EFFECTS OF CYTOKINES AND EXTRACELLULAR MATRIX ON PERIPHERAL GLIA

by

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ABSTRACT

This thesis deals with: (A) The regulation of the major histocompatibility complex class II molecules on Schwann cells in vivo and in vitro, (B) the induction of the major histocompatibility complex class II molecules on oligodendrocytes and (C) the effect of collagen type I, which is a major component of the extracellular matrix, on the function of enteric glial cells.

The expression of class II molecules on Schwann cells in vivo and in vitro was investigated, in particular the question of whether Schwann cells are able to present mycobacterial antigen to sensitized lymphocytes. When Schwann cells were cocultured together with antigen and T cells they could support the lymphoproliferative responses of mycobacteria-reactive T lymphocytes without pretreatment with interferon-γ. After the incubation period essentially all of the Schwann cells expressed class II antigen. T cell derived interferon-γ and tumor necrosis factor appeared to mediate the T cell induced class II expression on the Schwann cells. It was also found that the injection of cytokines or mycobacterial antigens into the living sciatic nerve, and crushing the nerve induced class II expression on some Schwann cells. Another molecule which is important in the T cell mediated immune response is interleukin-1. It was found that Schwann cells produce interleukin-1 when they are incubated with mycobacterial antigens or cytokines, and the interleukin-1 activity was seen both in the Schwann cell supernatant and in the cell lysate. These results further support the view that Schwann cells can function as antigen presenting cells and may participate in neuroimmunological responses within peripheral nerves.

When rat oligodendrocytes derived from the optic nerve were incubated with interferon-γ in the presence of dexamethasone they expressed the major histocompatibility complex class II molecules.

Collagen is a major component of the extracellular matrix and there is now evidence from several studies, particularly on epithelial cells but also on muscle cells, that collagen type I can influence cell proliferation, differentiation, migration and specific gene expression. A possible role for type I collagen in the induction of differentiation of neural cells was investigated, using the enteric nervous system of the gut. When the myenteric plexus of a newborn guinea pig is taken into culture and grown on a 2-dimensional substrate, the glia start to divide and migrate away from the neurons, and the network-like arrangement of the plexus breaks down. To test whether collagen type I could prevent these changes, the myenteric plexus freshly dissected from the gut was embedded in a 3-dimensional collagen gel and grown in a defined medium containing 0.5% FCS. In this case the glial cells did not migrate away from the neurons and the plexus stayed as a network. When a disaggregated culture of the myenteric plexus was embedded in a 3-dimensional collagen gel the cultures rearranged into a network of small ganglia and interconnecting strands, very similar to the myenteric plexus in situ. Electron microscopy showed that the ultrastructure of a reformed plexus was very similar to that seen in situ. These results show that type I collagen prevents the breakdown of the myenteric plexus and also induces network formation in a disaggregated culture.

FIGURES

PUBLICATIONS

The folowing papers and conference presentations have been published and presented based on work from this thesis.

Papers:

Kingston, A. E., Bergsteinsdottir, K., Jessen, K. R., Van der Meide, P. H., Colston, M. J., Mirsky, R. (1989) Schwann cells co-cultured with stimulated T cells and antigen express major histocompatibility complex (MHC) class II determinants without interferon-γ pretreatment: synergistic effects of interferon-γ and tumor necrosis factor on MHC class II induction. Eur. J. Immunol. 19, 177-83.

Bergsteinsdottir, K., Kingston, A.E., Mirsky, R., Jessen, K.R. (1991) Rat Schwann cells produce interleukin-1. J. Neuroimmunol. 34, 15-33.

Bergsteinsdottir, K., Kingston, A.E., Jessen, K.R. (1991) Schwann cells can be induced to express major histocompatibility complex class II molecules in vivo. J. Neurocytology. In press.

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Bergsteinsdottir, K., Kingston, A.E., Jessen, K.R., Mirsky, R. (1990) Regulation of MHC class II expression on rat Schwann cells in vitro and in vivo. Eur. J. Neurosci. Suppl. 2, 71.14.

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CHAPTER 1

GENERAL INTRODUCTION

This thesis deals with: (A) The regulation of the major histocompatibility complex (MHC) class II molecules on Schwann cells in vivo and in vitro and the question of whether Schwann cells are able to produce interleukin-1 (IL-1). (B) The expression of the major histocompatibility complex class II molecules by oligodendrocytes in vitro. (C) The effect of collagen type I, which is a major component of the extracellular matrix, on the function of enteric glial cells.

Schwann cells of peripheral nerves

The Schwann cell is the major cell type of peripheral nerve, since the cell bodies of neurons are confined to the central nervous system (CNS) or peripheral ganglia. Schwann cells originate from the neural crest (Le Douarin et al.,1991), and in the rat sciatic nerve, Schwann cell precursors can be identified at day 14 to 15 of embryonic development (Jessen and Mirsky, 1991). Morphological observations on developing nerves in vivo and in vitro studies, indicate that myelin-forming and non-myelin-forming Schwann cells derive from a common precursor cell (reviewed in Mirsky and Jessen 1990). During development Schwann cells proliferate along axons, as they grow to form nerves in the developing limb (Keynes 1987), and

there is evidence that at this developmental stage the axon may control both Schwann cell proliferation and survival (Jessen and Mirsky 1991). Subsequently Schwann cells stop dividing and differentiate, giving rise to either the myelin-forming or the non-myelin-forming phenotype.

Proliferation is essential for expanding the Schwann cell population, and in development Schwann cells are generated during a phase of proliferation, followed by growth inhibition, which is a prerequisite to their differentiation (Peters and Muir, 1959; Morgan et al., 1990). The axonal surface may be a source of Schwann cell mitogens both in development and also in regeneration following Wallerian degeneration (Ratner et al., 1988). In the latter situation inhibition of Schwann cell division markedly impairs axon regrowth (Hall and Gregson, 1975). In vitro studies have shown that several growth factors (including glial growth factor (GGF)), stimulate Schwann cell DNA synthesis in vitro, (Raff et al., 1978; Brockes et al., 1980b) although whether they are present during PNS development is uncertain. Recent results have shown that transforming growth factor-β (TGF-β), acidic and basic fibroblast growth factors (FGF) and platelet-derived growth factor (PDGF) are also capable of stimulating Schwann cell DNA synthesis (Eccleston et al., 1987, 1989a, 1990; Davis and Stroobant, 1990; Lemke, 1990). FGF and a PDGF-like activity have been isolated from normal peripheral nerves (Eccleston 1990; Eckenstein 1991). Cultured Schwann cells are also stimulated to divide by fibronectin (Baron Van Evercooren et al., 1982; 1986), laminin (McGarvey et al., 1984; Eccleston et al.,1987), and type IV collagen (Eccleston et al.,

1987). Since cessation of division is a prerequisite for Schwann cell differentiation, it is interesting that three proteins have been shown, at least in rat, to inhibit Schwann cell proliferation under some conditions: type I collagen, TGF-β and IFN-γ (Eccleston et al., 1989a and b). Among these only type I collagen, which is a component of the PNS extracellular matrix, has been shown to inhibit DNA synthesis in neonatal Schwann cell cultures maintained in serum-containing medium. Schwann cell proliferation may be controlled by both stimulatory and inhibitory growth factors, and during development an alteration in the balance between stimulators and inhibitors of cell division may take place (for review see Lemke, 1990).

During development of PNS nerves, the myelin-forming Schwann cells differentiate first. They attain a 1:1 relationship with large axons and they do not divide once they have begun to form the myelin-sheath, which happens around the time of birth (Peters and Muir, 1959). The cells destined to become non-myelin-forming Schwann cells proliferate during the period of myelination and in mixed nerves these cells differentiate several weeks after myelination has commenced (Diner, 1965; Friede and Samorajski, 1968). In the rat sciatic nerve, mature unmyelinated fibers, in which several axons are enclosed in individual furrows in the surface of a single Schwann cell, appear first during the third postnatal week, and are fully differentiated 2-3 weeks later. The non-myelin-forming Schwann cells remain associated with smaller axons, and extend long thin processes that encircle and separate smaller axons from one another (Pannese et al., 1988 for review).

Axonal control of myelination has been clearly demonstrated

(Weinberg and Spencer, 1976; Aguayo et al., 1976), but how the axon instructs its associated Schwann cells to make and then to maintain myelin is not known. It has been suggsted that specific signal molecules initiate myelin formation, or alternatively that the axon diameter acts as a signal for myelination (Friede, 1972; Voyvodic, 1989). There is also evidence that the ECM is important in Schwann cell differentiation and myelination. Surrounding all Schwann cell-axon units is an endoneurial extracellular matrix, which consists of a basal lamina surrounding the surface of the Schwann cells and an interstitial matrix rich in collagen fibrils (Olson 1990). Schwann cells are able to synthesize and secrete a range of ECM molecules incuding collagens type I, II, IV and V (Carey et al., 1983; Bunge and Bunge, 1983), laminin (Cornbrooks et al.,1983; McGarvey et al., 1984) proteoglycans (Mehta et al., 1985; Eldrige et al., 1986) and entactin (Bunge et al., 1986).

Formation of a basal lamina depends upon the synthesis and secretion of procollagen type IV (Carey et al., 1983; McGarvey et al., 1984; Bunge et al., 1983, 1986), which is a major structural component of the basal lamina, and a sulfated proteoglycan (Mehta et al., 1985) and in culture these proteins are only produced at high levels when the Schwann cell is in contact with an axon. In vitro studies on Schwann cell myelination have shown that Schwann cells are unable to ensheath and myelinate axons unless they are able to assemble basal lamina (Bunge et al.,1983; 1986). It is possible that basal lamina is required for the Schwann cell to generate the plasma membrane polarity and cytoskeletal organization needed for the shape changes which myelin formation requires.

As described earlier, it seems probable that in the rat, both myelin-forming and non-myelin-forming Schwann cells derive from a common precursor cell. During development they acquire distinctive and well defined molecular phenotypes in addition to the morphological differences. Myelin-forming Schwann cells express major myelin proteins, including the glycoprotein P_0, myelin basic protein (MBP) and a subpopulation of myelin-forming cells express the enzyme P_2 and a lipid antigen defined by the monoclonal antibody O11 (reviewed in Mirsky and Jessen, 1990). Non-myelin-forming cells express proteins that are absent from myelin-forming cells. These include glial fibrillary acidic protein (GFAP), the cell adhesion molecules N-CAM and L1, the cell surface proteins A5E3, Ran-2, and low affinity NGF receptors (Mirsky and Jessen, 1990). The two Schwann cell variants also express several glial-associated antigens in common, including the calcium binding protein S100. This is not expressed by fibroblastic or perineurial cells. Both myelin-forming and non-myelin-forming Schwann cells express the intermediate filament protein vimentin, the glycolipid galactocerebroside (Gal C), and the lipid antigens O4, O8, and O9. The ECM molecules mentioned before are produced by all Schwann cells in peripheral nerve fibres (reviewed in Mirsky and Jessen, 1990).

Myelin-forming Schwann cells of the peripheral nervous system share some similarities in molecular phenotype with oligodendrocytes, the myelin-forming cells of the central nervous system (for review see Hudson, 1990), and non-myelin-forming cells show many similarities with astrocytes, the non-myelin-forming glia of the CNS and with enteric glial cells (Mirsky and Jessen, 1990).

The mature Schwann cell phenotype depends upon a constant

interaction beteen the cell and the axon (Mirsky et al., 1980; Mirsky and Jessen, 1990). When Schwann cells are deprived of axonal contact, either by nerve transection or when the Schwann cells are maintained in neurone-free culture, they dedifferentiate and take on an immature phenotype, very similar to that of the early Schwann cells first seen in rat nerves at embryo day 16.

Research on the biology of Schwann cells has shown that they fulfil several important roles in addition to the provision of myelin sheaths round the larger axons. This includes regulation of the extracellular environment and synthesis of trophic factors and adhesion molecules which are important during development and also for the maintenance of mature nerves and in regeneration (for review see Mirsky and Jessen, 1990). Schwann cells may also be able to interact with the immune system by expressing molecules which are important in the initiation of immune responses, such as the major histocompatibility complex class II antigens and interleukin-1 (see Chapters 3 and 4).

The major histocompatibility complex class II molecules

The major histocompatibility complex (MHC) class II molecules are transmembrane glycoproteins, which consist of two non-covalently associated peptides, β chains and α chains of molecular weights 29,000 to 34,000 and 25,000 to 28,000 daltons respectively. These molecules have a key role in presenting foreign antigens to T lymphocytes and thus in the initiation of the immune response. Lymphocytes with a T helper phenotype recognize peptide fragments of antigens only in association with MHC class II molecules in the

membrane of antigen presenting cells (APCs). Some T cells, including T cells with the cytotoxic phenotype and T killer cells, recognize foreign antigen in the context of MHC class I antigens (Hirsch et al., 1983; Fontana et al., 1984; Wong et al., 1984). Many nucleated cells express MHC class I antigens, but the distribution of MHC class II antigens is much more limited. Antigen presenting cells (APCs) are essential for the initiation of an immune response (Steinman et al., 1980) and almost all tissues outside the nervous system contain professional APCs that constitutively express high levels of MHC class II antigens (Hart and Fabre 1981). In addition, several cell types, that do not express MHC class II antigens continuously, can be induced to express MHC class II molecules and to act as APCs, by stimuli such as interferon-γ (IFN-γ) (Natali et al., 1981; Pober et al., 1983). IFN-γ is a lymphokine usually produced by T cells in response to proliferative signals. It induces MHC class II antigens on certain cell types and also has other immunomodulatory effects (Basham and Merigan, 1983, Pober et al., 1983).

Macrophages, the most important professional APC's play a central role in immune responses. They are involved not only in the initiation of immune responses as APC's, but also in inflammatory, tumoricidal and microbicidal responses since they secrete factors such as TNF which can have direct cytotoxic effects on susceptible cells (Brosnan et al., 1988). Although some macrophages express MHC class II antigens constitutively, others do not and expression is upregulated by IFN-γ (Unanue 1981; Perry et al., 1987). Their ability to present antigen to helper (CD4+) T cells is directly proportional to the density of MHC class II antigens on their

surface (Zlotnic et al., 1983; Janeway et al., 1984). Although most T cells with this phenotype carry out helper functions without being cytotoxic, it is now well established that some cells with the helper surface phenotype are cytotoxic and kill the presenting cell in a MHC class II restricted manner (Tite et al., 1985; Sun and Wekerle, 1986).

It is now recognised that dendritic cells (sometimes thought of as fixed macrophages) present in lymph nodes and spleen and in skin as Langerhans cells, normally express high levels of MHC class II molecules and it has recently been suggested that they may be important in the initial stages of antigen presentation (Zamvil and Steinman 1990), while macrophages are more likely to be involved in the proliferative phases of the T cell response.

Since MHC class II molecules are essential for immune recognition, it is important to investigate the control of the expression of these antigens and look at their tissue distribution. It has been demonstrated that IFN-γ can increase or induce MHC class II expression on the surface of cultured human endothelial cells, human dermal fibroblasts (Pober et al., 1983), on astrocytes (Fontana et al., 1986) and on Schwann cells (Samuel et al., 1987a, b). This can be correlated with an increased ability of the cells to stimulate T cell proliferation in lymphoproliferative assays. Furthermore, addition of anti-MHC class II antibodies inhibits the ability of astrocytes to present MBP to MBP-specific T cells showing that induction of MHC class II antigens on these cells is essential for the antigen specific T cell response (Wekerle et al., 1986).

Interleukin-1

In addition to MHC class II associated presentation of antigen to T lymphocytes, the release of the co-stimulatory factor IL-1 is an essential function of accessory cells for T cell activation (Gery et al., 1972; Unanue et al., 1981; Oppenheim et al., 1986).

IL-1 is important for the initiation of the immune response, since it stimulates T lymphocytes to express interleukin-2 receptors and to secrete interleukin-2 (IL-2) which drives antigen activated T cells into proliferation (Gery et al., 1972). IL-1 is also involved in other physiological responses in several different systems (March et al., 1985; Oppenheim et al., 1986; Mochizuki et al., 1987).

Two distinct forms of IL-1 molecules, IL-1α and IL-1β have been isolated from monocytes, and the existence of membrane-associated IL-1 has been revealed in activated murine macrophages and in human monocytes (Kurt-Jones et al., 1985). It is thought that most IL-1α is membrane bound but most IL-1β is intracellular and acts as a soluble factor (Conlon et al., 1987; Fuhlbrigge et al., 1988). IL-1α and IL-1β elicit similar systemic and cellular responses (Oppenheim et al., 1986) and they bind to the same receptor (Dower et al., 1987). Both forms of IL-1 are produced, in vitro and in vivo, in response to a wide range of stimuli, including exposure to cytokines (Boraschi et al., 1984; Arenzana-Seisedos et al., 1985), bacteria, viruses and other infectious agents (for review see DiGiovine 1990). Disruption of cells from their normal environment also induces IL-1 secretion by some cells (Kurt-Jones et al., 1985; Dinarello et al., 1989).

IL-1 is secreted by monocytes/macrophages and is found in both

culture supernatant and cell lysate (Mizel et al., 1979; Gery et al., 1981). IL-1 is also secreted by several other cell types, including Langerhans cells, keratinocytes (Luger et al., 1982), smooth muscle cells and endothelial cells (for review see DiGiovine 1990). Within the nervous system, IL-1 secretion has been reported from astrocytes (Fontana et al., 1982), microglial cells (Giulian et al., 1986) and glioma cells (Fontana et al., 1984b; Oppenheim et al., 1986).

The possible role of glial cells as antigen presenting cells

Whether glial cells and capillary endothelial cells can take part in processing and / or presentation of antigen has been the subject of considerable debate in recent years. Antigen presentation by nonimmune cells in some other tissues has gained acceptance, raising the possibility that presentation of foreign or self antigen by these cells may result in abnormal immune responses leading to autoimmune diseases (for discussion see e.g. Rodriguez, 1987). As mentioned above several types of nonimmune cells can be induced to express MHC class II molecules by incubation with IFN-γ and to present antigen to T lymphocytes. IFN- γ and viral infection are the stimuli for increased MHC class II production, which have recieved the greatest attention in this context.

Until recently, the deficit of APCs in nervous tissue and the protection against antigen and cell invasion offered by the blood-tissue barriers of the nervous system (Steinman and Nussenzweig, 1980; Schwartz, 1984) were thought to be responsible for the relative lack of immune reactivity in this tissue. It has now been shown that activated T lymphocytes can cross the blood-brain barrier

(Wekerle et al., 1986a). Furthermore, astrocytes can be induced to express MHC class II antigens in vitro by exposure to IFN-γ or to certain viral antigens (Hirsch et al., 1983; Wong et al., 1984; Massa et al., 1986) and they can present myelin basic protein (MBP) to encephalitogenic MBP-specific T cell lines (Fontana et al., 1984a; Fierz et al., 1985;). As mentioned previously astrocytes can produce interleukin-1 and also prostaglandin-E (Fontana et al., 1982), both of which take part in regulation of immune responses.

In the great majority of previous studies, oligodendrocytes have been reported not to express MHC class II molecules in vitro after incubation with IFN-γ. Most of these studies have been carried out in vitro using neonatal or foetal tissue cultured in DMEM containing 10% foetal calf serum (FCS) or in supplemented media containing 0.5% FCS. Astrocytes (Hirsch et al., 1983; Wong et al., 1984; Fierz et al., 1985; Fontana et al., 1986) and O2-A progenitor cells (Calder et al., 1988) have been found to express class II molecules in response to IFN-γ under these conditions, but no class II expression was seen on the oligodendrocytes, except in one study on human brain cells, not using IFN-γ (Kim et al., 1985). In the in vivo studies it has been more difficult to definitively identify the class II positive cells found in the brain and MHC class II molecules have only been positively identified in one study involving Theiler's murine virus (Rodriguez et al., 1987). In this immunoelectron microscopic study MHC class II molecules were found on astrocytes, oligodendrocytes, endothelial cells and microglial cells. The difficulty in demonstrating class II expression on oligodendrocytes in vitro could be due to lack of necessary environmental factors in the culture medium. Glucocorticoids, which

are present in the CNS but absent from routine media, are one candidate for such agents.

It has been shown that there is a differential regulation for class II expression in some tissues. In the pancreas (Mauerhoff et al., 1988) the ductal and the exocrine cells are easily induced by IFN-γ to express class II molecules (Pujol-Borrell et al., 1987), while islet endocrine cells require IFN-γ in combination with TNF. Glucocorticoids, which are not normally included in culture media, potentiate various aspects of oligodendrocyte differentiation and myelinogenesis during neonatal development (Kumar et al., 1989). Glucocorticoids also increase the IFN-γ induced class II expression on human monocytes and endothelial cells (Shen et al., 1986; Manyak et al., 1988).

Neither of the major Schwann cell types express detectable levels of MHC class II antigens in situ in the normal, uninfected rat sciatic nerve (Samuel et al., 1987a). Similarly, Schwann cells in dissociated cell cultures, which have a different morphological and molecular phenotype from that found in situ, are MHC class II negative. Cultured rat and human Schwann cells can be induced to express MHC class II antigens by treatment with IFN-γ (Pollard et al., 1987; Samuel et al., 1987 a,b) and IFN-γ treated Schwann cells are able to present MBP antigen to MBP specific T cell lines (Wekerle et al., 1986b). However, events in vitro do not necessarily reflect in vivo behaviour of Schwann cells, a point that is discussed more fully later in this chapter. One weakness of these studies is that the concentration of IFN-γ is relatively high and may not be within the physiological range.

Microglial cells of the central nervous system

In the CNS the main glial cell types within the parenchyma of the brain are astrocytes and oligodendrocytes (macroglia) and microglia. Whereas the macroglia originate during development from the neural tube, there is good evidence that microglia are derived from blood monocytes and that they can be regarded as the resident macrophage-like cells of the brain (for review see Perry and Gordon, 1988). In the adult CNS microglia are small process bearing cells which express several cell surface antigens also expressed by macrophages, and morphologically they resemble Langerhans cells in epithelia. They form a network throughout the gray and white matter of the brain. Unlike dendritic cells (e.g. Langerhans cells) which may act in the earliest stages of antigen presentation in some other tissues, microglia in the normal brain are not reported to be strongly MHC class II positive, a prerequisite for them to act in this role (Williams et al., 1980; Wong et al., 1984, 1985). In the lesions of both experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS), MHC class II positive macrophages/microglia have been reported to be present (Traugott and Raine 1985; Hofman et al., 1986; Lee and Raine, 1989), in addition to MHC class II positive astrocytes, which have also been reported in these plaques (Hofman et al., 1986; Lee and Raine 1989). They are therefore theoretically capable of presenting antigens to T cells and because of phenotypic and morphological similarities to macrophages in other tissues microglia are often considered to be the most likely cells within the brain parenchyma to do so. Nevertheless, even in tissues outside the brain, the sequence of cells involved in T cell activation in vivo is only beginning to be understood and from

present evidence it is extremely difficult to predict which cells within the brain are involved in interaction with the immune system cells in the various stages of chronic diseases like MS.

During Wallerian degeneration in the optic nerve, following nerve transection, myelin breakdown in the distal stump is much slower than in the PNS, despite the presence of microglia in the nerve and few macrophages are recruited from outside the CNS. In the PNS macrophages actively phagocytose myelin debris and secrete cytokines (see above). The slowness of myelin breakdown in the CNS suggests that microglia cannot function in this role and that their properties are distinctly different from those of incoming macrophages (Perry et al., 1987).

The role of macrophages in Wallerian degeneration and in inflammatory diseases in peripheral nerves

Although normal peripheral nerves are reported to contain relatively few macrophages, substantial numbers of macrophages invade the distal stump of the nerve after axotomy (Perry et al., 1987; Clemence et al., 1989; Brown et al., 1991). There is plenty of evidence for interactions between macrophages and the resident cells and axons in the nerve, some of which will be discussed below. The experiments of Beuche and Friede (1984) suggest that macrophages are involved in triggering the Schwann cell mitosis and degeneration of myelin that occurs after peripheral nerve transection classically described as Wallerian degeneration (for review see Fawcett and Keynes, 1990). Experiments in vitro suggest an interaction between myelin and macrophages is involved in triggering Schwann cell

mitosis (Bigbee and DeVries 1988; Baichwal et al., 1989). Furthermore it is known that cell division in the distal stump of axotomized nerves with a high proportion of myelinated fibers is greater than that in nerves in which the proportion of myelinated fibers is low (Bradley and Asbury, 1970; Romine et al., 1970). Non-myelin-forming Schwann cells in the distal stump of transected nerves divide more rapidly if the nerve is a mixed one containing many myelinated fibres (e.g.sciatic nerve) than if the nerve is largely unmyelinated (e.g. cervical sympathetic trunk). In the mixed nerves, cells derived from myelin-forming Schwann cells also contribute substantially to the cell division in the distal stump observed in the first week after axotomy (Clemence et al., 1989).

The C57BL/Ola mouse has provided further evidence for the role of macrophages in the axonal degeneration, myelin breakdown and Schwann cell mitosis that occurs in Wallerian degeneration. In this mouse, recruitment of macrophages into the lesioned nerve is much lower overall than in normal mice and in consequence axonal degeneration, myelin breakdown and Schwann cell mitosis all occur at much lower rates than in normal nerves (Lunn et al., 1989; Perry et al., 1990). There is also good evidence that interaction between incoming macrophages and resident cells of peripheral nerve contributes to the elevation of NGF synthesis seen in the distal stump after nerve transection. Macrophages secrete the cytokine IL-1 which increases NGF levels in cells within the nerve (Lindholm et al., 1987; Lindholm et al., 1990). In the C57BL/Ola mouse macrophage recruitment only occurs within the first 3 mm of the distal stump after axotomy and macrophage numbers in the rest of the distal stump are barely above normal levels. NGF levels in the

portion of the stump containing recruited macrophages are comparable to those in lesioned nerves of control mice, but much lower in the more distal portions of the stump. Regrowth of sensory axons is markedly impaired in these mice suggesting that NGF synthesis is important in the regrowth of this peripheral nerve population (Brown et al., 1991).

Macrophages may also be involved in immune responses within the nerve either after injury (transection or crush) or in diseases such as acute and chronic inflammatory demyelinating neuropathy (AIDP and CIDP respectively), Guillain Barre' syndrome (GBS), experimental allergic neuritis (EAN) or in mycobacterial infections such as leprosy, where MHC class II positive macrophages are seen in the nerve 5 weeks after injection of M. leprae into the nerve (Cowley et al., 1989). As professional antigen presenting cells they are likely to play a major role in T cell activation although the possibility that other cells in the nerve, in particular endothelial cells and Schwann cells can also play a part in immune responses (see above) is not precluded.

Experimental allergic neuritis

EAN is regarded an animal model of GBS, and is thought to involve an immune response directed against myelin components causing demyelination and nerve damage. EAN is induced by immunizing animals with myelin or the myelin protein P_2, and it can also be induced by adoptive transfer of T cells autoreactive for P_2 (Hughes et al.,1987). There is now clear evidence that EAN is a T cell-mediated disorder but macrophage-monocytes appear to be the

major effector cells (Linington et al., 1984; Hughes et al., 1987; Hartung et al., 1990). T cells activate macrophages to attack the myelin sheath, and cytokines, especially IFN-γ seem to have an important role in modulating cell-mediated responses (Nathan and Yoshida, 1988). The precise process by which demyelination is initiated is not well understood, but myelin or Schwann cells could be damaged by lymphokine-mediated cytotoxicity (Brosnan et al.,1989), antibody dependent cell-mediated cytotoxicity (ADCC) and complement mediated cytotoxicity (antibody dependent or independent) since Schwann cells have complement receptors on their surface in vivo (Wren and Noble, 1988; Scolding et al., 1990).

In EAN there is infiltration of helper T cells and MHC class II restricted cells are probably important in the progression of inflammation (Hughes et al., 1987). Hartung et al., (1990) investigated the possible role of IFN-γ in the pathogenesis of EAN in Lewis rats. They found that administration of IFN-γ produced enhancement of EAN, whereas application of a monoclonal antibody against IFN-γ suppressed clinical signs and morphological changes in EAN. In the IFN-γ treated animals there were much denser cellular infiltrates in the morphological lesion, and MHC class II expression was greatly increased. In tissue sections they found MHC class II expression on macrophages and T cells, but they state, referring to unpublished observations of Schmidt et al. (1989) that no MHC class II positive Schwann cells were found. Similarily, Schmidt et al., (1990) investigated MHC class II expression in ventral roots of animals with EAN 12-21 day after immunization, using 1μm-thick serial sections. In serial sections antiserum against S100 was used to identify Schwann cells and the ED1 antibody was used to identify

monocytes/macrophages. In these serial sections, MHC class II immunoreactivity was associated with macrophages (ED1 positive cells), but some MHC class II reactive cells and processes were ED1 negative and these cells were not identified (Schmidt et al 1990). These results may reflect an absence of MHC class II positive Schwann cells in this disease. Alternatively, it is possible that it reflects the relative insensitivity of the procedure used to visualize surface antigens which are often difficult to detect in fixed tissue sections as opposed to teased nerve preparations which allow better access of antibodies to the surface of unfixed cells. With respect to MHC class II molecules, in particular, it has been shown that even mild fixation radically reduces the detection, by antibodies or rosetting, on cell surfaces (Walker et al., 1984). The stage in the disease at which the nerves were examined may also be an important factor, since the levels of MHC class II molecules are likely to vary as the disease progresses.

Diseases of peripheral nerves

The pathogenesis of GBS, CIDP and AIDP remains unknown, but experiments on EAN have helped to elucidate many mechanisms of inflammatory demyelination. There is evidence that tissue damage in GBS and CIDP result from immune reactions within the peripheral nervous system, and in GBS cell-mediated immune reactions seem to be involved in lesion formation. Attempts to demonstrate disease-specific T cell sensitization in human conditions have, however, given inconsistent results (Hartung et al., 1988). It is possible to detect T cell activation by measuring serum concentration of IL-2

receptor, since T cells express a IL-2 surface receptor only after mitogenic or antigenic stimulation. There are reports of a significant elevation of IL-2 receptor in the serum of patients with GBS, although no elevation was seen in the serum of patients with CIDP (Hartung et al., 1988). In the case of AIDP, there seems to be a correlation of the disease with an infection, since the disease may follow a variety of bacterial, mycoplasmal and viral infections (Arnason, 1984).

Leprosy is a disease caused by Mycobacterium leprae (M.leprae), and the infection most usually occurs through the skin or the upper respiratory tract. The bacteria invade peripheral nerves and live mainly in Schwann cells, but are also found in macrophages, and in lesser numbers in perineurial cells and axons. Many individuals infected with the bacterium develop immunity to it and do not go on to develop leprosy, but in susceptible people one of the commonest symtoms is severe nerve damage, particularly in sensory fibres (for reviews see Sabin and Swift, 1984; Bloom, 1990).

Leprosy exists in three main forms, tuberculoid, lepromatous and borderline or dimorphous (which is intermediate between the two main forms). Patients with intermediate forms can convert to either the tuberculoid or lepromatous forms. The individual response does not depend on the bacterium but seems to be largely governed by the responsiveness of the immune system to the bacterial invasion. In tuberculoid leprosy, lesions, which include skin reactions and nerve damage, are typically confined to a small region in a cool area of the body. The body mounts a vigorous T cell mediated immune response, with the formation of a granuloma, bacteria are eliminated but severe nerve damage with axonal loss is common though limited in

extent. At the other end of the spectrum, in lepromatous leprosy, very little cellular immune response is seen, patients typically do not respond to M. leprae or other antigens when challenged intradermally and the Schwann cells are full of bacteria, with little sign of inflammatory reactions. In this form of leprosy the bacteria are carried from infected nerves via the bloodstream to nerves in widespread areas of the body, though as in tuberculoid leprosy, nerves in colder areas such as the ears and digits are more affected. Local demyelination occurs and in untreated cases widespread axonal loss eventually occurs (Sabin and Swift, 1984). Suppressor T cells have been isolated from lesions of patients with lepromatous leprosy and in vitro these cells can inhibit the proliferation of M. leprae specific T helper cells isolated from similar patients (Modlin et al., 1986; Ottenhoff et al., 1989; Bloom, 1990). Studies on the ratio of T helper to T suppressor cells in different forms of leprosy reveal a higher ratio of helper to suppressor cells in tuberculoid leprosy than in intermediate or lepromatous forms (Modlin et al., 1986). Humoral immunity is unaffected, levels of antibody specific for M. leprae are high in all forms, and B lymphocytes are found in nerve lesions from both tuberculoid, intermediate and lepromatous patients (Nilsen et al., 1986).

Experimental allergic encephalomyelitis

The animal model experimental allergic encephalomyelitis (EAE) is the prototype for antigen-induced T cell mediated autoimmune disease, and is considered to be the best available model for MS, a

spontaneous demyelinating disease in humans. EAE can be induced by injection of a relatively low number of MHC class II-restricted T lymphocytes ($CD4^+$), specific for MBP, which is one of the main proteins of central nervous system myelin (Bourdette et al., 1988). MBP has been identified as an encephalitogenic antigen in CNS tissue and injection of MBP induces acute EAE in mice, rats, guinea pigs, rabbits , monkeys, and in humans accidentally injected in the course of laboratory experiments (reviewed in Zamvil & Steinman 1990). Chronic forms of EAE, resembling MS more closely, can be generated in the Lewis rat by injection of PLP and in suseptible mouse strains by injection of MBP specific T cells.

The histological changes in the CNS of animals with EAE consist of perivascular cuffs of inflammatory cells, mostly T lymphocytes and macrophages, many of which are MHC class II positive (Polman et al., 1986). There is extensive demyelination and loss of oligodendrocytes in the chronic forms of EAE. Neurons are spared, at least initially, while astrocytes often respond by proliferation. Susceptibility to EAE is linked to MHC class II genes, and EAE can be prevented by injection of antibody against MHC class II antigens prior to immunization with spinal cord homogenate. This treatment also reduces the influx of radiolabelled lymphocytes into the CNS (Zamvil and Steinman, 1990).

It is likely that in EAE infiltrating T cells release their factors at the site of inflammation. One of the factors released by activated T cells is IFN-γ, which is a potent activator of macrophages. Another factor released from activated T cells is tumor necrosis factor (TNF) which has been shown to be toxic to oligodendrocytes in culture experiments (Massa et al., 1987b;

Brosnan et al., 1988), TNF might therefore play a part in oligodendrocyte damage in EAE.

Several reports indicate that MHC class II expression is important for the induction or the amplification of EAE, and it has been found that cultured astrocytes of rat and mouse strains susceptible to EAE express much higher levels of MHC class II molecules than astrocytes of resistant strains after treatment with IFN-γ (Massa et al., 1987a).

The human CNS disorder multiple sclerosis

The histological features of lesions seen in MS are essentially similar to those seen in chronic EAE. They are devoid of oligodendrocytes, axons display remarkable sparing and there is often an extensive astrocyte response. It has been suggested that the immunological response might be preceded by viral infection which causes repeated breakdown of the blood-brain barrier and sensitization of T cells to CNS antigens (Compston et al., 1991).

There is general agreement that the lesions also contain high numbers of T cells with both helper and cytotoxic subtypes. MHC class II antigens are seen in association with invading monocytes/macrophages and microglia and, to some extent, on astrocytes (Hofman et al., 1986; Lee and Raine, 1989). It has been suggested that astrocytes may have an important role in local antigen presentation in active MS lesions, but this has not been shown conclusively and is still a matter of controversy (for review see Wekerle et al., 1986).

In a recent detailed investigation by Traugott and Lebon (1988)

the expression of interferons (IFNs) (IFN-γ, IFN-α, IFN-β), and MHC class II antigens was examined in CNS tissue from MS patients and also from 3 cases of other neurological diseases. IFNs were detectable in active but not in inactive chronic MS lesions. In acute MS lesions astrocytes were labelled for IFN-γ and IFN-β, and MHC class I and class II antigens were detectable on many astrocytes and macrophages. There were extensive parenchymal and perivascular infiltrates of lymphocytes (CD8+ and CD4+ T cells) and macrophages. Macrophages were stained more frequently for IFN-α, and IFN-γ than for IFN-β, and some lymphocytes were IFN-γ and IFN-β positive, and IL-2 receptors were detected on lymphocytes and on some macrophages. Endothelial cells in plaques were occasionally positive for all three types of IFNs, but in unaffected areas they were mainly IFN-β positive. In normal CNS, IFN-γ and IFN-β were found on a few astrocytes but no MHC class II positive cells were found. These results were interpreted as showing that IFN-γ might play a role in active lesion growth in MS, whereas IFN-α and IFN-β might have some local immunosuppresive effect. In line with this suggestion is the observation that systemic administration of IFN-γ to MS patients causes a significant increase in the exacerbation rate (Panitch et al 1987). Thus, IFN-γ can enhance cell-mediated immune mechanisms, possibly by inhibiting suppressor cell function or by promoting MHC class II expression on various cell types (Basham and Merigan, 1983). IFN-α and IFN-β have been shown to increase the density of MHC class I molecules (May et al., 1986) and to have some suppressive effects. As a consequence of higher density of MHC class I and class II molecules, antigen can be presented more effectively to CD8+ and CD4+ (helper/inducer) T cells respectively.

The enteric nervous system

The enteric nervous system (ENS) of the gut was originally classified by Langley (1921) as the third division of the autonomic nervous system. While functionally the ENS might overlap with the sympathetic and parasympathetic divisions, Langley emphasized its distinctive nature. The main features distinguishing the ENS from other parts of the autonomic nervous system are its ability to carry out its major functions relatively independently of control by the CNS, the diversity of neuronal types, and the presence of complete reflex pathways consisting of sensory neurons, interneurons and motorneurons. The ENS is a complex and an integrative nervous network and many authors have pointed out that it shows striking histological, biochemical and functional similarities to the CNS (Gabella 1972, 1976, 1981; Gabella and Blundell, 1979; Furness and Costa, 1980).

The ENS consists of small intramural ganglia joined by connecting strands which are gathered into two ganglionated plexuses, the myenteric and the submucosal plexus. The myenteric plexus is a network of nerve strands and small ganglia that lie in between the external longitudinal and circular muscle coats of the intestine. The network is continuous around the circumference of the gastrointestinal tract and extends without interruption from the esophagus through the stomach, small and large intestine to the anus.

The enteric ganglia are very compact in structure. They consist of the cell bodies of neurons, enteric glia and nerve and glial cell processes. The ganglia are ensheathed by glial cells and the

ensheathment pattern differs substantially from the usual pattern found in other parts of the PNS (Gabella, 1981; Jessen and Mirsky, 1980, 1983). Whereas elsewhere, each nerve cell and its processes are separated from other neurons and axons by ensheathment by satellite cells or Schwann cells, within the small ganglia of the enteric plexuses the neuronal elements receive communal ensheathment by thin layers of externally applied glial cell sheets and processes. Internal to this sheath, the neuronal cell bodies and axons come into direct contact with one another and the close packing of neurons and glia give it an appearance similar to the CNS.

In the myenteric plexus of the guinea pig the glial cells outnumber neurons by approximately 2 to 1 and in the connecting meshes of the plexus, apart from the rare occurrence of neuronal perikarya, all the cells are glial cells. Glial cells of the intramural ganglia are structurally similar in all species studied. They are smaller in size than the neurons, the glial nuclei are elongated, about 2-3 μm in width and electrondense chromatin material is abundant. Neuronal nuclei are larger, and show lower electron-density. The glial cells are irregular in shape and carry long branching processes and spread over the surface of neuronal perikarya and in spaces between neuronal processes. They do not however form a complete capsule around a neuron, and large areas of the surface of perikarya and large dendrites are not covered by a cellular sheath, but are directly coated by the basal lamina surrounding the whole ganglion. Beyond this lie collagen fibrils, fibroblasts, interstitial cells and capillaries. In addition, glial cells do not cover the neuronal membrane at numerous sites where

nerve processes are directly apposed to the perikaryal surface. Other areas where glial covering is also missing are where two neuronal perikarya are in direct membrane to membrane contact; such areas are found occasionally in ganglia of adult animals, but are common during development. Some glial cells have long processes and relate mainly to nerve processes and completely surround small bundles of them.

In the connecting strands of the plexus, neurons are very rare. Glial cells have a characteristic arrangement, their small cell bodies are situated in the central part of the strand and send laminar expansions radially arranged between the neuronal processes. Many axons are tightly packed together without intervening glial processes.

As described above, the glial cells do not form a complete barrier between neurons and connective tissue or interstitium (Gabella 1981). Gershon and Burstajn (1978) reported that there is a blood-myenteric plexus barrier that resembles the blood-thymus barrier and may be functionally analogous to the blood-brain barrier. The main evidence for this is that the myenteric capillaries are non-fenestrated and have tight junctions and they resemble cerebral capillaries in this sense (Gershon and Bursztajn, 1978). There is some leakage of macromolecular tracers out of myenteric capillaries, but phagocytic cells remove this material and prevent the tracers leaking out of vessels from reaching detectable concentrations in the extravascular space or within the myenteric plexus (Gershon, 1981). This is similar to what is found in the thymus and functions as a part of the blood-thymus barrier (Raviola

and Karnovsky, 1972). The existence of a blood-myenteric barrier is however controversial, since Jacobs (1977) found that systemically injected horseradish peroxidase penetrated the myenteric plexus.

