with DNase I. Approximately 20 µg of RNA was added to 10 µl Tris-HCl (1 M, pH 7.5), 2 µl MgCl₂ (1 M), 16 U RNase inhibitor (RNasin, Promega), 5 U DNase I (Promega) and 190 µl dH₂O. The reaction was incubated at 37°C for 20 minutes. The enzyme was removed by phenol/chloroform (1:1) extraction and the RNA was ethanol precipitated. Pellets were washed in 70% ethanol, and air dried for 5 minutes before being resuspended in DEPC-treated water. The integrity of the RNA was checked by electrophoresis on a 1.5% agarose gel and staining with ethidium bromide to visualise ribosomal RNAs.

Approximately 5 µg of DNase-treated RNA was added to 1 µl of oligo d(T)₁₂₋₁₈ (0.5 µg/µl) and heated to 65°C for 10 minutes. The reaction was allowed to cool at room for 10 minutes before the cDNA master mix was added. This consisted of 5 µl 10x MMLV reverse transcription buffer (Stratagene), 5 µl 0.1 mM DTT (USB), 2.5 µl 20 mM dNTP mix (Pharmacia), 40 units of RNasin (Promega), and 50 units of reverse transcriptase (Stratagene) made up to 50 µl using DEPC-dH₂O. The reaction was incubated for 1 hour at 37°C, then the enzyme was heat inactivated at 95°C for 5 minutes and placed on ice. The cDNA was frozen and stored at -70°C until use.

2.7.3 Polymerase Chain Reaction

Primers specific for rat muscarinic acetylcholine receptor (mAChR) subtypes m1-m5 were previously reported by Wei *et al.* (1994). Reverse transcription and PCR of rat

β-actin was used as a positive control. The primers were designed based on the coding sequence obtained from the GenBank database (Table 2.1).

The PCR master mix contained: 5µl 10 x PCR buffer (Qiagen), 10 µl Q solution (Qiagen), 5 µl 2 mM dNTP, 1 µl of each primer (10 pmol of sense and anti-sense), and 22.5 µl of DEPC-dH₂O. cDNA (5 µl) and Taq polymerase (2.5 U) were added last and the reaction mixtures overlaid with mineral oil. The PCR programme was set at 94°C for 1 minute (denaturation), 57°C for 1 minute (annealing), 72°C for 1 minute 30 seconds (extension), for 30 cycles in a programmable heating block (Perkin Elmer).

Table 2.1. PCR primer sequences and expected product sizes.

Primer		Sequence	Amplified Band (bp)
m1	Sense	5'-GCACAGGCACCCACCAAGCAG-3'	373
	Antisense	5'-AGAGCAGCAGCAGGCGGAACG-3'	
m2	Sense	5'-CACGAAACCTCTGACCTACCC-3'	686
	Antisense	5'-TCTGACCCGACGACCCAATA-3'	
m3	Sense	5'-GTCTGGCTTGGGTCATCTCCT-3'	434
	Antisense	5'-GCTGCTGCTGTGGTCTTGGTC-3'	
m4	Sense	5'-TGGGTCTTGGCCTTTGTGCTC-3'	588
	Antisense	5'-TTCATTGCCTGTCTGCTTTGT-3'	
m5	Sense	5'-CTGGTCTCCTTCATCCTCTGG-3'	394
	Antisense	5'-CCTGGGTGTCTTTCCTGTTG-3'	
β-actin	Sense	5'-CCCCAGCCATGTACGTAGCCATCC-3'	400
	Antisense	5'-AGAGCCTCGGGGCATCGGAACCGC-3	

CHAPTER 3

The determination and localisation of parasite secreted acetylcholinesterases

3.1 Introduction

Acetylcholinesterase (AChE) activity has been localised to the oesophageal and subventral glands of *N. brasiliensis* using histochemical techniques (Lee, 1970; Nakazawa *et al.*, 1995). A similar pattern of localisation was observed in *Trichostrongylus colubriformis* (Rothwell *et al.*, 1973), but in *Oesophagostomum radiatum* and *O. venulosum* (nodular worms that infect cattle), enzyme activity was restricted to the subventral or the oesophageal glands respectively (Bremner *et al.*, 1973). McLaren and colleagues (1974) observed that AChE activity was localised to the oesophageal and the two large amphidial glands of the hookworm *Necator americanus*, but staining was absent in the subventral glands. The demonstration of AChE activity in specialised glands, located at the anterior of the parasite suggested a role other than the termination of acetylcholine (ACh) receptor signals at parasite neuronal synapses. It has been reported that ACh is a major excitatory neurotransmitter at neuromuscular junctions of the free-living nematode *Caenorhabditis elegans* (Rand & Nonet, 1997). Cholinergic motor neurons have been demonstrated in both free-living and parasitic nematodes (Alfonso *et al.*, 1993; Johnson & Stretton, 1985), and mutations in the *C. elegans* gene that encodes choline acetyltransferase (the enzyme which synthesizes ACh within neurons) *cha-1* or the vesicular ACh transporter *unc-17* result in worms with restricted movement that do not feed or grow, and die within a few days of hatching (Alfonso *et al.*, 1993; Rand, 1989).

It had previously been demonstrated that several species of parasitic nematode secrete AChEs when maintained *in vitro* (Ogilvie *et al.*, 1973). This initial study concentrated on nematodes that colonise the intestinal tract of its host. However, further studies have observed that this phenomenon is not entirely exclusive to gut dwelling

parasites. Examples of AChE secretion at alternative sites include the cattle lungworm *Dictyocaulus viviparus*, the filarial nematode *Brugia malayi*, and *Stephanurus dentatus*, which infect the kidneys of swine (McKeand *et al.*, 1994; Rathaur *et al.*, 1987; Rhoads, 1981).

The level and activity of AChE secreted by the different species varies greatly, with parasites such as *T. colubriformis*, *T. axis*, *O. radiatum* and *O. venosulum*, releasing considerably more enzyme *in vitro* than *N. brasiliensis* (Ogilvie *et al.*, 1973). As well as differences in enzyme secretion, it has been reported that the properties and isoforms of AChE vary between species. The human hookworm *N. americanus* secretes a single isoform of the enzyme, with an apparent mass of 66-70 kDa, existing as a hydrophilic (G₂) dimer (Pritchard *et al.*, 1994). In contrast, two forms (G₁ and G₂) were identified in *T. colubriformis* secretory products which had apparent molecular masses of 80 and 189 kDa respectively (Griffiths & Pritchard, 1994). Three major secreted isoforms of AChE have been identified from *N. brasiliensis* (A, B and C), with apparent masses of 74, 69 and 71 kDa, and all three isoforms have been identified as hydrophilic (G₁) monomers (Grigg *et al.*, 1997).

The AChEs secreted by *N. brasiliensis* have a unique pattern of expression that has yet to be observed in other parasites. The levels of enzyme produced steadily increase as the parasite develops in the intestinal tract (Sanderson & Ogilvie, 1971), with a loss of the A isoform, and exclusive expression of isoforms B and C during the second week of infection (Edwards *et al.*, 1971; Blackburn & Selkirk, 1992). The transfer of adult worms, yet to be damaged by the immune response, into naive recipients results in a reduction of AChE levels (Sanderson *et al.*, 1972; Sanderson *et al.*, 1976a). A reduction in the levels of parasite enzyme were also demonstrated when infected animals

were treated with the anti-inflammatory agent cortisone (Jones & Ogilvie, 1972). These data indicate that the expression of AChE by the parasite is modulated by the host immune response.

To date, all work on parasite secreted AChEs have utilised culture supernatants from worms maintained *in vitro*. Antibodies specific to parasite AChEs have been observed in the sera of infected humans and animals (Bremner *et al.*, 1973; Brown & Pritchard, 1993; McKeand *et al.*, 1993; Rothwell *et al.*, 1973), which implied that enzyme is secreted *in vivo* in these cases. Further evidence for *in vivo* secretion of AChEs comes from vaccination studies, where purified or partially purified enzyme confers a degree of protection to immunised animals (Griffiths & Pritchard, 1994; McKeand *et al.*, 1995). With the findings that: 1) there is isoform switching and subsequent parasite expulsion of *N. brasiliensis*, 2) the enzyme is highly immunogenic to the host, and 3) the enzyme is stored in highly specialised secretory glands, it has been speculated that the enzyme is important to the parasites survival (Lee, 1996). Before trying to ascertain the physiological importance of the enzyme, the *in vivo* secretion of the enzyme by the parasite, and the location of the enzyme within the parasitised intestine were investigated.

3.2 Detection of cholinesterase activity in intestinal flushes

Intestinal flushes were obtained from rats sacrificed at different times post-infection (day 0, 3, 4, 7 and 10 p.i.) with *N. brasiliensis*, concentrated and assayed for protein content and cholinesterase (ChE) activity. It has been previously documented (Blackburn & Selkirk, 1992) that only one isoform of parasite secreted AChE (isoform A) retained its enzymatic activity when resolved by SDS-PAGE. Therefore, samples of equivalent ChE activity were separated by non-denaturing gel electrophoresis, and cholinesterase activity visualised by the method of Karnovsky and Roots (1964). The species of ChE was determined by the use of BW 284C51 and iso-OMPA, specific inhibitors of AChE and BuChE respectively (Austin & Berry, 1953). For comparison, worm culture supernatants were obtained from parasites isolated at different times post-infection (day 3, 4, 7 and 10 p.i.), and electrophoresed alongside the intestinal flushes.

High levels of ChE activity were observed at all timepoints from intestinal flushes (Table 3.1), appearing in gels as a smear of activity which electrophoresed with low mobility (Fig 3.1; lanes 1-5). Two other forms of ChE were detected, with less activity than the major form (Fig 3.1; lanes 3-5). These isoforms were only observed in infected animals, and were detected at the highest levels at d7 and d10 p.i, with electrophoretic mobilities similar to those identified in parasite culture supernatants (lanes 6-9). Three isoforms of ChE were detected in culture supernatants recovered from worms isolated on days 3 and 4 p.i. (lanes 6 & 7) and these have been previously designated forms A, B and C (Edwards *et al.*, 1971). Isoform A was absent from culture supernatants recovered from animals on days 7 and 10 p.i. (lanes 8 & 9). Pre-incubation and staining of the gel in the presence of BW284C51 led to inhibition of AChE activity (Fig 3.2).

Timepoint (days post-infection)	Protein concentration (mg/ml)	ChE activity (μ mol/min/ml)
d 0	1.27	13.5
d 3	8.46	107.6
d 4	3.71	57.6
d 7	18.76	126.0
d 10	8.92	56.6

Table 3.1. Protein concentration and ChE activity of intestinal flushes from uninfected and *N. brasiliensis*-infected rats.

Intestines were dissected from animals sacrificed at different times post-infection with *N. brasiliensis* and repeatedly flushed with ice-cold 50 mM NaPO₄ buffer (pH 7.4) containing a cocktail of protease inhibitors. Samples were concentrated and assayed for protein content by the Bradford assay (1976), and for ChE activity using the method of Ellman *et al.*, (1961).

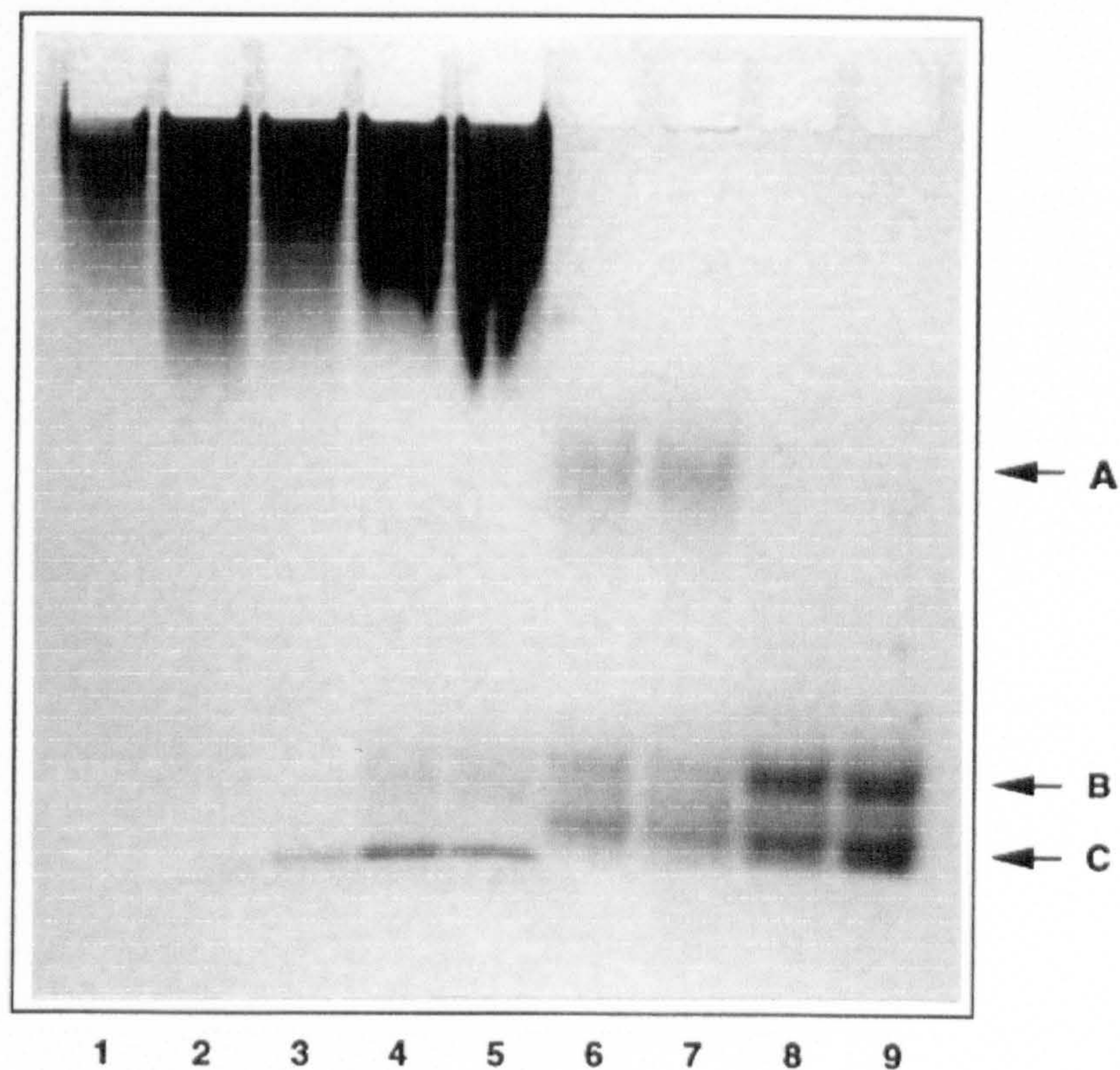


Figure 3.1. The identification of ChE activity within the intestinal flushes of rats infected with *N. brasiliensis* . Intestinal washes were separated by non-denaturing 7.5% PAGE in Tris-glycine buffer, and stained for cholinesterase activity by the method of Karnovsky and Roots (1964). Lanes 1-5 show the intestinal flushes from an uninfected rat, and rats at D3, D4, D7 and D10 p.i. respectively, while lanes 6-9 show culture supernatants from parasites isolated from D3, D4, D7 and D10 p.i.

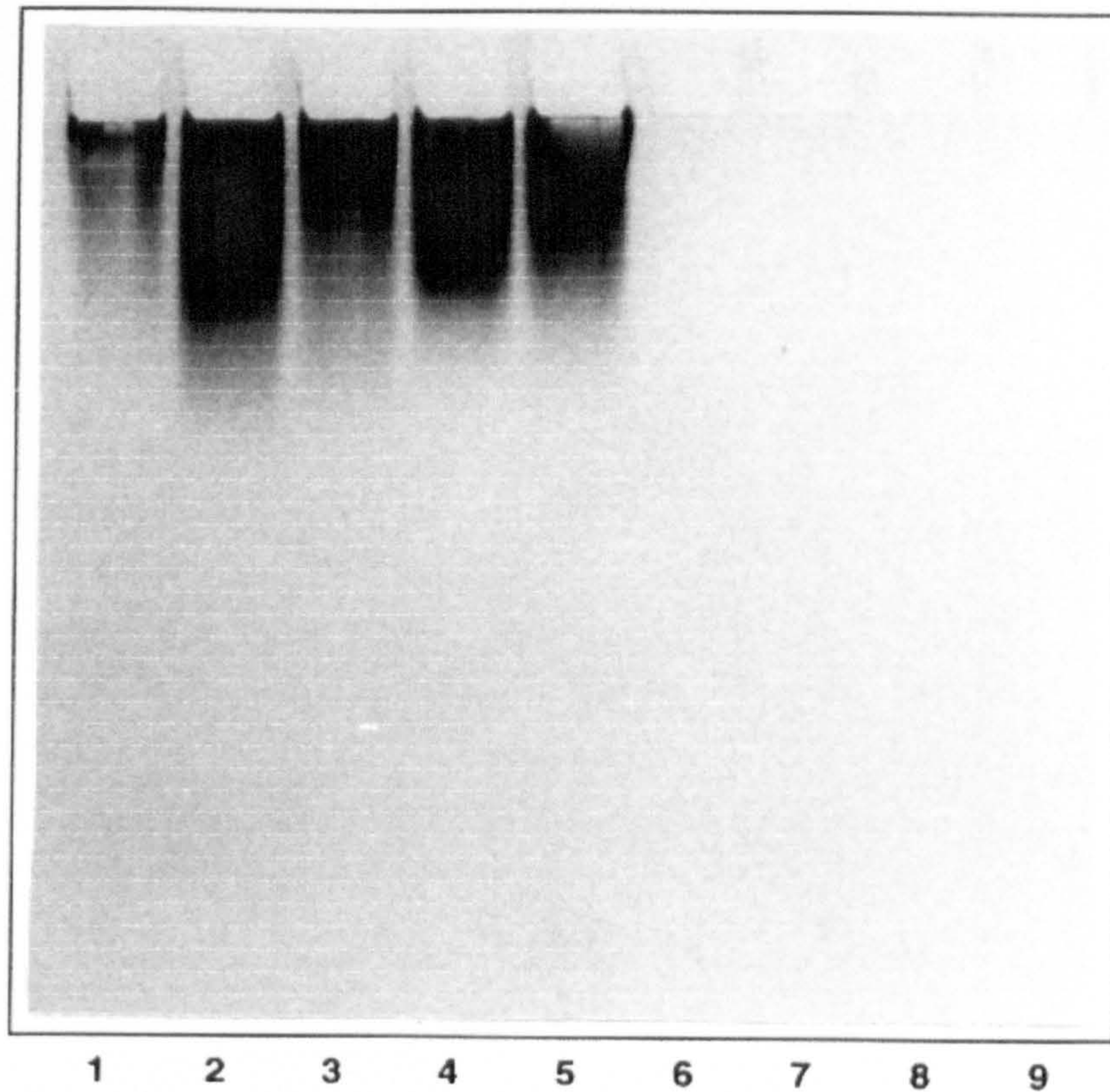


Figure 3.2. The identification of BuChE activity within the intestinal flushes of rats infected with *N. brasiliensis*. Intestinal washes were separated by non-denaturing 7.5% PAGE in Tris-glycine buffer, and stained for cholinesterase activity by the method of Karnovsky and Roots (1964). BuChE activity was determined by pre-incubating the gel in the AChE inhibitor BW284C51 (10^{-4} M). Lanes 1-5 show the intestinal flushes from an uninfected rat, and animals sacrificed on D3, D4, D7 and D10 p.i. respectively, while lanes 6-9 show culture supernatants from parasites isolated from D3, D4, D7 and D10 p.i.

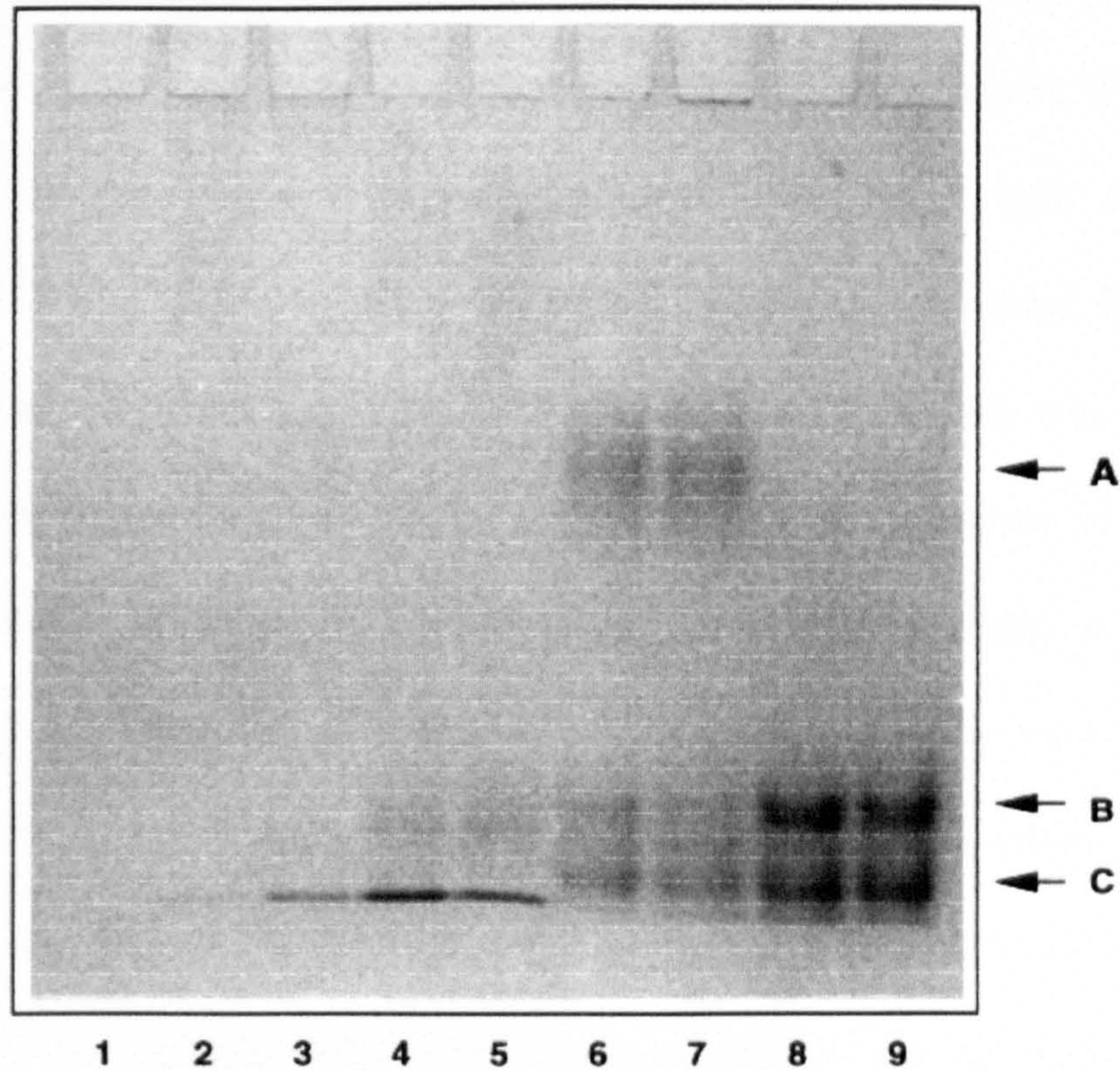


Figure 3.3. The identification of AChE activity within the intestinal flushes of rats infected with *N. brasiliensis* . Intestinal washes were separated by non-denaturing 7.5% PAGE in Tris-glycine buffer, and stained for cholinesterase activity by the method of Karnovsky and Roots (1964). AChE activity was determined by pre-incubation with the BuChE inhibitor iso-OMPA (10^{-4} M). Lanes 1-5 show the intestinal flushes from an uninfected rat, and animals sacrificed on D3, D4, D7 and D10 p.i. respectively, while lanes 6-9 show culture supernatants from parasites isolated from D3, D4, D7 and D10 p.i.

The slow migrating band observed at the top of the gel (lanes 1-5) was unaffected by this inhibitor. The fact that this isoform migrates slowly in non-denaturing gel electrophoresis, and its insensitivity to BW284C51 suggests this isoform is an amphiphilic BuChE. In contrast, the enzyme activity detected at the bottom of the gel in the intestinal flushes (lanes 3-5), and those detected in parasite culture supernatants (lanes 6-9) were totally abolished by incubation with BW284C51, indicating that these were true acetylcholinesterases. Incubation with the BuChE inhibitor iso-OMPA (Fig 3.3) removed the activity within the intestinal flushes that was located at the top of the gel, confirming the assumption that this was a BuChE. However, iso-OMPA had no effect on the enzyme activity at the bottom of the gel of the infected intestinal flushes (lanes 3-5), and parasite culture supernatants were also unaffected (Fig. 3.3; lanes 6-9). The inhibitor sensitivities of the parasite culture supernatants were in accordance with previous studies which identified the three isoforms of the enzyme, secreted by the parasite in culture, to be true AChEs (Grigg *et al.*, 1997). These studies demonstrate the presence of BuChE activity in the intestinal washes throughout the time course of infection, which complements previous work carried out by Sine and co-workers (1992) who isolated amphiphilic BuChEs from rat intestinal cells. More interesting is the appearance of AChE activity in the gut flushes. This phenomenon was limited to animals containing parasites in the intestine at the time of the study, and these enzymes had a similar migration pattern to those enzymes isolated from worms maintained in culture.

3.3 Histochemical localisation of ChE in the rat intestine

Segments of normal and parasitised rat jejunum were stained for ChE activity by the method of Karnovsky and Roots (1964). As mentioned above, the species of ChE was determined using the inhibitors BW284C51 and iso-OMPA.

In normal jejunum, high levels of ChE activity were localised to the intestinal epithelium, some secretory glands and the external muscle layers (Fig. 3.4). ChE activity was distributed throughout the cytoplasm of the epithelium and the secretory glands but was absent from the cells within the lamina propria. Inclusion of BW284C51, the AChE inhibitor had no effect on the levels of enzyme activity in the epithelial cells, the glands and the external muscle (Fig. 3.5). In comparison, the BuChE inhibitor iso-OMPA totally abolished enzyme activity in the epithelium and secretory glands (Fig. 3.6), and significantly reduced the levels of staining in the muscle layers to reveal discrete clusters of activity. The enzyme activity localised to the epithelium and secretory glands was therefore demonstrated to be entirely BuChE, whereas the external muscle layers contained both BuChE and AChE. In the majority of vertebrate species studied, the intestinal epithelial cells contain either AChE or BuChE activity (Sine *et al.*, 1988), with rat intestinal cells reported to possess amphiphilic BuChEs (Sine *et al.*, 1992). Infected sections of intestine showed similar patterns of ChE staining (Fig. 3.7), but intriguingly focal staining was also observed in the lamina propria. This activity was abolished in the presence of BW284C51 (Fig. 3.8), and unaffected by incubation with iso-OMPA (Fig. 3.9). These results show discrete, focal AChE staining within the lamina propria of infected animals which appeared to be localised in many cases along the external face of the basal lamina of the mucosal epithelial cells.

It is tempting to suggest that this is parasite derived enzyme which has penetrated the mucosa, but further work is required to rule out the possibility that this staining could be host derived enzyme, present as a result of intestinal inflammation

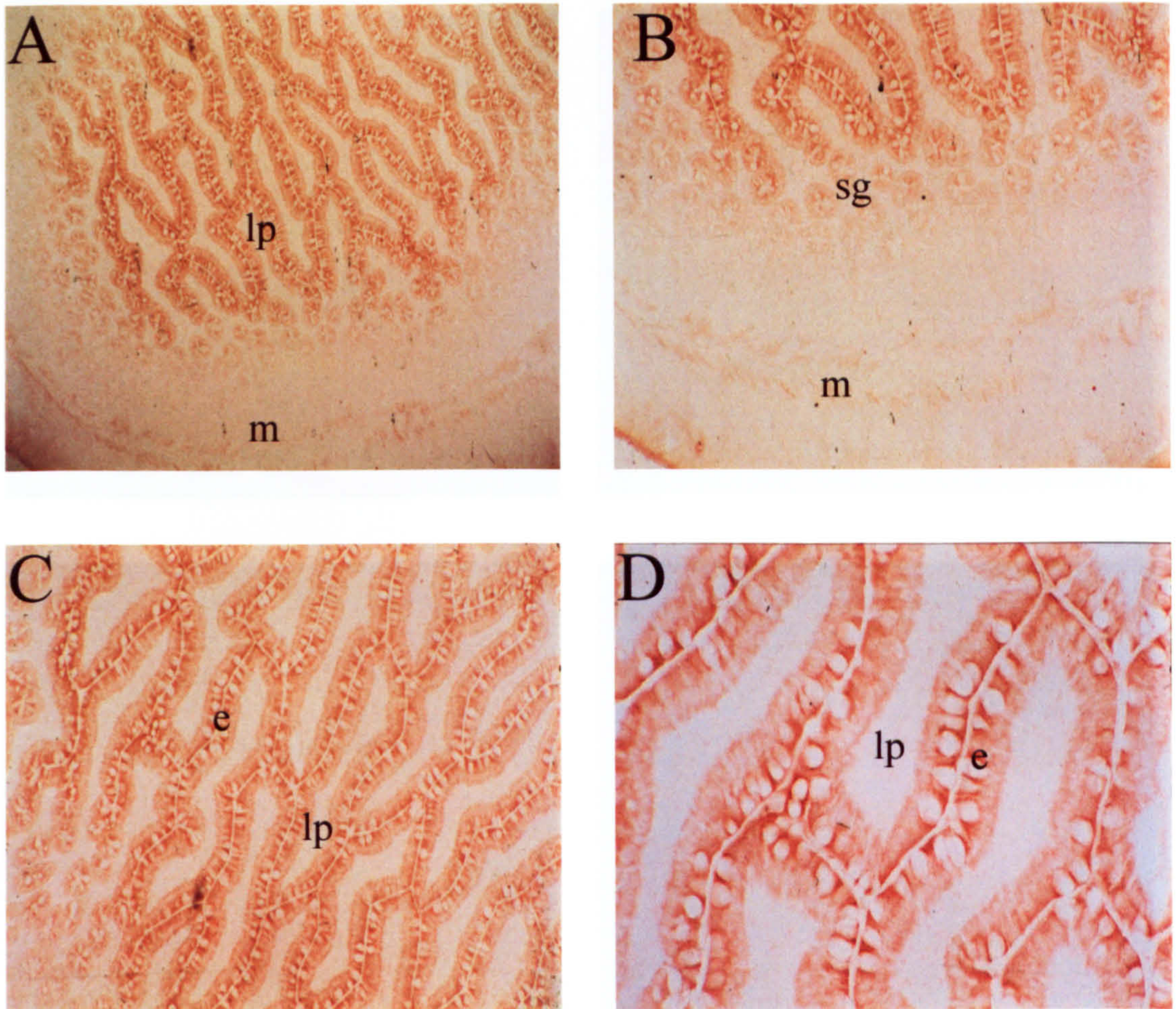


Figure 3.4. Histochemical localisation of ChE activity within the intestine of uninfected rats. Intestinal sections were stained for cholinesterase activity by the method of Karnovsky & Roots (1964). Strong enzyme activity was observed along the epithelium (e), within the secretory glands (sg) and in the external muscle (m). The lamina propria (lp) was devoid of staining. Magnification was x80 (A), x125 (B-C), and x312 (D).

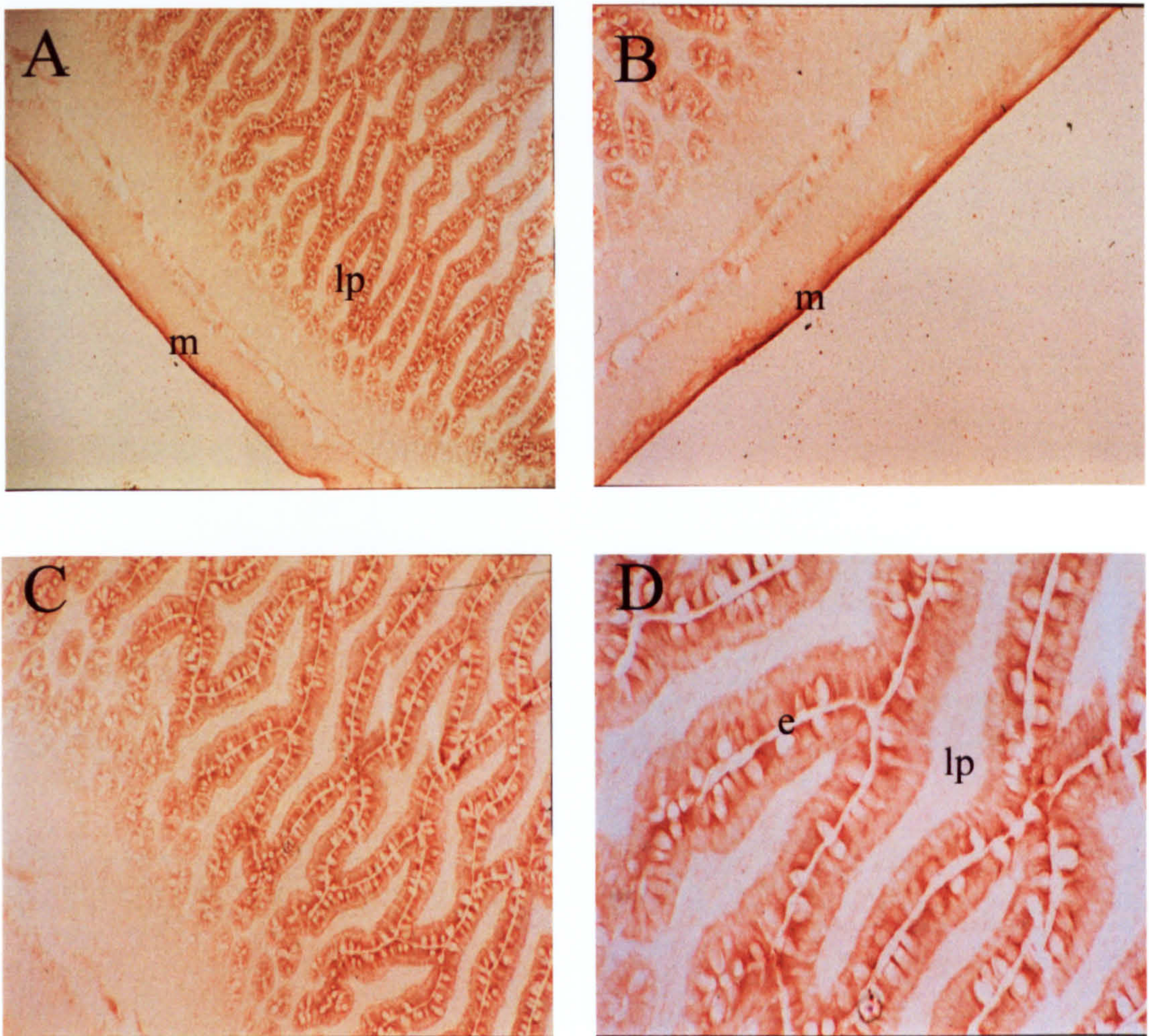


Figure 3.5. Localisation of BuChE activity in the intestine of uninfected rats. Sections were stained by the method of Karnovsky and Roots (1964), and BuChE activity was determined by pre-incubation with the AChE inhibitor BW284C51 (10^{-4} M). BuChE activity was localised to the epithelium (e) and the external muscle (m). Magnification was x80 (A), x125 (B-C), and x312 (D).

