ANTIGEN-PRESENTING CELLS OF THE GASTROINTESTINAL TRACT

By

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STATEMENT

All the experimental work reported in this thesis was performed by the author except as stated below:

Drs William Allan and David Hume performed the immunohistochemistry illustrated in Figures 1.2 and 3.13 respectively.

The splenic dendritic cells were prepared by Cathy Woodhams of the Division of Cell Biology of the John Curtin School of Medical Research.

Assistance was provided for the running of the samples in the flow cytometer by Derek Light, Cathy Kneale and Phil Newman at the John Curtin School of Medical Research, by the members of the staff of the Department of Immunology at the M.D. Anderson Hospital and Tumor Institute, Houston, Texas, and by Phillip Baron of the Department of Haematology of the Woden Valley Hospital, Canberra.

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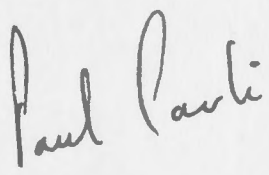
Ms Erika van de Pol provided assistance with the final human colonic disaggregation experiments and helped with the preparation of the illustrations in this thesis.

None of the material has been presented previously for the purpose of obtaining any other degree.



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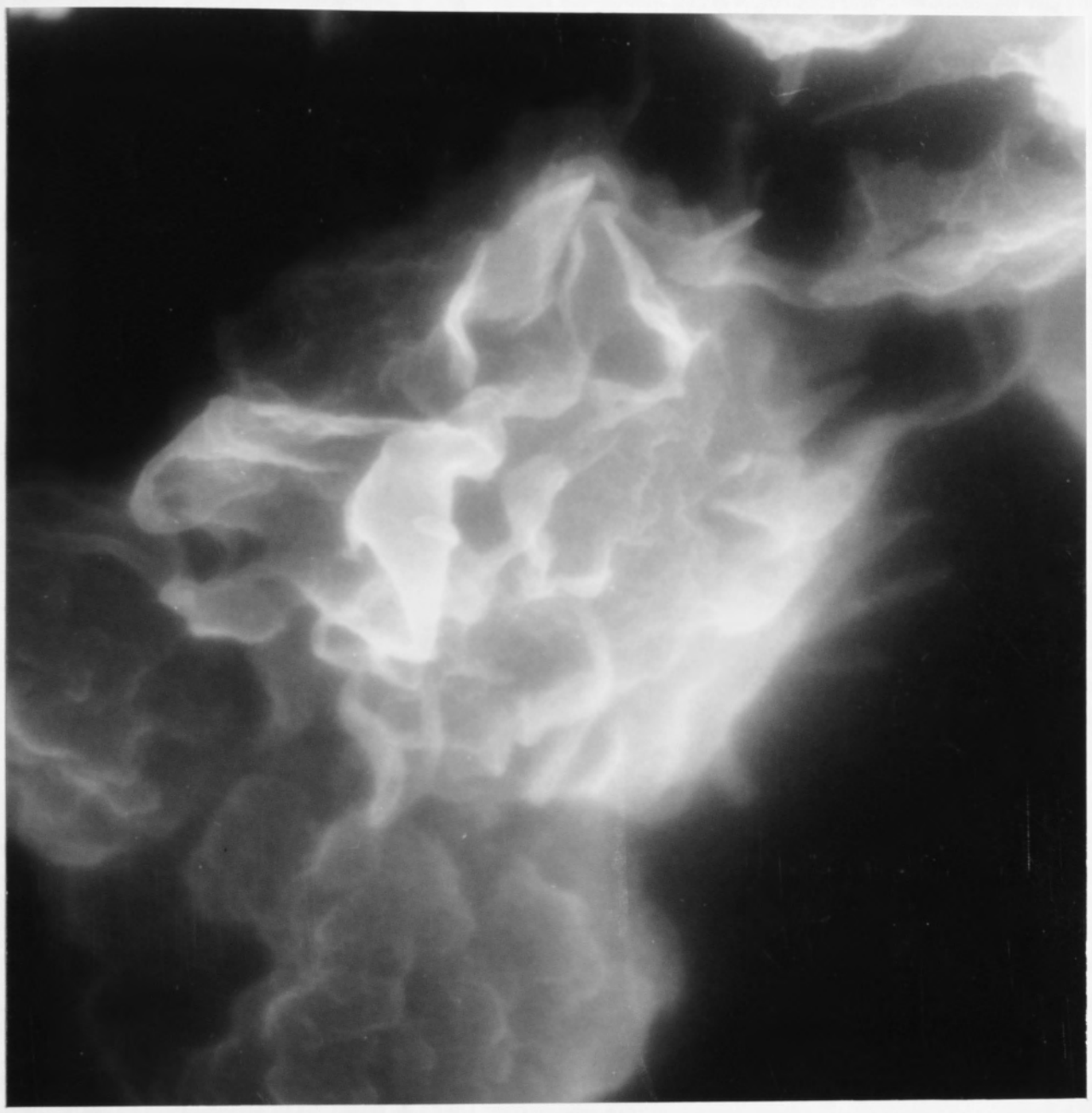

Frontispiece

Scanning electron micrograph of a human colonic lamina propria dendritic cell demonstrating the characteristic cytoplasmic processes or veils. Final magnification x 8,000

ACKNOWLEDGEMENTS

"The page of a thesis most likely to be read is the *Acknowledgements*."

Paul Pavli, "*Aphorisms*" (1990).

Why is it so?

Perhaps it is because the "Acknowledgements" are regarded as a form of light relief before the anticipated journey through the turgid prose or idiosyncratic style which may lie ahead.

Perhaps the reader is seeking to discover who provided the support necessary for the execution of this thesis. Naturally, there are many people who have made a significant contribution, and I thank them sincerely. In particular, many people gave me technical assistance including Phillip Baron of Woden Valley Hospital, Canberra (WVH), Derek Light, Cathy Kneale and Phil Newman of the John Curtin School of Medical Research (JCSMR) and the staff of the Department of Immunology at the M.D. Anderson Hospital and Tumor Institute (MDA) who helped with flow cytometry; Lesley Maxwell of JCSMR and Cora Bucana and Mike Dennis from MDA who performed the electron microscopy; Cathy Woodhams from JCSMR who provided murine splenic dendritic cells; and Erika van de Pol from JCSMR who assisted with the human colonic experiments and the illustrations. I am also grateful to Prof. Bill Doe, for his support and encouragement, and to the other people who gave me advice and engaged me in critical discussions (including Rhod Ceredig, Andrew Hapel and Peter Gibson from JCSMR, Hilary Warren from WVH and JoAnn Triall, Kim Jessup and Prof. Josh Fidler from MDA).

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course, my wife and children, who provided both the support and motivation for undertaking this work, and to whom I dedicate this thesis.

Whatever the reason you have taken up this thesis, dear reader, I welcome you, and hope that you will read on! If you know that these pages, although likely to be the first read, are the last written, you will understand my current sentiments and choice of quotation.

"Χαιρετε, νικωμεν."

Philippides at the end of his marathon c.490 B.C.

Lucian III.64 *"Pro Lapsu inter salutandun"* para iii.

"Hooray! We did it!" (Loosely translated by P. Pavli, 1990).

ABBREVIATIONS

The following abbreviations are used in the text:

ADCC	Antibody-dependent cellular cytotoxicity
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
$C3b_i$	Complement component
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation
CDR	Complementarity-determining region
CMF	Calcium- and magnesium-free
Con A	Concanavalin A
CR3	Complement receptor for component, $C3b_i$
CSF	Colony stimulating factor
DNase	Deoxyribonuclease
DTH	Delayed-type hypersensitivity reaction
DTT	Dithiothrietol
EDTA	Ethylenediaminetetraacetic acid
FAE	Follicle-associated epithelium
F_{ab}	F_{ab} fraction of the immunoglobulin molecule
F_c	F_c fraction of the immunoglobulin molecule
F_cR	Cell surface receptor for F_c
FCS	Foetal calf serum (heat inactivated)
GM-CSF	Granulocyte/macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxylpiperazine-N^1-2-ethane sulphonic acid
HLA	Human leucocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
LFA	Leucocyte function antigen
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
2-ME	2-mercaptoethanol
MHC	Major Histocompatibility Complex
MLN	Mesenteric lymph node
MLR	Mixed leucocyte reaction
Mls	Mixed lymphocyte stimulatory
mRNA	Messenger ribonucleic acid
NK	Natural killer
NSE	Non-specific esterase
PBS	Phosphate-buffered saline
PG	Prostaglandin
PHA	Phytohaemagglutinin
PMA	Phorbol-12-myristate-13 acetate
PWM	Pokeweed mitogen
RAR	Rabbit anti-rat (antibody)
SE or SEM	Standard error of the mean
SPF	Specific-pathogen free
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VLA	Very late proteins of activation

A note on style:

The style used conforms, wherever possible, to the recommendations of the Council of Biological Editors, Committee on Form and Style, 1972 [Council of Biological Editors, Committee on Form and Style. CBE Style Manual. Third Edition. American Institute of Biological Sciences, Washington, D.C.]. In particular, Latin terms in common usage are neither italicized nor underlined [op. cit. p. 183]

SUMMARY

The aims of the experiments recorded in this thesis were to identify and characterize the cell(s) responsible for T cell activation in the murine and human intestinal lamina propria.

Following the development of a method for the disaggregation of murine small intestinal lamina propria into single cell suspensions, antigen-presenting cell activity (as determined by the ability to stimulate the one-way primary allogeneic mixed leucocyte reaction {MLR}) was found to be comparable to that of splenic cell populations. Cell depletion experiments determined that the stimulatory cell expressed Class II Major Histocompatibility Complex antigens, but did not express features characteristic of macrophages, B cells or T cells.

A method was developed for isolating intestinal macrophages. These cells were compartmentalized, comprising 10% of the yields from non-Peyer's patch lamina propria tissue, but $<1\%$ of the yields from Peyer's patches. The expression of cell surface markers of intestinal macrophages indicated that they were in an "activated" state - they expressed Class II MHC antigens, and had low level expression of the macrophage-specific F4/80 antigen, the F_c receptor and the receptor for the complement component, $C3b_i$. Isolated intestinal lamina propria macrophages inhibited MLR stimulatory activity in an indomethacin-sensitive manner, suggesting a prostaglandin-mediated effect.

Unfractionated cell suspensions from the lamina propria were ten times more potent MLR stimulators per cell than unfractionated Peyer's patch cells. Dendritic cells were obtained from the intestinal lamina propria and from Peyer's patches by density gradient centrifugation following the removal of adherent cells (macrophages). Dendritic cell-enriched populations from both sites were potent MLR stimulators with activity indistinguishable from that of splenic dendritic cells.

Parallel experiments undertaken using human colonic lamina propria produced similar results. Isolated colonic macrophages were in an "activated" state and did not stimulate the allogeneic MLR, whilst enriched (30-50%) populations of dendritic cells were potent stimulators.

PUBLICATIONS

The work presented in Chapters 2, 3 and 4 has been accepted for publication in:

Pavli P, Woodhams CE, Doe WF and Hume DA. *Isolation and characterization of antigen-presenting dendritic cells from the mouse intestinal lamina propria.* **Immunology (1990**);

and has been published previously in abstract form in the following papers:

Pavli P, Doe WF and Hume DA. *Isolation and characterization of antigen-presenting cells from mouse intestinal lamina propria.* **Gastroenterology 1987; 92:1569**

Pavli P, Doe WF and Hume DA. *Inhibition of mouse intestinal lamina propria and Peyer's patch antigen-presenting cell activity by lamina propria macrophages.* **Gastroenterology 1987; 92:1569**

Pavli P, Doe WF and Hume DA. *Enrichment and characterization of dendritic cells from the lamina propria of murine intestine.* **Gastroenterology 1988; 94:A346.**

The work presented in Chapter 5 has been published in abstract form in:

Pavli P, Doe WF and Hume DA. *Isolation and enrichment of human colonic antigen-presenting dendritic cells (DCs).* **Gastroenterology 1989; 96:A385.**

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CHAPTER 6 DISCUSSION

CHAPTER 1

ANTIGEN-PRESENTING CELLS IN INTESTINAL IMMUNE RESPONSES

"The truth is rarely pure, and never simple."

From *"The Importance of Being Earnest"* (1895) Act I

Oscar Wilde (1854-1900)

1.1 INTRODUCTION

A distinctive characteristic of the vertebrate immune system is antigen specificity, a property conferred by well-characterized cell surface receptors present on T and B lymphocytes. The B cell antigen receptor (surface immunoglobulin) recognizes either bound or soluble antigen in a single interaction. In contrast, the T cell antigen receptor (TCR) has a requirement for dual recognition. That is, antigen must be "seen" in association with proteins coded by the Major Histocompatibility Complex (MHC) on specialized antigen-presenting cells. For this reason, the T cell repertoire is limited to those antigens that can bind to MHC molecules, and the net result is that immune responsiveness genes map to the MHC region. This introduction is concerned with the mechanisms involved in the regulation of T lymphocyte function.

Precursors of T cells arise in the bone marrow and migrate to the thymus where they differentiate to express antigen-specific receptors and other cell surface molecules [reviewed in 6]. During thymic maturation, T cells are also subjected to selective pressures resulting in the induction of tolerance to self-antigens and the phenomenon of MHC-restriction. After release from the thymus, mature T cells migrate through the peripheral blood and other tissues. If they come into contact with their cognate antigen in association with self-MHC molecules (MHC-restriction) on the surface of specialized antigen-presenting cells, T cell activation results [reviewed in 417]. In this process T cells undergo blastogenesis, proliferate and differentiate into immunological effector cells with functions including cytotoxicity, immune suppression and the provision of helper factors (for B cell antibody production, and for the control of other effector arms of the immune response [reviewed in 323]).

1.1.1 Molecules involved in T cell interactions

Interactions between T cells, foreign antigen and other components of the immune system are mediated by a number of cell surface molecules involved in

antigen-recognition, intercellular adhesion and/or signal transduction (Fig. 1.1). The majority are members of two families of structurally-related integral membrane proteins, the immunoglobulin gene superfamily (Table 1.1) [reviewed in 13, 39, 175, 511] and the integrin receptor superfamily (Table 1.2) [reviewed in 298, 408].

1.1.1.1 T cell antigen receptor

The TCR is a disulphide-linked heterodimer (comprising α and β polypeptides which contain the variant domains involved in antigen specificity) which is associated with a collection of at least five invariant proteins called CD3 [Reviewed in 30, 97, 297]. The receptor complex appears to be present on all helper and cytotoxic T cells. A second type of TCR heterodimer (γ:δ) has also been recognized. It is found on a small proportion of peripheral blood T cells, but is more common in certain anatomical locations eg. the dendritic T cells of the skin [243] and the intraepithelial lymphocyte in the mouse [51, 137]. The function of the γ:δ heterodimer is unknown. The four recognized TCR polypeptides are similar to the immunoglobulins in both structure and gene organization. Each has V, J and C regions and β and δ also have D regions. Diversity is apparent in three regions equivalent to the classical immunoglobulin hypervariable regions which form the points of contact with antigens - the complementarity-determining regions (CDRs). The V gene codes for the CDR1 and CDR2 regions whilst the CDR3-equivalent region is formed by the junction of V and J (in α and γ) and V, D and J (in β and δ).

1.1.1.2 Major Histocompatibility Complex gene products

The TCR ligand comprises MHC molecules complexed with a peptide fragment [7, 75]. MHC molecules are the products of highly polymorphic genes, termed Class I and Class II, within the major histocompatibility gene complex [reviewed in 209]. The nature of the crystalline structure of the Class I MHC molecule, Human Leucocyte Antigen (HLA)-A2 [44] and the hypothetical model

of antigen binding to Class II MHC molecules [66] suggest that the binding site for peptide is on the top surface of the molecules located between the α-helical regions of the polymorphic α_1 and α_2 domains in Class I MHC and the α_1 and β_1 domains in Class II MHC proteins.

A model for the alignment of the TCR over a peptide-MHC complex proposes that the V_α and V_β CDR1- and CDR2-equivalent regions contact the side chains of the MHC α-helices. The centrally located CDR3-equivalent regions would then be aligned with the bound peptide (Fig. 1.1) [97]. This model explains both antigen-specificity and MHC restriction. The formation of the trimolecular complex leads to signal transduction via the CD3 complex and, in association with other signals, induction of primary T cell responses.

1.1.1.3 Antigen processing

Antigen processing at the molecular level has recently been reviewed by Allen (1987) [9] and Benacerraf (1988) [30]. Unlike the B cell antigen receptor (the surface immunoglobulin molecule) which recognizes the tertiary configuration of an antigen (which may be in a soluble state), the T cell antigen receptor generally recognizes a denatured form of antigen in association with MHC molecules on the surface of a target or an antigen-presenting cell. The conversion of a native form to a non-native form is termed antigen-processing. For many exogenous antigens proteolytic degradation and contact with the acidic intracellular lysosmal compartment is presumably the major pathway of antigen processing. Another pathway involving nonendosomal proteases was originally believed to be accessible only to endogenous viral antigens synthesized in infected cells. Recent work examining the processing of a viral antigen showed that both pathways could handle antigen regardless of its source [202]. Thus, there are at least two pathways for antigen processing - an endosomal, chloroquine-sensitive and a non-endosomal, chloroquine-

insensitive. A third pathway utilizing a cell-surface, proteolytic "ectoenzyme" system also has been proposed [80].

1.1.1.4 CD4 and CD8 molecules

CD4 and CD8 glycoproteins are nonpolymorphic members of the immunoglobulin gene superfamily which are expressed on the surface of functionally distinct populations of peripheral T lymphocytes. The expression of CD4 and CD8 molecules on peripheral T cells correlates with the class of MHC determinant recognized (Class II MHC and Class I MHC respectively). In addition to the demonstration of direct binding between CD4 and Class II MHC molecules [100] and CD8 to Class I MHC molecules [404, 411], other evidence [reviewed in 39] suggests that this interaction increases adhesion between antigen-presenting cells and T cells. There is also evidence for an intrinsic, low affinity interaction between CD4 and the CD3-T cell receptor complex [410, reviewed in 13]. Thus, the CD4 molecule may adhere to the Class II MHC molecule and stabilize its association with the TCR. A role for these molecules in signal transduction is also suggested by the demonstration of their association with cytoplasmic protein tyrosine kinases [339].

1.1.1.5 CD2 and leucocyte function antigen-3 (LFA-3)

CD2 is a glycoprotein with limited tissue distribution (thymocytes, T cells and some natural killer {NK} cells) which plays an important role in cell adhesion and T cell activation [38]. Monoclonal antibodies against CD2 inhibit or stimulate T cells, depending on the epitope specificity.

Its natural ligand is a widely distributed glycoprotein, LFA-3. Both these molecules are generally considered to be members of the immunoglobulin gene superfamily, although there is some debate [175]. The interaction between these molecules, together with a co-stimulatory signal (anti-CD3 antibody or suboptimal doses of the plant lectin, phytohaemagglutinin) can stimulate T cell proliferation [38].

1.1.1.6 LFA-1 and intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2)

The LFA-1 molecule is a member of a family comprising three related heterodimers found on lymphoid and myeloid cells [reviewed in 39, 101]. These proteins share a common β chain (CD18) noncovalently associated with a unique α subunit. The α chain of LFA-1 is termed CD11a; the other proteins in the family are CD11b (CR3) and CD11c (the p150,95 protein). Binding of LFA-1 is temperature-sensitive and cation-dependent.

The ligand for LFA-1 is ICAM-1, a glycoprotein that is also widely distributed. A recent paper [11] demonstrated that co-expression of ICAM-1 is critical for effective MHC Class II-restricted and allospecific T cell activation in transfected cells expressing modest levels of HLA-DR antigens. This implies an important role for ICAM-1 in the induction of T cell responses. The presence of other ligands for LFA-1 was suggested by the failure of anti-ICAM-1 monoclonal antibodies to inhibit LFA-1 dependent adhesion to all cell types. An alternative ligand, ICAM-2, expressed on endothelial cells has now also been identified [439].

1.1.1.7 VLA proteins (very late proteins of activation)

Studies on the adhesion of cells to the extracellular matrix led to the discovery of a family of cell attachment receptors recognising similar amino acid structures in the matrix proteins (the RGD {arginine-glycine-aspartic acid} sequence) [reviewed in 408, 39]. These receptors include the fibronectin and vitronectin receptors, and VLA proteins 1-4 which bind other matrix proteins including laminin and collagen. Structural similarities with the LFA-1 family were identified, and together these proteins comprise part of the integrin receptor superfamily.

The VLA proteins -1 and -2 were initially identified on the surface of stimulated T cells, but are also found elsewhere. VLA-4 is found on all lymphocytes,

thymocytes and monocytes, whilst VLA-3 and VLA-5 (the fibronectin receptor) are not expressed on peripheral blood lymphocytes.

Antibody blocking studies have shown that the VLA proteins mediate cell adhesion to matrix proteins, but inhibition of T cell function has not been demonstrated. T cells, once activated, express increased levels of ICAM-1 [101] and VLA-1 and -2 [39].

1.1.1.8 Summary

Many cell surface molecules are involved in both the stabilization of interactions between T cells and antigen-presenting cells, and in signal transduction. The ultimate determinant of antigen-specificity, however, is the TCR.

1.1.2 Mechanisms and measurement of T cell activation

[Reviewed in 91, 177, 285, 505]

The specificity of T cell receptors can be bypassed using various physiological or non-physiological stimuli leading to non-specific T cell activation. Examples include calcium ionophores, plant lectins, and certain antibodies to either the T cell receptor, the CD3 molecule or the CD2 molecule. In addition, agents that activate protein kinase C (eg. phorbol myristate acetate {PMA}) are thought to reproduce the requirement for accessory cells and their products. The role of interleukin 1 (IL-1) in T cell activation is considered in Section 1.2.5.3.2.

The membrane and intracellular events initiated by these stimuli are similar to those induced by many hormones [8, 91, 278, 347]. Inositol phospholipids are hydrolysed, intracellular stores of calcium are mobilized and membrane and cytosolic proteins are phosphorylated. The process continues for at least 10 days and involves a complex sequence of gene activation resulting in the regulation of more than 70 molecules [91], and the acquisition of specialized effector functions. For at least a subset of T cells, these events result

in increased production of the T cell growth factor, interleukin 2 (IL-2), increased expression of the cell surface IL-2 receptor and an autocrine mode of proliferation.

The consequences of these cell surface interactions can therefore be measured by end-point assays that detect T cell functions (eg. proliferation, target cell cytotoxicity, help for antibody production or the production of lymphokines such as IL-2) or T cell activation markers (eg. Class II MHC antigens or receptors for IL-2 {CD25} or transferrin {CD71}).

1.1.2.1 Activation requirements for resting and sensitized T cells

Activation requirements for resting and memory T cells differ from those for sensitized or activated T cells [21, 442, 446]. Steinman (1988) [442] described two broad functions of accessory cells. The first is the "presentation" of antigen in association with Class II MHC products to form a complex on the accessory cell surface. This structure can then be recognized by an antigen-specific clonotypic T cell receptor. The subsequent response depends on the nature of both the accessory cell and the responding T lymphocyte. A resting T cell requires an additional "sensitization" or co-stimulatory signal. As a result the T cells "blast transform", secrete and become responsive to lymphokines, and proliferate vigorously. In the absence of such a signal there is no response. A previously sensitized T cell does not require this second function and will respond vigorously to the antigen-MHC complex alone.

The magnitude of this secondary response correlates with the concentration of antigen and the surface density of Class II MHC molecles on an antigen-presenting cell [265, 302]. The complex may be present on macrophages, resting B cells, endothelial cells, epithelial cells or fibroblasts.

Fundamental differences in activation requirements between resting and sensitized T cells were demonstrated using antibodies directed against the CD3 molecule - the signal transducing element for the T cell receptor molecule [176] -

and other cell surface molecules. In experiments measuring the activation of T cell clones (ie. sensitized cells), accessory cell activity was replaced by anti-CD3 coupled to Sepharose beads [321]. When resting T cells were used, accessory cells were necessary for IL-2 production [476, 506]. Similarly, whilst studying T cell activation, Odum et al (1988) [357] showed that, in contrast to unprimed cells, the proliferation of sensitized cells was independent of the interaction with the CD5 and CD11 molecules, and IL-1$_\beta$. Thus, activation requirements for sensitized and unsensitized T cells differ.

1.1.2.2 The primary allogeneic mixed leucocyte reaction (MLR)

The best physiological stimulus for studying primary T cell activation would be previously "unseen" antigen in association with syngeneic antigen-presenting cells. However, the frequency of a particular antigen-specific T cell in an unprimed population is very low, making the study of primary antigen-specific responses difficult. A relatively large proportion (>1-3% of unprimed T cells) is activated by allogeneic MHC products so that this model is often used for the study of T cell activation.

Co-culturing leucocytes which differ at the MHC loci results in cell proliferation and the development of cytotoxic T lymphocytes (reviewed in Steinman and Inaba (1986) [445]). Both Class I MHC and Class II MHC molecules can stimulate the MLR as can non-MHC encoded determinants, such as the product of the Mls locus (loci) in the mouse.

The interactions between antigen, Class II MHC gene products and the clonotypic T cell receptor have been studied recently by gene transfer studies. It is now clear that a single T cell receptor $\alpha\beta$ heterodimer can recognise self-MHC molecules complexed with fragments of antigen, allogeneic MHC molecules and an Mls-encoded determinant [221, 294]. These observations verify that the primary MLR is a valid and physiological model for studying T cell activation.

1.1.2.3 The oxidative mitogenesis model

Treatment with the oxidizing agent, sodium metaperiodate, induces extensive blastogenesis as the result of the oxidation of the terminal sialic residue of a specific membrane glycoprotein or glycolipid [reviewed in 174]. The effect of periodate can be reproduced by the removal of sialic acid with neuraminidase followed by oxidation of exposed galactose residues with galactose oxidase. Both these treatments cause cross-linking of membrane components and subsequent transformation and proliferation of a responder T cell population if accessory cells are present. Antigenic changes induced by oxidation of surface glycoproteins of a stimulatory cell population may be analogous to the MLR if the Class II MHC molecules are involved.