The glial cells found in the enteric ganglia have a number of unique structural features and are different in some essential aspects from the satellite cells of autonomic ganglia and from the Schwann cells of peripheral nerves (Gabella 1981; Jessen and Mirsky, 1983). The glial cells of the ENS and the astrocytic glia of the central nervous system (CNS) closely resemble each other in morphology, ultrastructure and in relationship to neuronal cell bodies and processes (Gabella, 1981). Although the enteric glial cell is of the same lineage as Schwann cells, its response to either different neuronal influences and/or different signals from extracellular matrix (ECM) compounds in the enteric tissues results in a unique course of differentiation. The resemblance between the enteric glia and astrocytes also extends to the molecular level, since the adult molecular phenotype of enteric glia resembles that of astrocytes. Thus, in situ most astrocytes and enteric glia express the Ran-2 antigen, A5E3 antigen and glial fibrillary acidic protein (GFAP) which is a predominant component of the 10-nm intermediate filaments (Jessen and Mirsky, 1980; Jessen and Mirsky, 1983). Enteric glial cells, but not Schwann cells express an astrocyte-associated determinant recognized by some anti-GFAP monoclonal antibodies, and enteric glial cells and astrocytes express glutamine synthetase. These data show that the enteric glia resemble astrocytes both morphologically and at the molecular level (Jessen and Mirsky, 1983). In culture some of the glial cell surface features change, reverting to those seen during development.

In particular, enteric glia in culture express Ran-1, now known to be the low affinity NGF receptor. Comparable changes are not seen in astrocytes, indicating that the enteric glia are more dependent than astrocytes on ongoing cell-cell signalling for the maintenance of full differentiation (Jessen and Mirsky, 1983).

It has been shown that in explant cultures of the myenteric plexus both glial cells and fibroblasts express the ECM component laminin. Both cell-surface and cytoplasmic laminin immunolabelling was detectable in enteric glia but the intracellular laminin expression was transient (Bannerman et al., 1986). The ability to produce laminin suggests that these cells may participate in forming the basement membrane which surrounds the enteric ganglia in situ, which is intensely laminin positive in immuno-histochemical tests (Bannerman et al., 1988). The observation that intracellular immunoreactivity is transient in culture, even in those glia that retain contact with neurons, may have implications for the possibility of basement membrane formation in ENS cultures.

Development of the enteric nervous system

The enteric nervous system originates from the neural crest (Gershon et al., 1981). The development of the enteric nervous system of the guinea pig has been analysed with morphological methods. Twenty five days of embryonic development marks the earliest time that primordial ganglia can be discerned. Already developed at this time are presumptive ganglion cells and supporting cells and neuritic processes form a small neuropil and growth cones. The circular muscle begins to form at this time. At 32 days of

gestation the longitudinal layer of smooth muscle can be discerned and, within the myenteric plexus, terminal axonal varicosities appear containing small electron-lucent synaptic vesicles. The gut has now a distinct myenteric plexus which has become much more densely packed with neurites than were the neuritic islands present at earlier ages. Synapses on ganglion cell somata first appear on gestational day 38 and are numerous on day 42, axodendritic synapses are also seen at this time. Between days 42 and 48 the developing neural tissue and the smooth muscle cells inter-digitate but after day 48, the plexus becomes ensheathed by supporting cells and the interdigitation is lost. It appears that the myenteric plexus has developed before a basement membrane has formed around the ganglia, since at day 32 the gut has a distinct plexus but at day 48 a basement membrane has formed judging by electron micrographs of the developing ENS. Cell division of neuroblasts within ganglia was found as late as 53 days of gestation, which shows how plastic even the mature appearing enteric nervous system can be.

Many of the characteristics of the mature myenteric plexus appear between gestational days 48 and 57. These include acquisition of different types of terminal varicosities and the projection of varicosities through the supporting cell sheath that covers the myenteric plexus. Contraction of the surrounding smooth muscle in response to acetylcholine appeared at day 48 although responses to electrical stimulation could not be elicited until days 50-56 (Gershon et al., 1981).

Extracellular matrix

The ECM is a complex network of proteins and carbohydrate polymers that surrounds most cells as an amorphous ground substance or as distinct structures such as fibrils and basement membranes. The two main classes of extracellular macromolecules that make up the matrix are glycosaminoglycans (GAGs) which are usually found covalently linked to protein in the form of proteoglycans, and fibrous proteins such as collagen, elastin, fibronectin and laminin. The glycosaminoglycans and proteoglycan molecules form a highly hydrated gel-like ground-substance in which the fibrous proteins are embedded (Kleinman et al., 1981).

The main ECM glycoproteins include collagen, fibronectin, laminin and entactin. Fibronectin (molecular weight 440,000 dalton) is composed of two similar but non-identical disulphide-bonded chains, it posesses several structural domains with strong binding sites for collagen, GAG, heparin and fibrin (Hynes 1981; Yamada, 1981). It is found in plasma and is present in some basement membranes and connective tissue (Yamada, 1981, Courtoy et al., 1982). Laminin is a glycoprotein (molecular weight 1,000,000 dalton) which is composed of three disulphide-bonded, N-glycosylated polypeptides: subunits A, B1 and B2 (Cooper et al., 1981). Like fibronectin, laminin has several domains which mediate different functions such as its ability to bind to type IV collagen, heparin, heparan sulphate proteoglycan and cells (Timpl et al., 1983b; Edgar et al., 1984). Laminin is primarily found in basement membranes (Foidart et al., 1980). Entactin is a sulphated glycoprotein (molecular weight 158,000 dalton) which is found in basement

membranes (Carlin et al., 1981). It forms an extracellular matrix complex with laminin and heparan sulphate proteoglycan (Edgar et al., 1984).

The collagens are a family of fibrous proteins found in all multicellular animals. They are secreted mainly by connective tissue cells and are the most abundant proteins in mammals, constituting approximately 25% of their total protein (Kleinman et al., 1981). Collagen molecules consist of three polypeptide chains, called α chains, which are wrapped around each other in a regular superhelix to form a rope-like collagen molecule about 300 nm long and 1.5 nm in diameter. About 20 distinct collagen α chains have been identified, each encoded by a separate gene and about thirteen types of collagen molecules have been found (Tikka et al., 1988). Collagens are extremely rich in proline and glycine, both of which are important in the formation of the triple-stranded helix. Selected proline and lysine residues are hydroxylated in the endoplasmic reticulum (ER) to form hydroxyproline and hydroxylysine and ascorbic acid has been shown to serve as a cofactor in these processes.

The individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and injected into the lumen of the ER as larger precursors called pro-α-chains. These precursors have the short aminoterminal signal peptide required to thread secreted proteins through the membrane of the ER. They also have other extra amino acids, propeptides, at both their amino- and carboxyl-terminal ends. Each pro-α-chain then combines with two others to form a hydrogen-bonded, triple-stranded helical molecule known as procollagen. The secreted forms of fibrillar collagens (but not of

type IV collagen) are converted to collagen molecules in the extracellular space by the removal of the propeptides (Hausman 1967).

Types I,II, III are the fibrillar collagens, they are the main types of collagen found in connective tissues, type I being by far the most common, accounting for 90% of body collagen. After being secreted into the extracellular space these three types of collagen molecules assemble into ordered polymers called collagen fibrils, which are thin cable-like structures, many micrometers long and clearly visible in electron micrographs (Bornstein et al., 1980; reviewed by Hay 1981, 1989).

Distribution of ECM in the nervous system

In the PNS of adult mammals, ECM components are associated with both loose connective tissue and basement membranes. In peripheral nerves the loose connective tissues, namely the epineurium and endoneurium contain fibronectin (Cornbrooks et al., 1983) and both thin and thick collagenous fibrils (Gamble, 1964) composed of types I and III collagen (Shellswell et al., 1979). The basement membranes surrounding Schwann cell/axon units, satellite cell/neuronal cell body units and the basement membranes associated with the perineurium contain laminin, fibronectin and types IV and V collagen (Carey and Bunge 1981; Bunge and Bunge, 1983). It has been demonstrated (Bannerman et al., 1986) that entactin is present in the basement membrane of rat and guinea pig Schwann cell and satellite cells and that heparan sulphate proteoglycan is present in the Schwann cell basement membrane in the rat and the guinea pig.

The distribution of ECM molecules in the myenteric plexus has been investigated. In the myenteric plexus of both rat and guinea pig the staining for laminin, fibronectin, entactin, type IV collagen and heparan sulphate proteoglycan was restricted to the periphery of individual ganglia. The immunostaining for laminin, entactin, type IV collagen and heparan sulphate proteoglycan was well defined and this discrete immunostaining pattern in the periphery of the enteric ganglia seemed to be due to localization within the basement membrane enveloping the ganglia. The weaker diffuse fibronectin immunostaining seen surrounding the ganglia appeared to be associated with the area of tissue containing the basement membrane and the loose connective tissue outside the plexus. Fibronectin is perhaps also a component of the lamina reticularis which is continuous with the underlying connective tissue zone. The basement membrane zone of smooth muscle, both circular and longitudinal and neighbouring blood vessels were also strongly labelled for all the five matrix components (Bannerman et al., 1986). The absence of these matrix molecules from within the enteric ganglia represents another aspect in which the tissue organization in the ENS resembles that of the CNS and is consistent with earlier electron microscopic observations.

Cell surface receptors for ECM molecules.

The ability of cells to adhere to the extracellular matrix is important for many aspects of cellular behaviour. This includes maintenance of tissue integrity, wound healing, morphogenetic movements, cellular migration, proliferation and cell shape. Many of the effects of ECM on cells are the result of an indirect linkage

of the ECM on the outside to the cytoskeleton on the inside of the cell. For many ECM molecules, membrane receptors have been identified which link specific matrix molecules to the actin cytoskeleton, which plays a central role in determining cell shape on one hand and in influencing gene expression on the other (Buck and Horwitz, 1987; Hay, 1989; Solursh, 1989). ECM components bind to the cell surface via special receptor glycoproteins. Some proteoglycans are integral components of the plasma membrane, and by binding to most types of extracellular matrix components these proteoglycans help link cells to the matrix (Saunders and Bernfield, 1988).

One large family of structurally related receptors, integrins, are known to mediate cell adhesion to a large variety of ECM matrix molecules and a specific domain within the cytoplasmic portion of the integrin β-chain appears to interconnect with microfilaments by binding to the actin-associated molecule talin (Buck and Horwitz, 1987; Hynes 1987; Ruoslahti and Pierschbacher, 1987) . The integrins are a superfamily of heterodimeric adhesion molecules consisting of noncovalently associated α and β chains. They function as transmembrane linkers to mediate interactions between the actin cytoskeleton inside the cell and the ECM, and they also have a role in cell-cell interactions. At present eleven different α chains and six different β chains have been identified (Sonnenberg et al., 1990).

There are multiple receptors for laminin and fibronectin within the integrin family (Elices and Hemler, 1989) and two types of integrin receptors have been shown to function as receptors for

collagen type I: VLA-2 and VLA-3. VLA-3 ($\alpha_3\beta_1$) is a multispecific integrin receptor which binds to fibronectin, laminin, collagen type I and type VI. Some of the integrin receptors seem to be monospecific for one ligand. VLA-2 ($\alpha_2\beta_1$) was thought to be monospecific for collagens (Kunicki et al., 1988) since this receptor, a member of the integrin β_1 subfamily, has been shown to act as a cell surface receptor for collagen in platelets (Santoro et al., 1988), fibroblasts (Wayner and Carter, 1987) and in melanoma cells (Kramer and Marks, 1989). Recently however VLA-2 has been shown to mediate attachment to laminin and to collagen in human melanoma cells (Kramer et al 1989), since antibody against the α chain blocked attachment to laminin. It seems therefore that VLA-2 can function as a collagen receptor in some cells and a collagen/ laminin receptor on others. Another possible mechanism for cell interaction with collagen is through other glycoproteins which bind to collagen. It has been shown that fibronectin (Klebe, 1974), laminin (Terranova et al., 1980) and chondronectin (Hewitt et al., 1982) can bind to collagen and can act as mediators of cell attachment to collagen-containing ECM (Yamada et al., 1983), and cell surface receptors which bind to collagen indirectly through these glycoproteins, have been found on several cell types (Pytela et al., 1985). It has also been demonstrated that various cell types including hepatocytes are able to attach directly to collagens although this probably involves an integrin receptor which is an RGD independent receptor (Rubin et al., 1981, 1986; Gullberg et al., 1989). The amino acid sequence Arg-Gly-Asp (RGD) has been shown to play an important role in the adhesion of cells to fibronectin (Pierschbacher and Ruoslahti, 1984a), to vitronectin (Suzuki et al.,

1985) and also to collagen type I. Many cell surface receptors, including some integrin receptors appear to interact specifically with this sequence (Hynes, 1987). Receptors with specificity for collagen type I, which are not related to the integrin family, have been found on human MG-63 osteosarcoma cells (Dedhar et al., 1987) and cell surface proteoglycans can act as attachment receptors, as exemplified by the attachment of non-trypsinized mouse mammary epithelial cells to both fibronectin and collagen (Saunders and Bernfield, 1988).

Collagen and differentiation

Until recently the vertebrate ECM was thought to serve mainly as a relatively inert scaffolding to stabilize the physical structure of tissues. But now it is clear that the matrix plays a far more active and complex role in regulating the behaviour of the cells that contact it, influencing their development, migration, proliferation, shape and metabolic functions (reviewed in Hay 1981, 1989; Ingber and Folkman, 1989). Cells interact with the matrix in specific ways using specific receptors in their cell membranes as discussed above. They secrete the matrix and then they react to this matrix and to matrix secreted by other cells. It has been suggested that embryonic induction, or tissue interaction in the embryo is mediated in many cases by ECM and that interaction between cells and ECM is also important for maintenance of differentiation in adult cells (reviewed in Hay, 1989).

Type I collagen is quantitatively the major ECM molecule and there is now evidence from several studies, mostly on epithelial

cells that type I collagen has a role in maintaining and inducing cellular differentiation (e.g. Bissel et al., 1982). Several studies have been carried out, using two-dimensional collagen subtrata or 3-dimensional collagen gels to investigate the role of cell-collagen interactions in morphogenic processes.

In 1971 Dodson and Hay reported a direct effect of frozen-killed lens capsule substratum (collagenous matrix, mostly type IV) on the ability of corneal epithelium to produce collagen. Gels of type I or type II collagen were just as effective as the type IV-rich lens capsule in enhancing epithelial differentiation (Meier and Hay, 1974). In these experiments the basal surface of the epithelium started to bleb as soon as it was separated from its basal lamina. When placed on collagen, ECM or on Millipore filters in medium containing soluble matrix molecules, the epithelium retracted the blebs, formed an organized basal actin cortical mat, and doubled its production of collagen (Svoboda and Hay, 1987). There is evidence that organization of the RER by the actin cytoskeleton mediates this stimulation of collagen production, and that actively translated mRNA is bound to the cytoskeleton. The ECM-induced organization of the cytoskeleton may enhance RER function in the differentiating epithelium by stabilizing mRNA (Hay 1989).

In the last few years it has been shown that several cell types can organize into tissue-like structures when grown inside 3-dimensional collagen gels. When monolayers of pancreatic endocrine cells were cultured in a 3-dimensional collagen gel, they reorganized into spheroidal aggregates and the cell distribution was the same as observed in islets of Langerhans in situ. Furthermore

capillary endothelial cells were grown inside a 3-dimensional collagen gel and the monolayer reorganised in 2 days into a network of branching and anastomosing cords of endothelial cells resembling blood capillaries, demonstrating that an interaction of the endothelial cells with collagen, plays a critical role in the organization of these cells into correctly-polarized capillary-like tubes (Montesano et al., 1986). It has also been shown that collagen induces morphological differentiation in a human colon carcinoma cell line (Pignatelli and Bodmer, 1988), in a canine kidney cell line and in a murine mammary gland cell line (Hall et al., 1982). There is also some evidence that collagen may have a role in the differentiation of neuronal cells, such as retinoblastoma cells (Tombran-Tink and Johnson, 1989).

In vitro and in vivo studies on glial cells

In vitro tissue culture systems can give valuable information about the biology f cells. The studies of Patterson and co-workers on the factors which influence the development of sympathetic neurons from cells with a potential to differentiate into adrenal chromaffin cells, SIF cells or sympathetic neurons are particularly good example of this approach applied to the nervous system (e.g. Patterson 1978; Wolinsky and Patterson, 1985). In the case of glial cells (including in this definition cells from both CNS and PNS), considerable progress has been made, using in vitro methods, in understanding the functional properties of these cells (for reviews see Mirsky and Jessen, 1990; Raff, 1989). It is now possible to make pure cultures of glial cells, which allow the study of the

behaviour and function of these cells in isolation, and give the possibility to analyse the effect of individual factors on these cells. The effect of one glial cell type on another (e.g. the effect of astrocytes on oligodendrocyte differentiation) or the effects of neurons on glia and vice versa can be studied and the effect of various factors and components evaluated. Results obtained must, however, always be referred back to the contexts in which the cells find themselves in vivo.

As described previously, there is now considerable knowledge about the molecular properties of Schwann cells both in vivo and in vitro. Myelin-forming Schwann cells and non-myelin-forming Schwann cells have distinctive molecular morphological phenotypes in adult nerves, and the molecular phenotype of the non-myelin-forming Schwann cell resembles that of Schwann cells in the nerve before myelination takes place. It is clear that the myelin-forming Schwann cells change their phenotype when they are taken into culture, since they stop expressing the myelin related proteins (Mirsky et al., 1980). Both variants of Schwann cells also stop making a basal lamina in culture unless they are in contact with neurons (Bunge et al., 1986). Most of the changes that happen when Schwann cells are taken into culture seem to be a reversal of the differentiation directed by axons that occur during Schwann cell development (Jessen and Mirsky 1991).

It has been shown that elevation of intracellular cAMP levels in cultured Schwann cells mimics many of the changes induced by axons in vivo. It increases the expression of the myelin related molecules, such as the lipids Gal C and O4 (Mirsky et al., 1990; Sobue and Pleasure, 1984), the myelin protein P_0 and it also

augments the synthesis of basal lamina components (Baron van Evercooren, 1986), but suppresses the expression of GFAP, N-CAM, A5E3 and NGF (Morgan et al., 1990). The up-regulation of P_0 and down-regulation of GFAP, N-CAM, A5E3 and NGF which is typical of the myelin phenotype in vivo, only occurs if cell division is inhibited in the cultures. This accords well with events in vivo where cell division in Schwann cells normally stops before myelin-sheath formation (Peters and Muir, 1979; Morgan et al., 1990). It has also been shown that rat Schwann cells cultured with dorsal root ganglion neurons in medium containing serum and ascorbic acid, differentiate and within a week they form basal lamina and a myelin sheath around the axon (Carey and Bunge, 1981; Eldridge et al., 1987).

With the possible exception of nerve growth factor (Lindholm et al. 1990; Brown et al., 1991) there is little evidence of for a qualitative difference in molecular expression between Schwann cells cultured in the absence of neurons, and Schwann cells in the distal nerve stump of a cut sciatic nerve. This suggests that it is not the in vitro conditions that are the reason for the change in Schwann cell function, but rather the fact that they are deprived of axonal contact.

The enteric nervous system has been studied both in vivo and in vitro, and the use of explant cultures has facilitated the analysis of cell types and their interactions in the ENS (Bannerman et al. 1987). The initial demonstration that GABA was likely to be a neurotransmitter in the PNS, subsequently confirmed in many laboratories using in situ methods, used explant cultures of myenteric plexus similar to those used in this thesis (Jessen et

al., 1979). These cultures have also been used to study the electrophysiological and biochemical characteristics of enteric neurons (Bannerman et al., 1987). In general these cultures have been grown in medium containing 10 % FCS, except for studies done by J. Saffrey et al (1984).

As mentioned above there is some evidence that enteric glial cells depend on axonal contact for the maintenance of their phenotype: the Ran-1 antigen (the low affinity receptor for NGF) is not found on rat enteric glia in situ but is expressed in culture, and the Ran-2 surface protein is present on rat enteric glia in situ but is lost after a short period in culture (Jessen and Mirsky, 1983). In future studies it would be interesting to look at the expression of these molecules and of the adhesion molecules, L1 and N-CAM (Mirsky et al., 1986) on enteric glial cells cultured in a 3-dimensional collagen gel in medium with low serum.

Oligodendrocytes, the myelin-forming cells of the CNS, seem to depend much less on axonal contact for their differentiation than both Schwann cells and enteric glial cells (Raff 1989). In contrast to Schwann cells, expression of myelin lipids and proteins is not lost from oligodendrocytes under neurone-free in vitro conditions (Mirsky et al., 1980). Furthermore in vivo studies have demonstrated a strict correlation between the structural and biochemical events of myelination and results from in vitro studies have shown that when oligodendrocytes from newborn rat brain or optic nerve are cultured in defined medium, they express Gal C and the myelin proteins MBP, MAG and PLP in a sequence very similar to that seen in vivo (Mirsky et al., 1980; Dubois-Dalcq et al., 1986). This suggests that cultured oligodendrocytes are suitable for

studies on factors which regulate expression of myelin proteins and it therefore seems likely that they would also be useful for studies on other aspects of oligodendrocyte biology, such as those presented in Chapter 5 of these thesis.

It is of great importance to carry out in vivo experiments, and to investigate if the results from the in vitro studies, are also true for the in vivo situation. Comparing the results we get from the in vitro studies with what we see in vivo can help us to find the best conditions for the in vitro experiments. The great complexity of the environment of both glia and neurons within the CNS and to a lesser extent within the PNS often makes it difficult to evaluate the direct effects of the many factors which may be at work. The in vitro tissue culture system clearly offers some advantages in this respect since we can analyse the effect of isolated factors or known combination of factors on individual cell types and on specific combinations of cells. In general, however, we have to be careful in the way we interpret our results from in vitro studies.

The content of this thesis

A) MHC class II expression and IL-1 production by Schwann cells

In Chapter 3, results from a lymphoproliferative assays are presented, in which cultures of neonatal rat Schwann cells are incubated with mycobacterial antigen and sensitized T cells. The neonatal rat Schwann cells were able to support the lympho-proliferative responses of mycobacteria-reactive T lymphocytes without IFN-γ pretreatment. After the process of antigen

presentation, essentially all of the untreated Schwann cells expressed class II antigens on their surface, while class II molecules are barely detectable in cultures incubated with T cells or antigen alone.

Specific antibodies against IFN-γ or against TNF-α inhibited the class II expression on the Schwann cells in these co-cultures, suggesting that endogenous IFN-γ and TNF have a role in the lymphocyte-induced MHC class II expression on the Schwann cells. TNF-α in combination with IFN-γ greatly enhanced class II expression on Schwann cells, but had no effect on its own, and antibody against IFN-γ blocked class II expression in the TNF-α plus IFN-γ treated cultures showing that effects of TNF-α are dependent on IFN-γ.

Studies done on the effect of collagen on Schwann cell function have suggested that collagen has an important role in Schwann cell differentiation and that Schwann cells are unable to function normally in their role in myelination and ensheathment unless they are able to produce and relate to extracellular matrix components (Bunge et al., 1986). It was therefore tested whether type I collagen preparations were able to modulate class II expression by Schwann cells. The results showed that type I collagen preparations affect the ability of embryonic rat Schwann cells to express MHC class II molecules.

Thus cultured Schwann cells can be induced to express class II molecules, simply by incubating them with antigen and activated T cells. Furthermore class II expression by cultured Schwann cells can be modified by extracellular matrix molecules.

Since Schwann cells in culture change their phenotype as a

result of removal from axonal contact. I investigated whether Schwann cells could be induced to express class II molecules in the living nerve. After an injection of lymphokines and mycobacterial antigens into the living sciatic nerve, class II positive Schwann cells were found in a teased preparation of the nerve. Crushing the sciatic nerve also induced class II molecules on some Schwann cells showing that nerve damage is sufficient to elicit MHC class II on Schwann cells. Most of the class II positive Schwann cells were myelin-forming cells but some non-myelin-forming Schwann cells expressed class II molecules, and class II positive mononuclear cells were also seen.

Taken together these results demonstrate that Schwann cells are able to express class II molecules in vivo and in vitro, and to support lymphoproliferative responses of sensitized T cells without IFN-γ pretreatment.

Chapter 4 deals with another important aspect of accessory cell function classically associated with macrophages and other antigen-presenting cells, namely the release of IL-1. The ability of Schwann cells to secrete IL-1, was tested by incubating purified cultures of neonatal or adult rat Schwann cells with lymphokines or bacterial antigens. The supernatants were collected from the Schwann cell cultures and cell lysates were made from these cultures and IL-1 assays were carried out by Dr Ann Kingston. The neonatal rat Schwann cells secreted IL-1 when they were incubated with mycobacterial antigens or with lipopolysaccharide and indomethacin. IL-1 production was also observed in adult rat Schwann cells. When untreated Schwann cells were examined with anti-IL-1 antibody, weak

staining was seen intracellularly while treated Schwann cells had bright intracellular staining.

B) Oligodendrocytes can be induced to express MHC class II molecules in vitro

In Chapter 5 results are presented from experiments on oligodendrocytes, which are the myelin-forming glial cells of the central nervous system. In the last few years, several studies have been done to look at the ability of these cells to express class II molecules. The results have been controversial, although the predominant view is that oligodendrocytes are unable to express class II molecules.

In Chapter 5, studies on the effect of glucocorticoids on MHC class II expression by oligodendrocytes are presented. I found that when oligodendrocytes, derived from the optic nerve, were grown in defined medium containing glucocorticoids (dexamethasone) and incubated with IFN-γ for 3 days, 52% of them expressed class II molecules on their surface, while no class II expression was seen in the absence of glucocorticoids. This provides an explanation for the absence of MHC class II molecules from oligodendrocytes in previous studies, since they were carried out in medium without dexamethasone.

C) The effect of collagen type I on the enteric nervous system

The subject of Chapter 6 is the effect of collagen type I on the differentiation of ENS of the gut. Hydrated collagen gels have been used to study the influence of specific ECM constituents on the morphogenic behaviour of various cell types. A major advantage of this technique over conventional monolayer cultures is that cells

can be embedded within a lattice of reconstituted collagen fibrils, and collagen gel mimics the 3-dimensional organization of connective tissue matrices. In this present work a possible role for type I collagen in the induction of differentiation of neural cells was investigated, using the myenteric plexus of the gut.

I tested whether a 3-dimensional gel made from collagen type I could prevent the breakdown of normal plexus morphology which occurs when the plexus in grown on a 2-dimensional collagen substrate. It was found that if the plexus was embedded in a 3-dimensional collagen gel and grown in a defined medium containing 0.5% FCS, the glial cells did not migrate away from the neurons and the plexus stayed as a network. Similarly, when a disaggregated culture of the myenteric plexus, grown on a 2-dimensional substrate for 5-7 days, was embedded in a 3-dimensional collagen gel, the cultures rearranged into a network of small ganglia and interconnecting strands, very similar to that seen in the myenteric plexus in situ. Electron microscopy showed that the ultrastructure of a reformed plexus was similar to that seen in situ. These results show that type I collagen prevents the breakdown of the myenteric plexus and also induces a plexus-like network formation in a disaggregated culture.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Sources of lymphokines, mycobacterial antigens and live M.leprae.

Whole cobalt irradiated M.leprae bacilli (ML) were prepared and purified from armadillo tissue by the method of Draper (1979). Soluble M.leprae cell-free extract (MLS) was prepared according to Smelt et al. (1981), and soluble M. tuberculosis cell-free extract was prepared as outlined previously (Kingston et al., 1987). All of these preparations and live M. leprae were provided by Drs. R. Rees and M.J. Colston (National Institute for Medical Research, Mill Hill, UK). Recombinant rat interferon-γ (IFN-γ) and mouse anti-rat IFN-γ antibody (DB1) were provided by Dr. P. Van der Meide (Van der Meide et al., 1986) (TNO Primate Centre, Rijswijk, The Netherlands). Recombinant tumor necrosis factor (TNF-α) was provided by Dr J. Taverne (Department of Immunology, University College and Middlesex School of Medicine, London, UK) and recombinant mouse interleukin-4 (IL-4) was supplied by DNAX (Palo Alto, CA).

Sources of antibodies

The S100 protein was used as a Schwann cell marker (Brockes et al., 1979) and detected by rabbit antiserum to bovine S100 (Dako Immunoglobulins, Copenhagen Denmark) used at a dilution of 1:800. Rabbit antiserum to glial fibrillary acidic protein (GFAP)(Dako

Immunoglobulins, Copenhagen, Denmark) diluted 1:100 was used to identify non-myelin-forming Schwann cells (Jessen et al., 1984). Rabbit antiserum against the major peripheral glycoprotein P_0 (Brockes et al., 1980b) a gift from Dr. J. Brockes, was used at a dilution of 1:200 to identify myelin-forming Schwann cells. Mouse monoclonal OX-6 and OX-4 antibodies (McMaster and Williams 1979) (Seralab, Crawley Down, UK), both directed against rat class II molecules were used at a dilution of 1:50. A monoclonal antibody against a macrophage marker ED1 (Dijsktra et al., 1985), a gift from Dr. C.D. Dijkstra was used at a 1:50 dilution. A mouse monoclonal antibody W3/13HLK (Brown et al., 1985) specific for thymocytes and T lymphocytes (Seralab, Crawley Down, UK) was used at a 1:50 dilution. Mouse IgM monoclonal antibody specific for Thy 1.1 (Serotec, Oxford, UK) diluted 1:500 was used to identify fibroblasts. Rabbit complement (Serotec, Oxford, UK) was used at a dilution of 1:20. Rabbit anti-murine TNF-α, used at a dilution of 1:240, was a gift from Dr A. Meager (NIBSC, UK). Sheep antibody against IL-1, a gift from Dr E. Zanders (Glaxo Research, Greenford, Middlesex UK), was used in a dilution of 1:50. Mouse monoclonal antibody to the carboxyterminal domain of type I procollagen, a gift from Dr. J.A. McDonald (St. Louis, Missouri 63110, USA), was used at a dilution of 1:50. Oligodendrocytes were identified by their expression of galactocerebroside (Gal C)(Raff et al., 1979). A polyclonal antibody against Gal C, a gift from Dr. P.A. Eccleston, was used at a dilution of 1:100. The following antibodies were used at a dilution of 1:100 for fluorescence labelling: rhodamine-conjugated goat anti-rabbit Ig (G-anti-RIg-Rd, Nordic Lab., Maidenhead, U.K.)

adsorbed with mouse Ig to remove cross-reacting antibodies; fluorescein-conjugated goat anti-mouse Ig (G-anti-MIg-Fl, Nordic Lab. Maidenhead UK) adsorbed with rabbit Ig to remove cross-reacting antibodies, and fluorescein-conjugated goat anti-sheep Ig (G-anti-ShIg-Fl) a gift from Dr. W. Smith (Lilly Research Ltd, UK).

Preparation of collagen gel

Type I collagen was extracted from rat tail tendons according to Bornstein (1958). Rat tail tendons were extracted in 0.1% acetic acid for a few days, the collagen solution was centrifuged at 13000 rpm for 2 h to remove gross insoluble material and then dialyzed. About 30 ml of the collagen solution was dialyzed for periods of 24 h against 2 lots of sterile water containing one-tenth strength Dulbecco's modified Eagle's medium (DMEM). The dialyzing solution was adjusted to pH 4.0 with HCl. The concentration of the collagen was measured according to Lowry et al., (1951), and was 0.8 mg/ml.

Collagen solutions are stable at low ionic strength and low pH, but raising the ionic strength and the pH to physiological levels causes the solution to gel. The collagen solution was placed on ice and mixed with 0.1 ml of 10 x DMEM and 0.1 ml of a solution of predetermined volumes of bicarbonate (0.285 ml) and water (0.715 ml) to bring the pH of the mixture to 7.4. When this collagen mixture was incubated at 37° C it formed a firm collagen gel. It has been shown that cells can move on and within collagen gels, and cells overlaid by collagen gel tend to release their attachments to the substrate and move a little way into the collagen, taking a pronounced spindle form (Elsdale and Bard 1972).

Preparation of collagen-coated coverslips

Fifty µl of collagen solution was placed on 13 mm glass coverslips and spread over the surface. Exposure to ammonia vapour for a few minutes, gelled the solution into firm adherent gel. The coated coverslips were washed in Minimal Eagle's Medium with 0.02 M Hepes (MEM-H) and then in several changes of water to remove ammonia. The gel was then dried down on the coverslip in a flow hood.

MICROINJECTION AND CRUSHING OF THE SCIATIC NERVE

Five, 31 day to 36 day old Sprague Dawley rats, six 31 day to 36 day old Wistar rats, and two 13 day old Wistar rats were anaesthetised with 0.045 % chloral hydrate (1 ml per 100 g body weight). The sciatic nerves were exposed and IFN-γ (10 µl of 2.4 x 10^5 U/ml) or TNF-α (5 µl of 10^6 U/ml) was pressure-injected into the nerve with a micropipette, causing minimum damage only, the wound was then closed by surgical suture. Three to 7 days later the rats were sacrificed and the sciatic nerve was excised, desheathed and teased into nerve bundles and individual fibres with fine syringe needles in a small amount of phosphate-buffered saline (PBS). Tissue was placed on gelatin coated microscope slides and allowed to dry before immunolabelling.

The sciatic nerves of four 30 day to 33 day old anaesthetised Wistar rats, and four 4 months old and two 10 weeks old anaesthetised AS rats were exposed and either soluble M.leprae (5 µl of 1 mg/ml), whole ^{60}Co irradiated (5 µl of 10^{10}/ml) or live M.

leprae (5µl of 5 x 10^8/ml) were microinjected into the living nerve. Six days later the rats were sacrificed and the nerves were excised and teased. Samples were allowed to dry before immunolabelling.

The sciatic nerves of six 31 day to 36 day old and two 9 day old anaesthetised Sprague Dawley rats and two 32 day old Wistar rats were exposed and crushed with fine watchmakers forceps three times for 1 min. Four days or 8 days later the rats were sacrificed and sciatic nerves were excised and teased.

TISSUE CULTURE

Schwann cell culture for the T cell assay

Schwann cells were prepared according to previously published methods (Brockes et al., 1979; Mirsky et al., 1980). Sciatic nerves were removed from 4 - 7 day old AS, Wistar, August or PVG strain rats bred at the National Institute for Medical Research, Mill Hill. The nerves were cut into small pieces and incubated in medium containing 0.2% collagenase and 0.125% trypsin in Ca^{2+} and Mg^{2+} free Dulbecco's modified Eagle's medium (DMEM) for 30 min at 37°C. After this period an equal volume of Minimal Eagle's medium with 0.02 M Hepes buffer (MEM-H) containing 10 % foetal calf serum (FCS) was added and the cells gently dissociated by repeated passage through a plastic pipette tip. The cells were centrifuged for 10 min at 500 g and resuspended in DMEM supplemented with penicillin (100 i.u/ml), streptomycin (100 µl/ml), glucose (2mg/ml) and glutamine (2mM) with 10 % FCS. The cells were seeded into 96-well flat bottom microtiter plates (Nunc, Roskilde, Denmark) at a density

of 2 x 10^4 cells/well. After overnight incubation at 37° C in a 5 % CO_2 humidified incubator, cytosine arabinoside (10^{-5} M, Sigma Chemical Co.,St Louis, Missouri, USA) was added for up to 48 h to kill rapidly dividing cells (mostly fibroblasts). After removal of cytosine arabinoside the cultures were maintained in DMEM containing 10 % FCS. Twenty-four h later some cultures were treated with rIFN-γ (100 U/ml) for 72 h, while others were maintained without adding IFN-γ.

Schwann cell cultures for immunolabelling

Schwann cell suspensions were prepared as described above, from sciatic nerves of 4 day old or 3 week old rats except that the 3 week old nerves were incubated with enzymes for 90 min. The cells were seeded onto poly-L-lysine-coated coverslips placed in 24-well plates (Sterilin, Ltd., Richmond, Surrey, UK) at a density of 2 x 10^4 cells/well. The cultures were treated with cytosine arabinoside (10^{-5} M) for 48 h to kill dividing cells. After removal of cytosine arabinoside the cultures were maintainded in DMEM for 24 h. Remaining fibroblasts in the adult Schwann cell cultures, were killed by using anti-Thy-1.1 antibody and rabbit complement as follows: the Schwann cell cultures were washed briefly in PBS and incubated with MEM-H containing anti-Thy-1.1 IgM antibody (dil 1:500) and rabbit complement (dil 1:10) for 50 min. The Schwann cell cultures were then washed in PBS.

Schwann cell cultures either untreated or previously treated with IFN-γ for 72 h were incubated with antigen and T lymphocytes for 4 days. When DB1 mAb against IFN-γ or a polyclonal antibody

against TNF-α was used, the antibodies were added to the cultures on the same day as the T lymphocytes and the antigen. Untreated cultures were also incubated with antigen alone or T lymphocytes alone for 4 days before immunolabelling. In some experiments, Schwann cell cultures were incubated with TNF-α alone for either 24 h or 72 h. Cultures incubated with a combination of TNF-α and IFN-γ were first incubated with IFN-γ for 72 h and TNF-α was added for the last 24 h. Some Schwann cell cultures were incubated with IL-4 (at 35 U/ml and 70 U/ml) alone or with IL-4 (35, 70 and 140 U/ml) and IFN-γ (300 U/ml) for 72 h. Reference Schwann cell cultures were also included and were incubated with IFN-γ alone. After the incubation period the cultures were immunolabelled.

Schwann cell culture for IL-1 assay

Schwann cell cultures were prepared from sciatic nerves from 4 day old neonatal or 3 week old Sprague Dawley rats as described above. Schwann cells were resuspended in DMEM containing 5% calf serum (CS) and the cells were seeded into a 35 mm plastic tissue culture Petri dish (Nunc, Roskilde, Denmark) coated with laminin (0.02 g/ml). After overnight incubation at 37° C in a 5% humidified incubator, cytosine arabinoside (10^{-5} M, Sigma Chemical Co, St Louis,Mo) was added for 48 h to kill dividing cells (mostly fibroblasts). After removal of cytosine arabinoside the cultures were maintained in DMEM containing 5% CS for 24 h after which the remaining fibroblasts were killed by incubation with mouse IgM mAb specific for Thy 1.1 (Serotec, Oxford, UK) used at a dilution of 1:500 and rabbit complement (Serotec, Oxford, UK) at a dilution of 1:20.

Schwann cells were removed from the Petri dishes by using 0.05% trypsin in PBS containing EDTA (5.95×10^{-5}M) and seeded into a 24 well plate at a density of 7×10^4/well. Forty-eight h later the Schwann cell cultures were incubated with soluble (10 µg/ml) or whole M.leprae (10^8 /ml), lipopolysaccharide (0.5 µg/ml), indomethacin (1 µg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml) or IFN-γ (100 U/ml) in combination with TNF-α (300/ml). Forty-eight or seventy-two h later the supernatant was collected from the cultures, new medium was added and a cell lysate was made by freezing and thawing three times. Some cultures seeded at 2×10^4 cells per well and incubated with M. leprae antigens were immunolabelled using antibody against IL-1.

Preparation of oligodendrocyte cultures

Cultures containing oligodendrocytes were prepared by dissociation of 7-day old rat optic nerves as follows essentially as described by Miller et al. (1985): optic nerves were removed from 4-6 rats under sterile conditions and taken from the point of entry into the optic canal to 1-2 mm behind the chiasma. They were digested at 37° C in 1 ml of Ca^{2+}- and Mg^{2+}- free Dulbecco`s modified Eagle`s medium (DMEM)(Imperial, Andover UK) containing trypsin (0.05%) and collagenase (0.04%), and after 30 min 1 ml of the same enzyme solution was added for another 30 min. After this incubation period 2 ml of PBS containing EDTA (0.02M) was added to the enzymes for 30 min at 37° C. Following centrifugation, the tissue was resuspended in 1 ml of DMEM containing 10% FCS, DNAse (0.04%) and trypsin inhibitor (0.25%), then the tissue was

triturated 4-5 times through a needle and filtered through gauze to remove debris. After centrifugation the cells were resuspended in a small volume of DMEM containing 10% FCS and plated out in 10 µl. After 1 h 400 µl of supplemented medium was added to the cultures. The medium consists of equal volumes of DMEM and Ham's F-12 medium supplemented with glucose (2mg/ml), insulin (5 µg/ml), selenium (0.16 µg/ml), triiodothyronine (0.1 µg/ml), transferrin (100 µg/ml), putrescine (16 µg/ml), thyroxine (0.4 µg/ml), progesterone (60 ng/ml), penicillin (100 U/ml), streptomycin (100 µg/ml)(Sigma, Chemical Co, St Louis, Missouri, USA), 30% BSA (ICN, Immuno-biologicals) (0.3 mg/ml), and glutamine (2 mM) (Flow, Irvine Scotland). 0.5% FCS was added to the medium. When optic nerve cells are grown in medium containing low serum the O-2A progenitor cells differentiate into oligodendrocytes (Raff et al., 1983). After 2 days of culture, cytosine arabinoside (10^{-5} M, Sigma Chemical Co) was added for up to 48 h to kill rapidly dividing cells, mostly meningeal cells and astrocytes. Oligodendrocytes were grown in defined medium with or without dexamethasone (38 ng/ml)(Sigma Chemical Co, St Louis, Missouri, USA) containing 0.5% FCS After 3 to 7 days in culture the cells were incubated with rat recombinant IFN-γ (100 U/ml) for 72 h, prior to staining the cells by indirect immunofluorescence.

Myenteric plexus from guinea pig taenia coli.

Tissues were obtained from newborn to 3 day old guinea pigs (Dunklin-Hartley). Guinea pig myenteric plexus explant cultures were prepared as described previously (Jessen et al., 1978, 1983; Saffrey and Burnstock 1984). The taenia libera or taenia mesocolica

together with the myenteric plexus, were dissected from the caecum by cutting into the underlying circular muscle layer without penetration of the gut wall. Tissue segments 2-5 mm long, were placed in MEM-H containing collagenase (0.1%) and trypsin inhibitor (0.03%) and kept overnight at 4° C and for 40 min at 37° C. After the enzyme treatment, the tissue was transferred to fresh MEM-H and the myenteric plexus was dissected free from the muscle layer under the dissecting microscope, using watchmakers forceps.

Explantation and culture of the myenteric plexus.