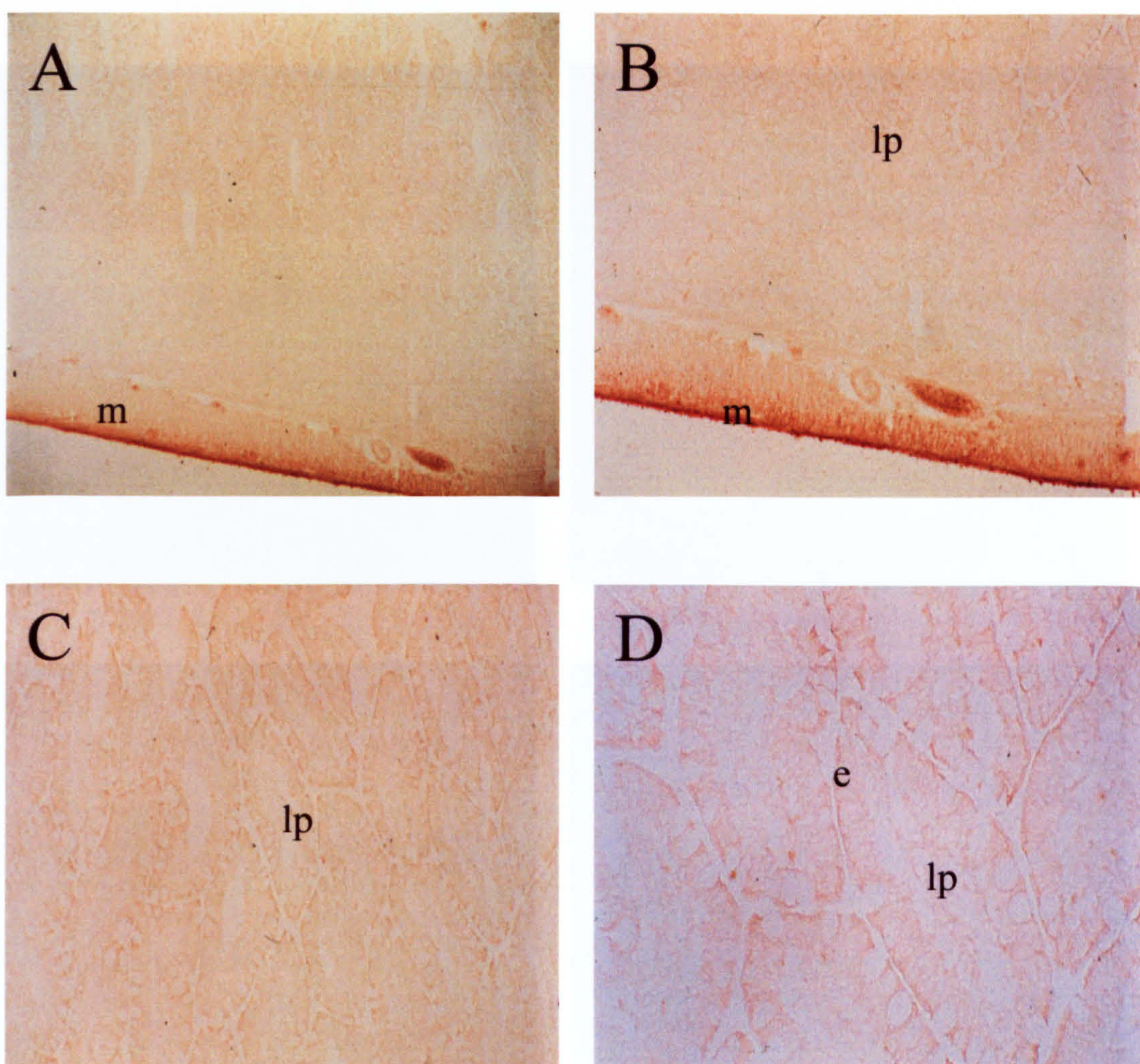


Figure 3.6. Localisation of AChE activity in the intestine of control (uninfected) rat. Intestinal sections were stained by the method of Karnovsky and Roots (1964), with AChE activity determined by pre-incubation with the BuChE inhibitor iso-OMPA (10^{-4} M). No activity was observed in the epithelium (e) or lamina propria (lp), but strong staining was present in the external muscle (m). Magnification x80 (A), x125 (B-C), and x312 (D).

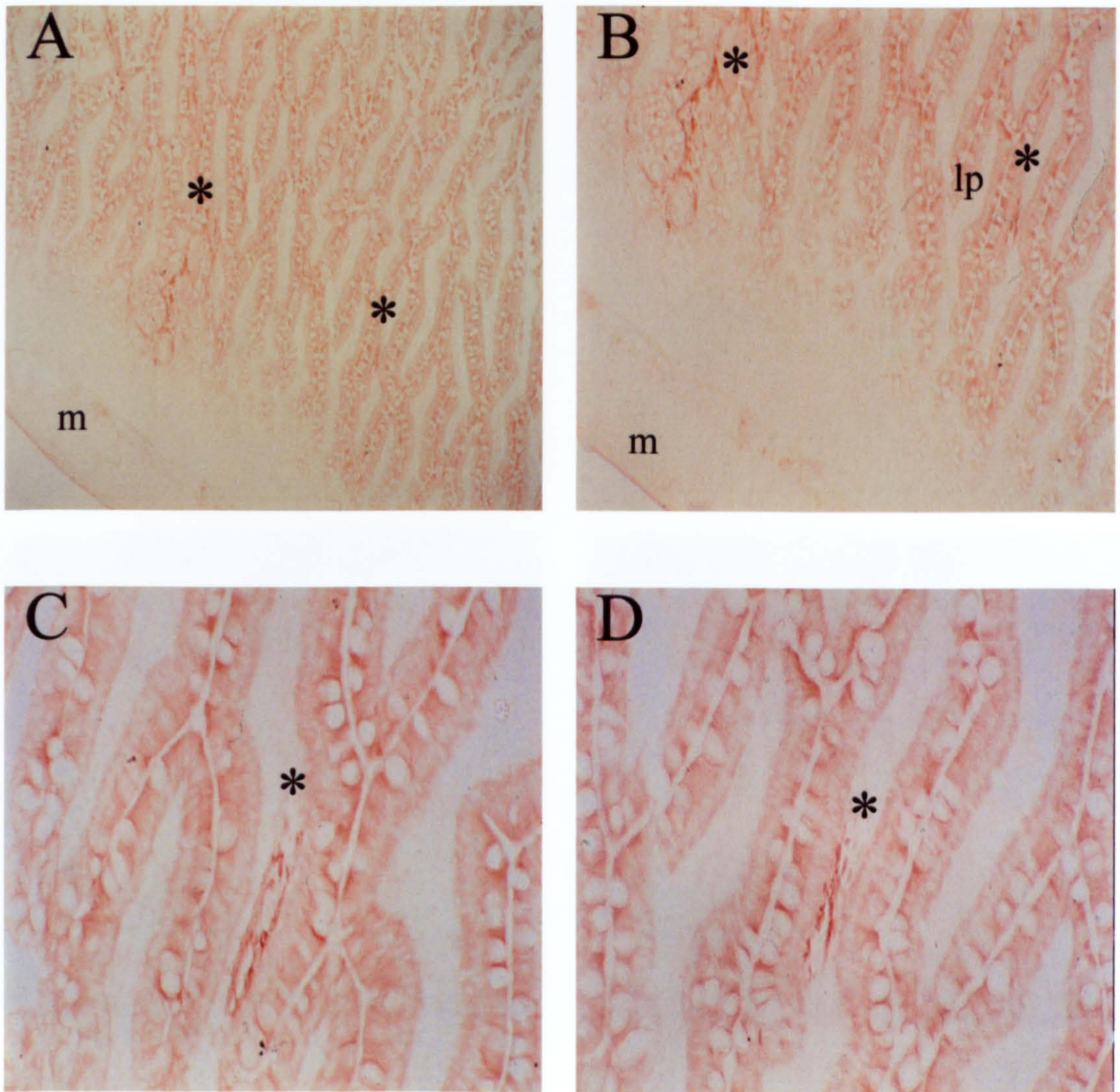


Figure 3.7. Histochemical localisation of ChE activity within the intestine of *N. brasiliensis*-infected rats. Intestinal sections were stained for cholinesterase activity by the method of Karnovsky & Roots (1964). Strong enzyme activity was observed along the epithelium (e), within the secretory glands (sg) and in the external muscle (m). Focal staining (*) was also observed in the lamina propria (lp). Magnification was x80 (A), x125 (B), and x312 (C-D).

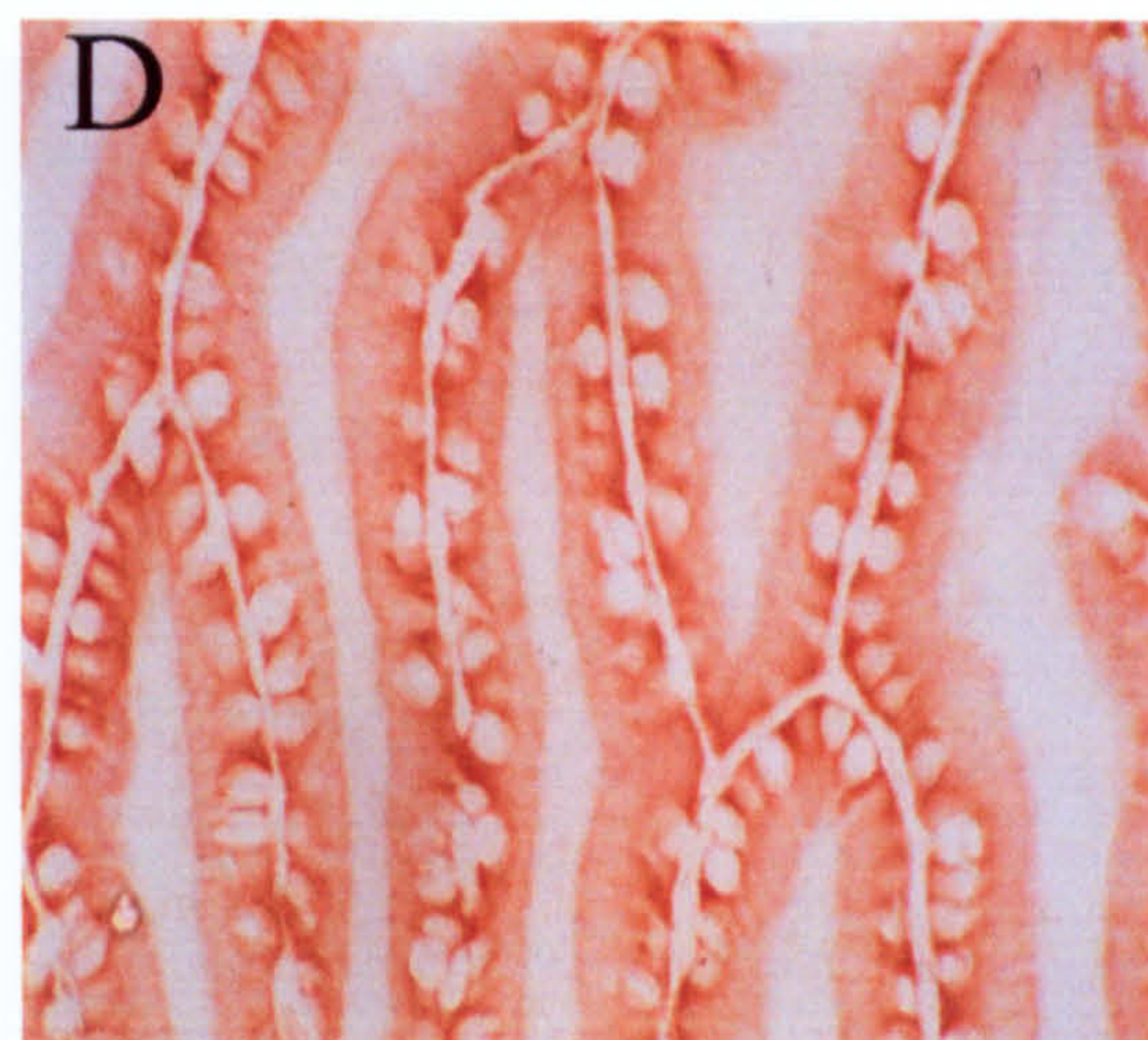
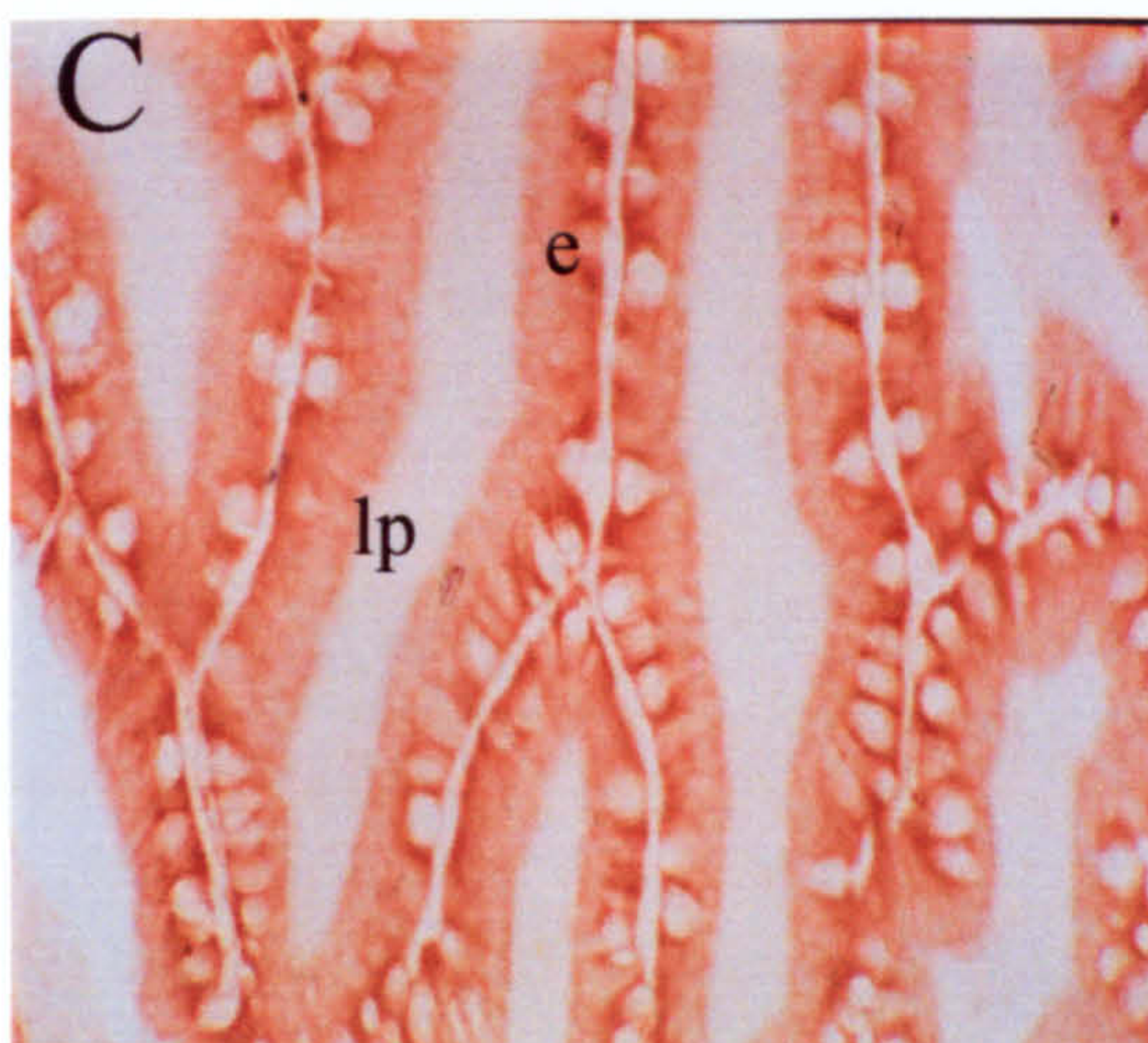
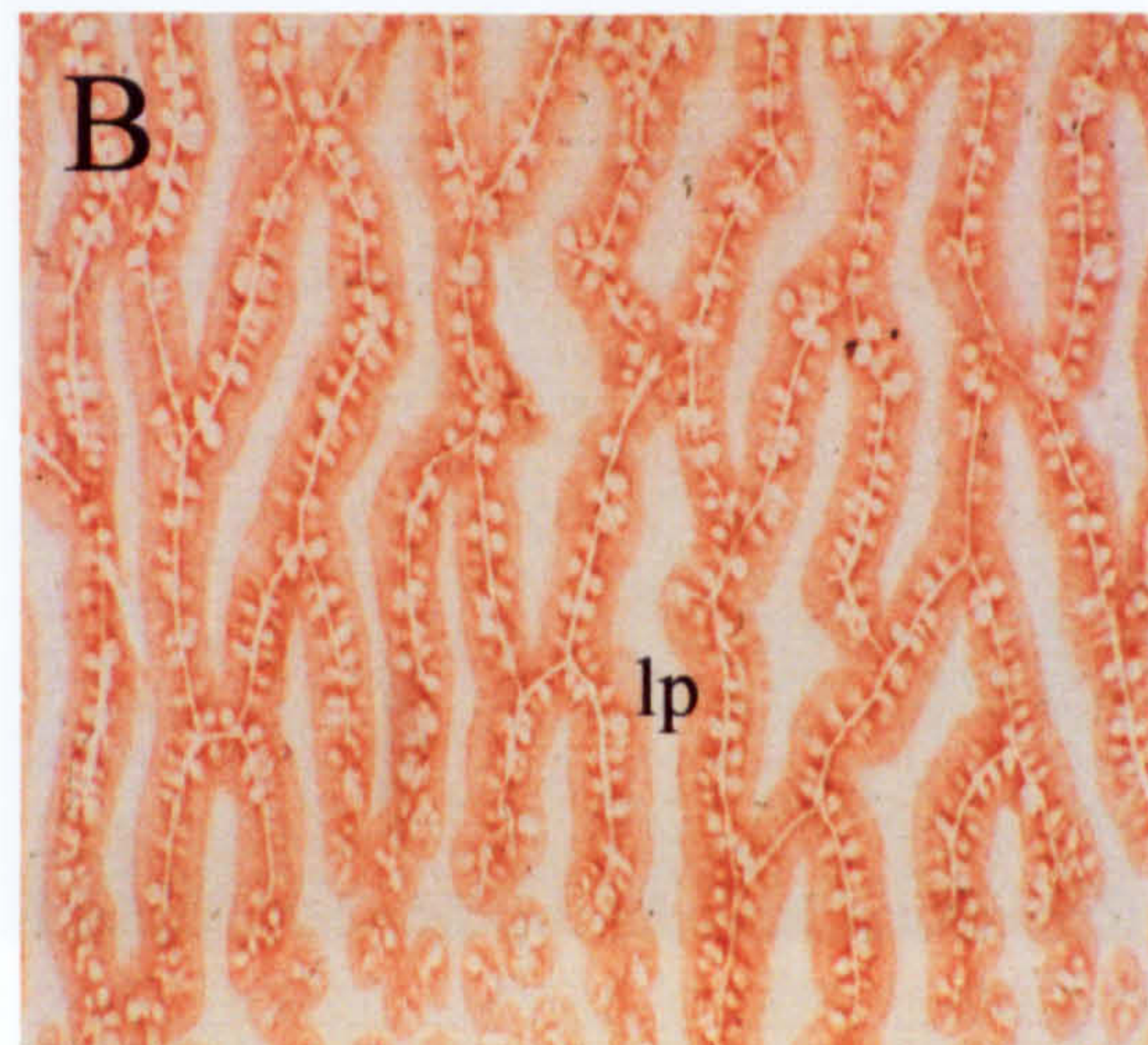
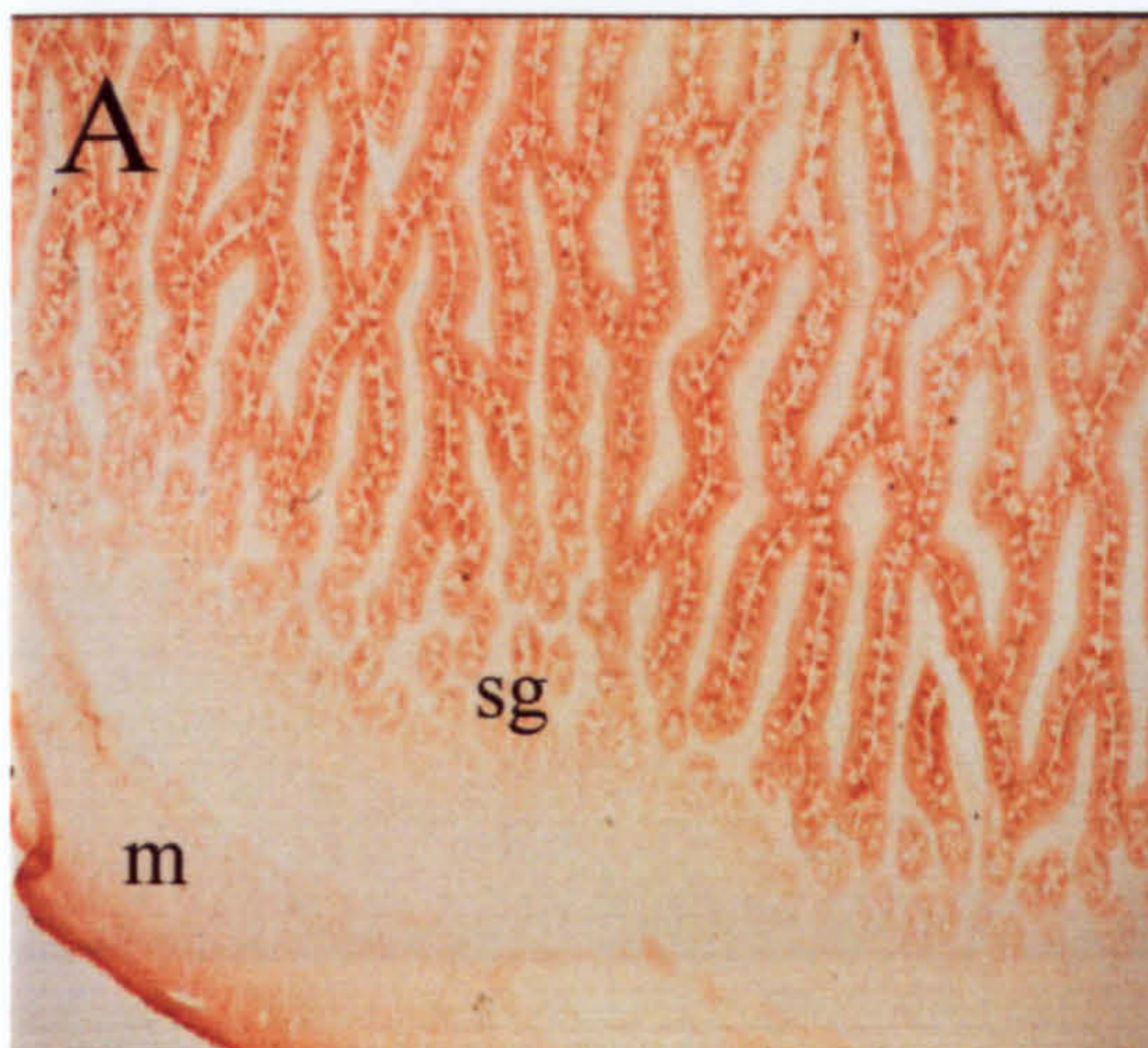


Figure 3.8. Localisation of BuChE activity in the intestine of infected rats. Sections were stained by the method of Karnovsky and Roots (1964), and BuChE activity was determined by pre-incubation with the AChE inhibitor BW284C51 (10^{-4} M). BuChE activity was localised to the epithelium (e), the external muscle (m), and the secretory glands (sg). No activity was observed in the lamina propria (lp). Magnification was x80 (A), x125 (B), and x312 (C-D).

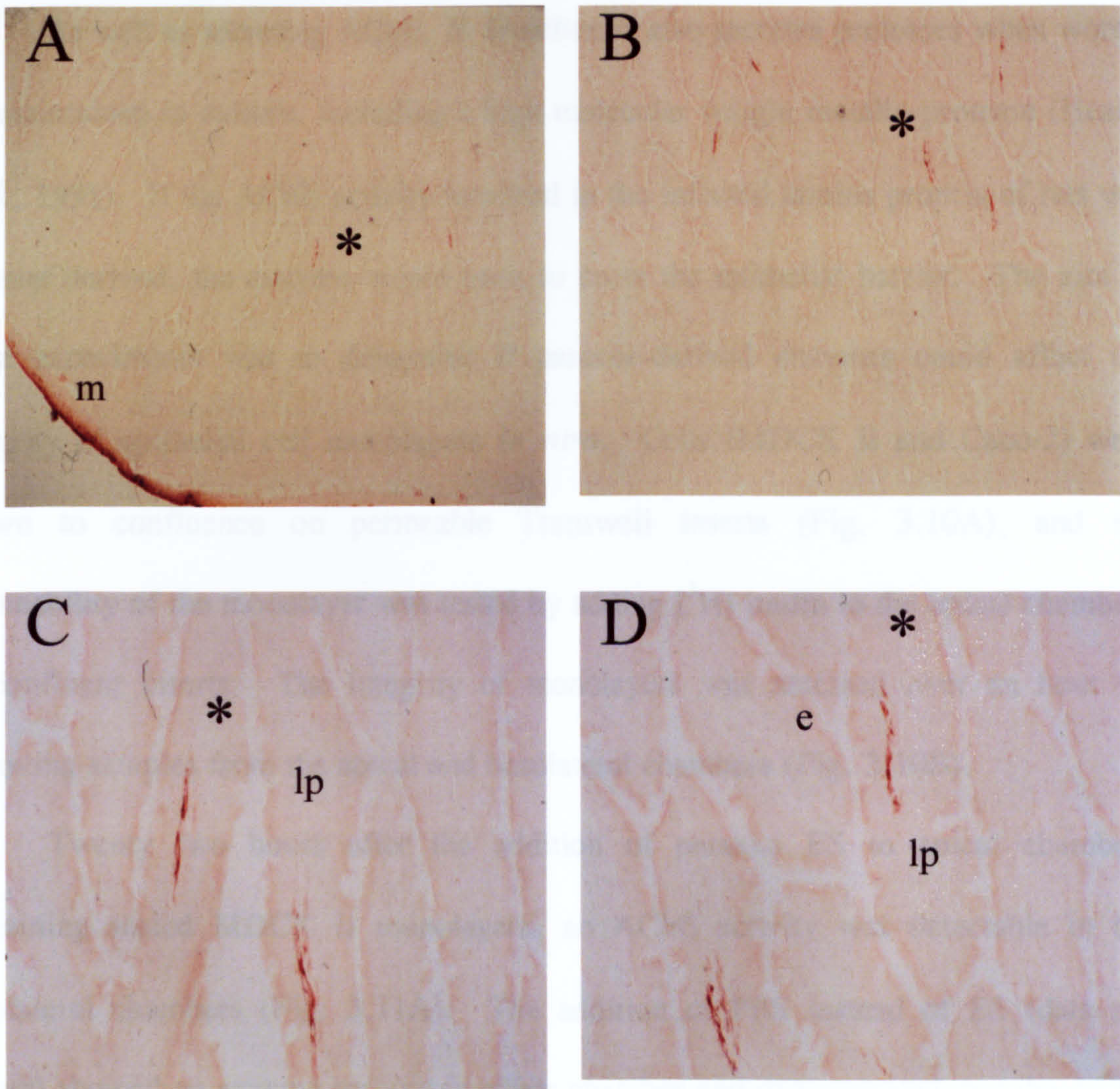


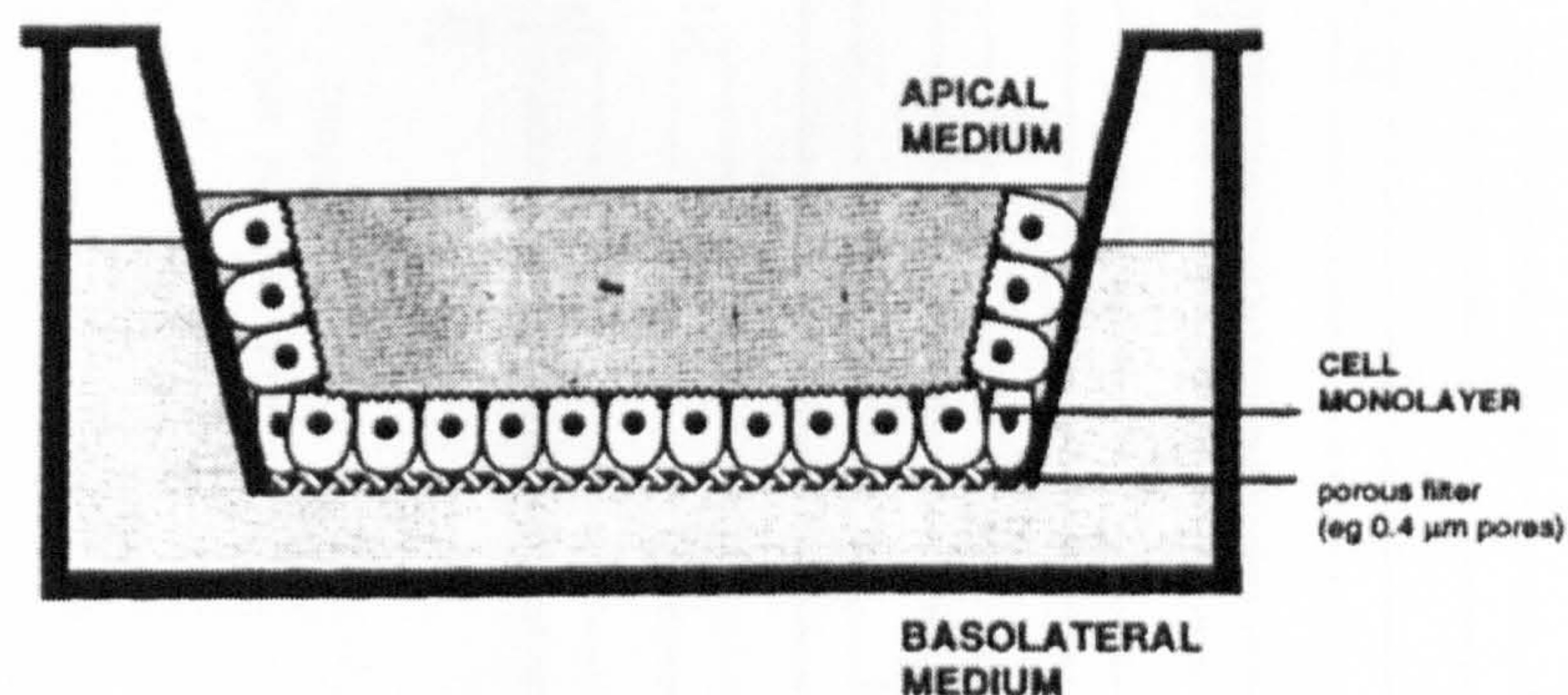
Figure 3.9. Localisation of AChE activity in the intestine of *N. brasiliensis*-infected rat. Intestinal sections were stained by the method of Karnovsky and Roots (1964), with AChE activity determined by pre-incubation with the BuChE inhibitor iso-OMPA (10^{-4} M). Focal activity was observed (*) in the lamina propria (lp), closely apposed to the basement membrane of the epithelium (e). Magnification x80 (A), x125 (B), and x312 (C-D).

3.4 The effects of parasite ES on epithelial monolayer integrity

As well as secreting AChE, *N. brasiliensis* also secretes proteases when worms are maintained in culture, including a high molecular weight metallo-protease (Healer *et al.*, 1991). If the AChE activity localised in the infected lamina propria of rats was parasite derived, the enzyme would have to cross the epithelial barrier. The aim of these experiments was to determine if parasite-derived enzymes could affect the integrity of epithelial cell monolayers *in vitro*. Cells (MDCK II and Caco-2) were grown to confluence on permeable Transwell inserts (Fig. 3.10A), and the permeability of the monolayer was tested by adding [³H] inulin to the apical chambers of confluent inserts. The integrity of monolayers was assessed over an hour by removing samples from the apical and basolateral chambers (Fig. 3.10B).

Twenty two hours after the addition of parasite ES to apical chambers containing sealed MDCK II monolayers, no AChE activity was detectable in the basolateral chambers (Fig. 3.11A). The addition of PBS instead of ES (data not shown) showed no enzyme activity in either chamber and demonstrated that the AChE assay (Ellman *et al.*, 1961) did not react with components of the cell culture medium. In Transwell inserts containing no cells, enzyme activity in the basolateral chamber rose steadily over the time course studied (Fig. 3.11B). Addition of parasite ES to the apical chambers of confluent Caco-2 cell monolayers resulted in no detectable enzyme activity in the basolateral chamber (Fig. 3.12A). Addition of ES to the basolateral chambers resulted in no enzyme activity detectable in the apical chambers.

A



B

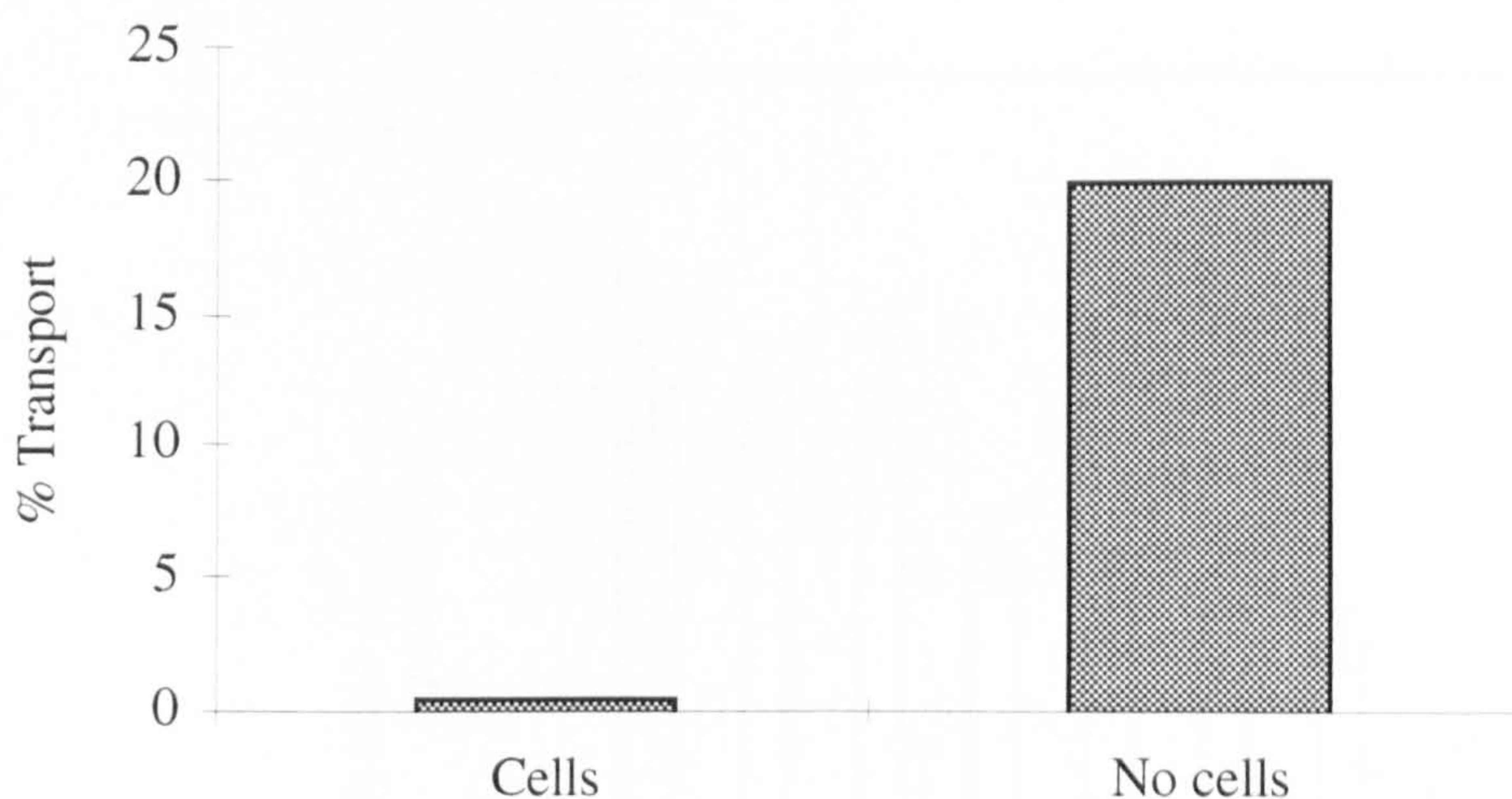


Figure 3.10. Determination of the permeability of epithelial cell monolayers. Panel (A) shows a diagrammatic representation of cells grown on Transwell filters. Panel (B) shows representative data, with results expressed as the mean. Monolayer permeability was determined by the inulin assay. [^3H]-inulin was added to the apical chambers of inserts containing cells, with samples taken from each chamber and counted. Cell monolayers were assessed as tightly sealed when less than 0.5% of the initial [^3H]-inulin could be detected in the basolateral chamber.