1.1.3 Putative intestinal lamina propria antigen-presenting cells

Cell types that express Class II MHC antigens, and might therefore act as antigen-presenting cells in the intestinal lamina propria (at least in a secondary immune response), include dendritic cells [446], macrophages [479], B cells [84], activated T cells [204], endothelial cells [352], and epithelial cells [48, 308].

In order to generate a T-cell-mediated immune response in the intestinal mucosa, antigen must cross the epithelial barrier. The major emphasis in studies of intestinal mucosal immunity has focused on Peyer's patches and their associated specialized epithelium which is believed to be the main site of antigen contact and sampling. A number of observations, however, suggest an important role for the non-Peyer's patch epithelium and associated lamina propria lymphoid tissue. Firstly, presumptive antigens have been demonstrated in the lamina propria or within lamina propria macrophages. Examples include enterotoxin (by receptor-mediated transport [467]); infectious agents (Shigella, Salmonella [467], and Campylobacter antigens [407]), soluble protein antigens (eg. bovine serum albumin [37]); and carageenan [3]. Secondly,

immunohistochemical techniques have identified all of the putative antigen-presenting cells described above in the small intestinal lamina propria. In particular, there are large numbers of Class II MHC antigen-bearing macrophages in the intestinal lamina propria of mouse, rat and human [169, 170, 172, 197, 312, 437] and some evidence has been presented concerning a population of large irregularly-shaped antigen-presenting cells which lack macrophage markers (presumptive dendritic cells) [169, 172, 197, 312] (Figs. 1.2a, 1.2b). Thirdly, the lamina propria contains T cells responsive to stimulation by the lectins, phytohaemagglutinin and concanavalin A, and by alloantigens in the mixed leukocyte reaction (MLR) [reviewed in 310].

1.1.4 Aims:

The aims of the experiments reported in the following chapters of this thesis were to isolate and characterize the antigen-presenting cell from the intestinal lamina propria. This first chapter will review the current information on the nature of putative intestinal lamina propria antigen-presenting cells, in particular, the dendritic cell, the macrophage, the B cell and the epithelial cell.

1.2 THE DENDRITIC CELL

Since the initial recognition of the dendritic cell by Steinman and Cohn (1973) [443], evidence has accumulated to suggest that it plays a unique role in the initiation of T cell dependent immune responses. Several recent papers have reviewed the properties and functions of these cells, and their proposed mechanisms of action [21, 442, 446, 448]. This review will concentrate on the following aspects of dendritic cells:

1.2.1 Physical and phenotypic features;

1.2.2 Functional properties;

1.2.3 Origin, tissue distribution and pattern of circulation;

1.2.4 Ontogeny and cell lineage; and

1.2.5 Mechanisms of action.

Because of the vast literature on dendritic cells wherever possible the evidence will be summarized in tabular form.

1.2.1 Properties and phenotype of dendritic cells

The initial studies of in vitro T cell activation identified a requirement for accessory cells, which were derived from the adherent fraction of murine lymphoid cell suspensions [reviewed in 446]. The study of the cellular composition of these adherent populations identified dendritic cells which were potent activators of T lymphocytes. Prior to these studies, all adherent cells were regarded as macrophages and the antigen-presenting cell activity of the adherent cell fraction was therefore thought to be a macrophage-mediated function. Over many years, the relationship between dendritic cells and macrophages has been the focus of considerable debate.

Several properties distinguish the dendritic cell from the mononuclear phagocyte system. Dendritic cells are large, irregular in shape, low-density, non-phagocytic and become non-adherent after overnight culture. They lack features

characteristic of other cell lineages including F_c receptors, non-specific esterase (NSE), membrane adenosine triphosphatase (ATPase), and the F4/80 antigen (mononuclear phagocytes), and surface markers of the T and B cell lineage. The dendritic cell constitutively expresses high levels of both Class I and Class II MHC molecules which do not change with time in culture and are not inducible by IFN-γ [442]. The kinetics of dendritic cells are different from the mononuclear phagocyte system [482]. A rat IgG_{2b} anti-murine splenic dendritic cell-specific antibody has been raised, and is useful when used with complement lysis to deplete dendritic cells [356].

Using immunohistochemical techniques, cells with the phenotype of dendritic cells have been identified in the interstitial tissues of all non-lymphoid organs studied except for the brain [150, 312]. The isolation and enrichment of dendritic cells from different tissues has also been possible. Recent extensive phenotypic analysis of dendritic cells obtained from three different tissues confirmed previous observations regarding the absence of key macrophage and lymphocyte antigens [93]. Of interest was the demonstration of heterogeneity amongst the dendritic cell populations studied (see Table 1.3). These phenotypic differences may reflect functional differences in dendritic cells or may be due to varying stages of development eg. migratory dendritic cells vs fixed tissue interdigitating dendritic cells [93]. These observations also raise the question of the relationship between phenotype and tissue distribution ie. does the phenotype determine the destination of the dendritic cell or does the phenotype develop after a common precursor is localized at a particular site?

1.2.1.1 Electron microscopic appearances of dendritic cells

Transmission electron microscopy of dendritic cells demonstrates an extremely irregular nucleus with a peripheral or rim heterochromatin pattern, few nuclear pores, and small nucleoli. The cytoplasm contains well developed mitochondria, rough endoplasmic reticulum and scattered smooth vesicles. The

Golgi region lacks typical lysosomes and secretory granules but contains a variety of multivesicular bodies comprised of large vacuoles surrounded by, or containing, smaller vesicles. Phagolysosomes are absent [447].

Scanning electron microscopy of cultured dendritic cells shows an array of bulbous cytoplasmic protrusions of varying size and shape, including the characteristic veils associated with the veiled cell of afferent lymph [378, 311].

1.2.2 Dendritic cell function

Before summarizing the functional properties of dendritic cells, it is important to emphasize a number of principles in the study of T cell activation using the MLR to explain the inconsistencies in the published literature. Firstly, accessory cell requirements for resting T cells differ from those for sensitized or activated T cells (see 1.1.2.1).

Secondly, given the potent stimulatory activity of dendritic cells, it is important to determine whether a small contaminating population could explain the observed experimental results. This is best done by removing possible contaminants and determining their relative contribution eg. by using the anti-dendritic cell monoclonal antibody, 33D1, with splenic adherent cells in the mouse [444].

Finally, the accessory cell requirements of a responder population must be absolute. The presence of accessory cells in the responding T cells may result in a syngeneic MLR and the production of helper factors eg. IL-2 [178]. This will be of even greater magnitude if xenogeneic antigens (eg. foetal calf serum) are used in the culture medium [353]. There is also the theoretical possibility of "back-stimulation" ie. the "responder" accessory cells may interact with T lymphocytes in the stimulator population and cause sensitization. Although these stimulator cells could not proliferate themselves (eg. if treated with mitomycin C or irradiation), the signal may "spill over" onto the responder cells which would then proliferate. Depletion of accessory cells from the

responder population is best undertaken by removing Class II MHC-bearing cells using specific monoclonal antibodies. Methods using adherence or nylon-wool adherence alone are unreliable. Comparisons of different methods for obtaining responder cells showed that Ia^+ cell-depleted T cell populations had more stringent requirements for accessory cells than conventional T cell preparations [217]. For example, the removal of a small population (3%) of Class II MHC positive responder cells using monoclonal antibodies and complement lysis resulted in abrogation of Ia^+-macrophage-induced T cell proliferation. This indicated that the Ia^+-macrophages induced proliferation not as autonomous accessory cells, but by acting synergistically with dendritic or other cells in the responder T cell population.

1.2.2.1 Dendritic cell functions

The functional properties of dendritic cells as determined by many independent groups are summarized in Table 1.4 [adapted from 446 and 21] and will not be discussed further. The evidence for MLR stimulatory activity of other putative accessory cells is considered below.

1.2.3 Origin and tissue distribution of dendritic cells

Studies on the origin and tissue distribution of dendritic cells indicate a definite circulatory pattern. In summary, dendritic cells (lymphoid dendritic cells, Langerhans cells and veiled cells) are bone-marrow derived [139, 216, 366, 378, 449] and migrate to peripheral tissues via the blood. In tissues, they may be functionally immature, eg. Langerhans cells, but following exposure to antigen develop the ability to act as potent T cell activators whilst migrating in the afferent lymphatics as veiled cells to draining lymph nodes. Trapped as the interdigitating cells in T cell dependent areas, they interact with antigen-specific T cells in the initiation of T cell responses. In certain inflammatory exudates, eg. the synovial fluid of rheumatoid arthritis or the lamina propria of inflammatory

bowel disease, dendritic cells are recruited by mechanisms which remain to be determined.

1.2.3.1 Human peripheral blood dendritic cells

Human peripheral blood contains a small proportion of dendritic cells which have been enriched to varying degrees [41, 118, 129, 187, 237, 251, 322, 384, 412, 413, 485, 486, 487, 492, 521, 522].

These cells possess properties similar to those of murine dendritic cells including:

a) characteristic morphology at light- and electron microscopy;

b) low density and poor adherence with time in culture;

c) constitutively high level expression of Class I and Class II MHC antigens (including HLA-DR, -DP and -DQ);

d) absence of other cell surface markers and functions associated with macrophages, B cells, T cells and NK cells, including phagocytosis, F_CR expression, surface immunoglobulin and the CD1, 3, 4, 8, 14 and 16 epitopes [521. CD nomenclature reviewed in 82, 123]. (There is conflicting evidence about the presence [522] or absence [412, 494] of the CR3 antigen (CD11b). The expression of this receptor on murine Langerhans cells is lost in culture [416], so these differences may reflect in vitro manipulation);

e) potent stimulatory activity for many T cell responses including:

i) oxidative mitogenesis [485, 487];

ii) mitogen- and antigen-induced T cell proliferation [41, 322, 487];

iii) T cell-dependent antibody production [187];

iv) syngeneic and allogeneic MLR [237, 251, 384, 412, 413, 485, 487]; and

v) cluster formation with T cells [118, 251, 521].

1.2.3.2 Langerhans cells

[Reviewed in 57, 426, 453]

Langerhans cells are Class II MHC antigen-bearing leucocytes found in the suprabasal region of the epidermis. Their dendritic processes form an extensive but non-overlapping network [173] which appears to be ideally suited for the trapping of antigens passing through the epidermis. In vitro and in vivo experiments using both enriched and purified epidermal Langerhans cells have demonstrated the ability of these cells to present antigen to T cells in various systems [2, 40, 111, 156, 181, 211, 365, 371, 416, 424, 441, 460, 461, 515]. Recent work in vitro has shown that the ability of Langerhans cells to stimulate primary immune responses is weak upon initial isolation, but increases considerably in culture under the influence of GM-CSF and IL-1 [156, 181, 371, 416, 424, 515]. Associated with this functional change is a change in phenotype (see Table 1.3) and morphology so that cultured Langerhans cells become indistinguishable from dendritic cells.

There is evidence to suggest that, in the development of contact hypersensitivity in vivo, Langerhans cells acquire antigen in the epidermis, migrate via the afferent lymph to the draining lymph nodes and, having matured, present antigen to T cells as dendritic cells [239, 281, 282, 426]. Macatonia et al (1987) [282] after painting the skin of mice with fluorescein-isothiocyanate demonstrated a rapid increase in the number of dendritic cells in lymph nodes, the preferential location of antigen on dendritic cells and potency of antigen-bearing dendritic cells in inducing syngeneic T cell responses. In addition, they showed "transitional" forms of dendritic cells containing Birbeck granules, generally thought to be characteristic of Langerhans cells.

1.2.3.3 Other tissue dendritic cells

1.2.3.3.1 Thymic dendritic cells

Human and murine thymic dendritic cells have been isolated and characterized in vitro [28, 93, 255, 259, 367]. The properties they share with the dendritic cell lineage include morphology, physical properties (low density and non-adherent), high level expression of HLA-DR surface antigens, the expression of the CD1a (OKT6) and CD4 (weakly) molecules (also expressed by Langerhans cells), the expression of the S-100 protein (found on interdigitating cells in T cell-dependent areas of human lymph nodes [367] and on tonsillar dendritic cells [504]) and potent stimulation of the MLR.

1.2.3.3.2 Tonsillar dendritic cells

There are several reports on the isolation of dendritic cells from human tonsillar tissue [63, 149, 227, 317]. These cells had the features described above. Purified populations of tonsillar dendritic cells expressed the following markers: HLA-A, -B, -C; HLA-DR, -DP, -DQ; CD4 (Leu3 only), CD11a, CD13, CD18, CD39, CDw40, CD45 and CD45R and lacked epitopes characteristic of T cells, B cells, macrophages and NK cells.

1.2.3.3.3 Synovial dendritic cells

Dendritic cells have been isolated from the synovial fluid of patients suffering from inflammatory joint diseases (in particular, rheumatoid arthritis) [147, 478, 490, 491, 493, 494, 495, 496, 522, reviewed in 492] and, in lesser numbers, from joints affected by non-inflammatory arthritis [147, 478]. These cells had features characteristic of lymphoid dendritic cells. They expressed high levels of Class II MHC antigens (HLA-DR, -DP and -DQ), even after 3-5 days in culture, bore the common leucocyte antigen (CD45) indicating bone-marrow origin [494], and had the typical features of dendritic cells, including characteristic veils at electron microscopy [478, 493, 496]. Functional studies demonstrated the ability of dendritic cell-enriched populations to stimulate oxidative mitogenesis

[522], antigen- [495] and mitogen- [478, 493, 496] induced proliferation of autologous peripheral blood lymphocytes, and both the syngeneic and allogeneic MLR [147, 494, 496]. As has been demonstrated in the mouse and the rat, the incubation of synovial dendritic cells with autologous T cells resulted in the formation of dendritic cell-T cell clusters which contained the proliferating cells. The addition of antibodies to the HLA-DR molecule on the dendritic cell or the CD2 molecule on the T cell inhibited the formation of these clusters [494].

Dendritic cells are not phagocytic and are negative for the enzyme markers, peroxidase and NSE [147, 492, 522]. Apart from some question about weak expression of the CR3 complement receptor [494, 522], they do not express cell surface markers found on mononuclear phagocytes, T cells, B cells or NK cells [147, 494, 522].

1.2.3.3.4 Mucosal dendritic cells

Dendritic cells have been identified histologically in many tissues (see above) including human lung [423]. Isolation from rat lung has also been reported [167, 396]. The literature regarding intestinal mucosal dendritic cells is considered in Chapters 4 and 5.

1.2.3.3.5 Functional maturity of tissue dendritic cells

One, as yet, unresolved question relates to the functional maturity of the tissue dendritic cell in comparison to lymphoid dendritic cells (splenic, lymph node or veiled cell). Does the tissue dendritic cell, like the Langerhans cell, need to contact antigen and migrate to draining lymph nodes in order to achieve functional maturity or is the Langerhans cell in a specialized micro-environment? Are tissue dendritic cells as competent as lymphoid dendritic cells?

This question is difficult to answer because of the difficulties involved in obtaining purified populations of dendritic cells from tissues and the possible effects of the isolation process on function. For example, maturation may be induced by cytokines during culture. Apart from evidence presented above relat-

ing to murine Langerhans cells and splenic dendritic cells, the only direct functional comparisons are between murine splenic and Peyer's patch dendritic cells [433, 435], murine splenic or lymph node and thymic dendritic cells [368, 519], rat lymph node and lung dendritic cells [396], rat thymic and lymph node dendritic cells [519], and human peripheral blood and synovial fluid dendritic cells [see above]. (Dendritic cells from the thymus and Peyer's patches should probably be considered as lymphoid-derived, but for the purpose of this discussion will be considered non-lymphoid). (A number of other investigators have indirectly compared dendritic cells from different tissues. For example, liver dendritic cell-enriched populations were found to have equivalent stimulatory activity/cell when compared to splenic dendritic cells [231]; thymic and splenic lymphostromal clusters had equivalent antigen-presenting cell function [255], and thymic dendritic cells were found to have MLR stimulatory activity similar to that which would have been expected from splenic dendritic cells [93]).

The only clear difference in the direct comparisons was that thymic dendritic cells generated lower levels of alloreactive cytotoxic T lymphocytes in vitro than those from the spleen [368]. The significance of these findings is unclear. All other comparisons of function found equivalent dendritic cell activity per cell. The comparisons of peripheral blood and synovial fluid dendritic cells found, if anything, greater activity in peripheral blood dendritic cells (which would be expected to have the less mature precursors) [493, 492], but the use of impure populations makes this finding hard to interpret.

The matter is as yet unresolved. Studies on the archetypal tissue dendritic cell, the Langerhans cell, consistently showed a thirty fold increase in antigen-presenting cell function during several days in culture (Section 1.2.3.2). The evidence on dendritic cells from other tissues is scarce, and will of necessity, have any interpretation clouded by the need to dissociate and culture cells during the enrichment process.

1.2.3.4 Veiled cells

The veiled or non-lymphoid cells of afferent lymphatics are migratory forms of dendritic cells en route from the peripheral tissues to the draining lymph nodes. They are normally found in very small numbers in thoracic duct lymph (0.03%-0.05% [300, 311, 387]). Lymphadenectomy results in increased numbers in the lymph, whilst irradiation causes a higher relative proportion by depleting radiosensitive lymphocytes [300, 387]. By using these methods, the phenotype and function of these cells has been studied in rodents [26, 233, 234, 288, 289, 300, 311, 341, 378, 387, 388, 389] as well as in other species [72, 168].

The reported properties of veiled cells are similar to those of dendritic cells. They are bone-marrow derived, non-phagocytic, low density, and have irregular surface morphology. Various forms including the "classical" veiled cell have been described [234, 311, 378, 387]. They express high levels of Class II MHC surface proteins, and are potent stimulators of various primary and secondary T cell responses [234, 300, 311, 341]. The demonstration of veiled cells bearing bacteria and bacterial antigens in the afferent lymph of orally infected rats suggests a significant role in the transport of antigen [311].

1.2.3.5 The migration patterns of dendritic cells and their relationship to interdigitating cells

Using enriched populations of labelled dendritic cells a number of investigators have examined their migration pattern in vivo [22, 120, 236, 252]. When injected into the footpads of mice or rats, labelled dendritic cells were carried by the lymphatics to the draining lymph nodes [252] where they were identified as interdigitating cells in the paracortex [120]. Studies in nude mice showed that this process was not dependent on the presence of T lymphocytes [120, 236, 252].

When injected intravenously, dendritic cells localized preferentially in the spleen and the liver after brief sequestration in the lungs. They were unable to

enter lymph nodes [120, 252], except by an indirect route through the lymphatics of the liver [120]. The principal site of localization was the spleen and this process depended on the presence of T cells. The dendritic cells were not just "homing" to their tissue of origin since mesenteric lymph node-derived dendritic cells also localized to the spleen [252].

Shortly after injection the dendritic cells were found in the red pulp of the spleen where they were thought to bind to the endothelium in the marginal zones. By 24 hours after injection the majority had migrated to the T cell areas of the white pulp where they may have developed into interdigitating cells. It is likely that these differences in location are reflected by differences in phenotype and possibly even function. Witmer and Steinman (1984) [514] demonstrated staining with an anti-dendritic cell antibody, 33D1, only in the marginal zone of the spleen. Crowley et al (1989) [93] showed that two populations of dendritic cells are present in the spleen: a major, $33D1^+$ population in the marginal zone (which may represent a migratory cell), and a minor (10-20%), $33D1^-$ population corresponding to a fixed, longer-lived interdigitating cell. Rhodes and Agger (1987) [388] also found phenotypic differences between dendritic cells from different locations.

1.2.4 Ontogeny and cell lineage of dendritic cells

There is considerable evidence that the lymphoid dendritic cell, fixed tissue dendritic cell, Langerhans cell, veiled cell and interdigitating cell represent different developmental stages of the same bone marrow-derived precursor and that there are significant differences between this cell lineage and the mononuclear phagocyte.

For example, studies on the appearance of accessory cells in human and rat foetal development consistently indicate an intrinsic developmental heterogeneity. Janossy et al (1986) [197] detected, in human foetal tissue, a major population of accessory cells which expressed typical macrophage surface

markers and were found at sites of accumulation of classical tissue macrophages. These cells did not bear Class II MHC antigens except for a subset present in the gut and the liver. A minor population of Class II MHC-positive accessory cells localized to sites characteristic of dendritic cells. These cells were the first cells in the body to express HLA-DR antigens and constitutively expressed all subsets of Class II MHC antigens by 23 weeks gestational age. Differences between macrophage and dendritic cell lineages were maintained throughout foetal development. Others came to similar conclusions after studying the distribution of non-lymphoid cells in the human [437] and the rat foetal gut [312, 484].

Immunohistochemical studies of dendritic cells and macrophages in adult tissues have also demonstrated distinctive differences in their phenotype and, by implication, their lineage [15, 71, 135, 375, 383, 422], although not all authors would agree [124, 291].

Although it has been suggested that dendritic cells and macrophages are members of the same cell lineage, contradictory findings have been reported. For example, diametrically opposed differentiation pathways have been proposed (dermal macrophages into Langerhans cells [338] and, conversely, veiled accessory cells into macrophages [369]). The finding that injected dendritic cells did not give rise to sinus macrophages, tingible body macrophages or follicular dendritic cells [120] implies, at least, that dendritic cells do not differentiate into macrophages. The in vitro differentiation of macrophages into dendritic cells has yet to be demonstrated convincingly.

Overall, there is little evidence to suggest that these cells are members of the same cell lineage although an understanding of the nature of the relationship between the dendritic cell and the mononuclear phagocyte is of importance. The evidence presented above suggests that dendritic cells differ from the mono-

nuclear phagocyte system in terms of ontogeny, morphology, phenotype, function and behaviour in vitro and in vivo.

1.2.5 The mechanism of action of dendritic cells

1.2.5.1 Antigen processing

Although dendritic cells do not phagocytose particles in vitro there is compelling evidence to suggest that they are able to induce primary T cell responses to both soluble and particulate antigens (See Table 1.4) [eg. 12, 80, 223, 322]. A recent report demonstrated that the ability of a dendritic cell population to present intact exogenous protein antigens is greatest in immature epidermal Langerhans cells, and is inversely related to its ability to stimulate the MLR [401].

Studies of the mechanism of antigen processing have yielded conflicting results. Dendritic cell chloroquine-sensitivity was reported by some authors [12, 80, 144], whereas others [213] could not detect any inhibitory effect. Two factors may explain these differences. Firstly, in the doses used, chloroquine may be toxic [213]. A dose greater than 15 mM in continuous culture [76] or pretreatment with doses of 300 mM [213] has a suppressive effect on T cell proliferation. It is also possible that chloroquine has effects other than on antigen processing, eg. on the function of the Class II MHC molecules [184]. In experiments on dendritic cell-T cell clustering, antigen-independent clustering at 37^{o}C was unaffected by chloroquine. Nor did chloroquine appear to inhibit T cell receptor-antigen-Class II MHC complex interaction, since its presence did not alter accessory-T cell binding at 4^{o}C. However, when any antigen-presenting cell was pretreated with chloroquine, the ability to bind helper T blasts in an antigen-dependent manner was inhibited for both soluble and allo-antigens at 4^{o}C. Proliferation of primed blasts in the secondary MLR was also blocked by pre-exposure to a low dose of chloroquine, whilst primary MLR responses were unaffected [114, 144, 184]. These observations imply a reversible effect of

chloroquine on Class II MHC protein function. The mechanism of any effect has yet to be explored.

There are also differences in the reported efficacy of dendritic cells to process insolubilized protein antigens. T cell proliferative responses to ovalbumin bound to latex beads or methylated bovine serum albumin were similar to controls in one study [213], while in another, proliferation was enhanced by a hapten bound to large, soluble and insoluble antigens (polystyrene beads) [12]. Possible reasons for these differences include the use of populations of dendritic cells at different stages of differentiation, the degree of spontaneous dissociation of the antigens from the carrier and the accessibility of the proteins to the putative "ectoenzyme" system. Clearly, there is a need for further investigation into the method by which dendritic cells process antigen.

To explain synergistic effects between dendritic cells and macrophages, some authors have suggested that dendritic cells present peptide antigens following metabolic degradation by macrophages [144, 213]. In vitro mixing experiments using macrophages and dendritic cells gave variable results - some showed synergy [324], some suppression [242] and others showed no effect [223]. Increased macrophage production of the cytokine, IL-1, may amplify the function of dendritic cells [241], but there are many other factors which may affect their interaction. This area has yet to be systematically analysed.

1.2.5.2 Dendritic cell-T cell cluster formation

One unique feature of dendritic cells is their ability to form clusters with unsensitized antigen-reactive cells in culture [24, 118, 142, 184, 185, 188, 190, reviewed in 21, 442, 446]. These cells may be $CD4^+$, $CD8^+$ [190] or B cells in association with $CD4^+$ cells [188]. Cluster formation precedes, and seems essential for, mitogenesis. In the periodate model (Section 1.1.2.3), Austyn et al (1988) [24] found that clustering between dendritic cells and responding T cells occurred within 2 hours, was associated with T cell release and responsiveness

to IL-2 within 18 hours, and with DNA synthesis at 24 hours. Proliferative activity was confined to the cluster fraction at all times.

Studies on the human MLR demonstrated (allo)-antigen-specificity. Clustered T cells were depleted of reactivity to a third party but responded rapidly to rechallenge with leucocytes from the original donor. The non-clustered cells were depleted in specific reactivity to the original donor but responded normally to a third party [118].

Inaba and Steinman [180, 184, 185] studied the mechanisms of T cell-accessory cell interaction. All Class II MHC-bearing cells studied (dendritic cells, Langerhans cells, B cells, macrophages) interacted with T cells at 4°C or 37°C in an antigen-dependent fashion. The mechanism presumably involved the recognition of the antigen-Class II MHC complex on the accessory cell by the T cell receptor. In contrast, antigen-independent clustering occurred primarily with dendritic cells and was greatest at 37°C. This implies a requirement for some metabolic process rather than a simple receptor-ligand interaction. This property was unique to dendritic cells, was inducible in cultured Langerhans cells and paralleled their ability to induce primary responses in vitro.