The plexuses were transferred onto coverslips using a Pasteur pipette and grown in modified Rose Chambers (Rose 1954) at 37° C in a humid atmosphere containing 5% CO_2 in air. Plexuses were explanted onto glass coverslips coated with polylysine or with collagen type I. All explants were grown in DMEM containing 10% FCS for the first 12 h to allow the cultures to attach to the substrate. When the myenteric plexus is rinsed free of the muscle layer before it is explanted, only three major cell types are put into culture, neurons, glial cells and fibroblasts. Cytosine arabinoside (10^{-5} M, Sigma Chemical Co) was added to the medium for up to 72 h to kill rapidly dividing cells, mostly fibroblasts. The explant cultures were either maintained in DMEM + 10% FCS or transferred to supplemented medium.

Growth of the myenteric plexus inside a 3-dimensional collagen gel

Myenteric plexuses were explanted onto collagen coated coverslips for about 5 days, during which the individual ganglia

have spread into a monolayer of neurons and glial cells. A mixture of collagen solution A was put on top of the culture and at 37° C the collagen solution forms a firm gel and the plexus becomes embedded in a 3-dimensional collagen gel. These cultures were grown in either DMEM + 10% FCS or in chemically defined medium containing 0.5% FCS.

Freshly dissected explants were put straight into a 3-dimensional collagen gel. A segment of freshly dissected plexus was transferred in 50 µl of MEM-H and put onto a collagen coated coverslip in a 4 well tray, most of the solution was removed from the coverslip and 50 µl of a collagen solution A was put on top of the plexus and allowed to gel at 37° C and the plexus became embedded in a 3-dimensional collagen gel. These cultures were grown in either DMEM + 10% FCS or in defined medium containing 0.5% FCS.

IMMUNOHISTOCHEMISTRY

Antibodies were diluted in MEM-H plus 10% FCS if they were applied to living cells, or in PBS containig 0.1 M lysine, 0.02 % azide and 10% FCS if they were applied to fixed or dried cells.

Immunolabelling of Schwann cell cultures

All antibody incubations were carried out for 30 min at room temperature. Living Schwann cells on coverslips were incubated with OX-6 mAb to detect class II antigens. To visualize class II antigens the OX-6 mAb was followed by G-anti-MIg-Fl. To detect IL-1 Schwann cells on coverslips were labelled by using antibody against IL-1, raised in sheep. The cells were fixed in acetone for 10 min at -20° C, washed in PBS and incubated with anti IL-1 antibody

followed by G-anti-shIg-Fl. To identify Schwann cells the cultures were then double labelled with S-100 antibody. After the IL-1 labelling the cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature then in 5% acetic acid in ethanol for 15 min at -20° C followed by anti-S100 antibody and G-anti-RIg-Rd. To visualize fibroblasts the cultures were immunolabelled with anti-Thy-1.1 antibody (IgM) which was applied to living cultures, followed by G-anti-M-Fl to visualise Thy-1.1. To visualize macrophages the cultures were immunolabelled with the ED1 mAb which was applied to permeabilized cells to detect intracellular antigen. The cultures were fixed in formaldehyde in PBS for 20 min at room temperature followed by methanol for 10 min at -20° C and then further permeabilized with 0.3% Triton X-100 for 10 min. After washing in PBS the cells were incubated with ED1 mAb followed by goat G-anti-MIg-Fl.

Immunolabelling of oligodendrocytes

Rat oligodendrocyte cultures were incubated for 30 min with heat-inactivated normal rat serum diluted 1:10 in Eagles minimum essential medium containing 20 mM Hepes buffer (MEM-H) to block any potential sites of interaction with the F_c portion of immunoglobulin. Living cells on coverslips were labelled by indirect immunofluorescence using either OX6 or OX4 mAb to detect class II antigens. To visualize class II antigens the OX6 and OX4 mAb were followed by G-anti-MIg-Fl. The cells were then incubated with anti-Gal C antibodies followed by G-anti-RIg-Rd to identify oligodendrocytes. After the immunolabelling the cells were fixed in

formaldehyde in PBS for 20 min at room temperature and washed in PBS prior to mounting.

Immunolabelling of myenteric explant cultures

Cultures of the myenteric plexuses were labelled by indirect immuno-fluorescence using mAb against pro-collagen type I combined with S-100 antibody to identify glial cells. When cultures grown on coverslips were labelled the antibody incubations were carried out for 30 min at room temperature, but cultures grown inside collagen gels were incubated with antibodies overnight at 4° C. The cells were fixed in 5% acetic acid in ethanol for 15 min at -20° C, washed in PBS and treated with anti-pro-collagen type I antibody followed by G-anti-MIg-Fl to visualize intracellular pro-collagen. The cultures were then fixed in 4% formaldehyde in PBS for 20 min at room temperature, followed by 5% acetic acid in ethanol for 15 min at -20° C and incubated with S-100 antibody followed by G-anti-RIg-Rd. The cultures were washed in PBS prior to mounting.

Immunolabelling of teased nerve preparations

The expression of class II antigens on Schwann cells in the teased nerve preparations was determined by immunolabelling using OX6 mAb to detect class II antigens. Antibody against the intermediate filament protein GFAP was used to specifically label the non-myelin-forming Schwann cells and antibody against P_0 was used to identify the myelin-forming Schwann cells. The teased nerve preparations were allowed to dry for several hours before application of antibodies. The OX-6 was applied directly to the teased nerves for 30 min, followed by G-anti-MIg-Fl. Fibres were

then fixed and immunolabelled with either GFAP or P_0. Prior to immunolabelling with GFAP antibodies the teased nerves were treated with 95% ethanol/5% acetic acid for 10 min at - 20° C, washed with PBS and then incubated with 0.3% triton X-100 in PBS for 30 min. Anti-GFAP antibodies were visualized by G-anti-RIg-Rd. Before immunolabelling with anti-P_0 the teased nerve preparations were treated with 95% ethanol/5% acetic acid for 10 min at -20° C, washed in PBS and then incubated with 0.3% triton X-100 in PBS for 10 min. Anti-P_0 were visualized by G-anti-RIg-Rd. To detect macrophages the teased nerve preparations were immunolabelled with the ED1 mAb. The teased nerve preparations were fixed in 4% formaldehyde in PBS for 20 min, followed by methanol for 10 min at -20° C and then incubated with triton X-100 for 10 min. After washing in PBS the preparations were incubated with ED1 mAb followed by G-anti-MIg-Fl.

To visualize T lymphocytes the teased nerve preparations were incubated with mAb against rat T lymphocytes (W3/13HLK) followed by G-anti-MIg-Fl. After washing in PBS the preparations were fixed in 4% paraformaldehyde in PBS for 20 min and washed in PBS prior to mounting.

All preparations were mounted with Citifluor (Chemistry Department, City University, London,UK) and sealed with nail varnish. They were examined in a Zeiss microscope using x 40 and x 63 objectives with fluorescence and phase contrast Zeiss (Oberkochen, FRG) optics. Photographs were taken with Ilford HP5 film.

FIXATION AND EMBEDDING OF CULTURES FOR ELECTRON MICROSCOPY

Segments of the myenteric plexus were grown inside a 3-dimensional collagen gel as described earlier. For electron microscopy, the cultures were fixed in modified Karnovsky's fixative containing 2.5% glutaraldehyde and 2.7% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2 for 2 h on ice. The cultures were then rinsed in the same buffer for 30 min and post-fixed in 2% osmium tetroxide for 2 h on ice. After rinsing with distilled water, they were block-stained with a saturated aqueous solution of uranyl acetate overnight at 4° C, washed in distilled water, dehydrated in a graded series of ethyl alcohols and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert OMU 2 microtome and double-stained with uranyl acetate and lead citrate for observation in a Philips 301 electron microscope.

APPENDIX

Immunization and preparation of T lymphocytes, the lympho-proliferative assays and the IL-1 assays were carried out by Dr Ann Kingston.

Immunization and preparation of T lymphocytes

NIMR bred female Wistar or AS strain rats (200-250g) were immunized with 2 x 10^8 ^{60}Co-killed M. leprae intradermally in the shaved hind flank region. After 21 days animals were given a repeat dose of antigen and killed 7-10 days later. Draining lymph nodes were removed and a single-cell suspension was made. Cells were

enriched for T cells by nylon wool column incubation (Julius et al., 1973) and further treated with anti-MHC class II monoclonal antibody (mAb OX6; Seralab, Sussex, UK) at a 1:40 dilution plus rabbit complement (1:6 dilution, Buxted, Sussex, UK) to remove class II positive cells. Purified T cells were resuspended at 4×10^6/ml in complete RPMI 1640 medium containing L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-mercaptoethanol (5×10^{-5} M) and 10% FCS.

Lymphoproliferative assays

T lymphocytes (4×10^5/well) were added to 96-well flat bottom microtiter plates which contained rat sciatic nerve Schwann cells (2×10^4/well). Antigens were added at given concentrations and cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. When OX6 mAb was used, it was added to the cultures on the same day as the T lymphocytes.

After 5 days, cultures were pulsed with (^{3}H)-thymidine (1 µCi/well = 37 kBq/well, spec. act. 2 Ci/mmol, Amersham International, Amersham, UK) and harvested 18 h later with a Skatron harvester (Flow Labs., McLean, VA). (^{3}H)-thymidine incorporation was determined by liquid scintillation counting.

IL-1 assays

a) Thymocyte proliferative assay.

Thymuses from female BALB/c mice (6 weeks old), were removed and single cell suspensions prepared by the method of Oppenheim et al. (1976). Thymocytes were resuspended in complete RPMI-medium

containing L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 µ/ml), 2-mercaptoethanol (5×10^{-5} M) and 10% FCS. Cells were cultured in 96-well round bottom microtiter plates (Nunc, Roskilde, Denmark) at a density of 1.5×10^6 cells per well. A predetermined suboptimal concentration of phytohemagglutin (0.1 µ/ml) was added and one volume of Schwann cell culture supernatant or medium (100 µl) was added to the cells. Cultures were incubated for 72 h in 5 % CO_2/ 37° C incubator and pulsed with [^{3}H]-Thymidine (1 µ Ci/well) for the last 18 h of culture.

Cultures were harvested using a semi-automatic Skatron harvester and radioactivity was determined by standard liquid scintillation counting procedures.

b) EL-4 NOB-1 thymoma co-culture assay

Il-1 was assayed according to the method of Gearing et al. (1987). Briefly, a mouse thymoma line EL-4 NOB-1 obtained from the PHLS tissue culture collection Porton, U.K. was cultured in 96 well round bottom microtitre plate (Nunc, Roskilde, Scotland) at 2×10^5 cells per well. An IL-2 dependent mouse cytotoxic T cell line (PHLS, Porton, U.K.) was also added at 4×10^3 per well followed by a 100 µl of either an appropriate IL-1 standard (Genzyme, Boston, USA) or of supernatant sample taken from Schwann cell cultures to give a final volume of 200 µl. Cells were incubated for 24 h and were pulsed with 0.5 µCi (^{3}H)-thymidine (Amersham, s.a. 5 Ci/mmol) during the last 4 h of culture. Cells were then harvested and radioactivity was determined by standard liquid scintillation counting procedures.

CHAPTER 3

REGULATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II MOLECULES ON SCHWANN CELLS IN VITRO AND IN VIVO

SUMMARY

1 The regulation of the MHC class II molecules on Schwann cells in vivo and in vitro was investigated, and whether Schwann cells are able to present mycobacterial antigens to sensitized T lymphocytes. Immunization and preparation of T lymphocytes and also the lymphoproliferative assays were carried out by Dr. Ann Kingston.

2 Neonatal rat Schwann cells were able to support the lymphoproliferative responses of M.leprae antigen-reactive T lymphocytes in the presence of soluble antigen, without IFN-γ pretreatment. After incubation with T cells and MLS antigen, essentially all the Schwann cells expressed class II molecules. The accessory cell function of the untreated Schwann cells was dependent on class II expression.

3 T cell-derived TNF and IFN-γ appeared to act as mediators of the T cell-induced expression of MHC class II by Schwann cells, since antibodies against IFN-γ and TNF-α suppressed class II expression on the Schwann cells in the co-cultures.

4 When TNF was used in combination with rIFN-γ class II expression by the Schwann cells was substantially increased relative to that induced by IFN-γ alone. Antibody against TNF-α reduced class II expression in the TNF-α plus IFN-γ containing cultures to the same level as was seen with IFN-γ alone, and antibody against rIFN-γ inhibited class II expression in these cultures showing that the effects of TNF are directly dependent on IFN-γ.

5 Injection of lymphokines or mycobacterial antigens and crushing the sciatic nerve induced class II expression on Schwann cells in vivo. Strong class II immunolabelling was obtained on many myelin-forming Schwann cells, but very few non-myelin-forming Schwann cells expressed class II antigens. Class II positive Schwann cells were most numerous in nerves injected with IFN-γ or whole M.leprae.

6 To test whether cultured adult rat Schwann cells were able to process and perhaps present whole M.leprae to T cells, adult rat Schwann cells were incubated with mycobacteria-reactive T cells and whole M.leprae. The T cells formed clusters around the adult Schwann cells and immunolabelling showed that 98% of the Schwann cells expressed class II molecules on their surface.

7 The conclusion from this work is that Schwann cells fulfil several of the essential criteria necessary for antigen presentation, thus further supporting the view that in some pathological conditions, Schwann cells may interact with the immune system in T cell mediated immune responses.

INTRODUCTION

The nervous system has long been considered to be an immunologically privileged site, since access of immune cells and macromolecules is restricted by the blood-brain barrier and the blood-nerve barrier. Recent studies have challenged this view. It has been shown that activated T lymphocytes can cross the blood-brain and the blood-nerve barrier (Wekerle et al., 1986a), there is also evidence that astrocytes can be induced to express MHC class II antigens in vitro (Hirsch et al., 1983; Wong et al., 1984; Massa et al., 1986) and in vivo (Wong et al., 1985) and they can present antigen to myelin basic protein reactive T cell lines in vitro (Fontana et al., 1984; Fierz et al., 1985).

In peripheral nerves, the main glial elements are Schwann cells. In normal nerves these cells do not express MHC class II antigens. Schwann cells are also MHC class II negative in culture, but can be induced to express MHC class II molecules by exposure to high concentrations of IFN-γ (Samuel et al., 1987a, b; Wekerle et al., 1986b). IFN-γ treated Schwann cells can present antigen to T cell lines in vitro and this function is class II dependent (Wekerle et al., 1986b). Expression of class II antigens on Schwann cells could be relevant in pathological situations. Therefore it is important to gain a thorough knowledge of which factors might take part in regulating class II expression by Schwann cells and to define the circumstances under which they act.

In the in vitro studies quoted above, high concentrations of recombinant IFN-γ have been used to induce MHC class II antigens and

one of the points of interest is therefore to investigate whether class II antigens can be induced on Schwann cells in vitro under more physiological conditions. In man, M. leprae is the major bacterium to have special affinity for Schwann cells, and causes severe nerve damage. In the present study I used rats immunized with M. leprae antigen as a model system to examine the interaction of Schwann cells with antigen-reactive T lymphocytes. I have tested whether pretreatment with exogenous IFN-γ is required for antigen presentation by Schwann cells and have investigated the roles of T cell derived IFN-γ, TNF and IL-4 in regulating the expression of class II antigens by Schwann cells. Since the extracellular matrix, including type I collagen, can affect various aspects of Schwann cell behaviour and differentiation (Bunge et al., 1986; Eccleston et al., 1989b), the capability of collagen type I to regulate class II expression by cultured embryonic Schwann cells was also investigated.

In these in vitro studies the Schwann cells are removed from axonal contact. This has profound effects on the morphology and molecular composition of these cells, which, in culture, resemble embryonic Schwann cells more than they do mature non-myelin- or myelin-forming cells in vivo (Mirsky and Jessen 1990). To show that class II expression is potentially relevant for nerve pathology of adult mature nerves, it is also neccessary to demonstrate that class II expression can also occur on Schwann cells in vivo. I have therefore investigated the effect of intraneural challenge with soluble M. leprae antigens or whole M.leprae on the expression of MHC class II, and tested whether Schwann cells express class II molecules after an injection of INF-γ or TNF and after nerve crush.

Schwann cells present mycobacterial antigens to T lymphocytes without pretreatment with IFN-γ

To test whether Schwann cells could support the lymphoproliferative responses of mycobacteria-reactive T lymphocytes, cultures of early postnatal Schwann cells were incubated with MLS antigen and purified T cells derived from sensitized lymph nodes. In some experiments the Schwann cells were pretreated with IFN-γ to induce MHC class II antigens prior to incubation with T cells.

The proliferative responses to antigen were compared with those of unfractionated lymph nodes. Fig 3.1 shows the results of a representative experiment in which both untreated or IFN-γ treated Schwann cells were able to support the lymphoproliferative responses of M.leprae antigen-reactive T lymphocytes. Three separate assays were performed, all of which gave similar results. It was evident by direct observation that T cells formed rosette-like clusters around Schwann cells in culture demonstrating characteristic cell-cell interactions normally associated with T cells and APC (Fig. 3.2). Moreover, we consistently observed that T cells cultured with untreated Schwann cells showed better response to antigen, over a range of concentrations, than those cultures containing IFN-γ treated Schwann cells.

T lymphocytes induce class II expression on Schwann cells in the presence of antigen

We used OX6 mAb, which is directed against MHC class II molecules, and immunofluorescence to directly examine class II expression on Schwann cells which had been incubated with T cells and/or antigen under the conditions used in the lymphoproliferative assays. The results of two experiments using triplicate cultures in each one showed that essentially all of the untreated Schwann cells expressed class II antigen after incubation with T cells and MLS antigen (Fig. 3.3), while class II molecules were barely detectable in cultures incubated with T cells or antigen alone (single experiment using triplicate cultures). These results are summarized quantitatively in Table 3.1. These findings were confirmed in experiments involving different rat strains and in experiments involving the use of antibodies against TNF (see below).

IFN-γ is involved in the lymphocyte-induced class II expression on Schwann cells

To investigate whether IFN-γ was involved in the induction of MHC class II on untreated Schwann cells we added mAb against rat IFN-γ, DB1, to co-cultures of Schwann cells and activated T lymphocytes in the presence of MLS antigen. Two independent experiments were carried out each involving triplicate cultures. Immunofluorescence labelling showed that when DB1 was added to MLS-stimulated co-cultures on day 0, the expression of class II on the surface of Schwann cells was reduced from 98% (n=2) to 4% (n=2) of the total cells after 4 days in culture, indicating a role for endogenous IFN-γ in MHC class II induction.

Synergistic effects of TNF, but not IL-4, on IFN-γ induction of class II expression

Since class II antigens were induced on a greater number of Schwann cells when cultured with T lymphocytes and antigen than when they were cultured with IFN-γ alone (Table 3.1), it was conceiveable that other T cell-derived lymphokines such as TNF or IL-4, might also be involved in the mechanism of MHC class II induction on Schwann cells.

Schwann cells were incubated with different concentrations of either TNF or IL-4 alone or in combination with IFN-γ. After a 72h culture period it was evident by immunofluorescence labelling that TNF alone induced class II on approximately 1% of Schwann cells whereas IL-4 had no effect (results not shown). When TNF was used in combination with IFN-γ class II expression was substantially increased relative to that induced by IFN-γ alone (p values determined by Student's t-test ranged from $p<0.05$ to $p<0.0005$ when the response to INF-γ at varying concentrations with and without 300 U/ml TNF were compared) and approached the levels observed in Schwann cell cultures containing T cells and antigen. Addition of DB1 mAb inhibited class II expression in the TNF-α plus IFN-γ containing cultures further supporting the previous observation that effects of TNF are directly dependent on IFN-γ (Fig. 3.4).

Role of class II antigen in T lymphocyte and Schwann cell interaction

To show that the mechanism whereby untreated Schwann cells support T cell responses was directly dependent on the expression of

class II antigen, experiments were carried out to test the effects of the anti-MHC class II mAb OX6 on antigen-induced T cell proliferation. The antigens used for these experiments were soluble mycobacterium tuberculosis antigens (MTBX). When OX6 was added to the cell cultures at the beginning of co-culture, the T cell proliferative response to mycobacterial antigen was abrogated, demonstrating that the accessory cell function of the untreated Schwann cells was dependent on MHC class II expression (Fig. 3.5).

To test whether T lymphocyte and Schwann cell interaction is MHC restricted, Schwann cells from the non-MHC-compatible PVG or August strain of rats were cultured with sensitized AS strain T cells and MTBX antigen to see whether induction of class II antigen on Schwann cells would occur. Immunofluorescence labelling of Schwann cells from the various cultures showed that only syngeneic AS Schwann cells expressed measurable levels of class II antigens after 4 days of culture with AS T cells (Table 3.2). These results are supported by an experiment in which AS T cells did not show a lymphoproliferative response to antigen when cultured with PVG Scwhann cells (data not shown). While these results need to be substantiated by further experiments, they strongly suggest that T and Schwann cell cooperation is class II restricted.

TNF may be involved in the lymphocyte-induced class II expression on Schwann cells.

To further investigate whether endogenous TNF was contributing to lymphocyte-induced MHC class II expression on Schwann cells, a polyclonal antibody against murine TNF-α (dil 1:250) was added to co-cultures of neonatal rat Schwann cells and sensitized T

lymphocytes. In co-cultures to which anti-TNF-α antibody had been added the lymphoproliferative response to MLS antigen was reduced compared to that in co-cultures to which TNF-α was not added (Fig. 3.6). To test whether similar results would be obtained using antigens from a related bacterium, another experiment was carried out using MTBX. In the co-cultures to which anti-TNF-α was added, lymphoproliferative response to MTBX was also reduced compared to what was found in co-cultures without anti-TNF-α (Fig. 3.7). To test this further Schwann cells were co-cultured together with T cells and either MLS (Fig. 3.8) or MTBX (Fig 3.9) and anti-TNF-α antibody was added to some of the cultures. Immunolabelling showed that the expression of MHC class II on the surface of both neonatal and adult rat Schwann cells was reduced by 60% approximately after 4 days in culture.

The effects of anti-TNF-α antibody on the synergistic action of TNF-α on INF-γ induced expression of MHC class II were also investigated (Fig. 3.10). Fifty-one percent of neonatal Schwann cells expressed class II antigens when incubated with IFN-γ alone, while IFN-γ in combination with TNF-α induced class II antigens on 83.5% of the cells. When anti TNF-α antibody was added to Schwann cell cultures before they were exposed to a combination of TNF-α and IFN-γ, the class II expression was reduced to levels similar to those seen with INF-γ, indicating that anti TNF-α antibody blocks the effect of TNF-α on class II expression.

Taken together, the results presented here strongly suggest that endogenous TNF has a role in the lymphocyte-induced MHC class II expression on Schwann cells in lymphocyte-Schwann cell co-

cultures.

Type I collagen enables embryonic Schwann cells to respond to INF-γ by expressing class II antigens.

When Schwann cells are grown on polylysine, their ability to respond to IFN-γ by class II expression was found to increase with developmental age (Fig. 3.11). Thus, if embryonic day 19 (E-19) Schwann cells were grown on polylysine and incubated with IFN-γ for 3 days, 3.9% of the Schwann cells expressed class II antigens. When E-21, newborn or 4 day old rat Schwann cells were treated in the same way, class II expression is obtained on 15.9%, 19.0% and 48% of cells respectively. When E-19 Schwann cells were, however grown on gelled or dried type I collagen substrata and cultured for 2 days prior to incubation with IFN-γ, about 50% of the Schwann cells expressed class II on their surface. This enhancement of class II expression was also observed when embryonic Schwann cells were grown on heat denatured type I collagen, and when cells were grown on polylysine in medium containing soluble type I collagen (see below, Fig.3.13). Fibronectin was not necessary, since the embryonic Schwann cells expressed class II molecules when grown on collagen substrate in medium containing serum with or without fibronectin (data not shown).

To test whether rabbit antiserum (Gullberg et al., 1989) against the β_1 chain of integrin matrix receptor would bind to the surface of unfixed cultured Schwann cells, embryonic Schwann cells were immunolabelled and they showed a typical speckled cell surface immunostaining (Fig. 3.12). In preliminary studies,it was tested in two ways whether the effect of collagen on class II expression by

embryonic Schwann cells was mediated via integrin receptors. The anti-β_1 chain antibodies were added to Schwann cells grown on collagen substrate (Fig. 3.13) or on polylysine in medium containing soluble type I collagen (Fig. 3.14). In both experiments the IFN-γ induced class II expression on Schwann cells was reduced to the same percentage as that on Schwann cells grown on polylysine without soluble type I collagen. Irrelevant monoclonal antibody Ran-2 against a 140 kD Schwann cell surface protein had no effect.

Injection of lymphokines or mycobacterial antigens induces MHC class II expression on Schwann cells in vivo.

To test whether Schwann cells in vivo could be induced to express MHC class II molecules, nerves injected with IFN-γ were examined 3 days later, since maximum levels of MHC class II expression on cultured Schwann cells is seen after 3 days of incubation with IFN-γ. Strong MHC class II immunostaining was obtained on several Schwann cells, in the vicinity of the injection site (Fig.3.15). While it was impossible to assess accurately the number of class II positive fibers, I estimated, on the basis of counts from several injection experiments, that about 10% of the fibers expressed clear class II labelling at least along some part of their length. Most of the MHC class II positive Schwann cells were myelin-forming Schwann cells, identified by their characteristic morphology, by absence of GFAP immunoreactivity, and by the P_o immunolabelling. When sciatic nerves injected with TNF-α, MLS, whole irradiated M. leprae or live M. leprae were examined 3 days after the injection, many mononuclear cells were found near the

injection site and a patchy MHC class II staining was seen on some myelin-forming Schwann cells (Fig.3.16, 3.17). If nerves were examined six days after the injection of TNF-α or MLS, MHC class II expression was found on about 5% of the Schwann cells and in nerves injected with irradiated M. leprae or live M.leprae, MHC class II expression was found on about 10% of the Schwann cells (Fig.3.18; Table 3.3). Very few non-myelin-forming Schwann cells, identified by morphology and by immunostaining with GFAP, expressed MHC class II antigens (Fig.3.19).

In the sciatic nerve the MHC class II expression was seen on a much lower percentage of cells than in culture. The perineurial sheath which surrounds a bundle of nerve fibers, very likely forms a barrier to the diffusion of factors in the nerve and it is impossible to know with any certainty about the concentration of the injected factors in the nerve. Several MHC class II positive mononuclear cells, identified by their morphology, were present in the injected nerves and by using specific antibodies macrophages (ED1+ cells) and a few T lymphocytes (W3/13HLK+ cells) were also identified. Endothelial cells of blood vessels expressed high levels of MHC class II antigens, judging by the intensity of the immunostaining. No MHC class II was seen in control nerves injected with PBS in agreement with previous work (Samuel et al., 1987a).

Crushing the sciatic nerve induces MHC class II expression on Schwann cells.

Crushed sciatic nerves from adult rats were examined 1, 2, 3, 4, and 7 days after the nerve crush. When the distal stumps of crushed sciatic nerves were examined 1 or 2 days after crush, only

very few mononuclear cells were found and the Schwann cells did not express MHC class II molecules. Three or four days after the crush many mononuclear cells were found and a patchy MHC class II staining was seen on a low number of Schwann cells (Fig. 3.20). Six days after the nerve crush a few Schwann cells still expressed MHC class II molecules. In crushed sciatic nerves, from 9 day old rats, several GFAP positive Schwann cells were class II positive (Fig.3.21). Some mononuclear cells were seen, and endothelial cells of capillaries expressed high levels of MHC class II antigens. These results show that nerve damage is sufficient to elicit MHC class II expression on Schwann cells, 4 to 7 days after crush.

Adult rat Schwann cells express class II molecules when incubated with sensitized T cells and whole M.leprae.

In our previous work (Kingston et al., 1989), we showed that when neonatal rat Schwann cells were incubated with soluble M.leprae antigen and T cells, they were able to support a lymphoproliferative response and essentially all the Schwann cells expressed class II molecules after the process of antigen presentation. If soluble M. leprae antigens were replaced with whole M. leprae, in this type of experiment, no lymphoproliferative response was seen, and only 34.4% of the Schwann cells expressed class II molecules. This contrasts with the experiments above, in which whole M. leprae were more effective than soluble M.leprae antigens in triggering class II expression in adult Schwann cells in vivo. It seemed possible that this discrepancy was related to the difference in developmental age of the cells, adult cells responding better, perhaps because of

greater ability to process antigens, than the neonatal cells used in the in vitro experiments.

To test whether cultured adult rat Schwann cells were able to process and perhaps present whole M.leprae to T cells we incubated adult rat Schwann cells with mycobacteria-reactive T cells and whole M. leprae for 4 days. In contrast to the results seen with neonatal cells, the T cells formed clusters around the adult Schwann cells in these co-cultures (Fig. 3.22), and immunolabelling using OX6 mAb showed that 98% of the Schwann cells expressed class II molecules on their surface.

Figure 3.1

Effects of rat Schwann cells on mycobacterial antigen-induced stimulation of purified T cells. Schwann cells ($2x10^4$) were co-cultured with purified T lymphocytes ($4x10^5$) in the presence of MLS antigen. Assays were carried out as described in Chapter 2. (▲) represents whole unfractionated lymphocyte cultures; (■) represents T lymphocytes cultured with untreated 4-7 day-old Schwann cells; (●) represents T lymphocytes cultured with rIFN-γ -treated Schwann cells and (□) shows responses for T cells alone. Mean cpm (±SD) values of quadruplicate cultures are shown.

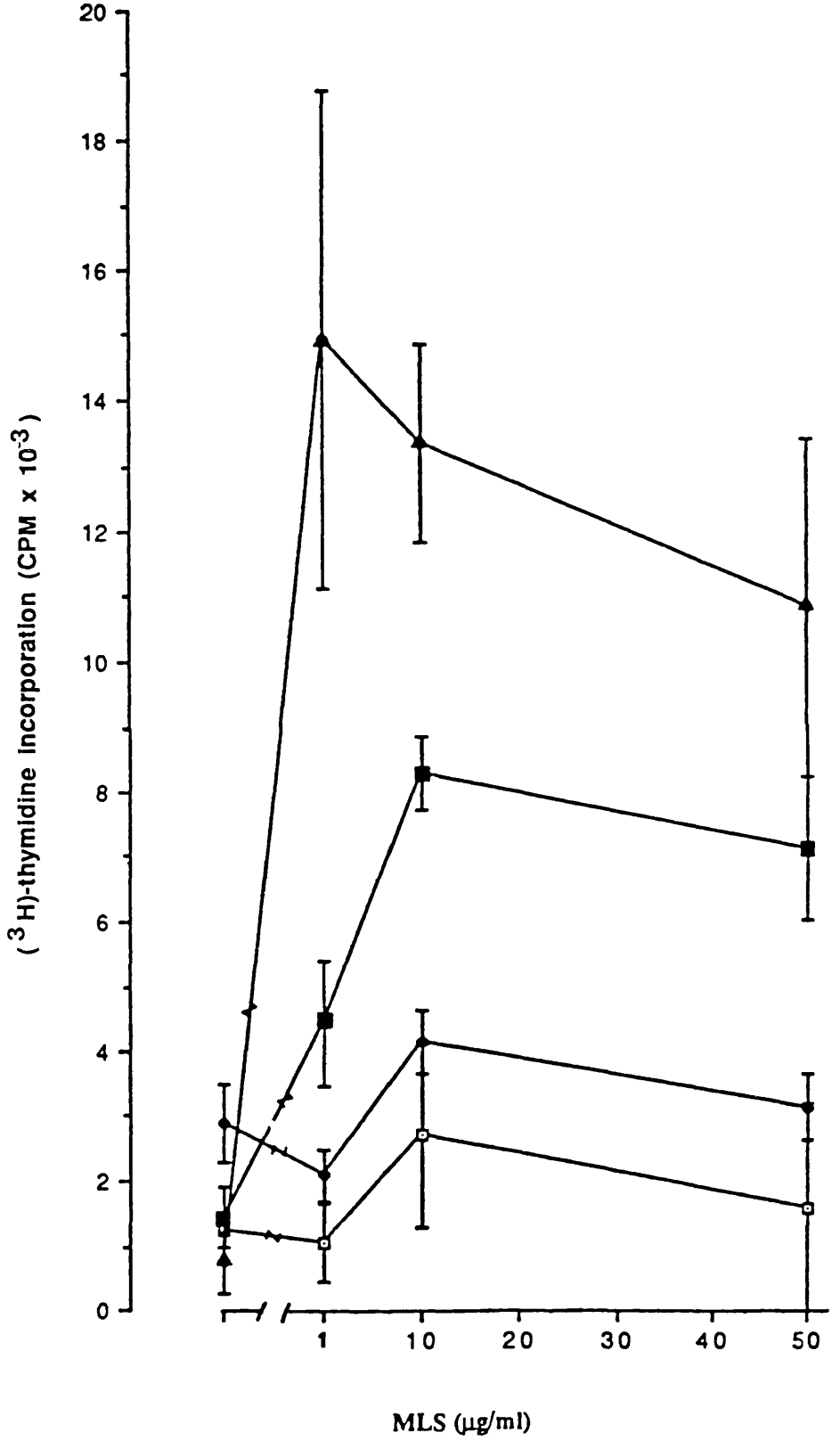

20
18
16
14
12
10
8
6
4
2
0
(^{3}H)-thymidine incorporation (CPM x 10^{-3})
1
10
20
30
40
50
MLS (μg/ml)

Figure 3.2

Phase contrast photomicrograph of neonatal rat Schwann cells and T lymphocytes. The T cells (small arrows) form clusters around the Schwann cells (large arrow) in the presence of MLS antigen. Magnification x 500.

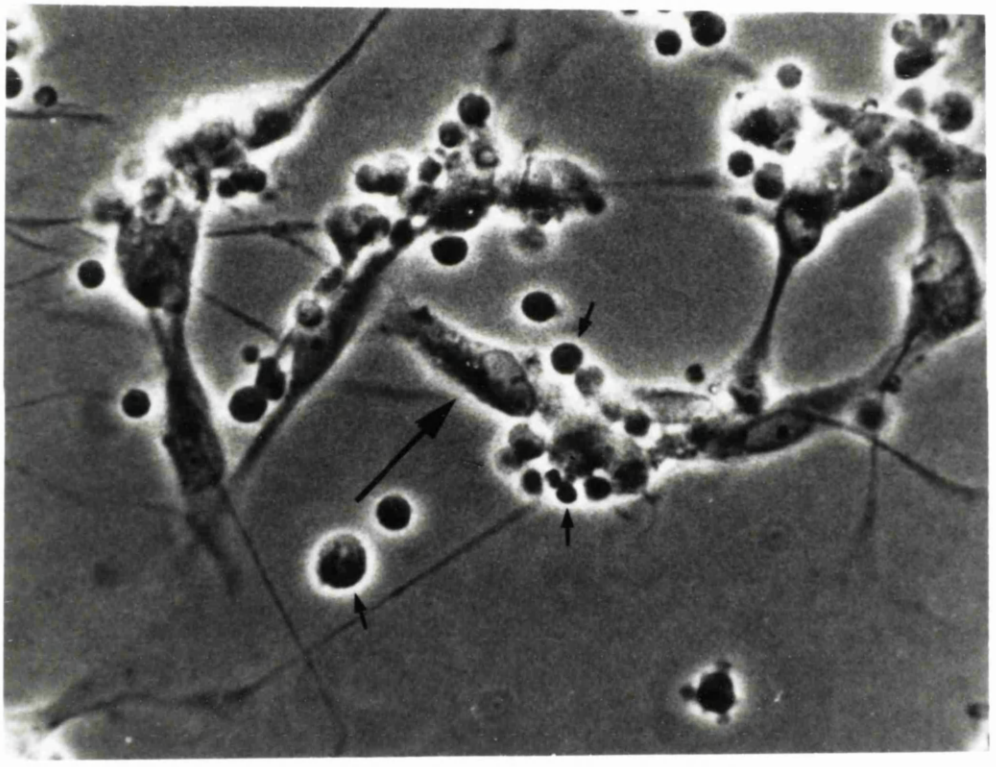


Figure 3.3

Expression of class II antigens by Schwann cells using double-label immunofluorescence. a) Phase contrast picture of Schwann cells (large arrow) and T cells (small arrow) after 4 days of culture in the presence of MLS antigen; b) expression of class II seen with fluorescence optics and c) the Schwann cell marker S100 detected intracellularly with rhodamine optics. Magnification x 500.

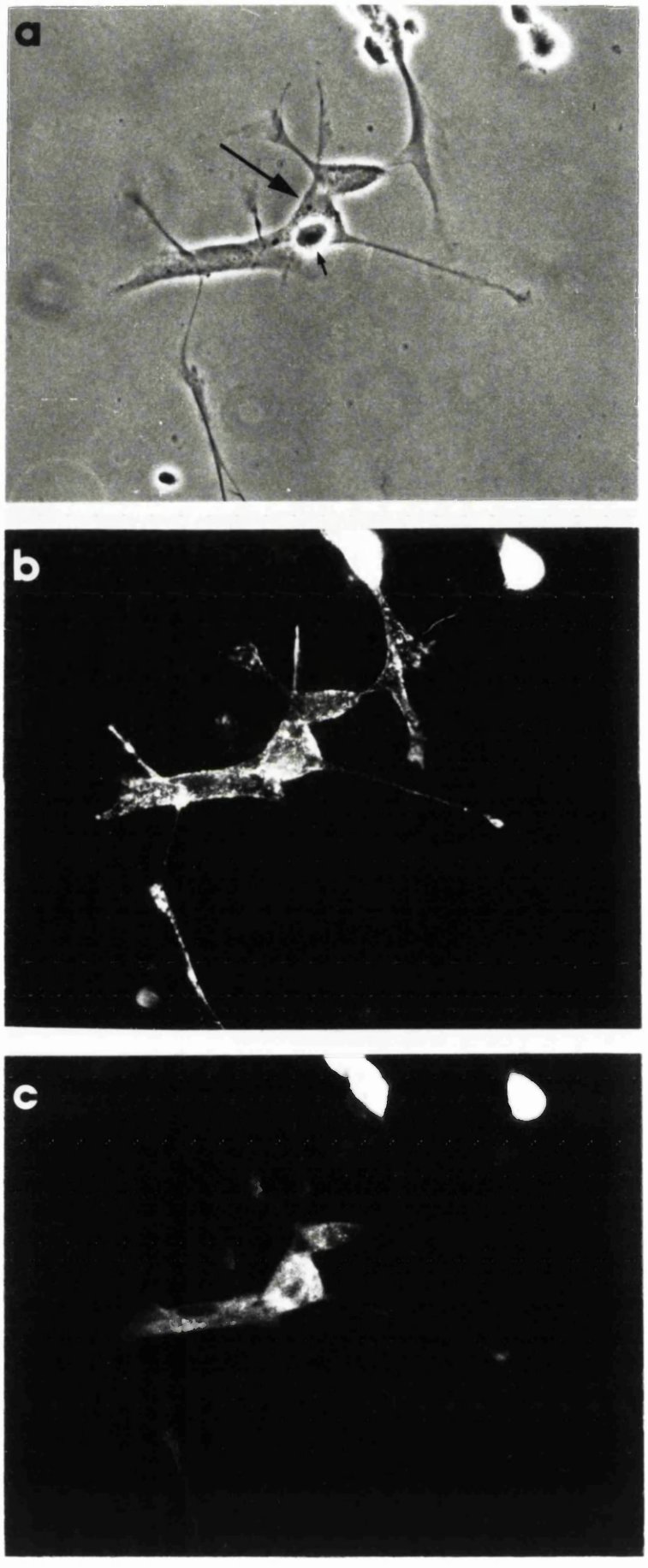

a
b
c

Figure 3.4

Induction of class II on Schwann cells incubated with different concentrations of IFN-γ and TNF. Schwann cells were cultured on coverslips as described in Chapter 2, and incubated with IFN-γ for 72 h adding TNF-α for the last 24 h. Cultures were then immunolabeled. Mean values for three separate experiments, each involving duplicate cultures, are shown; SD were within 10% of mean.