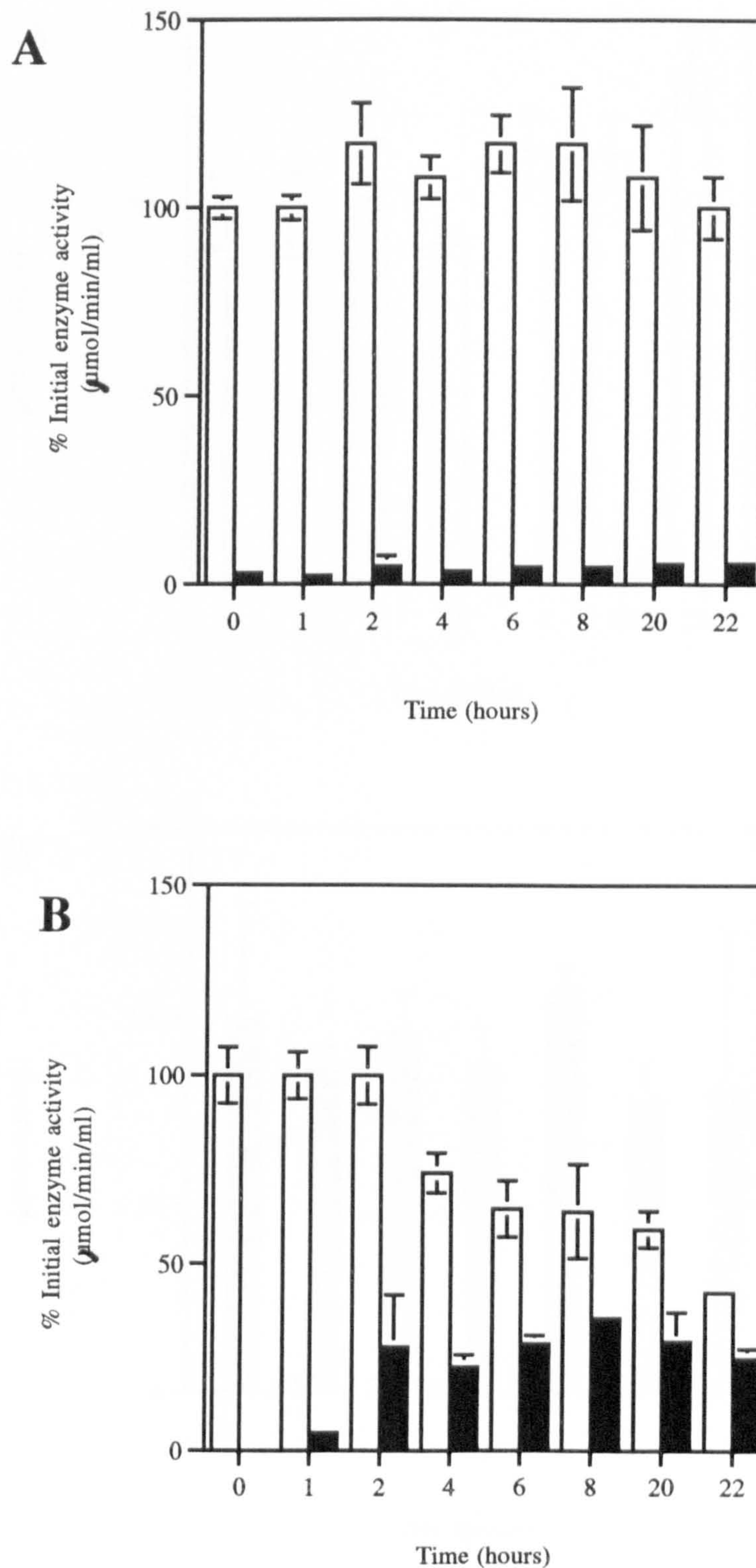


Figure 3.11. The effect of parasite secreted enzymes on MDCK II monolayer integrity. Parasite ES was added to the apical chambers of Transwell inserts containing tightly sealed MDCK II cells (A) or Transwells without cells (B). Enzyme activity was determined in the apical (open bar) and basolateral (closed bar) chambers and results are expressed as the mean $\pm$ standard deviation of triplicate assays at each time point.

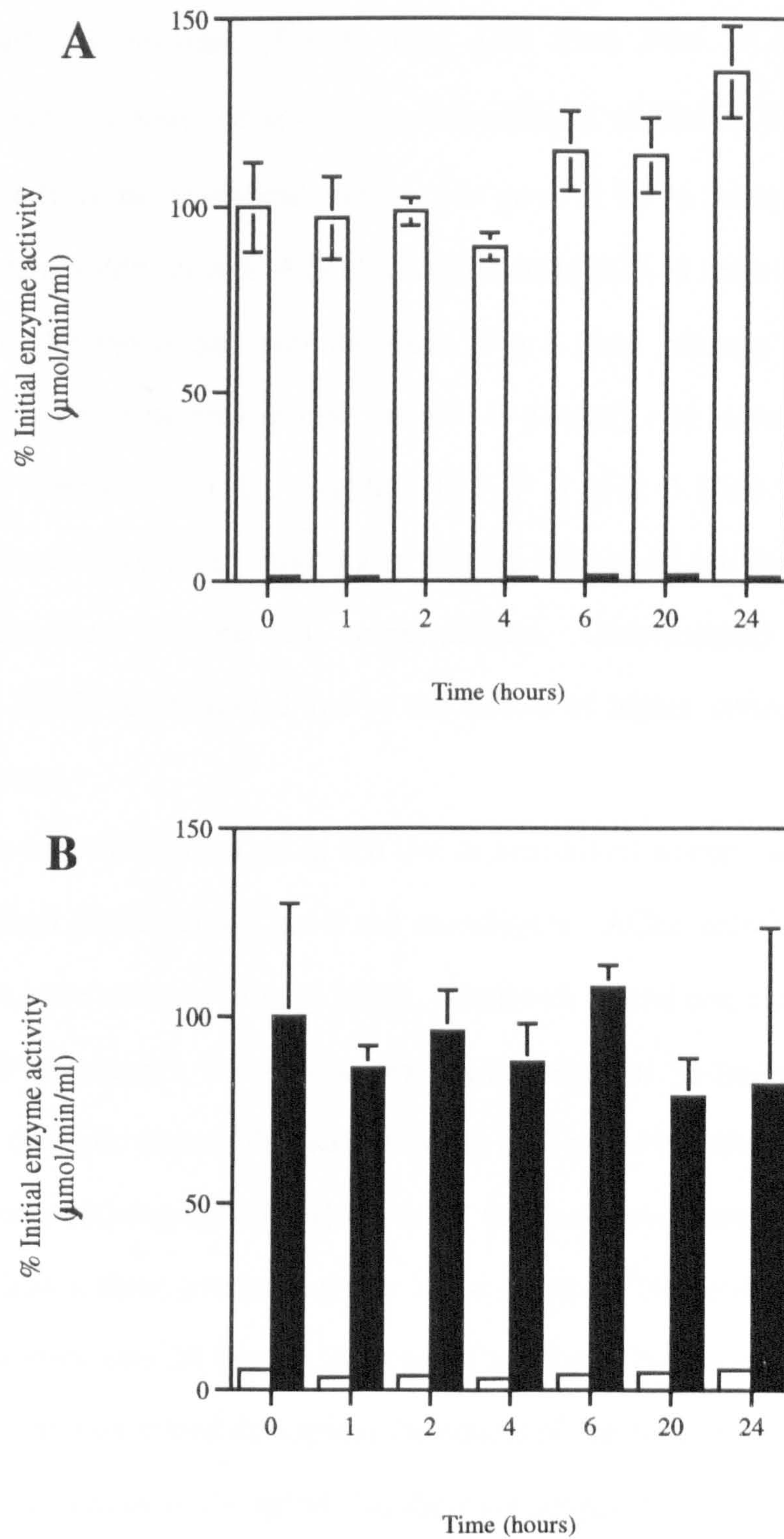


Figure 3.12. The effect of parasite secreted enzymes on Caco-2 monolayer integrity. Parasite ES was added to the apical (A) or basolateral (B) chambers of Transwell inserts containing tightly sealed Caco-2 cells. Enzyme activity was determined in the apical (open bar) and basolateral (closed bar) chambers, with results expressed as the mean $\pm$ standard deviation of triplicate assays at each time point.

Experiments to investigate the effects of rat mast cell protease II (RMCP II) and sheep mast cell protease (SMCP; kind gifts from Prof. H.R.P Miller) on epithelial permeability were carried out, by the addition of RMCP II (10 µg/ml) or SMCP (50 µg/ml) to the basolateral medium and parasite ES to the apical medium of Transwell inserts. Addition of RMCP II to confluent MDCK II cells had no effect on the integrity of the monolayer over 24 hours (Fig 3.13A) whereas the addition of parasite ES to inserts containing no cells resulted in a steady rise in enzyme activity in the basolateral chamber (3.13B). Adding RMCP II (Fig 3.14A) or SMCP (Fig 3.14B) to confluent Caco-2 cell monolayers resulted in enzyme activity being retained in the apical chambers over the time course studied. Unfortunately the supplies of RMCP II and SMCP were limited and so the effects of higher concentrations could not be investigated.

Further studies were carried out using 100 live or heat-killed worms, which were added to the apical chambers of Caco-2 cell monolayers. AChE activity and the ³H-inulin transport were assessed over 24 hours. Transwell inserts containing live worms but lacking cell monolayers, show a sharp rise in the levels of ³H-Inulin detected in the basolateral chamber and equilibration between both chambers after 24 hours (Fig. 3.15A). Inserts containing tightly sealed Caco-2 cell monolayers and live or heat-killed worms show a slow, gradual increase in the levels of ³H-Inulin detected in the basolateral chambers after 24 hours. However, Caco-2 cell monolayers containing no worms remained tightly sealed throughout the course of the experiments (Fig. 3.15). AChE levels rose steadily in the apical chambers containing live worms (Table 3.2), with a slight increase also detected in the apical chambers containing heat-killed worms. The basolateral chambers from inserts containing live or heat-killed worms demonstrated a small rise in AChE activity, which at 24 hours was 3- and 6-times lower than the activity detected in the basolateral chambers of inserts containing no cells.

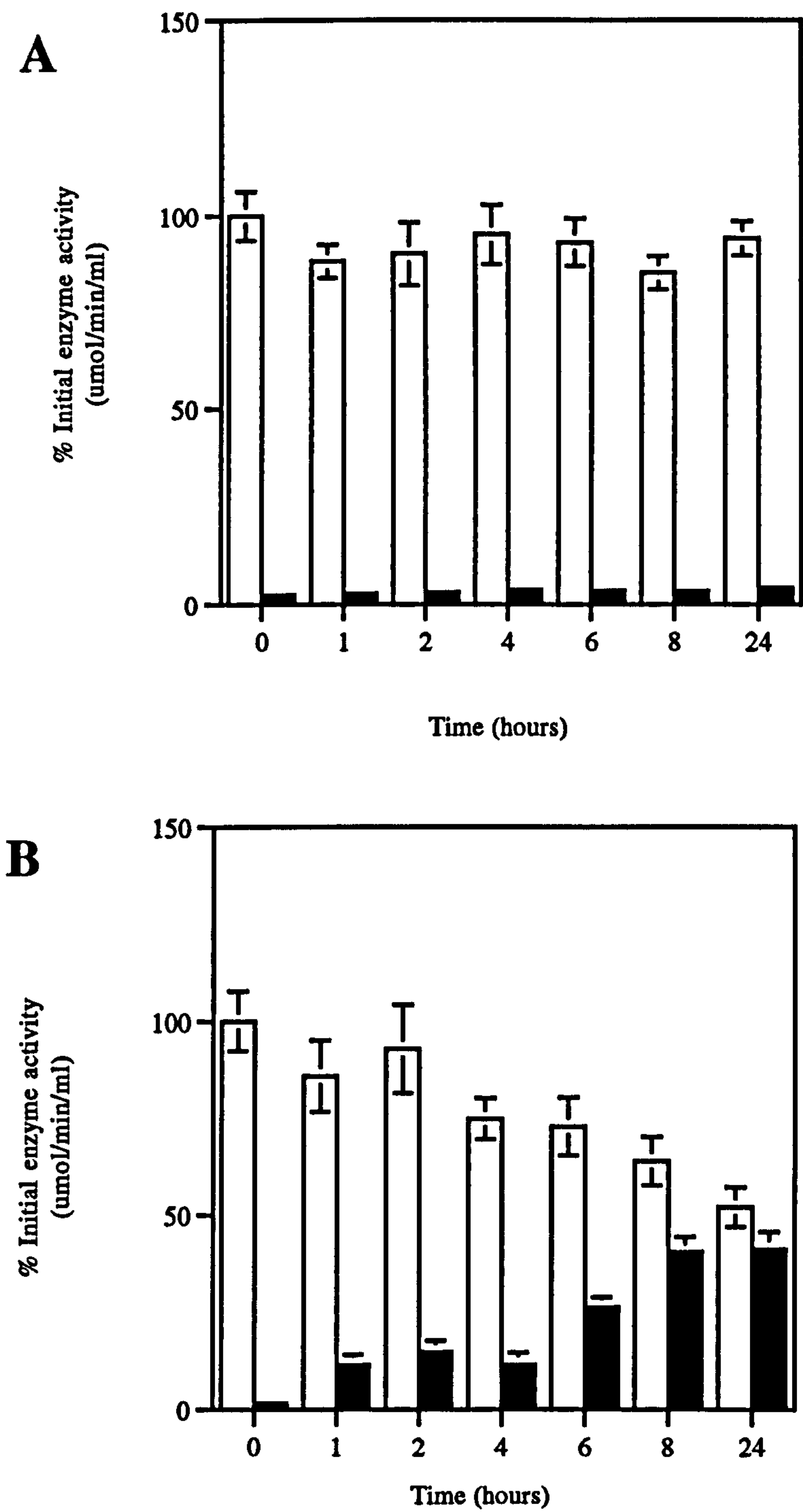


Figure 3.13. The effects of parasite ES and RMCP II on MDCK II monolayer integrity. Parasite ES was added to the apical chambers and RMCP II to basolateral chambers of tightly sealed monolayers (A). Parasite ES was also added to the apical chambers of inserts containing no cells (B). Enzyme activity was determined in the apical (open bar) and basolateral (closed bar) chambers, with results expressed as the mean $\pm$ standard deviation of triplicate assays at each time point.

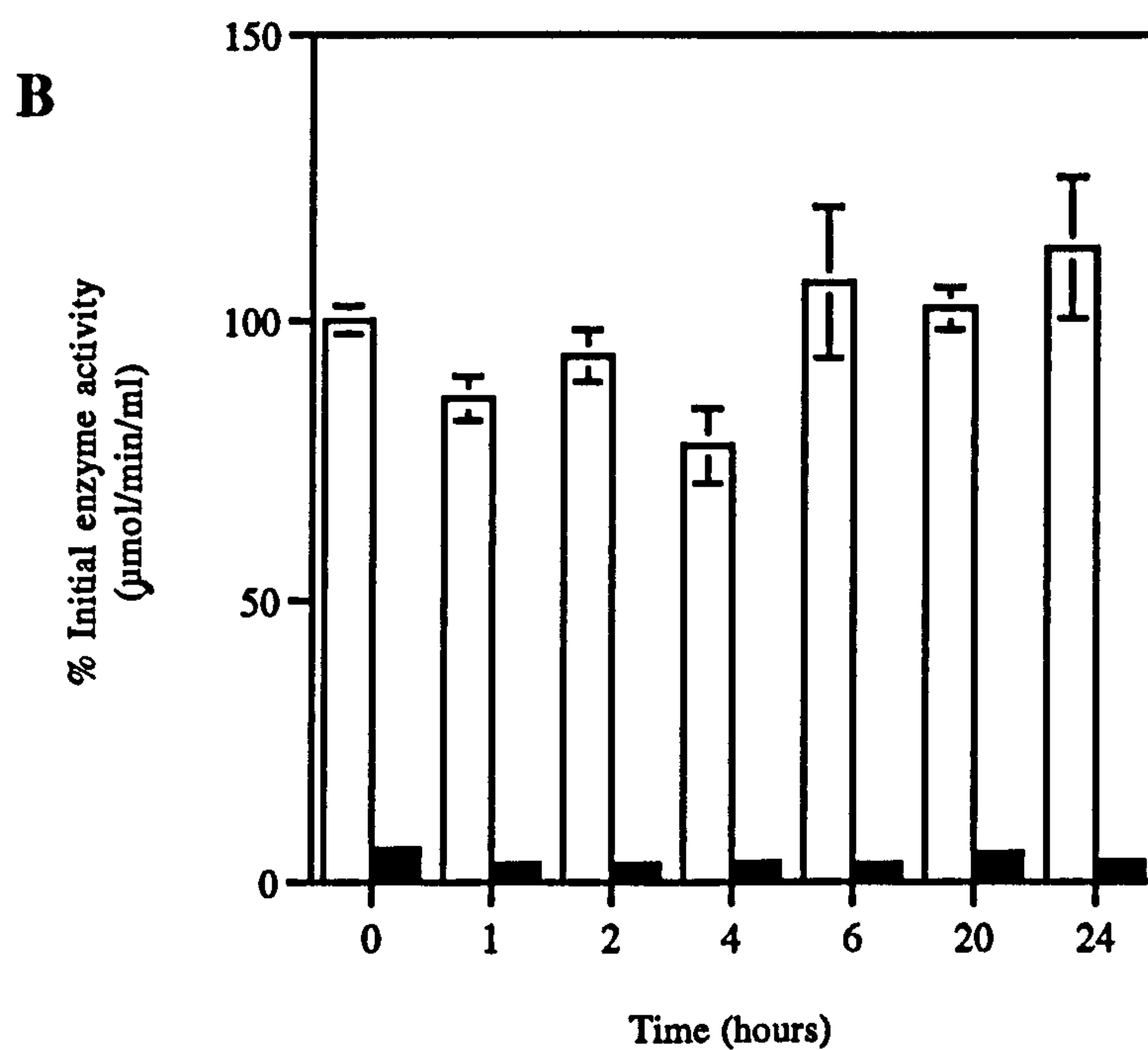
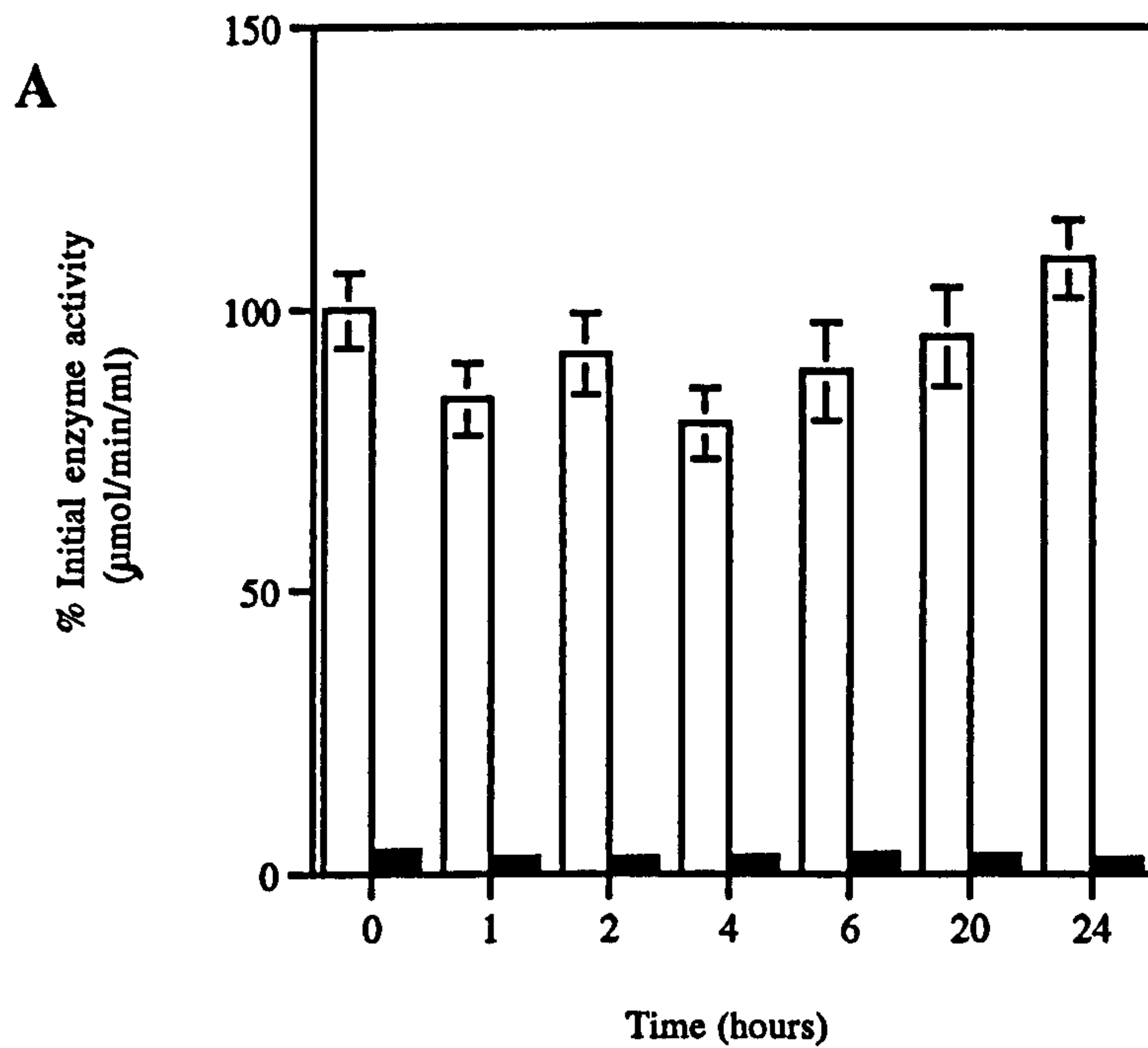


Figure 3.14. The effects of parasite ES on Caco-2 cell monolayer integrity. Parasite ES was added to apical chambers and RMCP II (A) or SMCP (B) was added to basolateral chambers. Enzyme activity was determined in the apical (open bar) and basolateral (closed bar) chambers and results were expressed as the mean $\pm$ standard deviation of triplicate assays at each time point.

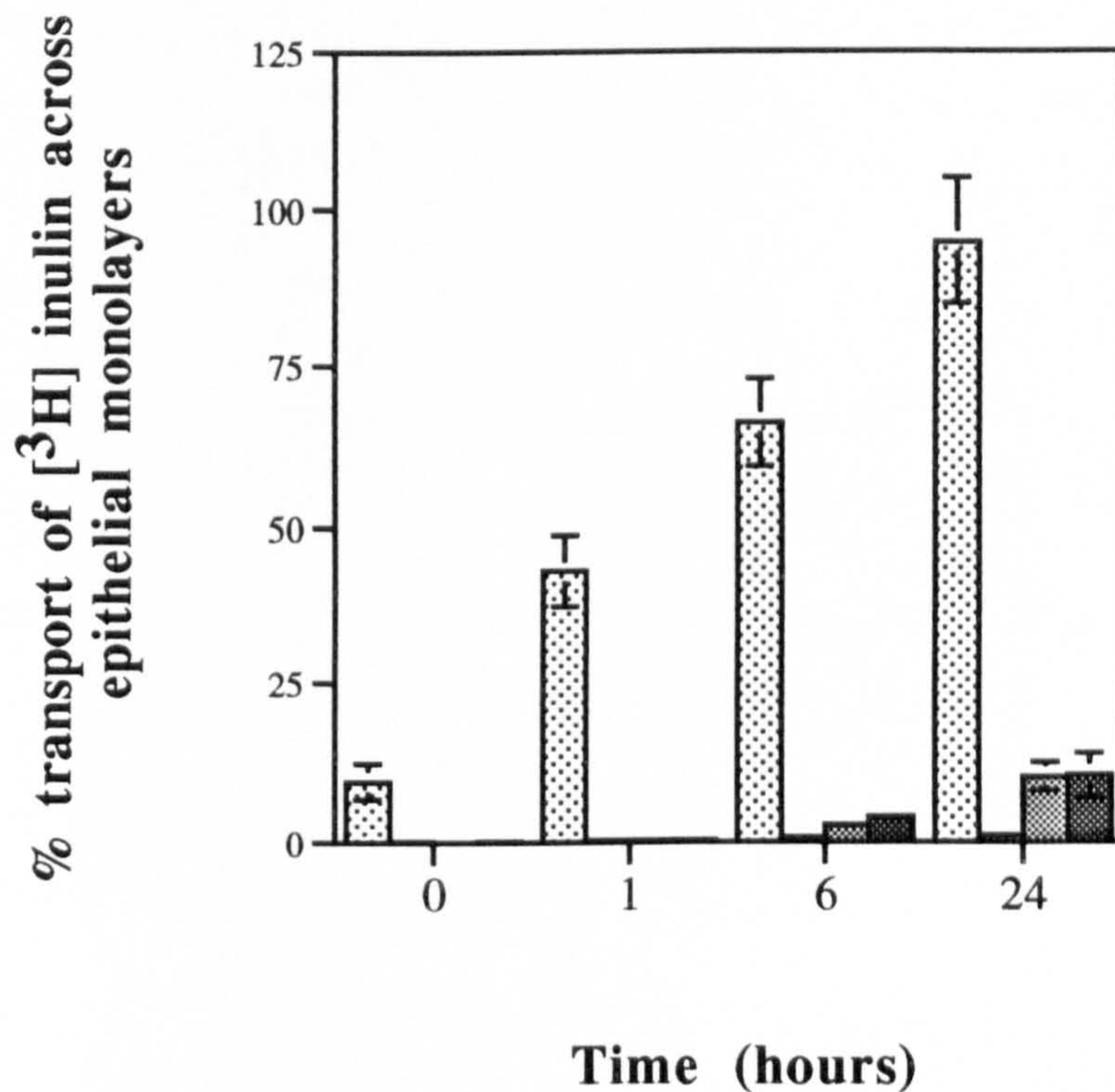
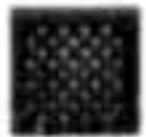


Figure 3.15. The effects of *N. brasiliensis* on Caco-2 cell monolayer integrity. Live or heat-killed worms were added to the apical chambers of tightly sealed monolayers containing [^3H] inulin. Samples were taken over 24 hours from both chambers and counted. Monolayer permeability was expressed as a percentage of the initial activity in the apical chamber. Controls consisted of apical chambers with live worms in the absence of Caco-2 cells. Results were expressed as the mean $\pm$ standard deviation of triplicate assays.

-  Inserts containing no cells
-  Confluent Caco-2 cells, no worms
-  Confluent Caco-2 cells, live worms
-  Confluent Caco-2 cells, dead worms

Incubation Time (hr)	Live worms		Dead worms		Control (live worms without cells)	
	Apical	Basolateral	Apical	Basolateral	Apical	Basolateral
1	6.4 ± 3.1	0.8 ± 0.3	3.4 ± 0.4	0.6 ± 0.1	3.2 ± 0.4	0.8 ± 0.1
6	7.8 ± 2.2	1.0 ± 0.4	3.8 ± 0.7	0.3 ± 0.2	3.1 ± 0.6	1.2 ± 0.5
24	31.2 ± 13.7	2.6 ± 1.2	8.5 ± 3.4	1.4 ± 0.2	39.6 ± 16.2	8.6 ± 3.2

Table 3.2 The effect of parasites on Caco-2 cell monolayer permeability.

AChE was detected by the method of Ellman (1961), with enzyme activity expressed as nmol/min/ml. The results are expressed as the means ± stardard deviation of triplicate assays at each time point.

3.5 Discussion

The secretion of AChE by parasitic nematodes *in vivo* has been speculated upon for many years. It has been demonstrated that several species of gastrointestinal nematode, along with some non-gut dwelling parasites, secrete high levels of AChE into culture medium (Ogilvie *et al.*, 1973; McKeand *et al.*, 1994; Rhoads, 1981), and that the gut dwelling nematodes have evolved highly specialised glands that store and secrete the enzyme (Bremner *et al.*, 1973; Lee, 1970; McLaren *et al.*, 1974; Nakazawa *et al.*, 1995; Rothwell *et al.*, 1973). It has been observed that these enzymes are highly immunogenic to their host (Bremner *et al.*, 1973; Brown & Pritchard, 1993; McKeand *et al.*, 1993; Rothwell *et al.*, 1973), and that purified or partially purified enzyme may confer a degree of immunity when used to immunise animals (Griffiths & Pritchard, 1994; McKeand *et al.*, 1995). Results presented in this chapter demonstrate an AChE activity within the intestinal flushes of parasitised animals that when analysed by non-denaturing polyacrylamide gel electrophoresis, migrated at a similar rate to parasite AChEs isolated from *in vitro* culture. The enzyme activity was only observed at time points where the parasite was resident within the intestine, with no AChE present in the uninfected gut flushes, and very faint staining found on day 3 p.i (a time where the majority of the worms are immature L4s, in transit from the lungs to the intestine). It can therefore be concluded that *N. brasiliensis* secretes high levels of AChE *in vivo* as well as *in vitro*.

Putative roles for parasite secreted AChEs include modulation of the immune responses (Rhoads, 1984), and the inhibition of secretory responses within the intestine (Selkirk *et al.*, 1998). It has been demonstrated that ACh enhances the release of lysosomal enzymes from polymorphonuclear leukocytes and neutrophils during uptake of

immune complexes (Ignarro & Colombo, 1973; Ignarro & George, 1974), enhances phagocytosis and chemotaxis by neutrophils (Hill *et al.*, 1975; Ignarro & Cech, 1976), and stimulates lymphocyte-mediated antibody-dependent cellular cytotoxicity (ADCC; Gale & Zigelboim, 1974). ACh and AChR agonists have also been shown to stimulate fluid and mucus secretion from enterocytes and goblet cells respectively (Bradbury *et al.*, 1980; Cooke, 1984). Therefore, the hydrolysis of ACh may reduce inflammation and down-regulate the secretory mechanisms that are thought to play a role in parasite removal. For parasite AChEs to have any effect on host immunological/physiological responses they would be required to access the mucosa where they would be in intimate contact with their target cells.

Sine and co-workers (Sine *et al.* 1988, 1989, 1990, 1991, 1992) have extensively examined the ChEs expressed in intestinal tissue of various mammalian species. Most species contain either AChE or BuChE, with the exception of human intestine which possesses both (Sine *et al.*, 1991). The expression of BuChEs in the rat intestine was first demonstrated using the method of Karnovsky and Roots (1964), and subsequent analysis showed the enzyme to be a class II, amphiphilic dimer (Sine *et al.*, 1992). The localisation of BuChE activity in normal uninfected animals demonstrated in this chapter were in agreement with those results observed in previous studies (Karnovsky & Roots, 1964; Sine *et al.*; 1988; 1992). High levels of BuChE activity were found in the intestinal epithelial cells, secretory glands and the external muscle layers, with some AChE activity also demonstrated in the external muscle coat. In stark contrast, the lamina propria of infected animals contained focal AChE activity, which suggests that parasite secreted AChE gains access to the intestinal mucosa. Jones and Knox (1990) observed AChE activity in mucosal homogenates from sheep infected with *T. colubriformis*. Isolated

AChEs from adult *T. colubriformis* homogenates resolved into 5 isoforms when analysed by gel electrophoresis and enzyme staining. Two of these isoforms were present in the mucosal homogenates of *T. colubriformis*-infected sheep and absent from worm-free controls. These results further increase the possibility that parasite secreted AChEs are able to enter the intestinal mucosa and perturb cholinergic stimulation.

Another possible explanation is that this enzyme activity was host derived. *N. brasiliensis* infection leads to marked inflammation within the intestine, leading to degeneration and further remodeling of the nerves that innervate this tissue. During this inflammatory response, staining for the neuronal marker neuron specific enolase (NSE) was observed to be diffuse, suggesting that NSE was leaking out of damaged nerves (Stead *et al.*, 1991). One could therefore speculate that during the inflammatory response within the intestine cholinergic nerve fibres are damaged, resulting in the diffusion of AChE into the local environment. However, preliminary studies determined that animals infected with *T. spiralis* showed no increase in AChE activity within the mucosa, and a significant reduction in enzyme activity within the muscle layers (Masella *et al.*, 1995). Further work by Palmer and Koch (1995) observed that AChE activity within the outer muscle layers was reduced from day 6 p.i., returning to normal levels by day 30 p.i., and that the levels of ChAT were increased significantly at 30 days p.i. As well as inducing changes in the cholinergic enzymes, infection with *T. spiralis* also resulted in impaired ACh release from rat myenteric plexus preparations. It was demonstrated that suppression of ACh release was dependent on the inflammatory response, but was T-cell independent (Collins *et al.*, 1992). Masson and colleagues (1996) studied the neuronal alterations in rats infected with *N. brasiliensis* and their subsequent effects on intestinal fluid secretion. It was shown that during parasitic

infection cholinergic nerve function was defective in stimulating fluid secretion, leading to the induction of non-cholinergic compensatory mechanisms. The secretory responses returned to control levels by day 35 p.i., but the cholinergic component was still absent. The possibility of the epithelial cells becoming insensitive to ACh was ruled out by the addition of exogenous cholinergic agonists, which resulted in a secretory response comparable to uninfected controls (Masson *et al.*, 1996). Concomitant with these changes in cholinergic function were the increases in substance P reactive-nerves and substance P content within the mucosa. Substance P is a pro-inflammatory neuropeptide that has a number of effects on the immune system, and has been demonstrated to induce fluid secretion from rat intestinal segments (Wang *et al.*, 1995). It can be concluded that inflammatory and neuronal responses initiated against intestinal nematodes are complex, where the 'switching off' of the cholinergic component possibly acts as a braking mechanism to prevent the deleterious effects of continuous fluid secretion.