Subsequent studies on the nature of dendritic cell-T cell interaction in the primary MLR examined the effect of monoclonal antibodies directed at T cell adhesion molecules (specifically anti-H-2^k, anti-I-A/-E, anti-LFA-1 and anti-CD4, amongst others). No antibody tested interfered with antigen-independent binding [185]. The last three groups of antibodies inhibited proliferation and IL-2 production. Antigen-dependent binding was inhibited by antibodies to Class II MHC products as would be expected. Antibodies to CD4 and LFA-1 did not affect the early steps of cluster formation but seemed to retard blastogenesis (and IL-2 release) and cluster stability, respectively.

Odum et al (1988) [357] examined the effect of monoclonal antibodies directed at various adhesion molecules and T cell antigens in both primary and

secondary responses in the human. In summary, they found that the primary MLR was inhibited by antibodies to CD2, 4, 5, 11a, 11c and Class II MHC antigens, whereas suppression of secondary proliferation occurred only in the presence of anti-CD2, -CD4 and -Class II MHC antibodies.

King and Katz (1989) [227] also used monoclonal antibodies directed against cell surface antigens to study human tonsillar dendritic cell-induced T cell responses. In the oxidative mitogenesis model, antibodies to Class I and Class II MHC antigens, CD2, CD4, CD11a, CD18, LFA-3 and ICAM-1 inhibited proliferation (whereas anti-CD8 and -CD16 antibodies did not). Dendritic cell-T cell clustering was inhibited by anti-CD2, -CD11a, -CD18, -LFA-3 and ICAM-1 whereas antibodies to Class I and Class II MHC antigens and CD4 had no effect. Antibodies against the CD45 molecule did not affect cluster formation but did weaken their stability, suggesting a role in cell-cell adhesion. With these and other experimental data, some conclusions about the role of the intercellular adhesion molecules in cluster formation and the nature of the antigen-independent interaction may be drawn.

The observed effect of anti-CD4 antibody implies a role in signal transduction, either directly or in concert with the CD3-T cell receptor complex, as well as in intercellular adhesion. Further support for such a role is provided by the association of CD4 with a cytoplasmic protein tyrosine kinase [339].

LFA-1-ICAM interactions contributed to the stability of the clusters without affecting initial binding, since after treatment with anti-LFA-1_{α}, aggregates were easily disrupted and did not reassemble [185]. The effect on proliferation was variable [Table II, 185] and probably depends on many factors including the dosage and the time of administration. Others [483] showed that the binding of ligand to LFA-1 in the human resulted in the transduction of regulatory signals across the plasma membrane (anti-LFA-1_{α} antibodies enhanced proliferation whilst those directed against LFA-1_{β} inhibited proliferation).

CD2/LFA3 adhesion [reviewed in 39] also plays an important role in the stabilization of dendritic cell-T cell clusters. However, it is unlikely to account for the antigen-independent dendritic cell-T cell clustering because the ligand, LFA-3 is not unique to the dendritic cell and the interaction is operative in the absence of divalent cations and functions at 4°C. VLA interactions (very late antigens of activation) [reviewed in 39, 408] are also unlikely to play a role in antigen-independent binding in primary responses because of their widespread distribution and their appearance late in lymphocyte activation. These antigens may play a role in secondary immune responses.

Evidence suggests that antigen-independent dendritic cell-T cell binding precedes antigen recognition [180, 184]. This may be a means of surveying T cells with subsequent responses depending on the interaction between the T cell receptor and the antigen-Class II MHC complex. The mechanism of this interaction is unknown and is an exciting area of future research.

One possible factor is the degree of desialylation of MHC molecules. Dendritic cell-associated MHC molecules carried fewer sialic acid residues than the same molecules from other putative antigen-presenting cells [54]. It was proposed the the low net negative charge at the dendritic cell surface, together with the tendency to form veils may explain the ability to form antigen-independent clusters. Neuraminidase treatment of thioglycollate-elicited macrophages enabled them to stimulate $CD8^+$ (but not $CD4^+$) T cells in the presence of IL-2 [159] and B cell antigen-presenting activity has also been shown to be enhanced by treatment with neuraminidase [126, 159, 224, 248]. The role of sialic acid residues in intercellular reactions is unclear and further work needs to be undertaken in this area. Several groups have proposed that other characteristics of the dendritic cell contribute to its potent antigen-presenting cell activity, for example, the expression of subclasses of Class II MHC antigens subserving different functions [238]. Dendritic cells express the

antigens HLA-DR, -DP and -DQ constitutively [65, 130, 149, 494, 522], but it is unlikely that this factor is critical for accessory cell activity - in spite of high level expression of -DR and -DQ, both B cells [394] and alveolar macrophages [272] are poor stimulators of resting T cells.

As discussed in Section 1.1, the main factor determining the mapping of immune responsiveness to a particular MHC gene is the ability of the MHC molecules to bind antigen. Class II MHC polymorphism in an individual confers survival advantage by minimizing the number of antigens which do not bind and to which the T cell cannot respond. Class II MHC polymorphism in a species confers even greater benefits [406]. For this reason the suggestion that Class II MHC subregion molecules act as restriction elements for the generation of either immune responsiveness (HLA-DR and I-E) or suppression (HLA-DQ and I-A) [158] is unlikely to be proven. Arguments against this proposition are, firstly, that inbred strains of mice which do not express the surface proteins coded for by the I-E locus (HLA-DR equivalent) [301] survive and, secondly, that T cell proliferation [464] and antigen-dependent dendritic cell-T cell binding in the mouse was blocked only in the presence of antibodies to both I-A and I-E determinants [185].

1.2.5.3 Dendritic cells and cytokines

Recent interest in the role of cytokines in inflammatory responses, as well as in haemopoietic cell differentiation, has provided some information as to the possible mechanism of action of dendritic cells. I will review the literature in three areas: the production of lymphokines by T cells activated by dendritic cells; the production of cytokines by dendritic cells themselves; and the effect of cytokines on dendritic cell maturation, differentiation and function.

1.2.5.3.1 Dendritic cell-induced lymphokine production

Dendritic cells activate resting T lymphocytes by a process which results in the release of IL-2 and the expression of IL-2 receptors on the T cell surface

(see Table 1.4 for references). Other lymphokines detectable in both the primary and secondary MLR include IL-4 and other B-cell growth factors [381]. A novel IL-2-enhancing factor produced by co-culturing dendritic cells with periodate-treated splenic T cells has also been described recently [108].

The site of origin of a tissue dendritic cell may influence the particular lymphokines secreted. For example, Peyer's patch dendritic cell-T cell clusters preferentially induce polyclonal IgA responses when mixed with either Peyer's patch or splenic B cells, whilst splenic dendritic cell-T cell clusters do not augment IgA secretion by either splenic or Peyer's patch B cells [434, 435].

1.2.5.3.2 Dendritic cell cytokine production

T cell activation was thought to require the delivery of two signals: the presentation of antigen in conjunction with the products of the Class II MHC gene locus and the production of antigen-nonspecific factors, presumably IL-1 [325]. Although IL-1 is a well documented costimulator, an absolute requirement for IL-1 has not been demonstrated [34, 99, 126]. IL-1 has been shown not to directly influence the proliferation of isolated T cells after mitogen-induced stimulation or in the MLR, even in the presence of allogeneic Class II MHC-bearing cells (peritoneal macrophages) [241]. Similarly, antibodies to IL-1_{α} and IL-1_{β} did not inhibit proliferation in dendritic cell-induced oxidative mitogenesis [King PD and Katz DR, cited in 227] or in the MLR [317].

Evidence suggests that IL-1 mediates its effects on the immune system by amplifying proliferative responses to limiting doses of dendritic cells, which cluster more efficiently with T cells before the onset of mitogenesis [241]. IL-1 is not required to be present continuously in culture since IL-1 pretreatment of dendritic cells generally resulted in greater proliferation [241] which was not inhibited by the addition of anti-IL-1 antibody [442]. Other cytokines have been tested and either had no effect (TNF, IL-2, IL-3) or decreased dendritic cell function (IFN-γ) [241].

IL-1 production by dendritic cells has been examined in a number of different systems. Murine splenic dendritic cells did not produce detectable IL-1 (D10.G4 T cell clone bioassay) or IL-1_α RNA (IL-1_α cDNA probe) in response to a wide range of stimuli (LPS, PMA, IFN-γ, con A, PHA) [240]. Similarly, human dendritic cells did not produce detectable IL-1_α or -$_\beta$ using immunolabelling with both anti-IL-1_α and anti-IL-1_β antibodies [149, 240, 317].

In contrast to these experiments using purified populations of dendritic cells, other investigators have demonstrated IL-1 production in human peripheral blood- or synovial fluid-derived dendritic cells. Enriched populations of synovial dendritic cells were shown to produce IL-1 spontaneously and after stimulation with LPS, when assayed by both the mouse thymocyte proliferation assay and the murine T helper clone (D10.G4.1) proliferation assay [490]. The spontaneous production of an IL-1-like factor (detected by mouse thymocyte proliferation) by cloned, adherent, rheumatoid synovial dendritic cells in long-term culture has also been described [140]. These cells were classified as dendritic cells on the basis of morphology, the absence of phagocytosis and (weak) reactivity with antibody to HLA-DR (which intensified after treatment with IFN-γ). Functional T cell stimulation assays using these cells were not reported. Both these studies have methodological flaws. For example, in the former study [490], contaminating synovial fluid mononuclear phagocytes could have produced the detectable IL-1. To show that IL-1 activity decreased with decreasing ratios of dendritic cells to monocytes, peripheral blood monocytes were used instead of the appropriate control, synovial fluid mononuclear cells. The cells in the second study were not adequately characterized and in fact, were not typical dendritic cells.

Subsequent studies have confirmed by double-labelling with anti-IL-1 and monocyte-specific antibodies that the IL-1-producing cells in rheumatoid

synovial fluid were monocytes and that dendritic cells did not produce IL-1, either constitutively or in response to a number of stimuli [35].

In rodents, two other papers have described IL-1 production. Pereira et al, (1986) [368] demonstrated IL-1 production by both splenic and thymic dendritic cells using the thymocyte stimulation assay. These findings are difficult to reconcile with the data presented above and may be due to the production of another cytokine which is stimulatory in the bioassay or the presence of contaminating macrophages. Nagelkerken and Vriesman (1986) [342] could not detect secreted IL-1 from rat dendritic cells and claim to have demonstrated membrane-associated IL-1-like activity. They showed that dendritic cells stimulated the EL4 murine thymoma cell line by a mechanism which involved cell-cell contact and which did not require metabolically active stimulator cells. Although suggesting that this mechanism involved passively acquired IL-1, they acknowledged that other membrane structures distinct from the secreted form of IL-1 may have been involved in T cell activation. There is no evidence to suggest that other cytokines are produced by dendritic cells.

The evidence suggests therefore that IL-1 is not produced by dendritic cells but that it amplifies dendritic cell function by unknown mechanisms.

A recent paper using the anti-CD3 model of T cell mitogenesis in dual chamber experiments showed that soluble lymphocyte-activating factors were not required for T cell activation [180].

1.2.5.3.3 Effect of cytokines on dendritic cell differentiation and function

IL-1

As discussed above, IL-1, although not produced by splenic dendritic cells, acts upon them to cluster T cells more efficiently, thereby amplifying proliferative responses. IL-1 also enhanced thymocyte proliferative responses to thymic dendritic cell-enriched populations [189]. These responses were not due

to a carry over effect of IL-1 on T cells since pretreatment of dendritic cells resulted in levels of proliferation which were similar to those induced by the continuous presence of IL-1 and which were not blocked by monoclonal antibodies to IL-1.

IL-1 also acted upon thymic precursor cells which were Ia$^-$ and nylon wool-adherent to give rise to typical dendritic cells. Other cytokines (IL-2, IL-3, IL-4 and GM-CSF) had no effect. Similar IL-1-inducible Ia$^-$ precursors were not detectable in bone marrow, spleen or lymph node. Thus IL-1 appears to have an additional effect on dendritic cell precursors (at least in the thymus) which results in their differentiation into functionally competent dendritic cells.

GM-CSF

A series of papers recently examined the relationship between Langerhans cells and dendritic cells [156, 181, 416, 424, 515, 516]. In summary, these papers demonstrated that freshly isolated Langerhans cells were able to present antigen to primed T cells but were weak stimulators of the MLR; over several days in culture, Langerhans cells lost features found in freshly isolated and in vivo Langerhans cells (eg. F4/80 and 2.4G2 (F_C receptor) antigens, nonspecific esterase and ATPase activity and pinocytosis of exogenous horseradish per-oxidase), and at the same time developed morphological and functional characteristics of splenic dendritic cells. This maturation was dependent upon factors present in keratinocyte-conditioned medium, in particular, GM-CSF, whilst IL-1 enhanced this effect.

Other workers [327] showed that GM-CSF augments primary antibody responses by enhancing the function of murine splenic antigen-presenting cells. These cells were obtained by short-term adherence and were a heterogeneous population, so the results are difficult to interpret. GM-CSF did not increase the function of either mature Langerhans cell or splenic dendritic cells [515], but it is possible that immature dendritic cells were present in the splenic antigen-

presenting cell population and that their function was augmented by exposure to GM-CSF. Another recent paper suggests that GM-CSF enhances the MLR initiated by relatively low numbers of dendritic cells, an effect that is inhibited by anti-GM-CSF antiserum [336].

In summary, it is clear that control of dendritic cell function and maturation is, in part, dependent on the release of cytokines in peripheral tissues. The complex cellular and molecular interactions are only just beginning to be unravelled.

1.3 MACROPHAGES AS ANTIGEN-PRESENTING CELLS

The immunological literature is replete with studies demonstrating that macrophages are the predominant antigen-presenting cell in vitro and in vivo. There is no doubt that macrophages can express Class II MHC molecules and can present antigen to sensitized T cells [eg. 179, 182], but a critical appraisal of the many reports consistently fails to establish a role for macrophages as primary T cell activators.

The main experimental flaws have been described above in detail (Section 1.2.2). They include the use of sensitized responder T cells (ie. the assessment of secondary rather than primary responses), the failure to adequately determine the proportion of "contaminating" dendritic cells (and to see primary antigen-presenting cell activity co-purified with dendritic cell or macrophage enrichment) and the use of "nonstringent" responder cell populations.

When antigen-presenting cell activity has been assessed rigorously using either macrophages alone or in comparison with dendritic cell-enriched populations, macrophages have been less potent or suppressive [163, 245, 272, 280, 313, 414, 473]. The results of in vivo studies are similar [64, 281].

1.3.1 The relationship between dendritic cells and macrophages

Macrophages are a heterogeneous group of cells which may vary considerably in size, morphology, phenotype and function [eg. 266, 267, 309, 438]. There are some similarities with dendritic cells in that they are both bone-marrow derived, generally of low density, and dendritic in morphology. The question then arises, are dendritic cells a subset of macrophages? (This question is also considered in Section 1.2.4)

The Langerhans cell is in some ways a hybrid between the macrophage and the dendritic cell. In situ, it expresses the mouse macrophage-specific antigen detected by the F4/80 antibody, as well as the $C3b_i$ and F_c receptors

[400, 416]. In culture, the F4/80 antigen and F_cR are lost, the expression of Class II MHC determinants is increased [516] and the cells more closely resemble the phenotype of dendritic cells. Freshly isolated Langerhans cells are more effective at presenting protein antigens to T cell clones than cultured Langerhans cells, which, in turn, are very potent stimulators of the primary MLR [401]. Thus, the Langerhans cell in situ more closely resembles the macrophage but, in culture or after contact with antigen, develops the characteristics of the dendritic cell.

In contrast, there are very distinct differences between macrophages and dendritic cells. These include the constitutively high level expression of Class II MHC molecules, the ability to form long-term clusters with antigen-specific lymphocytes and to induce lymphokine (IL-2) secretion and responsiveness, and the failure to secrete IL-1 after stimulation with LPS [240].

These differences together with the evidence for a separate cell lineage (discussed in Section 1.2.4) strongly suggest that dendritic cells and macrophages are not different stages in the same differentiation pathway. A more likely explanation is that they are divergent pathways extending from a common precursor.

1.4 B CELLS AS ANTIGEN-PRESENTING CELLS

The B cell possesses many of the properties of a potential antigen-presenting cell. These include the ability to take up antigen by a specific receptor (cell surface immunoglobulin), to process and to display protein antigens in association with Class II MHC molecules, and to release growth factors [reviewed in 1, 18, 20, 84, 319]. A review comparing the properties of dendritic cells and B cells as antigen-presenting cells has been published recently [318].

In the following review, I will present the evidence that activated, but not resting, B cells can initiate in vitro primary T cell responses. Different models have been used in vivo to explore this question, but the balance of evidence suggests no role for resting B cells in the induction of primary T cell responses.

1.4.1 B cell antigen-presenting cell function in vitro

The observation that the interaction between T and B cells was genetically restricted to the I region of the MHC gene complex [296], and the phenomenon of hapten-carrier-linked recognition between T and B cells suggested to Chesnut and Grey (1985) [84] that antigen served as a bridging unit in these interactions. This, in turn, led to the study of the ability of B cells to process and present the hapten-carrier complex to T cells and their capacity to induce antigen-specific T cell responses [83].

1.4.1.1 Resting B cells

The role of resting B cells in inducing primary T cell responses is still debated [18, 19, 20, 138, 198, 397]. Most studies examining B cell antigen-presenting activity have used secondary responses. Studies examining resting B cells in the primary MLR have shown poor stimulation [249, 319] except when Mls determinants were disparate [501]. Particular problems pertain to obtaining pure populations of resting B cells since the isolation procedure (eg. the use of anti-mouse immunoglobulin) may result in the initiation of the activation process. Similarly LPS may be present in media additives [126]. When care is taken to

eliminate activated cells, small resting B cells appear to be inefficient accessory cells [250, 319].

1.4.1.2 Activated B cells

There is experimental evidence [126, 249, 250, 319, 501] indicating that the ability to stimulate T cells in primary responses depends on the state of activation (and hence size or density) of the B cell. Greatest stimulatory activity was seen in large, low-density, LPS- or anti-immunoglobulin stimulated B cell blasts whereas small, dense, resting B cells were non-stimulatory or, at best, weak stimulators. The state of activation also determined the radiosensitivity of antigen-presenting cell function. For example, exposure to LPS resulted in both increased activation and increased radioresistance [84, 319]. In contrast, the weak stimulation of resting B cells was radiosensitive [19, 84, 126, 319].

A number of differences in the properties of resting B cells and B cell blasts may account for their varying capacity to stimulate T cells. For example, cluster formation is not seen when resting B cells are mixed with allogeneic T cells in the primary MLR [118, 182, 319]. B cell blasts do form clusters and once bound, are able to subserve the accessory functions required for T cell proliferation. This property is critically dependent on the LFA-1 molecule [319].

Other mechanisms by which activated B cells may be more active inducers of T cell responses are reviewed in Chesnut and Grey (1985) [84] and include:

a) the uptake of antigen

Resting B cells are less efficient in their capacity to take up antigen either by fluid-phase pinocytosis or by adsorptive binding when compared to activated B cells [84, 88]. These differences however, do not explain the inability of resting B cells to stimulate T cells in either the MLR or the rabbit anti-mouse immunoglobulin model.

b) antigen processing

Both resting and activated B cells degrade protein antigens effectively, so this is unlikely to account for their different stimulatory activity [84, 138].

c) density of Class II MHC molecules

Class II MHC molecule density increases during activation of B cells and then gradually falls to levels found in resting B cells. There is evidence to suggest that Ia density is not a critical factor in the antigen-presenting cell activity of activated B cells so this mechanism is unlikely to account for the observed differences.

At present the major difference between resting and activated B cells in terms of T cell activation is the ability of B cell blasts to form clusters with T cells.

1.4.1.3 Differences between B cell blasts and dendritic cells

B cell blasts differ from dendritic cells in being less efficient at forming clusters and by being more dependent on the LFA-1 molecule for initial cell-cell adhesion [319]. Another important difference in the mouse is the expression of the mixed lymphocyte stimulatory (Mls) determinants [195, 395]. These antigens were first described because of their ability to stimulate MHC-identical lymphocytes to proliferate in vitro. In certain combinations, disparities at the Mls locus (loci) stimulate 20% of T cells in primary mixed leucocyte reactions (compared to <3% in allogeneic-MHC interactions [445]). The nature of the gene products has not yet been determined. There is evidence for both a direct interaction of these products with the MHC antigens [210, 286] and as accessory molecules expressed on antigen-presenting cells (with a complementary T cell Mls-reactive molecule distinct from the T cell receptor) [502]. B cells differ from both dendritic cells and macrophages in expressing these determinants [319, 502].

1.4.2 In vivo studies of B cell antigen-presenting cell function

1.4.2.1 The anti-μ-treated mouse model

One model for the study of B cell antigen-presenting cell function in vivo is the anti-μ-treated mouse model [196, 253, 403, reviewed in 18]. Mice given repeated injections of rabbit or goat anti-mouse μ-chain antibodies from birth showed an almost total lack of Ig^+ B cells whilst T cell numbers and responses to lectins appeared normal. To study T cell sensitization (and, indirectly, antigen-presenting cell activity), lymph nodes or splenic cells from immunized mice were assayed for their secondary proliferative responses when cultured in the presence of antigen, and for their ability to act as helper cells in plaque-forming cell assays. After immunization, the proliferation of antigen-stimulated lymph node (but not spleen) T cells in anti-μ-treated mice was impaired [196] unless the animals were reconstituted with B cells before immunization [403]. Although initially reported as suggesting that B cells were the initiating antigen-presenting cell in peripheral lymph nodes [196], subsequent experiments suggested that the T cells had been sensitized but had not been clonally expanded. Ron and Sprent (1987) [403] argued that unprimed T cells were sensitized to antigen by dendritic cells in lymph nodes, but that as dendritic cell numbers became limiting, the interaction between antigen-specific T cells and B cells resulted in clonal expansion. When lymph node T cells were examined for secondary proliferative responses in B cell deficient mice, the proportion of sensitized cells would be so low that no proliferation would be detectable. This hypothesis was supported by the findings of significant T helper cell function from primed anti-μ-treated or irradiated mice [403].

Kurt-Jones et al (1988) [253] used the anti-μ-treated mouse model with the adoptive transfer of antigen-specific B cells to show that T cell "priming" only occurred when transferred B cells were specific for the immunizing hapten-carrier conjugate. Using parental to F_1 cell transfer, they also showed that

"primed" T cells were restricted to Class II MHC recognition of the donor strain if memory B cells were used, but to both host and donor strain if activated B cells were used. It was argued that in the latter situation, anti-hapten antibody enhanced presentation of antigen by host non-B cell antigen-presenting cells, whereas in the former experiments, the transferred B cells functioned as antigen-presenting cells in vivo. These experiments also failed to differentiate between sensitization and clonal expansion and may be consistent with the hypothesis stated above. Thus, the main conflict is whether the B cells are the sensitizing antigen-presenting cell or whether they contribute to the clonal expansion of sensitized lymphocytes.

1.4.2.2 The chicken chimera model

Chickens treated neonatally with cyclophosphamide will generate their own T cells and antigen-presenting cells but will not develop B cells because they no longer have stem cells capable of homing to the bursa [261]. Donor B cell precursors can reconstitute a mature B cell compartment with resultant tolerance to the donor alloantigens. When host and donor strains expressed different Class II MHC determinants, the antibody responses of these animals to T-independent antigens was normal, showing that they had functional B cells, yet their T-dependent responses were greatly impaired. These data suggested a breakdown in T-B cell communication in the allogeneic chimeras. The antibody responses were restored when donor strain splenic antigen-presenting cells were injected simultaneously with antigen, whereas host strain antigen-presenting cells had little effect. Thus the defect in the chimeras was not a lack of T cells able to recognize antigen associated with donor MHC. Nor was the defect due to the inability of the B cells to display antigen and receive T cell help. The defect was a lack of donor antigen-presenting cells capable of providing the triggering signals to activate virgin T cells. Donor strain splenocytes were effective whereas donor strain B cells were not. Bottomly and Janeway (1989)

[59] subsequently argued that these experiments demonstrated the failure of B cells to prime the subset of T cells responsible for providing help for B cell antibody production (T_{h2}). They reasoned that the experiments did not demonstrate a failure to prime the $CD4^+$ T cells which proliferate in response to antigen and which actively suppress B cell responses (T_{h1}).

1.4.2.3 Other models

Macatonia et al (1987) [282] showed that B cells which expressed antigen after skin painting failed to transfer sensitization for delayed-type hypersensitivity. Dendritic cells possess this property [239] and are able to stimulate T cells in vitro [282].

1.4.3 Overview

The in vitro data suggest that activated B cells are capable of initiating primary immune responses and that any activity of resting B cells is attributable to "contaminating" activated B cells, or B cells activated during the isolation procedure. Clearly then, B cells must be activated by some mechanism before they can sensitize resting T cells. This precludes an initiating role for B cells in antigen presentation.

The in vivo data is controversial. The evidence from the anti-μ-treated mouse model is consistent with a priming role for B cells as well as a function as a clonal expander of T cells. Unfortunately, the function of dendritic cells has not yet been examined in this system, so indirect effects of the anti-μ-treatment on dendritic cells as well as other explanations may account for the observed differences.

The elegant and more biologically "clean", chicken chimera model suggests that B cells are not able to turn on virgin T cells. However not all the properties of different subsets of T cells were examined so further experiments need to be undertaken.

In Mother Nature's human experimental model, patients suffering from B cell deficiencies appear only to be susceptible to those infections handled by humoral immune mechanisms. Infections which are normally controlled by cell-mediated immune responses, such as those due to viruses, fungi and intracellular bacteria, do not occur more frequently than would be expected. In a mouse model of immunodeficiency, antigen-presenting cell activity is equivalent to that of normal mice when tested in vitro, in spite of the B cell deficiencies [186]. This suggests that the initiation of T cell responses is unaffected by the B cell deficiency. Another soft argument is teleological. Examination of the immunoglobulin supergene family gives some clues to the evolutionary development of T and B cells. The immunoglobulin heavy chain may have arisen as a duplication of the T cell receptor and may, in fact, be regarded as a secreted T cell receptor. (This would have conferred a survival advantage in that the actions of immunoglobulins would occur at sites distant from, and possibly, inaccessible to the cellular components of the immune system. The requirement for T cell help in the production of antibodies to most antigens is consistent with more recent development of B cells, and possibly, the co-evolution of a subset of T cells concerned with antibody synthesis, isotype switching etc.) The question therefore arises, what turned on T cells before B cells had evolved? Assuming that B cells evolved after T cells, it suggests that at least at one stage in evolution, B cells were not present to initiate T cell responses. Although there are conflicting claims for the role of B cells as antigen-presenting cells in vitro and in vivo, the bulk of evidence points to a secondary role.