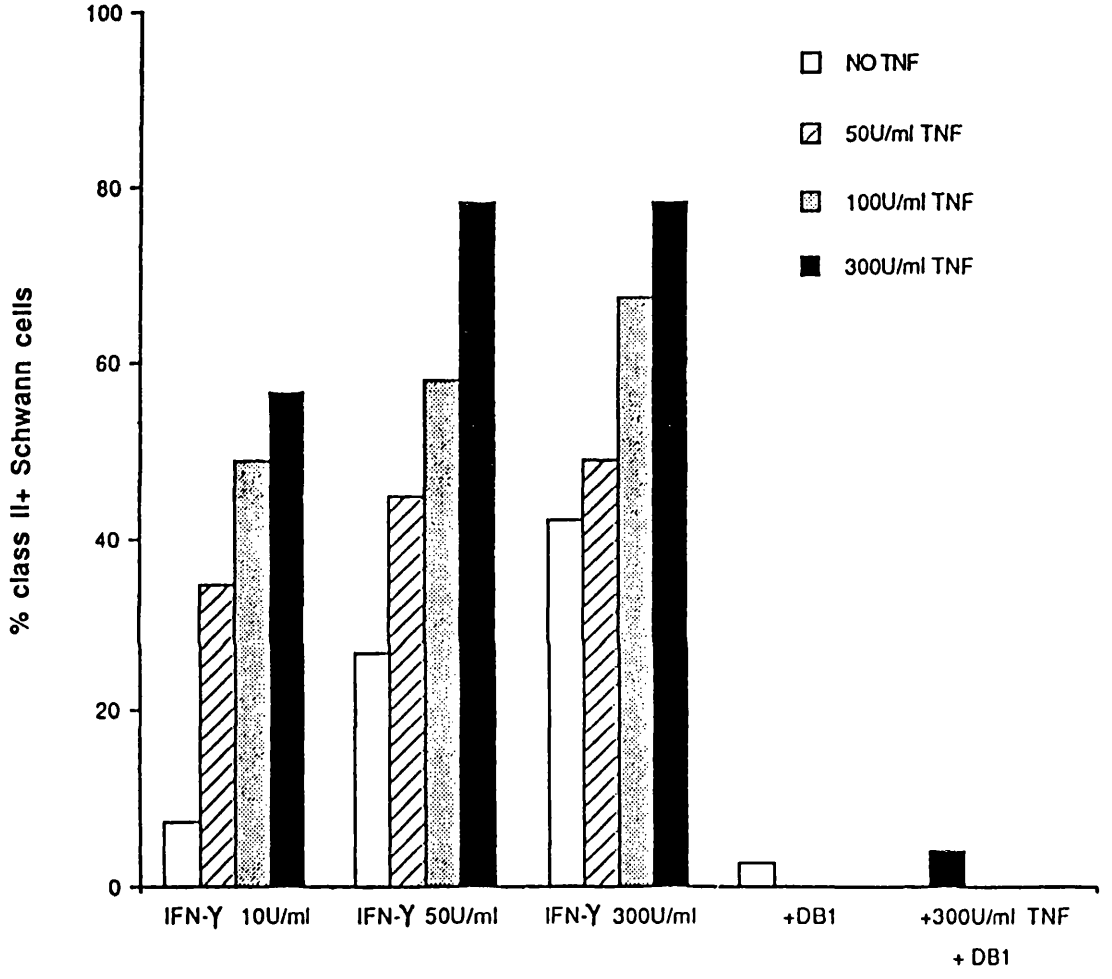

100
80
60
40
20
0
% class II+ Schwann cells
NO TNF
50U/ml TNF
100U/ml TNF
300U/ml TNF
IFN-γ 10U/ml
IFN-γ 50U/ml
IFN-γ 300U/ml
+DB1
+300U/ml TNF
+ DB1

Figure 3.5

Effects of anti-class II antibody on mycobacterial antigen presentation by Schwann cells to sensitized T lymphocytes. MTBX (10 μg/ml) was added to cultures of purified T cells; purified T cells co-cultured with Schwann cells (S.C.) and cultures of unfractionated lymph node lymphocytes. Assays were carried out as described in Chapter 2. Cross hatched bars represent responses in cultures to which anti-class II OX6 mAb was added at the start of culture (1:100 dilution). Mean cpm value of quadruplicate cultures are shown for one representative experiment; SD were within 10 % of mean. Three independent experiments were carried out.

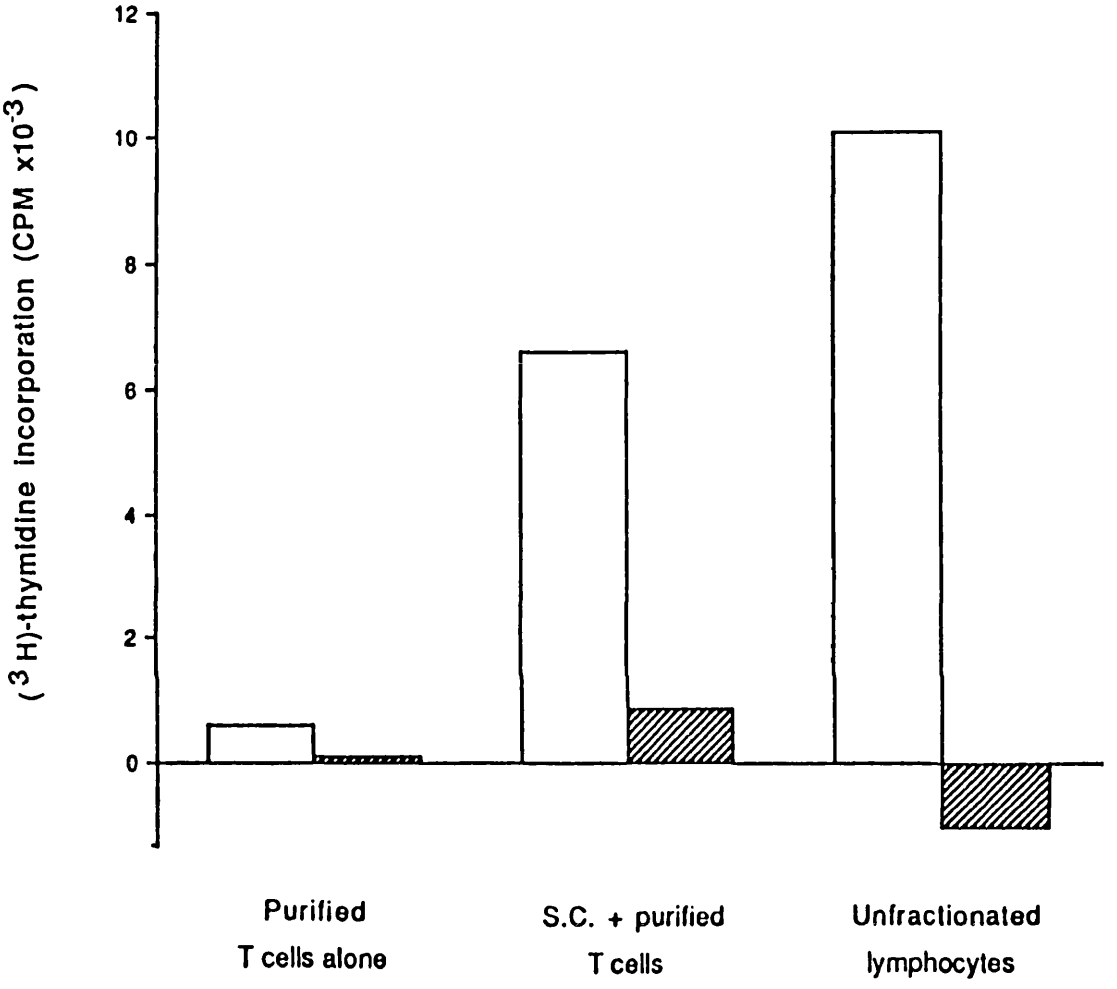

12
10
8
6
4
2
0
(^{3}H)-thymidine incorporation (CPM x10^{-3})
Purified
T cells alone
S.C. + purified
T cells
Unfractionated
lymphocytes

Figure 3.6

Effects of anti-TNF antibody on mycobacterial antigen presentation by Schwann cells to sensitized T lymphocytes. Schwann cells were co-cultured with purified T lymphocytes in the presence of three different concentrations of MLS antigen. Assays were carried out as described in Chapter 2. Each point represents the average (± SD) of quadruplicate cultures from one experiment.

(□) represents T lymphocytes cultured with Schwann cells; (●) shows responses in cultures to which anti-TNF-α antibody (1:250 dilution) was added at the start of culture.

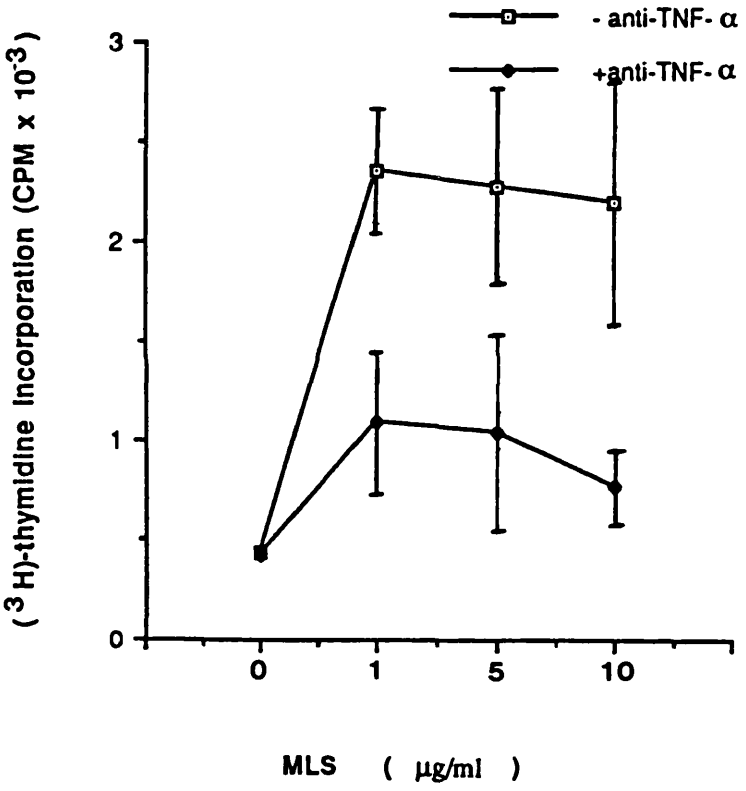

- anti-TNF- α
+anti-TNF- α
(^{3}H)-thymidine Incorporation (CPM x 10^{-3})
3
2
1
0
0
1
5
10
MLS (µg/ml)

Figure 3.7

Effects of anti-TNF-α antibody on mycobacterial antigen presentation by Schwann cells to sensitized T lymphocytes. Schwann cells were co-cultured with purified T lymphocytes in the presence of three different concentrations of MTBX antigen. Assays were carried out as described in Chapter 2. Each point represents the average (± SD) of quadruplicate cultures from one experiment. (●) represents T lymphocytes cultured with Schwann cells; (□) shows responses in co-cultures to which anti-TNF-α antibody (1:250 dilution) was added at the start of culture.

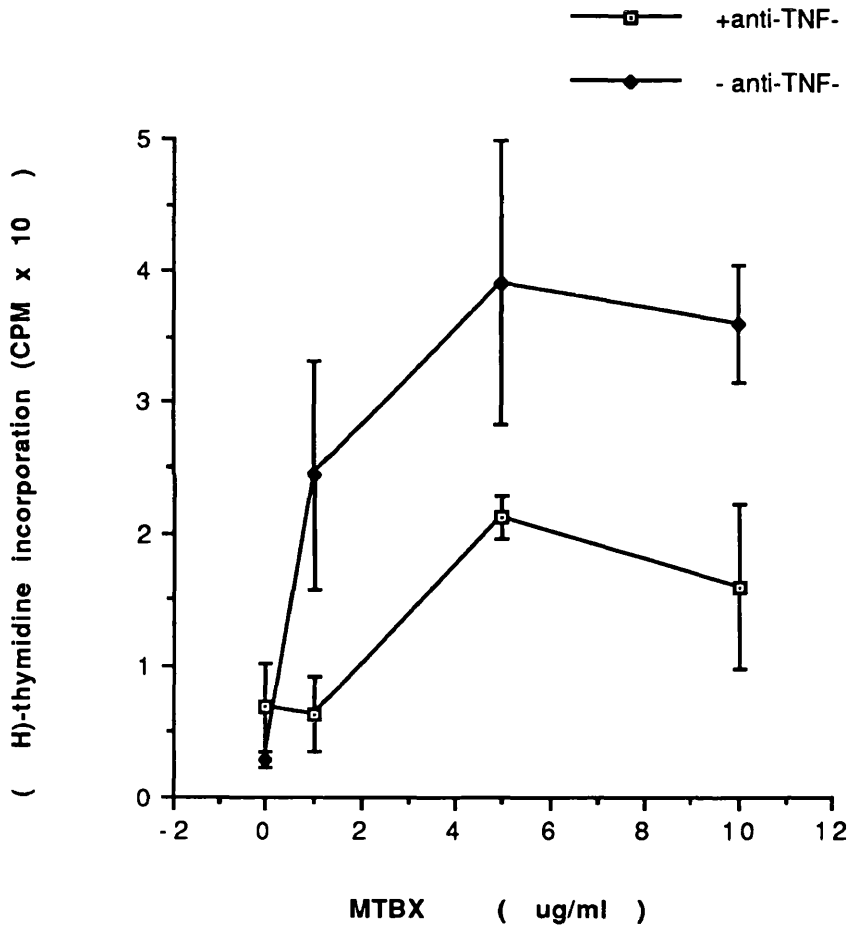

+anti-TNF-
- anti-TNF-
(H)-thymidine incorporation (CPM x 10)
5
4
3
2
1
0
-2
0
2
4
6
8
10
12
MTBX (ug/ml)

Figure 3.8

Effects of anti-TNF-α antibody on class II expression by Schwann cells (adult and neonatal), using double-label immunofluorescence. Schwann cells were co-cultured with purified T lymphocytes in the presence of MLS antigen (10 μg/ml) for 4 days.Anti-TNF-α antibody (diluted 1:250) was added to some of the cultures at the start of the co-culture period. The Schwann cell cultures were then immunolabelled using OX6 mAb. For neonatal Schwann cells each column represents an average (± SD) of a total of five coverslips derived from three independent experiments, and for the adult Schwann cells each column represents an average (± SD) of three coverslips from one experiment.

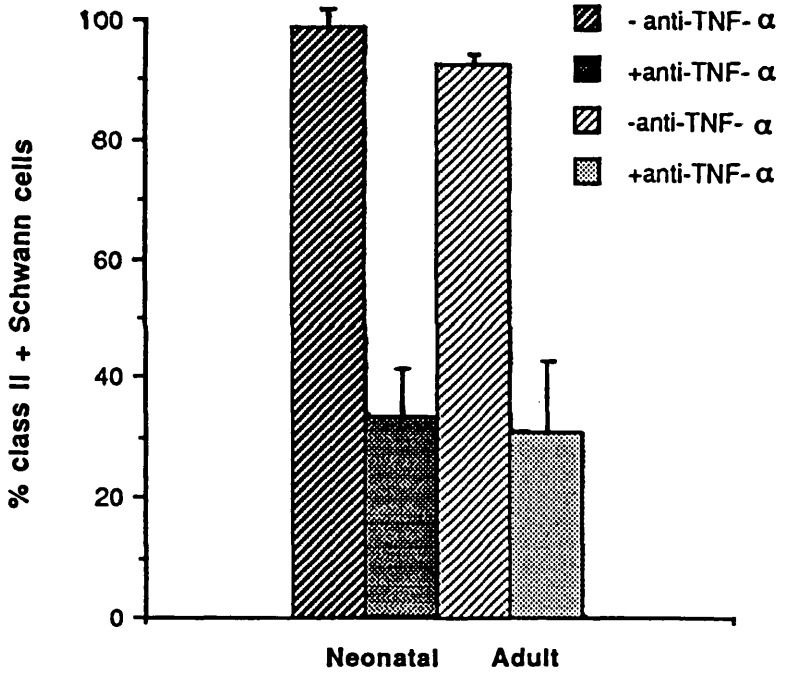

100
80
60
40
20
0
% class II + Schwann cells
- anti-TNF- α
+anti-TNF- α
-anti-TNF- α
+anti-TNF- α
Neonatal
Adult

Figure 3.9

Effects of anti-TNF-α antibody on class II expression by neonatal Schwann cells, using double-label immunofluorescence. Schwann cells were co-cultured with purified T lymphocytes in the presence of MTBX antigen (10 μg/ml) for 4 days. Anti-TNF-α antibody (diluted 1:250) was added to some cultures at the start of the co-culture period. The Schwann cell cultures were then immunolabelled using OX6 mAb. Each column represents an average (± SD) of three coverslips from one experiment.

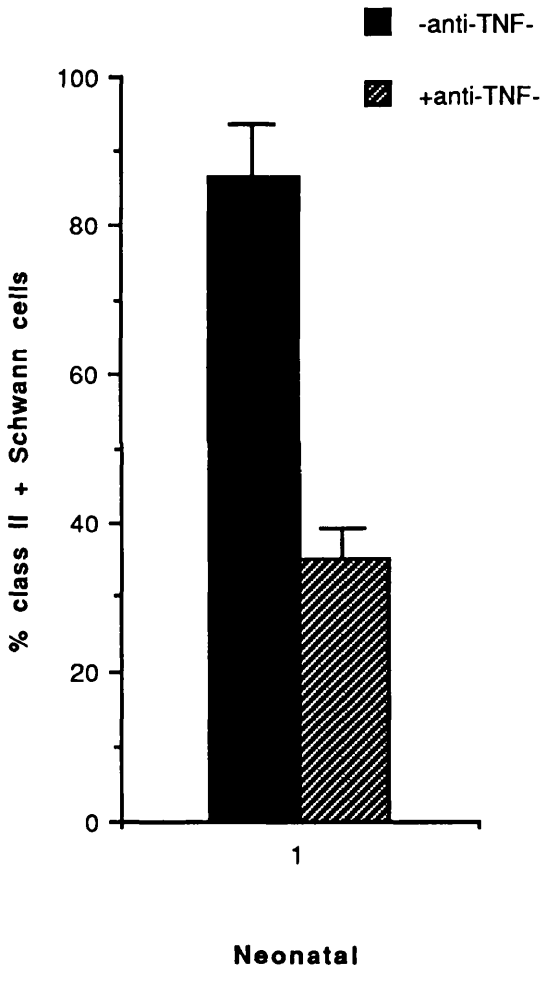

-anti-TNF-
+anti-TNF-
100
80
60
40
20
0
% class II + Schwann cells
1
Neonatal

Figure 3.10

Effects of anti-TNF-α antibody on class II expression, induced by IFN-γ in combination with TNF, on neonatal rat Schwann cells, using double-label immunofluorescence. Schwann cells were cultured on coverslips as described in Chapter 2, and incubated with IFN-γ (100 U/ml) for 72 h, adding TNF (300 U/ml) for the last 24 h. Anti-TNF-α (diluted 1:240) was added to some cultures before TNF-α was added. Cultures were then immunolabelled. Each column represents an average (± SD) of a total of four coverslips derived from two independent experiments each involving duplicate coverslips.

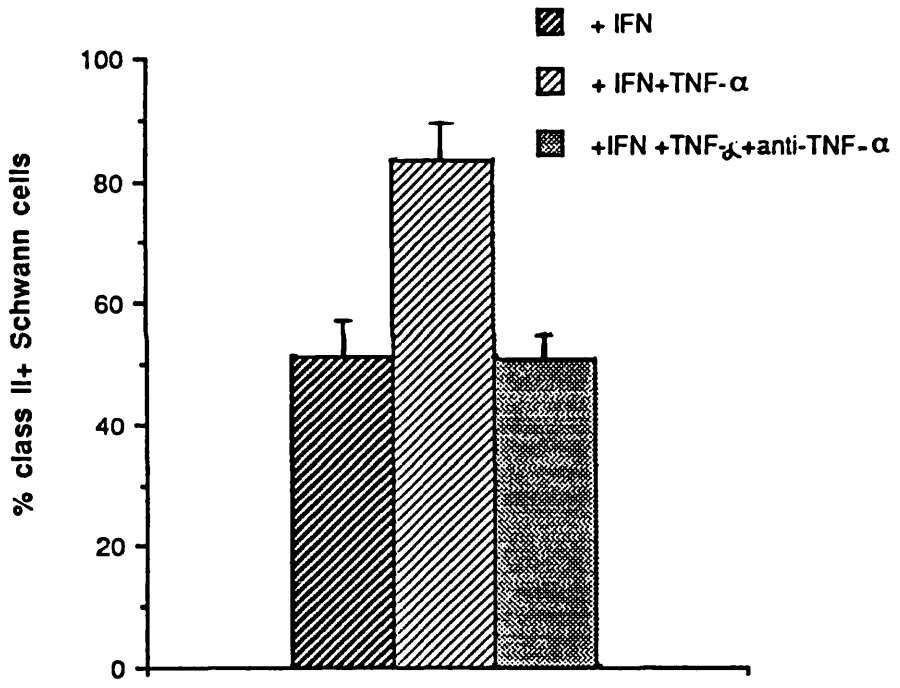

+ IFN
+ IFN+TNF-α
+IFN +TNF-α+anti-TNF-α
% class II+ Schwann cells
100
80
60
40
20
0

Figure 3.11

IFN-γ induction of class II antigens on Schwann cells at different developmental stages, using double-label immunofluorescence. E-19, E-20, E-21, newborn, and 4 day old neonatal rat Schwann cells were grown on polylysine or on collagen coated coverslips for 2 days, and then incubated with IFN-γ (100 U/ml) for 3 days. Cultures were then immunolabelled with OX6 mAb. In the case of E-19 and the 4 day time points, three separate experiments were carried out each involving duplicate coverslips, for the E-20 time point one experiment was carried out involving two coverslips and for time points E-21 and newborn two experiments were carried out each involving two coverslips. The columns represent the average (±SD) of the total number of coverslips at each time point.

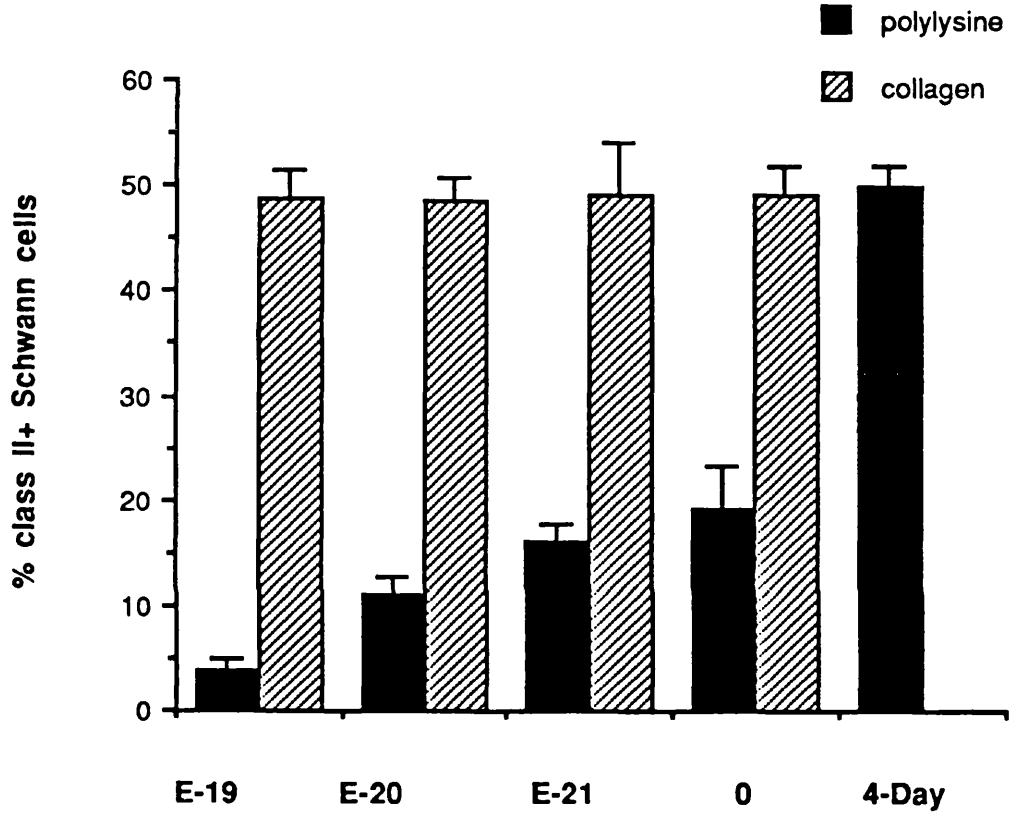
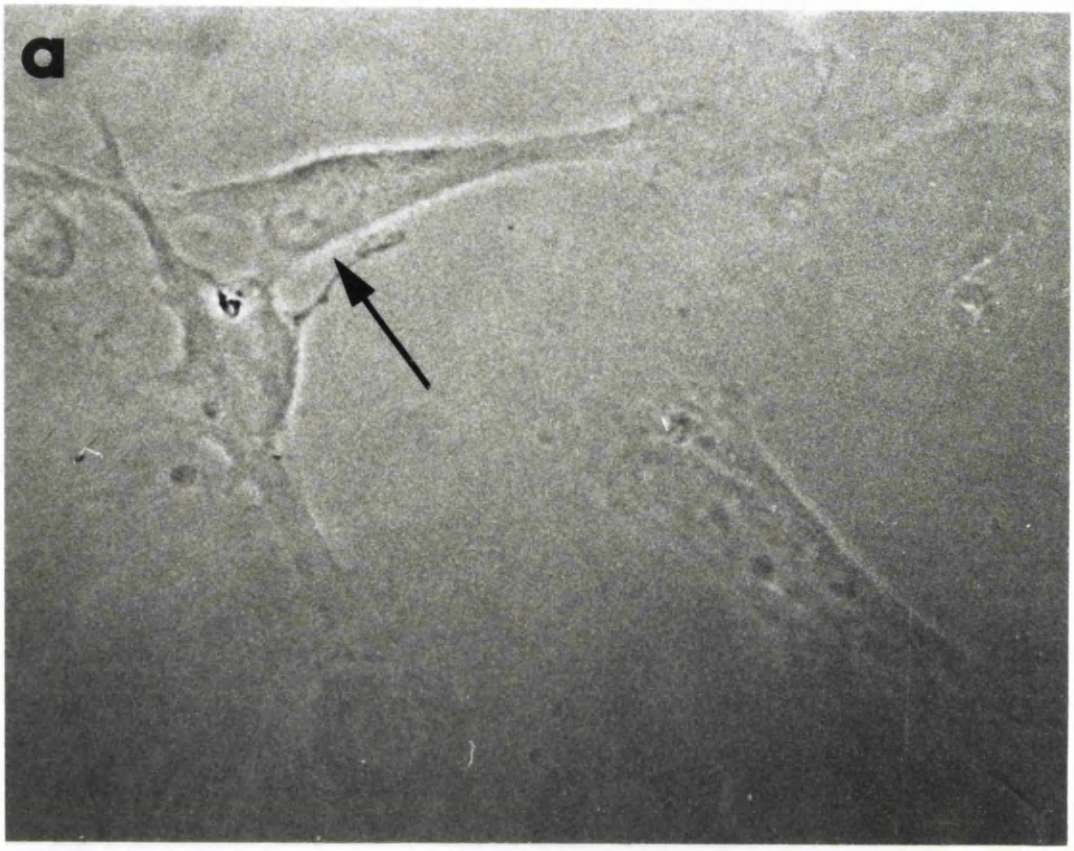

polylysine
collagen
% class II+ Schwann cells
60
50
40
30
20
10
0
E-19
E-20
E-21
0
4-Day

Figure 3.12

Expression of the β_1 chain type integrin receptor on embryonic Schwann cells. a) Phase contrast picture of embryonic Schwann cells (arrow); b) expression of integrin receptor seen with fluorescein optics . Magnification x 800.


a

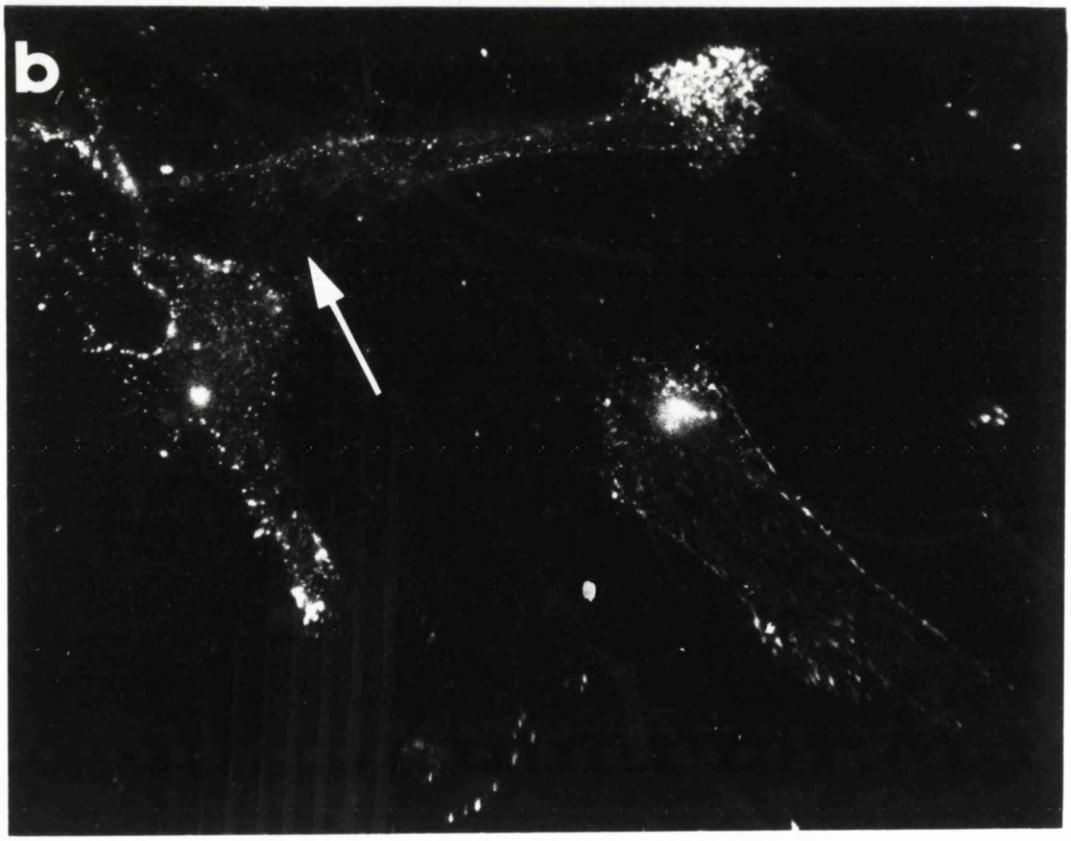
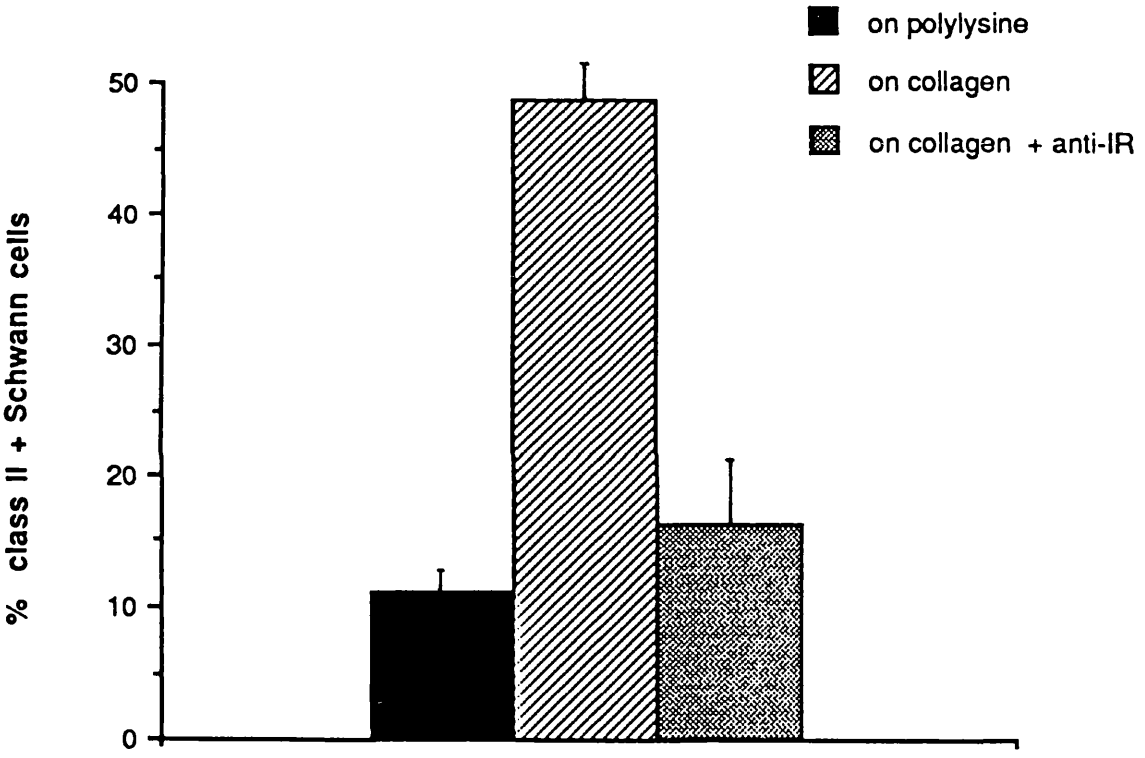

b

Figure 3.13

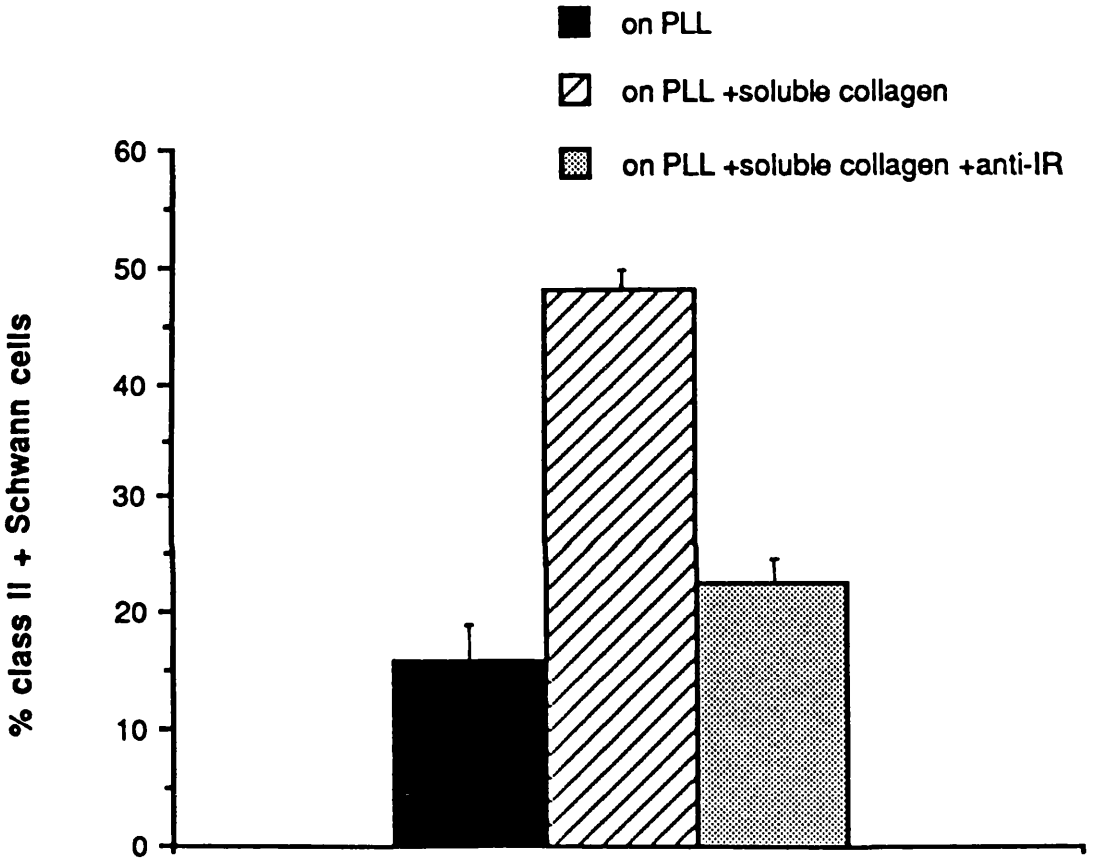
Effects of anti-integrin-receptor antibody on class II expression by Schwann cells. E-20 Schwann cells were grown on polylysine or on collagen coated coverslips, anti-integrin-receptor antiserum (diluted 1:20) was added to some cultures at the same time as they were plated out on the coverslips. The antibody did not prevent the Schwann cells from attaching to the substrate. After 2 days of culture the Schwann cells were incubated with IFN-γ (100 U/ml) for 3 days and then immunolabelled with OX6 mAb. Each column represents an average (± SD) of duplicate cultures from one experiment.


on polylysine
on collagen
on collagen + anti-IR
% class II + Schwann cells
50
40
30
20
10
0

E-20

Figure 3.14

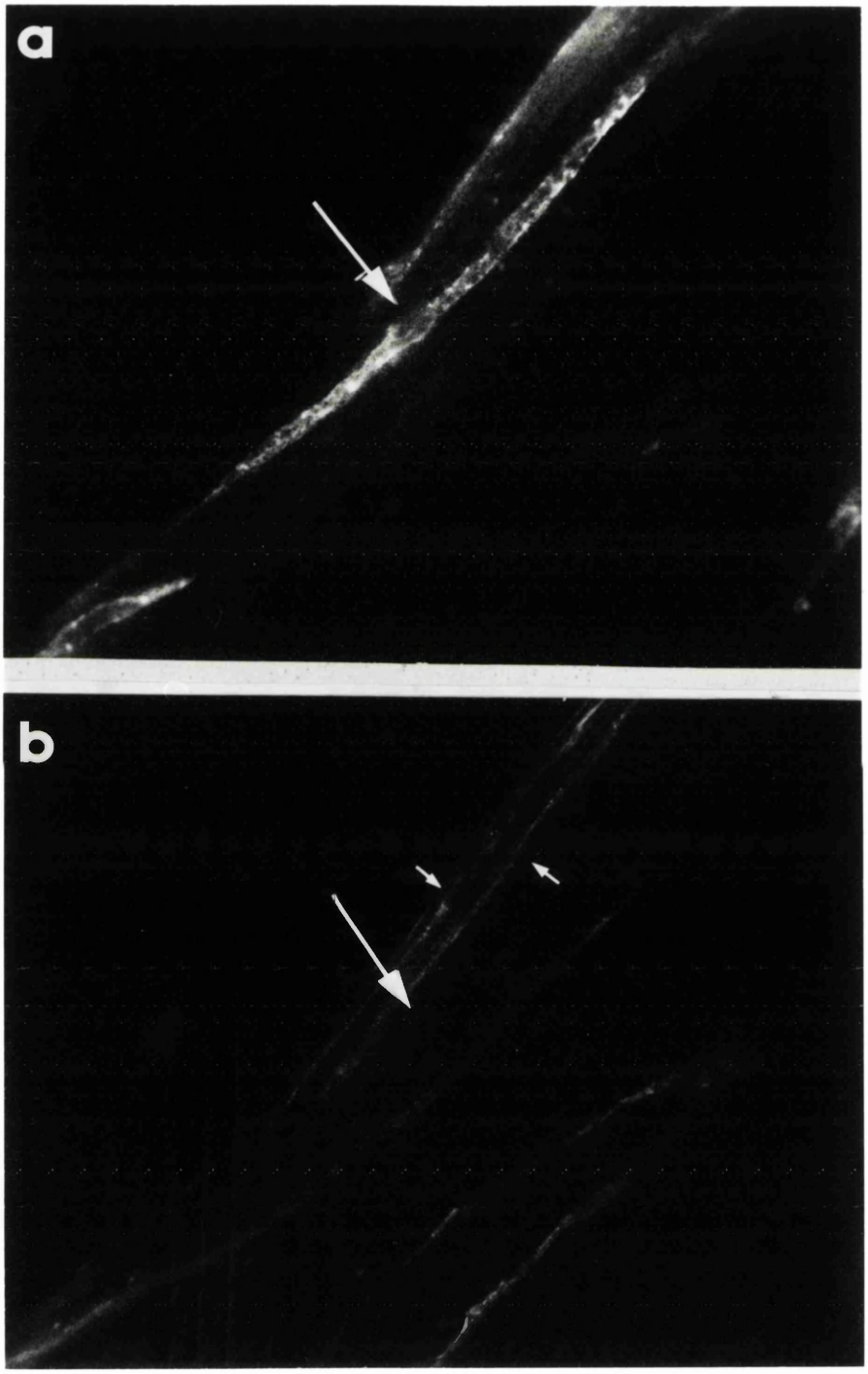
Effects of anti-integrin-receptor antibody (diluted 1:20) on class II expression by Schwann cells. E-21 Schwann cells were grown on polylysine, and some cultures were grown in medium containing soluble collagen (80 µg/ml) with or without anti-integrin-receptor mAb. After 2 days of culture the Schwann cells were incubated with IFN-γ (100 U/ml) for 3 days and then immunolabelled using OX6 mAb. Each column represents an average
(± SD) of duplicate cultures from one experiment.


on PLL
on PLL +soluble collagen
on PLL +soluble collagen +anti-IR
% class II + Schwann cells
60
50
40
30
20
10
0

E-21

Figure 3.15

Expression of class II in a teased nerve preparation of a sciatic nerve injected with IFN-γ, using double label immunofluorescence. A 5 to 6mm nerve segment centered on the injection site was teased and examined. a) Expression of class II in a bundle of Schwann cells, seen with fluorescein optics, one of the Schwann cells is clearly class II positive (large arrow, the same cell is arrowed in b) while two other positive cells lie partly out of the plane of focus; b) GFAP, a marker of non-myelin-forming Schwann cells, detected intracellularly with rhodamine optics (e.g small arrows); the class II positive Schwann cell (large arrow) is GFAP negative. Magnification x 800.


a
b

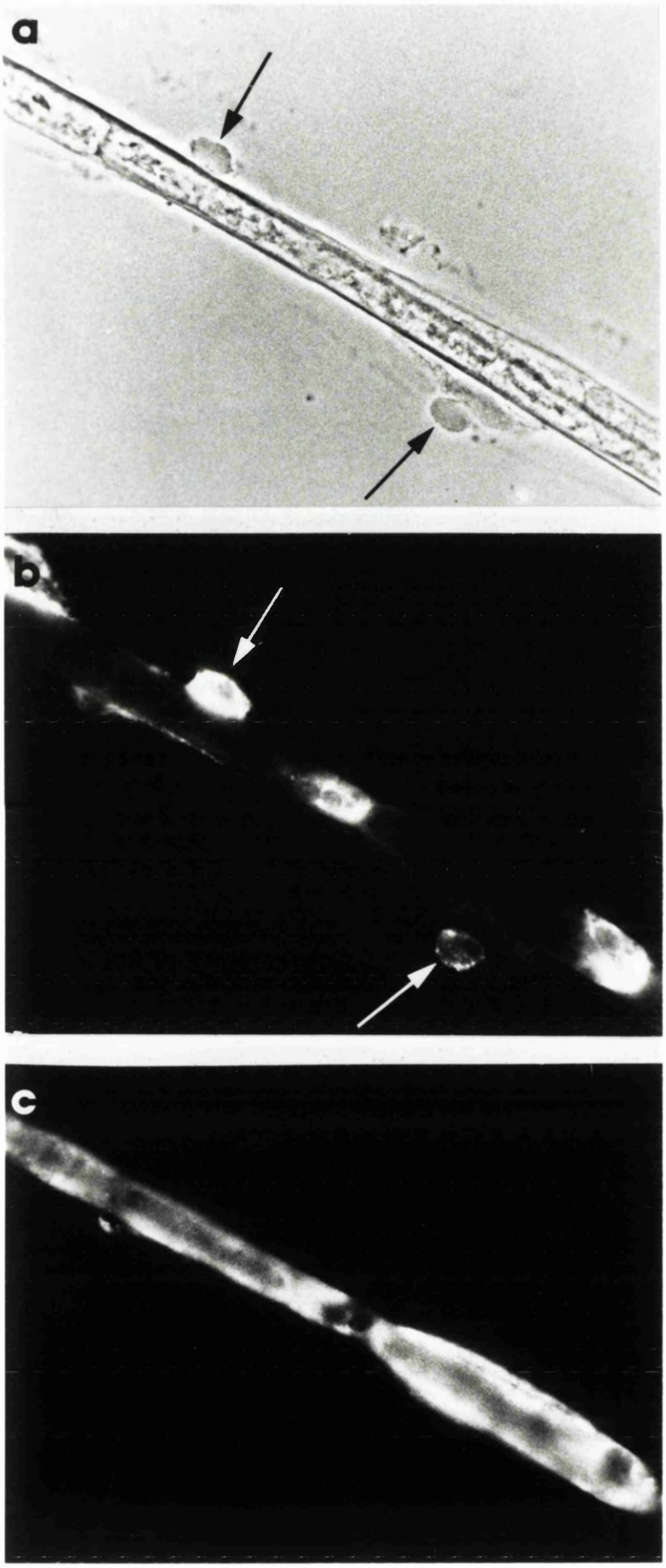

a
b
c

Figure 3.16

Expression of class II antigens in a teased nerve preparation of a sciatic nerve injected with irradiated M.leprae, using double label immunofluorescence. a) Phase contrast picture of a single myelin-forming Schwann cell and associated mononuclear cells (arrows) b) expression of class II seen with fluorescein optics, the mononuclear cells are class II positive and the Schwann cell shows a patchy class II staining, often seen in the injected nerves c) P_0 detected intracellularly with rhodamine optics. Magnification x 1000.