As discussed earlier, for parasite AChEs to enter the intestinal mucosa they must traverse the epithelial barrier. A simplistic view of the intestinal epithelium is as a sheet of cells, which are joined together at their latero-apical interfaces by junctional complexes. These junctional complexes include tight junctions (zona occludens; ZO), belt desmosomes (zona adherens), desmosomes and gap junctions. One of the functions of these inter- and intracellular structures is to regulate the passage of molecules between the apical and basolateral domains of the epithelium (Farquhar & Palade, 1963). The ZO is considered to act as a permeability barrier between the two domains, and at these zones adjacent cells are at their closest apposition (Stevenson *et al.*, 1988). The transport of electrolytes and non-electrolytes between the two compartments can occur via one of two pathways. In the paracellular pathway, solutes cross the tight junctional

complexes, diffuse through the intercellular space, traverse the basement membrane and enter the bloodstream. Transcellular transport involves diffusion through the glycocalyx that covers the apical membrane and active transport into, and through the cell (Lewis *et al.*, 1995).

Unsurprisingly, the transport proteins and junctional complexes of the small intestine are resistant to the actions of pancreatic proteases. This was confirmed by Azghani *et al.* (1993), who observed that elastases isolated from porcine pancreas and human leukocytes had no effect on the permeability of confluent MDCK cell monolayers. However, elastases purified from the bacterium *Pseudomonas aeruginosa* caused an increase in membrane permeability, with no marked morphological changes to the cells. Immunocytochemistry revealed a decrease in levels of the ZO protein ZO-1. Bacteria have proved useful in the study of epithelial function, with the diverse array of toxins they secrete able to compromise the epithelium by several mechanisms. These included the release of toxins which detach epithelial sheets, damage to cell membranes which increases cellular permeability, traversing the membrane and killing cells (Lewis *et al.*, 1995).

The results presented in this chapter demonstrated that parasite secreted ES, which contained AChE and several other proteases (Healer *et al.*, 1991), were unable to disrupt the tightly sealed monolayer formed by MDCK II and Caco-2 cells. It is therefore more likely that transport of AChE across the epithelium would be as a result of increased epithelial permeability induced by the inflammatory responses to the parasite. There was a marked increase in mucosal permeability in the small intestine of animals infected with *N. brasiliensis*, and a correlation between worm burden and the degree of epithelial permeability (Nawa, 1979). An increase in intestinal permeability

was first observed 8 days p.i.. One of the inflammatory mediators released during anaphylaxis is RMCP II, which can be detected in the systemic circulation during primary infection with *N. brasiliensis*, and is also released in the lumen of the intestine indicating a breakdown in the integrity of the epithelial barrier. The addition of RMCP II to the basolateral chamber of Transwell inserts containing MDCK II cells had no effect on permeability of the monolayer to AChE. Unfortunately the supply of RMCP II was limited, and so experiments to determine the effects of higher doses to the monolayer were not studied. Scudamore and colleagues (Scudamore *et al.*, 1995a & b) used an *ex vivo* perfusion model, where segments of rat jejunum were ligated and perfused through the cranial mesenteric artery, and assessed the role of RMCP II during anaphylaxis. They observed a 10-fold increase in the translocation of RMCP II into the jejunal lumen after 2 minutes of challenge using *N. brasiliensis* antigen in sensitised animals (Scudamore *et al.*, 1995a). The translocation of RMCP II was associated with macromolecular leakage into the lumen, with no significant alteration in mucosal histology, suggesting that RMCP II increases epithelial permeability via a paracellular route (Scudamore *et al.*, 1995a & b).

The effects of cytokines on the permeability of intestinal epithelial monolayers have also been studied. Madara and Stafford (1989) demonstrated that IFN- γ induced a decrease in the tight junctional resistance of the human colonic carcinoma cell line (T84), which was both time- and dose-dependent. Work by Adams *et al.* (1993) observed that this response was specific for IFN- γ , with IFN- α having no effect on cell permeability, and only addition of IFN- γ to the basolateral side resulted in increased permeability. More interesting were the observations that both IL-4 and IL-13 disrupt the barrier function of T84 cell monolayers, and these effects were shown to be dose dependent

(Zünd *et al.*, 1996). High levels of both these cytokines are produced during *N. brasiliensis* infection of mice, with IL-13 required for parasite elimination (Barner *et al.*, 1998; McKenzie *et al.*, 1998; Urban *et al.*, 1998). It is therefore inviting to speculate that the host immune response disrupts the epithelial barrier, allowing the passage of parasite secreted enzymes into the host mucosa.

CHAPTER 4

Identification of muscarinic acetylcholine receptors in the gastrointestinal tract

4.1 Introduction

As discussed in the previous chapter, putative roles for parasite secreted AChEs include modulation of the immune (Rhoads, 1984) and the secretory responses within the intestine (Selkirk *et al.*, 1998), whereby the hydrolysis of ACh would prevent the engagement of, and subsequent stimulation of AChRs. The gastrointestinal tract is highly innervated by the nervous system, containing sympathetic, parasympathetic and enteric nerve fibres (Cooke, 1986). The enteric nervous system (ENS) represents a rapid signalling system which coordinates and programmes the appropriate effector responses throughout the entirety of the gut. The ENS can function independently of the central nervous system (CNS). However, sensory information is processed in the CNS and transmitted to the ENS through sympathetic and parasympathetic pathways via interfaces with enteric neurons (Cooke, 1994). The submucosal neurons of the intestinal tract are rich in cholinergic nerves, which can possess nicotinic and/or muscarinic ACh receptors (nAChR/mAChR; Cooke, 1986; Isaacs *et al.*, 1982). The nAChRs are thought to be associated with neuron/neuron interactions and have yet to be demonstrated on epithelial cells, whereas mAChRs have been observed throughout the intestinal mucosa and muscle layers (Goyal, 1988; Isaacs *et al.*, 1982), and have been localised to epithelial cells (Rimele *et al.*, 1981; Tien *et al.*, 1985).

Muscarinic AChRs are members of a large family of neurotransmitter, hormone and light receptors which contain seven transmembrane domains and transduce their intracellular signals via coupling to guanine nucleotide-binding regulatory proteins (G-proteins). Receptors were first separated into two classes of receptor, defined by high or low affinity for the antagonist pirenzepine, M1 and M2 respectively (Hammer *et al.*, 1980). Subsequent work identified further classes of receptor from binding studies in rat

cerebral cortex, myocardium and salivary gland, with the classification extended to M1, M2, and M3 (Doods *et al.*, 1987). The further development of muscarinic ligands, with selective affinities, has resulted in the classification of four pharmacological receptor subtypes (M1-M4). However, these ligands do not possess a high degree of selectivity for one subtype over any other, with receptor subtype identification performed by generating a rank order of affinity for a range of ligands (Caulfield, 1993; Eglen *et al.*, 1994). It is therefore difficult to identify receptor subtypes in tissue which contains heterogenous population of receptors, with the predominant subtype more likely to be determined. Molecular biological techniques have identified a family of mAChR genes (m1-m5), which have been cloned and encode distinct receptor subtypes (Kubo, *et al.*, 1986; Bonner *et al.*, 1987, 1988; Peralta *et al.*, 1987). Chinese hamster ovary cells (CHO-K1) have been stably transfected with the human mAChR genes (m1-m5), and used for antagonist binding studies in an attempt to correlate the pharmacological data with five receptor genes identified. It has been demonstrated that m2 and m3 receptors correlate well with the pharmacologically defined M2 and M3 subtypes (Peralta *et al.*, 1987; Buckley *et al.*, 1989). More recent work by Dörje and colleagues (1991a), using an extensive range of antagonists, demonstrated that although none of the antagonists showed high selectivity for one receptor over the others, each receptor had a unique antagonist binding profile. These studies also observed a general correlation between the cloned m2 and m3 receptors with the M2 and M3 subtypes defined pharmacologically, and both m1 and m4 receptors possessed similar binding profiles to the M1 subtype. The current nomenclature defines the pharmacologically derived M1-M4 subtypes correspond to those receptors genetically identified as m1-m4, with the m5 receptor gene having no equivalent pharmacological profile (Caulfield, 1993).

Rimele and colleagues (1981) provided the first direct evidence for the presence of mAChRs on intestinal epithelial cells, using the mAChR antagonist [3 H]-Quinuclidinyl benzilate ([3 H]-QNB). It was observed that receptor density was five-fold higher in rat colonic epithelial cells when compared to epithelial cells isolated from the ileum and jejunum. Tien and co-workers (1985) isolated epithelial and intestinal muscle cells from various regions of the rat small and large intestine, and compared their binding characteristics to [3 H]-QNB in the presence of a range of muscarinic agonists and antagonists. The inhibition constants obtained with the non-selective antagonist atropine were similar between muscle and epithelial cell preparations. However, the inhibition constants from muscle cell preparations using the antagonist pirenzepine were significantly higher than those obtained from epithelial cells. In contrast, the non-selective agonist oxotremorine (Oxo-M) produced lower inhibition constants in muscle cell preparations when compared to those obtained from epithelial cells. It was therefore suggested that the muscarinic binding sites of intestinal epithelial and the external muscle cells contained different populations of muscarinic receptors, with the epithelial cells thought to express the M1 receptor subtype. Further work by Kachur and co-workers (1990) observed that carbachol-induced electrolyte transport in guinea pig ileum was significantly reduced by the M3 antagonist, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), leading to the suggestion that M3 receptors mediated secretory responses in this system.

Although ligand binding and pharmacological studies have implied the presence of mAChRs within the intestinal tract, very little work has been published using other techniques to confirm these data. Several groups have reported the generation of antibodies raised against monoclonal antibodies, which have been used to determine and

localise receptor subtypes within the brain and some peripheral organs (André *et al.*, 1984; Levey *et al.*, 1993; Wall *et al.*, 1991b). Molecular biological techniques have also been developed to identify the presence of mRNAs for the receptor subtypes, however very few investigators have examined the expression of mAChR mRNA within the intestinal tract. In this study a range of techniques were used to identify, and localise the expression of mAChRs within uninfected and *N. brasiliensis*-infected intestine. This parasite secretes high levels of AChE *in vivo* and *in vitro*, and it therefore interesting to speculate that this enzyme would prevent cholinergic nerve stimulation within the host intestine and possibly interfere with the host immune response.

4.2 Localisation of mACh receptors in the intestine

In this study, mAChRs were localised in the intestine of uninfected and *N. brasiliensis*-infected rats using the monoclonal antibody M35, which was raised against purified calf forebrain mAChRs (André *et al.*, 1983, 1984). This antibody does not discriminate between receptor subtypes (m1-m5), and has been widely used in different species and tissues (Carsi-Gabrenas *et al.*, 1997).

In the jejunum of uninfected animals there was a high degree of mAChR-immunoreactivity within the external muscle layers, with strong binding also detected within the submucosa, possibly ganglia of the myenteric plexus (Fig 4.1). Some weaker staining was also observed in the lamina propria, which extended along the intestinal epithelium. Infected jejunum also exhibited a high degree of immunoreactivity within the external muscle layers, however in contrast to uninfected tissue, there was an increase in the degree of mAChR-positive staining in the lamina propria, with staining closely apposed to the epithelial basement membrane (Fig. 4.2).

Intestinal sections were also stained for the lymphocyte markers CD4 and CD8 in an attempt to identify the mAChR-positive cells within the lamina propria. CD4⁺ cells were localised within the lamina propria of uninfected jejunum (Fig. 4.3), with increased immunoreactivity observed in infected jejunum (Fig. 4.4). In uninfected intestine a small population of CD8⁺ cells were localised within the lamina propria, with cells in close apposition to the epithelium (Fig. 4.5), whereas infected jejunum displayed an increase in CD8⁺ cells throughout the lamina propria (Fig 4.6).

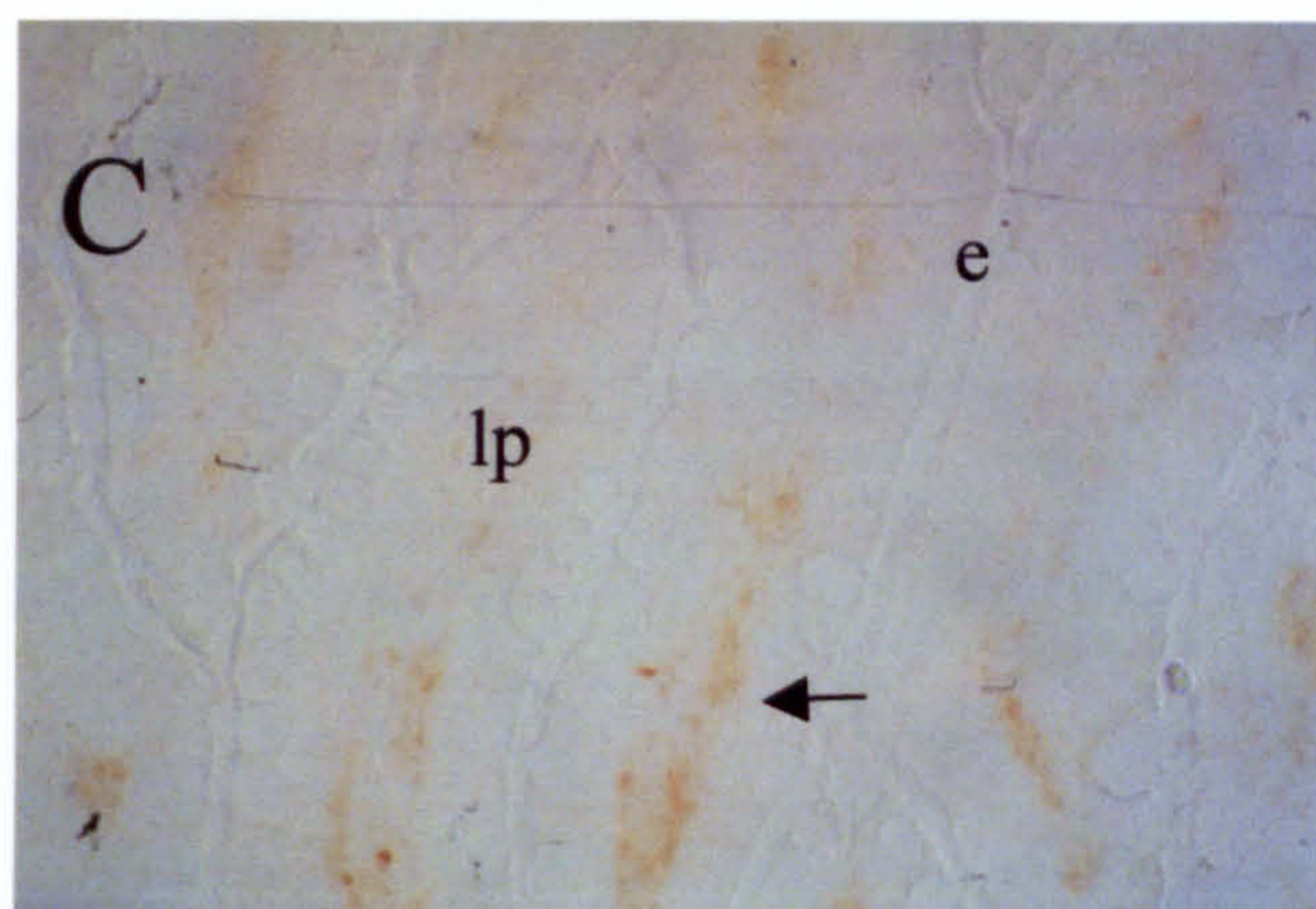
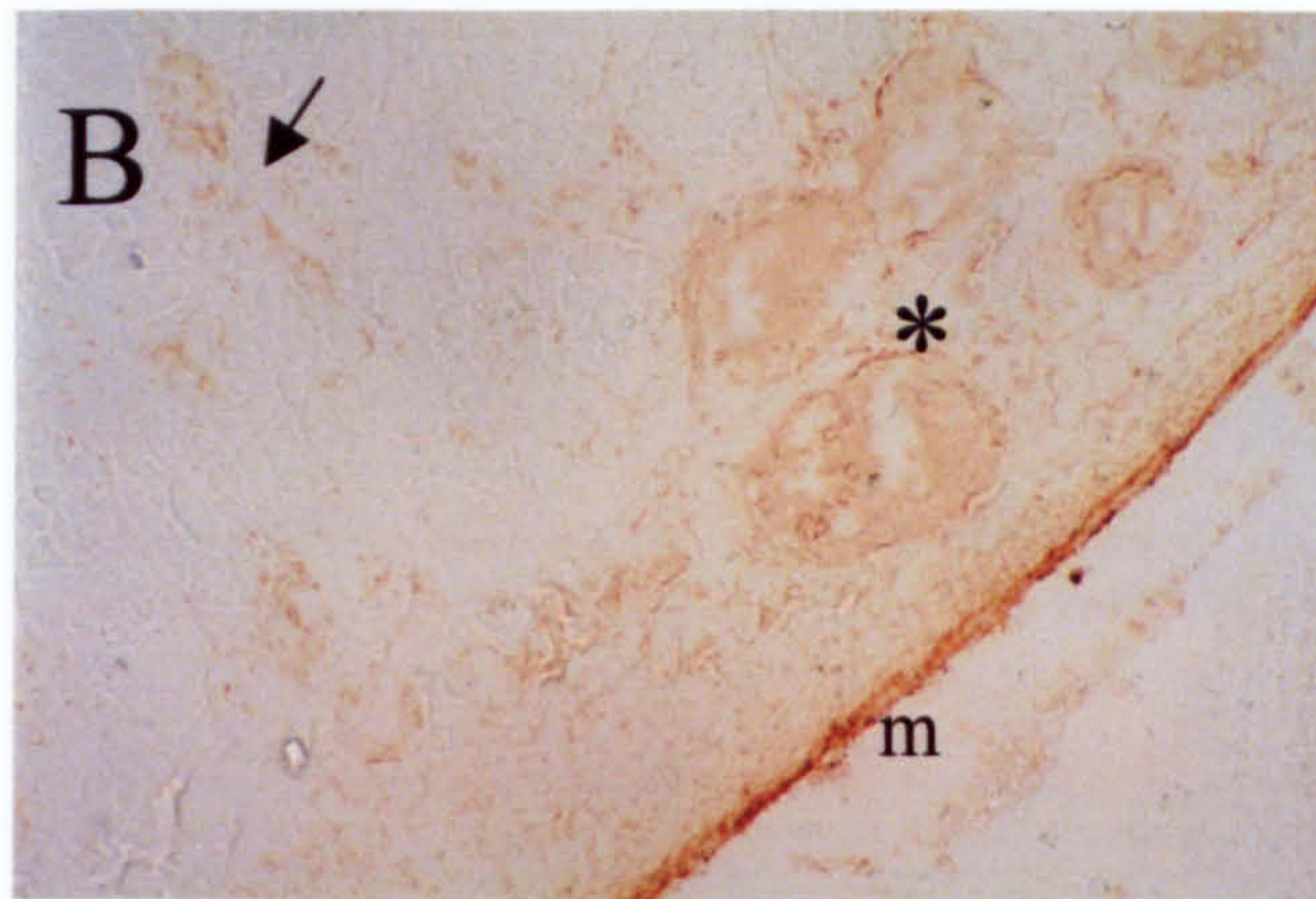
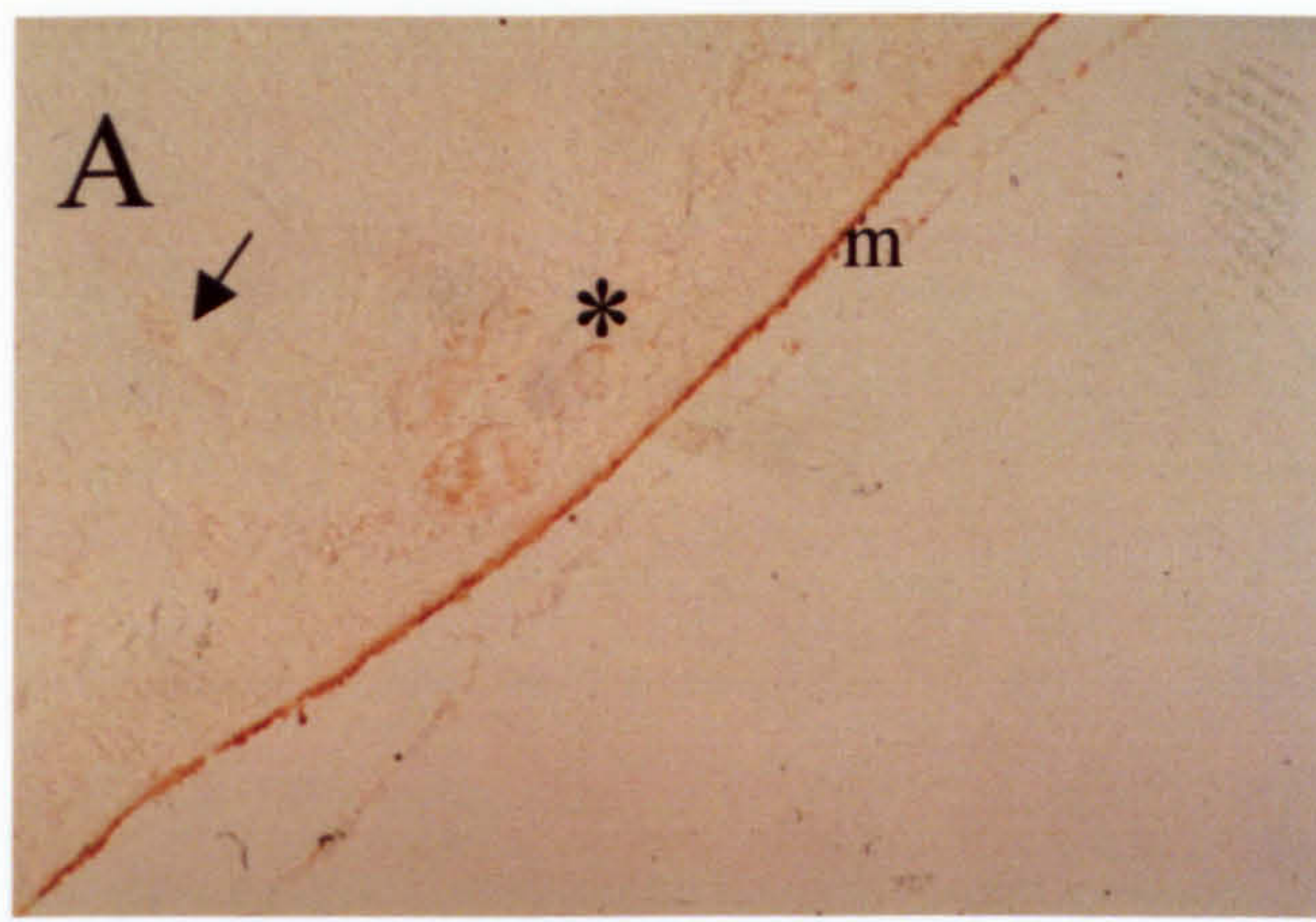


Figure 4.1. Immunolocalisation of mAChRs within uninfected rat intestine. Strong staining was observed in the external muscle (m), and possibly ganglia of the myenteric plexus (*). Faint staining (arrowed) could be detected in the lamina propria (lp), which extended along the epithelium (e). Magnification was x80 (A), x125 (B), and x312 (C).

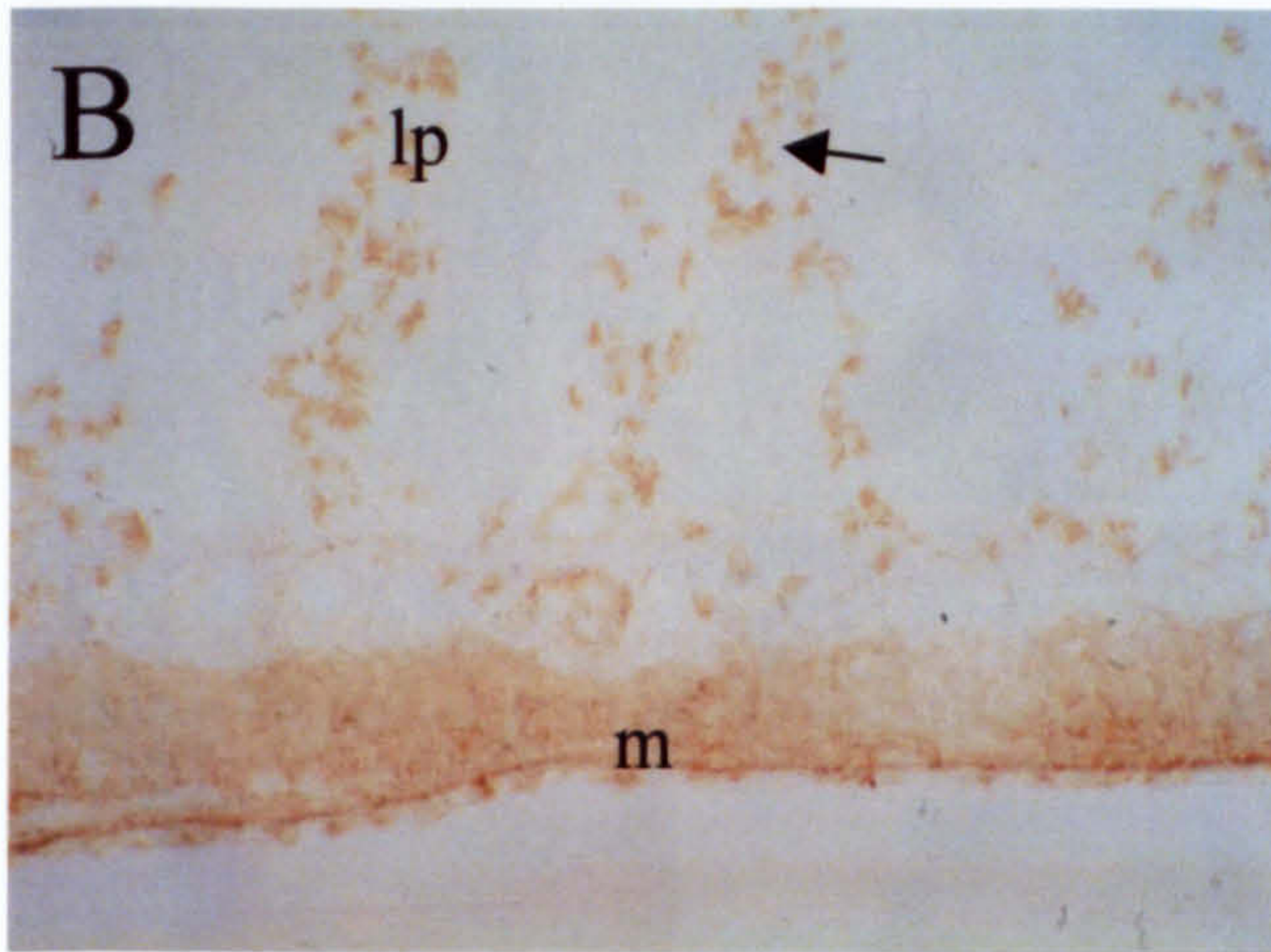
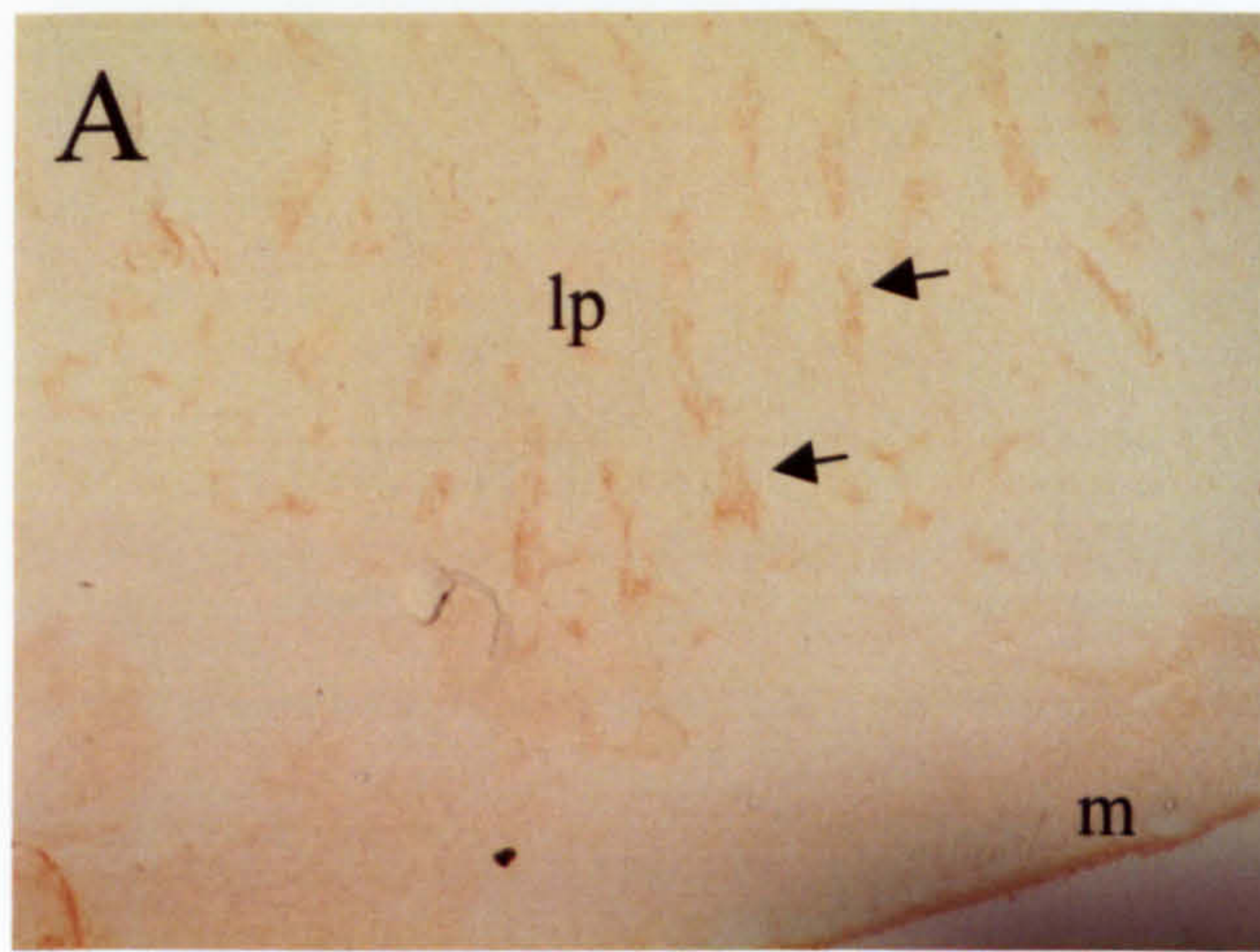


Figure 4.2. Distribution of mACHR-positive staining within *N. brasiliensis*-infected intestine. Immunoreactivity was observed in the external muscle (m), the lamina propria (lp, arrowed) and in close apposition to the basement membrane of the epithelium (e*). Magnification was x80 (A), x125 (B), and x312 (C).