1.5 EPITHELIAL CELL ANTIGEN PRESENTATION

Since the initial demonstration of Class II MHC protein expression on guinea pig [512], murine [363] and human [418] small intestinal epithelium, there has been considerable interest in the possible role of such cells in the induction and control of lamina propria and intra-epithelial immune responses [reviewed in 45, 62, 310]. The following discussion will consider the normal and abnormal expression of these molecules on epithelial cells, and their possible role in antigen presentation. It will be argued that aberrant expression of Class II MHC proteins is a response to inflammation rather than being an initiator of it. These molecules may play a role in immune regulation, either positively or negatively, but play a minor role in the induction of primary T cell responses.

1.5.1 Normal expression of Class II MHC molecules on gut epithelium

Class II MHC surface antigens are either not detectable [312, 437, 484] or expressed weakly [358] on the gastrointestinal epithelium during foetal development. After birth the expression of these molecules increases and reaches adult levels at about one month of age [312, 345]. In some species, increasing expression coincides with weaning [312]. A role for environmental immunological stimuli in the induction of epithelial Class II MHC expression is also suggested by the absence of these determinants in germ-free animals [79], and in foetal gut grafts placed under the renal capsule [312]. Expression is also temporally related to the ability to induce oral tolerance [45].

In adult animals of most species studied, the pattern of distribution is similar. The Class II determinants are expressed by small intestinal villous epithelium [418, 420, 436], and follicle-associated epithelium [42]. The molecules are present on the distal two-thirds of the villi, whilst the crypt epithelium normally does not have detectable levels [418], unless directly adjacent to lymphoid nodules [436]. Both immunofluorescence and electron

demonstrated expression along the basolateral membrane and on the microvilli of the apical surface [157, 418], as well as intra-cytoplasmic staining.

The epithelia of the stomach and the colon are generally negative [110, 157, 418, 420, 421, 314], again with the exception of cells directly associated with lymphoid tissue in the human [436].

1.5.2 Function of epithelial Class II MHC determinants

The pattern of staining of Class II MHC molecules on the intracellular granules of mature absorptive cells of the villus in the small intestine was observed to be similar to that of absorbed protein in the gut [312]. These similarities suggested that Ia molecules were involved in the normal pinocytic uptake and transport of macromolecules across the epithelial cell barrier in the phagolysosome system. Specialized molecules with structural similarities to Class I MHC molecules mediate the uptake of IgG from maternal milk [427] and are involved in the transepithelial transportation of immunoglobulins (Table 1.1). If Class II antigens did play a role in the absorption of luminal macromolecules, polymorphism and regional variations in expression could result in differences in their handling. These, in turn, may confer susceptibility to hypersensitivity diseases involving the gastrointestinal tract [312]. The recent elucidation of the structure of the Class I MHC molecule, HLA-A2 [44], makes it unlikely that the Class II MHC molecules (which probably have a similar antigen-binding site [66]) play a role in the transportation of macromolecules. However, it is still possible that epithelial cells mediate genetically-restricted Class II-dependent transportation and presentation of luminal antigens to intra-epithelial lymphocytes or the cells of the lamina propria [45, 62].

1.5.3 Abnormal expression of Class II MHC molecules

A large number of natural and experimental stimuli result in increased levels or de novo expression of Class II MHC determinants in the gut epithelium. Examples include graft versus host disease [25, 299], graft versus host reaction

[145], Trichinella spiralis-induced inflammation [25], topical application of a contact allergan [200], coeliac disease [16, 419], inflammatory bowel disease [110, 314, 374, 421] and infections eg. Campylobacter pylori gastritis [362]. The expression of Class II MHC appears to be mediated by IFN-γ [79] and may be modulated by intra-epithelial lymphocytes [79, 419].

Similar observations have been made in many other epithelia. For example, keratinocyte Class II MHC expression is induced by graft versus host disease [25, 32, 299], by the topical application of contact sensitizing agents [200], and by infections [472]. There are many more examples of aberrant Class II MHC antigen expression in the literature.

1.5.4 Expression of Class II MHC subregion products

In human foetal gut epithium, HLA-DR antigens may be expressed weakly, if at all. There is no evidence however, that the other subregion products (-DP or -DQ) are expressed [358]. In contrast to the universal presence of HLA-DR in histologically normal adult gut epithelium, HLA-DP is seen rarely, and -DQ is almost never seen [419]. In gluten-sensitive enteropathy, there is increased expression of the subregion products in the order -DR> -DP> -DQ, with -DQ expression only occurring in patients who have a high epithelial density of T cells.

1.5.5 Functional significance of aberrant Class II MHC antigen expression

The interest in aberrant expression of Class II MHC has arisen because of the theoretical possibility that epithelia expressing these molecules could act as "non-professional" antigen-presenting cells. The immune responses initiated in this manner may be normal and result in the elimination of a luminal pathogen or result in systemic hyporesponsiveness ie. the induction of oral tolerance, for example, by T_S cell induction. Alternatively, the responses may be abnormal and

result in uncontrolled reactions and disease eg. inflammatory bowel disease or auto-immune disease [58, 146, 193].

There is a paucity of information on the functional role of aberrant Class II MHC molecules on the intestinal epithelial cell, so I will first examine the evidence for a role of the epithelial cell in auto-immune disease.

1.5.5.1 Thyroid auto-immune disease

Hanafusa et al (1983) [146] observed that thyroid epithelial cells from thyroidectomy specimens of patients suffering from auto-immune disease had aberrant expression of HLA-DR antigens. A hypothesis about the induction of endocrine auto-immunity was then proposed [58]: an unidentified environmental stimulus (eg. a viral infection with resultant IFN-γ production) would induce epithelial HLA-DR expression. These molecules would then present auto-antigens (eg. hormone receptors) to the immune system with subsequent activation of auto-reactive effector T and B cells. Other factors such as abnormalities of T_S pathways, may also be permissive in the development of overt auto-immune disease. This hypothesis was supported by observations that Class II MHC-bearing thyroid epithelial cells presented viral peptide antigens (but not intact virus) to antigen-specific cloned human T cells [277]. Furthermore, clones of T cells derived from thyroid glands of patients with Graves' disease were auto-reactive [276].

A number of factors, however, suggested that the aberrant expression was a secondary phenomenon related to the release of IFN-γ by T cells. Although the initial proposition was that lectins such as PHA, Con A or pokeweed mitogen (PWM) could induce Class II MHC expression on thyrocytes directly [379], it became apparent that this effect was mediated by IFN-γ [102, 193, 503]. Furthermore, in contrast to initial reports [146], in vivo thyroid follicular cell Class II MHC antigen expression was always associated with lymphocytic infiltration [143, 503]. Finally, aberrant expression was not an exclusive property

of thyrocytes in auto-immune disease, but could be observed in other diseases [143]. Regardless of the underlying disease, the antigen-presenting cell activity of Class II MHC antigen-bearing thyrocytes was similar (assayed in the MLR under non-stringent conditions). This resulted in a modification of the original hypothesis: that Class II MHC expression was an essential but insufficient factor for the induction of auto-immunity [143]. Since cultured intra-thyroidal T lymphocytes proliferated specifically in response to autologous Class II MHC$^+$ thyroid follicular cells only in Graves' disease, it was postulated that auto-antigen presentation occurred only in auto-immune disease. The nature of the other necessary components is unclear.

Other investigators have demonstrated weak autologous MLR stimulatory activity [96] and weak antigen-presenting activity [103] of Class II MHC$^+$ thyroid cells or thyroid cell lines [160]. However it is possible that these results were due to contaminating cells in either the stimulating or responding cell populations. For example, Davies (1985) [96] induced Class II MHC expression on thyroid cell monolayers with the lectin, PHA, implying at least a significant T cell contamination. Dendritic cells have been demonstrated histologically in the thyroid [203] and may be the cell responsible for the observed stimulation. Subsequent work from the same group showed that a cloned rat thyrocyte line was able to stimulate the syngeneic MLR [160]. In these experiments there was considerable background proliferation in the responder cell populations raising some questions about methodology.

In contrast to the work on human thyroid follicular cells, workers using murine cells could not demonstrate antigen presentation in vitro (testing both antigen-specific responses and the allogeneic MLR), in spite of the IFN-γ-induced expression of Class II MHC surface proteins [102]. A murine thyroid epithelial cell line, which had been induced to express Ia antigens with IFN-γ, was also ineffective in stimulating a primary allogeneic MLR in the absence of a

co-stimulatory signal (which was PMA and not IL-1) [440]. Other researchers have suggested that the role of Class II MHC expression on non-lymphoid cells may be to induce tolerance against self-antigens and that auto-immune disease is the result of an antigen-specific T_S cell defect [193, 194, 258]. Alternatively, it is possible that enhanced expression of Class II MHC antigens in auto-immune disease merely reflects the involvement of effector T cells in tissue pathology [329] and has no pathogenic role.

1.5.5.2 Histological studies

Further evidence against a role for aberrant expression on non-lymphoid cells in the induction of immune responses is provided by histological studies examining the time course of Class II MHC expression in various disease states. For example, in cutaneous graft versus host disease [32] and gut graft versus host reaction [145], there was no evidence to suggest that the induction of Class II MHC antigens triggered the target tissue phase of the disease. The data suggest that the HLA-DR expression was the result of the infiltrates. Similarly, in skin biopsies from patients suffering from alopecia areata, an organ-specific disorder thought to involve auto-immune mechanisms, lymphocyte and macrophage infiltration consistently preceded ectopic HLA-DR expression on keratinocytes [226]. In addition in the human colon, epithelial expression of Class II MHC antigens was a non-specific response to inflammation and only occurred in association with lymphoid infiltrates in both infective colitis and inflammatory bowel disease [314].

1.5.5.3 Studies in transgenic mice

A recent paper from Markman et al (1988) [295] provides further evidence against a role for epithelial cells in antigen presentation. In a transgenic mouse model, beta cells of the pancreatic islets expressed Class II MHC molecules (I-E) whilst bone marrow-derived cells did not. Foetal pancreatic grafts from $I\text{-}E^+$ transgenic mice into naive $I\text{-}E^-$ mice were accepted indefinitely,

indicating that the I-E$^+$ beta cells were incapable of initiating an immune response. If the recipients were primed with splenic cells from wild-type I-E$^+$ mice, the grafts from the I-E$^+$ transgenic mice were rejected, whilst their I-E$^-$ littermates survived. This showed that the I-E proteins could be recognized by the host immune cells when presented by effective antigen-presenting cells.

In vitro the I-E$^+$ beta cells were unable to stimulate T cells reactive to I-E plus a peptide antigen. In fact, antigen-specific unresponsiveness was induced. These observations suggested that the expression of Class II MHC antigens on non-lymphoid cells played a regulatory role and was involved in the maintenance of self-tolerance. T cell activation would be induced if other factors overcame the T cell paralysis.

More recent papers [50, 274] also cast doubt on the Bottazzo-Feldmann hypothesis of autoimmunity. These authors used transgenic mice expressing I-A or I-E, on pancreatic islet or acinar cells respectively, to show that the expression of Class II MHC antigens did not independently trigger an auto-immune attack. In contrast to Markham et al (1988) [295] and Lo et al 1989 [274], Boehme et al (1989) [50] did not demonstrate tolerance to the "allogeneic" Class II molecules.

1.5.6 In vitro studies of gut epithelial cell antigen presentation

Considering the interest in the expression of Class II MHC molecules on intestinal and other epithelia and their putative function in vivo, there are surprisingly few reports examining intestinal epithelial cell antigen presentation [48, 49, 308, 431]. Bland (1988) [45] attributes this to methodological problems, namely that enterocytes are friable, terminally differentiated, highly polarized and do not survive in culture for more than a few hours.

Bland and Warren (1986) [48] showed that isolated rat enterocytes in continuous culture with sensitized lymph node-derived T cells did not stimulate proliferation in the presence of the specific antigen. They proposed that epithelial

cells activated antigen-specific T_S cells or other suppressive factors which inhibited proliferation. By using discontinuous cultures (ie. by removing the presumably dead enterocytes after 18 h), antigen-specific T cell proliferation was demonstrated. Anti-Ia anti-sera inhibited these secondary responses. Significant proliferation in the absence of stimulator epithelial cells indicated "contamination" of the responder cell population. Subsequent experiments demonstrated that enterocytes mediated suppression by antigen-dependent and -independent mechanisms. Antigen-specific inhibition was conferred by the induction of $CD8^+$ cells and was also blocked with antibodies to Class II MHC antigens. The origin of these cells was not determined.

The authors suggested that epithelial cell presentation of antigen to the intra-epithelial lymphocyte was a possible mechanism of oral tolerance induction. Furthermore, increased expression of epithelial cell Ia antigens during inflammation would result in aberrant presentation of enterocyte antigens and the subsequent generation of cytotoxic activity.

Mayer and Schlien (1987) [308], using freshly isolated human colonic epithelial cells induced both primary (allogeneic MLR) and secondary (antigen-specific) activation in responder lymphocytes. These results are surprising given the failure of other investigators to demonstrate Class II MHC expression on the normal colonic epithelial cell (see Section 1.5.1).

In further experiments, a colonic adenocarcinoma cell line (DLD1) stimulated a primary MLR following the induction of Class II MHC molecules by IFN-γ. Like Bland and Warren (1986) [48], they demonstrated preferential stimulation of $CD8^+$ T_S lymphocytes but these cells inhibited both T and B cell responses in an antigen-non-specific manner. Of even more interest was the observation that colonic epithelial cells obtained from patients suffering from inflammatory bowel disease (and with increased expression of HLA-DR, -DP and -DQ) preferentially

stimulated $CD4^+$ cells with the implication that the enterocyte played a role in immunoregulation [307].

Other workers using a colonic carcinoma cell line (HT-29) after inducing HLA-DR (-DP and -DQ), could not detect MLR stimulatory activity [431]. It is not clear whether these conflicting results are a manifestation of the properties of different cell lines or whether they represent methodological problems.

In any case, the arguments that Class II MHC antigen expression on the small intestinal epithelial cell plays a role in oral tolerance induction or, alternatively, in the initiation of T cell-mediated immune responses fail to explain the following observations:

(i) oral tolerance can be induced by intrarectal instillation of antigen [S. Strober, PhD Thesis, cited 330].

(ii) T cell-mediated immune responses directed against gluten in patients suffering from coeliac disease can also be elicited by intrarectal administration of the appropriate antigen [275]. Neither the colonic nor the rectal epithelial cell expresses Class II MHC antigens, so they are apparently not essential for the responses described above.

Arguments that antigen could be presented by the follicle-associated epithelium (FAE) which does express Class II MHC antigens would need to account for the observed interspecies differences, eg. rat FAE does not express Class II MHC [312] and the patchy or absent expression of Class II MHC antigens on M cells [43, 61].

1.5.7 Conclusions

What then is the function of epithelial cell Ia expression? It is appealing to propose immunoregulatory positive or negative feedback loops activated by Class II MHC molecules on epithelial cells. An alternative explanation is that

increased expression of these determinants enhances retention of sensitized T cells at sites of antigen deposition [442]. In secondary immune responses, $CD4^+$ lymphoblasts are responsive to antigens presented with Class II MHC molecules on any cell, so that local induction of Ia focuses even further the effector responses at the inflammatory site.

Intestinal (and other) epithelial cells lie at the interface between self and non-self where they are in an ideal position to regulate immune responses. Supporting evidence for such a role is provided by the detection of Class II MHC surface antigens and the ability to produce IL-1 [46]. The weight of experimental evidence in four separate epithelial systems (thyroid, skin [128], pancreas and gut) mitigates strongly against a role for the epithelial cell in primary T cell activation. A role in secondary immunoregulatory circuits is yet to be proven.

TABLE 1.1

IMMUNOGLOBULIN GENE SUPERFAMILY

[Modified from 511]

Molecule	Ligand or molecular association	Function
Immunoglobulins:	Antigen	Antigen recognition
Heavy chains		
Kappa light chain		
Lambda light chain		
T cell receptor complex:	Antigen/MHC protein complex	Antigen recognition
α chain		
β chain		
γ chain		
δ chain		
CD3:	T cell receptor (TCR)	Signal transduction
γ chain		
δ chain		
ϵ chain		
MHC proteins:	TCR	Antigen presentation
Class I (α chain)	α chain - CD8	
Class II (α, β chains)	- CD4	
β_2-microglobulin	Class I α-chain	

T cell adhesion molecules:		Cell adhesion
CD2	LFA-3	CD2 present on T cells,
LFA-3 (CD58)	CD2	LFA-3 on accessory or target cell
T cell subset markers:		Stabilization of
CD4	MHC Class II	TCR - antigen - MHC
CD8	MHC Class I-α_3 domain	protein complex and signal transduction
Neural molecules:		
Neural adhesion molecule (NCAM)	NCAM (CD56)	Neural cell adhesion
Myelin associated glycoprotein (MAG)		? Myelination
P_O myelin protein		Myelin protein
Intercellular adhesion molecules:		
ICAM-1 (CD54)	Leucocyte function	Adhesion
ICAM-2	antigen (LFA)-1	
β_2-microglobulin-associated-proteins:		
Tl heavy chain	Not known	Not known
Q_a heavy chain		
CD1a heavy chain		
Brain/lymphoid antigens:		
Thy-1	Not known	Not known
MRC OX-2		
Immunoglobulin receptors:		
Poly Ig R	Multimeric IgA, IgM	Epithelial transport
$Fc_{\gamma 2b/\gamma 1}R$	Aggregated IgG	

Tumour antigen:		
Carcinoembryonic antigen (CEA)	Not known	Not known
Growth factor receptors:		
Platelet-derived growth factor (PDGF receptor)	PDGF	Cell division, etc
Colony stimulating factor-1 receptor	CSF-1	Cell division, etc.
Link protein:		Binding molecule between proteoglycan and hyaluronate chain

Vaccinia virus haemagglutinin [201]

Cytomegalovirus proteins [29]

TABLE 1.2

INTEGRIN RECEPTOR SUPERFAMILY

[Modified from 408]

Molecule		Ligand	Function
LFA-1 family:			
Common β chain	(CD18)		
LFA-1 α chain	(CD11a)	ICAM-1,-2	Cell-Cell adhesion
Mac-1	(CD11b)	$C3b_i$	Complement binding
p150,95	(CD11c)		Cell-Cell adhesion
VLA (very late antigens of activation) **family**:			Cell adhesion, phagocytosis
Common β chain	(CD29)		Platelet gpIIa
VLA-1			
-2	(CDw49b)		Adhesion to collagen
-3			
-4	(CDw49d)		
-5			Fibronectin receptor
-6	(CDw49f)		Laminin receptor
Glycoproteins IIb/IIIa		Fibrinogen	Platelet aggregation
		Fibronectin	
		von Willebrand factor	
		Vitronectin	

TABLE 1.3

PHENOTYPE OF MURINE DENDRITIC CELLS

Antibodies	Specificity	Origin of Dendritic Cell					
		Spleen	VC	LN	Thymus	Skin	PP
33D1	DC	S	S	+	-	-	+
TIB120							
B21-2	Class II MHC	+	+	+	+	+	+
10-2.16							
11-5.2							
M1/42	Class I MHC	+			+	+	
NLDC 145	IDC	S	+	+	+	+	+
2D2C	Pgp	+			+	+	
F4/80	F4/80 antigen	+	-	-	-	+	
M1/70 (Mac-1)	$C3b_iR$	+	-	-	-	+	
2.4G2	F_cR	-	-		+	+	

J11d	"B cell"	S			+	+
RA3-3A1/6.1	B cell, B220	-	-	-	-	-
I3/2	Common leucocyte	+				+
53-6.7	CD8	S			+	-
GK1.5 (CD4)						
B5.3 (Thy1)	Other T cell	-	-	-	-	-
30-H12	antigens					
(Thy 1.2)						
S100		+		+	+	+
MAC-2	Macrophage	S	S	S		+
MAC-3	Macrophage	S	S	S		+

[Adapted from References:

93, 244, 319, 355, 388, 400, 402, 405, 416, 433]

Abbreviations:

DC	dendritic cell
IDC	interdigitating cell
VC	veiled cell
LN	lymph node dendritic cell
PP	Peyer's patch dendritic cell
S	Subset of cells

TABLE 1.4

Dendritic Cell Properties	References
Oxidative mitogenesis (periodate)	23, 60, 136, 227, 231, 232, 241, 487, 519
Mitogen-induced T cell proliferation	12, 24, 136, 142, 184, 212, 235, 241, 521
Antigen-induced T cell proliferation	12*, 80, 184, 213, 217, 223*, 322*, 462, 463, 469, 487 (Particulate antigens *)
Syngeneic MLR	142, 149, 178, 231, 241, 251, 353, 384, 485, 487
Allogeneic MLR	12, 142, 149, 179, 182, 185, 190, 231, 237, 241, 251, 259, 317, 319, 343, 381, 384, 412, 413, 444, 451, 463, 485, 487, 519, 521
Formation of T cell clusters	24, 118, 142, 179, 180, 182, 184, 185, 186, 227, 241, 245, 246, 319, 353
Induction of lymphokine release	23, 24, 108, 178, 179, 182, 184, 185, 186, 188, 190, 241, 319, 381, 462
Induction of cytotoxic T lymphocytes	215, 283, 354, 398
Direct activation of $CD8^+$ T cells	52, 53, 190

LEGENDS TO FIGURES

Figure 1.1 Cell surface molecules

Figure 1.1a

Representation of the alignment of complementarity determining regions (CDRs) in a hypothetical T cell receptor (TCR) (top of figure) over a peptide-MHC complex (bottom of figure). This figure demonstrates the complementarity between the two surfaces and the alignment of CDR-1 and CDR-2 (green, top figure) with the helices of the MHC molecule (yellow, bottom figure) and of CDR-3 (pink, top figure) with peptide determinants (pink, bottom figure). [Adapted from 97].

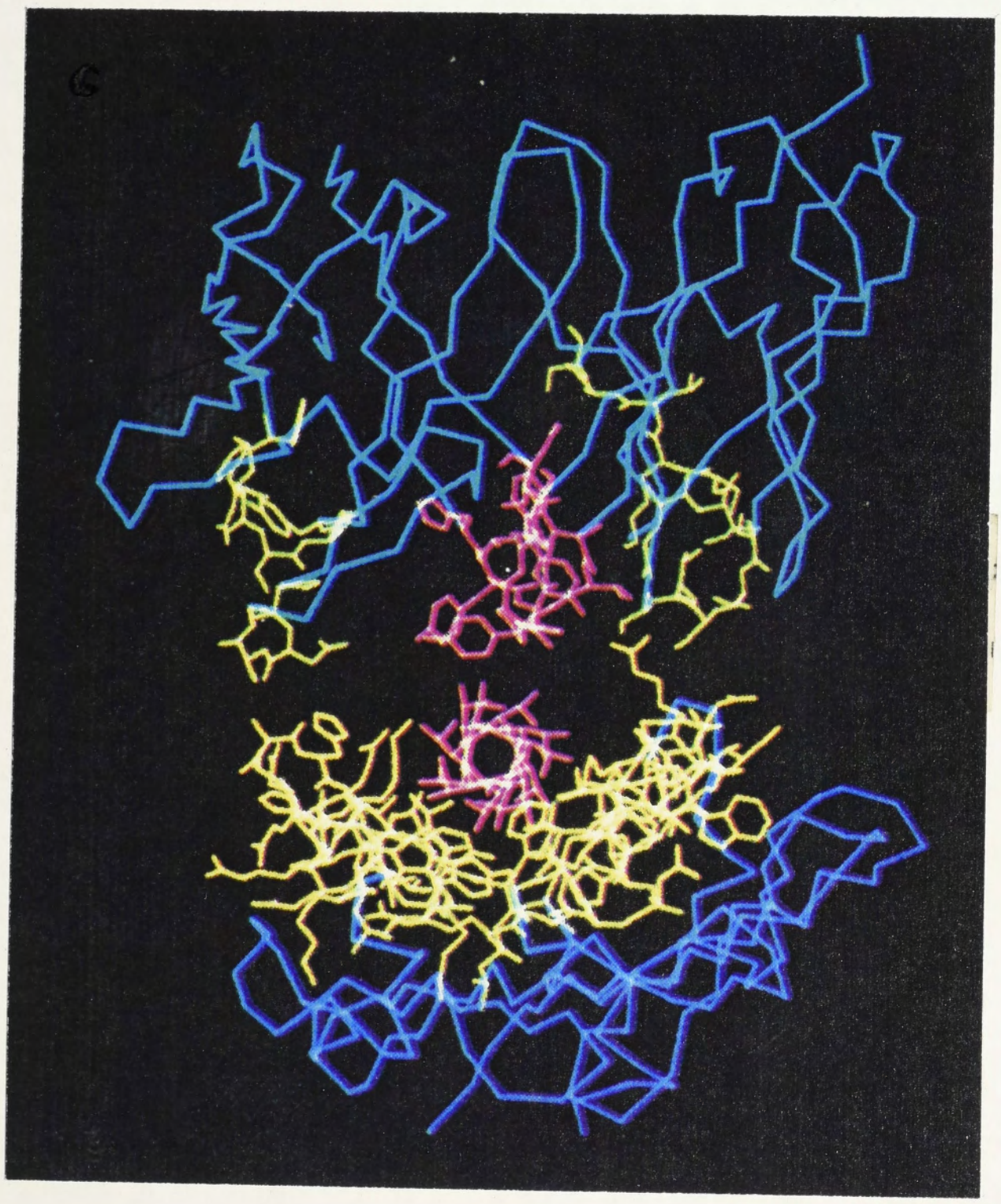

G

Figure 1.1b

Binding of T cell surface molecules to specific ligands on the antigen-presenting cell. The T cell receptor (TCR)-CD3 complex interacts with antigen in association with MHC molecules on the antigen-presenting cell. CD4/8 interact with MHC Class II/I molecules respectively. LFA-1 interacts with ICAM-1 and -2 whilst CD2 interacts with LFA-3. [Modified from 39].