Figure 3.17

Expression of MHC class II antigens in a teased nerve preparation of a sciatic nerve 3 days after an injection of TNF, using double-label immunofluorescence. a) expression of MHC class II seen with fluorescein optics, the mononuclear cells are MHC class II positive, and the Schwann cells have a patchy staining. b) Phase contrast picture of Schwann cells and associated mononuclear cells (arrows). Magnification x 1000.

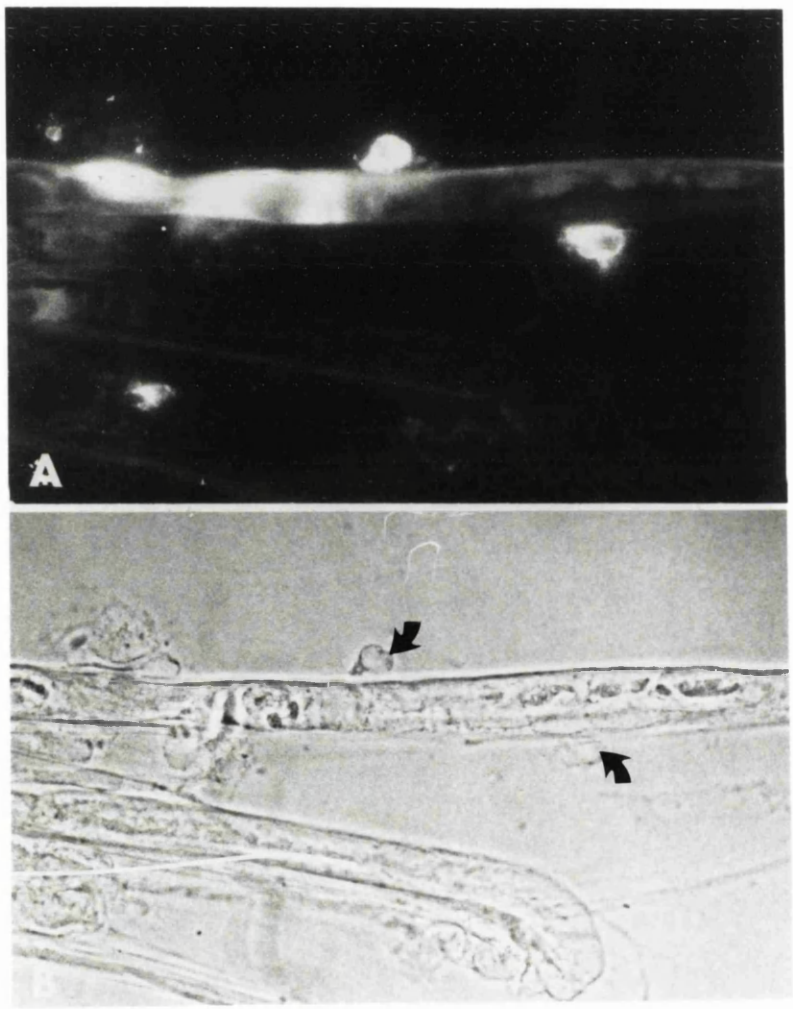

A

Figure 3.18

Expression of class II antigens in a teased nerve preparation of a sciatic nerve injected with whole M.leprae, using double-label immunofluorescence. a) Phase contrast picture showing a single myelin-forming Schwann cell b) expression of class II seen with fluorescein optics c) P_0 detected intracellularly with rhodamine optics. Magnification x 1000.

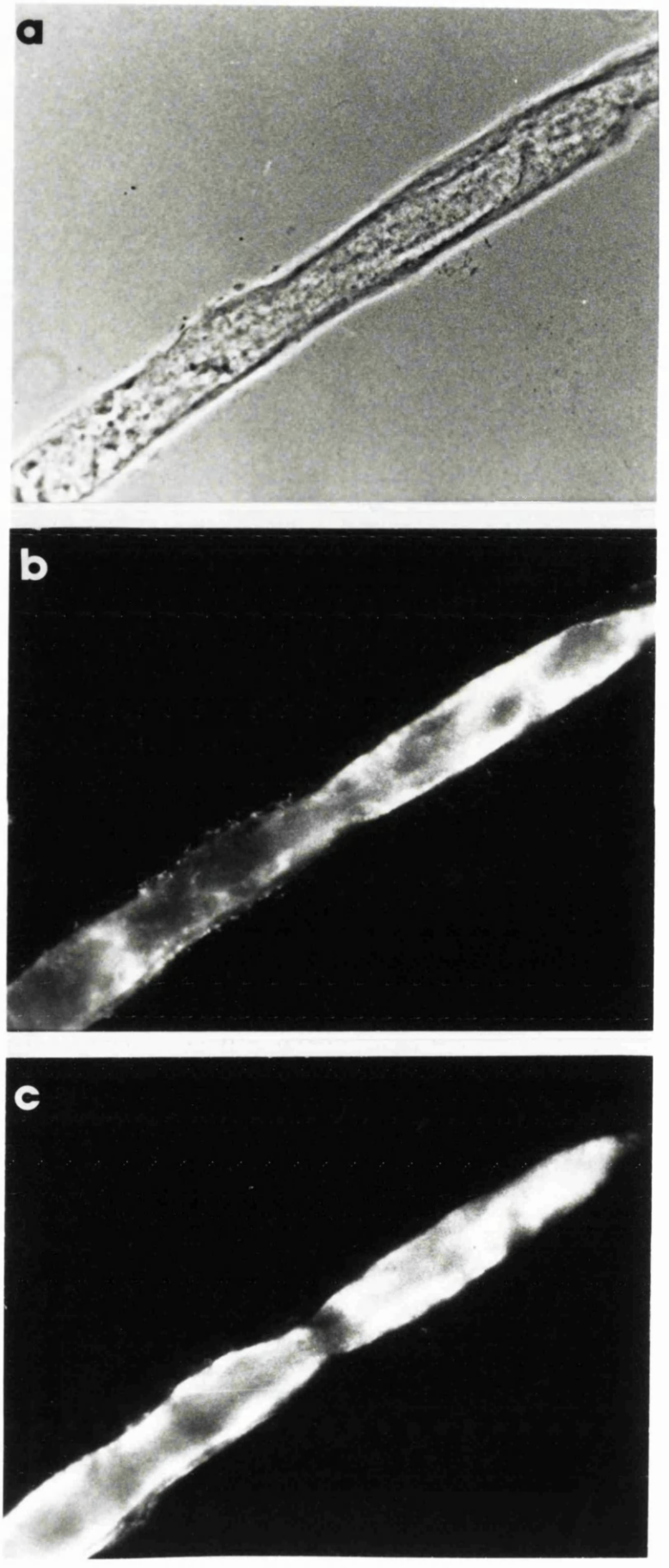

a
b
c

Figure 3.19

Expression of class II antigens in a teased nerve preparation of a sciatic nerve injected with whole M.leprae using double-label immunofluorescence. a) Expression of class II in a small bundle of Schwann cells, seen with fluorescein optics, one of the Schwann cells is clearly class II positive (arrow), other class II positive cells lie out of the plane of focus b) GFAP detected intra-cellularly with rhodamine optics, the class II positive Schwann cell is also GFAP positive (arrow), showing that on some occasions non-myelin-forming Schwann cells can express class II antigens. Magnification x 800.

Figure 3.20

Expression of MHC class II antigens in a teased nerve preparation of an adult sciatic nerve 4 days after crush, using double-label immunofluorescence. a) Phase contrast picture showing a bundle of Schwann cells b) expression of MHC class II seen with fluorescein optics, one of the Schwann cells is clearly MHC class II positive (arrow) c) P_o detected intracellularly with rhodamine optics, the MHC class II positive cell is also P_o positive. Magnification x 1000.

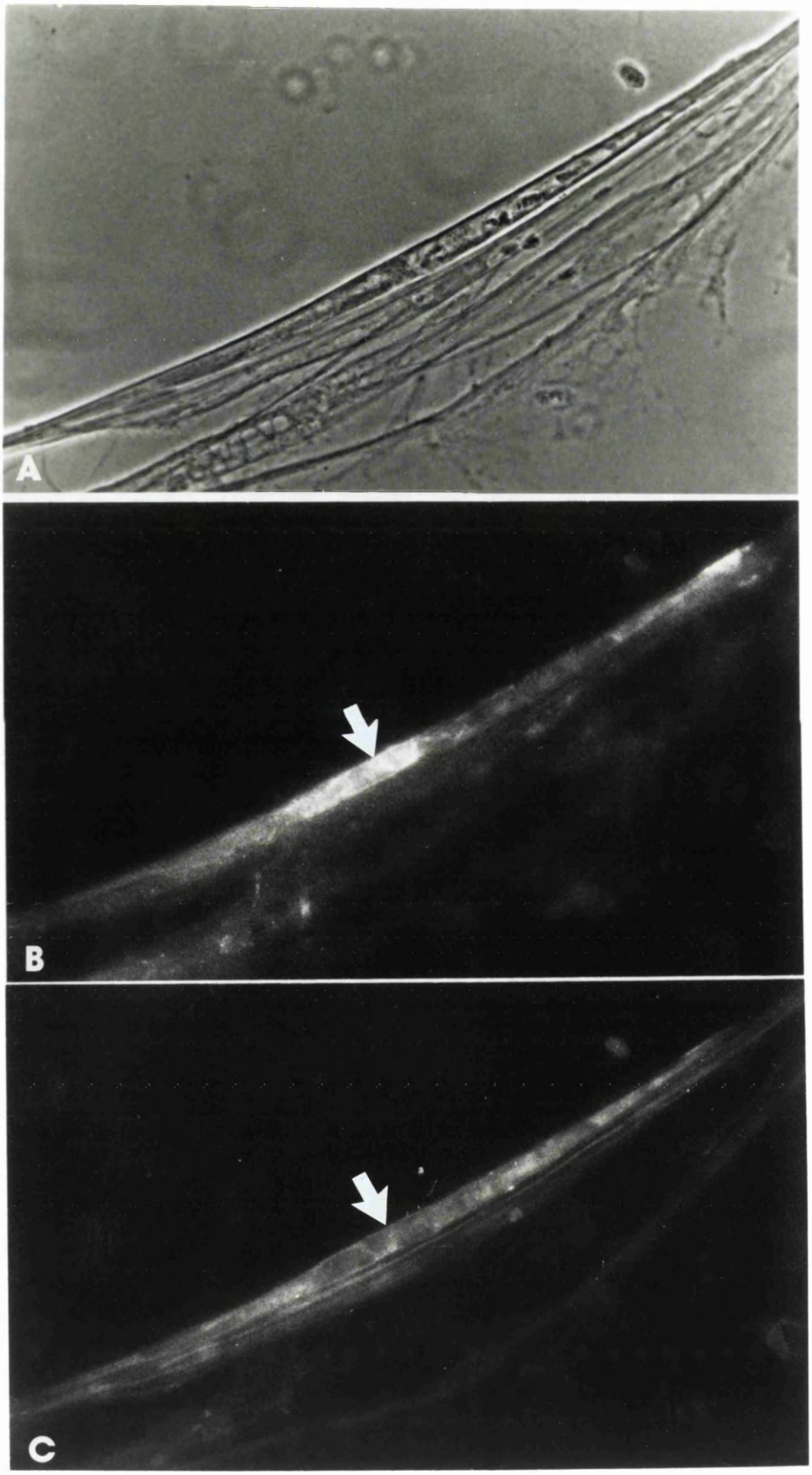

A
B
C

Figure 3.21

Expression of MHC class II antigens in a teased nerve preparation of a 9 day old sciatic nerve 7 days after crush, using double-label immunofluorescence. a) Phase contrast picture showing a single non-myelin-forming Schwann cell b) expression of MHC class II seen with fluorescein optics c) GFAP detected intracellularly with rhodamine optics. Magnification x 1000.

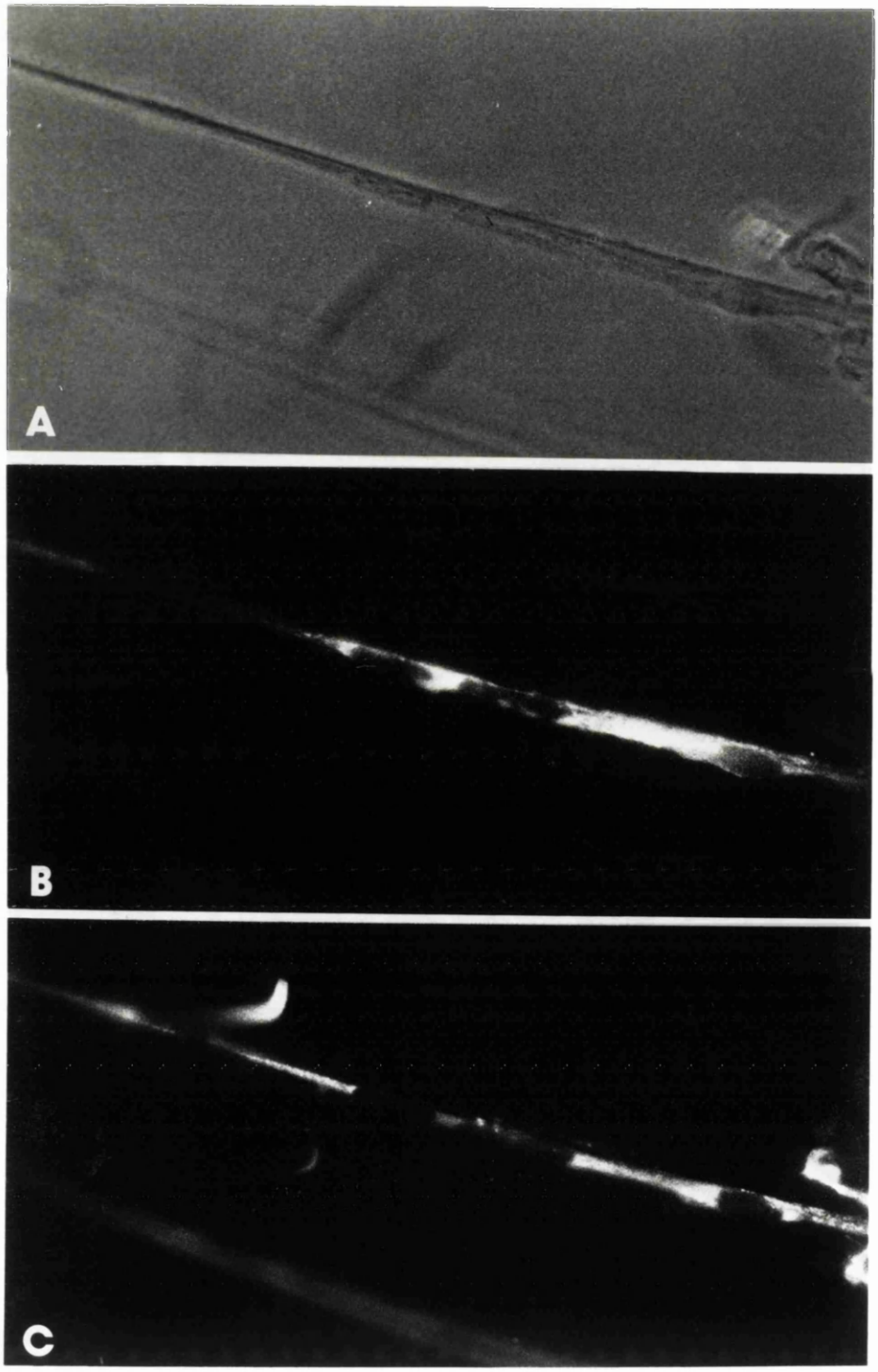

A
B
C

Figure 3.22

Phase contrast photomicrograph of adult rat Schwann cells and T lymphocytes. The T cells (small arrows) form clusters around the Schwann cells (large arrow) in the presence of whole M.leprae antigen. Magnification x 500.

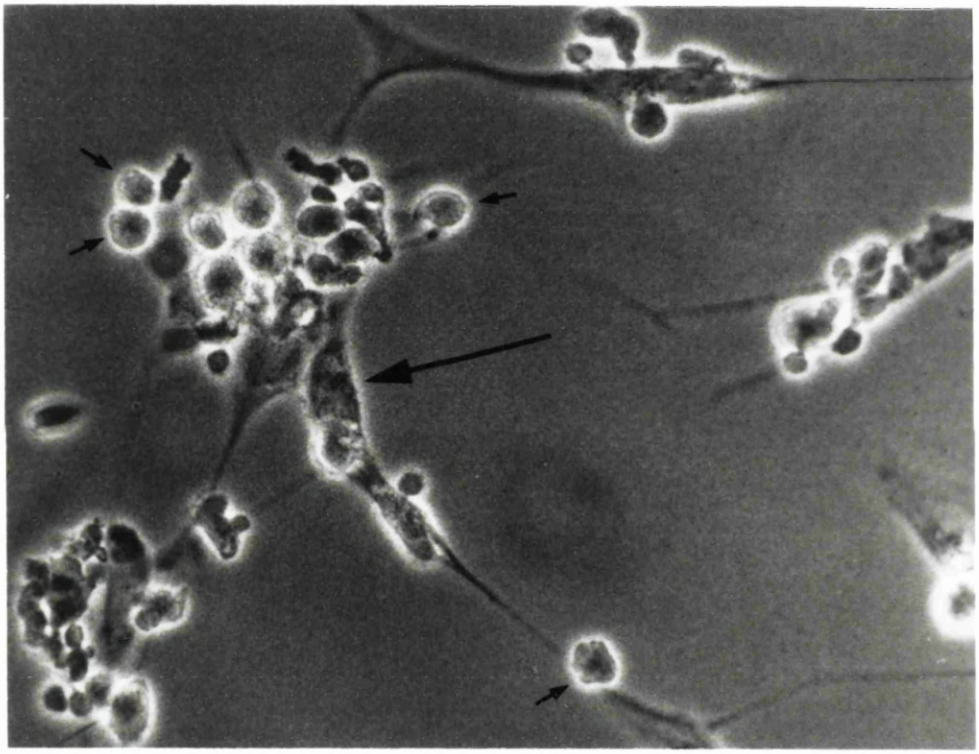


Table 3.1

Comparison of MHC class II antigen expression in untreated and IFN-γ pretreated cultures of Schwann cells.

a) Results show mean ± SD for two experiments. In each experiment at least three coverslip cultures were examined and between 100-200 S100 positive Schwann cells /coverslip were counted.

b) Not determined.

c) Results are for one experiment using three coverslips.

d) MLS (10 μg/ml).

Comparison of MHC class II antigen expression in untreated and γ-interferon pretreated cultures of Schwann cells.

	Mean percentage MHC class II positive Schwann cells[a]			
	No addition	Plus Antigen[d]	Plus T cells	Plus antigen plus T cells
Untreated Schwann Cells	0	0.75(±0.5)	0	97.75(±0.9)
rIFN-γ treated Schwann cells	48(±4.0)	ND[b]	ND[b]	83.7(±7.0)[c]

Table 3.2

Comparison of class II antigen expression in different strains of rat Schwann cells co-cultured with activated AS rat T lymphocytes.

a) Results show mean ±SD of triplicate cultures. An average of 190, S100 positive Schwann cells/coverslip were counted.

b) MTBX (10 µg/ml).

Comparison of MHC class II antigen expression in different strains of rat Schwann cells co-cultured with AS rat lymphocytes.

	Mean percentage MHC class II positive Schwann cells[a]		
	No addition	Plus AS T cells	Antigen [b] plus AS T cells
August Schwann cells	0	0	0
PVG Schwann cells	0	0	8.0 (±5.0)
AS Schwann cells	0	0	74.5 (±10.6)

Table 3.3

Comparison of class II expression by Schwann cells, in teased nerve preparations of nerves 3 to 6 days after injection with live M.leprae, soluble M. leprae (MLS), whole irradiated (^{60}Co) M.leprae antigens or cytokines. Phosphate buffered saline was used as a control. A 5 to 6mm nerve segment centered on the injection site was teased and examined. Some specimens contained no positive cells (-). The rest were divided into two broad categories, those with several class II positive Schwann cells (approx. 10%; ++;) and those with significantly fewer positive cells (approx. 5%; +).

Expression of MHC class II antigens on Schwann cells in vivo

Factors injected into the sciatic nerve.	MHC class II + Schwann cells
PBS Control	-
Lymphokines:	
Interferon-γ	++
Tumor necrosis factor	+
Bacterial antigens:	
Soluble M.leprae	+
Whole ^{60}Co M.leprae	++
Bacteria:	
M.leprae	++

DISCUSSION

The present work confirms and extends a recent series of studies which support the view that glial cells participate in immune responses. I found that when Schwann cells were incubated with sensitized T lymphocytes and antigens, essentially all the Schwann cells were stimulated to express class II antigens even without any added IFN-γ. This is an important observation, since the high concentrations of IFN-γ used in earlier studies to induce class II on Schwann cells have cast doubt on whether this response can be considered physiological. The ability of Schwann cells to express class II antigens was dependent on the presence of both antigen and T lymphocytes. It appeared to require MHC compatibility, since Schwann cells from neonatal rats of strains other than AS were not induced to express class II molecules when cultured with mycobacterial antigen and AS rat T cells although this needs to be confirmed by further experiments. In addition to expressing class II antigens, Schwann cells untreated with exogenous IFN-γ were seen in close association with T cells and acted as antigen presenters when co-cultured with T cells in vitro. Under these conditions, endogenous IFN-γ and TNF, presumably secreted by the T cells, play a key role in inducing high levels of class II molecules on the Schwann cells, since we found that anti-IFN-γ mAB DB1 suppressed the T cell-induced class II expression and anti-TNF-α antiserum reduces the proportion of Schwann cells expressing class II antigens by around 65%. Comparable levels of class II expression could not be induced by exposing Schwann cell cultures to IFN-γ alone, but were obtained in the presence of IFN-γ and TNF-α.

The induction of class II molecules by foreign antigen and T cells in vitro suggests a subtle mechanism for T cell and Schwann cell interaction. It is possible that antigen first binds to Schwann cells via an interaction with undefined ligands and is then taken up and internalized to be expressed later on the surface of the Schwann cells in association with class II antigens. We observed that antigen alone stimulated expression of class II molecules on a very small number of Schwann cells which may be sufficient to at least initiate T cell binding and recognition of specific antigen. As reported for macrophages, this may be an event first mediated nonspecifically through the binding of a molecule such as LFA-1 on T cells to Schwann cell surface ligands (Dougherty et al., 1987). This is supported by a study which has shown that such ligands are present on the Schwann cell surface membrane (Edelman et al., 1987). As a consequence of T cell binding and recognition of the class II antigen complex expressed on the Schwann cell membrane, T cells presumably become activated and produce lymphokines which up-regulate expression of class II on more Schwann cells and thereby amplify further recruitment of T cells.

In order to define the lymphokines which, apart from IFN-γ, may also regulate and enhance class II expression, we chose to investigate the effects of two factors, TNF and IL-4, which have been shown to induce expression of class II antigens on other cell types (Chang et al., 1986; Noelle et al., 1984; Stuart et al., 1988). Incubation of Schwann cells with TNF-α or IL-4 alone did not induce expression of class II molecules. When TNF-α was added together with IFN-γ however, it was evident that stimulation of class II expression was greater than when IFN-γ was added alone. On the other hand, IL-4 did not affect IFN-γ induction of class II antigens. The synergistic actions of TNF

and IFN-γ have already been reported for a variety of cell types including a murine myelomonocytic cell (Chang et al., 1986), rat thyroid cells (Stuart et al., 1988) and human islet cells (Pujol-Borrell et al., 1987). It has been shown that IFN-γ sensitizes some cell types to the effects of TNF by receptor upregulation (Aggarwal et al., 1985), which, in turn, stimulates further expression of class II determinants. The fact that IL-4 had no effect may reflect a more restricted tissue specificity of action than that of TNF.

It was interesting to observe that the level of T cell proliferative responses in Schwann cell cultures pretreated with IFN-γ were consistently lower than those observed in untreated cultures. It is conceivable that the dose of exogenous IFN-γ used to pretreat may have had a negative or down-regulatory influence on Schwann cell accessory cell function such as synthesis and secretion of soluble factors which promote cell activation (Mond et al 1986).

I found that type I collagen modulated the response of Schwann cells to IFN-γ and preliminary studies suggested that the effects of collagen were mediated via the β_1 family of integrin receptors. The significance of these findings for Schwann cells in vivo or for pathological states in peripheral nerves is unclear. It is possible that the positive effect of collagen on class II expression reflects a more general maturational promoting effect of collagen.

The ability of a cell to present epitopes antigenic to T cells on its surface does not always coincide with its ability to process and degrade complex antigen intracellularly. The neonatal rat Schwann cells were able to present soluble, but not whole, M.leprae to sensitized T cells. It is possible that Schwann cells, like dendritic

cells, may only be able to present macrophage-processed fragments of multideterminant antigens, such as whole mycobacteria because they lack the lysosomal processing apparatus necessary for degradation of complex antigen particles (Kaye et al., 1985). It is, however, established that Schwann cells both in situ and in culture are capable of taking up antigens such as M.leprae intracellularly (Boddingius 1974; Samuel et al., 1987b), and that mycobacterial antigen fragments have been detected within the nerves or leprosy patients in the absence of intact bacilli (Ridley et al., 1987).

I also found that Schwann cells in the living sciatic nerve could be induced to express MHC class II antigens by injection of IFN-γ, TNF-α, MLS and irradiated or live M. leprae and that injuring the nerve is also sufficient to induce Schwann cells to express class II antigens. It was very difficult to obtain a meaningful quantitation of this response for two main reasons: the immunostaining was generally seen on shorter or longer segments of a fiber, leaving other areas of the same fiber unlabelled. More importantly, several class II positive fibers were often seen in the vicinity of one another, leaving other areas of teased fibers on the microscope slide immunonegative. This probably reflects uneven diffusion of INF-γ within the nerve following injection. The quantitative estimations made here (5% and 10% depending on treatment) represent only approximations and must therefore be treated with caution. In the injected nerves some macrophages and lymphocytes were found, while essentially no cells of this kind were detectable in normal nerves using the ED1 and W3/13HLK antibodies. Most of the class II positive Schwann cells were myelin-forming cells, although a few of the non-myelin-forming Schwann cells were also class II positive in some experiments.

These experiments showed that in vivo whole M.leprae was effective in inducing MHC class II on adult Schwann cells. We therefore wanted to test whether adult rat Schwann cells in culture had the ability to process and degrade whole M. leprae, and if they would interact with mycobacteria-reactive T lymphocytes in the presence of whole M. leprae. When Schwann cells from three week old rat sciatic nerve were incubated together with sensitized T cells and whole M. leprae, lymphocytes formed clusters around the Schwann cells and essentially all of the Schwann cells expressed class II molecules after the incubation period. These results suggest that, unlike neonatal cells, adult rat Schwann cells in culture may be able to process and present whole M. leprae to T cells, but this needs further investigation. It would be particularly interesting if a significant difference in the behaviour of adult and neonatal Schwann cells was maintained in culture, in view of the well established observation that Schwann cells lose many of the key characteristics of their adult phenotype when they are removed from axonal contact (Jessen and Mirsky, 1991; Mirsky et al., 1980).

Schwann cells in normal nerves do not express MHC class II antigens (Samuel et al., 1987a), but, in certain pathological conditions at least, some Schwann cells appear to become class II positive. In an immunohistochemical study on nerve lesions from 44 patients with the histological diagnosis of leprosy 14 individuals had a positive diagnosis of leprosy in the nerve biopsy but not in the skin, and 8 patients had multibacillary leprosy in the nerve biopsy but a paucibacillary leprosy in the skin biopsy (Nilsen et al., 1986). In frozen sections from radial cutaneous and the sural nerves of these patients, T lymphocytes of both helper and suppressor/ cytotoxic

subtype, B lymphocytes and macrophages were found in the leprous lesions. Schwann cells in all types of lesion showed a strong staining for the anti-MHC class II antigens in contrast to Schwann cells in normal nerves, but in this study the Schwann cells were not identified by using a specific Schwann cell marker although morphologically the labelled cells look like Schwann cells. The expression of MHC class II molecules has also been investigated in the sural nerve of patients with the Guillain-Barré syndrome (Cadoni et al., 1986). Frozen serial sections were stained with antibody against MHC class II and with S100 to identify Schwann cells. In these nerves, both endoneurial cells and Schwann cells expressed MHC class II molecules. In another study Pollard et al. (1987) used immunoelectron microscopy to localize MHC class II on cells in the inflammatory infiltrate of 2 patients with acute GBS. In these nerves the inflammatory infiltrate consisted mainly of MHC class II positive cells of the monocyte-macrophage lineage, but also of T lymphocytes, and in the more severely affected patient, MHC class II molecules were expressed on endothelial cells, perineurial cells, and on Schwann cells. MHC class II expression has also been reported in nerves from patients with CIDP (Pollard et al., 1986; Mancardi et al., 1988). These studies indicate that human Schwann cells express MHC class II molecules in the living nerve, under some conditions. The expression of MHC class II molecules on Schwann cells in these human diseases raises the interesting question of whether these cells may be involved in antigen presentation and may have a role in the local immunoregulation in these diseases. It has been suggested (Samuel et al., 1987b) that the failure of the immune response, within nerve trunks in lepromatous leprosy, could possibly be caused by deficient class II expression by Schwann cells and that this

deficiency in class II expression may be caused by reduced IFN-γ production characteristic of lepromatous leprosy (Nogueira et al., 1983). Infection with M. leprae of cultured Schwann cells does not by itself induce MHC class II expression but the infected Schwann cells can be induced by IFN-γ to express class II molecules (Samuel et al., 1987a).

Several previous studies have investigated MHC class II expression on Schwann cells in animal models. Schmidt et al., (1990) studied MHC class II expression in animals with EAN. Serial sections of the ventral root of rats 12-21 days after immunization with myelin, were immunolabelled with antibody against MHC class II, the macrophage marker ED1, and S100 to identify Schwann cells. MHC class II was found on macrophages and on some lymphocytes, but not on Schwann cells. In some sections, however, MHC class II positive cells and processes were negative for the macrophage marker ED1, showing that MHC class II expression on Schwann cells can not be absolutely excluded.

Hartung et al (1990) investigated the role of IFN-γ in the pathogenesis of EAN. They found that the administration of IFN-γ enhanced the disease severity in Lewis rats afflicted with EAN, but application of antibody against IFN-γ suppressed clinical signs and morphological changes in the disease. Serial sections of the sciatic nerves of these rats were immunolabelled using antibodies against MHC class II antigens, ED1 was used to identify macrophages and T cell markers specific for T helper/inducer cells and for T suppressor/ cytotoxic cells were also to identify T cell subsets. In the nerve lesions, dense cellular infiltrates were seen, and immunostaining for MHC class II antigen revealed a dramatic increase especially in the

IFN-γ injected animals. In serial sections stained with ED1 antibody and anti-MHC class II antibody, the immunostaining for MHC class II seems much more widespread than the ED1 immunostaining judging the light microscopic pictures. Since in this study Schwannn cells were not identified, no conclusion can be made regarding MHC class II expression on Schwann cells in these nerves.

Cowley et al. (1989), investigated the expression of MHC class II antigens in granulomatous lesions in nerves. They injected M.leprae into the sciatic nerve of guinea pigs and looked at MHC class II expression 5 weeks later using immunoelectron-microscopical techniques. Infiltrating mononuclear cells and endoneurial fibroblast-like cells were class II positive, but no MHC class II Schwann cells were found. This is in contrast with our results and the discrepancy may be due to species differences or to the differences in the time interval between the injection and examination of the nerves in these two studies. Rapid loss of class II expression has been observed by Steiniger et al (1988), who studied the effects of recombinant rat IFN-γ on class II expression in rats after continuous intravenous infusion of IFN-γ for 3 days. Although IFN-γ induced class II molecules on previously negative cells, such as duct epithelial cells and endothelial cells in big vessels, the induced expression was rapidly lost from most cells within one or two days after interferon withdrawal. This rapid loss of induced class II antigens might help to protect parenchymal cells against autoimmune processes being initiated during systemic immune reactions which involve high interferon levels (Steiniger et al., 1988). It has also been shown that most fixatives abolish or diminish MHC class II antigens on cell surfaces (Walker, et al., 1984), and this fact makes it more difficult to investigate the expression of this

molecule in sections. I therefore decided to investigate the expression of MHC class II antigens in the living sciatic nerve by using teased nerve preparations which allows immunolabelling to be carried out on unfixed tissue; this represents a significant difference between the present work and previous experiments in this area.

The results presented here show that Schwann cells can express high levels of class II antigen and support a T lymphoproliferative response when cultured with foreign antigen and sensitized T cells. In addition they can express MHC class II antigens in vivo after an injection of mycobacterial antigens, IFN-γ and TNF-α. This further supports the view that, in leprosy and other pathological conditions, Schwann cells may interact with the immune system in T cell mediated immune responses.

CHAPTER 4

PRODUCTION OF INTERLEUKIN 1 BY ADULT AND NEONATAL RAT SCHWANN CELLS

SUMMARY

1 The ability of Schwann cells to produce IL-1 was investigated. Purified cultures of neonatal and adult rat Schwann cells were incubated with LPS, bacterial antigens or lymphokines. Supernatants and cell lysates were collected from the cultures and IL-1 assays were carried out by Dr A. Kingston.

2 In two different experiments on neonatal Schwann cells, each involving two time point determinations (48 h and 72 h), elevation of IL-1 activity was obtained, at both times, in the lysates of cells stimulated with soluble or whole M. leprae or with LPS in combination with indomethacin. IL-1 was also found in the supernatants of these cultures at 48 h, and of cells stimulated with whole M. leprae or LPS at 72 h. In a third set of experiments elevation of IL-1 expression was confirmed in Schwann cells exposed to soluble M.leprae antigens by using double-label immunofluorescence and antibodies to IL-1 and S100 to identify Schwann cells. No IL-1 elevation was seen in response to cytokines in any of these experiments.

3 In a single experiment on adult Schwann cells IL-1 elevation at 48 h and 72 h occurred in lysates of cells stimulated with TNF-α in the presence of IFN-γ. IL-1 activity was also obtained in the lysate of cells stimulated with TNF-α alone for 48 h. Significant

elevation of IL-1 activity in supernatants was only seen following stimulation with TNF-α for 72h. Elevation of IL-1 expression in adult Schwann cells was confirmed in a separate set of experiments using immunohistochemistry.

4 These results further support the view that Schwann cells may function as antigen presenting cells taking part in immune responses within peripheral nerves.

The cytokines are a large group of polypeptides, which include the interleukins, TNF and interferons. They have numerous actions on many cell types, but best known are their effects on cells of the immune system (for review see Dinarello 1989). The actions of different cytokines are often complex, as a result of the synergistic effects of cytokines, and also since their synthesis and release is interdependent, e.g. IL-1 expression can be induced by TNF-α, IFN-γ and by IL-1 itself (reviewed in DiGiovine and Duff 1990). This may be a form of biological amplification that leads to the activation of host defence systems. Cytokines may also have a role in endocrinology, since it has been shown that several cytokines including IL-1, are produced by endocrine glands and can act at sites distant from their synthesis (reviewed in Rothwell 1991). Macrophages, lymphocytes and fibroblasts are the major sites of IL-1 synthesis, but several other cell types are able to produce IL-1.

In the brain, glial cells (microglia and astrocytes) and neurons synthesize IL-1 (Fontana et al. 1982; Giulian et al, 1986, 1987). IL-1-like immunoreactivity has also been described in pituitary, gastrointestinal tract, kidney and adrenal glands (reviewed in Rothwell 1991). IL-1 is important in the initiation of the immune response, and IL-1 may also have a role in regeneration of peripheral nerves, since it has been known that IL-1 induces NGF synthesis in non-neuronal cells in the nerve (Lindholm et al., 1987). It has also been demonstrated that IL-1 can cause fever, hepatic acute-phase protein synthesis, neutrophilia and increased

levels of various hormones. IL-1 induces hypoferremia and hypozincemia. Bacteria and tumor cells need large amounts of iron for cell growth and the ability of the host to remove iron from tissues seems to be an important host defence mechanism (reviewed in Dinarello 1989). IL-1 also acts on vascular endothelium and is able to alter endothelial cell plasma membranes, making them more adhesive for neutrophils, monocytes and lymphocytes (Bevilacqua et al., 1985).

In view of the evidence that Schwann cells can present antigens to T cells, and since IL-1 is important in the initiation of T cell mediated immune responses, it was clearly of interest to investigate whether Schwann cells could produce IL-1 when incubated with bacterial antigens or cytokines. In the present work I have investigated IL-1 production by purified neonatal or adult rat Schwann cells in response to incubation with soluble (MLS) or whole (ML) M. leprae, LPS plus indomethacin, IFN-γ, TNF-α, or IFN-γ in combination with TNF-α.

3 RESULTS

3.1 Production of IL-1 activity by neonatal rat Schwann cells.

To test whether neonatal rat Schwann cells could produce IL-1, purified Schwann cells maintained in culture for one week, were incubated with various stimulatory agents: MLS, ML, LPS in combination with indomethacin, IFN-γ, TNF-α or IFN-γ in combination with TNF-α. In two experiments neonatal rat Schwann cells produced IL-1 when they were incubated with MLS, ML or with

LPS and indomethacin. IL-1 activity was found both in the supernatant and the cell lysate. In an experiment using the IL-1 assay method of Gearing et al. (1987) the Schwann cells showed statistically significant elevation of IL-1 activity when incubated for 48 h with MLS, ML, and LPS in combination with indomethacin (Fig. 4.1). The IL-1 activity was seen both in cell lysates and in the supernatant, with highest activity obtained in the supernatant of MLS and LPS stimulated cells. Significant response was not obtained on 48 h stimulation with IFN-γ, TNF-α or IFN-γ in combination with TNF-α. Essentially the same results were obtained following 72 h stimulation with the same agents, with the exception that supernatants from MLS stimulated cells did not show statistically significant elevation (Fig 4.2). This experiment was repeated using the method of Oppenheim (1976) to detect IL-1 activity. This confirmed the two most striking observations from the previous experiments, i.e. marked elevation of IL-1 activity in lysates from MLS and LPS stimulated cells. This was seen both at the 48 h (Fig 4.3) and 72 h (Fig 4.4) time points. Statistically significant increase in IL-1 activity was also seen in supernatants from LPS stimulated cells at both time points. On the other hand, this experiment failed to confirm elevation of IL-1 activity in response to ML and no IL-1 elevation was seen in supernatants of MLS stimulated cells, seen in the previous experiment at the 48 h time point only. All of these experiments were carried out on parallel cultures to those used for the T cell proliferation assays (Chapter 3) where I had ascertained that the percentage of macrophages, a potential source of IL-1, was less than 1 %.

To further confirm that the IL-1 elevation seen in the above experiments was not due to release from macrophages only, a third set of experiments was carried out to demonstrate IL-1 elevation in Schwann cells, this time using antibodies to IL-1 and immunohistochemistry.

Exposure to MLS for 48 h was used as a stimulant. Strong elevation of IL-1 immunoreactivity was obtained in MLS stimulated cells. The Schwann cells were identified by anti-S100 antibodies in double-label experiments (Fig 4.5 and 4.6), the unstimulated cultures appeared immunonegative. All of the immunoreactive IL-1 was intracellular since no IL-1 immunostaining was obtained when IL-1 antibodies were applied to living cultures. In the stimulated cultures over 50% of S100 labelled cells showed clear elevation of IL-1 immunoreactivity.