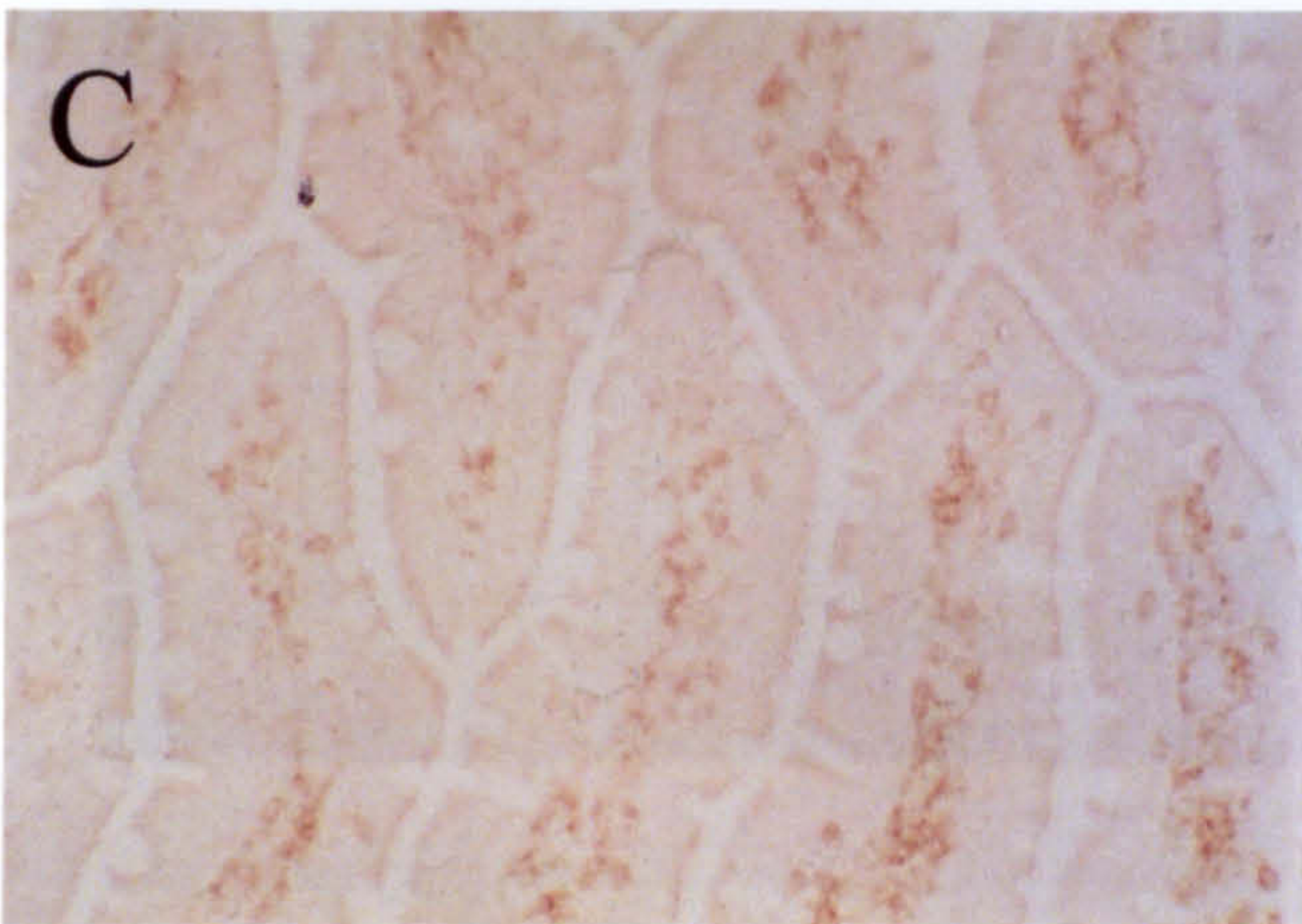
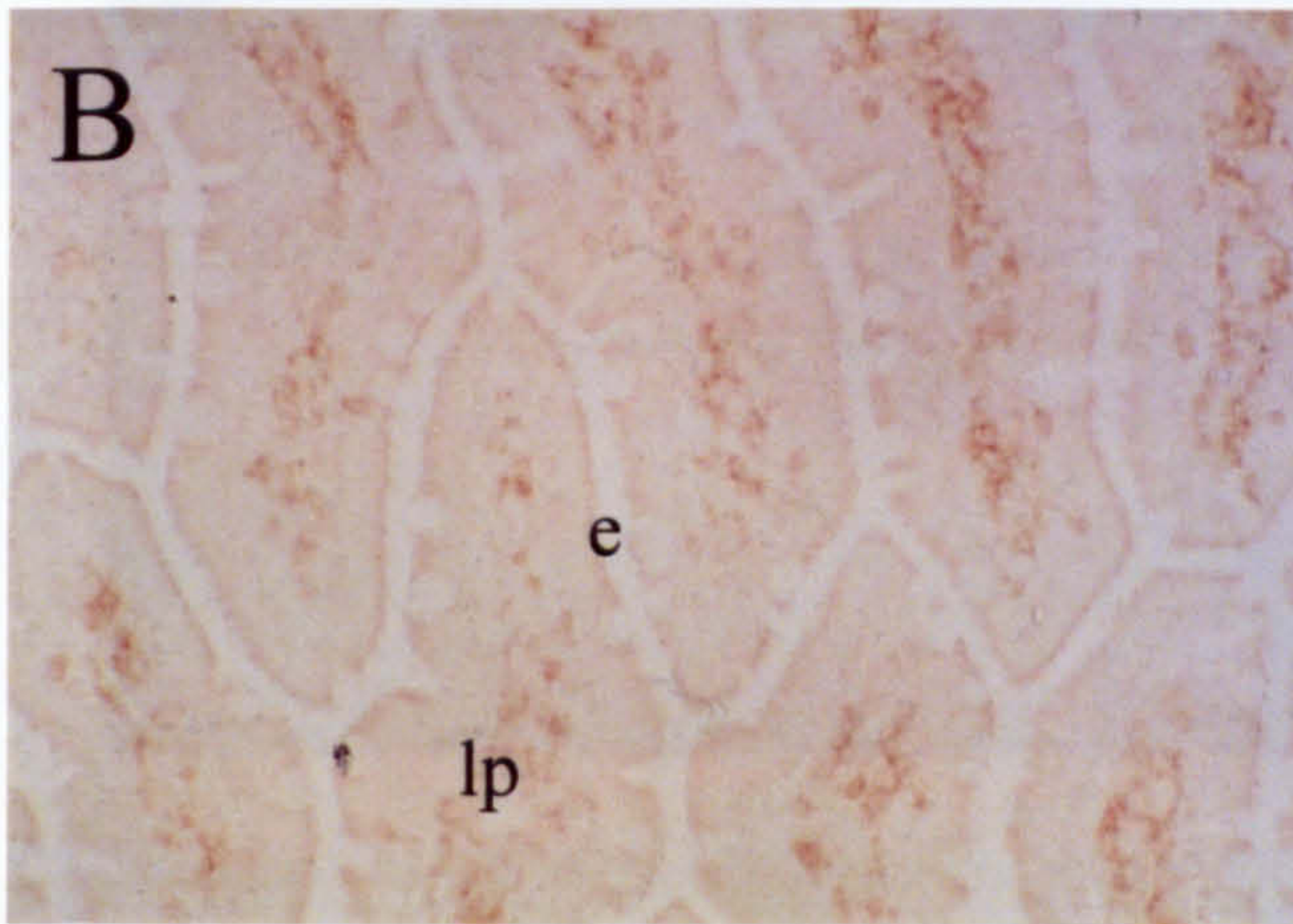
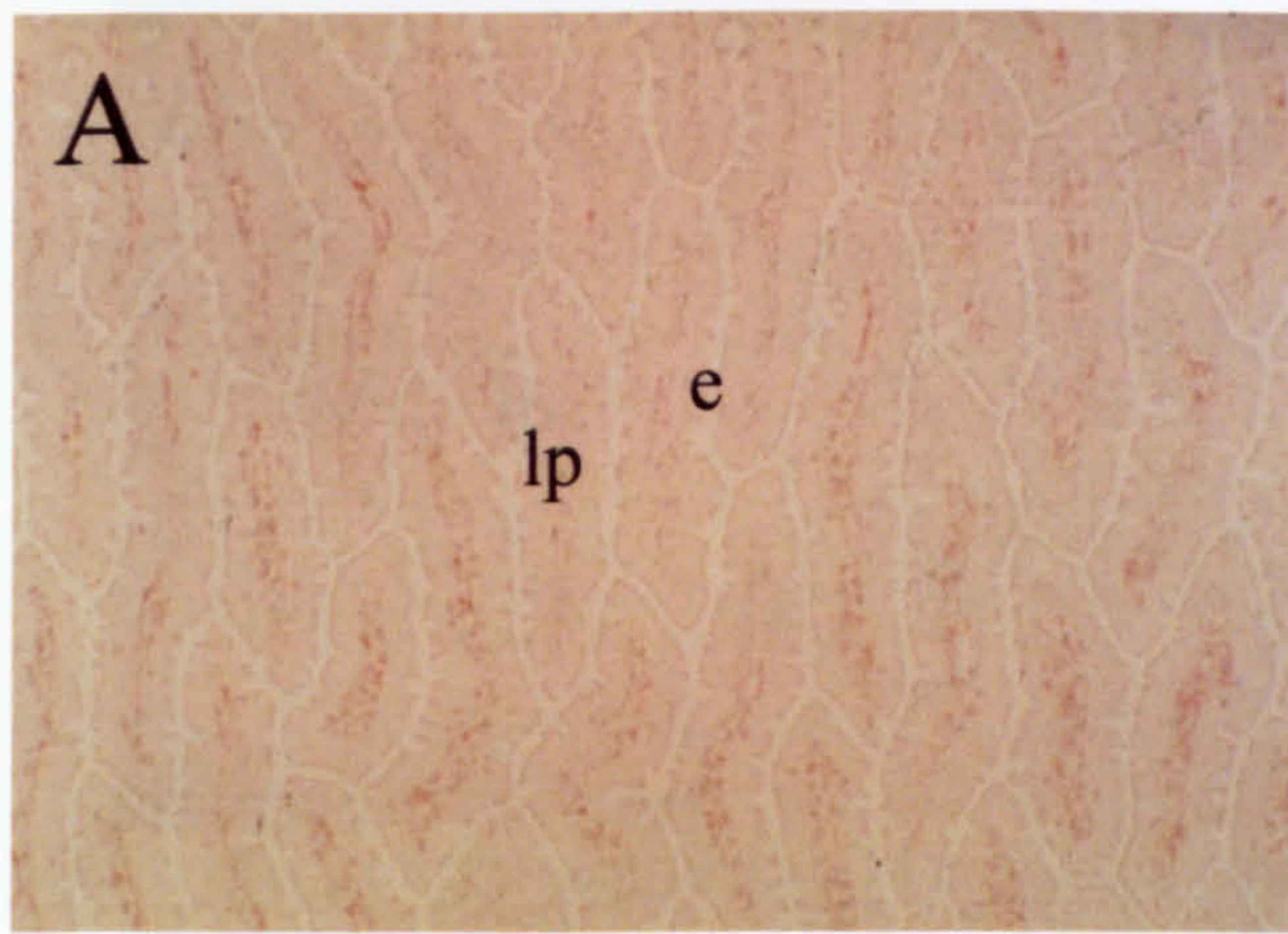


Figure 4.3. Localisation of CD4⁺ cells in control (uninfected) rat intestine. Staining for CD4⁺ cells was distributed throughout the lamina propria (lp). Magnification was x80 (A), x312 (B-C).

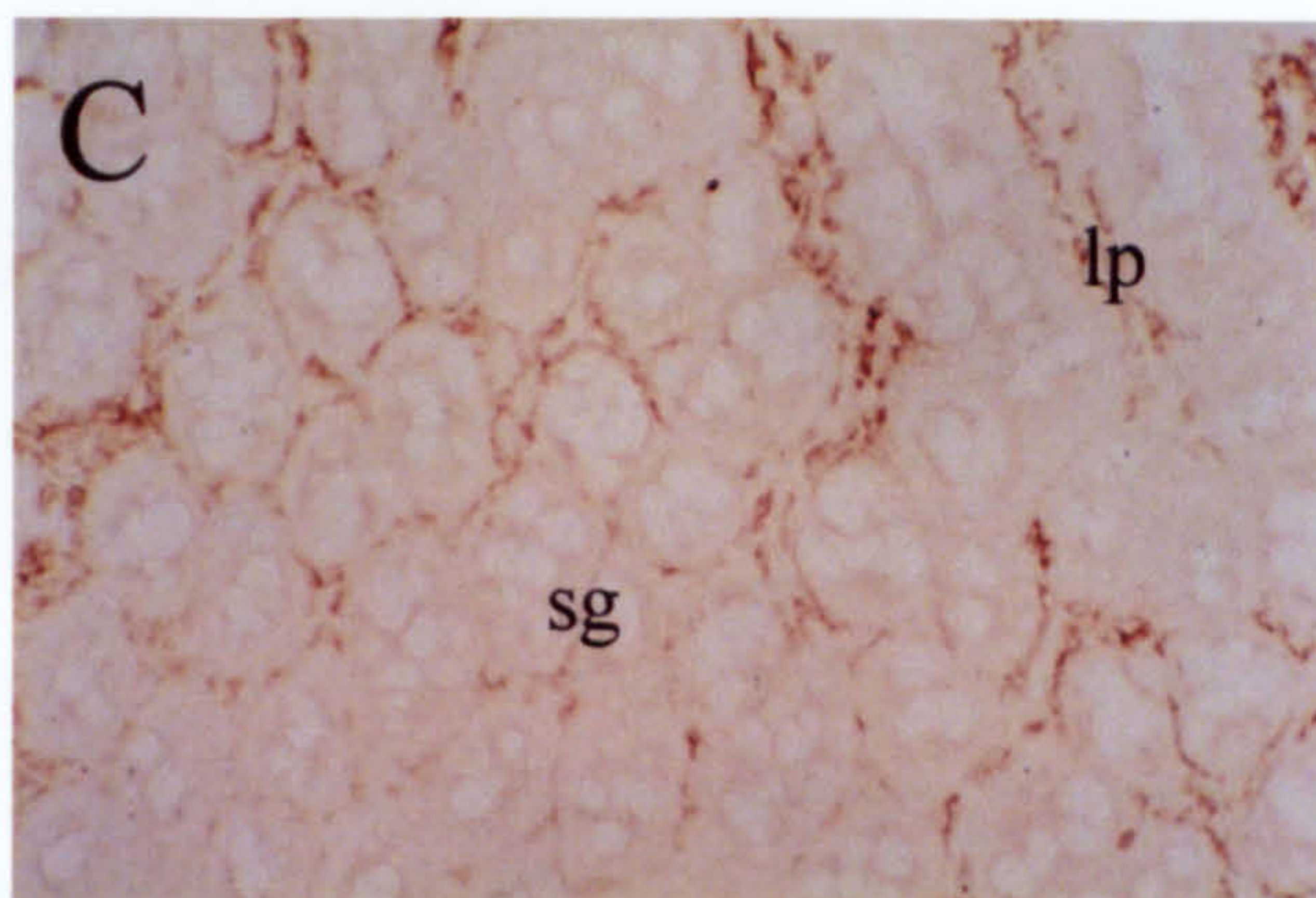
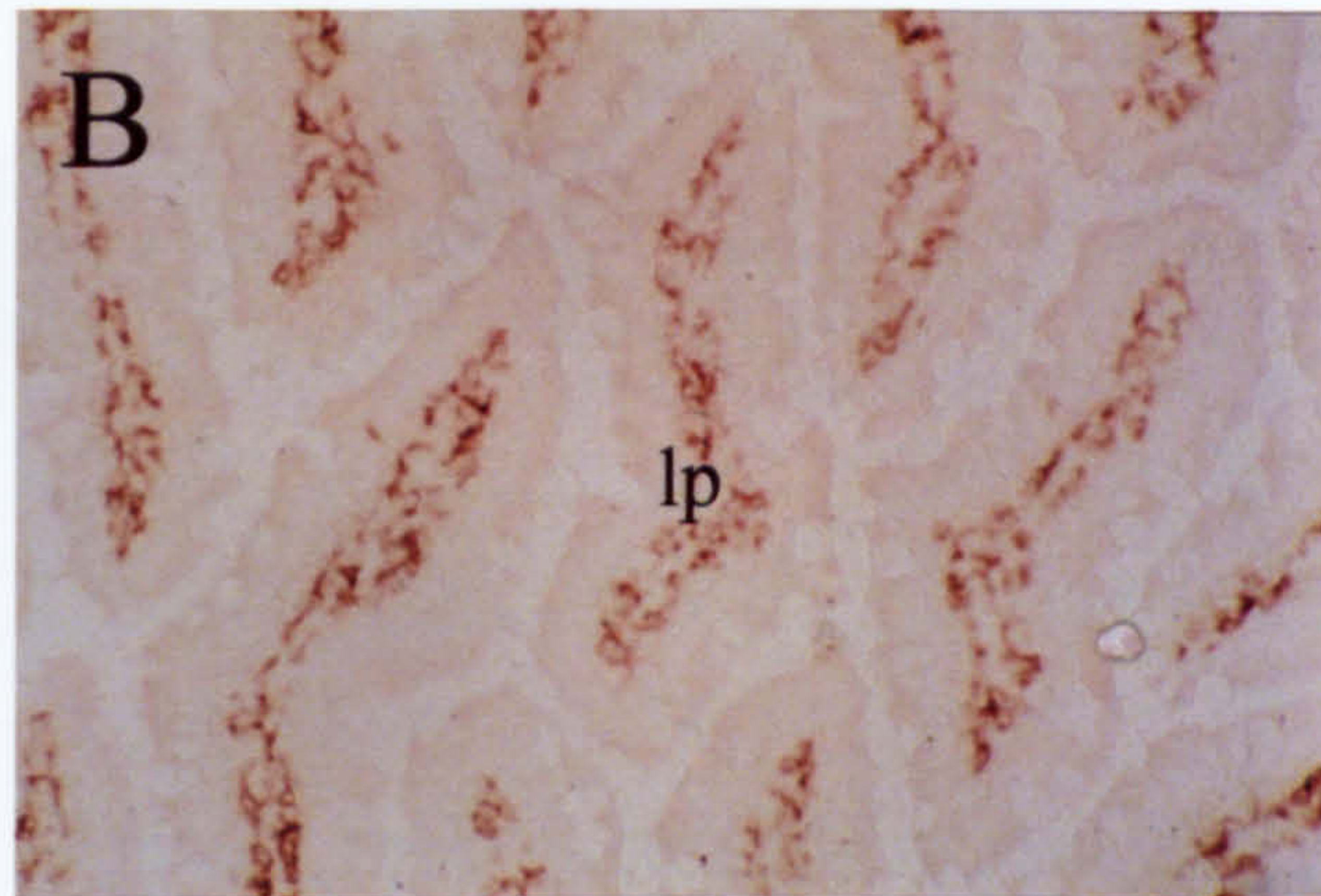
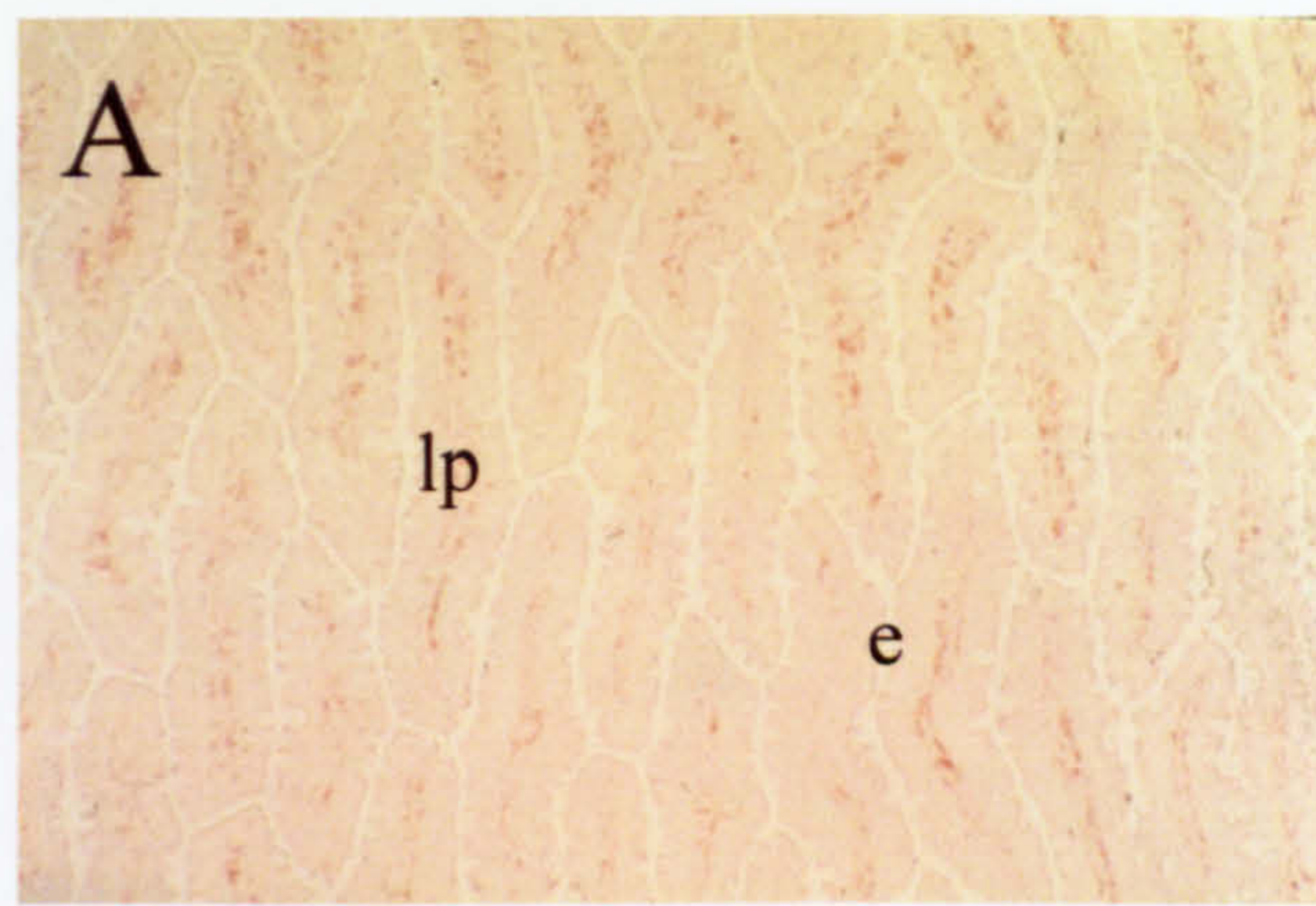


Figure 4.4. Localisation of CD4⁺ cells in the intestine of *N. brasiliensis*-infected rats. Intense staining for CD4⁺ cells was observed throughout the lamina propria (lp) of the intestine. Magnification was x80 (A), x312 (B-C).

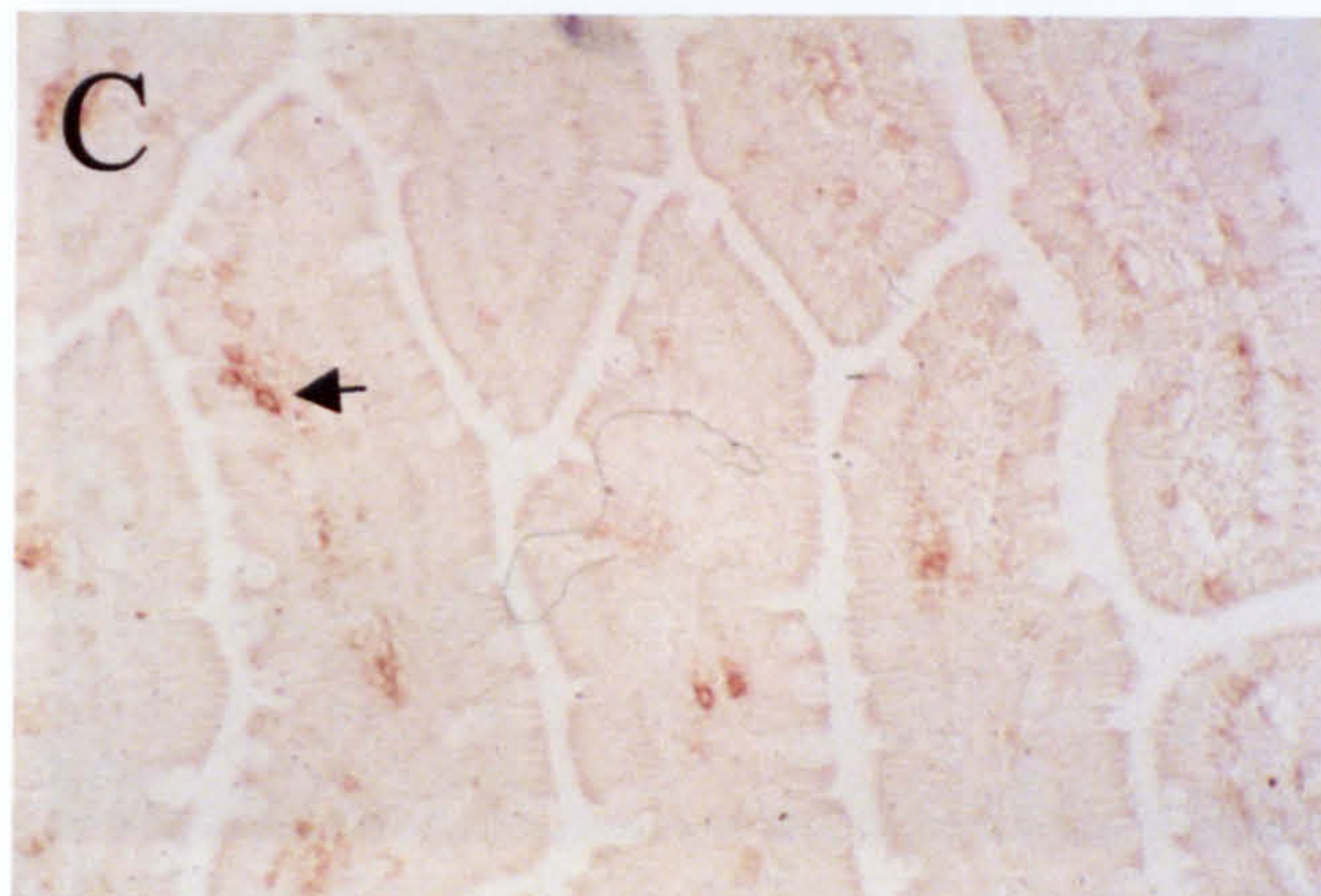
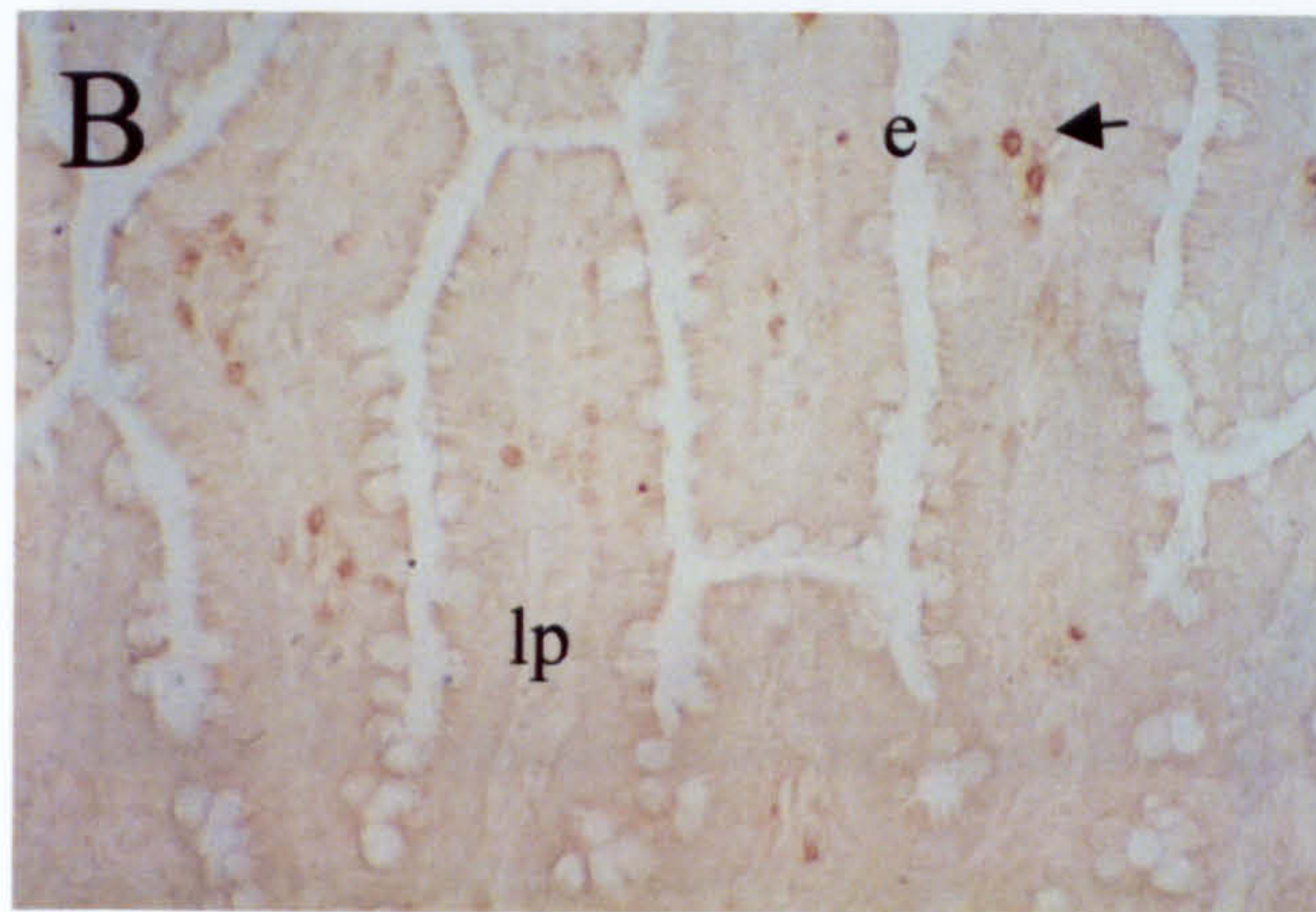
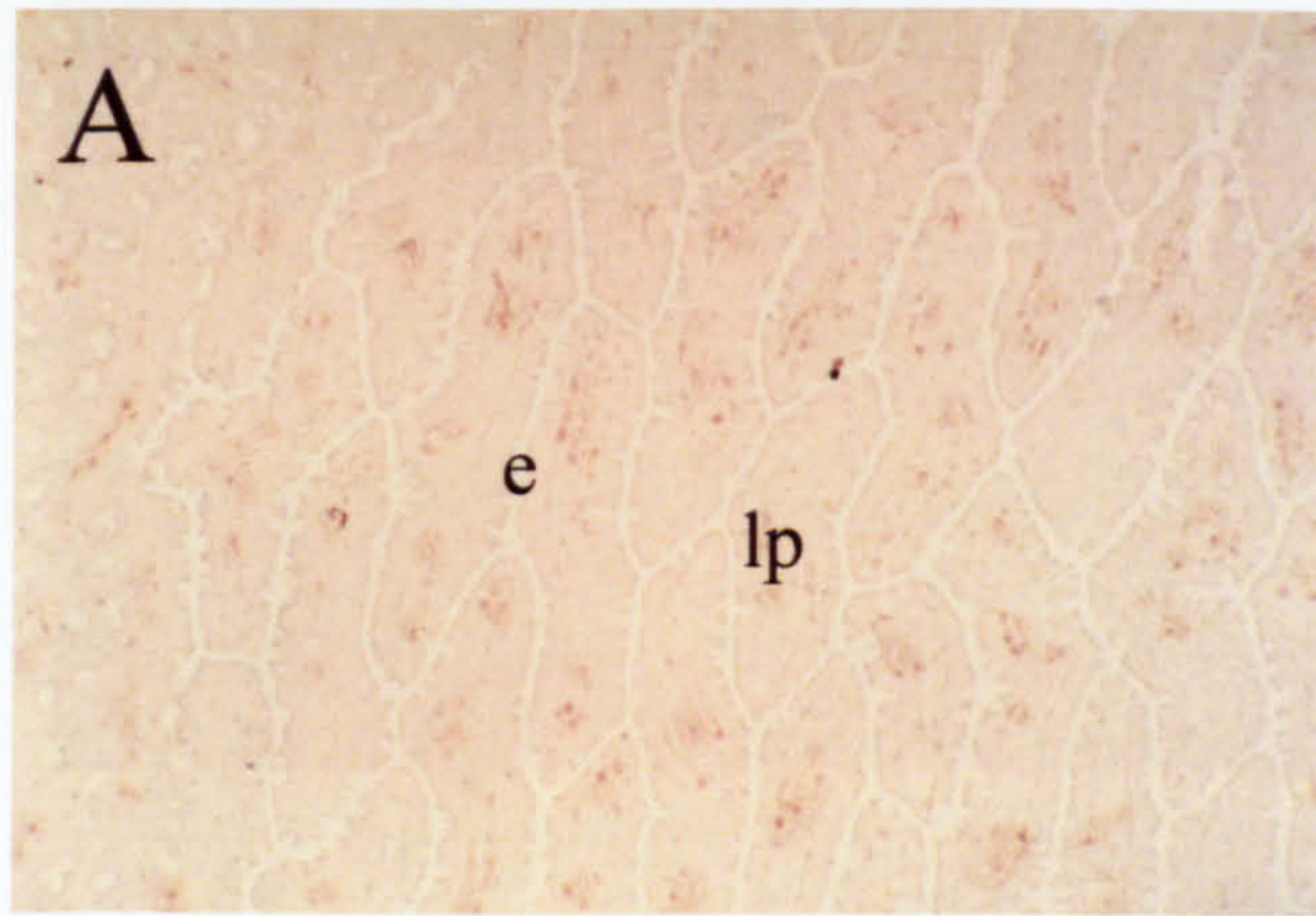


Figure 4.5. Distribution of CD8⁺ cells in uninfected rat intestine. CD8⁺ cells were localised in the lamina propria (lp), with some cells closely apposed (arrow) to the intestinal epithelium (e). Magnification was x80 (A), x312 (B-C).

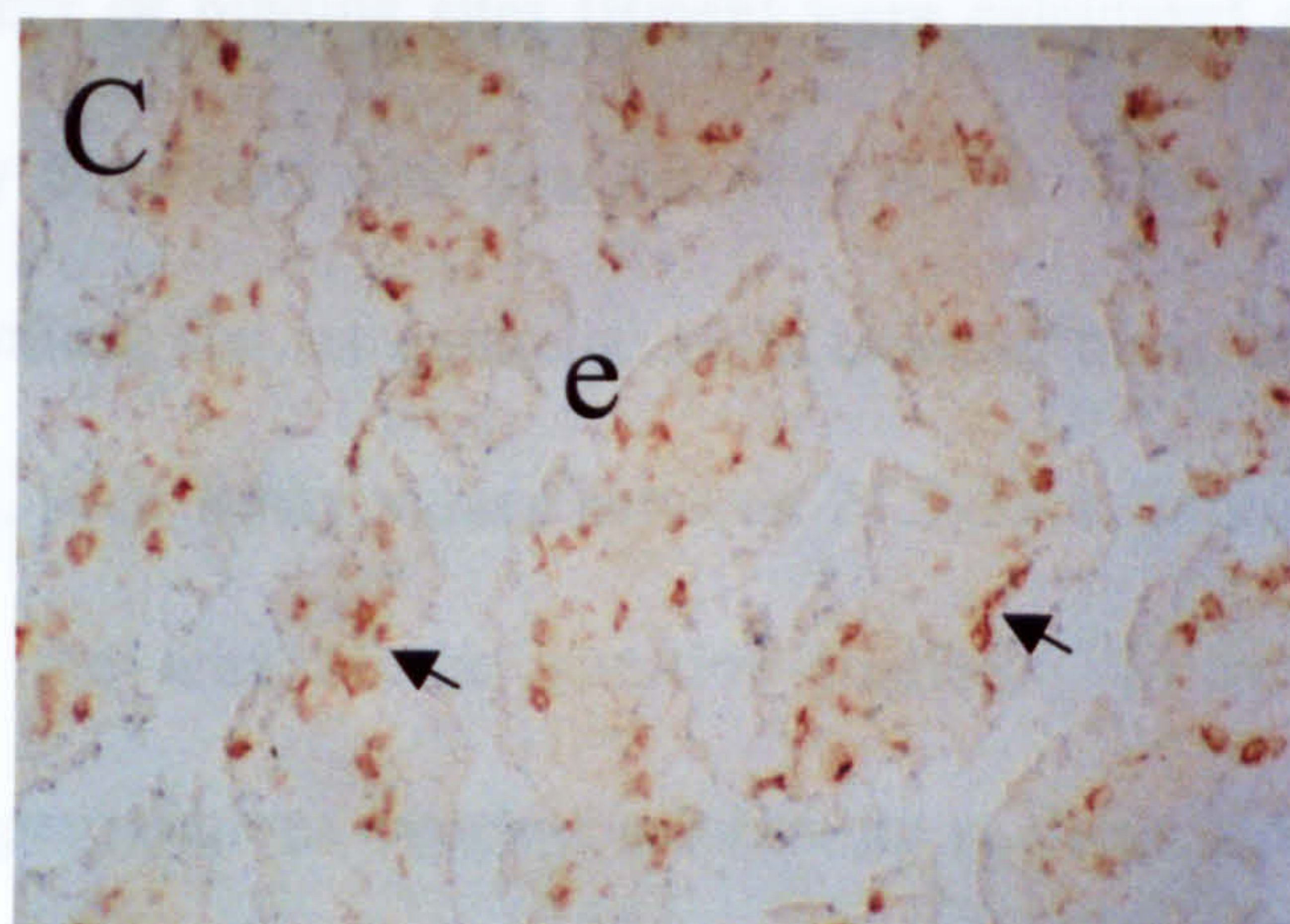
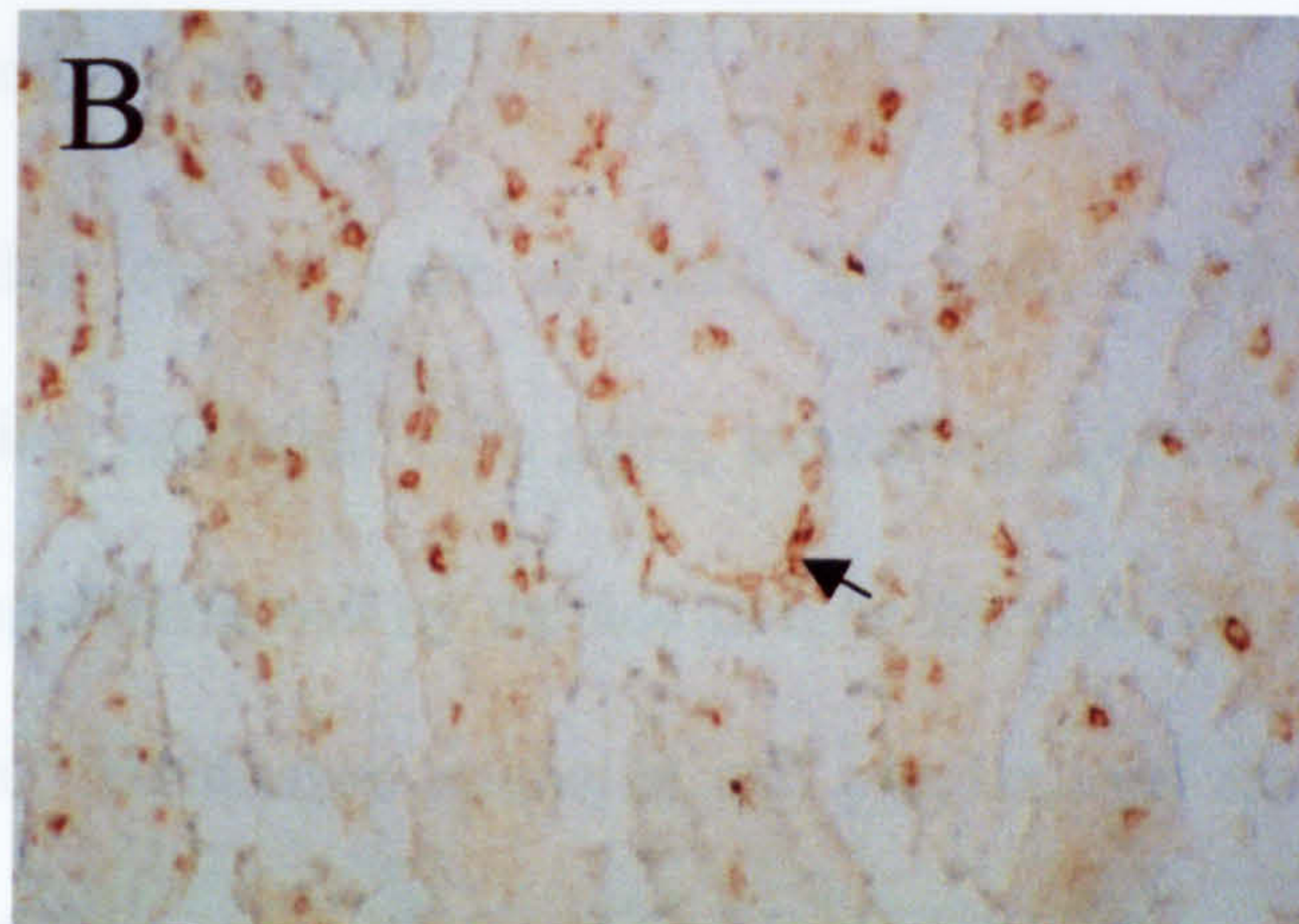


Figure 4.6. Immunolocalisation of CD8⁺ cells in the intestine of *N. brasiliensis*-infected rats. Increased CD8⁺ immunoreactivity was observed in the lamina propria (lp), with numerous cells closely apposed (arrow) to the intestinal epithelium (e). Magnification was x80 (A), x312 (B-C).