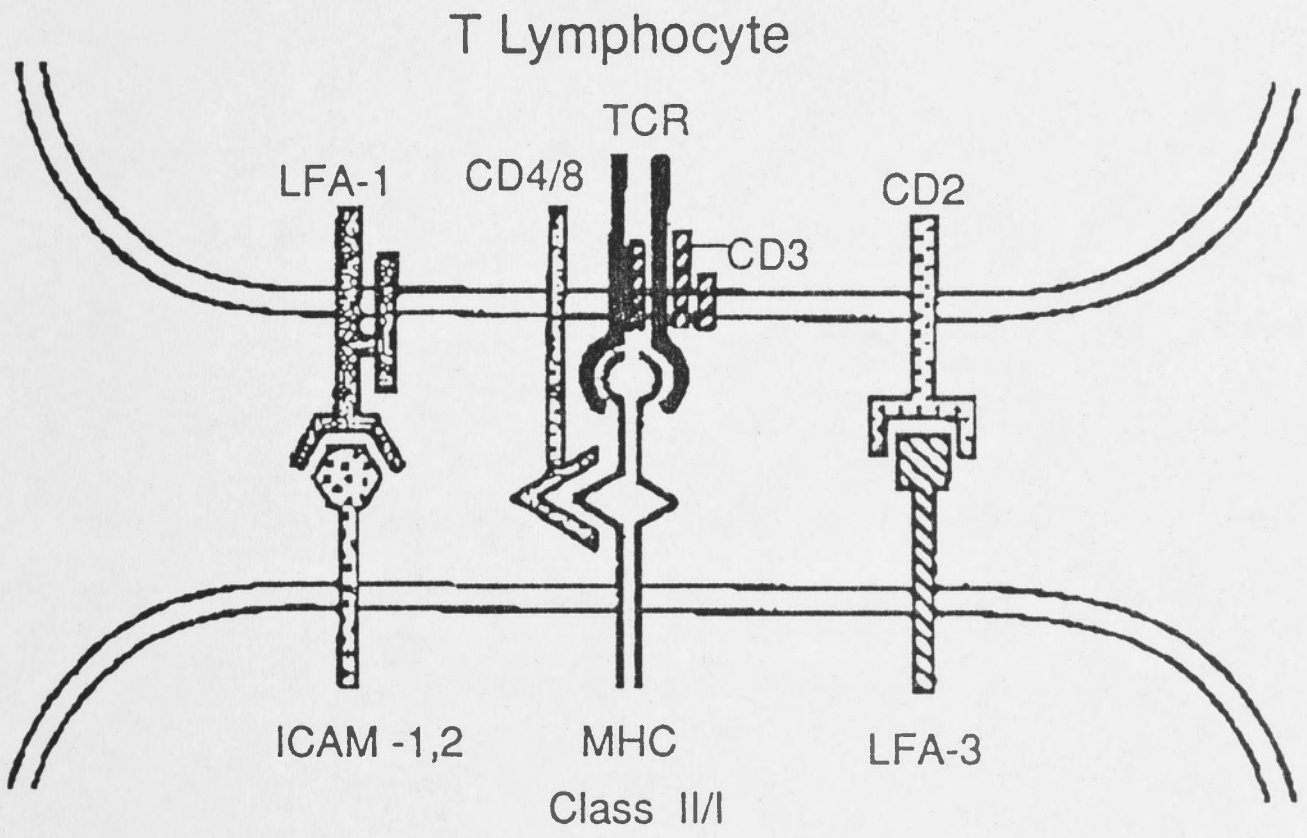
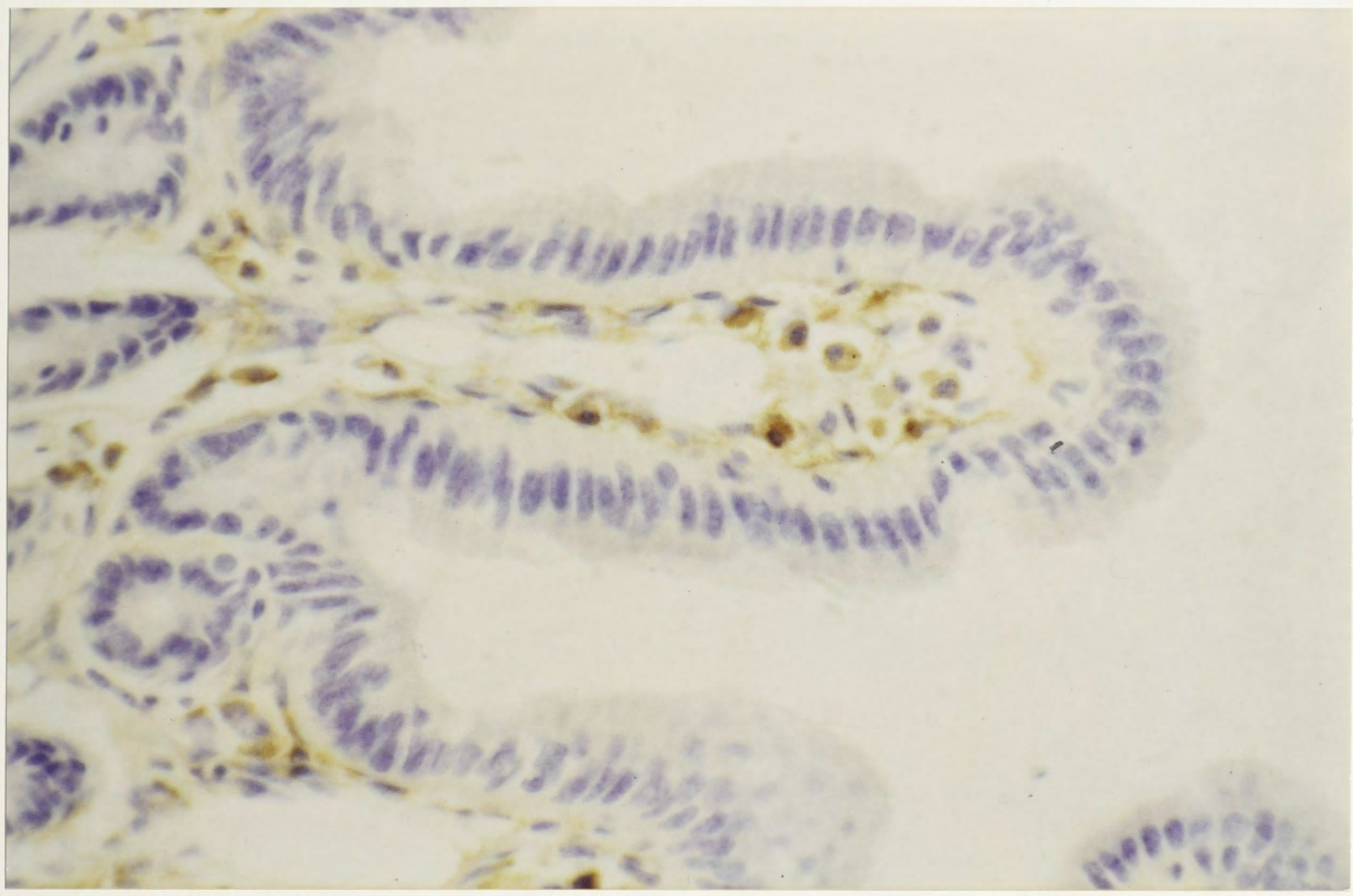

T Lymphocyte
TCR
LFA-1
CD4/8
CD2
CD3
ICAM -1,2
MHC
Class II/I
LFA-3
Antigen-presenting Cell

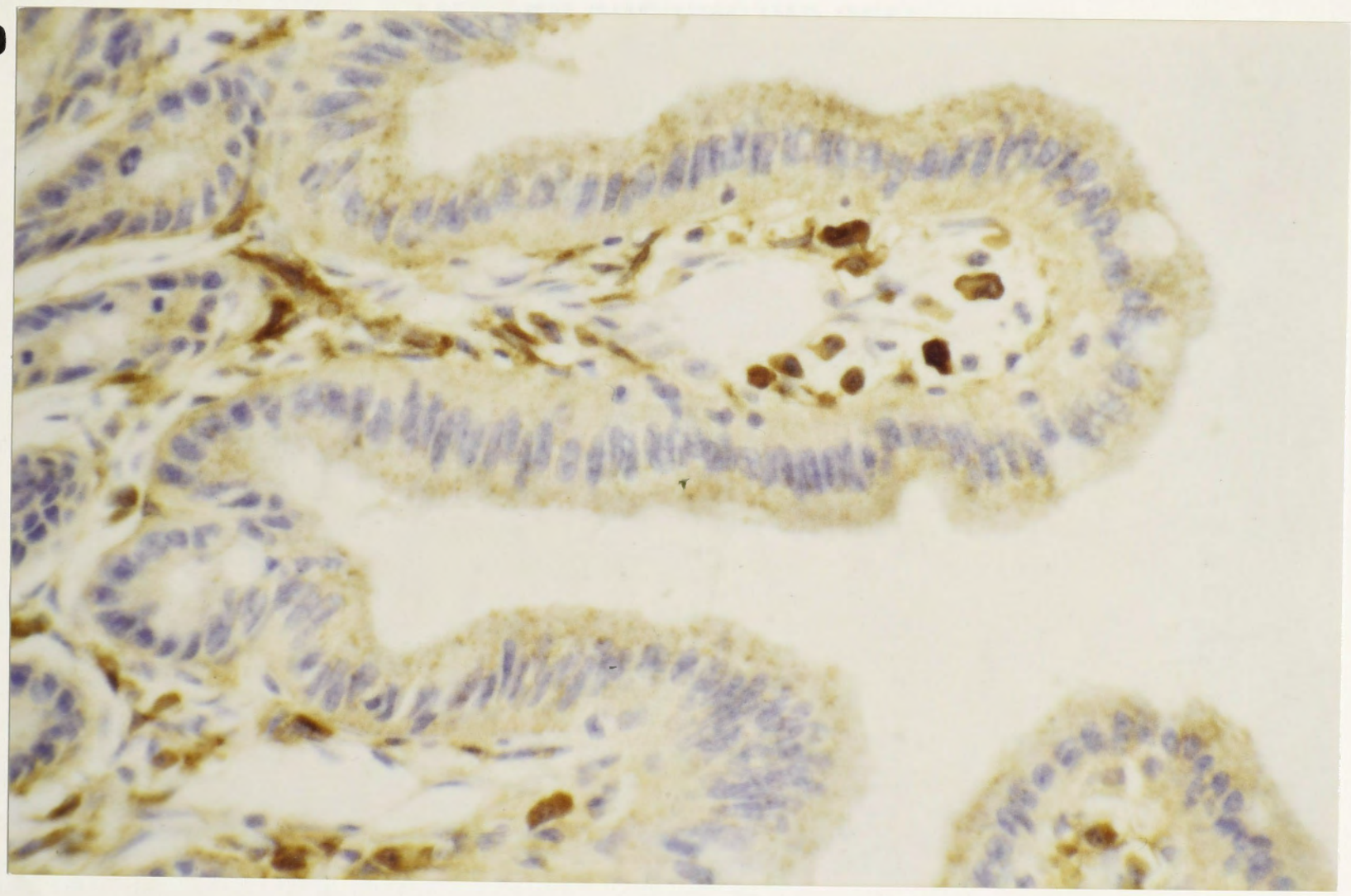
Figure 1.2

Immunoperoxidase staining of parallel sections of murine small intestine using the antibodies: (a) F4/80 (anti-macrophage); and (b) TIB120 (anti-Class II MHC). The methods are described in references 169, 172, 173. In brief, the tissues were fixed in periodate-lysine-paraformaldehyde (2%), embedded in O.C.T. compound and stored at -20°C. Immunoperoxidase staining was carried out using the avidin-biotin-peroxidase (ABC) method with reagents supplied by Vector Laboratories (Burlingame, CA). The slides were prepared by Dr W. Allan. Both antibodies, F4/80 and TIB120, stain the intestinal lamina propria macrophages, whilst TIB120 appears to stain an additional population of irregular intestinal lamina propria cells (presumptive dendritic cells) and the epithelium. Both antibodies are rat IgG_{2b}.

a



b



CHAPTER 2

THE DEVELOPMENT OF THE DISAGGREGATION TECHNIQUE AND PRELIMINARY CHARACTERIZATION OF THE INTESTINAL ANTIGEN-PRESENTING CELL

"Take your hare when it is cased..."

From "*The Art of Cookery*"

Hannah Glasse fl 1747

Usually misquoted as "First catch your hare".

2.1 INTRODUCTION

Prerequisites for the demonstration of antigen-presenting cell activity in disaggregated intestinal lamina propria cell suspensions include the development of the methodology for obtaining representative, viable and functional lamina propria cells and the characterization of an assay system, in this case the allogeneic MLR. This chapter describes the method for disaggregating intestinal lamina propria and outlines the functional characteristics of the MLR.

The disaggregated cell suspension was initially shown to contain cells which could both induce and respond to mitogen-induced T cell activation. The MLR stimulatory activity of the cells was then assessed and found to be comparable to that of splenic cell preparations. Using cell depletion techniques, the MLR stimulatory cell was shown to express Class II MHC surface antigens but did not bear the surface markers or have other characteristics of T cells, B cells or macrophages. Finally, lamina propria cell suspensions were found to have greater antigen-presenting cell activity when directly compared to Peyer's patch cells.

2.2 MATERIALS AND METHODS

2.2.1 Mice

C57BL/6 and BALB/c mice were bred and maintained under specific pathogen free (SPF) conditions in the Animal Breeding Establishment of the John Curtin School of Medical Research, Australian National University or were purchased from the Animal Production Area of the NCI-Frederick Cancer Research Facility, Frederick, MD and maintained specific pathogen free at the Department of Cell Biology, M.D.Anderson Hospital and Tumor Institute, Houston, TX. Mice of both sexes aged between 8-16 weeks were used.

2.2.2 Media

Calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS), supplemented with penicillin (100 U/ml) and gentamycin (60 U/ml) was used for isolation of cells from the lamina propria. Where indicated 0.75 mM EDTA (Fluka, Buchs, Switzerland) was added. RPMI 1640 was supplemented with 2 mM L-glutamine (Sigma, St Louis, MO), penicillin (100 U/ml), gentamycin (60 U/ml) and 10% heat-inactivated foetal calf serum (FCS) (Flow Laboratories, Melbourne, Victoria). For cell culture experiments, 0.01 mM 2-mercaptoethanol (2ME) (BDH Chemicals Pty Ltd, Sydney, NSW) was added.

2.2.3 Mouse tissues

The small intestine or colon was dissected from C57BL/6 mice. The luminal contents were expressed, and where indicated the Peyer's patches were removed under a dissecting microscope. The intestine was split lengthwise and then cut into 1 cm segments. After brief washing in CMF-HBSS to remove remaining luminal contents, the intestines were placed in a Wheaton stirring flask and washed gently for 2-2.1/2 h in CMF-HBSS with EDTA at 37°C. During this time, the medium was changed every 10-15 min until no increase in particulate matter was visible in the supernatant. Finally, after a 5 min wash in RPMI, the tissue was finely chopped, weighed and added to an enzyme cocktail containing

1.2 U/ml Dispase II (Boehringer-Mannheim, Tutzing, FRG), 10 U/ml collagenase CLSPA (Cooper Biomedical, Malvern, PA) and 5 U/ml DNase Type II (Calbiochem, Behring Diagnostics, La Jolla, CA) in glass Petri dishes siliconized with Coatasil (Ajax Chemicals, Sydney, NSW). Maximal yields were obtained when <500 mg tissue was digested with 20 ml enzyme mixture. After 2-3 h incubation at 37°C under 5% CO_2 with occasional gentle agitation, the digested tissue was mechanically disrupted by passage through a fine mesh stainless steel sieve. The cell suspension was then passed through six layers of cotton gauze to remove any particulate matter and washed three times. If necessary, the cells could be pelletted in RPMI 1640 containing 20% FCS and kept overnight at 4°C for use the next day without loss of antigen-presenting activity.

Epithelial cells to be used as MLR stimulators were obtained by harvesting the supernatants from the EDTA-CMF-HBSS washes (after discarding the first two or three which contained excessive mucus and debris). FCS was added immediately to the supernatants which were centrifuged, resuspended in culture medium and pooled for use in MLR stimulation experiments.

Spleen cells were obtained by passing spleens from C57BL/6 mice through a fine mesh stainless steel sieve, then centrifuging over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) (600g for 30 min at 20°C). The cells from the interface were washed, treated with mitomycin C (see below) and used in the MLR.

Mesenteric lymph node (MLN) cells to be used as responders were removed from BALB/c mice, pushed through a fine mesh stainless steel sieve, washed, counted and viability assessed using Trypan blue. They were depleted of cells bearing Class II MHC molecules by incubating with a monoclonal antibody directed against murine Class II MHC antigens (see Table 2.1) and

using the technique of panning (Section 2.2.4). In the initial experiments, Ia^+ cells were not removed.

2.2.4 Panning to remove cells expressing particular antigens

Bacteriological grade 90 mm Petri dishes were pretreated with 5 ml 1/1000 rabbit anti-rat heavy and light chain IgG (RAR), (Nordic, Tilburg, The Netherlands), in phosphate-buffered saline (PBS) for 45 min at 4°C. The unbound antibody was removed by washing four times in PBS. Cells were incubated with a saturating amount of monoclonal antibody for 30 min at 4°C. After three washes, the antibody-binding cells were removed by panning [520]. After removing the unbound monoclonal antibody, $20\text{-}30x10^6$ of the labelled cells suspended in 5 ml PBS/5% FCS were added to the RAR antibody-coated plates. Plates were kept for 75 min at 4°C and the non-adherent cells removed by gentle agitation and aspiration, centrifuged, then reapplied to a second RAR-coated dish for another 75 min at 4°C. The remaining non-adherent cells were removed, washed, counted and used immediately or pelletted and kept overnight at 4°C.

Antibodies used for cell depletion, immunocytochemistry and flow cytometry included the culture supernatants of hybridoma cell lines secreting monoclonal antibodies listed in Table 2.1.

2.2.5 Mitogen-induced proliferation of lamina propria cell suspensions

Concanavalin A (con A) (Sigma, St Louis, MO) and phytohaemagglutinin (PHA) (Commonwealth Serum Laboratories, Melbourne, Victoria) were added to constant numbers of disaggregated lamina propria cells ($1x10^5$ - $1x10^4$ well) in varying concentrations (con A 0.05-50 mg/ml; PHA 0.4-400 mg/ml) and cultured for 2-3 days in flat-bottom 96-well plates. One microCurie of tritiated thymidine was added to each culture for the final 8 h. The contents of the wells were freeze-thawed and the cells harvested automatically onto glass fibre discs

(Whatman, Maidstone, UK) using a Dynatech CH-103 cell harvester. The assay was performed on a Packard TriCarb K60 scintillation counter following the addition of 5ml scintillant (0.5% 2,5-diphenyloxazole (PPO) in xylene).

2.2.6 Mixed Leukocyte Reaction

Cultures were performed in round-bottom 96-well plates (Linbro Flow Laboratories, McLean, VA). A constant number of responder MLN cells ($2x10^5$) was incubated with varying numbers of stimulator cells (from the intestine or spleen). Cell proliferation in the stimulator population was prevented by pretreatment with mitomycin C (Sigma, St Louis, MO.) ($5x10^6$ cells/ml were incubated with mitomycin C at a final concentration of 25 mg/ml for 45 min at 37^o, then washed three times) or exposed to 2,500-3,000 Rads irradiation. The reaction was carried out in a total volume of 0.2 ml medium (see above). After four days, 1 μCi tritiated thymidine (Amersham Australia, Surry Hills, NSW or New England Nuclear, Boston, MA.) was added to each well and the incubation continued for a further 16 h. The plates were freeze-thawed and the cells harvested automatically onto glass fiber discs and counted as described in Section 2.2.5.

2.3 RESULTS

2.3.1 Development of the disaggregation technique

Application of previously published techniques for obtaining lamina propria cell suspensions (eg. Davies and Parrott, 1981 [95]) gave inconsistent results, so the methods were modified. The many variables examined included the use of mucolytic agents eg. dithiothrietol (DTT), type and concentration of enzymes, the length of incubation, the receptacle used for disaggregation, the medium used, the presence or absence of FCS, and the amount of tissue digested in a given volume of enzyme mixture. Figure 2.1 summarizes data from 40 experiments examining cell yields in relation to the type and concentrations of enzymes and the length of incubation. Effective removal of the epithelial monolayer by washing with EDTA was confirmed by histological examination of the specimens on several occasions. The final technique is described in Section 2.2.3.

Factors critical to the successful application of the isolation procedure included: careful dissection of the intestines to ensure removal of blood vessels and fatty tissue; frequent washing in low concentrations of EDTA, the inclusion of DNase (to prevent cell clumping) and FCS (presumably to inhibit cytotoxic proteases [164]); the use of Petri dishes (rather than a stirring bar which was associated with poor viability); and siliconization to prevent loss of adherent cells during the enzymatic digestion. One of the most important factors was the amount of tissue digested (PG Holt. Personal communication). When more than 500 mg tissue was digested in 20 ml enzyme mixture, cell yields and viability dropped considerably. For example, in one experiment, adding 400 mg tissue to 20 ml enzyme mixture yielded $53x10^6$ viable cells/g with 78% viability; adding 1.02 g to the same volume resulted in $4x10^6$ viable cells/g with $<10\%$ viability.

2.3.2 Recovery of lamina propria and mesenteric lymph node cells

Using the optimal procedure described in Section 2.2.3, disaggregation of intact small intestinal mucosa resulted in yields of $17\pm4x10^6$ per mouse (mean $\pm$ standard deviation, n=204 mice) with $78\pm16\%$ viability as determined by Trypan blue exclusion. When the lamina propria and Peyer's patches were disaggregated separately, the average yield was $11.5\pm4x10^6$/mouse (n=56) for Peyer's patches and $7\pm3x10^6$/mouse (n=59) for the lamina propria. The yield from the colon was $7x10^6$ cells/mouse (n=18) and from mesenteric lymph nodes was $39x10^6$/mouse. Following panning to remove cells expressing Class II MHC antigens (TIB 120), the average yield of mesenteric node cells was $52\pm7\%$.

The yields of cells differed between the two institutions where the work was performed (the John Curtin School of Medical Research {JCSMR} and the MD Anderson Hospital and Tumor Institute {MDA}). Yields of cells were compared using the Student's t test. The differences were found to be statistically significant. Lamina propria yields were $7.0\pm3.0x10^6$/mouse at JCSMR versus $3.0\pm1.3x10^6$/mouse at MDA ($p<0.001$) and Peyer's patch yields $11.5\pm4.0x10^6$/mouse at JCSMR versus $7.2\pm1.9x10^6$/mouse at MDA ($p<0.02$). These data supported the visual observation that the number and size of Peyer's patches was substantially greater at JCSMR than at MDA. This variable could reflect differences in feeding, or the nature of normal flora in mice in the two institutions. There was no clear functional difference between dendritic cells isolated in the two institutions, however.

2.3.3 Mitogen-induced stimulation

Isolated lamina propria cell suspensions proliferated in response to the mitogens, con A and PHA. Maximal proliferation with con A occurred with concentrations between 0.8-3.0 μg/ml for $1x10^5$ cells/well and with PHA at concentrations between 25-50 μg/ml (Fig. 2.2).

2.3.4 Characterization of the MLR

In the initial experiments, untreated or nylon-wool treated MLN cells were used. These cells gave unacceptably high background levels of stimulation (the syngeneic MLR) reaching a peak of 5-20,000 cpm between days 6 and 7 of culture. Removal of Ia^+ cells by panning following incubation with TIB120 (anti-Class II MHC) consistently gave background levels of proliferation of <500 cpm, whereas treatment with TIB120 alone (without panning) reduced the proliferation to half the control value.

The time course of the MLR was determined using both unfractionated spleen and lamina propria cell suspensions as stimulators. Maximum levels of proliferation were comparable and occurred between 96-120 h of culture (Fig. 2.3). To determine whether the MLR stimulatory cells were adherent to plastic, splenic or lamina propria cells were cultured overnight and MLR stimulatory activity compared to cells left as a pellet overnight in RPMI containing 20% FCS at 4°C (Fig. 2.4). Rather than decreased activity, overnight culture resulted in either increased or unchanged MLR stimulatory activity.

2.3.5 Characteristics of mucosal cells stimulating the MLR

Panning alone, which depletes surface immunoglobulin-bearing cells because the second antibody used cross-reacts with mouse immunoglobulin, resulted in removal of 41±6% of the mucosal cells; pretreatment with anti-Ia, 57±14%. Other antibodies used in combinations (Lyt2, L3T4, F4/80, Pgp-1 and anti-mouse immunoglobulin), resulted in removal of 76±3%. Removal of Ia^+ cells resulted in a shift in the stimulator dose-response curve so that, at low cell numbers, four to five times more cells were required to achieve the same level of tritiated thymidine uptake (Fig. 2.5). In addition, there was a 90-95% reduction in peak MLR stimulation (10.9 vs 113.5×10^3 cpm at maximal stimulator cell number, Table 2.2). Treatment with other antibodies did not reduce the maximal MLR response. Cell depletion experiments using panning with 33D1 were

ineffective because it is not detectable on intestinal dendritic cells (see Chapter 4). Complement lysis experiments were not undertaken. In general, removal of the T cell (Lyt 2, L3T4, Thy 1-positive) or B cell (surface Ig-positive) cells enriched for stimulator cells to the expected extent (given the proportion of lamina propria cells expressing T or B cell markers). Overnight plastic- or fibronectin-adherence decreased the proportion of macrophages to <2% as judged by morphology. Removal of macrophages resulted in a significant increase in MLR stimulatory activity of the remaining non-adherent lamina propria cells (Fig. 2.6). These results suggest that the MLR stimulator cells in the intestinal lamina propria expressed Class II MHC antigens, but lacked the characteristics of macrophages, B cells or T cells.

2.3.6 Epithelial cells as MLR stimulators

Viability of small intestinal epithelial cells after 3-4 h in culture was <30%. These cells were used in MLR experiments but did not stimulate proliferation (not shown).