3.2 Production of IL-1 activity by adult rat Schwann cells.

The production of IL-1 by neonatal rat Schwann cells was compared with cells prepared from 3 week old rats in one experiment, Schwann cells from these rats did not produce significant amounts of IL-1 after incubation with bacteria or bacterial antigens. Significant elevation of IL-1 activity was, however, found in the cell lysate in response to a combination of IFN-γ and TNF-α both at 48 h (Fig. 4.7) and 72 h (Fig. 4.8). Strong IL-1 response was also obtained in the lysate of TNF-α stimulated cells at 48 h, but at 72 h IL-1 elevation was only detectable in the supernatants.

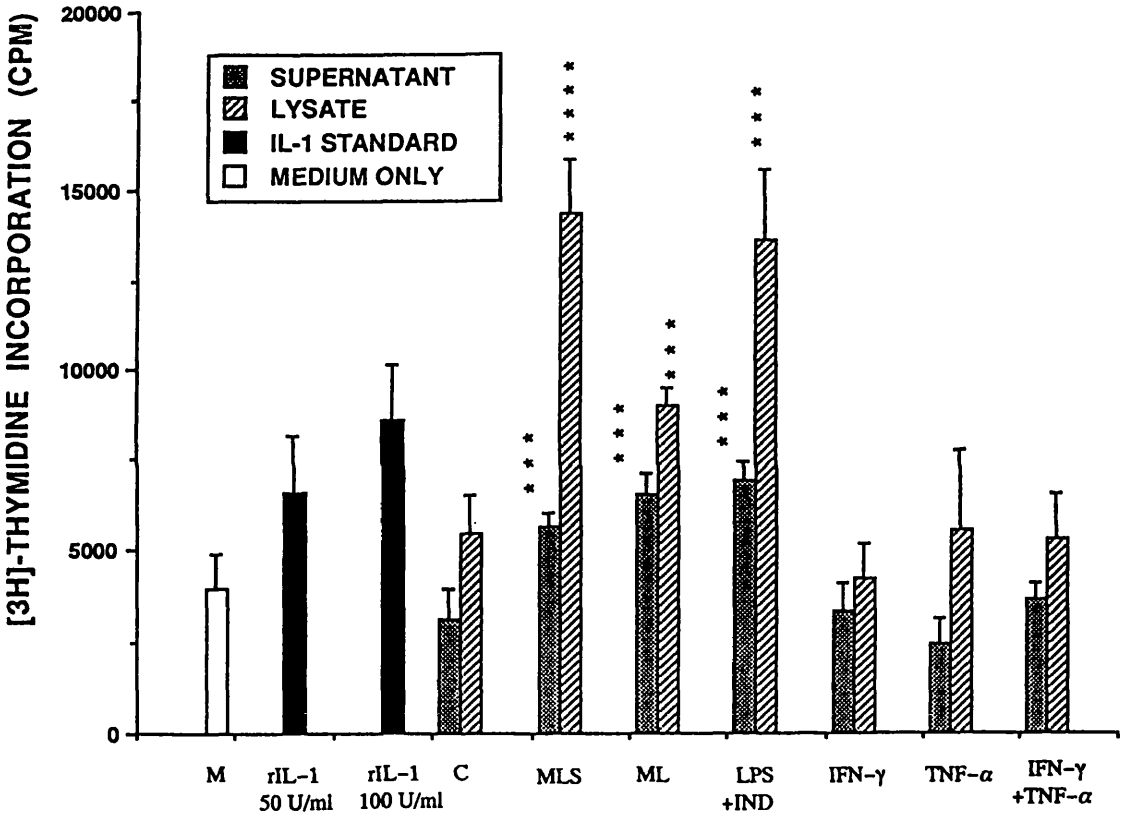
When adult rat Schwann cells were examined with IL-1 antibody, strong IL-1 labelling was seen intracellularly in cells stimulated

with M.leprae antigens as well as those incubated with cytokines (not shown). Weak staining only was observed in untreated cells. No cell surface IL-1 was detected in any of the cultures.

Figure 4.1

EL-4 NOB thymoma co-culture assay for IL-1 activity. IL-1 activity in supernatants and cell lysates from neonatal Schwann cell cultures incubated with bacterial antigens or cytokines for 48 h. Untreated Schwann cell cultures were used as a control (C) and recombinant murine IL-1 (rIL-1), 50 U/ml and 100 U/ml, was used as a standard. MLS (10 μg/ml), ML (10^8), indomethacin (1 μg/ml) and LPS (0.5 μg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml) or IFN-γ (100 U/ml) in combination with TNF-α (300 U/ml). Each column represents an average of at least triplicate measurements.

$P<0.02$ = **, $P<0.01$ = *** $P<0.001$ = ****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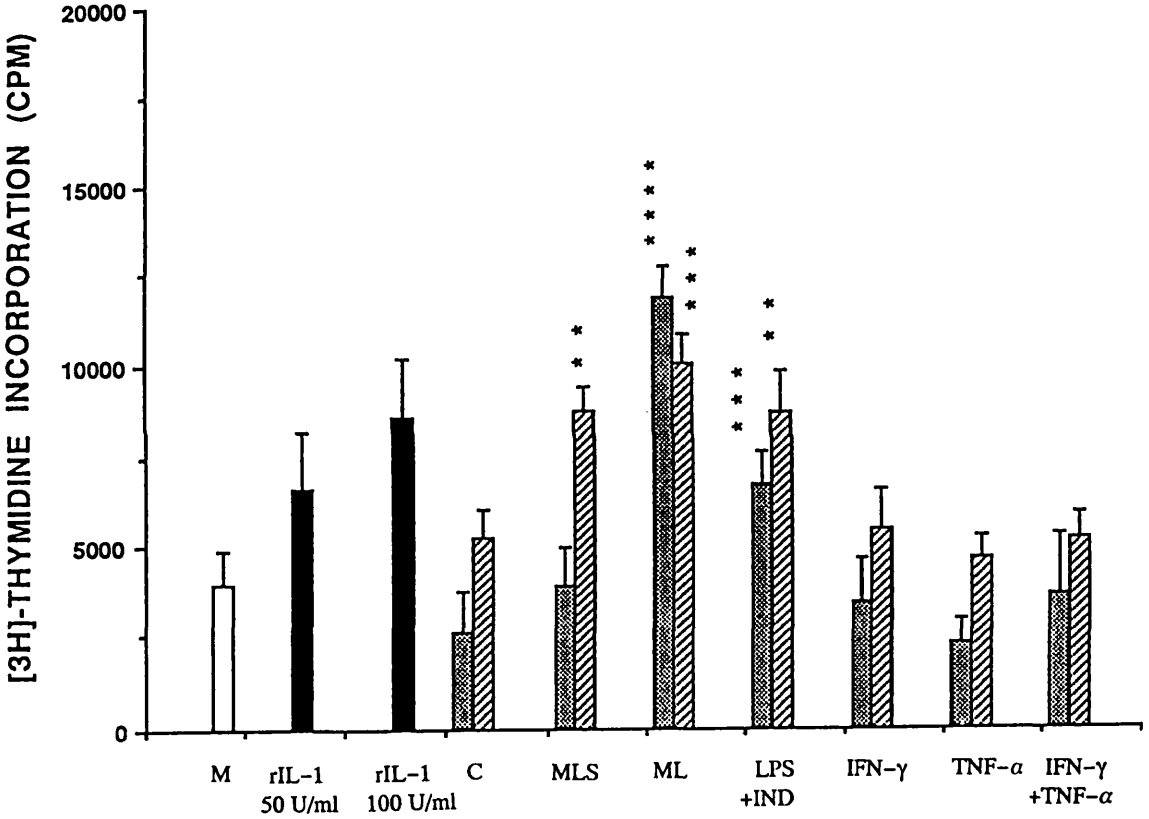
10000
5000
0
SUPERNATANT
LYSATE
IL-1 STANDARD
MEDIUM ONLY
M
rIL-1 50 U/ml
rIL-1 100 U/ml
C
MLS
ML
LPS +IND
IFN-γ
TNF-α
IFN-γ +TNF-α

Figure 4.2

EL-4 NOB thymoma co-culture assay for IL-1 activity. Interleukin-1 activity in supernatants and cell lysates from neonatal Schwann cell cultures incubated with bacterial antigens or cytokines for 72 h. Untreated Schwann cell cultures were used as a control (C) and recombinant murine IL-1 (rIL-1), 50 U/ml and 100 U/ml, was used as a standard. MLS (10 μg/ml), ML (10^8), indomethacin (1 μg/ml) and LPS (0.5 μg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml) or IFN-γ (100 U/ml) in combination with TNF-α (300 U/ml). Each column represents an average of at least triplicate measurements. For statistical significance see legend to Fig. 4.1.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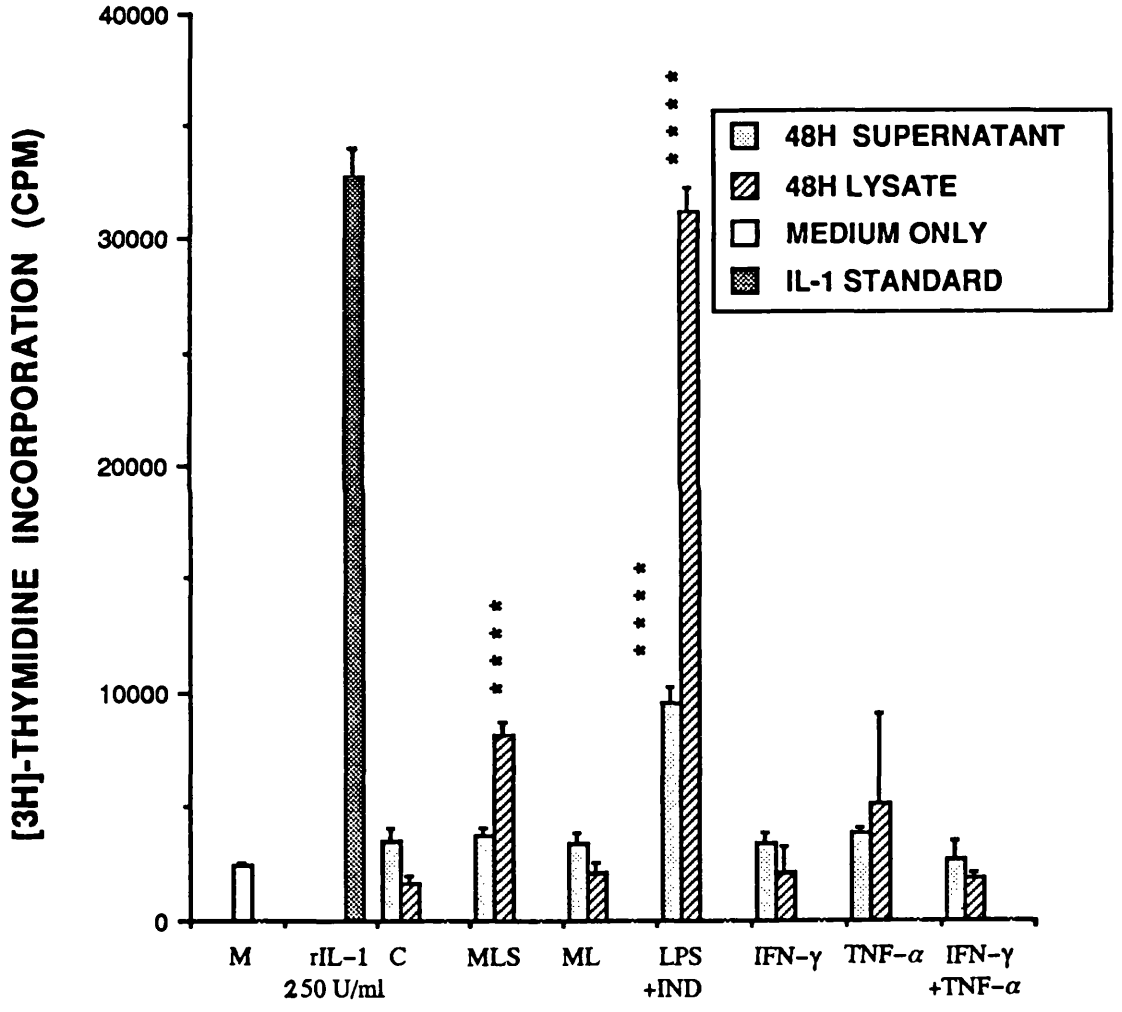
M
rIL-1 50 U/ml
rIL-1 100 U/ml
C
MLS
ML
LPS +IND
IFN-γ
TNF-α
IFN-γ +TNF-α

Figure 4.3

Thymocyte proliferative assay for IL-1 activity. IL-1 activity in supernatants and cell lysates from neonatal Schwann cell cultures incubated with bacterial antigens or cytokines for 48 h. Untreated Schwann cell cultures were used as a control (C) and recombinant murine IL-1 (rIL-1) 250 U/ml was used as a standard. MLS (10 μg/ml), ML (10^8), indomethacin (1 μg/ml) and LPS (0.5 μg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml), IFN-γ (100 U/ml) in combination with TNF-α (300 U/ml). Each column represents an average of at least triplicate measurements.

$P < 0.1$ = *, $P < 0.01$ = ***, $P < 0.001$ = ****.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10000
0
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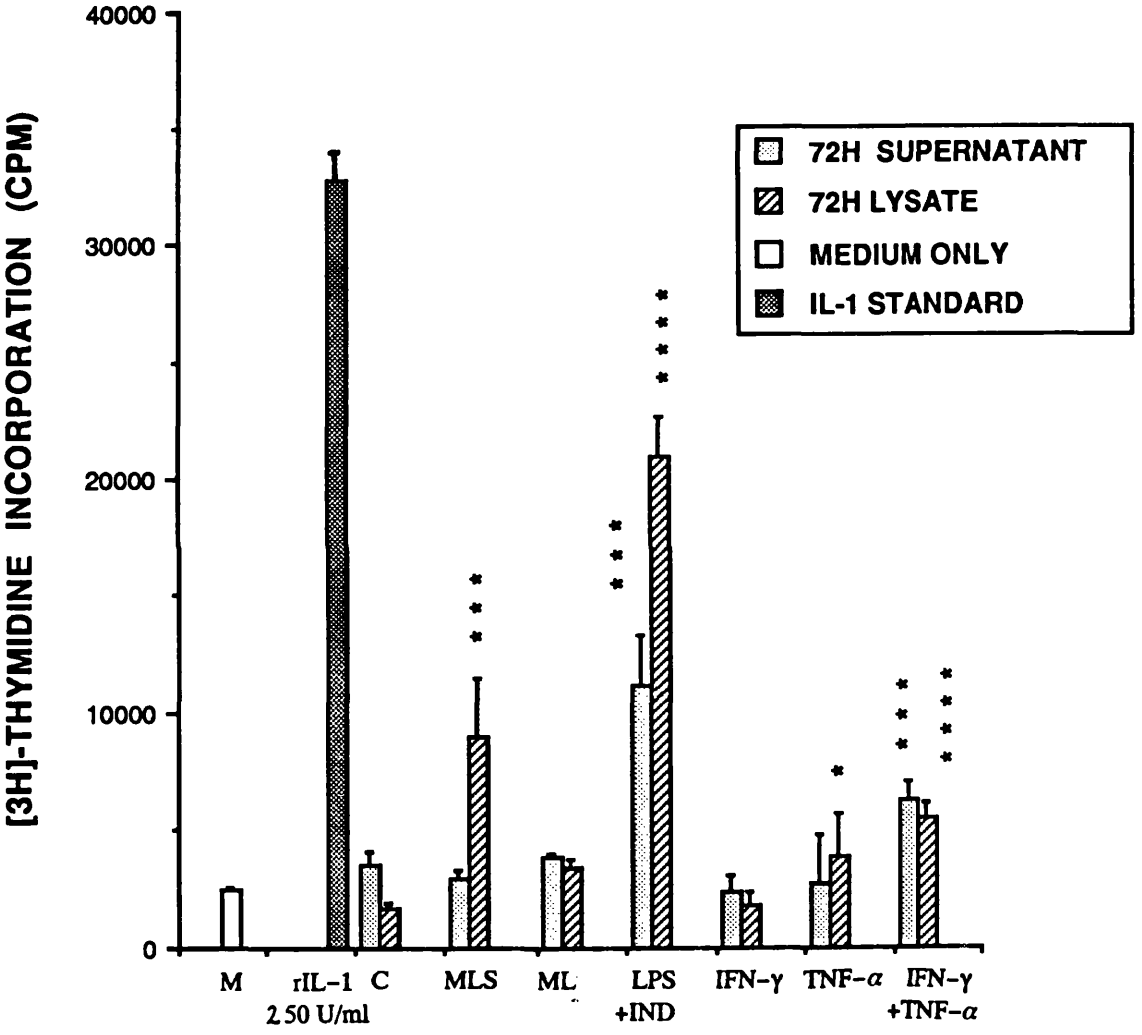
48H LYSATE
MEDIUM ONLY
IL-1 STANDARD
M
rIL-1 C
250 U/ml
MLS
ML
LPS
+IND
IFN-γ
TNF-α
IFN-γ
+TNF-α

Figure 4.4

Thymocyte proliferative assay for IL-1 activity. IL-1 activity in supernatants and cell lysates from neonatal Schwann cell cultures incubated with bacterial antigens or cytokines for 72 h. Untreated Schwann cell cultures were used as a control (C) and recombinant murine IL-1 (rIL-1) 250 U/ml was used as a standard. MLS (10 μg/ml), ML (10^8), indomethacin (1 μg/ml) and LPS (0.5 μg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml), IFN-γ (100 U/ml) in combination with TNF-α (300 U/ml). Each column represents an average of at least triplicate measurements. For statistical significance see legend to Fig. 4.3.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72H LYSATE
MEDIUM ONLY
IL-1 STANDARD
M
rIL-1 C
2.50 U/ml
MLS
ML
LPS
+IND
IFN-γ
TNF-α
IFN-γ
+TNF-α

Figure 4.5

Expression of interleukin-1 by Schwann cells using double-label immunofluorescence. a) Phase contrast picture of neonatal Schwann cells; b) expression of IL-1 in the same cells, visualized intracellularly with fluorescence optics, and c) the Schwann cell marker S100 detected intracellularly with rhodamine optics. Magnification x 500. Unstimulated cells photographed using the same exposure time appeared immunonegative.

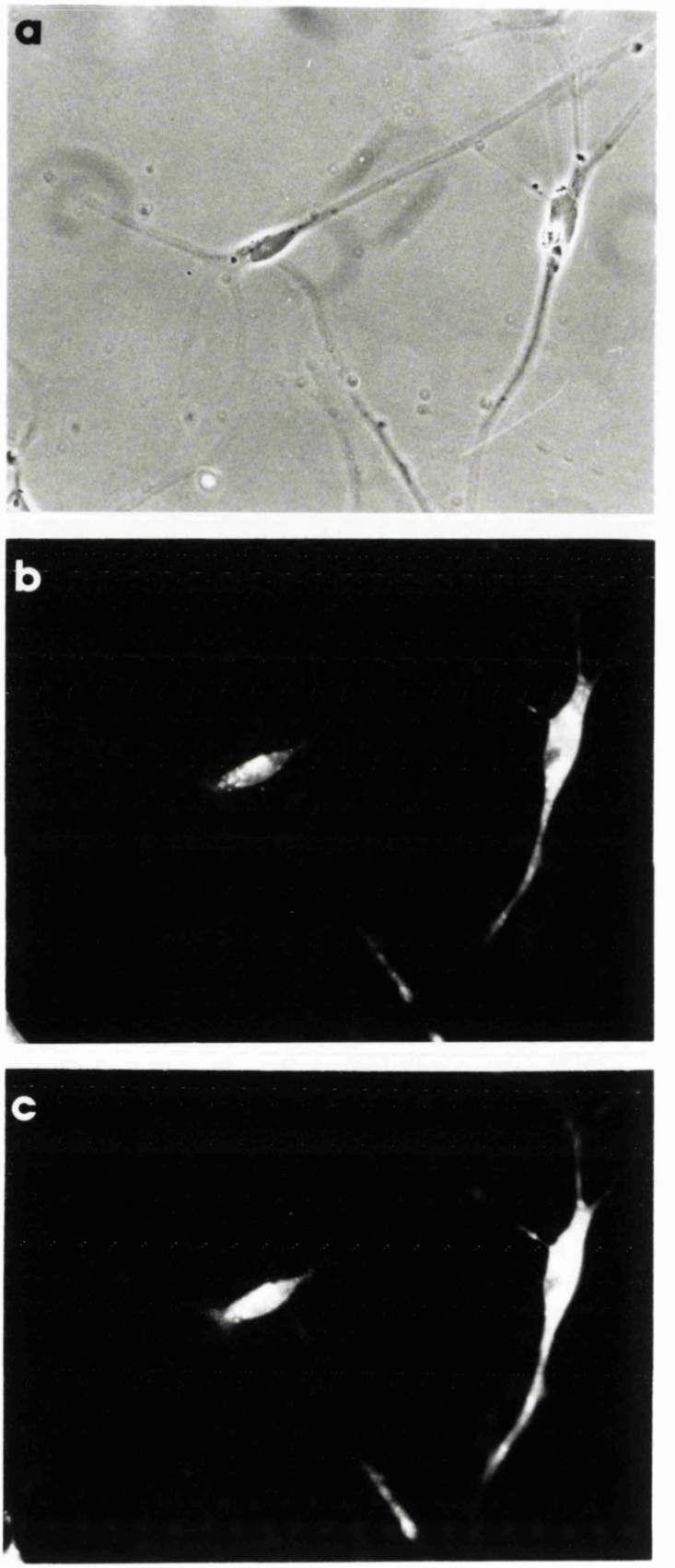

a
b
c

Figure 4.6

Expression of interleukin-1 intracellularly by Schwann cells. Expression of IL-1 visualized with fluorescein on a Biorad Lasersharp MRC-500 confocal microscope. The granular nature of the interacellular labelling pattern and the presence of IL-1 in fine Schwann cell processes can be seen much more clearly than with conventional fluorescence optics. Magnification x 600.

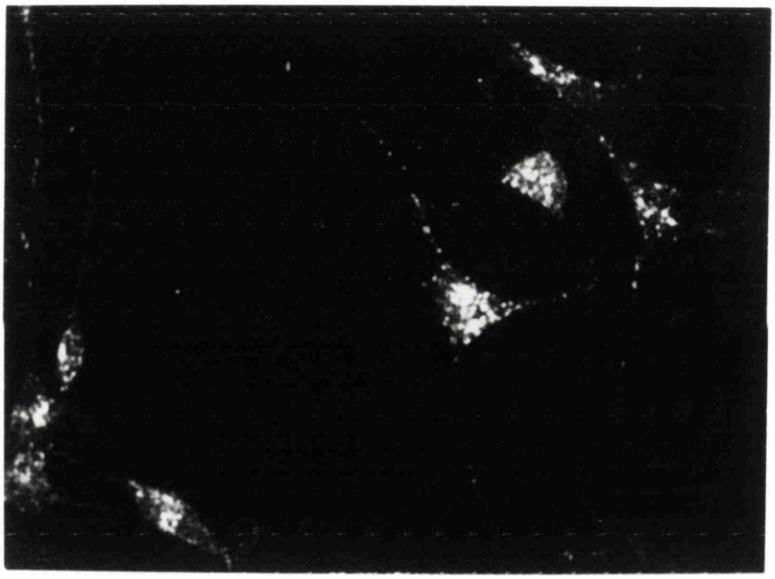
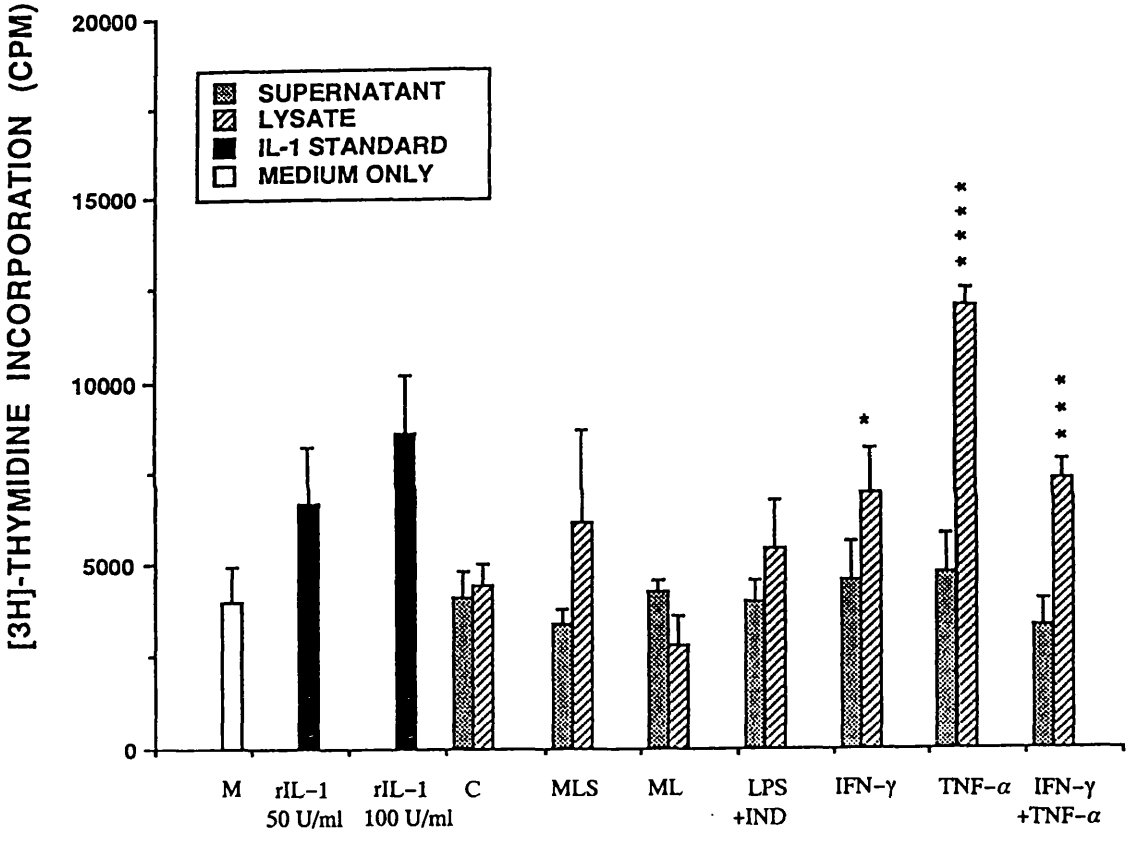


Figure 4.7

EL-4 NOB thymoma co-culture assay for IL-1 activity. Interleukin-1 activity in supernatants and cell lysates from adult Schwann cell cultures incubated with bacterial antigens or cytokines for 48 h. Untreated Schwann cell cultures were used as a control (C) and recombinant murine IL-1 (rIL-1), 50 U/ml and 100 U/ml, was used as a standard. MLS (10 μg/ML), ML (10^8), indomethacin (1 μg/ml) and LPS (0.5 μg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml) or IFN-γ (100 U/ml) in combination with TNF-α (300 U/ml). Each column represents an average of at least triplicate measurements. For statistical significance see legend to Fig. 4.1.


[3H]-THYMIDINE INCORPORATION (CPM)
20000
15000
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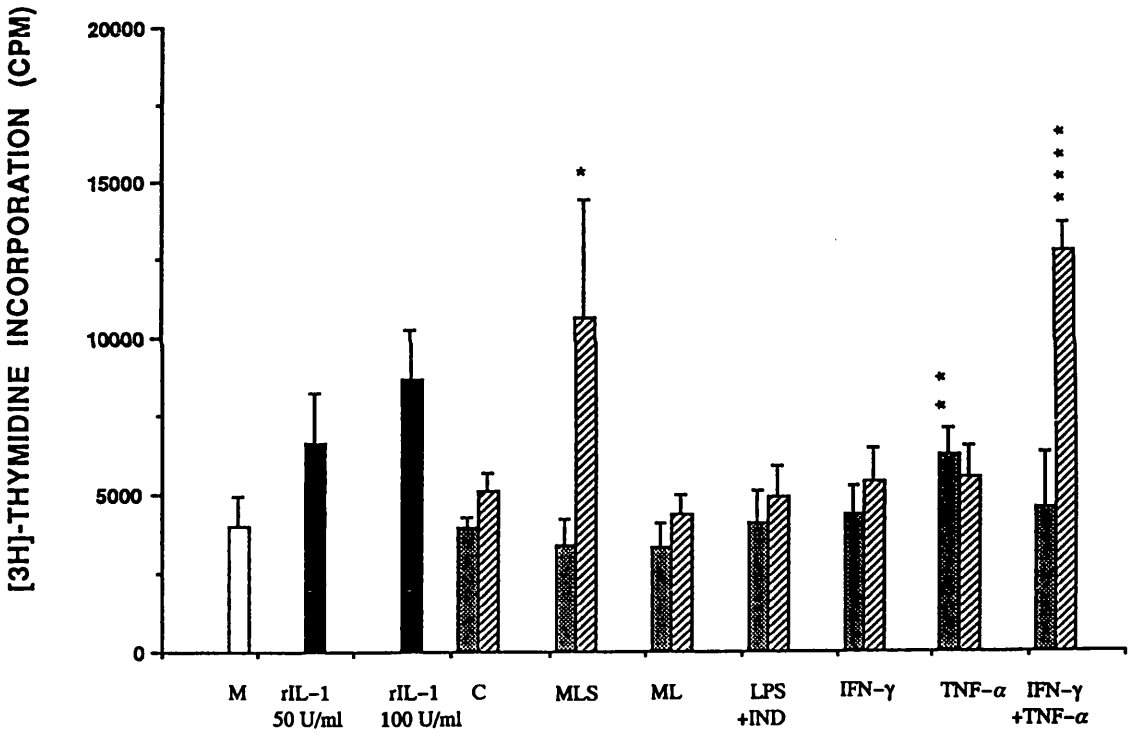
SUPERNATANT
LYSATE
IL-1 STANDARD
MEDIUM ONLY
M
rIL-1 50 U/ml
rIL-1 100 U/ml
C
MLS
ML
LPS +IND
IFN-γ
TNF-α
IFN-γ +TNF-α

Figure 4.8

EL-4 NOB thymoma co-culture assay for IL-1 activity. Interleukin-1 activity in supernatants and cell lysates from adult Schwann cell cultures incubated with bacterial antigens or cytokines for 72 h. Untreated Schwann cell cultures were used as a control (C) and recombinant murine IL-1 (rIL-1), 50 U/ml and 100 U/ml, was used as a standard. MLS (10 μg/ml), ML (10^8), indomethacin (1 μg/ml) and LPS (0.5 μg/ml), IFN-γ (100 U/ml), TNF-α (300 U/ml) or IFN-γ (100 U/ml) in combination with TNF-α (300 U/ml). Each column represents an average of at least triplicate measurements.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20000
15000
10000
5000
0
M
rIL-1 50 U/ml
rIL-1 100 U/ml
C
MLS
ML
LPS +IND
IFN-γ
TNF-α
IFN-γ +TNF-α

In this Chapter I have examined whether Schwann cells can produce IL-1 in response to various stimuli in vitro. It was found that a statistically highly significant response to soluble bacterial antigens was obtained in two separate experiments, each involving measurements at two time points and triplicate cultures for each determination. While further repeats of these experiments were unfortunately not possible (due to the changed circumstances of Dr A. Kingston) the IL-1 response was confirmed, with respect to MLS antigens, in a third set of experiments using immunohistochemistry. Additional support for the notion that Schwann cells, at least in vitro, synthesize IL-1 in response to bacterial antigens comes from immunohistochemical experiments of Schwann cells derived from adult nerves. Strongly elevated IL-1 immunoreactivity was obtained in cells exposed to MLS antigens. When IL-1 activity was measured in cell lysates from a similar experiment with adult cells (exposure to MLS for 72 h) the variability between the triplicate cultures precluded the observed rise (very close to 100 % increase on the bases of averages) from being statistically significant. When taken together these observations provide good evidence in favour of the conclusion that IL-1 levels in rat Schwann cells can be strongly elevated by exposing the cells to soluble bacterial antigens. These experiments also provide evidence that IL-1 production by adult Schwann cells may be more responsive than neonatal cells to cytokines in particular TNF-α. This conclusion need to be treated with caution, however, and clearly requires further experimental support.

Since IL-1 plays an important role in the initiation of immune

responses, these observations support the view that Schwann cells may function as antigen presenting cells (for refs. see Introduction) and thereby participate in neuroimmunological responses within peripheral nerves.

IL-1 has chemotactic activity for both T and B lymphocytes (Luger et al., 1983; Miossec et al., 1984) and IL-1 release by Schwann cells could play a role in amplifying inflammatory responses in the endoneurium by attracting lymphocytes and monocytes to the site of initial inflammation or infection. IL-1 also acts on vascular endothelium causing it to become selectively adhesive for leukocytes (Bevilacqua et al., 1985). These processes may play an important role in attracting lymphocytes into the nerve from the blood and contribute to the formation of the dense foci of inflammation seen in some peripheral neuropathies like Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy and in leprosy.

When the sciatic nerve is lesioned in vivo there is a dramatic increase in the amount of NGF synthesis by nonneuronal cells in the nerve and it has been shown that this increase can also be obtained by adding macrophages or IL-1 to transected distal nerve stumps placed in culture (Lindholm et al., 1987). It has therefore been suggested that IL-1 secreted by macrophages which invade the nerve following transection, is responsible for stimulating NGF synthesis in Schwann cells (Lindholm et al., 1987), although more recent work has implicated fibroblasts in NGF synthesis in response to IL-1 (Lindholm et al., 1990). The present results suggest, that lymphoid and non-lymphoid derived cytokines such as TNF-α and IFN-γ could provide the necessary trigger for such a cascade of events, by inducing IL-1

production in Schwann cells, which in turn would lead to elevated NGF levels in the nerve. A precedent for IL-1-induced NGF production by glia is seen in the case of astrocytes (Spranger et al., 1990), which resemble Schwann cells without axonal contact in many aspects of their molecular phenotype (Jessen et al., 1984; Jessen et al., 1990).

The ability of Schwann cells to function as antigen presenting cells might be particularly relevant in leprosy. The host immune responses toward the bacteria play a dominant role in determining the clinical type of the disease. We have shown that Schwann cells can support T cell responses to M. leprae antigen in culture and Schwann cells are able to express MHC class II antigens in vitro and in vivo (Chapter 3).

Here we show that neonatal Schwann cells are also able to produce IL-1 in response to mycobacterial antigens. It is possible, therefore, that in M. leprae infected nerves, Schwann cell derived IL-1 could attract lymphocytes into the nerve, and subsequently support their proliferation via antigen presentation in the context of MHC class II antigens.

In one experiment both bacterial antigens and cytokines induced IL-1 production by adult rat Schwann cells. In peripheral neuropathies such as Guillain-Barré syndrome which is not associated with bacterial infection, Schwann cells may be induced to produce IL-1 by IFN-γ and TNF-α released from activated macrophages and lymphocytes that have infiltrated the nerves (Hughes et.al. 1987). Thus it is conceivable that Schwann cells could contribute directly to inflammatory processes that cause tissue damage under situations where either antigens derived from infectious agents or cytokines per se are involved.

CHAPTER 5

THE INDUCTION OF MHC CLASS II MOLECULES ON RAT OLIGODENDROCYTES

SUMMARY

1 The ability of oligodendrocytes to express MHC class II molecules in vitro was investigated, using cell cultures from the rat optic nerve.

2 When oligodendrocytes were grown in supplemented medium containing dexamethasone, and exposed to IFN-γ for 72 h, immunolabelling using OX-6 antibody showed that 43 ± 3.2% (SEM) of the Gal C positive oligodendrocytes expressed class II antigens on their surface. TNF did not increase the IFN-γ induced class II expression on oligodendrocytes in these cultures. No significant MHC class II expression was seen on cells grown in dexamethasone containing medium without IFN-γ or on cells exposed to INF-γ without dexamethasone.

3 MHC class II molecules are found mainly on cells involved in immune responses and these results raise the question of whether oligodendrocytes may be able to take part in immune reactions within the brain. In particular, they open the possibility that oligodendrocytes may interact directly with $CD4^+$ lymphocytes.

As already mentioned (Chapter 1), the brain has long been considered a site of relative immune deficiency, since there is very little lymphocyte traffic and the blood brain barrier provides a physical barrier to the entry of both pathogens and immune factors (Schwartz et al., 1984). The expression of MHC antigens, both class I and class II is very low in the normal brain (Vitetta et al., 1978; Williams et al., 1980; Wong et al., 1985; Vass et al., 1986) and dendritic cells expressing class II antigens are not found in the CNS. In certain diseases, such as EAE and MS, MHC class II expression has been observed in close association with endothelial cells of capillaries, on invading monocytes/macrophages and on microglia, which are endogenous to the CNS. Lymphocytes are also found within the brain parenchyma in EAE and MS, showing that immune reactions do occur in the brain (Traugott et al., 1983; Hoffman et al., 1986). This raises the question of which cells endogenous to the brain are able to take part in immune responses.

Although MHC class II antigens are constitutively expressed on very few cells in the normal uninfected brain, induction of MHC class II occurs on astrocytes and on some microglia in vitro and in vivo after treatment with IFN-γ (Hirsch et al., 1983). In addition it has been shown that astrocytes express class II molecules after incubation with murine corona virus (Massa et al., 1986) or with measles virus (Massa et al., 1987b) and that tumor necrosis factor amplifies class II expression induced by these viruses (Massa et al., 1987b). It has been shown that astrocytes are able to present antigen to T cell lines and that this function is class II dependent

(Fontana et al., 1984; Wekerle et al., 1986). Astrocytes can also be induced to secrete IL-1, and prostaglandin E (Fontana et al., 1982). These results support the view that astrocytes can function as antigen presenting cells and may be able to interact with activated T cells initiating an immune response within the CNS parenchyma. The brain lacks lymphatic drainage but it has been shown that activated T lymphocytes, both myelin basic protein and ovalbumin-specific T lymphoblasts, can cross the blood-brain-barrier and invade the CNS (Wekerle et al., 1986).

Several studies have been done to investigate whether oligodendrocytes are able to express these molecules. Wong et al (1984) used cell cultures prepared from entire brains of 2 day old mice. After incubation with IFN-γ about 10% of the cells expressed MHC class II antigens, these cells were also positive for the astrocyte marker GFAP showing that they were astrocytes, but no class II antigens were detected on oligodendrocytes. Mauerhoff et al. (1988) used cell cultures derived from human fetal brains, after incubation with IFN-γ for 4 days, astrocytes expressed class I and class II antigens, and oligodendrocytes expressed class I but, not class II on their surface. These experiments were carried out in medium containing 10% FCS. Calder et al. (1988) looked at class II expression in cultures derived from the rat optic nerve. The cells were grown in chemically defined medium containing 0.5% fetal calf serum in the presence of mouse recombinant IFN-γ 200-500 U/ml for 24-48 h. They found that both type 2 astrocytes and O2-A progenitor cells expressed class II and also class I antigens after the incubation period. The Gal C positive oligodendrocytes did not

express class II antigens, but class I was induced on all of the oligodendrocytes after the IFN-γ treatment indicating that these cells possess functional receptors for IFN-γ. It has been noted that in cultures of oligodendrocytes and astrocytes derived from adult human brains, grown in medium containing 5% FCS, but without IFN-γ, a low percentage of presumed oligodendrocytes expressed class II antigens (Kim et al., 1985).

Thus, the predominant view is that oligodendrocytes are unable to express MHC class II molecules, although some evidence exists to the contrary. The present experiments suggest that the difficulty in demonstrating class II expression on oligodendrocytes in vitro, could be due to lack of environmental factors in the culture media.

Recently, it has been shown that glucocorticoids potentiate oligodendrocyte differentiation and myelinogenesis during neonatal development, by regulating the expression of myelin basic protein (MBP), proteolipid protein (PLP), and also glycerol phosphate dehydrogenase (GPDH) which plays an important role in phosphatide biosynthesis. It has also been found that dexamethasone, a potent glucocorticoid agonist, increases the expression of CNPase (2',3'-cyclic-nucleotide 3'-phosphodiesterase), both in reaggregating and dissociated rat brain-cell cultures in a serum-free medium (Kumar et al., 1989). It has also been shown that dexamethasone enhances the effect of IFN-γ on class II expression on human monocytes (Shen et al., 1986) and human endothelial cells (Manyak et al., 1988). In man, the natural glucocorticoid is cortisol. It plays a critical role in differentiation and development, in regulating metabolism and in modulating the responses to many hormones and growth factors. Cortisol acts on almost all organ systems of the body and

physiological levels (5-25 µg/dl plasma) of the hormone are required for optimal cortisol function in these responses. Cortisol diffuses through the membrane of the target cell and binds to specific receptors in the cytosol and these receptors determine cortisol activity. The presence of receptors for cortisol in a cell defines that cell as a cortisol target. The concentration required to half-saturate the receptors is 3 to 10 nM. Free cortisol concentration varies between approximately 1 to 50 nM during the day, and changes in the circulating concentration of cortisol leads to changes in the receptor occupancy, and to changes in the magnitude of the cortisol response (reviewed in Feldman, 1989).

Corticosterone is the major glucocorticoid in rats, and it has been demonstrated that after an intravenous injection of radiolabelled corticosterone, the radiolabelled material was found in many regions of the brain. It was most apparent in neuronal cell nuclei, but significant accumulation was also found in the neuropil, in non-neuronal cells and in the cerebrospinal fluid (Kraulis, 1975; Birmingham 1984).

In view of the ability of glucocorticoids to regulate expression of some oligodendrocyte proteins, and the uncertainty concerning class II expression on these cells, the ability of oligodendrocytes to express class II antigens was investigated in the presence of dexamethasone. Oligodendrocytes derived from the rat optic nerve were grown in supplemented medium containing 0.5% FCS, dexamethasone and incubated with IFN-γ to trigger MHC class II expression.