4.3 Radioligand binding studies

In an attempt to quantify the mACh receptor density, radioligand binding studies with the non-selective mAChR agonist [^3H] quinuclidinyl benzilate ([^3H] QNB) were performed on isolated intestinal epithelial cells from uninfected and infected rats. Membrane preparations were made from isolated epithelial cells, and 50 μg were used per reaction.

Saturation curves were obtained for both uninfected and *N. brasiliensis*-infected intestinal epithelial cells using increasing concentrations of [^3H] QNB, with specific binding determined by the addition of the mAChR antagonist atropine (1 μM) to the reaction. Uninfected epithelial cell preparations reached saturability at about 1.5 nM (Fig 4.7A), with an apparent dissociation constant (K_D) of 0.21 nM. The maximal number of binding sites (B_{max}) were calculated at 27 fmol/mg of protein, determined by Scatchard plot analysis (Fig 4.7B). Rimele and co-workers (1981) reported K_D values for rat epithelial cells isolated from the small intestine between 0.08-0.14 nM, with B_{max} values between 21.9-32.1 fmol/mg of protein, which was dependent on the area of the intestine studied. In contrast, infected intestinal epithelial cell preparations reached saturability at about 3 nM [^3H] QNB (Fig 4.8A), with K_D and B_{max} values calculated at 0.72 nM and 40 fmol/mg of protein respectively (Fig 4.8B). Intestinal epithelial cells taken from infected animals showed a three-fold increase in dissociation constant and approximately two-fold increase in maximal binding, in comparison to uninfected animals. This suggests that during infection with *N.brasiliensis*, there is an increase in the number of mAChRs within the intestine, which results in the shift in receptor affinity for the mAChR agonists observed in this study.

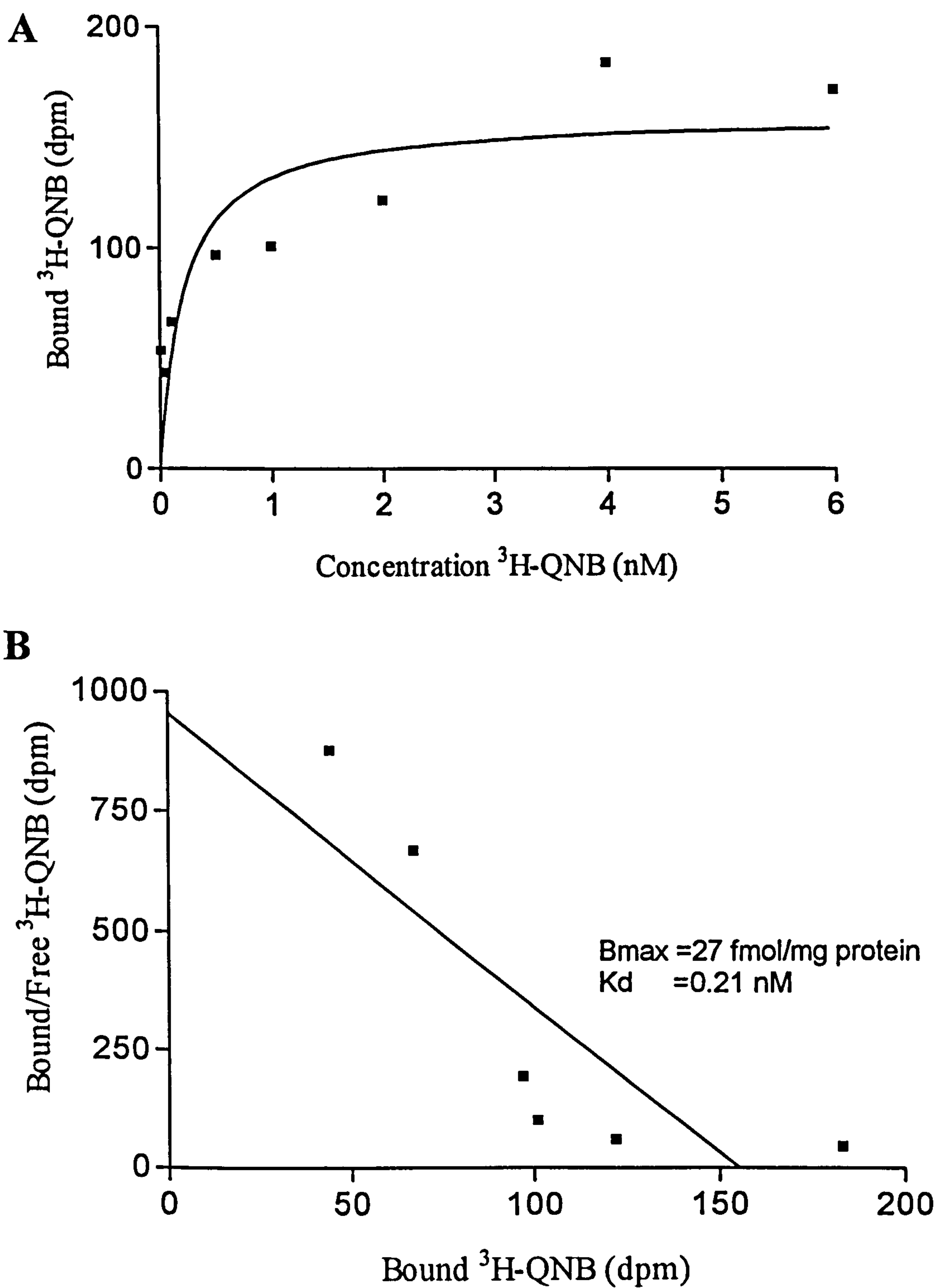


Figure 4.7. Determination of mAChR expression on isolated intestinal epithelial cells membranes from uninfected animals. Membrane preparations (50ug) were incubated in a range of [^3H]-QNB concentrations in the presence (non-specific binding) or absence (total binding) of atropine (10^{-4} M) to determine specific binding. Panel (A) shows specific saturation binding to epithelial membranes, with Scatchard analysis (B) used to calculate the K_d and B_{max} .

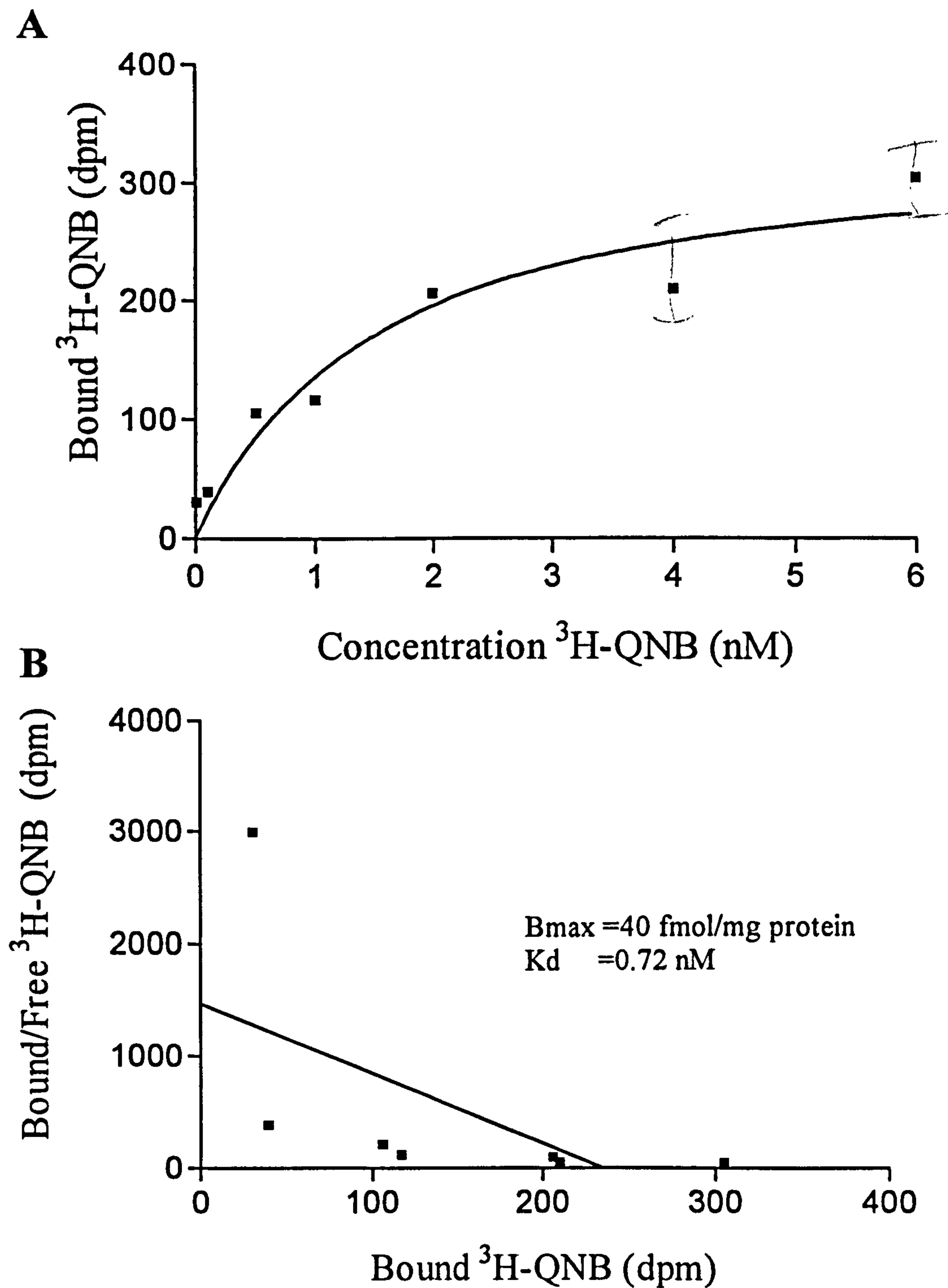


Figure 4.8. Determination of mAChR expression on isolated epithelial cells from rats infected with *N. brasiliensis*. Membrane preparations (50ug) were incubated in a range of [^3H]-QNB concentrations in the presence (non-specific binding) or absence (total binding) of atropine (10^{-4} M) to determine specific binding. Panel (A) shows specific saturation binding to epithelial membranes, with Scatchard analysis (B) used to calculate the Kd and Bmax.

4.4 Identification of mAChR subtypes during infection

Analysis of mAChR distribution within peripheral tissues by Northern blotting ²⁷² has revealed mRNA for m2 and m3 from whole small intestine samples, ^{specie} with m1 and m3 detected in exocrine glands, and m2 and m3 found in smooth muscle (Maeda *et al.*, 1988; Peralta *et al.*, 1987), whereas the presence of mRNA for all mAChR subtypes has previously been reported in brain by Northern blotting and RT-PCR techniques (Maeda *et al.*, 1988; Wei *et al.*, 1996). Primers specific for rat mAChR subtypes were adapted from Wei and colleagues (1996), and used to detect the expression of mRNA in isolated intestinal epithelial and lamina propria samples for these receptors by RT-PCR.

Specific primers were first used on samples isolated from rat whole brain to check for efficiency and to determine the RT-PCR conditions to be used. Subsequent PCR reactions were performed for 30 cycles (94°C denaturation, 1 min; 57°C annealing, 1 min; 72°C extension, 1.5 min), and bands were visualised by 1.5% agarose gel electrophoresis and ethidium bromide staining. PCR products were detected for all five mAChR subtypes in rat brain (Fig. 4.9), with apparent sizes in accordance with those previously reported (Wei *et al.*, 1996: m1, 373 bp; m2, 683; m3, 434 bp; m4, 588 bp; m5, 394 bp). Primers were designed to the housekeeping gene β -actin (see Materials & Methods) to check the efficiency of the reverse transcription and PCR reactions, giving PCR product of 400 bp.

Total RNA was extracted from cells isolated from the intestinal epithelium and lamina propria of uninfected and infected animals, reverse transcribed and used in PCR reaction with the relevant primers.

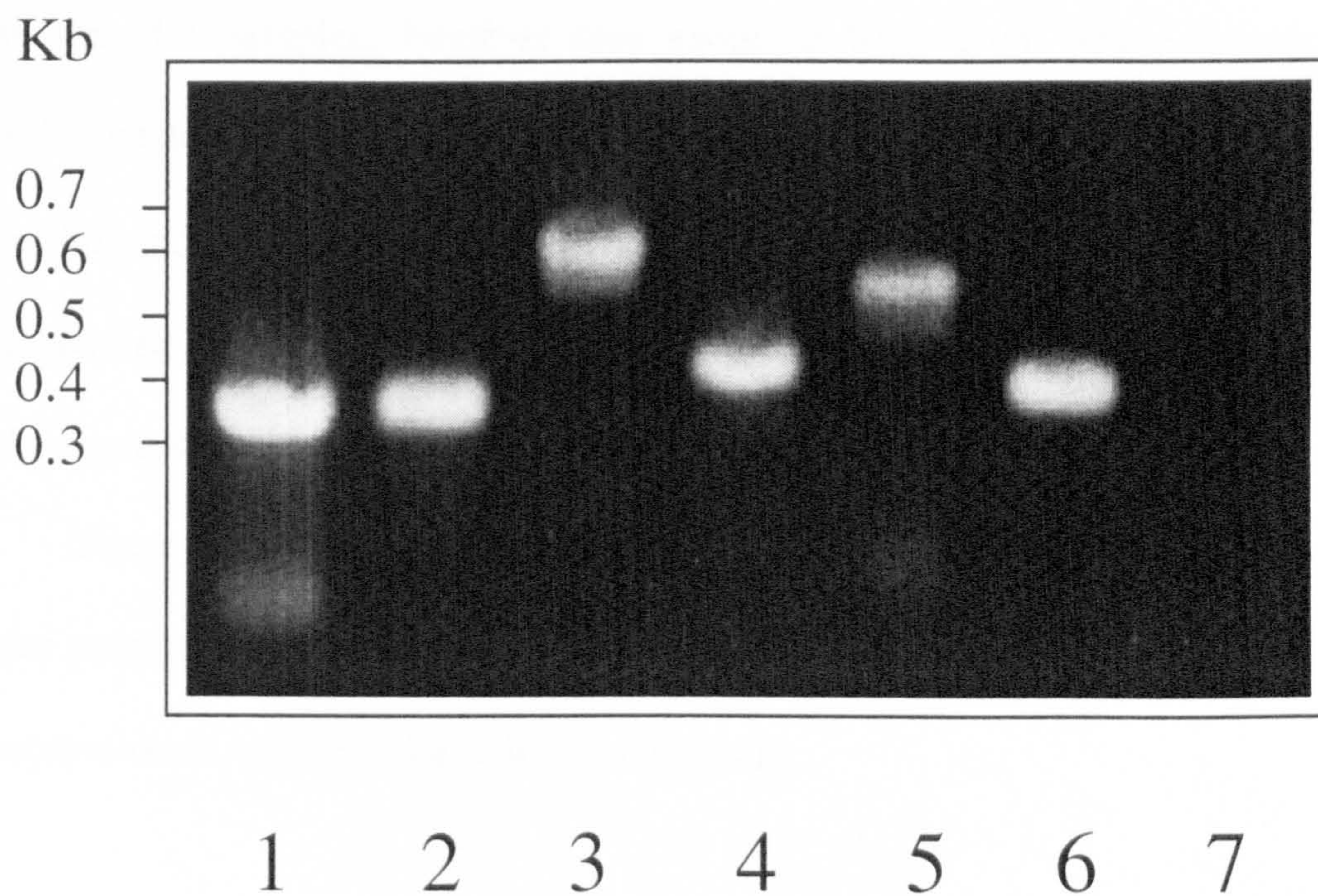


Figure 4.9. Expression of mRNA for mAChRs in rat brain detected by RT-PCR. cDNA samples were amplified with specific primers for β -actin (1), m1 (2), m2 (3), m3 (4), m4 (5), m5 (6) and untranscribed RNA using β -actin primers (7). PCR products were separated by agarose (1.2%) gel electrophoresis and stained with ethidium bromide.

In both uninfected and infected samples, products for β -actin were observed demonstrating efficient transcription and amplification of RNA samples. Both uninfected epithelial and lamina propria cells possess mRNA for the m3 receptor subtype, with no signal observed for the other mAChRs (Fig. 4.10). Maeda and co-workers (1987) previously reported both m2 and m3 transcripts were present in rat small intestine samples, however they extracted RNA from whole intestine which would contain smooth muscle and mucosal tissue. RNA samples extracted from epithelial and lamina propria cells isolated from *N. brasiliensis*-infected animals demonstrated the same pattern of receptor gene expression as uninfected animals, solely expressing the m3 transcript (Fig. 4.11).

These results demonstrate the presence of m3 mRNA in both epithelial and lamina propria samples, however they do not quantify the differences between levels of expression in uninfected and infected animals.

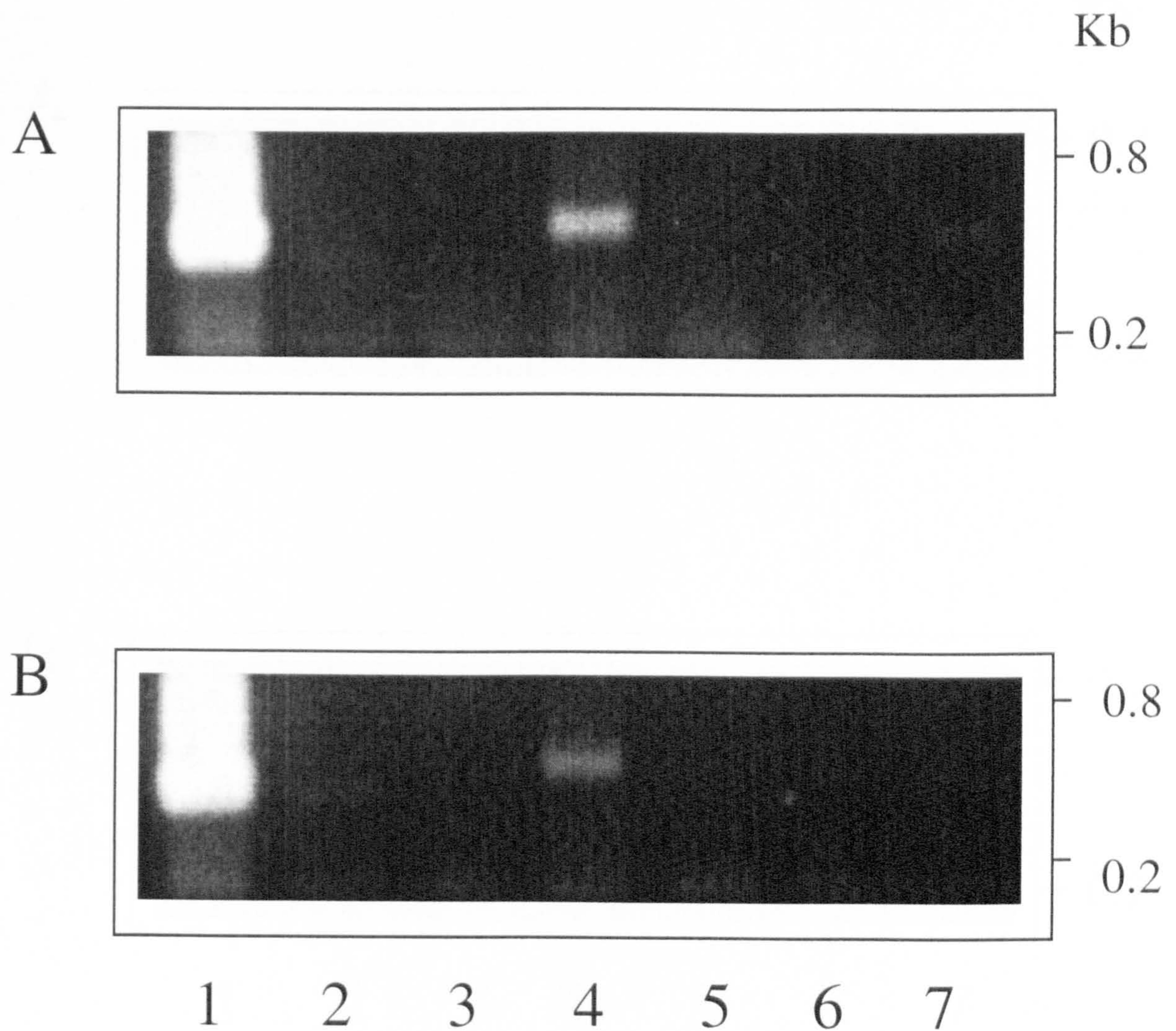


Figure 4.10. Expression of mAChR subtypes in intestinal epithelial (A) and intestinal lamina propria (B) cells from control (uninfected) rats. Reverse transcribed RNA was used in a PCR reaction with primers for β -actin (1), m1 (2), m2 (3), m3 (4), m4 (5), m5 (6) and untranscribed RNA using β -actin primers (7).

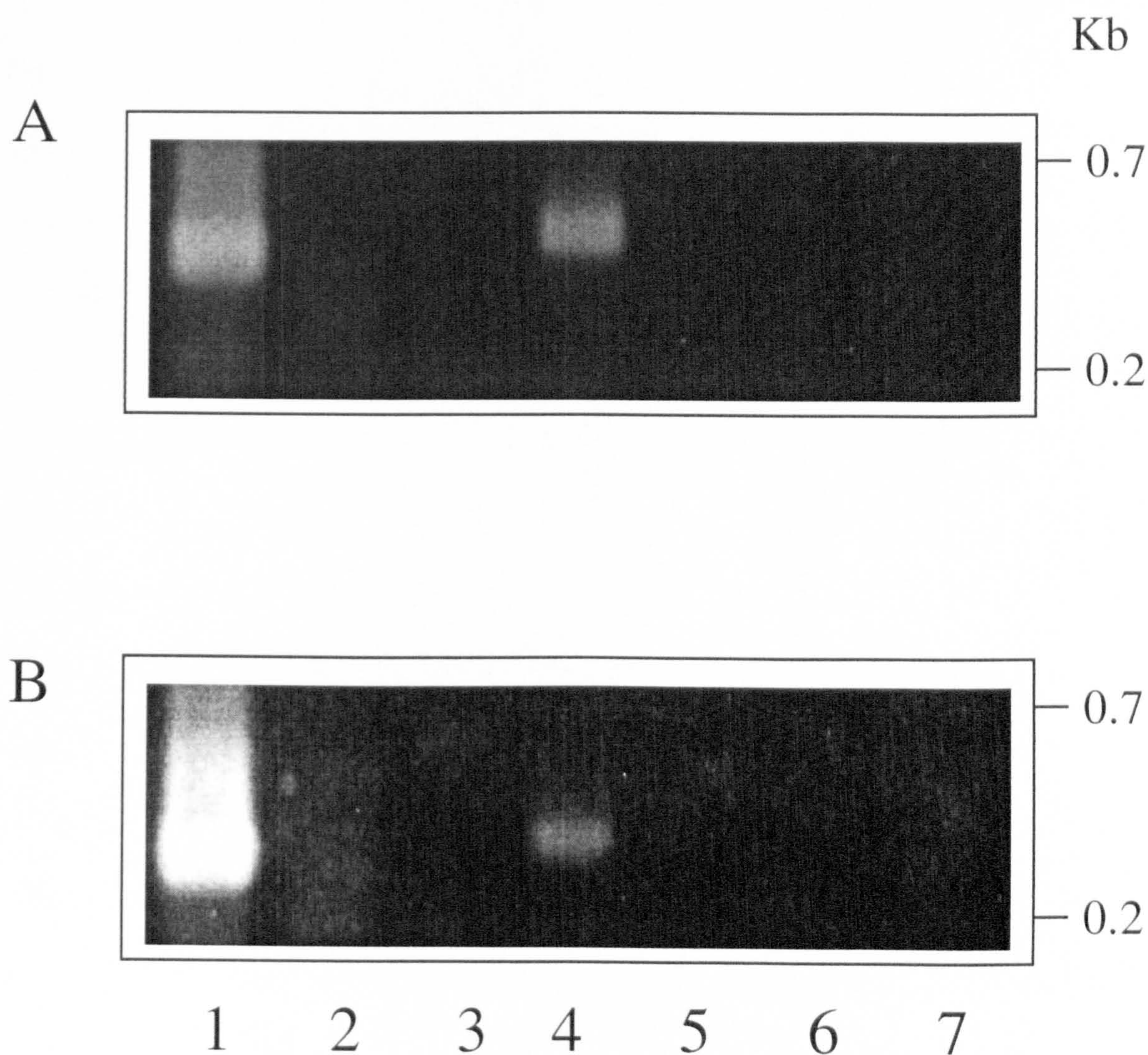


Figure 4. 11. Expression of mAChR subtypes in intestinal epithelial (A) and lamina propria cells (B) from rats infected with *N. brasiliensis*. RNA was reverse transcribed and used in PCR reactions containing primers for β -actin (1), m1 (2), m2 (3), m3 (4), m4 (5), m6 (6) and untranscribed RNA using β -actin primers (7).

4.5 Discussion

The identification and localisation of mAChRs within peripheral organs of the body has previously been studied using pharmacological, immunological and molecular biology techniques. Receptors were first demonstrated by the use of mAChR ligands, with receptor subtypes identified by the relative potencies of a range of receptor antagonists (Reviewed by Caulfield, 1993). However, receptor antagonists have revealed only four putative mAChR subtypes, and the difficulties in interpreting results from tissues and organs containing mixed populations of mAChRs led to inconclusive receptor subtype localisation (Caulfield, 1993). More recently, the existence of five different genes encoding for mAChRs were reported (Bonner *et al.*, 1987, 1988; Peralta *et al.*, 1987), leading to localisation studies of mRNA for mAChR subtypes by Northern blotting, and RT-PCR techniques (Maeda *et al.*, 1988; Peralta *et al.*, 1987). Receptor subtype-specific antibodies have been generated, and used in immunoprecipitation studies (Levey *et al.*, 1990, 1991; Li *et al.*, 1991; Wall *et al.*, 1991a, b). Comparing results obtained from different techniques revealed some discordance between the studies. Immunoprecipitation of mAChRs using receptor specific antisera reported high levels of m2 protein in rabbit and rat ileal homogenates, with m4, m3 and m1 proteins also detected (Dörje *et al.*, 1991; Wall *et al.*, 1991). In contrast, mRNA has only been detected for m2 and m3 receptors (Maeda *et al.*, 1988; Peralta *et al.*, 1987), which correlated well with results obtained from ligand binding studies showing the presence of M2 and M3 receptors within ileal smooth muscle (Michel & Whiting, 1988; Candell *et al.*, 1990), and M3 receptors in the intestinal mucosa (Chandan *et al.*, 1991; Kachur *et al.*, 1990). It has been suggested that the disparity between these studies could be due to regional differences in the tissues studied, sensitivities of the techniques used, and the

translational efficiencies of individual mRNAs (Buckley *et al.*, 1988). Locational differences maybe encountered where the nerve cell bodies are distant from the nerve synapse, such as where the tissue is innervated extrinsically, leading to the detection of mAChR protein but not mRNA (Levey, 1993).

Due to the problems of generating specific antibodies for mAChR immunocytochemistry and their general availability, very little work has been published on the immunolocalisation of these receptors in peripheral organs. In this study mAChRs were localised within the intestine using the monoclonal antibody M35, which does not discriminate between receptor subtype (Carsi-Gabrenas *et al.*, 1997). In agreement with previous pharmacological and molecular biology data (Michel & Whiting, 1988; Candell *et al.*, 1990; Maeda *et al.*, 1988), the external muscle layers of the intestine of both uninfected and *N. brasiliensis*-infected rat were highly immunoreactive for M35. The mucosa of uninfected intestine has only weak staining for M35 which extends into the lamina propria, whereas in infected animals there is strong immunoreactivity along the basolateral domain of the epithelium and upon cells within the lamina propria. Lammerding-Köppel and colleagues (1995) using M35 identified immunopositive staining within the external muscle layers of the prenatal mouse embryo intestine, with some M35-positive staining observed close to the epithelium. Subtype selective antibodies demonstrated localisation of m1 to the gastric mucosa, and m4 immunoreactivity within the musculature and submucosa of the colon (Levey, 1993). More recent work by Schäfer and co-workers (1998) using an antibody specific to the vesicular acetylcholine transporter (VAChT), demonstrated cholinergic innervation of the external muscle layers, with some immunoreactivity extending into the lamina propria and running close to the intestinal epithelium.

Using antibodies which are specific for CD4⁺ and CD8⁺ lymphocytes, it was observed that the mAChR-positive cells within the infected lamina propria more closely resembled the localisation of CD4⁺ rather than CD8⁺ cells. Unfortunately co-localisation of M35- and CD4⁺-cells could not be performed on tissue sections because the antibodies required different fixation regimes, with lymphocyte markers fixed using acetone and M35 requiring paraformaldehyde fixation. Techniques for the isolation and purification of intestinal epithelial and lamina propria have now been successfully set up in the laboratory, therefore the localisation of a range of lymphocyte and intestinal markers in conjunction with M35 using flow cytometry would overcome these technical problems, and should be more easily investigated.

The radioligand binding data reported in this chapter shows only a slight increase in receptor sites, but a three-fold increase in dissociation constant between uninfected and *N. brasiliensis*-infected intestinal epithelial cells. These findings are in stark contrast to the immunocytochemical data, which suggests a significant increase in mAChR-positive cells within the lamina propria and epithelium of infected intestine. The first direct evidence for the presence of mAChRs on intestinal epithelial cells was reported by Rimele and colleagues (1981), with colonic cells having a dissociation constant similar to jejunal and ileal epithelial cells (0.08-0.14 nM), but the receptor density was about five-fold higher. Isaacs and co-workers (1982) observed differences in receptor density between ileal mucosal homogenates and those obtained from duodenal and jejunal samples, with differences also shown between mucosal and muscle fractions. Taken together these reports suggest regional differences in mAChR density along the intestine. Since *N. brasiliensis* inhabits the anterior third of the intestine (Jenkins, 1973; Miller *et al.*, 1981) and the radioligand binding studies used epithelial cells isolated from whole

intestine, discrete regional changes in mAChR expression may be masked. It would therefore be interesting to look at the receptor binding densities and dissociation constants of epithelial cells isolated from uninfected and infected jejunum.

Analysis of mRNA expression for mAChR subtypes by RT-PCR revealed the presence of m3 in epithelial and lamina propria preparations from uninfected and infected animals. These results are in accordance with the results of Maeda and colleagues (1988), who observed m2 and m3 receptor subtypes in rat small intestine by Northern blotting. These workers used whole intestine preparations containing smooth muscle and mucosa, with smooth muscle reported to predominantly express the m2 receptor subtype by several experimental techniques (Maeda *et al.*, 1988; Michel & Whiting, 1988; Wall *et al.*, 1991). With the development of reliable methods for the quantitation of mRNA transcripts, the measurement of mRNA expression for the m3 transcript in epithelial and lamina propria cell populations during parasite infection may become a valuable tool for the determination of receptor upregulation. However, as discussed for radioligand binding studies, care must be taken to ensure that regional differences in the expression and localisation of receptors in the gut are taken into account.

Many physiological studies have demonstrated that the addition of ACh or ACh receptor agonists to isolated intestinal sheets evokes chloride and mucus secretion. Incubation in the presence of the muscarinic antagonist atropine abrogates these responses, leading to the suggestion that mAChRs play a role in these secretory responses (Chandan *et al.*, 1991; Javed *et al.*, 1992; Kachur *et al.*, 1990; Phillips *et al.*, 1984; Specian & Neutra, 1980, 1982; Tapper *et al.*, 1978). Work by Kachur and colleagues (1990) using guinea pig ileum, and Chandan and co-workers (1991) with pig distal jejunum have suggested that the M3 receptor subtype mediates the effects of ACh

on enterocytes. Immediate type hypersensitivity reactions (anaphylaxis) in the gut have been shown to coincide with antigenic challenge of previously-sensitised animals (Castro, 1989; Castro & Arntzen, 1993). These responses are characterised by a rapid rise in fluid secretion into the intestinal lumen, which in *T. spiralis*-sensitised animals was observed to be bi-phasic. The first phase occurred within two minutes following antigen challenge and appeared to be caused by histamine and 5-hydroxytryptamine (5-HT) derived from mast cells (Zhang *et al.*, 1991), whereas the second phase lasted for twenty minutes, peaked after eight minutes and appeared to be generated by prostaglandin E₂ (PGE₂) synthesized from mast cells (Castro, 1989). Work by Javed and colleagues (1992) suggested a role for cholinergic neurons during the secretory responses of bovine milk sensitised-guinea pig colon to re-challenge with β -lactoglobulin. ACh release from re-challenged animals paralleled the increase in fluid secretion, with histamine and prostaglandin inhibitors reducing these effects. Using different model systems, investigators have observed a significant reduction in fluid secretion in the presence of atropine, which further implies the role for cholinergic innervation in these responses (Castro *et al.*, 1987; Javed *et al.*, 1992; Wang *et al.*, 1991). However, during infection with *N. brasiliensis* it has been demonstrated that the cholinergic component of the nervous system appears to be defective (Masson *et al.*, 1996). Jejunal segments from rats infected ten days previously with *N. brasiliensis* displayed a reduced secretory response to nerve stimulation, which was insensitive to atropine. Exposure to the cholinergic agonist, bethanechol, resulted in secretory responses comparable to those of control animals, which suggests that the epithelium was able to respond to exogenous cholinergic stimulation (Masson *et al.*, 1996). It is therefore possible that parasite derived AChE could influence the efficiency of cholinergic processing and down-regulate

potentially harmful immune responses. The increase in mAChR expression during parasite infection may be a result of ineffective cholinergic input, with increases in receptor number rendering the intestinal epithelium hypersensitive to ACh stimulation.