2.3.7 Enrichment of MLR stimulatory cells

Following the demonstration of MLR stimulatory activity in the lamina propria cell suspensions, attempts were made to enrich the antigen-presenting cells. Density gradient centrifugation using various concentrations of Percoll (Pharmacia, Uppsala, Sweden) in both discontinuous and continuous gradients was unsuccessful, probably because of co-enrichment of low density suppressive cells (see Chapter 3).

Other attempts at enrichment involved the use of a cocktail of antibodies for panning (Lyt2, L3T4, F4/80 and Pgp-1), and the use of temporarily adherent cells (ie. cells which were adherent to fibronectin-coated gelatinized flasks at 3 h but which became non-adherent during overnight culture). Neither approach resulted in significant or consistent enrichment.

2.3.8 Comparison of MLR stimulatory activity of lamina propria cells and Peyer's patch cells

A comparison of Peyer's patch and non-Peyer's patch lamina propria cell suspensions in over 10 experiments consistently showed that the lamina propria fraction had 5-10 times greater MLR stimulatory activity per cell (Fig. 2.7). Similar results were noted when the incubation times were varied, suggesting that these differences were not due to different kinetics of MLR stimulation (Fig. 2.8).

2.4 DISCUSSION

2.4.1 The development of the disaggregation technique

Initial studies on immune responses in Peyer's patches failed to demonstrate accessory cell activity in preparations obtained by mechanical dissociation [206], so that it was concluded that Peyer's patches were deficient in functional accessory cells. Similar observations were made from the study of lung-derived cell suspensions [reviewed in 164]. With the development of techniques for the enzymatic dissociation of tissue, it was clear that this deficiency was due to the failure of mechanical methods to release accessory cells from the connective tissue stroma [433]. When enzymatic and mechanical methods were directly compared, the latter resulted in lower yields, the selection of particular cell types and the liberation of suppressive factors eg. prostaglandin E_2 [47,92].

On the other hand, the use of enzymatic methods in tissue disaggregation is also associated with particular problems. For example, trypsin treatment, amongst other effects [reviewed in 115], resulted in the loss of cell surface proteins [399], and impaired cellular cytotoxicity [425]. Other enzymes such as pronase and papain had variable effects depending on their concentration and duration of treatment [92]. Generally, the impairment of cellular function is completely reversible with time in culture [132, 225].

In spite of these possible adverse effects, enzymatic treatment of murine Peyer's patch with the neutral protease, Dispase, yielded cell suspensions that were capable of accessory cell activity in vitro. Furthermore, Dispase treatment of murine splenic dendritic cells did not alter their stimulatory activity [433]. Treatment with collagenase generally has little effect on cellular function [132] or phenotype [166], although occasional batches may be overtly toxic [164]. Since broad spectrum proteases and/or mixtures of enzymes are more efficient for tissue disruption than single enzymes alone [409], it was decided to examine the

use of multiple enzymes and the combination was found to give higher yields (Fig. 2.1).

The final yields obtained were comparable with those published at the time, although methods reporting greater yields have since been published [reviewed in 481]. One important difference between our method and that of van der Heijden and Stok (1987) [481] is that our final proportion of epithelial cells was very low (<5%) compared to approximately 25% overall in their study. In our experiments the epithelial cells co-purified with other low density cells and caused cell losses by clumping, whereas van der Heijden and Stok (1987) were concerned with plaque-forming cell assays which presumably were unaffected by epithelial cells.

There are many factors affecting the yield of cells using a particular dis-aggregation technique. Examples include the age and strain [164], but possibly the most important is antigen-exposure. This may affect both the size and the cellular composition of Peyer's patches [90, 373, 428]. The use of Peyer's patches from specific pathogen-free mice resulted in lower yields when directly compared with mice housed in a conventional environment [eg. 287] and greater yields were obtained following inoculation with a specific micro-organism [77]. Other evidence [98] also points to selective effects of certain infections on immune function eg. the ability of dendritic cell-T cell clusters to induce poly-clonal immunoglobulin secretion by cultures of Peyer's patch lymphoid cells is abrogated if the cultured stimulator cells are derived from mice infected with mouse hepatitis virus [78].

Our results demonstrated a difference in yields between "SPF" facilities. In addition, over the three year period during which the mouse experiments were performed, two viral diseases (ectromelia and mouse hepatitis virus) infected the laboratory animals for an unknown period before their discovery.

Intestinal lamina propria cell suspensions had MLR stimulatory activity and were capable of both stimulating and responding to mitogen-induced proliferation. (These observations raise the possibility that significant T cell activation occurs in situ rather than, or as well as, in draining lymph nodes, but this question was not explored.)

Attempts to characterize the MLR stimulatory cell confirmed the requirement for Class II MHC molecule expression and suggested that the cell was a dendritic cell. Attempts to enrich for this cell type were unsuccessful until the macrophages were removed from the cell suspension (See Chapter 3).

An unexpected finding was the consistently greater MLR stimulatory activity in the unfractionated cell suspensions from the lamina propria when compared to Peyer's patches. In addition, there was clear evidence of inhibition of proliferation when large numbers of lamina propria cells were added. This raised the possibility of a suppressor cell population and led to the experiments in the following chapter.

TABLE 2.1

Antibodies Used in Murine Experiments

Name	Determinant	Class	Reference
TIB120	Class II MHC $I\text{-}A^{b,d,q}$, $I\text{-}E^{d,k}$	IgG_{2b}	J Immunol 1981; 127: 2488
F4/80	F4/80 antigen	IgG_{2b}	Eur J Immunol 1981; 11: 805
M1/70 (Mac-1)	$C3b_i$ receptor	IgG_{2b}	Eur J Immunol 1978; 8: 539; 1979; 9: 301
2.4G2	F_C receptor	IgG_{2b}	J Exp Med 1979; 150: 580
IM7	Pgp-1		J Biol Chem 1983; 258: 1014
33D1	Dendritic cell antigen	IgG_{2b}	Proc Natl Acad Sci USA 1982; 79: 161
AT83	Thy 1	IgG_{2b}	Dr R Ceredig
30-H12	Thy 1.2	IgG_{2b}	Immunol Rev 1979; 47: 63
53-6.72	Lyt 2	IgG_{2a}	Immunol Rev 1979; 47: 63
HO-2.2	Lyt 2.2	IgM	Immunogenetics 1980; 10: 545
GK-1.5	L3T4	IgG_{2b}	Immunol Rev 1983; 74: 29

RA3-3A1/6.1	B cell glycoprotein (B220)	IgM	Nature 1981; 289: 681
J11d	"B cell"	IgM	J Immunol 1981; 127: 2496
SAM	Surface Ig (sheep antimouse immunoglobulin)		

All antibodies except SAM were rat-derived

Table 2.2

MLR stimulation: Comparison of different treatments of stimulator and responder cells.

Stimulator cells ($1x10^5$/well) and responder cells ($2x10^5$/well) were either untreated (control) or treated by incubating with TIB120 at 4°C for 30 min and washing three times before plating (anti-Ia); by "panning" on rabbit anti-rat immunoglobulin-coated plastic Petri dishes without prior incubation with primary antibody (panning); or by incubating with TIB120 together with the "panning" treatment as described in the text (anti-Ia/panning) (Section 2.2.4). The figures in parentheses refer to the yields following the specified treatment as a percentage of the starting cell number. The MLR was performed as described in the text (Section 2.2.6).

The first column and the first row indicate the uptake of tritiated thymidine when the stimulator and responder cells respectively were cultured alone. The other values in the Table indicate the uptake of tritiated thymidine when the stimulator and responder cells subjected to the specified treatments were cultured together in the allogeneic MLR.

Table 2.2 MLR stimulation: Comparison of different treatments of stimulator and responder cells

Stimulator cell treatment {1 x105 cells/well} (yields after treatment)		Uptake of [^{3}H] thymidine (±SD) (cpm x 10^{-3})			
		8.0±1.4	4.0±3.0	0.9±0.3	0.2±0.1
Control (100%)	0.2±0.04	92±3	41±4	89±5	88±4
Anti-Ia (84%)	1.0±0.5	58±7	56±8	74±6	66±3
Panning (63%)	0.7±0.1	114±32	56±6	86±8	82±8
Anti-Ia/ panning (51%)	0.3±0.01	11±2	8±1	4±0.3	8±5
		Control (100%)	Anti-Ia (99%)	Panning (58%)	Anti-Ia/ panning (52%)

Responder cell treatment {2 x10^5 cells/well} (yields after treatment)

LEGENDS TO FIGURES

Figure 2.1

Disaggregation technique: Effect of enzyme concentration and length of incubation on cell yields. Mouse small intestines were removed, washed in EDTA to remove the epithelial cell monolayer and incubated with different enzyme types and concentrations for the indicated times. The points are the means of between three (at time 16 h) and eleven (at time three hours) experiments. The variability ranged from <10% to 400% and depended on factors which were not initially recognized, eg. the amount of tissue added per 20 ml enzyme cocktail (see discussion). The method was otherwise as described in the text. Dispase 0.12 U/ml, inverted triangles; 0.24 U/ml, circles; 0.48 U/ml, triangles; 0.96 U/ml, open squares; Dispase 0.48 U/ml closed diamonds; with collagenase 10 U/ml, closed squares.

Figure 2.2

Mitogen-induced lamina propria cell proliferation. Disaggregated lamina propria cells from C57BL/6 mice ($1x10^5$/well) were incubated for 72 h in flat-bottom 96-well plates in 200 ml medium with increasing concentrations of concanavalin A (con A) and phytohaemagglutinin (PHA). Tritiated thymidine was added for the final 8 h of culture. The plates were freeze-thawed and the cells harvested automatically onto glass fibre discs. The results are the means $\pm$ SE of triplicate assays. PHA-stimulated, squares; con A-stimulated, triangles. The optimal stimulatory concentrations for PHA and con A were 25-50 μg/ml and 0.8-3.0 μg/ml respectively.

Figure 2.1

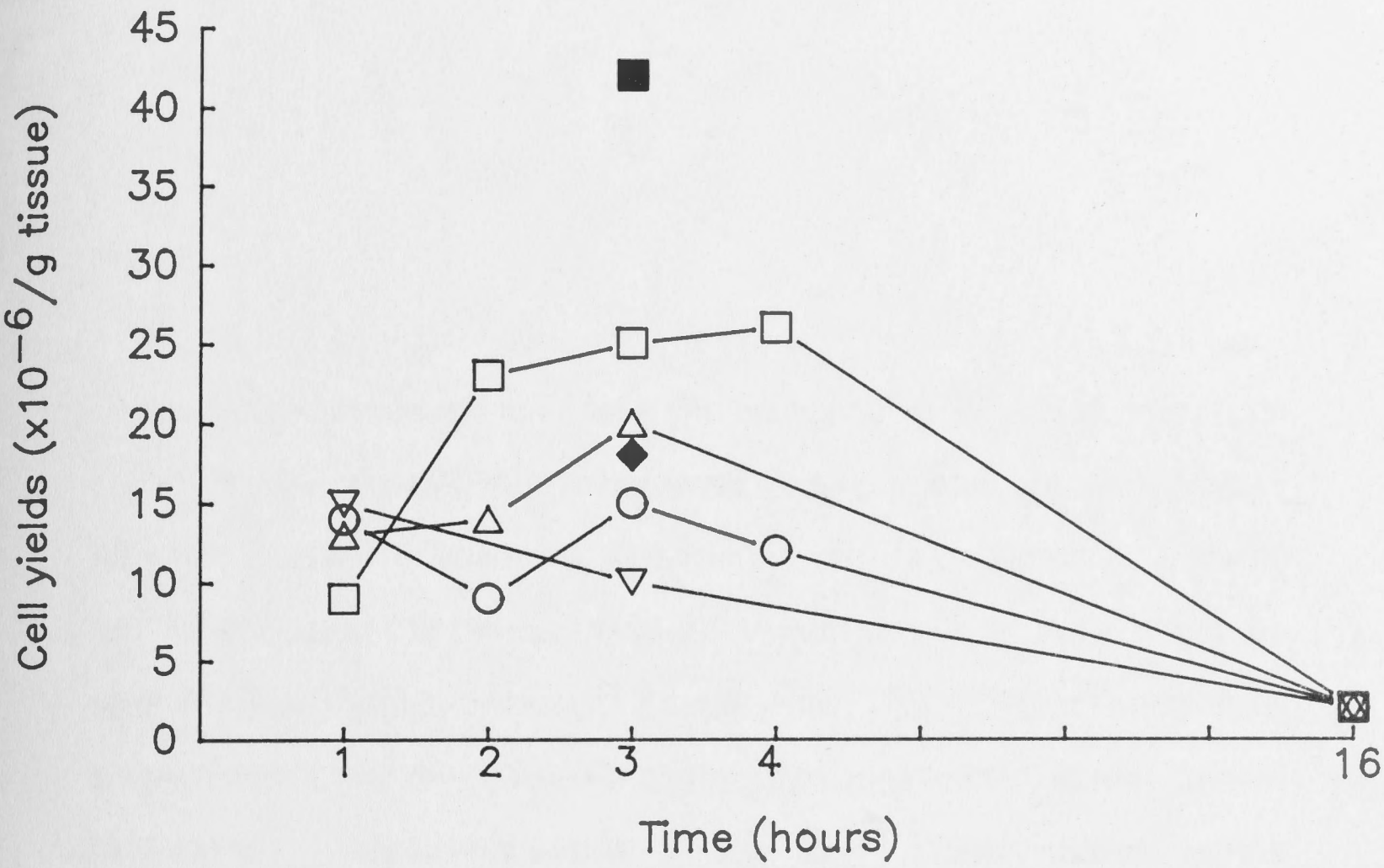


Figure 2.2

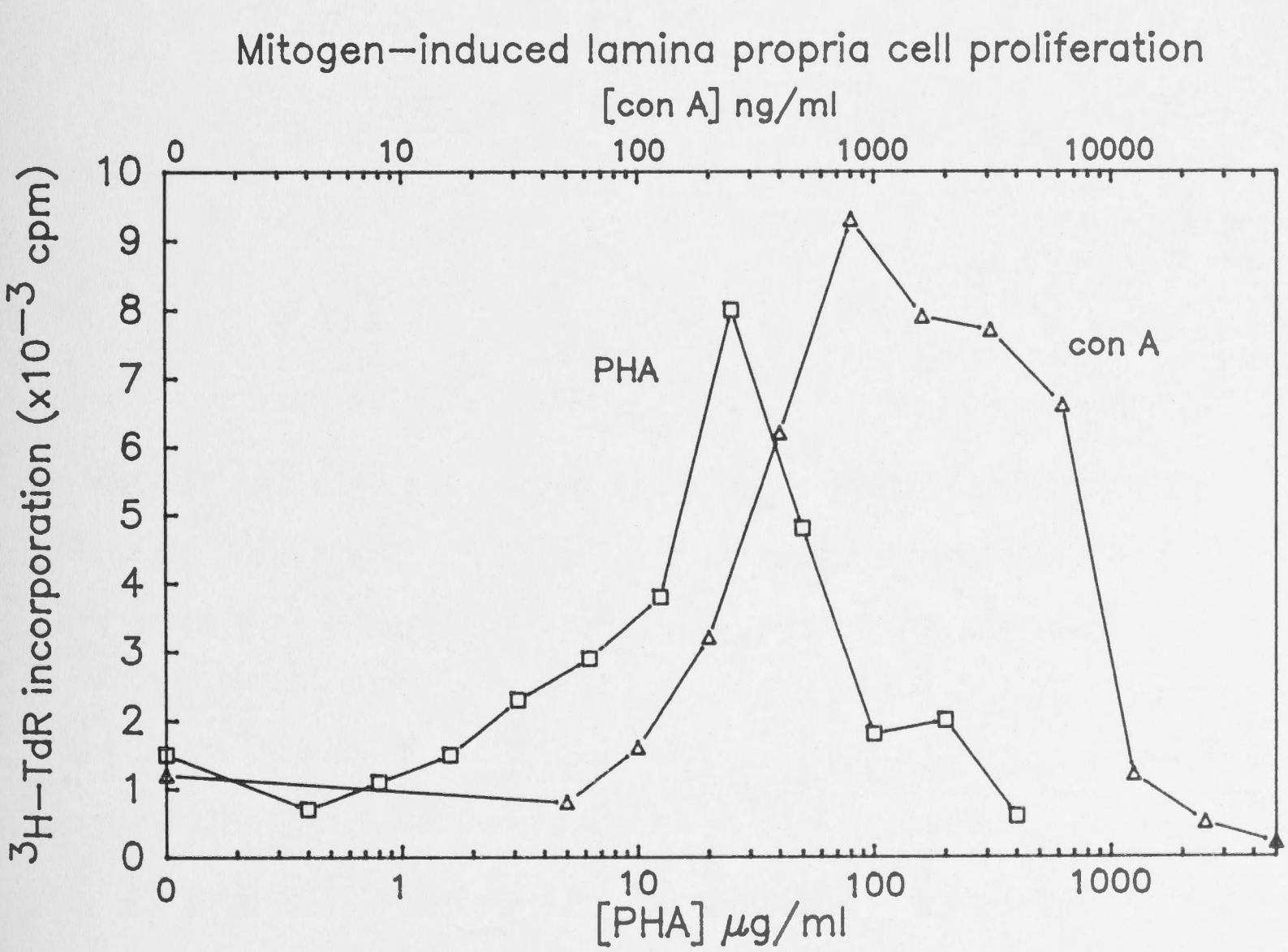


Figure 2.3

MLR stimulation: Comparison of intestinal lamina propria and spleen cells. Spleen or lamina propria cells from C57BL/6 mice ($2x10^5$ cells /well) were added to $2x10^5$ untreated MLN cells from BALB/c mice for the indicated times. For the last 16 h of incubation 1 μCi tritiated thymidine was added to each well. The plates were freeze-thawed and the cells harvested automatically onto glass-fibre discs. Spleen and lamina propria controls incorporated $<1,000$ cpm. Values are the mean $\pm$ SE of triplicate assays. Lamina propria (LP) cells, triangles; spleen cells, squares; mesenteric lymph node (MLN) cells, circles.

Figure 2.4

MLR stimulation: Effect of pelletting or culturing cells overnight. The experiment was performed as for Figure 2.3. Open symbols, cells cultured overnight; closed symbols, cells pelletted overnight at 4°C in RPMI containing 20% FCS.

Figure 2.3

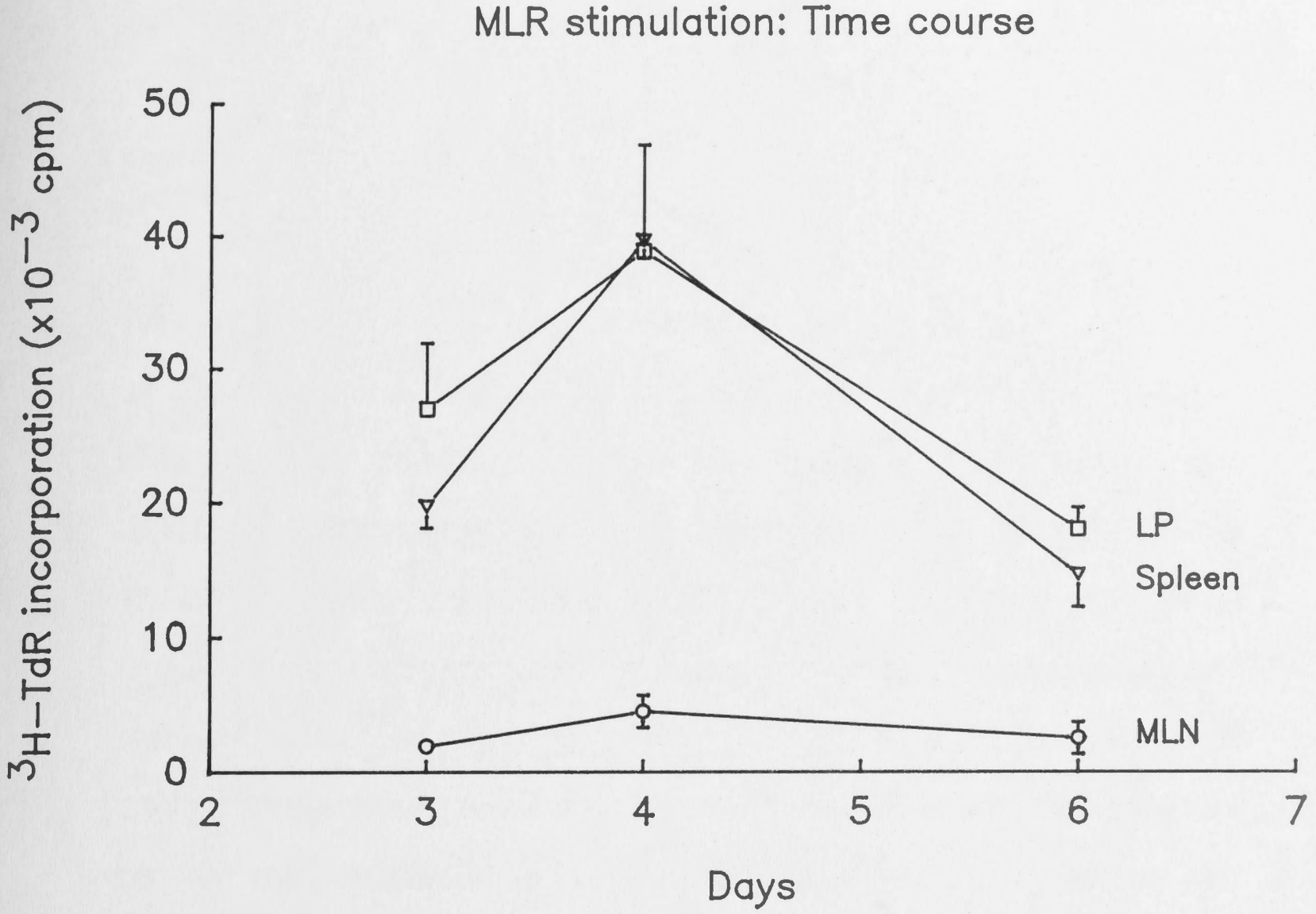


Figure 2.4

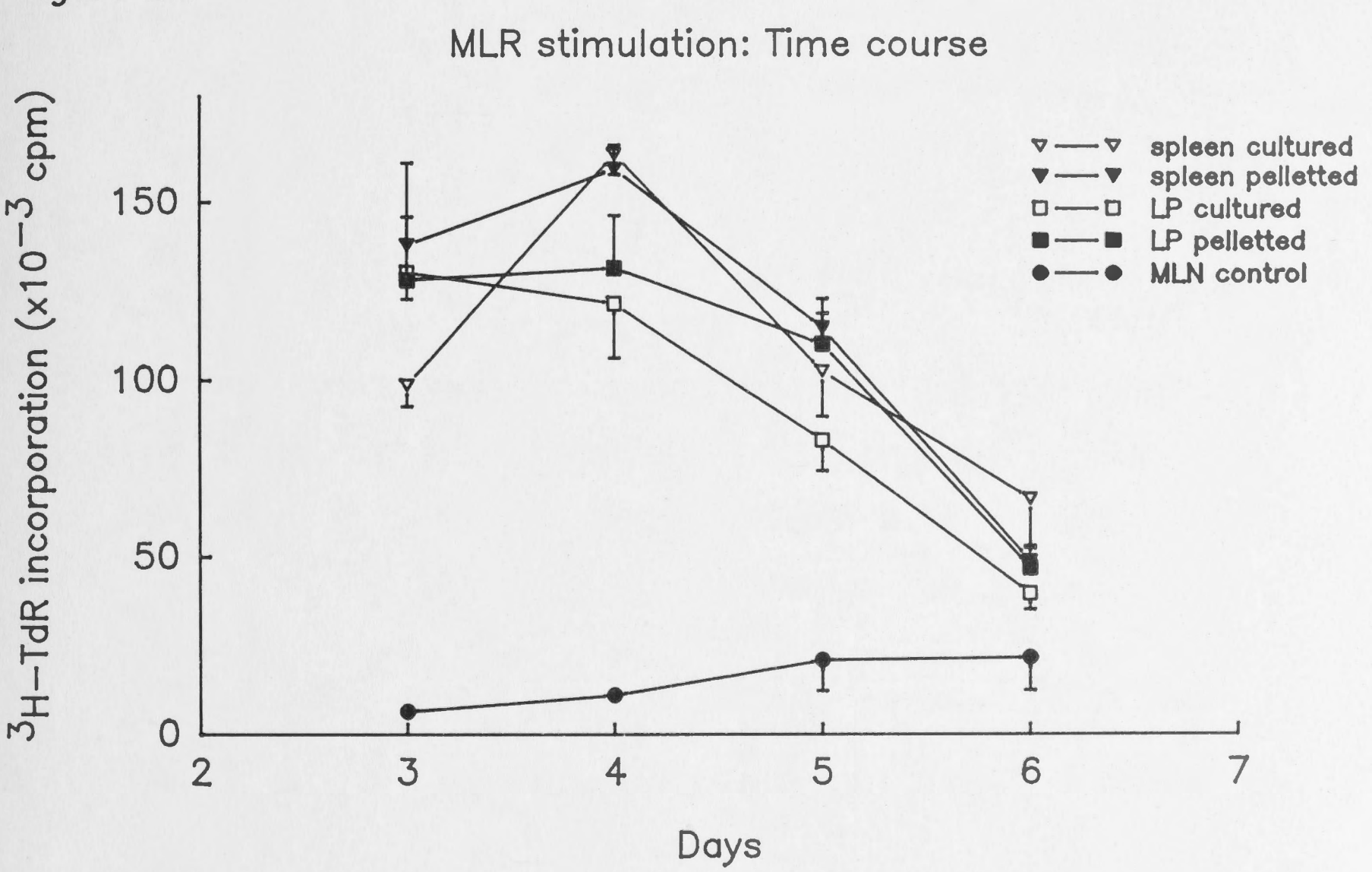


Figure 2.5

MLR stimulation by lamina propria cells. The abscissa represents increasing numbers of unfractionated small intestinal lamina propria cells (open squares) or lamina propria cells that were depleted of cells expressing class II MHC antigens (Ia) by panning (closed squares). Lamina propria cells from C57BL/6 mice were added to $2x10^5$ mesenteric lymph node cells, depleted of Ia-bearing cells, from BALB/c mice. After 4 days, 1 μCi of [^{3}H]-thymidine was added to each microculture and the cells were freeze-thawed and harvested onto glass fibre discs after a further 16 h. Unstimulated responder cells incorporated <1000 cpm whilst irradiated stimulator controls incorporated <500 cpm.