Oligodendrocytes express MHC class II molecules when incubated with IFN-γ in the presence of dexamethasone

To investigate the ability of oligodendrocytes to express MHC class II antigens when treated with IFN-γ, optic nerve cells were cultured in chemically defined medium containing 0.5% FCS. Under these culture conditions the O-2A progenitor cells differentiate into oligodendrocytes, which can be identified with antibodies against Gal C, the major glycolipid in myelin, which is expressed in the CNS only by oligodendrocytes (Raff et al., 1983). In cultures prepared in an identical way to those used in the experiments described here, and used for similar studies, 90 ± 4.6% (SEM) of the cells were Gal C positive oligodendrocytes, 2 ± 0.9% (SEM) were A2B5 positive O-2A progenitor cells and 6 ± 1.4% (SEM) were GFAP positive astrocytes at the start of the experiment prior to addition of INF-γ (A. Brennan and R. Mirsky, personal communication). When these oligodendrocytes were grown in defined medium containing dexamethasone (10^{-7}M) and exposed to IFN-γ (100 U/ml for 3 days), 43 ± 3.3% (SEM) (n=5) of the Gal C positive oligodendrocytes expressed MHC class II antigens on their surface (Fig. 5.1).

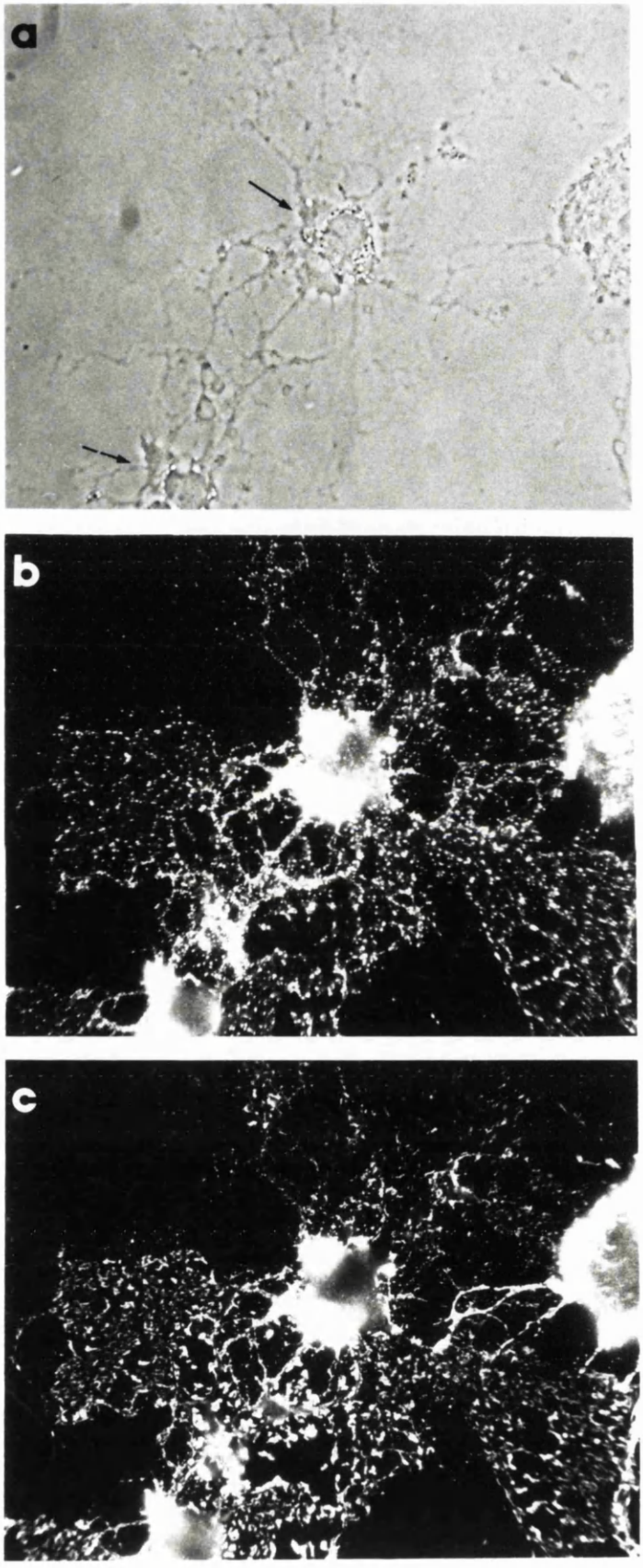
If oligodendrocytes were cultured in medium without dexamethasone no MHC class II expression was seen on the cells after 3 days of incubation with IFN-γ. Without IFN-γ treatment no MHC class II expression was seen on cells grown in the presence of dexamethasone.

Since it has been shown that TNF-α synergises with IFN-γ in inducing MHC class II antigens on some cell types including Schwann

cells (Kingston et al., 1989), the effect TNF-α on the MHC class II expression by oligodendrocytes was investigated. Oligodendrocyte cultures were grown in defined medium containing dexamethasone (10^{-7} M) and treated with IFN-γ (100 U/ml) for 72 h and TNF-α (300 U/ml) was added the last 24 h. However, TNF-α did not increase the IFN-γ induced class II expression on oligodendrocytes in these cultures.

Figure 5.1

Expression of class II antigens by oligodendrocytes, using double-label immunofluorescence. a) Phase contrast picture of oligodendrocytes incubated with IFN-γ for 3 days in the presence of dexamethasone; b) expression of class II seen with fluorescein optics; c) Gal C expression seen with rhodamine optics. Magnification x 1300.


a
b
c

DISCUSSION

These results show that oligodendrocytes can be induced to express MHC class II antigens in vitro by exposure to IFN-γ if they are cultured in medium containing dexamethasone. It has previously been suggested by some authors that oligodendrocytes can be induced to express MHC class II molecules in vitro and in vivo but this has been controversial. In many previous experiments oligodendrocytes do not express MHC class II after incubation with IFN-γ; the present experiments suggest that this is due to inappropriate culture conditions.

Further work in the laboratory has recently shown that the number of oligodendrocytes that express MHC class II antigens in response to 72 h exposure to IFN-γ (100 U/ml) is dose dependent with respect to dexamethasone in the concentration range of 10^{-10} M to 10^{-6} M. Similarly, in the presence of 10^{-7} M dexamethasone, the number of MHC class II positive oligodendrocytes is dose dependent with respect to IFN-γ in the concentration range of 20 U/ml to 200 U/ml. Furthermore, using in situ hybridization methods, it has been established that IFN-γ induced upregulation of MHC class II antigens in oligodendrocytes in the presence of dexamethasone occurs at the level of transcription (R. Mirsky and A. Brennan, personal communication).

In vivo circulating glucocorticoids are part of the normal environment of CNS cells (for review see Funder and Sheppard, 1987) and their actions are believed to be mediated by interactions with intracellular receptors, which are widely distributed throughout the

brain (Evans et al., 1989; Luttge et al., 1989). Glucocorticoids have been shown to influence the developmental expression of genes in rat brain, a rise in the levels of glucocorticoid receptors was seen during the first two weeks of development, and primary cultures of rat oligodendrocytes and astrocytes have high levels of glucocorticoid receptors (for review see Kumar et al., 1989). Dexamethasone has been shown to augment the effect of IFN-γ on human monocytes (Shen et al., 1986) and endothelial cells (Manyak et al., 1988), and studies on the binding of IFN-γ to human monocytes showed that there was a twofold increase in the number of IFN-γ molecules bound to human monocytes after treatment with dexamethasone, suggesting that dexamethasone may modulate the effect of IFN-γ on monocytes by changes in receptor number (Strickland et al., 1986). In contrast, glucocorticoids reduce the IFN-γ induced class II expression on murine macrophages (Fertsch-Ruggio et al., 1988) indicating that the interplay between lymphokines and glucocorticoid hormones may be more complex than previously thought. The importance of IFN-γ as an immunomodulator is becoming more evident and knowledge of agents that modulate the effect of IFN-γ on cells will be of increasing value.

As discussed previously MHC class II molecules are found mainly on cells involved in immune responses, and the first step in the induction of most immune responses is the MHC class II restricted presentation of antigens by antigen presenting cells to T lymphocytes. This function has been attributed to macrophages, dendritic cells (McDevitt et al., 1976) microglial cells in the CNS and also to other cell types, which can be induced by IFN-γ to express class II antigens, including Schwann cells (Wekerle et al.,

1986, Samuel et al., 1987) and astrocytes (Hirsch et al., 1983, Wong et al., 1984, Fontana et al., 1984). The present results therefore raise the question of whether oligodendrocytes as well as astrocytes may be able to function as facultative antigen presenting cells, taking part in immune responses within the brain.

The induction of MHC class II on glial cells may cause local antigen presentation in the CNS, and some disorders within the brain are correlated with abnormal immune responses, which are anti-viral or autoimmune reactions. In animal experiments, it has been shown that injection of CNS proteins such as MBP (Raine, 1984) induces an autoimmune disease, which serves as a model for demyelinating diseases of the CNS. In EAE autoreactive T cells (helper/inducer) are present which allow adoptive transfer of the disease, characterized by inflammatory brain lesions and demyelination. The lesions in corona or measles virus-infected rats with subacute encephalomyelitis (SAE) are very similar to those seen in EAE, and autoreactive T cells, with specificity for rat MBP, can be isolated from virus-infected rats with SAE. (Watanabe et al., 83, Liebert et al., 1988). Inducibility of MHC class II molecules on cultured astrocytes by murine coronavirus correlates with susceptibility to demyelinating disease. Thus, astrocytes isolated from the brain of rat or mouse strains susceptible to EAE express much higher levels of MHC class II than astrocytes from resistant strains after incubation with either IFN-γ or with murine coronavirus (Massa et al., 1987a). It has been suggested that MHC class II positive antigen presenting cells are important in the induction of EAE, since MHC class II positive cells are found in brain tissue during

the development of EAE (Sakai et al., 1986), and EAE can be prevented by treatment with monoclonal anti-class II antibodies (Sriram et al., 1983).

There is some evidence that the expression of MHC class II antigens stimulated by IFN-γ plays an important role in some immunologically mediated diseases in humans, since treatment with antibodies against MHC class II in vivo can suppress certain autoimmune diseases (Steinberg et al., 1984) such as MS, a major demyelinating inflammatory CNS disorder of humans (reviewed by Waksman and Reynolds, 1984). The pathogenesis of the inflammatory destructive lesions of MS is explained by many as an immunological reaction to tissue antigen triggered by viral infection in early life in genetically predisposed individuals. Demyelination and destruction of oligodendrocytes are the primary pathological features in MS (Prineas et al., 1989). In MS patients lesions are found in the brain, spinal cord and the optic nerve, and they are characterized by patchy inflammation and demyelination. Lymphocytes, both T helper/inducer and T suppressor/cytotoxic and monocytes are found in the lesions (Traugott et al., 1985; Hofman et al., 1986). The monocytes invade the parenchyma and differentiate into macrophages, which actively phagocytose myelin (Raine et al., 1984). MHC class I and II positive glial cells have been reported in the MS lesions although these observations are still somewhat controversial. They are most frequently found in active lesions and may play a role in local antigen presentation (Traugott, 1987, Hofman et al., 1986). The class II positive cells show a wide range of morphologies from small round cells to large, cytoplasmic multi-processed cells. In double -staining experiments, tissue sections

from MS patients were stained with both the astrocyte marker anti-GFAP and anti-class II antibody. Many of the class II positive cells also expressed GFAP indicating that they were astrocytes, but many of the class II positive cells were GFAP negative and these cells were not identified by double-labelling (Hoffman et al., 1986). In view of the present results it is possible that some of these unidentified MHC class II positive cells were oligodendrocytes.

Activated T lymphocytes have been found around lesions in the brain of MS patients (Hofman et al.,1986), and IFN-γ secreted by these cells, may induce class I and class II on brain cells, which would then facilitate the recognition of foreign (or auto) antigens by T cells. In the case of viral infection this would enable virus specific cytotoxic T cells to kill infected brain cells (Nyland et al., 1982; Traugott et al., 1985b). IFN-γ may well occur in the brain, measurable amounts have for instance been found in the brains of patients who died of HSV encephalitis (Legapsi et al., 1980). It has been shown that the production of IFN-γ and TNF increases before the onset of neurological symptoms (exacerbation) in MS patients. This suggests that there are activation processes in MS at early stages involving lymphocytes and macrophages, which are able to produce high amounts of IFN-γ and TNF (Beck et al., 1988). Interestingly, the MCH class II antigens are clearly seen on brain cells in sectioned material only in certain disease states such as MS. It therefore seems that the brain may be immunologically privileged most of the time, although low levels of MHC class II expression, e.g. on microglia, would not be detected in the pre-

fixed material used in most of the relevant studies.

There is considerable evidence that an early step in oligodendrocyte damage which occurs in MS, is a complement mediated antibody independent attack resulting from a repeated breakdown of the blood-drain barrier (Compston et al., 1991). Further oligodendrocyte damage could occur by antibody-dependent complement mediated cytotoxity and lymphokine mediated killing (Brosnan et al., 1988), and expression of MHC class II antigens on oligodendrocytes might increase their vulnerability to attack by these mechanisms. Another possibility opened up by the present work is the direct killing of oligodendrocytes by MHC class II restricted T cells of the $CD4^+$ (lyt^+) helper phenotype (Tite et al., 1985; Sun and Wekerle, 1986). This would provide a new potential mechanism for selective killing of oligodendrocytes, and would therefore be of considerable interest in the context of MS, where oligodendrocyte death is a central feature.

Compared with astrocytes, which have been shown to be able to function as antigen presenting cells in vitro, the role of oligodendrocytes as antigen presenting cells has been much less investigated. The results presented here demonstrate that under appropriate conditions oligodendrocytes like astrocytes are capable of expressing MHC class II molecules in vitro.

It will now be important to study further the ability of oligodendrocytes to express molecules, which are important in immune responses and to look for the involvement of oligodendrocytes in immune reactions in vivo.

CHAPTER 6

THE EFFECT OF COLLAGEN TYPE I ON THE STRUCTURAL ORGANIZATION OF THE ENTERIC NERVOUS SYSTEM IN CULTURE

SUMMARY

1 The possible role of collagen type I in the induction of differentiation of neural cells was investigated, using the enteric nervous system of the gut. The myenteric plexus is a network of nerve strands and small ganglia, which are compact structures consisting of the cell bodies of neurons, glia and nerve processes. When the myenteric plexus is taken into culture and explanted on a 2-dimensional substrate, the network-like arrangement of the plexus breaks down and individual ganglia spread into a semi-monolayer of neurons and glial cells.

2 To test whether type I collagen could prevent the disaggregation of the plexus, a freshly dissected myenteric plexus was embedded inside a 3-dimensional collagen gel. When the plexus was grown inside a 3-dimensional collagen gel and cultured in medium with 0.5 % FCS the glial cells did not migrate away from the neurons and the plexus stayed as a network.

3 To test if collagen could induce the rearrangement of the plexuses into a network-like structure, disaggregated plexuses were grown inside 3-dimensional collagen gels. When the plexuses were grown inside a 3-dimensional collagen gel they rearranged and formed a network-like structure of small ganglia and interconnecting

strands, similar to the myenteric plexus in situ. Electron microscopy showed that the ultrastructure of these reformed plexuses is very similar to the ultrastructure of the myenteric plexus in situ.

4 Serum is clearly important in this process since collagen induced the morphological differentiation of the enteric plexuses only if they were grown in medium containing low serum but not if the medium contained 10 % FCS.

5 Myenteric plexuses were grown on polylysine, 2-dimensional collagen substrates or inside a 3-dimensional collagen gel with or without ascorbic acid and some plexuses were put on gelatin-coated slides and dried. The plexuses were then immunolabelled using antibody against procollagen type I. In plexuses freshly dissected or cultured for one day most of the glial cells showed positive immunolabelling but this expression was transient since after 3 days in culture only very few of the glial cells still expressed intracellular procollagen type I.

6 The conclusions from this work is that the collagen surrounding the myenteric plexus in vivo is important (presumably via binding to glial matrix receptors) for inducing and maintaining normal neuro-glia interactions and glial differentiation in the myenteric ganglia. This is likely to be partly an autocrine effect since enteric glia synthesize and presumably secrete type I collagen.

Methods for the preparation and maintenance of explant cultures from the myenteric plexus have been developed previously (Jessen et al., 1978, 1983; Saffrey and Burnstock, 1984). Segments of plexuses made up of several ganglia interconnected by axon bundles are explanted. During dissection, they are rinsed free of the smooth muscle in which they are embedded, so that only 3 major cell types are put into culture: neurons, glial cells and a small number of fibroblasts, which can be killed with cytosine arabinoside. The development of the plexuses in culture has been studied in detail for the myenteric plexus from the guinea pig taenia coli and can be divided into four stages.

Stage 1: after the initial adhesion of the explant to the coverslip, the network-like appearance of the plexus disappears and a flattened single explant area is formed, consisting of neurons, glia and a variable number of fibroblasts. Stage 2: flattened sheet-like processes of glia cells emerge at the periphery of the explant area and fine neurites grow on top of the glial sheet. Stage 3: the glial cells continue to migrate out and the explant becomes surrounded by an extensive carpet of glial cells. Mitoses are frequently observed among these cells. The neurites grow from the explant onto the sheet as single fibres or in slender bundles, forming a very fine and irregular meshwork of fibres on top of the glial cells. The organization of the enteric neurons into discrete ganglia and interconnecting strands is now completely lost and the explant itself forms a continuous monolayer of clearly visible neuronal and glial cell bodies. The neurites show a marked

preference for the surface of glial cells, rather than the surface of fibroblasts and neurites only occasionally grow out onto the coverslip (Jessen et al., 1983). Stage 4: myenteric neurons and glial cells form small aggregates which resemble the myenteric ganglia in situ (Gabella 1972; Cook and Burnstock, 1976), but the ensheathment of neuronal cell bodies and neurites is not as complete as seen in situ and the culture aggregates do not have a basement membrane.

It was suggested that connective tissue cells or some ECM molecules have a role in the development and histotypic organization of the ENS, since the formation of aggregates is only seen in cultures which contain fibroblasts (Baluk et al., 1983). In the present work I have pursued this idea by investigating whether collagen type I has an effect on the differentiation of enteric glial cells and on the morphological arrangement of enteric neurons and glia. I tested whether 3-dimensional collagen gel can prevent the breakdown of the network, which happens when the plexus is taken into culture and grown on 2-dimensional substrate. I also tested if 3-dimensional collagen gel could induce network formation in a disaggregated culture.

RESULTS

Collagen gel prevents the disaggregation of the myenteric plexus in culture.

When the myenteric plexus was taken into culture and explanted on a 2-dimensional substrate it lost its characteristic in vivo morphology and the normal neuro-glia relationship was changed. The

glial cells started to divide, they migrated away from the neurons and the network-like arrangement of the plexus broke down confirming previous observations (Jessen et al., 1983).

When, however, the freshly dissected myenteric plexus was embedded in a 3-dimensional collagen gel and cultured in defined medium containing 0.5% FCS, all the changes seen when the plexus was grown on a 2-dimensional substrate were prevented. The glial cells did not migrate away from the neurons and the plexus stayed as a network of small ganglia and interconnecting strands. To quantitate the effect of collagen on the myenteric plexus, the rate of the filling of the holes in the meshwork was measured by counting the remaining holes, the first 3 days after the plexus was taken into culture. After 3 days in culture 86% of the holes still remained (Fig 6.1), and after 4 weeks in culture the plexus still remained as a network of small ganglia and interconnecting strands. If the plexus was embedded in 3-dimensional collagen gel but cultured in medium containing 10% FCS, only 28% of the holes remained after 3 days of culture (Fig 6.2). Later such cultures formed one big aggregate with an extensive outgrowth of axons.

These results indicate that type I collagen may have a role in maintaining the fully differentiated morphology of the myenteric plexus. Serum seems to be important since collagen prevents the breakdown of the network-like structure of the plexus only if it is cultured in defined medium containing low serum but not in DMEM containing 10% FCS.

Collagen type I induces network formation in disaggregated cultures of enteric glia and neurons

I also investigated whether collagen could induce the rearrangement of the plexuses into a network-like structure, by growing disaggregated cultures of enteric neurons and glia in 3-dimensional collagen gel in the presence of 0.5% serum. Myenteric plexuses, which had been grown on a 2-dimensional collagen substrate for about 5 days and had formed a monolayer of neurons and glia, were embedded in a 3-dimensional collagen gel as described in Chapter 2. When these disaggregated cultures were grown inside a 3-dimensional collagen gel they rearranged and formed, in about 4 weeks, a network of small ganglia and interconnecting strands, very similar to the myenteric plexus in situ (Fig 6.3). The first stage in the network formation was the migration of neurons and formation of small neuronal islands (Fig 6.3 a, b). This resulted in an uneven distribution of neurons instead of the uniform monolayer. The glial cells migrated towards the neuronal aggregates, suggesting that the neurons might be secreting chemotactic factors, which the glial cells responded to. At this stage holes had formed in the glial carpet (Figs. 6.3 c,d) and later the small aggregates of neurons and glial cells became so compact that individual cells could not be distinguished. After about 4 weeks the cultures had formed a network of small ganglia and interconnecting strands (Fig 6.3 e,f). To test the effect of serum on this re-aggregation the experiments were repeated in the preence of 10% FCS. In this case the glial cells migrated towards the neurons, but instead of forming a network of small ganglia and interconnecting strands, they formed

one large aggregate and there was an extensive outgrowth of neurites (Fig. 6.4).

Cultures that had rearranged to form many small interconnected ganglia in low serum were fixed and embedded for electron microscopy. The ultrastructure of these cultures was very similar to that seen in situ, except they lacked a basement membrane. The ganglia contained a compact meshwork of glial and neuronal cell bodies, and nerve cell processes. The ganglia were without any connective tissue, but glial cells formed a lining around the ganglia (Fig. 6.5). The nucleus of the neurons was pale with some peripheral dense chromatin. The cytoplasm of the neurons in these ganglia showed the characteristics of metabolically active cells, since there were many mitochondria and much rough endoplasmic reticulum and lysosomes. Free ribosomes were also present and Golgi apparatus were observed in the perinuclear regions (Fig 6.5). The interconnecting strands contained glial cell bodies and bundles of axons ensheathed by glial cells (Fig 6.6), and glial processes divided the axon bundles up to some extent. Glial cell bodies were seen both in the ganglia (Fig 6.5) and in the interconnecting strands (Fig 6.6), they were smaller than the neurons and had a thinner rim of cytoplasm around the nucleus, the glial nucleus was much denser and smaller then the neuronal nucleus. The cytoplasm of the enteric glial cells was denser than the axoplasm (Fig. 6.6) and showed many free ribosomes, lysosomes and a few small mitochondria. Microtubules were clearly visible in axons and clusters of intermediate filaments could be seen. Several vesicle-containing varicosities were seen, most of which contained agranular vesicles while dense cored vesicles were also seen. Electron microscopy of

single large aggregates formed in the presence of 10% serum showed that these aggregates did not have the compact structure which is characteristic of the enteric ganglia in situ. The aggregate contained neuronal and glial cell bodies and many nerve cell processes, but the glial cells did not ensheath the aggregate. The cellular arrangement was loose and the extracellular spaces relatively large.

These results show that collagen not only retarded the breakdown of the plexus but also induced network formation in a disaggregated culture. Again, serum is clearly important since this network formation is only seen if the cultures are grown in defined medium containing 0.5% serum.

Enteric glial cells express pro-collagen type I

The myenteric plexus of a newborn guinea pig , freshly dissected or cultured for one day, was immunolabelled using a monoclonal antibody against pro-collagen type I (Fig 6.7). Most of the glial cells showed positive immunolabelling but this expression was transient since after 3 days in culture only very few of the glial cells still expressed intracellular pro-collagen type I.

Figure 6.1

The effect of collagen in medium containing 0.5 % FCS on the disaggregation of the myenteric plexus. Hatched bars represent the percentage of remaining holes in the meshwork for the first 3 days in culture when the plexus is grown inside a 3-dimensional collagen gel. Each column represents an average of eight cultures. Black bars show the remaining holes in the meshwork when the plexus is grown on a 2-dimensional substrate. Each column represents an average of three cultures.

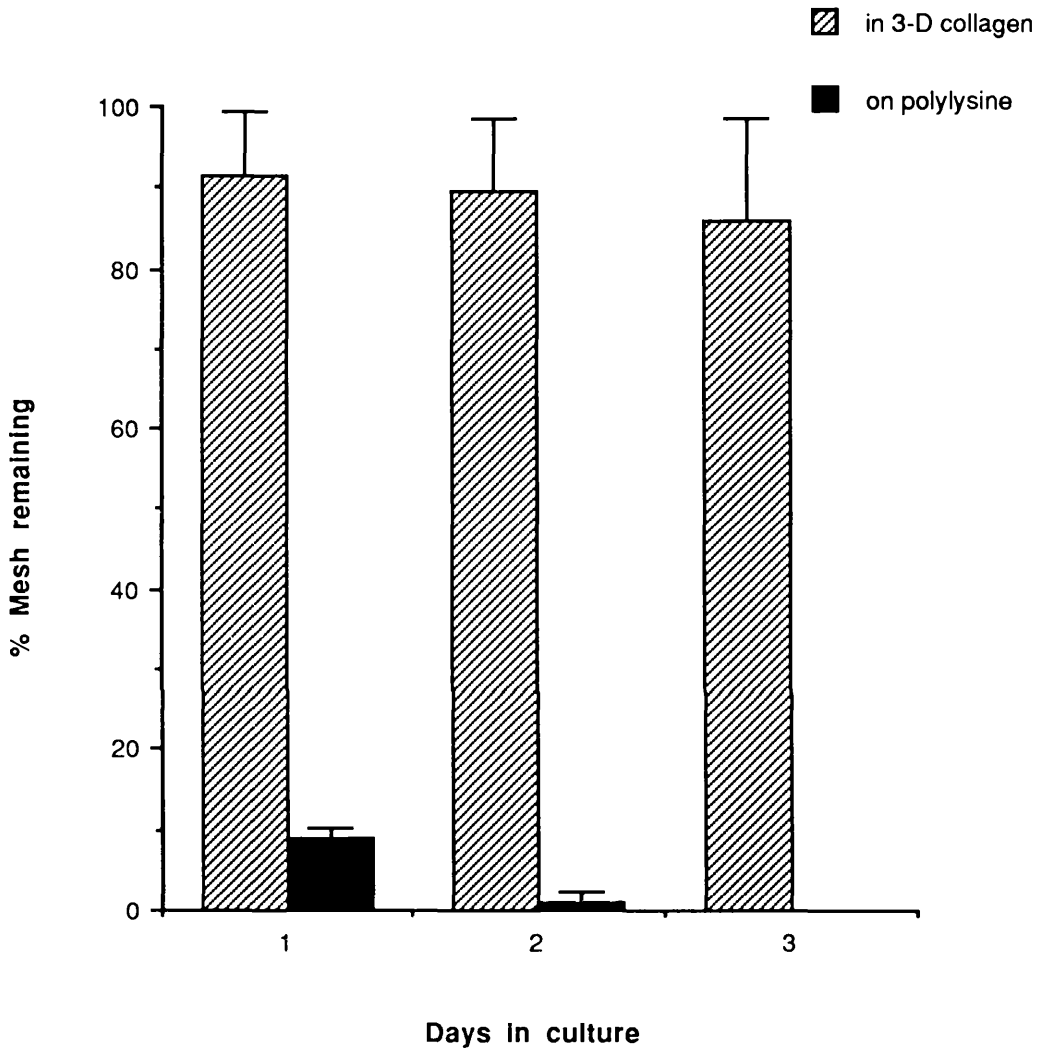
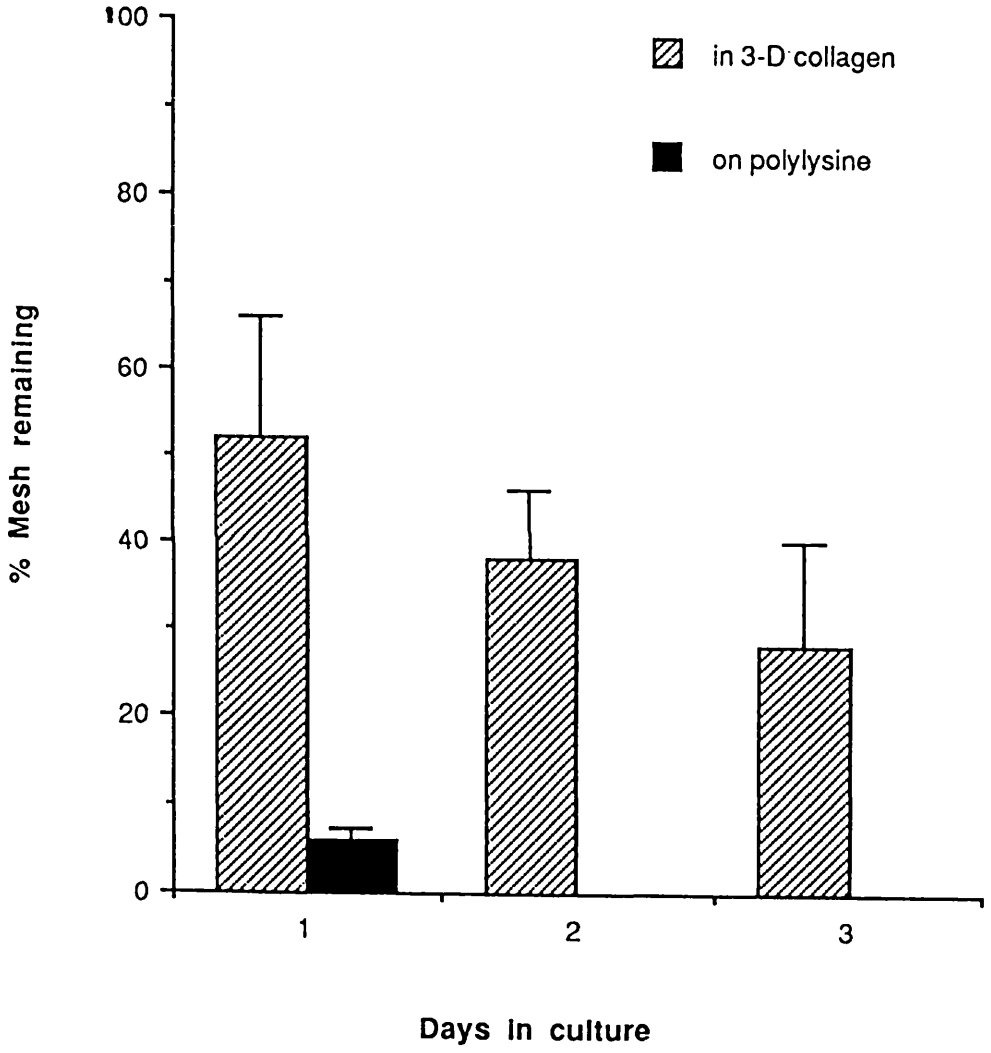

in 3-D collagen
on polylysine
100
80
60
40
20
0
% Mesh remaining
1
2
3
Days in culture

Figure 6.2

The effect of collagen in medium containing 10 % FCS on the disaggregation of the myenteric plexus. Hatched bars represent the percentage of remaining holes in the meshwork during the first 3 days in culture when the plexus is grown inside a 3-dimensional collagen gel. Each column represents an average of five cultures. Black bars show the remaining holes in the meshwork when the plexus is grown on a 2-dimensional substrate. Each column represents an average of three cultures.


100
80
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% Mesh remaining
in 3-D collagen
on polylysine
1
2
3
Days in culture

Figure 6.3; a,b. Network formation in myenteric explant cultures from guinea pig taenia coli grown inside a 3-dimensional collagen gel in medium containing 0.5 % FCS.

a) Plexus grown on a 2-dimensional collagen substrate. The field shows some nerve cell bodies (arrowheads), and the glial cells (arrows) form a continuous outgrowth zone, covered by a meshwork of neurites. Magnification x 650.

b) The first stage in network formation when the disaggregated myenteric explant cultures are grown inside a 3-dimensional collagen gel. Instead of the uniform monolayer of neurons, there are now signs of cell bodies grouping together (arrowheads). Magnification x 650.

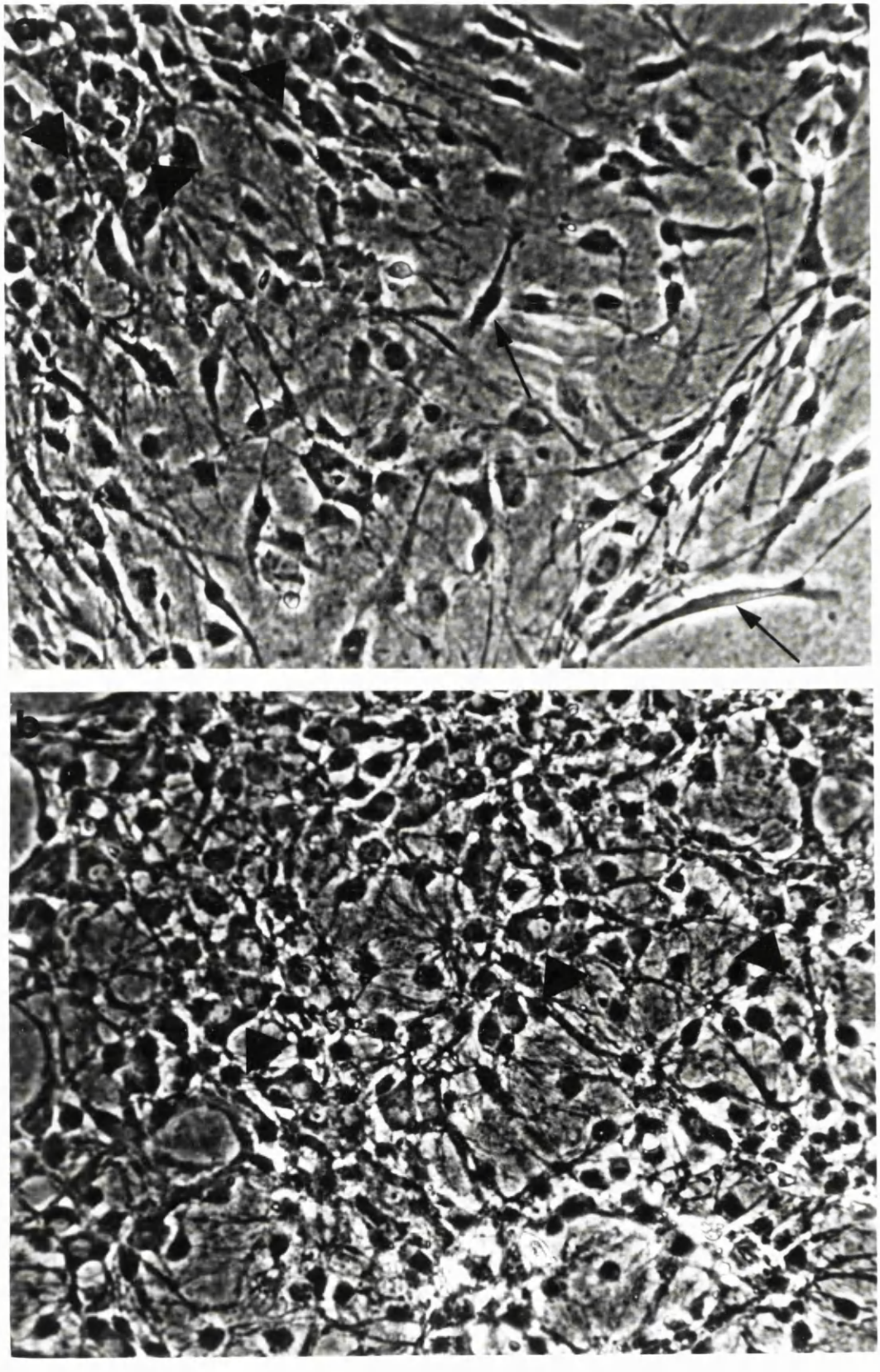
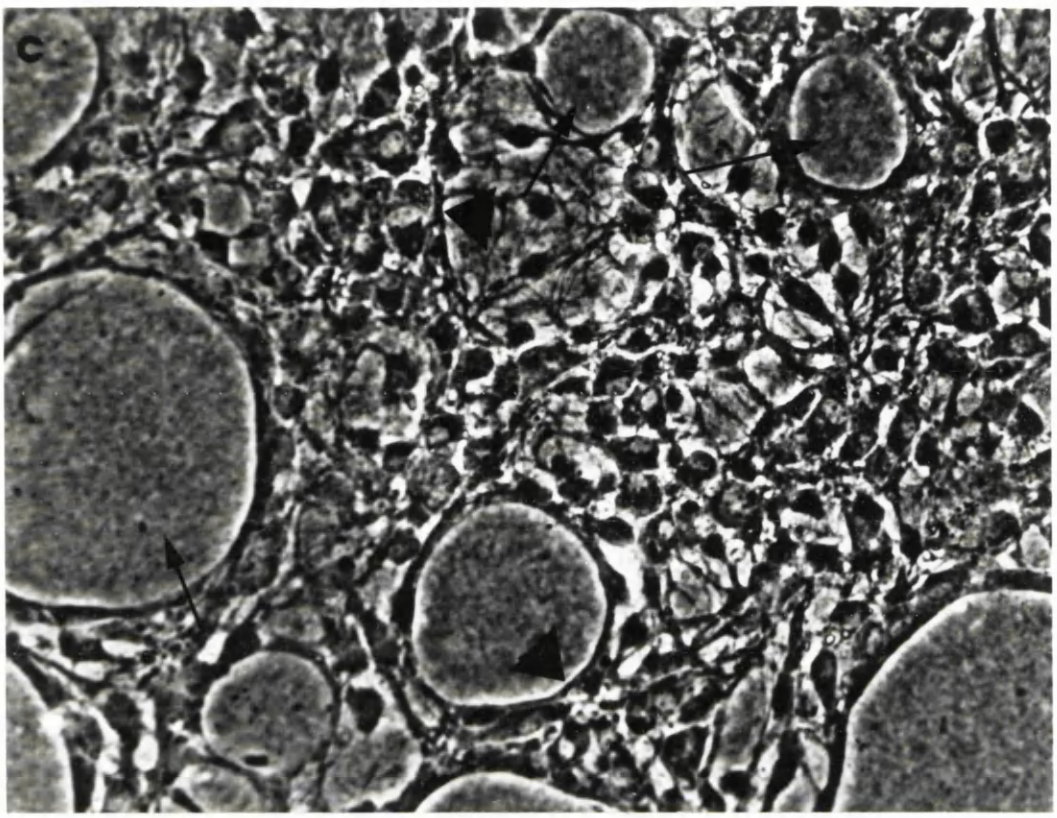


Figure 6.3; c,d c) The second stage in network formation of the disaggregated cultures. Small islands of neurons are seen (arrowheads) and holes have formed in the glia sheet (arrows). Magnification x 650.

d) Network formation is more advanced then in Fig c. The holes in the glia sheet have become bigger (arrows). Magnification x 650.


c

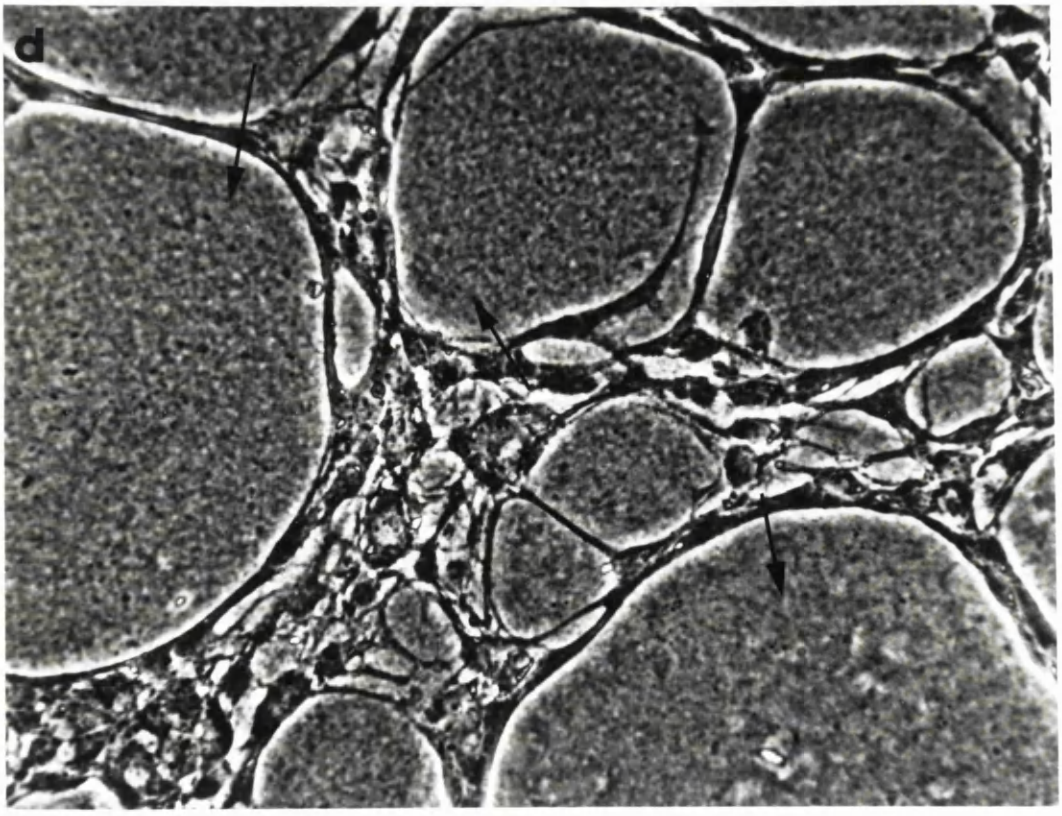
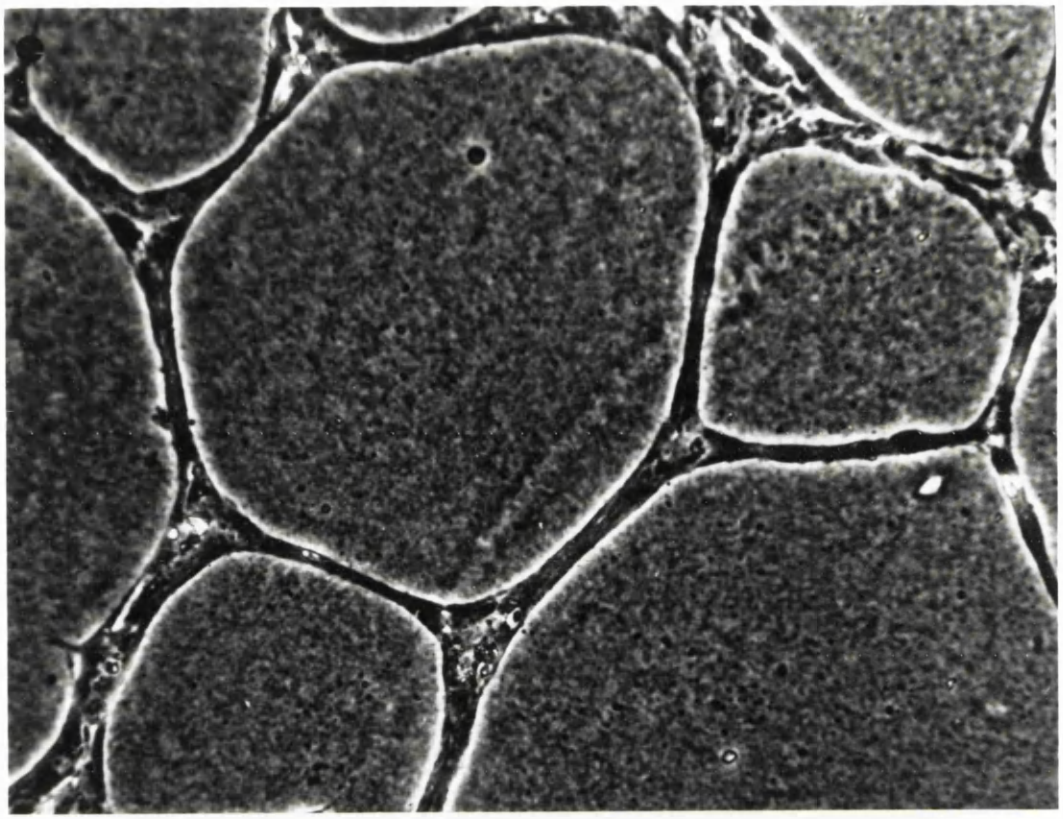

d

Figure 6.3;e,f e) The disaggregated cultures have rearranged and formed a network-like structure of small ganglia and interconnecting strands, very similar to the myenteric plexus in situ. Magnification x 650.

f) Picture of the network-like structure taken under lower magnification. Magnification x 400.