The data reported in this chapter demonstrates the presence of m3 mAChRs within the rat intestinal mucosa, with an increase in receptor expression during infection with *N. brasiliensis*. Unfortunately, these techniques do not elucidate the functional significance of an increase in receptor expression, and this is more likely to be determined by carefully designed pharmacological/physiological experiments.

CHAPTER 5

Effects of muscarinic receptor agonists on lymphocyte proliferative responses

5.1 Introduction

There is growing evidence to suggest that the nervous and immune systems interact and co-ordinate physiological responses to potential pathogens (Stead *et al.*, 1987b; Mathison *et al.*, 1994; Madden & Felten, 1995; Savino & Dardenne, 1995). Until recently the demonstration of muscarinic acetylcholine receptors (mAChRs) utilized radioligand binding assays using the mAChR antagonists [^3H] quinuclidinyl benzilate (^3H -QNB) or [^3H] *N*-methyl scopolamine (^3H -NMS). These studies have identified mAChRs on mouse, rat and human lymphocytes (Atweh *et al.*, 1984; Bronzetti *et al.*, 1996; Costa *et al.*, 1988; Eva *et al.*, 1989; Strom *et al.*, 1981). However, it has been noted that lymphocyte mAChRs have lower affinity for muscarinic antagonists than other tissues, and that in certain studies binding does not reach saturation (Adams *et al.*, 1992), which raises questions regarding the physiological significance of these receptors (Costa *et al.*, 1988; Eva *et al.*, 1989). Molecular biological techniques have identified genes encoding for 5 receptor subtypes (m1-m5; Bonner *et al.*, 1987, 1988; Peralta *et al.*, 1987), whereas only 4 receptor subtypes have been identified pharmacologically (M1-M4). The current nomenclature states that receptor subtypes M1-M4 correspond to the m1-m4 genes, with the expressed product from the m5 receptor gene yet to have a defined pharmacological profile (Caulfield, 1993).

Pharmacological analysis, using a range of antagonists which have a rank order of potency for each receptor subtype, implied that human peripheral blood lymphocytes expressed mainly M2 and M3, and to a lesser extent M4 subtype mAChRs (Bronzetti *et al.*, 1996). Using the reverse transcription-polymerase chain reaction (RT-PCR) technique, it has been observed that mRNA could only be detected for m3, m4, and m5 receptor subtypes in human blood mononuclear cells (Costa *et al.*, 1995; Hellström-

Lindahl & Nordberg, 1996). It has also been demonstrated by RT-PCR and Southern blotting, that rat blood mononuclear cells possess low levels of m3 and m4 receptor subtype mRNA (approximately 100 and 10,000 times lower than levels found in rat brain; Costa *et al.*, 1994b). mRNAs for mAChRs were also detected in human leukaemic T-cell lines, with the m3 subtype observed by Northern blot analysis in Jurkat cells (Kaneda *et al.*, 1993), and detection of mAChRs by RT-PCR in Peer cells and HL-60 (a myeloid leukaemic precursor cell line) cells (Hellström-Lindahl & Nordberg, 1996).

The functional significance of expression of mAChRs in lymphocytes has yet to be clearly understood. Several studies have observed increases in cyclic 3',5'-guanosine monophosphate (cGMP) levels, activation of phospholipase C (PLC), enhanced DNA and protein synthesis, and mobilization of calcium ions (Ca^{2+}) in response to cholinergic agonists (Strom *et al.*, 1974; Masturzo *et al.*, 1985; Kaneda *et al.*, 1993). The muscarinic agonist oxotremorine-M (Oxo-M) induced transient increases in intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) in the human leukaemic helper T lymphoma cell line, Jurkat. Cholera, pertussis and botulinus type C toxin had no affect on the Oxo-M-induced rise in $[\text{Ca}^{2+}]_i$, which lead to the suggestion that Ca^{2+} mobilization occurred via bacterial toxin-insensitive GTP binding proteins (Kaneda *et al.*, 1993). The work of Fujino and colleagues (1997) indicated that pretreatment of human peripheral blood mononuclear cells (hPBMLs) with Oxo-M or ACh, enhanced phytohaemagglutinin (PHA- a T-cell mitogen that activates the TCR/CD3 complex)-induced DNA synthesis, transcription of IL-2 mRNA and increased IL-2 production. These effects were not observed using the nicotinic receptor agonist nicotine, nor in the presence of Oxo-M alone. It has been suggested that cholinergic agonists can modulate immune function. ACh and muscarinic receptor agonists enhance the release of lysosomal enzymes from polymorphonuclear

leukocytes and neutrophils during uptake of immune complexes (Ignarro & Colombo, 1973; Ignarro & George, 1974; Zurier *et al.*, 1974), enhance phagocytosis and chemotaxis by neutrophils (Hill *et al.*, 1975; Ignarro & Cech, 1976), and stimulate lymphocyte-mediated antibody-dependent cellular cytotoxicity (ADCC; Gale & Zighelboim, 1974). Zurier and colleagues (1974) have suggested that the increase in lysosomal enzyme release from human neutrophils is augmented by cGMP, and that muscarinic receptor agonists are able to modulate enzyme release by elevating the intracellular levels of cGMP.

Although there is increasing evidence to suggest the presence of mAChRs on lymphocytes and that activation of these receptors by ACh or AChR agonists leads to detectable physiological and functional changes, the demonstration of cholinergic innervation of the primary and secondary lymphoid organs has yet to be established. Bellinger and co-workers (1993) localised AChE activity in the rat spleen, with enzyme reactivity observed in neuronal and non-neuronal elements. However, choline acetyltransferase (ChAT) the synthetic enzyme for ACh could not be detected by radiochemical assay, and surgical denervation of the spleen by removal of the vagus nerve had no effect on AChE activity. Removal of sympathetic innervation to the spleen using 6-hydroxydopamine (6-OHDA) lead to the removal of noradrenergic nerve profiles and a significant reduction in AChE activity. These findings lead the authors to speculate that there was little or no cholinergic innervation of the spleen. More recently, Schäfer and colleagues (1998) attempted to localise cholinergic innervation of the peripheral organs immunohistochemically using polyclonal antibodies raised against rat vesicular acetylcholine transporter (VAChT). This protein is expressed on the ACh-containing synaptic vesicles which are abundant in nerve terminals and cell bodies. Their work was

in accordance with the previous findings of Bellinger *et al.* (1993), with little or no VAChT-positive staining found in the vasculature or parenchymal tissue of the lymphoid organs.

In order to investigate the possible presence of mAChRs and determine the effects of mAChR agonists, lymphocytes were isolated from the spleen and mesenteric lymph nodes (MLN) of uninfected and *N. brasiliensis*-infected animals, and the proliferative responses to cholinergic agonists were examined.

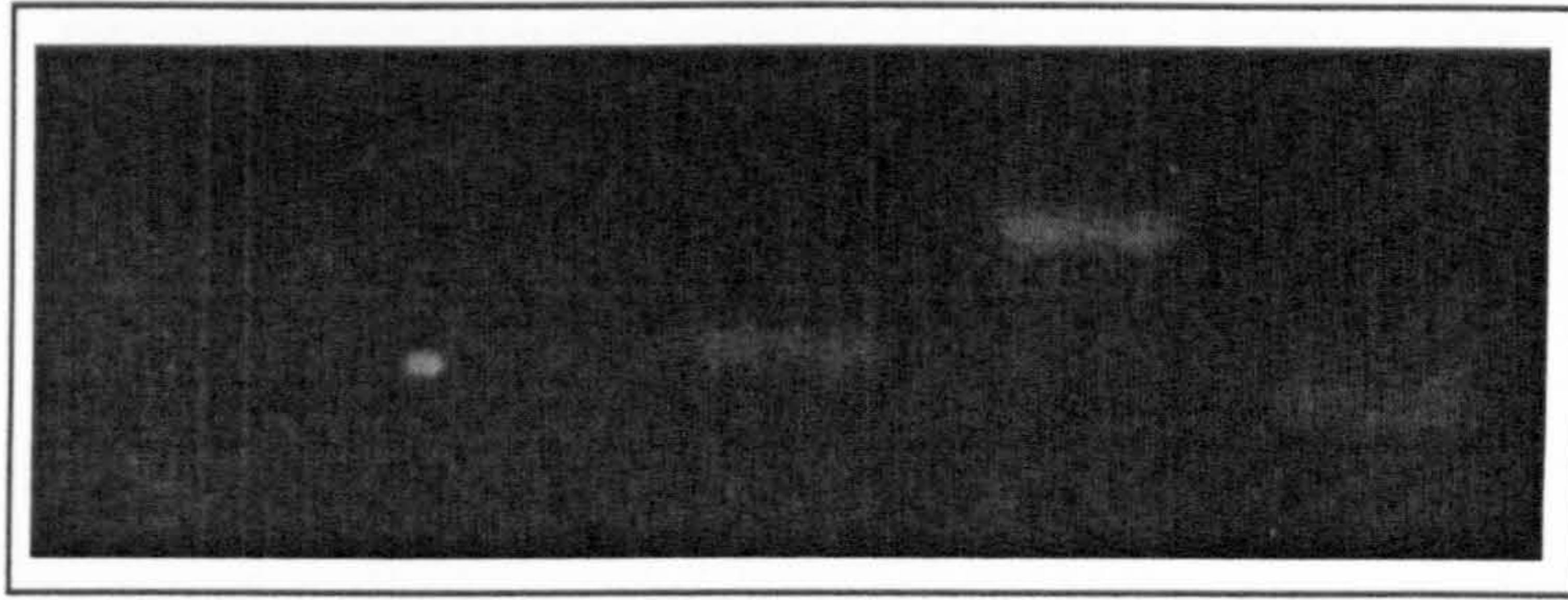
5.2 Analysis of lymphocyte mAChRs by RT-PCR

Mononuclear cells were isolated from the spleen and mesenteric lymph nodes (MLN) of uninfected animals, and infected animals (7 days post-infection). RNA samples were extracted, DNase-treated, and reverse transcribed for the determination of mAChR subtypes by PCR, with the efficiency of the PCR reaction assessed using primers specific for the housekeeping gene beta actin (β -actin).

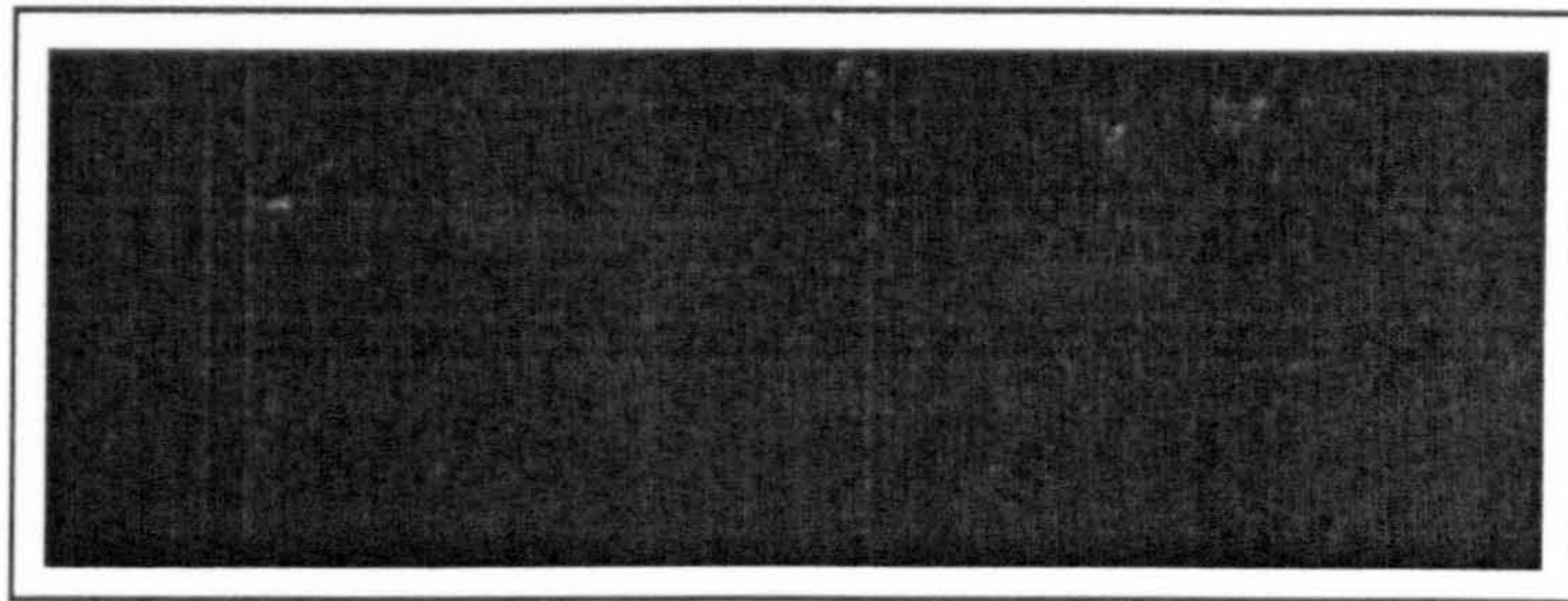
mRNA for mAChRs were detected in both splenocytes and MLN cells from uninfected animals (Fig 5.1 & 5.2), with variation between animals in the receptor subtypes expressed. The predominant mAChR subtype observed in uninfected spleen was m4 (Fig 5.1), with no m2 receptor detected. MLN cells expressed mRNA for several receptor subtypes (Fig 5.2), with m4 detected in all samples studied and m3 and m5 observed in the majority of samples. β -actin primers were used as a positive control for the efficiency of the reverse transcription reaction. cDNA was amplified with these primers and the expected 400 base pair (bp) product was detected. In splenocytes isolated from infected animals (Fig 5.3), mRNA was detected for m1, m2 and m4

receptor subtypes, but no animal expressed a full complement of mAChRs. It is interesting that during *N. brasiliensis*-infection, rat spleen cells appear to express the m2 receptor. This receptor subtype was not observed in uninfected splenocytes, and has previously been reported to be absent from rat blood and spleen mononuclear cells using Northern blot analysis (Costa *et al.*, 1994). Infected MLN cells (Fig 5.4) appear to predominantly express the m3 and m4 receptors, but again the pattern of receptor subtype expression varied between animals.

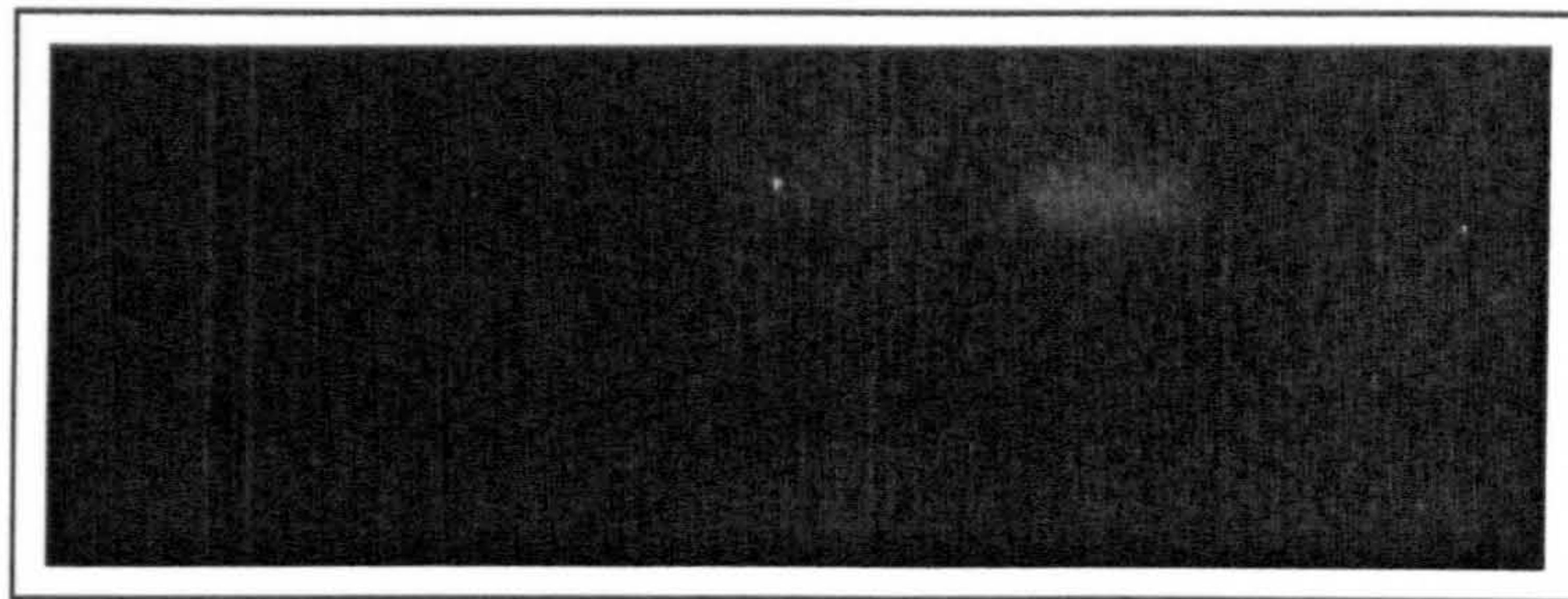
A



B



C



1 2 3 4 5

Figure 5.1. Expression of mAChRs in mononuclear cells isolated from the spleen of uninfected rats. cDNA samples were taken from individual animals (A, B, C) and amplified with specific primers for m1 (1), m2 (2), m3 (3), m4 (4), and m5 (5). PCR products were separated by agarose (1.2%) gel electrophoresis and stained with ethidium bromide.

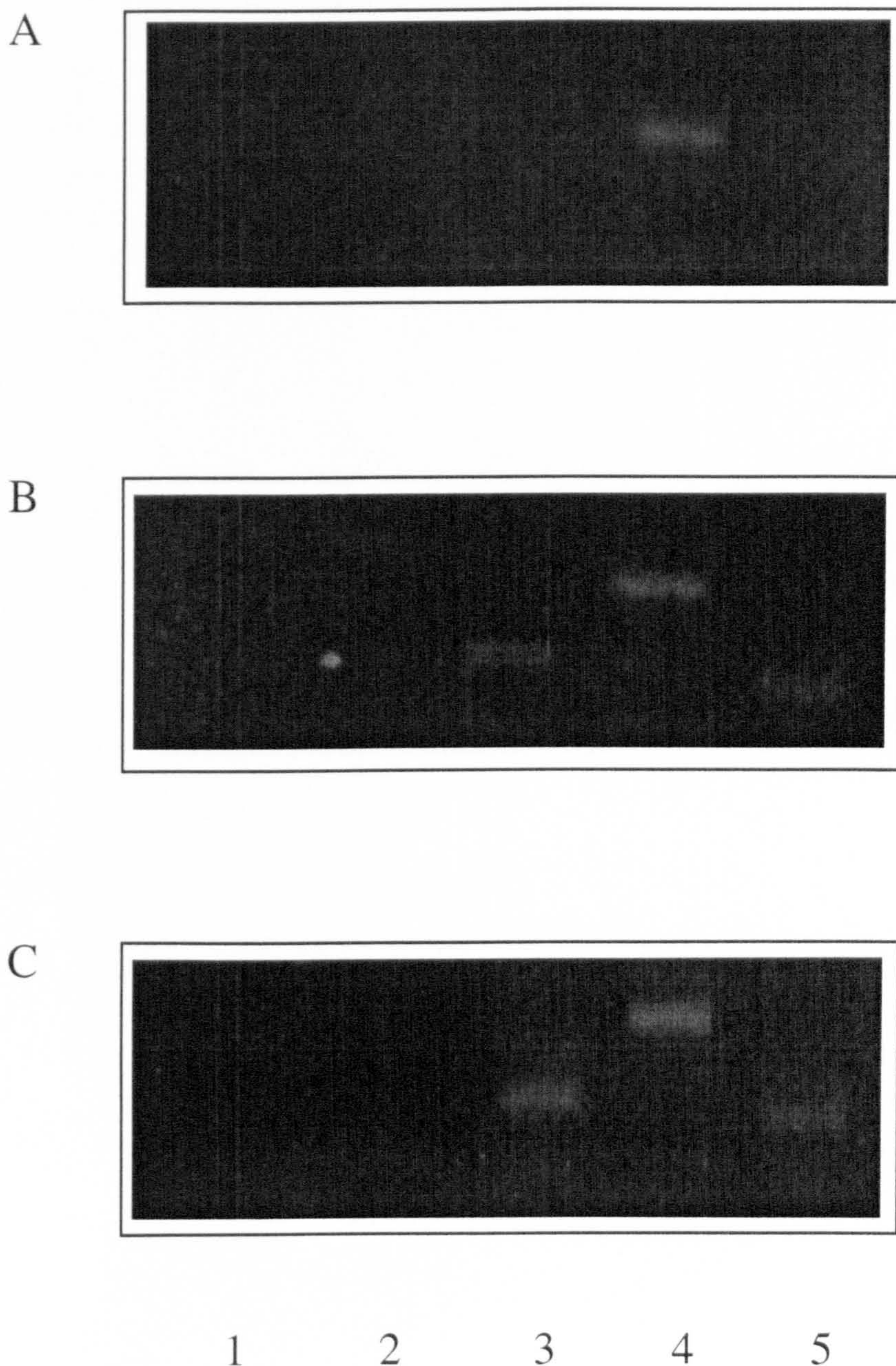


Figure 5.2. Expression of mAChRs in mononuclear cells isolated from the mesenteric lymph nodes of uninfected rats. cDNA samples were taken from individual animals (A, B, C) and amplified with specific primers for m1 (1), m2 (2), m3 (3), m4 (4), and m5 (5). PCR products were separated by agarose (1.2%) gel electrophoresis and stained with ethidium bromide.

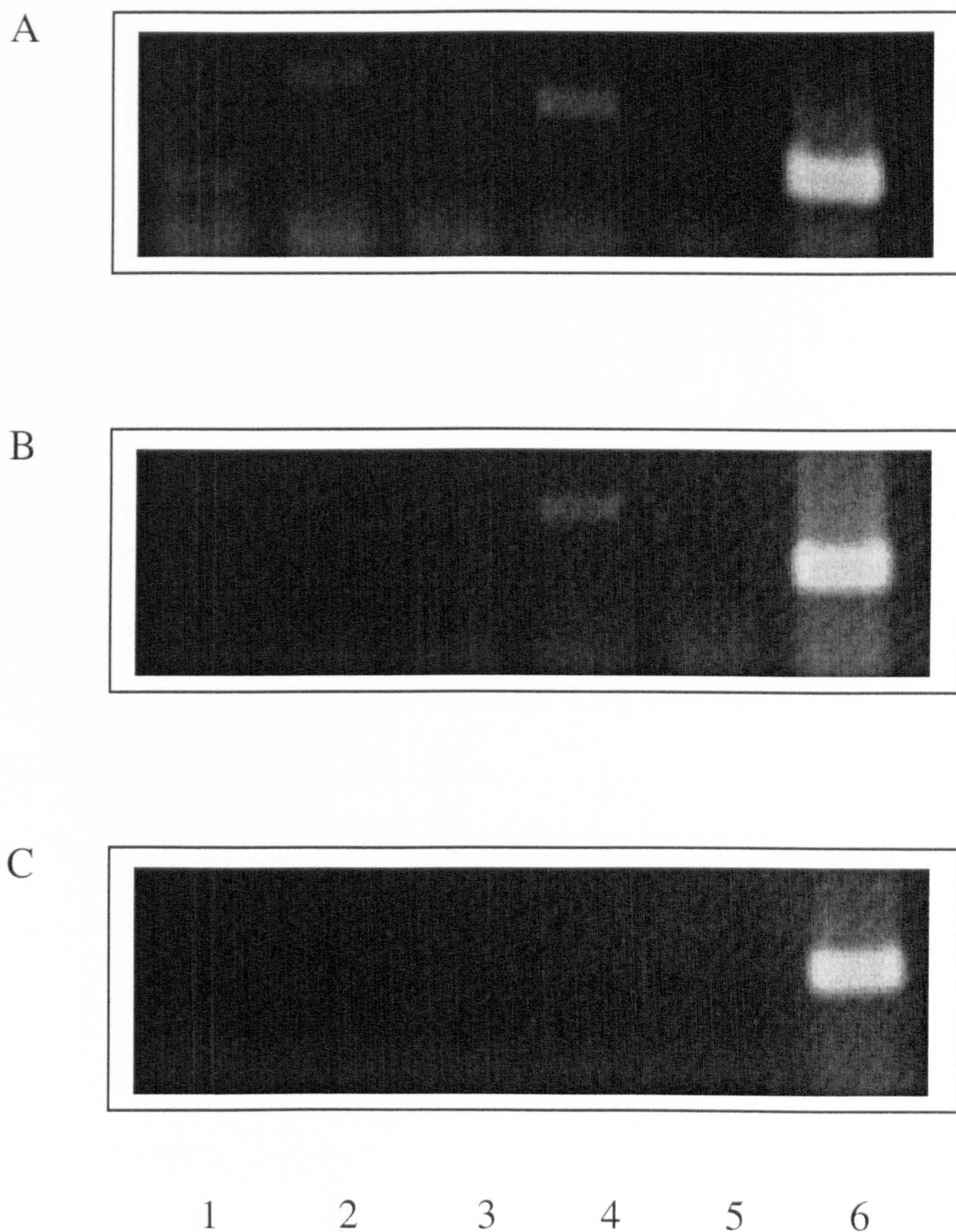


Figure 5.3. Expression of mAChRs in mononuclear cells isolated from the spleen of *N. brasiliensis* -infected rats (D7 p.i.). cDNA samples were taken from individual animals (A, B, C) and amplified with specific primers for m1 (1), m2 (2), m3 (3), m4 (4), m5 (5) and β -actin (6). PCR products were separated by agarose (1.2%) gel electrophoresis and stained with ethidium bromide.

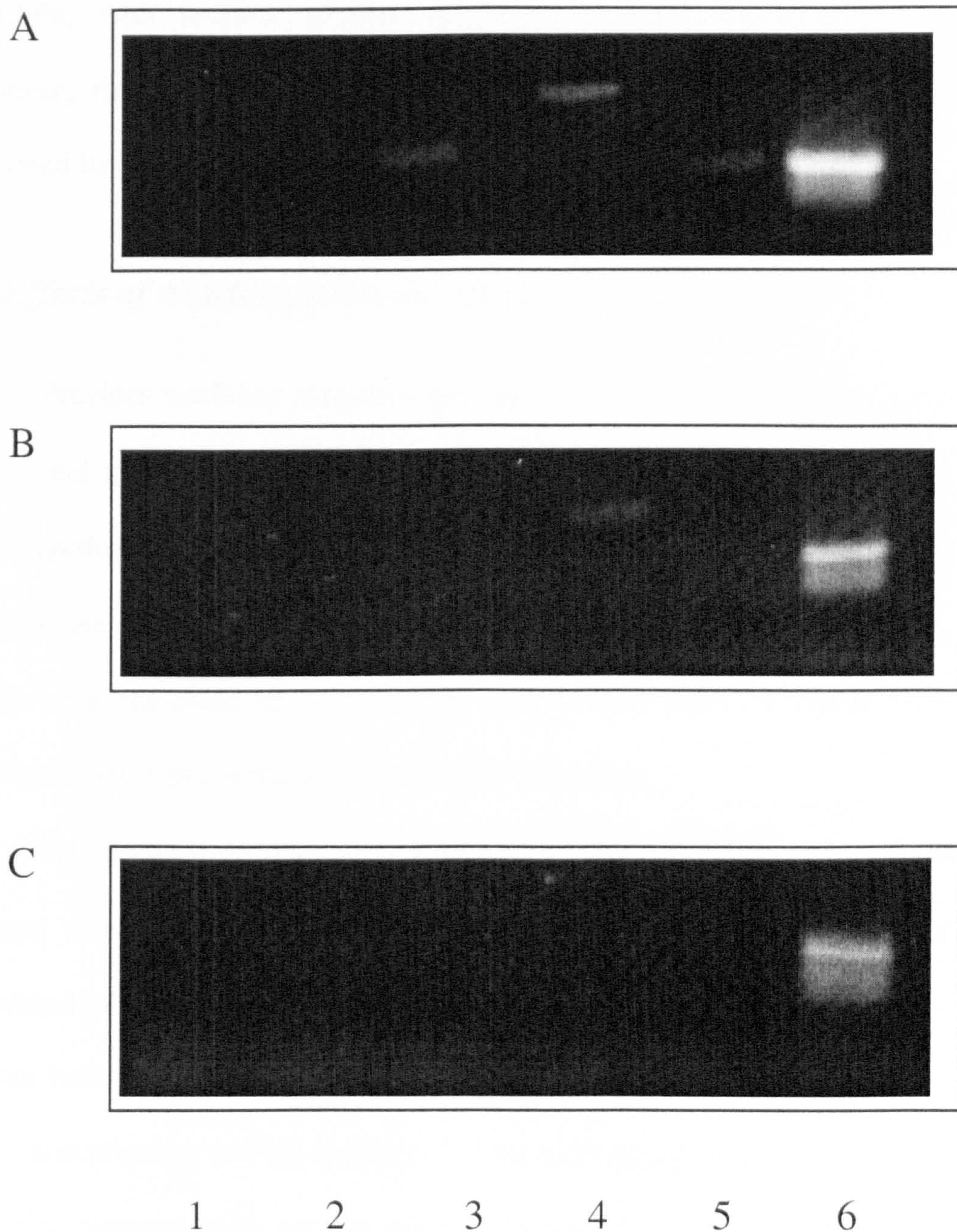


Figure 5.4. Expression of mAChRs in mononuclear cells isolated from mesenteric lymph nodes of *N. brasiliensis* -infected rats (D7 p.i.). cDNA samples were taken from individual animals (A, B, C) and amplified with specific primers for m1 (1), m2 (2), m3 (3), m4 (4), m5 (5) and β -actin (6). PCR products were separated by agarose (1.2%) gel electrophoresis and stained with ethidium bromide.

These results suggest that both splenocytes and MLN cells express mRNA for mAChRs, with receptor subtype differences detected from animal to animal. However, further studies are required to completely define the receptor subtypes expressed by these cell populations.

5.3 Effects of AChR agonists on lymphocyte proliferation

Previous work has suggested that mAChR agonists evoke increases in $[Ca^{2+}]_i$ and inositol trisphosphate ($InsP_3$) in Jurkat cells (Kaneda *et al.*, 1993), and enhance DNA synthesis and IL-2 production in PHA-stimulated human peripheral blood lymphocytes (Fujino *et al.*, 1997). The effects of mAChR agonists on the proliferative responses of mononuclear cells isolated from *N. brasiliensis*-infected spleen and MLN preparations were therefore investigated.

To demonstrate that rat lymphocytes proliferate in response to the T-cell mitogen concanavalin-A (Con A), splenocytes from uninfected animals were stimulated with increasing doses of Con A in a 96 well plate for 24 hours. Con A induces lymphocyte proliferation in a dose-dependent manner (Fig 5.5A), assessed by DNA incorporation of [3H] thymidine. The stimulatory effects of mAChR agonists were first assessed using agonist alone. Treatment of uninfected splenocytes over a range of concentrations (0-100 μM) with the mAChR agonist oxotremorine M (oxo-M) resulted in no significant increase in proliferation (Fig 5.5B).

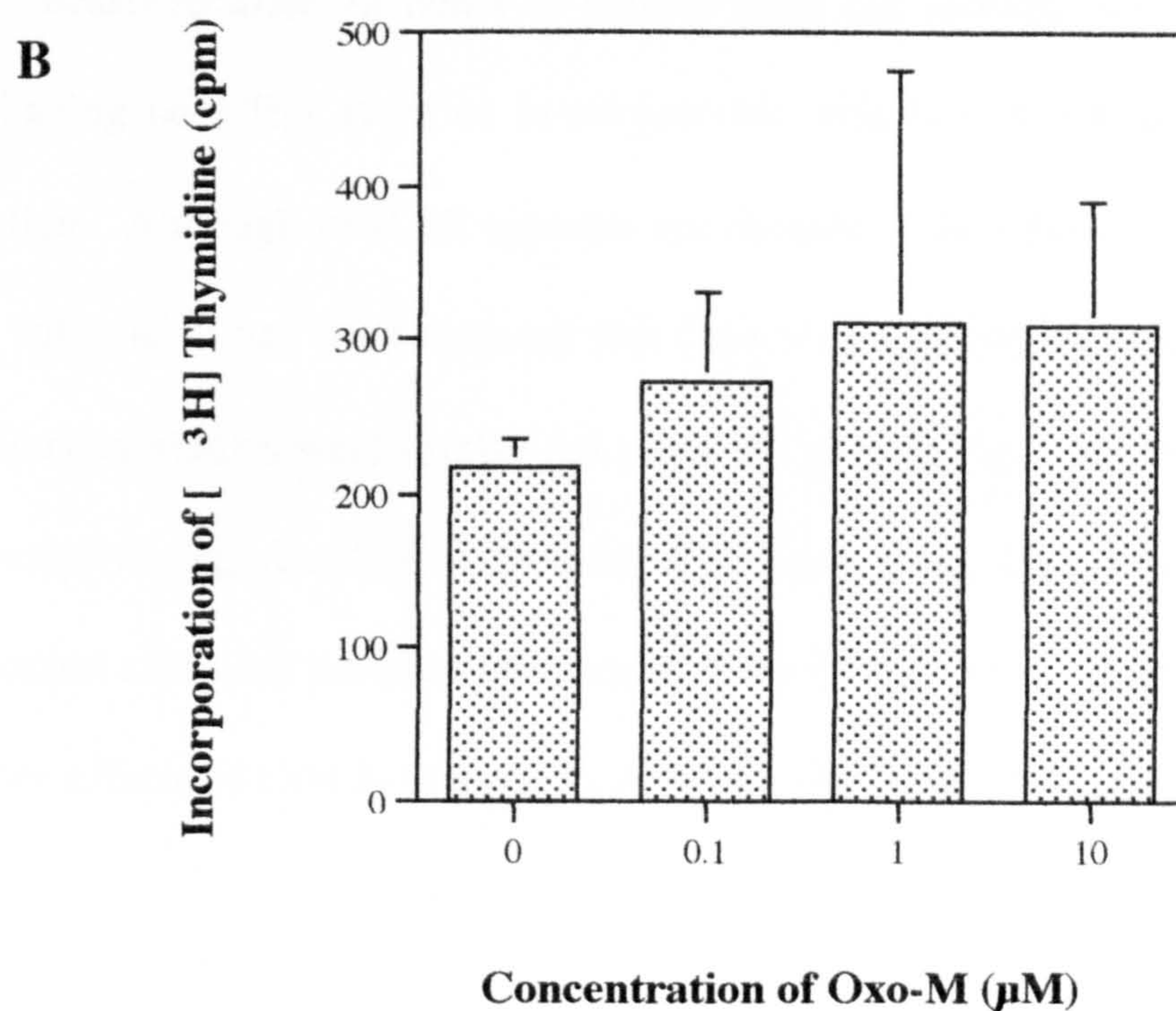
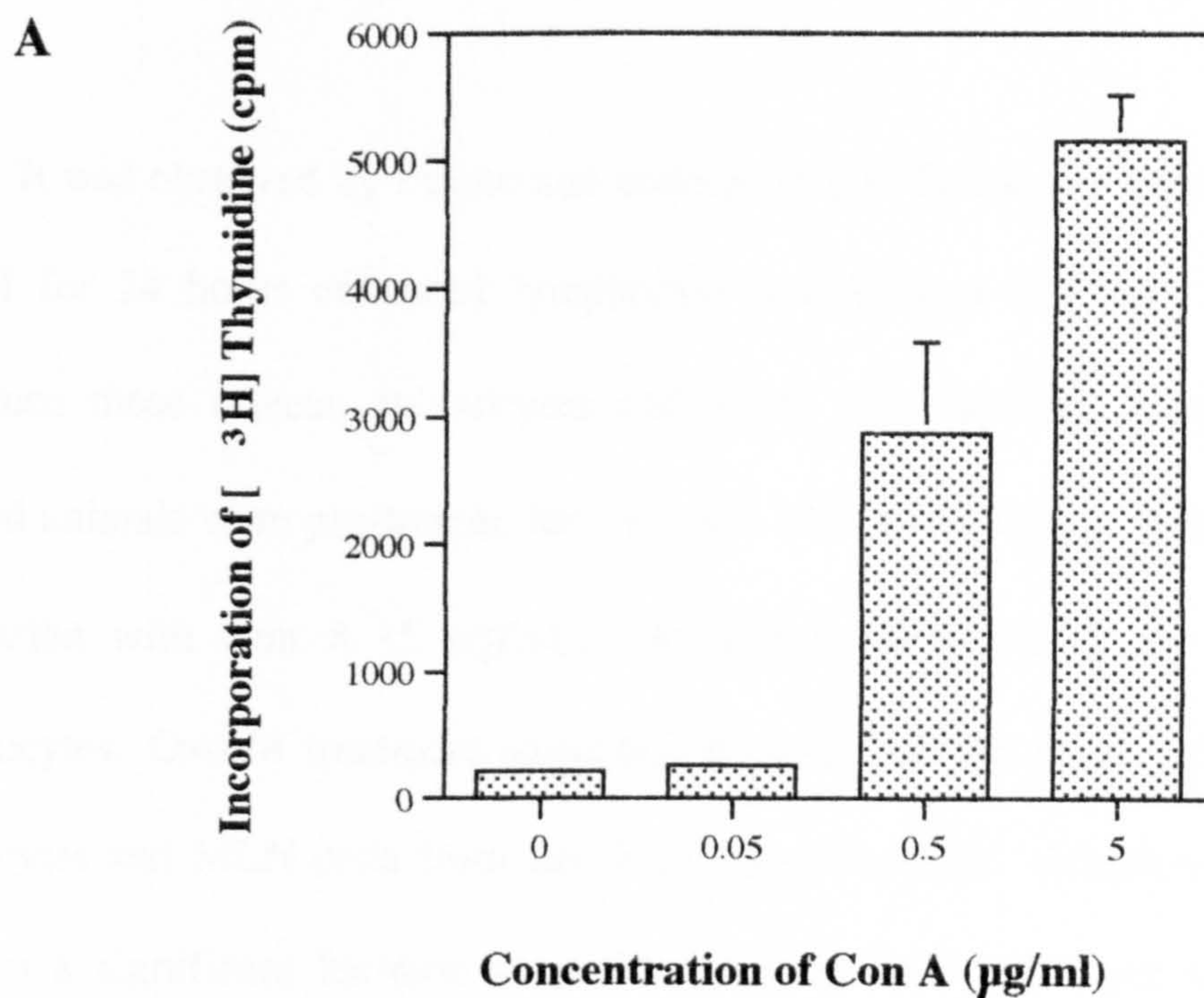


Figure 5.5. The effect of various concentrations of Con A (A) and Oxo-M (B) on lymphocyte proliferation. Splenocytes were isolated from uninfected animals and cultured in the presence of different concentrations of Con A or Oxo-M for 24 hours. DNA synthesis was assayed by [³H] thymidine incorporation during the last 8 hours of incubation. All experiments were carried out in triplicate and are expressed as the mean + standard deviation.