Figure 2.6

MLR stimulation: Effect of removing fibronectin-adherent cells. The experiment was performed as described in Figure 2.5. Open squares, unfractionated small intestinal lamina propria cells; closed squares, fibronectin-non-adherent cells.

Figure 2.5

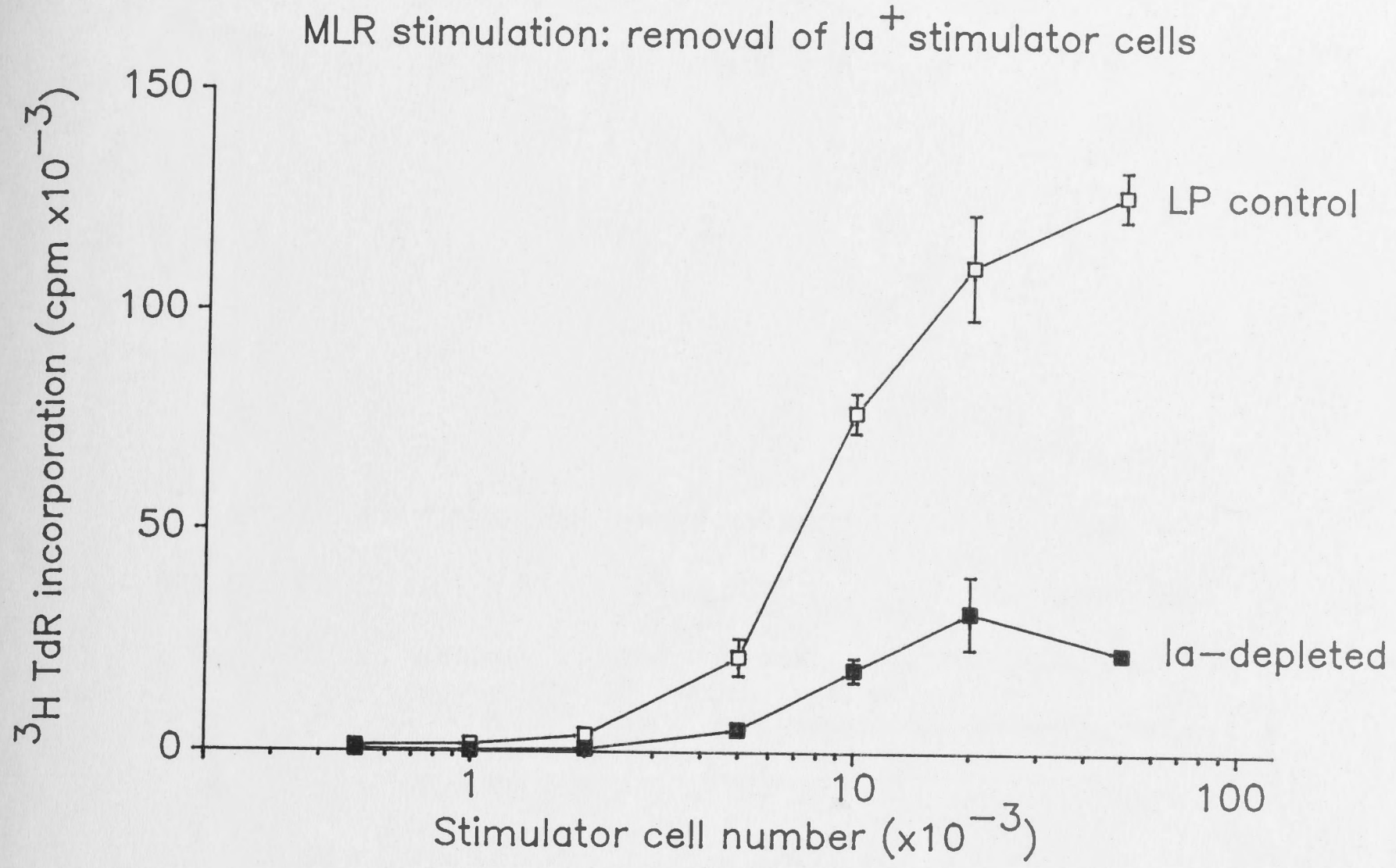


Figure 2.6

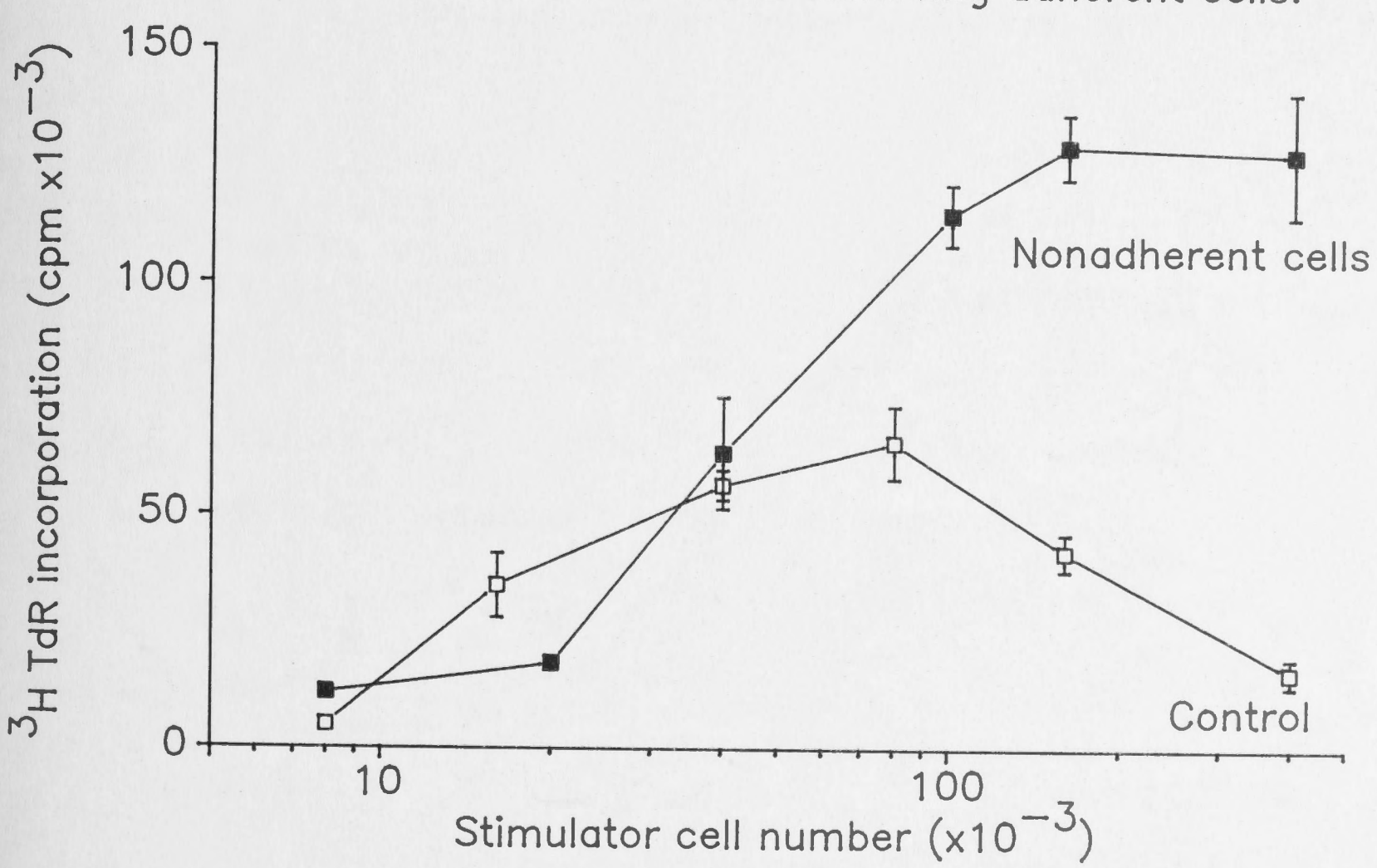


Figure 2.7

Comparison of MLR stimulation by Peyer's patch and lamina propria cells. The experiment was performed as described in Figure 2.5. Squares, unfractionated small intestinal lamina propria cells; triangles, unfractionated Peyer's patch cells.

Figure 2.8

Time course of MLR stimulation by Peyer's patch and lamina propria cells. The experiment was performed as described in Figure 2.5. Unfractionated lamina propria cells (squares) or Peyer's patch cells (triangles) were incubated for three days (open symbols) or five days (closed symbols) with $2x10^5$ Ia-depleted MLN cells.

Figure 2.7

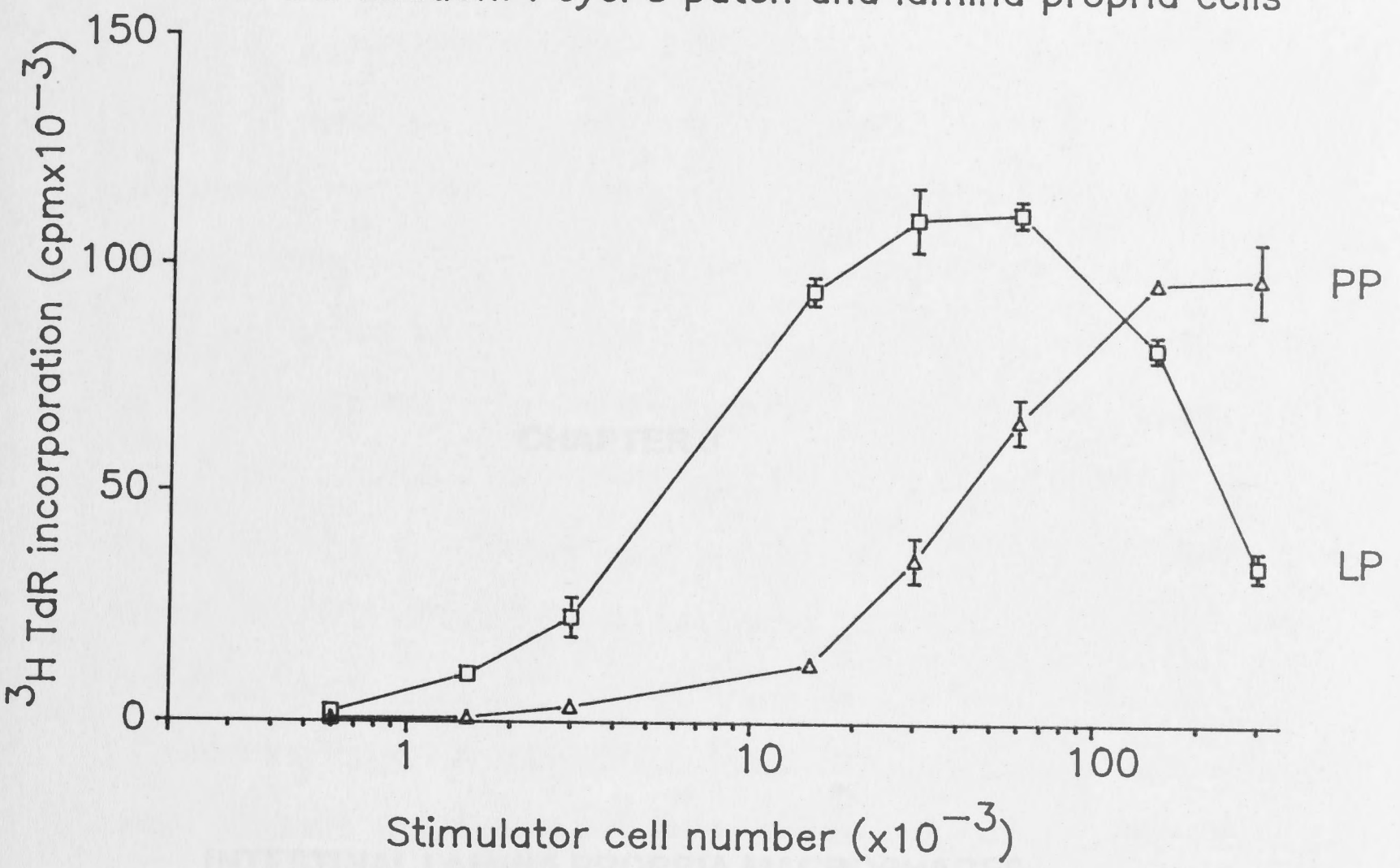
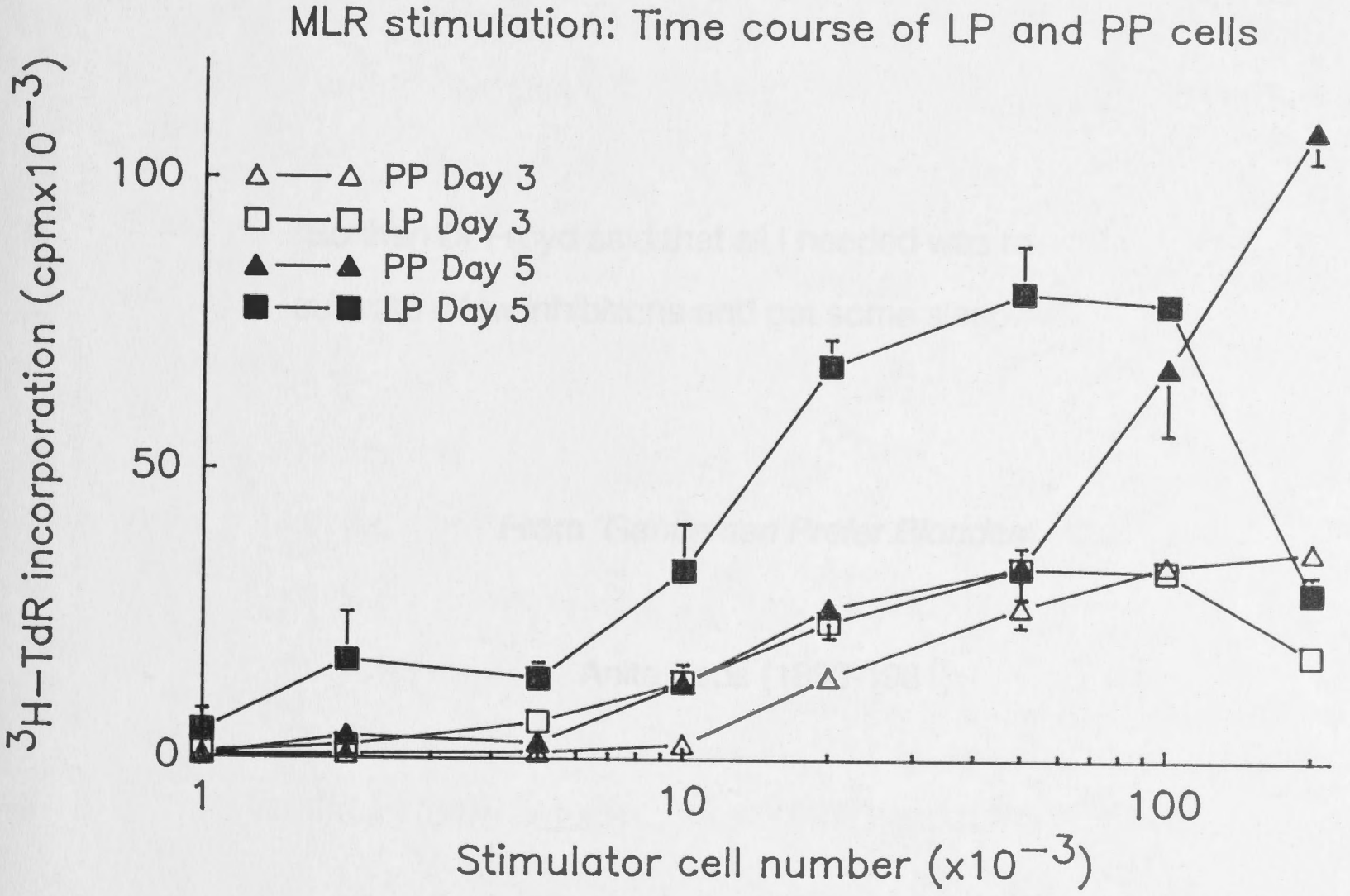


Figure 2.8



CHAPTER 3

INTESTINAL LAMINA PROPRIA MACROPHAGES

"So then Dr Froyd said that all I needed was to cultivate a few inhibitions and get some sleep."

From *"Gentlemen Prefer Blondes"*

Anita Loos (1893-1981)

3.1 INTRODUCTION

The role of macrophages in the regulation of immune responses is complex and reflects the morphological, phenotypic and functional diversity of the mononuclear phagocyte lineage in vivo [268, 270, 348, 349, 370]. Additional variability is added by the isolation procedure and by in vitro manipulation. Reports attributing antigen-presenting cell activity in primary T cell responses to macrophages often failed to consider other factors which were discussed in Sections 1.1.2.1 and 1.2.2. Although macrophages do stimulate activated T lymphoblasts [179, 182], there is no evidence for a role in the initiation of primary T cell responses.

There is, however, considerable evidence to suggest that macrophages are modulators of T cell activation. Effects in vitro are seen in two phases. In low numbers, macrophages enhance responses [182, 217, 242, 273, 311, 343], either by releasing IL-1 [217] which amplifies dendritic cell function [241], or by improving cell viability [445]. At high cell numbers, macrophages are suppressive. Examples are found in all species studied including the mouse [152, 182, 187, 190, 313, 343, 353, 354], the rat [163, 165, 231, 311, 341], the rabbit [212] and the human [107, 116, 390, 473, 477].

Particular functions which may be affected include:

i) the primary MLR [343, 450, 477];

ii) the syngeneic MLR [353, 377];

iii) stimulation of T lymphoblasts [182];

iv) antigen- or mitogen-induced T cell proliferation [107, 116, 117, 161, 162, 163, 165, 212, 245, 311, 341, 390, 473];

v) the induction of cytotoxic T cells [190, 354];

vi) oxidative mitogenesis [231, 396]; and

vii) B cell antibody production [113, 187, 242, 279, 313, 414].

One mechanism of high-dose inhibition involves the release of eicosanoids, in particular, prostaglandin E_2 (PGE_2) [116, 212, 326, 354, 414]. This is not a universal finding [107, 116, 272, 273, 341, 473, 477], and other mechanisms have been proposed eg. mediation by oxygen reactive species [320], by low molecular weight peptides [273, 359], by a contact-dependent mechanism [454] or by the secretion of suppressor T cell factors [113].

To study the role of macrophages in murine intestinal immune responses, a method for obtaining homogenous populations of macrophages was developed and the cells characterized. Using depletion and reconstitution experiments, intestinal macrophages were shown to mediate the high-dose suppression seen when lamina propria cells were used as stimulators in the MLR. The absence of high-dose supression seen when Peyer's patch cells were used, was a reflection of the small numbers of macrophages resident therein. The inhibition of MLR stimulatory activity was mediated by an indomethacin-sensitive mechanism.

3.2 METHODS

The materials and methods used for obtaining single suspensions of lamina propria or Peyer's patch cells and for performing the MLR was as described in Chapter 2. In order to minimize macrophage losses, one change was instituted to the protocol. Stimulator cell proliferation in the allogeneic one-way MLR was prevented by irradiation (2,500-3,000 Rads) rather than by treatment with mitomycin C at 37^{o}C.

3.2.1 Intestinal macrophages

The isolated lamina propria cell suspension at 5-10x10^{6}/ml was incubated for 2-3 h on fibronectin-coated gelatinized flasks [125]. Human serum from healthy donors, clotted at 37^{o}C was used as a source of fibronectin. Non-adherent cells were discarded or pelletted, and the adherent cells further incubated overnight. Any cells which were no longer adherent after overnight incubation were removed by washing with RPMI at 37^{o}C and pooled with the other non-adherent cells. Adherent cells were harvested by incubating with 10 mM EDTA in RPMI with 10% FCS for 10-15 min. These cells were washed three times, counted and viability assessed. Phagocytosis was assessed by incubating the cells for 2 h with 1μm diameter fluorescent microspheres (Polysciences Inc., Warrington, PA) after readhering the cells onto glass slides.

3.2.2 Resident peritoneal macrophages

Resident peritoneal cells were harvested by peritoneal lavage with CMF-HBSS and treated in parallel, including incubation overnight on fibronectin-coated gelatinized flasks.

3.2.3 Splenic macrophages

Spleens were removed from C57BL/6 mice and the pulp was extruded through a fine wire mesh. The resulting cells were vortexed, pelletted and centrifuged over Ficoll-Paque (Pharmacia, Uppsala, Sweden) (600 g for 30 min at 20^{o}C). The cells at the interface were washed three times in complete media

and allowed to adhere overnight to fibronectin-coated gelatinized flasks. The non-adherent cells were removed by washing three times with warm media and the adherent cells harvested after 10-15 min incubation in 10 mM EDTA in RPMI containing 10% FCS. These cells were washed three times and their viability assessed.

3.2.4 Alveolar macrophages

C57BL/6 mice were anaesthetized with ether and allowed to exsanguinate after the left renal artery and vein were severed. The trachea was isolated with careful haemostasis and a blunt needle inserted and secured. Five or six aliquots of CMF-HBSS (increasing from 50 to 200 μl) were injected and re-aspirated. The first aliquot was discarded and subsequent ones pooled. The cells were washed in RPMI containing 10% FCS, resuspended, counted and viability assessed. These cells were also incubated overnight on fibronectin-coated gelatinized flasks and the adherent cells harvested as above.

3.2.5 Immunocytochemistry

Cytocentrifuge slides were made using 2-10x10^4 cells in RPMI with 50% FCS on poly-L-lysine coated glass slides (0.1% poly-L-lysine (Sigma, St Louis, MO) for 10 min then air-dried). Specimens for cytology were stained using Diff-Quik (Lab Aids, Narrabeen, NSW).

For immunocytochemistry, the cytocentrifuge slides were air-dried overnight, and then blocked with horse serum (4 drops in 10 ml PBS) for 30 min. Primary antibodies (see Chapter 2) were added and incubated at 20^{o}C for 30 min. The slides were washed in PBS then biotinylated sheep anti-rat immunoglobulin (1/200)(Amersham, Amersham, UK) was added for 30 min. After washing, avidin-biotin-peroxidase complex (Vector Labs., Burlingame, CA) was added for 1 h and the slides developed for 10 min with 0.5 mg/ml 3,3'-diaminobenzidine (Sigma, St Louis, MO), 10 mM imidazole (BDH Chemicals, Poole, UK) and 0.3% hydrogen peroxide in PBS (pH 7.3). Except for 33D1 (with

which we could not demonstrate staining) appropriate positive and negative controls were included. The slides were lightly counterstained in Mayer's hematoxylin (BDH, Poole, UK), washed, air-dried and mounted. Photography was performed using a Zeiss Axiophot Microscope.

3.2.6 Cytofluorometry

Cells were suspended at a concentration of 2-10x10^6/ml in a solution containing the primary antibody for 30 min at 4^{o}C, washed three times, resuspended at a concentration of 2x10^7 in 1/100 fluorescein isothiocyanate-labelled sheep anti-rat immunoglobulin (Silenus, Melbourne, Australia) or 1/100 fluorescein-conjugated affinity-purified F(ab')$_2$ fragment goat anti-rat IgG (heavy and light chain specific) (Cappel, Cooper Biomedical, Cochranville, PA), washed three times then analysed in a FACS IV flow cytometer (Becton-Dickinson, Sunnyvale, CA), a FACScan (Becton Dickinson, Mountain View, CA) or a Coulter Epics C flow cytometer (Coulter Electronics, Hialeah, FL).

3.3 RESULTS

3.3.1 Yields

Yields of adherent cells from the intact small intestinal mucosa, the non-Peyer's patch lamina propria and the Peyer's patch were 3%, 10% and <0.1% respectively. Yields from the colonic lamina propria were also 10% (phenotypic and functional studies were not performed on these cells).

Yields of macrophages from the peritoneum and lung were 2-5x10^5/mouse and 1-2x10^5/mouse respectively. Splenic yields were not recorded. The proportion of adherent cells which were macrophages as judged by morphology was >90% from all three tissues and close to 99% from the lung. Viability in all cases was >90%.

3.3.2 Morphology and phenotype

The majority of the adherent intestinal cells were classical macrophages morphologically, with an oval nucleus, basophilic cytoplasm and numerous inclusions often giving a "foamy" appearance (Fig. 3.1). An average of 91% (3 experiments) phagocytosed fluorescent latex beads (Fig. 3.2). The phenotype of peritoneal and intestinal macrophages was compared by immunocytochemistry. Peritoneal macrophages were strongly positive for the F4/80 antigen and Pgp-1, while 20-29% (average 24%) were positive for Ia. In contrast, intestinal macrophages, although staining weakly for Pgp-1, had no detectable F4/80 antigen and were 45-55% (average 47%) positive for Ia. Other markers which could not be detected on intestinal macrophages were 2.4G2 (anti-F_cR) and 33D1 (anti-dendritic cell).

These observations were confirmed and extended using flow cytometry. When compared to peritoneal macrophages, intestinal macrophages expressed very low levels of the macrophage-specific markers, F4/80, 2.4G2 (the F_c receptor), (Fig. 3.3) and M1/70 (the $C3b_i$ receptor) at flow cytometry. The low level of F4/80 antigen expression does not appear to be due to the enzyme

digestion, since peritoneal cell expression of F4/80 antigen was unaffected by the enzyme cocktail (not shown). The effect of other steps of the disaggregation procedure on peritoneal macrophage F4/80 expression was not examined.

The majority of the intestinal macrophages expressed definite but low levels of Class II MHC antigens in keeping with previous observations using immunohistochemistry [169, 172]. A variable, but small subpopulation expressed high levels, probably reflecting varying degrees of activation of the macrophages in vivo, or the presence of small numbers of dendritic cells. The mean levels of expression were not as high as for lamina propria dendritic cells (see Chapter 4).