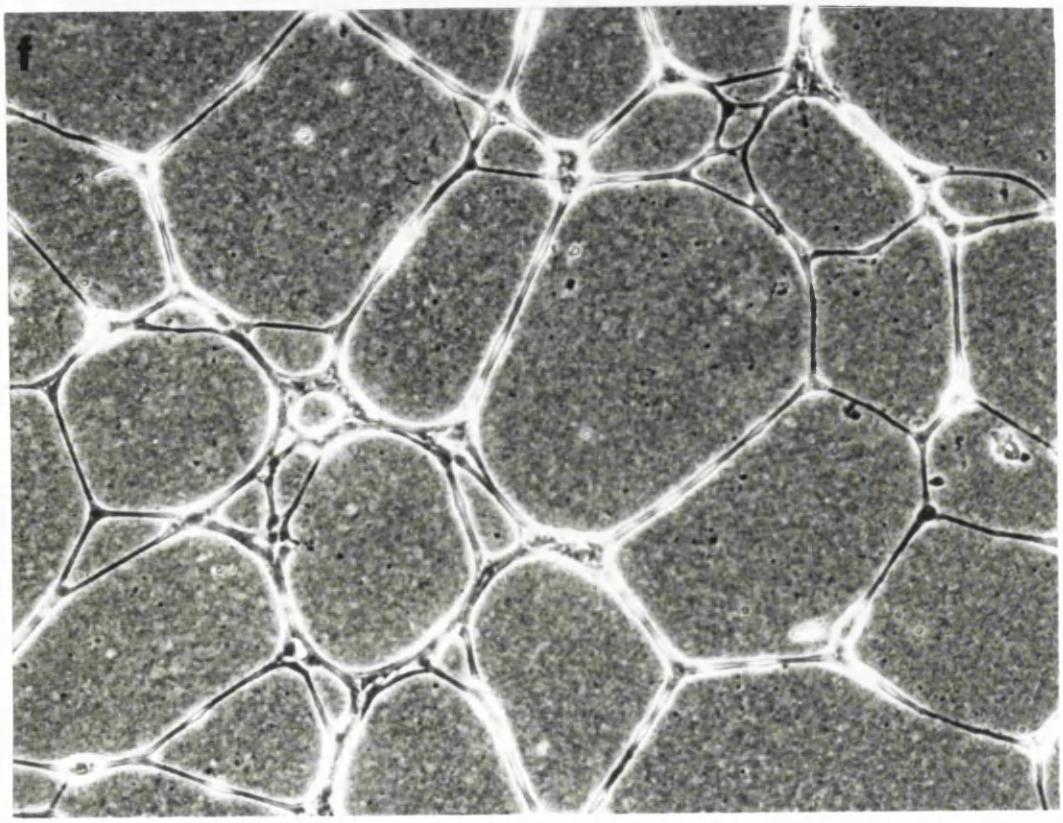


Figure 6.4; a,b Aggregation of a myenteric explant culture grown inside a 3-dimensional collagen gel in medium containing 10 % FCS.

a) The disaggregated plexus forms large aggregates (large arrows) and interconnecting strands. Individual glial cells are also seen (small arrows). Magnification x 400.

b) The aggregates migrate together into bigger aggregates (large arrows). A few starshaped glial cells are seen (small arrows). Magnification x 400.

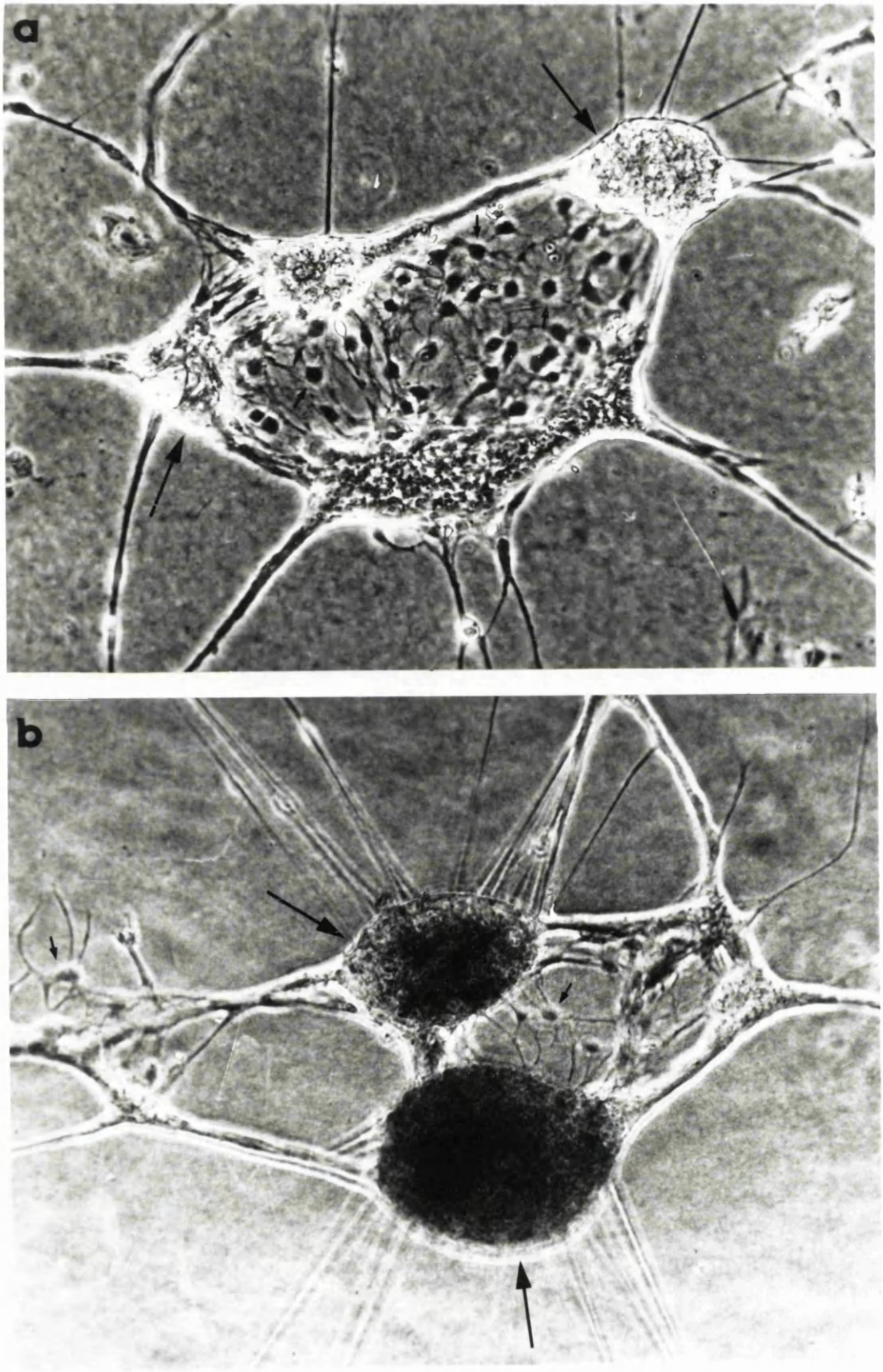

a
b

Figure 6.4; c) The culture forms in the end one very large aggregate (large arrow) and there is an extensive outgrowth of axon bundles (small arrows). Magnification x 400.

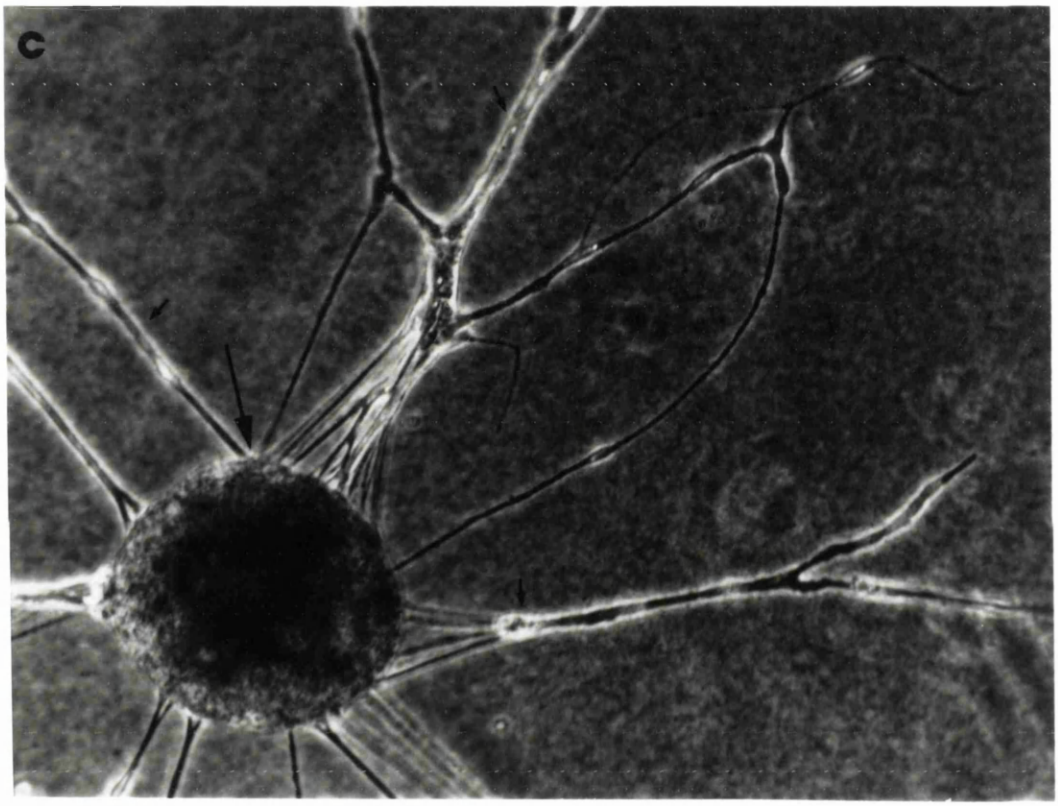

c

Figure 6.5

Transverse section through a ganglion of the rearranged network-like structure formed inside a 3-dimensional collagen gel. The ultrastructure is very similar to that seen in situ; the ganglion is a compact structure consisting of the cell bodies of neurons (N) and glia (G) and nerve cell processes (arrowhead). Some varicosities containing dense and agranular vesicles, can be seen (small arrows) and the glial cells form a lining around the ganglia (large arrows). Note the absence of basement membrane. Magnification x 51000.

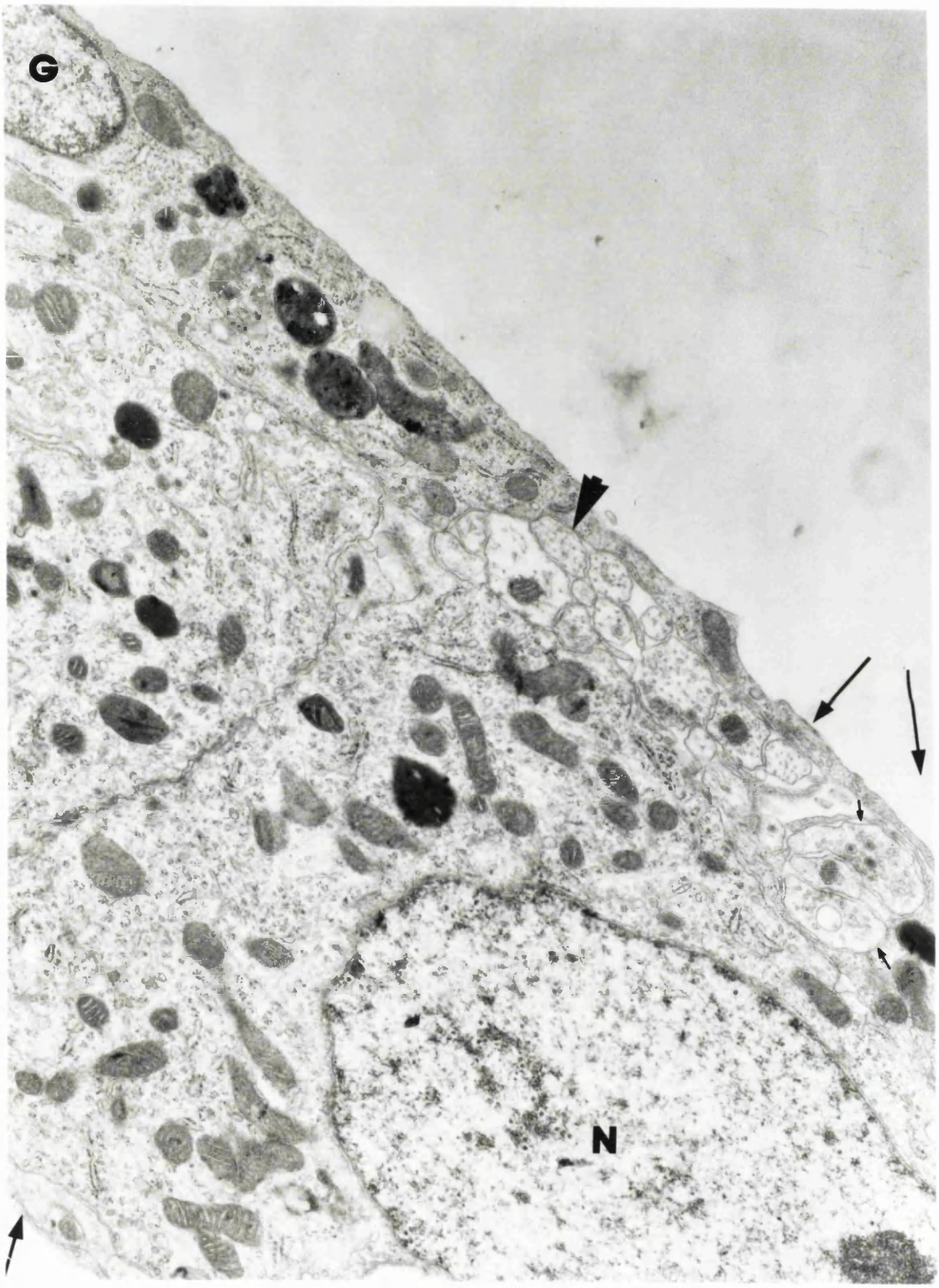

G
N

Figure 6.6

Transverse section through an interconnecting strand of the rearranged network-like structure formed inside a 3-dimesnional collagen gel. The ultrastructure is very similar to that seen in situ; several axons (arrowhead) are ensheathed by glial cells (arrows), but there is no basement membrane. Magnification x 51000.

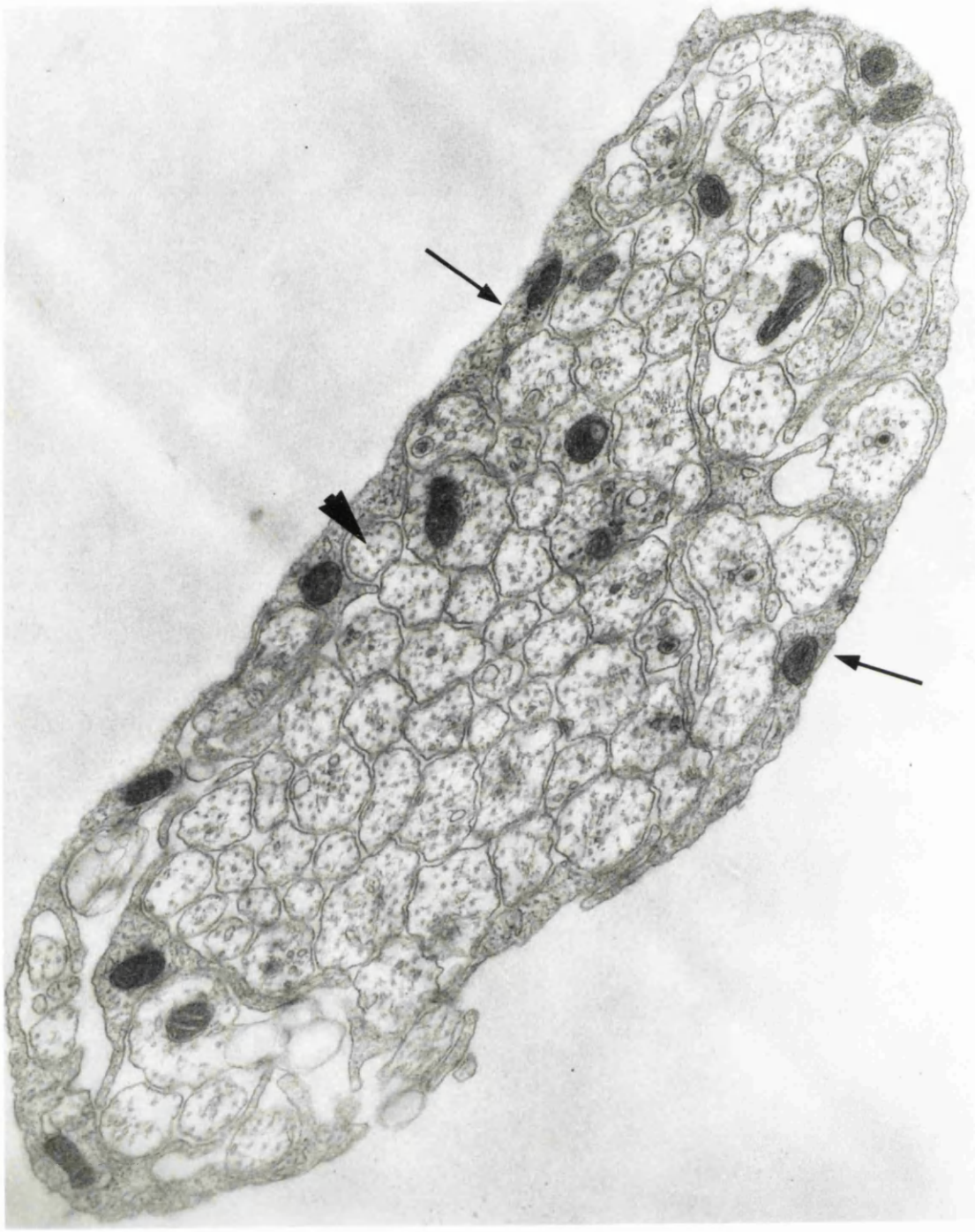
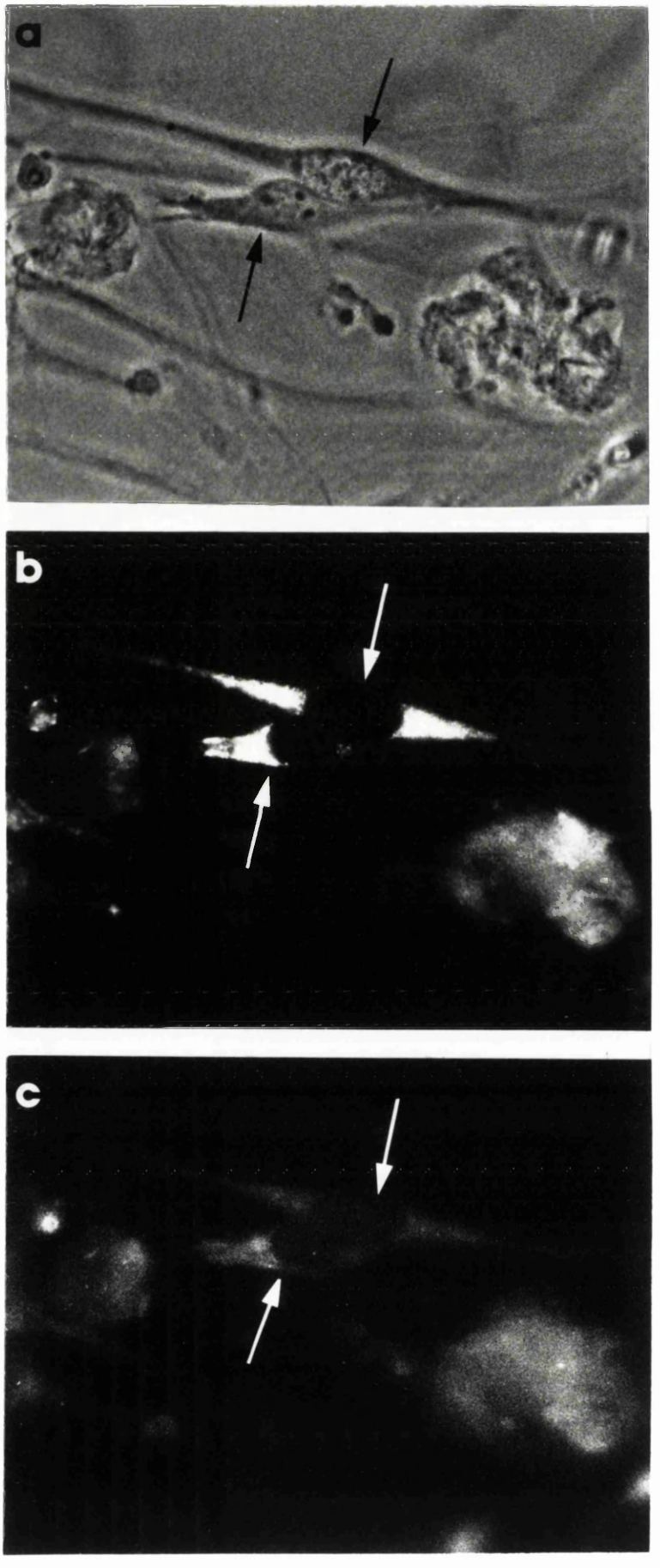


Figure 6.7

Expression of procollagen type I by enteric glial cells using double-label immunofluorescence. a) Phase contrast picture of glial cells in an explant culture of the myenteric plexus after one day in culture. The disintegration of the plexus into a monolayer of cells is incomplete. Two clusters of glial cells that are out of the plane of focus, lie at either side of the arrowed cells; b) procollagen type I seen with fluorescein optics. Note the strong procollagen immunoreactivity in the glial cell cytoplasm ; c) rhodamine optics to visualize S100 intracellularly. Magnification x 1000.


a
b
c

DISCUSSION

These results show that in vitro collagen type I is important for the maintenance of the differentiated morphology of the myenteric plexus, since collagen gel can prevent the disaggregation of the myenteric plexus, which happens when the plexus was grown on 2-dimensional substrate. This effect of collagen type I may be related to ability to inhibit the proliferation of enteric glial cells (Eccleston et al., 1989b). When the myenteric plexus is grown inside a 3-dimensional collagen gel, there was no indication of glial proliferation, although this was not measured directly, the glial cells did not migrate away from the neurons, and the characteristic in vivo morphology and the normal neuro-glia relationship were maintained. Serum was clearly very important, since collagen did not inhibit the breakdown of the network-like structure when it was grown in medium containing 10% FCS.

Collagen type I can also induce the rearrangement of disaggregated cultures of enteric neurons and glial cells into a network-like structure. When disaggregated cultures of enteric neurons and glia were grown inside a 3-dimensional collagen gel they rearranged and formed a network of small ganglia and interconnecting strands, very similar to the myenteric plexus in situ, provided that the serum concentration was kept low. Electron microscopy showed that the ultrastructure of these reformed plexuses was very similar to the ultrastructure of the ENS in vivo, except these rearranged plexuses do not form a basement membrane.

These results indicate that the collagen surrounding the myenteric plexus in vivo may be important for inducing and maintaining normal neuro-glia interactions and glial differentiation in the myenteric plexus. The effect of collagen is presumably mediated through specific glial matrix

receptors and may be in part an autocrine effect, since enteric glia synthesize and presumably secrete type I collagen, which then may act back on the cells and have an effect on their proliferation, differentiation and specific gene expression. The expression of an integrin receptor of the β_1 family, has been demonstrated on rat enteric glial cells (Eccleston, personal communication). Secretion of collagen type I (Bunge and Bunge, 1983) and type IV (Carey et al., 1983) has been demonstrated from Schwann cells.

Collagens can influence the ability of some cell types to maintain or to re-express a differentiated phenotype, cell polarity, and morphological organization (Hall et al., 1982) as discussed in Chapter 1, but the molecular events controlling cell-collagen interaction and their consequences have not yet been fully characterized. It has been proposed that either binding to collagen may induce a cell to become more responsive to soluble factors or that collagen itself may trigger a variety of changes in cell behaviour. (Bissel et al., 1982). It has also been shown that collagen inhibits glia cell division (Eccleston et al., 1989b), which in turn may switch glial cells from proliferation to differentiation (Morgan et al., 1990). In these enteric cultures, collagen may also exert its effects on differentiation by upregulating the expression of molecules important in normal neuro-glia interaction, which is crucial for the maintenance and also for induction of differentiation in these cultures. These molecules could be adhesion molecules, important in neuro-glia interactions, soluble factors or receptors for soluble factors or for ECM components. The membrane glycoproteins N-CAM and L1 play a crucial role in the mechanical adhesion between neural cells, both in development (Goridis et al., 1983) and also in adult animals (Edelman, 1984). It has been shown that these molecules are present on the surface of major categories of peripheral glia, including

enteric glia and neurons (Mirsky et al., 1986). Upregulation of these adhesion molecules and possibly also of soluble factors may occur in the collagen gel. Soluble factors and adhesion molecules are very likely to be important in the network formation seen in the collagen gel.

Serum inhibits the effect of collagen on the network formation in disaggregated enteric cultures, and also on the maintenance of the network-like structure in the collagen gel. It has been shown that serum has inhibitory effects on the differentiation of Schwann cells (Morgan personal comunication) and also of oligodendrocytes (Kumar et al., 1989). Serum contains a variety of factors, which have not been identified, some of these factors are mitogenic for some cell types including enteric glia (Eccleston et al.,1987). Serum may inhibit the differentiation of the enteric glial cells by causing them to divide. Alternatively the effect of serum could be unrelated to division.

The rearranged plexuses do not form a basement membrane but apart from this, the reformed cultures resemble the myenteric plexus in situ. The lack of basement membrane may be due to low serum content of the medium. This was inevitable since high serum prevents the network formation. It has been shown that Schwann cells cultured in the presence of neurons form basement membrane only if they are grown in medium containing high serum and ascorbic acid. Myelination is the best characterized function of Schwann cells and oligodendrocytes and it has been shown that Schwann cell differentiation, including myelin formation, is dependent upon the development of basal lamina which characteristically surrounds each axon-Schwann cell unit in peripheral nerve (Eldrige et al., 1989). In the presence of serum, ascorbic acid stimulates basal lamina assembly and myelin formation. In the presence of ascorbic acid the Schwann cells produce triple helical type IV collagen,

which may mediate the effect of ascorbic acid on basal lamina formation. If the Schwann cells are grown in serum free defined medium they fail to ensheath or myelinate the axons and they do not form a detectable basement membrane (Bunge et al., 1986). The formation of myelin by oligodendrocytes is little affected by ascorbic acid, suggesting that the biosynthesis and assembly of myelin per se does not require ascorbic acid. The results from this work show, that enteric glial cells do not need to form a basement membrane to be able to ensheath neurons and to take part in the formation of a network of ganglia and interconnecting strands. This is consistent with what is found in the development of ENS in vivo, since a distinct myenteric plexus has formed at day 32 of gestation, but the ganglia only become covered with a basement membrane at day 48 as judged by observation of electron micrographs (Gershon et al., 1981).

It has been suggested that the morphogenic action of ECM molecules depends on their capacity to both bind specific receptors and resist mechanical loads applied to those receptors. Experiments done on vascular endothelial cells, which form tubular network when they are grown inside collagen gel have shown that cell-generated tension has a role in ECM-dependent signalling. Simple alterations in the ability of a substratum to resist tensile forces generated by endothelial cells could switch these cells from proliferation to differentiation, in the presence of saturating levels of growth factor. Because of their insolubility, ECM molecules resist cell generated tensile forces and increase isometric tension within the cell when they bind to cell surface receptors. Mechanical stresses can only be transmitted over structural elements that are physically interconnected. Thus morphogenic signalling by transmembrane ECM receptors may be based on their ability to experience forces that soluble molecules cannot recognize (Ingber and Folkman, 1989). In agreement with this,

soluble collagen type I did not prevent disaggregation of myenteric plexuses, and did not induce network-formation in disaggregated cultures (data not shown).

The microenvironment is considered to have an important role in the final differentiation of the ganglion cells, which arrive in the gut, it is however unclear which factors are involved. The results presented here suggest that collagen type I, a major component of the ECM in the developing gut, is one of the factors which are important for the differentiation and the structural organization of the myenteric plexus. These results also indicate that for studying the differentiation and properties of enteric glial cells in culture, using supplemented medium containing low serum more closely mimics their environment in vivo than using medium containing high serum.

CHAPTER 7

GENERAL DISCUSSION

Immune related functions of Schwann cells

The first part of this work is an analysis of the interaction between Schwann cells and T cells when cultured together with or without antigen. It was found that in the presence of soluble mycobacterial antigen and without IFN-γ pretreatment, neonatal rat Schwann cells were able to interact with T cells and to support a lymphoproliferative response of mycobacteria-reactive T cells. Before the incubation period the Schwann cells were class II negative but essentially all the Schwann cells became class II positive during interaction with the T cells. Our results show that in the presence of antigen the Schwann cells were able to stimulate the T cells, and they in turn released factors which induced high levels of class II molecules on the Schwann cells. Schwann cells did not express class II molecules when incubated with T cells alone and only 1% of the Schwann cells expressed class II when incubated with antigen alone, but this may be sufficient to initiate T cell binding and recognition of specific antigens. It has been shown previously that IFN-γ induces class II expression on Schwann cells. The present work shows that when Schwann cells are incubated with IFN-γ in combination with TNF-α the class II expression is substantially increased. For example, when the Schwann cells were incubated with IFN-γ (50 U/ml) for 3 days class II was seen on

about 27% of the Schwann cells, but when TNF-α (300 U/ml) was added for the last 24 h, class II expression was increased up to almost 80% of the total number of the Schwann cells. The effect of TNF-α is directly dependent on IFN-γ. It has been shown that IFN-γ upregulates receptors for TNF-α on some cell types (Aggarwal et al., 1985) and this may be the mechanism behind the synergistic action of TNF and IFN-γ on the class II expression by Schwann cells.

As mentioned above, Schwann cells interacted with the T cells in Schwann cell T cell co-cultures and stimulated them to release factors, which in turn induced class II expression on the Schwann cells. The results from the antibody blocking experiments indicate that these factors are IFN-γ and TNF-α. This interaction between Schwann cells and T cells may be important for the initiation or augmentation of immune reactions within living nerves during inflammation, since Schwann cells can also be induced by lymphokines or by bacterial antigens to express class II molecules in the living sciatic nerve.

Most of the class II positive Schwann cells, found in the injected or in the crushed nerves, were of the myelin-forming type. Only a few class II positive non-myelin-forming Schwann cells were found in the injected or the damaged nerves. Class II positive mononuclear cells, were also found in these nerves. It may be that IFN-γ preferentially induces class II on the myelin-forming Schwann cells, and that the non-myelin-forming Schwann cells need another factor possibly TNF-β (released by lymphocytes) to express high levels of class II molecules.

Cultured Schwann cells were found to be able to produce IL-1 a molecule which is important in the initiation of the immune response. Neonatal rat Schwann cells produced IL-1 when they were incubated with bacterial antigens. Unstimulated Schwann cells expressed low levels of IL-1. It is an important question for future work whether Schwann cells express IL-1 in the normal living nerve or whether they can be induced by cytokines, bacterial antigens, or by injury to express this molecule in vivo. IL-1 has been shown to be chemotactic for leukocytes, and if infected or injured Schwann cells produce IL-1 it may attract both macrophages and lymphocytes into the nerve. These cells may become activated and secrete factors which in turn could induce class II expression on the Schwann cells and make them able to present antigen to the T cells. It is therefore possible that Schwann cells have a role in both immunoprotective and immunopathological responses in living nerves.

It was found that type I collagen substrate enabled embryonic rat Schwann cells to respond to IFN-γ by expressing class II molecules, and evidence was presented that the effect of collagen is mediated through integrin receptors. An interesting question is, by which mechanism collagen modulates the response of embryonic Schwann cells to INF-γ, and if collagen can affect the responsiveness of Schwann cells to other factors as well.

The neonatal rat Schwann cells were able to present soluble but not whole M.leprae to mycobacteria-reactive T lymphocytes. When adult rat Schwann cells were incubated together with irradiated M.leprae and sensitized T cells, the T cells formed clusters around the Schwann cells indicative of characteristic cell-cell

interactions normally associated with T cells and APCs. After the incubation period, essentially all of the Schwann cells expressed class II molecules on their surface, showing that in the presence of whole irradiated M.leprae the adult rat Schwann cells, unlike their newborn counterparts, were able to interact with the T cells. These results suggest that adult rat Schwann cells are able to process and present whole M. leprae to sensitized T cells, although this needs further investigation. The rat sciatic nerve is rather immature at birth. It is possible that the Schwann cells from the neonatal rat sciatic nerve are not fully immunocompetent cells and that rat Schwann cells become more immunocompetent as they become more mature. Another difference between adult Schwann cells and neonatal rat Schwann cells, is that the adult rat Schwann cells produce IL-1 in response to both cytokines and to bacterial antigens. Neonatal rat Schwann cells in contrast respond only to bacterial antigens.

The present work demonstrates that Schwann cells fulfil many of the essential criteria necessary for antigen presentation. It had been shown previously that IFN-γ induces class II expression on Schwann cells in vitro and that Schwann cells can present MBP to T cell lines. Now we have shown that another factor, TNF-α also affects class II expression on Schwann cells, but only in combination with IFN-γ. Cultured Schwann cells are also able to produce IL-1 and Schwann cells can be induced to express class II molecules in the living sciatic nerve. This work has also raised the question whether there is a difference between adult and neonatal rat Schwann cells, in their ability to function as accessory cells, the adult rat Schwann cells being more

immunocompetent than the neonatal rat Schwann cells. This could be important for future work in this field, since using cultures of neonatal rat Schwann cells would perhaps not give the right answers to important questions concerning Schwann cell functions in adult nerves.

MHC class II expression by oligodendrocytes

In experiments related to the above studies, the unexpected observation was made that oligodendrocytes were able to express class II molecules in vitro. Oligodendrocytes expressed class II molecules when they were incubated with IFN-γ in the presence of glucocorticoids.

This work shows that the regulation of class II expression varies between astrocytes and oligodendrocytes. Astrocytes are highly responsive and express both class I and class II molecules after incubation with IFN-γ in the absence of glucocorticoids. Oligodendrocytes also express class I molecules after incubation with IFN-γ but they require glucocorticoids in the medium to be able to express class II.

In the normal brain the expression of MHC antigens is very low and neither astrocytes nor oligodendrocytes express the MHC antigens. Now it is clear that both astrocytes and oligodendrocytes can be induced to express these molecules in vitro. The induction of class II molecules by IFN-γ may enable both cell types to support immune reactions within the brain, in diseases such as MS. Participation of astrocytes has still not been conclusively demonstrated in immune responses in vivo, although this is supported

by several investigations (e.g. Hofman et al., 1986).

It will now be important to look carefully for the involvement of oligodendrocytes in immune responses in vivo. The pathogenesis of the inflammatory destructive lesions of MS is explained by many as an immunological reaction to tissue antigen triggered by viral infection in early life in genetically predisposed individuals. Susceptibility to MS seems to be under multigenetic control, with links to HLA-D locus genes DW2 (DR2) on chromosome 6. The variable class II expression on certain cells, which do not express class II molecules constitutively, may be a regulatory mechanism for local immune reactions. This could be particularly important in the CNS in which class II antigens are normally expressed at very low levels.

It is an important question whether viruses can induce class II expression on oligodendrocytes. It will also be of interest to determine whether they can produce IL-1 and support lymphoproliferative T cell responses. Another interesting aspect is the question of which factors can inhibit the IFN-γ induced class II expression on glial cells both in the PNS and in the CNS, since the existence of such inhibitory factors has been demonstrated (Frohman et al., 1988; Schluessner et al., 1990).

The effect of collagen type I on the structural organization of the enteric nervous system

In the last part of this thesis the effect of collagen type I on the differentiation of the myenteric plexus was studied. For this purpose freshly dissected plexuses or disaggregated cultures of myenteric plexuses were embedded in 3-dimensional collagen gel, and

the cultures were grown in either DMEM containing 10% FCS or in supplemented medium containing 0.5% FCS. The results from this work indicate that collagen may be important in the maintenance of the fully differentiated morphology of the myenteric plexus, since collagen prevented the disaggregation of the myenteric plexus, which happens when the plexus is grown on 2-dimensional substrate. Collagen also induced the rearrangement of disaggregated cultures of enteric neurons and glial cells into a network-like structure, which resembled the myenteric plexus in situ. Electron microscopy showed that the ultrastructure of these reformed plexuses was very similar to the ultrastructure of the myenteric plexus in situ, except they did not form a basement membrane. The effect of collagen on the differentiation of the plexuses was only seen in supplemented medium containing low serum, but not if the cultures were grown in medium conating 10% FCS. Denatured, or soluble collagen type I did not prevent the disggregation of the myenteric plexus, or to induce network formation as was seen in the 3-dimensional collagen gel. Thus a 3-dimensional collagen framework seems to be needed to induce the network formation in these cultures, and collagen may have to be in triple helical conformation.

It is an important question, whether the effect of collagen on the differentiation of the myenteric plexus is mediated through special collagen receptors on the cell membrane of the enteric glial cells. It has been shown that rat enteric glial cells express integrin receptors of the β_1 type (P. A. Eccleston personal communication), but because of lack of cross-reacting antibodies it was not possible to investigate the expression of integrin receptors

on guinea pig cells. The effect of collagen is possibly an autocrine mechanism, since enteric glial cells of the newborn guinea pig produce collagen type I as was shown by using antibody against pro-collagen type I. In vitro collagen production is transient and disappears after few days both in cultures grown on 2-dimensional or in 3-dimensional collagen. Addition of ascorbic acid did not stimulate collagen production in these cultures as judged by immunohistochemical staining for pro-collagen. The reaggregated plexuses did not form a basement membrane, but apart from this, the reformed cultures resembled the myenteric plexus in situ, which suggests that collagen is inducing differentiation in these cultures. The lack of basement membrane may be related to the observation that enteric glial cells do not seem to sustain ECM production in culture (see also Bannerman et al., 1987). It should be further investigated which ECM components the enteric glial cells are able to produce, and which factors are needed for this expression and the assembly of basement membrane around the ganglia. It is also of interest to study if collagen upregulates the expression of some adhesion molecules and of soluble factors, which are important for normal neuro-glia interactions. When the disaggregated cultures were embedded in 3-dimensional collagen, glial cells migrated towards the neurons, indicating that the neurons were producing chemotactic factors and the glial cells were able to respond to these factors and migrate in the gel. Serum inhibits the effect of collagen on the network formation in disaggregated enteric cultures, and also prevents the long term maintenance of the network-like structure in the collagen gel. Serum may inhibit differentiation of the enteric glial cells either directly or indirectly by causing them to divide

(Morgan et al., 1990) since serum contains a number of unidentified growth factors, some of which are mitogenic (Eccleston et al. 1987).

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