It was observed by Fujino and colleagues (1997) that pre-treatment with 1 μ M Oxo-M for 24 hours enhanced lymphocyte proliferation to PHA. In an attempt to reproduce these effects, splenocytes and MLN cells isolated from *N. brasiliensis*-infected animals were pre-treated for 24 hours with 1 μ M Oxo-M prior to lymphocyte stimulation with Con A (5 μ g/ml). As demonstrated previously with uninfected lymphocytes, Oxo-M treatment alone had no effect on the proliferative responses of splenocytes and MLN cells from day 7 p.i. rats (Fig 5.6). Addition of Con A alone leads to a significant increase in proliferation in both cell types studied, but pre-treatment for 24 hours with Oxo-M did not augment this response. DNA synthesis was also measured after 72 hours in culture (data not shown), with the addition of Oxo-M having no effect alone or in conjunction with Con A stimulated lymphocyte proliferation. Although mAChR agonists are thought to be relatively non-selective of receptor subtype, it has been reported that Oxo-M is a potent M2 agonist (Caulfield, 1993). Further studies were carried out using the mAChR agonist carbachol (Fig 5.7) in an attempt to augment Con A-stimulated proliferation. Again, these results show that carbachol alone has no effect on lymphocyte proliferation and did not enhance the stimulatory effects of Con A when cells are pre-treated with this agonist for 24 hours.

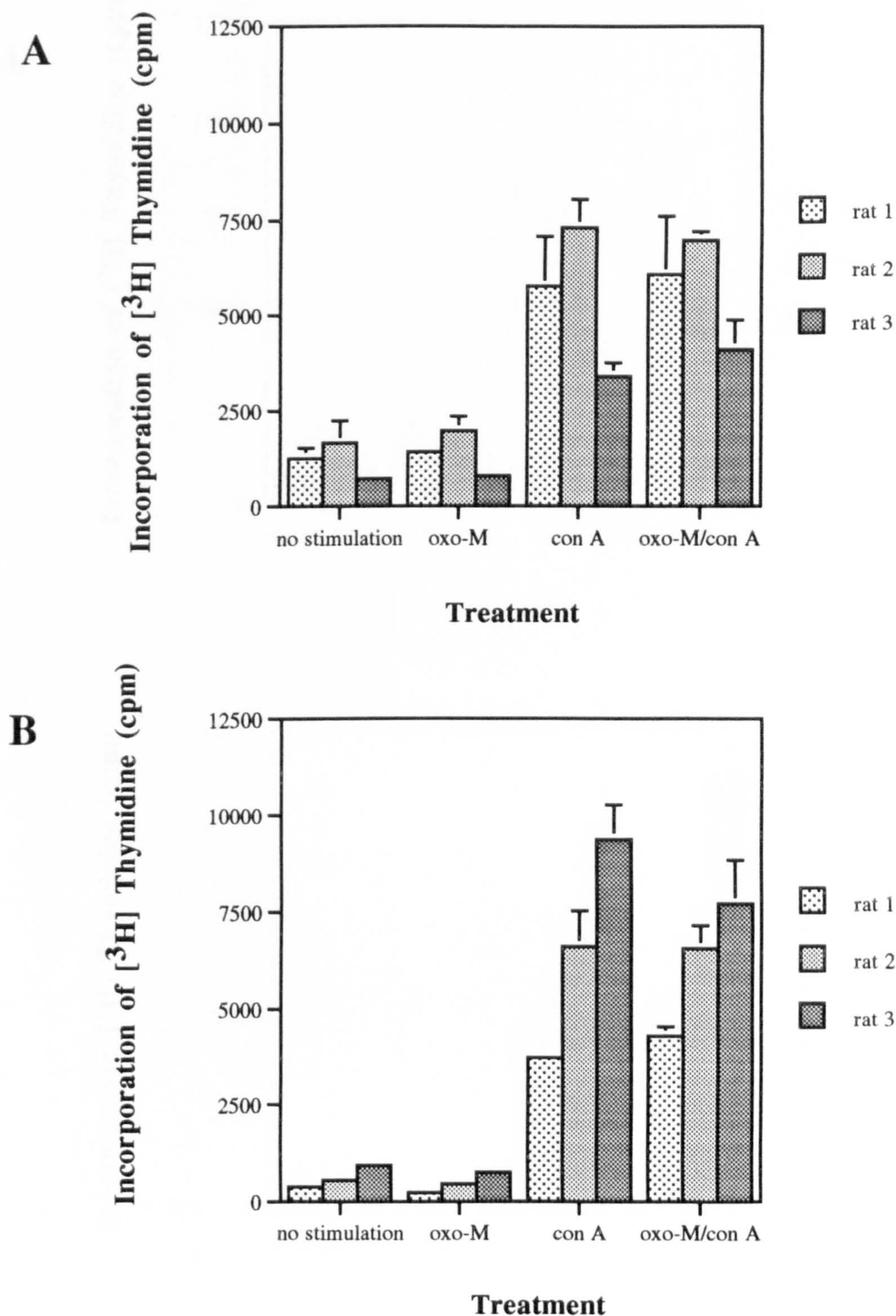


Figure 5.6. The effects of Oxo-M and Con A stimulation on proliferative responses. Mononuclear cells were isolated from *N. brasiliensis*-infected (D7 p.i) rat spleen (A) and MLN (B). 2×10^5 cells were cultured for 24 hours in the presence of Oxo-M ($1 \mu\text{M}$) or Con A ($5 \mu\text{g/ml}$) alone, or pre-treated with Oxo-M for 24 hours before addition of Con A. Proliferation was assayed by [^3H] thymidine incorporation during the last 8 hours in culture. All experiments were carried out in triplicate and are expressed as the mean + standard deviation.

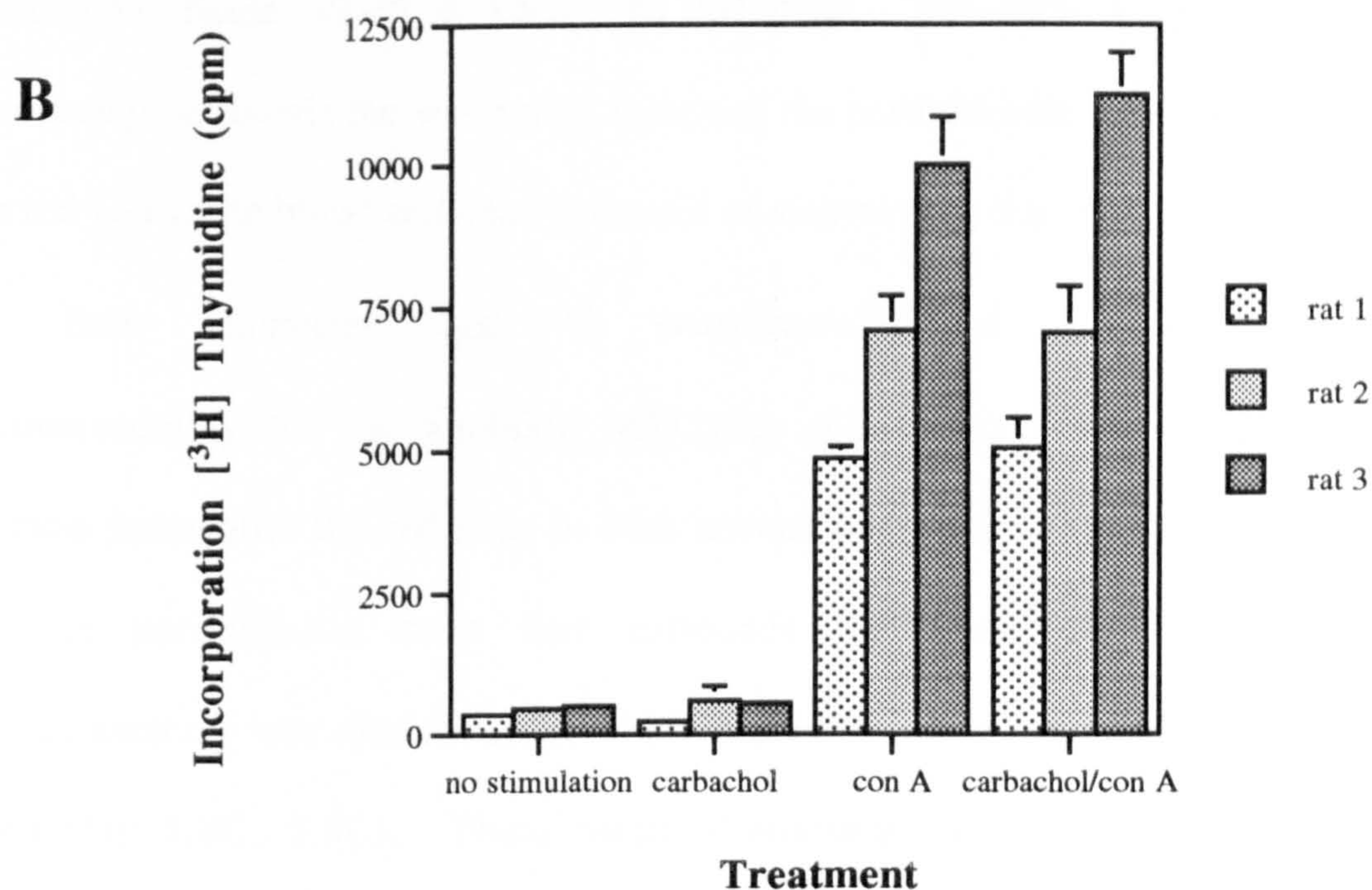
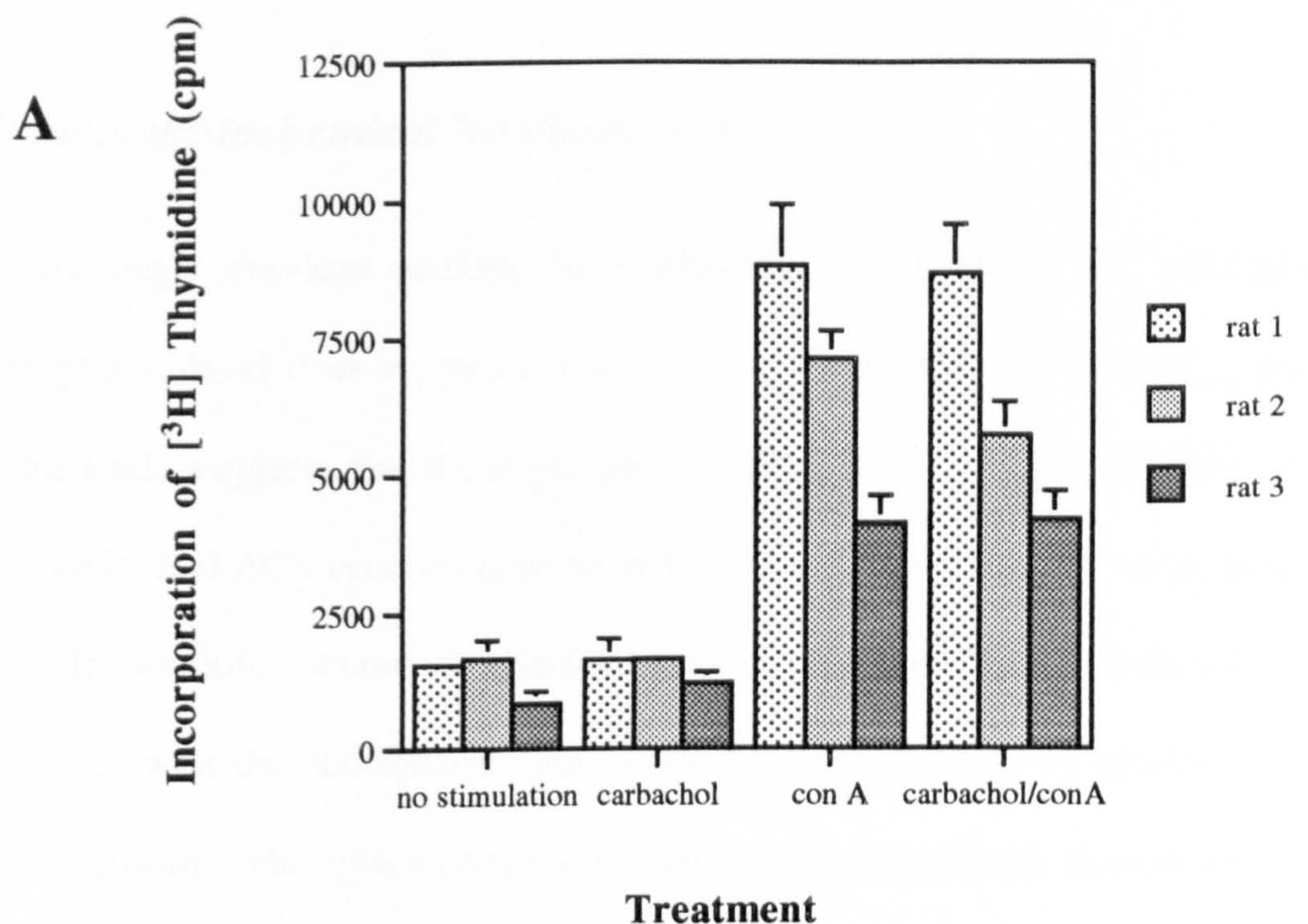


Figure 5.7. The effects of carbachol and Con A stimulation on proliferative responses. Mononuclear cells were isolated from *N. brasiliensis*-infected (D7 p.i.) rat spleen (A) and MLN (B). 2×10^5 cells were cultured for 24 hours in the presence of carbachol ($1 \mu\text{M}$) or Con A ($5 \mu\text{g/ml}$) alone, or pre-treated with carbachol for 24 hours before the addition of Con A. Proliferation was measured by [³H] thymidine incorporation during the last 8 hours in culture. All experiments were carried out in triplicate and are expressed as the mean + standard deviation.

5.4 Immunohistochemical localisation of mAChRs

Although previous studies have reported the presence of mAChRs on lymphocytes isolated from rat and mouse spleen (Strom *et al.*, 1975, 1981), the data from this study suggests that the expression of mRNA for mAChRs is relatively low in these cells, and ACh agonists have no effect on cell proliferation. In an attempt to clarify this situation, sections of spleen isolated from uninfected and infected animals were stained with the pan-specific anti-mAChR antibody (M35) to localise mAChRs within the spleen. The spleen consists of many lymphoid follicles termed white pulp, which are surrounded by a highly vascular network containing sinuses, capillaries and parenchymal tissue which is known as red pulp. The area of red pulp which immediately surrounds the white pulp is termed the perifollicular zone, which contains both red and white blood cells and is devoid of sinuses (Fig 5.8, 5.9).

Both uninfected and *N. brasiliensis*-infected spleens demonstrate immunoreactivity for the antibody M35 (Fig 5.9, 5.10). Intense staining was observed throughout the red pulp in both normal and infected tissue, and extended into the perifollicular zone that surrounds the white pulp. Faint M35 immunoreactivity was also localised within the white pulp of uninfected and infected spleen (Fig 5.8C, 5.9C). These results demonstrate no apparent changes in the expression or localisation of mAChRs within the spleen during *N. brasiliensis*-infection.

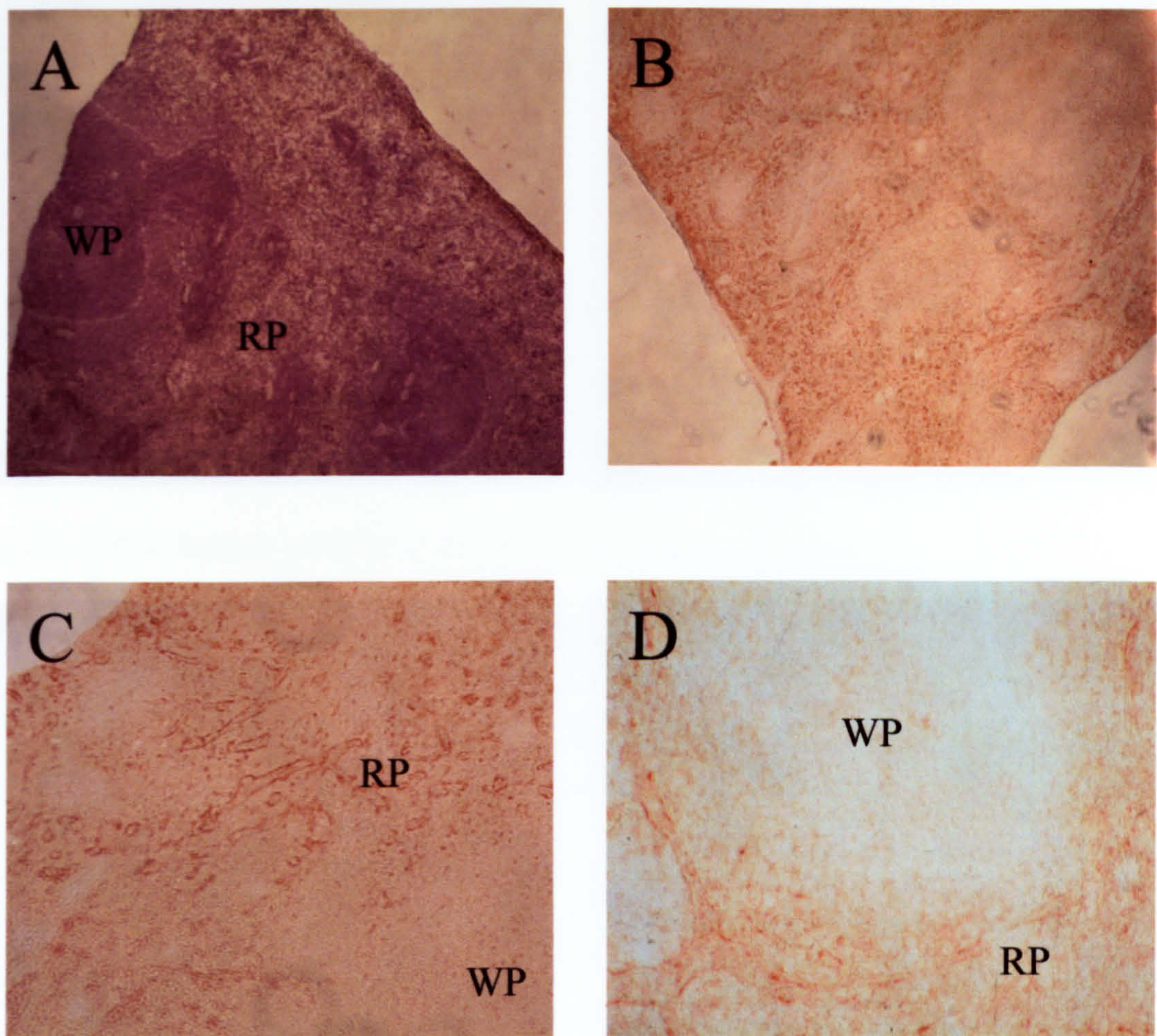


Figure 5.8. The localisation of mAChR-immunoreactivity in uninfected rat spleen. Sections were stained with haematoxylin (A) or with the monoclonal antibody M35 (B-D). Strong immunoreactivity was observed throughout the red pulp (RP), with faint staining detected in the white pulp (WP). Magnification was x80 (A-B), x125 (C), and x312 (D).

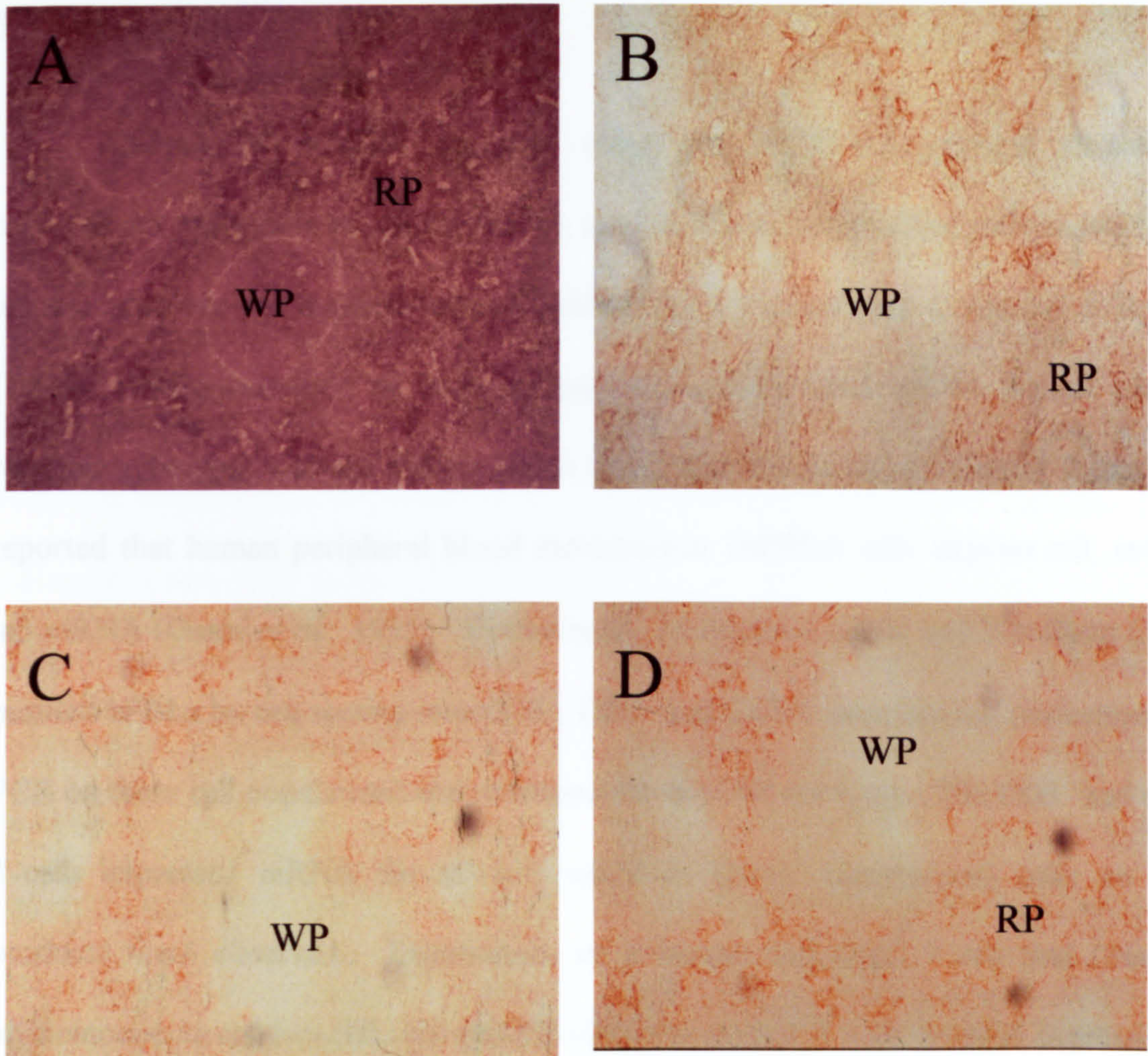


Figure 5.9. Determination of mAChR localisation in *N.brasiliensis*-infected rat spleen. Spleen sections were stained with haematoxylin (A), or the monoclonal antibody M35. mAChR-positive staining was observed throughout the red pulp (RP), with little immunoreactivity within the white pulp (WP). Magnification was x80 (A), x125 (B), x312 (C-D).

5.5 Discussion

Analysis of mAChR subtypes using the RT-PCR technique revealed a predominant expression of mRNA for m3, m4 and m5 in mononuclear cells isolated from uninfected spleen and mesenteric lymph node (MLN) preparations. Costa and colleagues (1994a, 1994b) examined rat mononuclear cells and detected mRNA for m3 and m4 receptor subtypes, with no mRNA for m1, m2, and m5 receptors found. It has been reported that human peripheral blood mononuclear (hPBLs) cells express m3, m4 and m5 mRNA (Costa *et al.*, 1995). Furthermore, Hellström-Lindahl and Nordberg (1996) purified hPBLs by cell sorting into CD3⁺, CD4⁺ and CD14⁺ populations, performed RT-PCR on these cell populations and examined the mAChR subtypes. The CD3⁺ and CD4⁺ T-cells expressed mRNA for m3-m5, whilst in CD14⁺ (monocytes) cells no PCR products were observed. Examination of a human leukaemic T-cell line (Peer), a myelomonocyte cell line (HL-60) and EB virus-transfected B cells revealed expression of m3 and m5 by Peer cells, with m4 and m5 in HL-60 cells, but no expression of m3-m5 in the transfected B-cells. This substantiates previous work by Eva and co-workers (1989) utilising radioligand binding studies, which reported a lack of binding by the mAChR agonist [³H] N methyl scopolamine (NMS) to human monocytes and B-cells.

Splenocytes isolated from *N. brasiliensis*-infected animals expressed some m1 and m2 mRNA, whilst MLN cells expressed the m3-m5 profile found in the uninfected animals. The PCR bands observed for mAChRs from both control and infected animals were fainter than those bands detected in rat brain, rat intestinal epithelium and lamina propria cell preparations observed in the previous chapter. Work utilising radioligand binding studies, RT-PCR, and Southern blotting techniques has suggested that mAChR

levels are lower in rat lymphocytes than those found rat brain and hPBMLs (Costa *et al.*, 1994b, 1995; Eva *et al.*, 1989; Strom *et al.*, 1981).

As described in previous reports, the addition of the T-cell mitogen Con A results in a significant increase in DNA synthesis, which is dose-dependent. However, the Con A-stimulated proliferative responses of mononuclear cells isolated from the spleen or mesenteric lymph nodes of *N. brasiliensis*-infected rats were unaffected by pre-incubation with the mAChR agonists Oxo-M, carbachol, and muscarine chloride. These findings contrast with those of Fujino and colleagues (1997), where pre-treatment of human peripheral blood mononuclear cells with Oxo-M resulted in a significant augmentation of proliferative responses to PHA. However, it should be noted that the literature contains conflicting reports on the effects of mAChR agonists on DNA synthesis. It has been reported that the proliferative responses of hPBLs to carbachol are dose-dependent, and incorporate both muscarinic and nicotinic properties. Both high (1 μ M) and low (0.1 nM) doses of carbachol increased cell proliferation via stimulation of mAChRs (determined by addition of the mAChR antagonist atropine), whereas concentrations of carbachol between 1 and 10 nM inhibited *in vitro* proliferation and were blocked by the nicotinic receptor antagonists α -bungarotoxin and *d*-tubocurarine (Richman & Arnason, 1979). The stimulation of DNA synthesis using mAChR agonists has also been reported in human lymphocytes (MacManus *et al.*, 1979) and mouse splenic lymphocytes (Strom *et al.*, 1975). In contrast, Arzt *et al.* (1989) reported inhibition of DNA and protein synthesis in PHA-stimulated human PBMLs by the mAChR agonist pilocarpine. The inhibitory effect upon lymphocyte proliferative responses to PHA was reversed by the addition of atropine or pirenzepine, leading the authors to suggest that this was mediated through the M1 muscarinic receptors.

Pilocarpine also inhibited DNA synthesis in unstimulated lymphocytes, whereas carbachol stimulated proliferation after 48 but not 72 hours in culture, and had no effect on PHA-induced lymphocyte proliferation. Richman and Arnason (1979) reported that carbachol-induced DNA synthesis was first observed after 48 hours in culture and peaked at 72 hours, whereas carbachol augmentation of PHA-induced proliferation was only detected after 24 hours. The lack of DNA synthesis in response to cholinergic agonists after 24 hours in culture was also observed by Hadden and colleagues (1974, 1976), but RNA and protein synthesis were increased. The situation is therefore confusing, but it is possible that in our model system, activation of lymphocytes by mAChR agonists leads to functional changes not examined in this study.

Strom and co-workers (1981) observed an increase in mAChR binding sites from rat and mouse splenocytes and hPBLs stimulated with Con A, and reported that cholinergic agonists augmented the effector function of cytotoxic T-cells. It has also been reported that the mAChR agonist pilocarpine significantly reduced the production of IFN- γ from PHA-stimulated hPBLs (Arzt *et al.*, 1989). Interestingly, ACh has been detected in whole blood and plasma from various mammals (Fujii *et al.*, 1995), human mononuclear lymphocytes (Kawashima *et al.*, 1993), and in several human leukaemic T-cell lines (Fujii *et al.*, 1996; Kawashima *et al.*, 1998). PHA stimulation of the T-cell lines HSB-2 and MOLT-3, or lymphocytes isolated from rat thymus, spleen and peripheral blood resulted in an increase in the levels of intracellular and released ACh (Fujii *et al.*, 1996; Rinner *et al.*, 1998), which was reduced using inhibitors of choline acetyltransferase (ChAT) and carnitine acetyltransferase (CarAT; Fujii *et al.*, 1996). Furthermore, mRNA for ChAT was observed in rat thymic, splenic and peripheral blood lymphocytes (Rinner

et al., 1998), human PBMLs (Fujii *et al.*, 1998), and human leukaemic T-cells (Fujii *et al.*, 1996).

In these studies immunohistochemical techniques were used to demonstrate mAChR-positive staining within normal and *N.brasiliensis*-infected spleen with the mAChR-specific antibody M35. mAChR reactivity was essentially localised to the red pulp and perifollicular zones in the spleen of both uninfected and infected animals, with some faint staining also demonstrated in the lymphocyte-rich white pulp. It has been reported that M35 does not discriminate between the 5 mAChR subtypes, and has been used to localise mAChRs in different species and tissues (Carsi-Gabrenas *et al.*, 1997).

Evidence for cholinergic innervation of primary and secondary lymphoid organs has yet to be established, primarily due to the lack of good anatomical markers for cholinergic nerves in peripheral organs (Bellinger *et al.*, 1993). AChE histochemistry had been widely used to determine cholinergic innervation of peripheral organs, however work by Bellinger *et al.* (1993) led to the speculation that the spleen lacked cholinergic innervation. AChE activity could be localised to the rat spleen, but this activity was not ablated by vagotomy, and showed a similar profile to noradrenergic (NA) nerve localisation after sympathetic denervation with 6-OHDA, suggesting that the AChE reactivity within the spleen was co-localised with NA nerve fibres and not cholinergic fibres. The authors also observed extremely low ChAT activity within the spleen, lymph nodes and thymus, which lead them to suggest that there is little, if any cholinergic innervation of the lymphoid organs. More recently, Schäfer and colleagues (1998) identified cholinergic innervation ^{of} the heart, lung, gastrointestinal tract and bladder of rat using antisera raised against the vesicular acetylcholine transporter (VAChT), and observed little VAChT-positive staining along the vasculature and within

the parenchymal tissue of the lymphoid organs, the kidney and liver. In an attempt to map the cholinergic distribution of rat superior olivary complex, Yoa and Godfrey (1997) compared AChE histochemistry, ChAT immunohistochemistry, mAChR (M35) immunohistochemistry and mAChR autoradiography ($[^3\text{H}]$ NMS). These studies suggested that ChAT-immunoreactivity and the majority of AChE staining was localised throughout cholinergic nerve bodies, but absent from cholinceptive sites (receiving cholinergic synapses), whereas M35-immunoreactivity was only observed at cholinceptive sites. Taken together, these data suggest that although the rat spleen receives no cholinergic innervation as revealed by AChE histochemistry and VChT-immunohistochemistry (Bellinger *et al.*, 1992; Schäfer *et al.*, 1998), mAChR-immunoreactivity is present within the red pulp and parafollicular zones.

Future work should concentrate on the identification of mAChR-positive cells using the monoclonal antibody (M35) in conjunction with *in situ* hybridisation techniques to determine the localisation and density of receptors, with the expression of mRNA for the receptor. Once these cells have been identified and isolated, the functional effects of adding receptor agonists such as carbachol or oxo-M should be easier to interpret.

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