Using flow cytometry, the phenotype of intestinal adherent cells was compared with that of resident peritoneal, splenic and alveolar macrophages (Fig. 3.4). Levels of expression of F4/80 antigen were highest in resident peritoneal macrophages, followed by alveolar and splenic macrophages which had similar levels, and finally intestinal adherent cells which had no detectable F4/80 antigen. The expression of Class II MHC antigens decreased in the opposite order with intestinal macrophages having the highest levels, followed by splenic, alveolar and finally peritoneal macrophages. Systematic analysis of the macrophages from these different sites was not undertaken since the objective was to demonstrate heterogeneity of F4/80 and Class II MHC expression.

To ensure stringent antigen-presenting cell requirements (see Sections 1.1.2.1 and 1.2.2) mesenteric lymph node responder cells were depleted of Ia-bearing cells (see Section 2.2.3). Cytofluorometric analysis of this MHC-II-depleted population demonstrated a homogenous population of T lymphocytes with $CD4^+$:$CD8^+$ = 70:30 (Fig. 3.5). The background levels of proliferation were always $<1{,}000$ cpm and generally between 200-300 cpm.

3.3.3 The role of adherent cells as modulators of immune responses

As discussed in Chapter 2, separated lamina propria cells stimulated maximal MLR responses at 5-10 fold lower stimulator:responder ratios than Peyer's patch cells from the same animals (Fig. 2.7). At high stimulator:responder ratios, the lamina propria cells were suppressive. Removal of cells adherent to fibronectin (macrophages) did not abrogate any stimulatory activity, but abolished the high dose suppression (Fig. 2.6). Suppression by lamina propria adherent cells was also reversible by indomethacin (2 μg/ml) (Figs. 3.6, 3.7). The MLR stimulatory activity of Peyer's patch cells was unaffected by both the removal of adherent cells (not shown) and by the addition of indomethacin (Fig. 3.6). The action of indomethacin was presumed to involve suppression of eicosanoid synthesis since prostaglandin E_2 (PGE_2) has been shown to inhibit lymphocyte activation [reviewed in 174]. We confirmed that addition of PGE_2 resulted in impairment of the MLR generated by both Peyer's patch and lamina propria cells (Fig. 3.8).

Adding increasing numbers of adherent cells to a constant number of either lamina propria or Peyer's patch cells (depleted of adherent cells) resulted in a dose-dependent inhibition (Figs. 3.9, 3.10). For both lamina propria and Peyer's patch cells, this suppression was overcome by adding indomethacin. When adherent cells and indomethacin were added to lamina propria cells, no increase in proliferation was seen when compared to lamina propria cells alone at both maximal (Fig. 3.9) and submaximal (Fig. 3.10) levels. In contrast, adding increasing numbers of adherent cells and indomethacin to cultures of Peyer's patch cells resulted in levels of proliferation greater than for Peyer's patch cells alone (Fig. 3.9).

Adherent cells also inhibited LPS-induced cell proliferation in a dose-dependent manner (Fig. 3.11).

3.3.4 Adherent cells as inhibitors of the MLR

Since fibronectin-adherence did not deplete MLR stimulators from the lamina propria, it appeared unlikely that the adherent cells would act as stimulators. This was found to be the case (Fig. 3.12). In the presence of indomethacin, some stimulator activity was revealed, but only at high stimulator:responder ratio. Whether this represents a weak stimulatory activity of all the cells, or is attributable to a minor contaminant is unclear. The latter explanation seems the more likely since, although the adherent cells are a relatively homogenous population of macrophages, cells with morphological features of dendritic cells were sometimes seen. Experiments assessing the effect of the removal of dendritic cells using the anti-dendritic cell antibody, 33D1 [356], and complement lysis were not performed.

The MLR stimulatory activity of peritoneal macrophages and intestinal adherent cells were found to be much less than that of unfractionated lamina propria cells, both in the presence and absence of indomethacin (Fig. 3.7).

3.4 DISCUSSION

Macrophages have been shown to have a specific magnesium-dependent receptor which binds fibronectin [33]. This property allowed the separation and recovery of macrophages from the lamina propria and an assessment of their function.

The lamina propria contained relatively large numbers of Ia^+ macrophages (10% of disaggregated lamina propria cell suspensions). Consistent with immunohistochemical observations [172, 173, 514, Fig. 3.13], and with previously described yields from disaggregation experiments [287], we found a paucity of macrophages in the Peyer's patch lymphoid tissue. In contrast to their presumptive counterparts in vivo [172], isolated lamina propria macrophages did not express detectable F4/80 antigen at cytofluorometry, in spite of exhaustive efforts to demonstrate it. Variables considered included the use of sera from different species as blocking agents, varying dilutions of both primary and secondary antibodies, the use of biotinylated and a number of different fluorescein-conjugated second antibodies and different methods of fixation. The failure to demonstrate detectable F4/80 expression may simply be a question of the sensitivity of the avidin-biotin complex (ABC) immunoperoxidase method in comparison with immunofluorescence. However, this does not explain the failure to detect F4/80 antigen on intestinal macrophages using immunocytochemistry and the ABC technique. It suggests that a factor in the isolation procedure (apart from the enzymes used) may affect the (presumably already low) F4/80 antigen expression. The mechanism probably involves the release of unspecified mediators during the disaggregation process, rather than being due to the various compounds used (EDTA, collagenase, Dispase, DNase etc.). This was not investigated further. An additional problem in assessing low level expression of markers on these cells was high autofluorescence and non-specific binding of both first and second antibodies.

A comparison of intestinal, splenic, alveolar and peritoneal macrophages showed considerable variation in levels of the F4/80 antigen consistent with other studies [348]. The expression of the F4/80 antigen tended to decline as Class II MHC antigen expression increased. Others have observed similar changes following macrophage activation, namely, down-regulation of the F4/80 antigen, the F_C receptor and the fucose-mannose receptor and increased Class II MHC antigen expression [109]. In spite of low levels of F4/80 antigen per cell on intestinal macrophages, quantitative studies of the level of F4/80 antigen in tissues suggest that the gut is the major repository of macrophages in the mouse [268].

In spite of the constitutive expression of Class II MHC antigens on lamina propria macrophages, these cells were weak stimulators of the MLR and were suppressive, in part, by an indomethacin-sensitive mechanism.

Initial studies on intestinal macrophages examined their morphological, ultra-structural, histochemical and immunohistochemical features and from these results function was inferred [reviewed in 310, 436, 468]. With the advent of techniques for the enzymatic disaggregation of intestinal mucosa their properties could be studied in vitro. The reported yields of macrophages from Peyer's patch are variable and range from <1% [218, 229, 287] to 10% [489]. Enzymatic methods of disaggregation resulted in up to ten times greater yields [122, 230, 287] and released cells capable of stimulating T and B cell responses [287, 393]. It is not clear why these large discrepancies should occur, but they are possibly related to the removal of surrounding lamina propria which does contain a large proportion of macrophages.

The surface antigen expression of isolated Peyer's patch macrophages has been examined in only two papers [247, 489]. The proportion expressing Ia molecules was 7-13% (immunofluorescence microscopy) and 65%

(immunoprecipitation) respectively. In comparison with peritoneal macrophages only a small proportion expressed F_c receptors.

Functional studies of Peyer's patch adherent cells are difficult to interpret. Some investigators have reported the ability to stimulate secondary T cell responses [287, 393] whilst others failed to demonstrate either primary or secondary responses [247]. These differences are partly explained by the observation that the MLR stimulator cells were non-adherent, low density cells [27, 433].

Prostaglandin-mediated suppression of immune responses

PGE_2 is a major product of the cyclo-oxygenase pathway of arachidonic acid metabolism in activated cells, particularly monocytes and macrophages. It binds to lymphocyte receptors and acts by stimulating adenylate cyclase and increasing intracellular cyclic AMP levels [372]. PGE_2 inhibits T cell activation both by inhibiting the increase in free calcium concentration in the cytosol [87] (with the resultant impairment of IL-2 production) and by inhibiting the activation of protein kinase C [87].

Lymphocyte functions inhibited by PGE_2 include the effector function of cytotoxic T cells and NK cells, and cellular proliferation in response to PHA and con A (but not PWM). Antigen- and mitogen-induced IL-2 release and IL-2-dependent proliferation are also inhibited. Preincubation of lymphoid cells with PGE_2 stimulates suppressor cells, an effect which may be mediated by a specific stimulatory effect of PGE_2 on the expression of IFN-γ receptors on $CD8^+$ cells [104]. The capacity of PGE_2 to inhibit proliferation may therefore be either due to a direct effect on lymphocyte proliferation or the result of the activation of suppressor cells [86].

Macrophages, the major source of PGE_2 in immune reactions, differ in their capacity to produce PGE_2 upon stimulation by activating agents. Systematic studies on intestinal macrophages have not been reported.

Indomethacin, in addition to inhibiting the cyclo-oxygenase pathway, also inhibits cAMP-dependent protein kinase and phosphodiesterase and may shunt the products of arachidonic acid metabolism to the lipoxygenase pathway.

An alternative means by which macrophages could inhibit MLR stimulatory activity is by the I-J-restricted induction of suppressor T cells and the production of T_S factors [17, 191, 337]. Particular problems relate to the nature of the I-J determinants. No polymorphic gene or coding element which maps to the I-J region has been identified and DNA probes spanning the I-J region fail to hybridize with mRNA in I-J$^+$ somatic T cell hybrids [reviewed in 337]. A number of mechanisms to explain the absence of an I-J-coded protein product have been suggested. These include the proposal that I-J molecules represent novel T cell receptors that recognize self-Class II molecules and/or receptors for Class II molecules, or that the I-J region controls the post-translational modification of Class II MHC antigens or other proteins. The mechanisms involved have not been elucidated and much work needs to be done to clarify these issues.

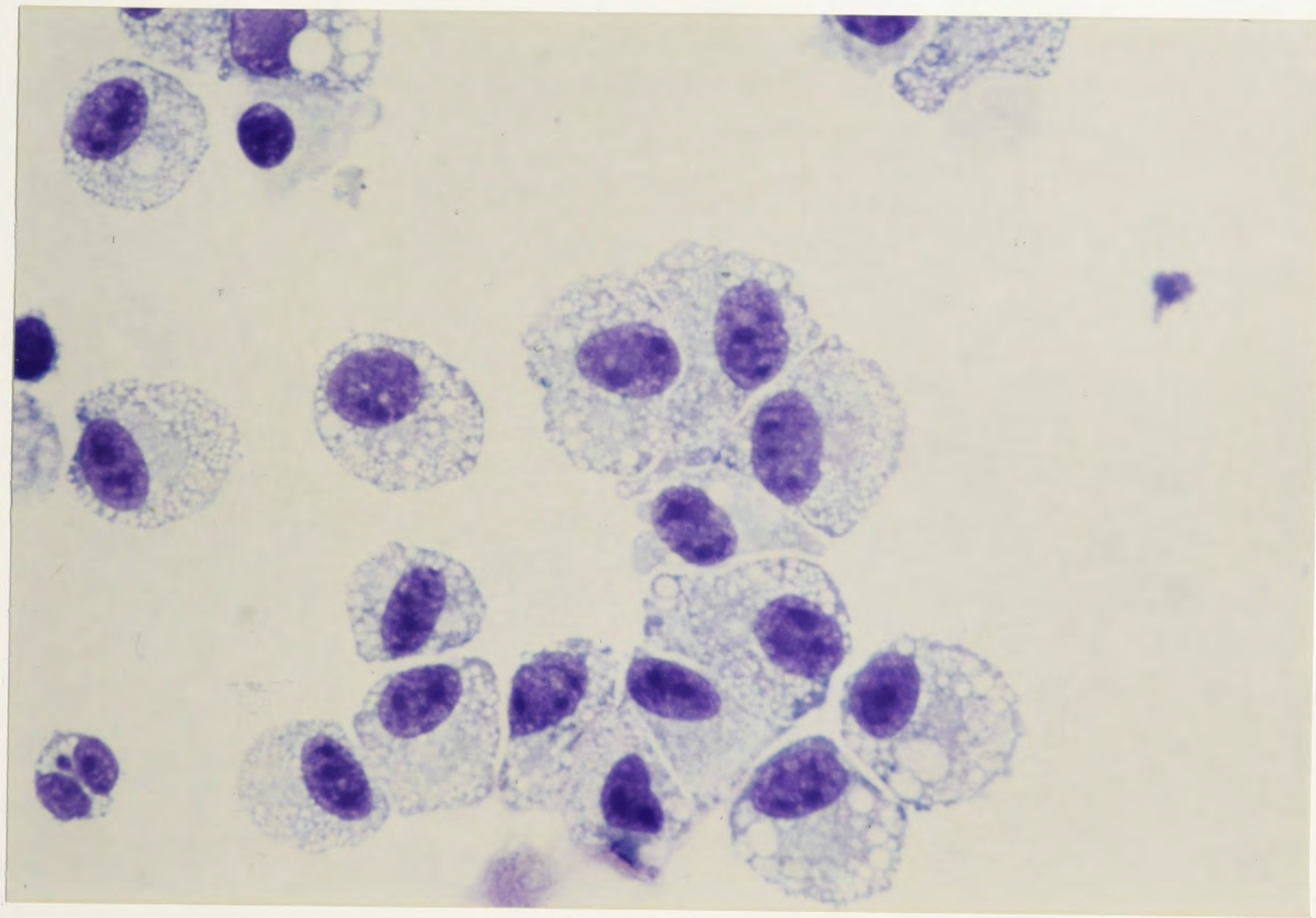
LEGENDS TO FIGURES

Figure 3.1

Cytocentrifuge slides of fibronectin-adherent lamina propria cells stained with Diff-Quik (x 630).

Figure 3.2

Immunofluorescence microscopy of fibronectin-adherent lamina propria cells. Cells were adhered onto glass slides and incubated for 2 h with fluorescent-labelled latex beads (1μ diameter). Over 90% of cells were phagocytic.



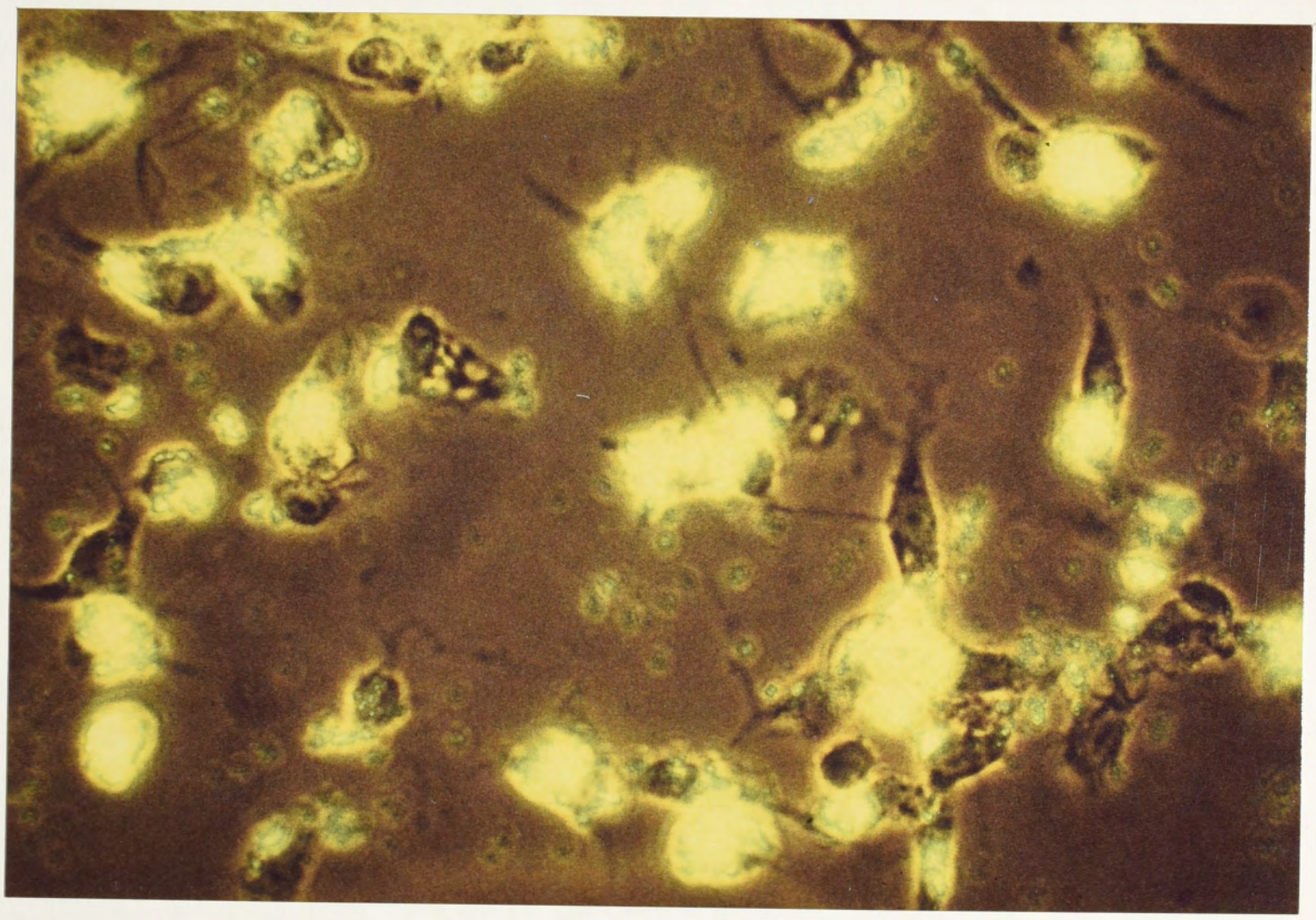
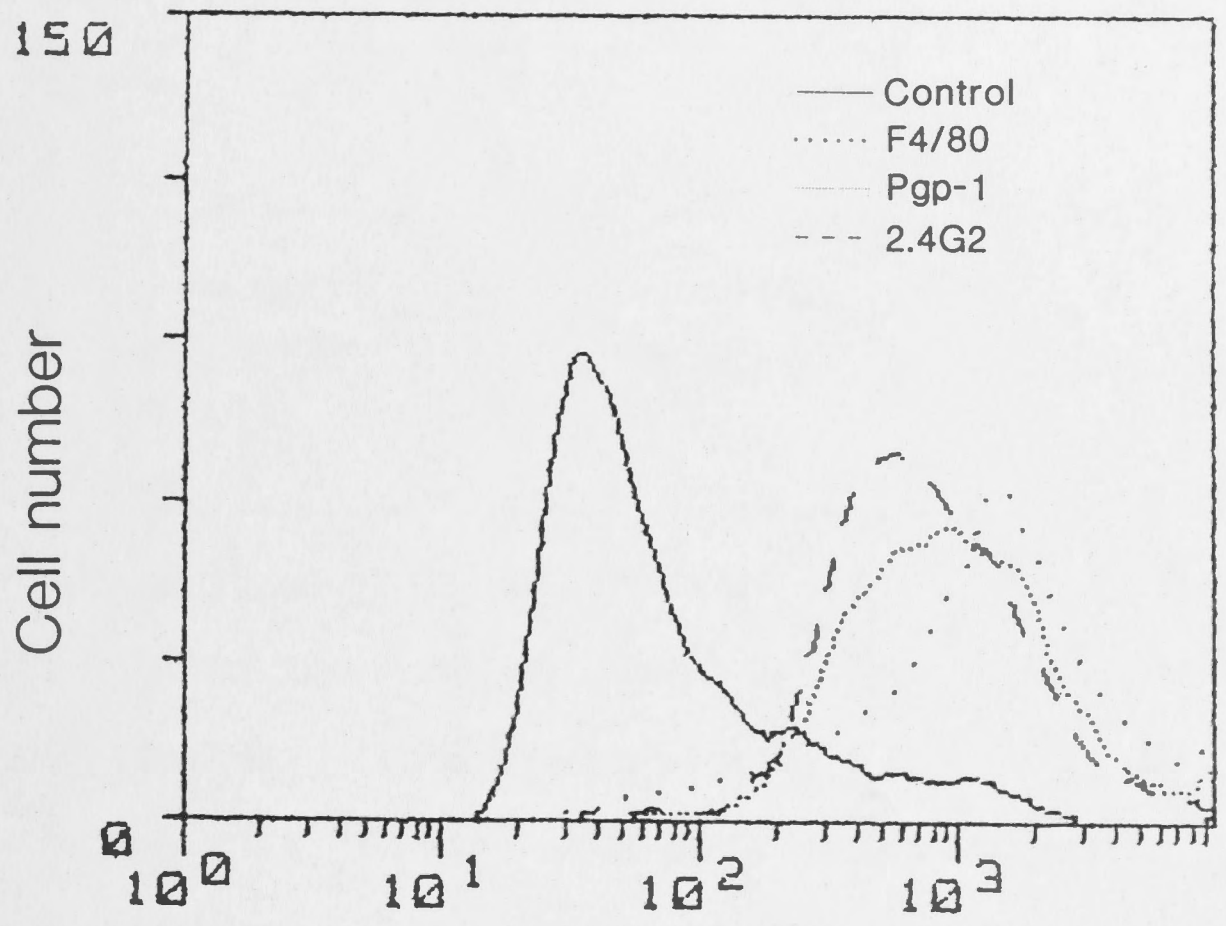


Figure 3.3

Flow cytometric analysis of antigen expression by fibronectin-adherent cells. (a) Fibronectin-adherent resident peritoneal cells from C57BL/6 mice were incubated with no antibody (control), F4/80, Pgp-1 or 2.4G2; then washed and labelled with FITC-conjugated $(Fab')_2$ goat anti-rat IgG and analysed by flow cytometry. Greater than 90% of the cells expressed high levels of F4/80, Pgp-1 and 2.4G2. (b) Fibronectin-adherent small intestinal lamina propria cells were labelled as for (a). Expression of F4/80 and 2.4G2 was much lower than on peritoneal macrophages and, if present, was difficult to detect because of high levels of nonspecific staining.

Peritoneal macrophages



Lamina propria macrophages

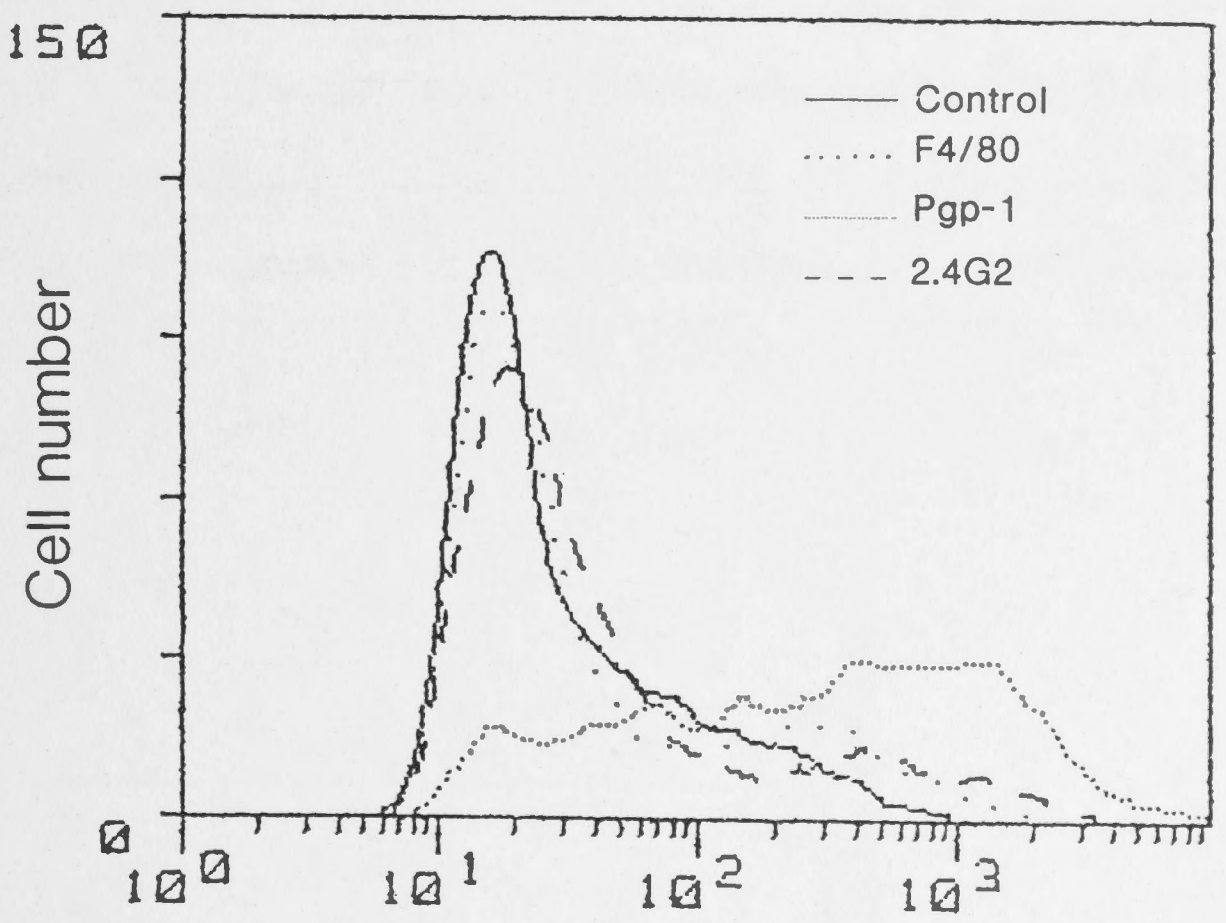


Figure 3.4

Phenotypic analysis of fibronectin-adherent splenic, alveolar and peritoneal cells using flow cytometry. (a) Fibronectin-adherent splenic macrophages were labelled as for Figure 3.3 using no antibody (control), TIB120 (anti-Ia) and F4/80. (An isotype-matched control gave binding identical to the no primary antibody control.) The mean fluorescence intensity for the whole population is indicated. The vertical line represents the fluorescence intensity above which only 5% of the no antibody and/or isotype-matched control cells were found. A cell expressing greater fluorescence intensity than this value was considered positive. (b) Fibronectin-adherent alveolar macrophages were labelled as in Figure 3.3 using no primary antibody (control), TIB120 and F4/80. (c) Fibronectin-adherent peritoneal cells labelled as above.

Macrophage Phenotype

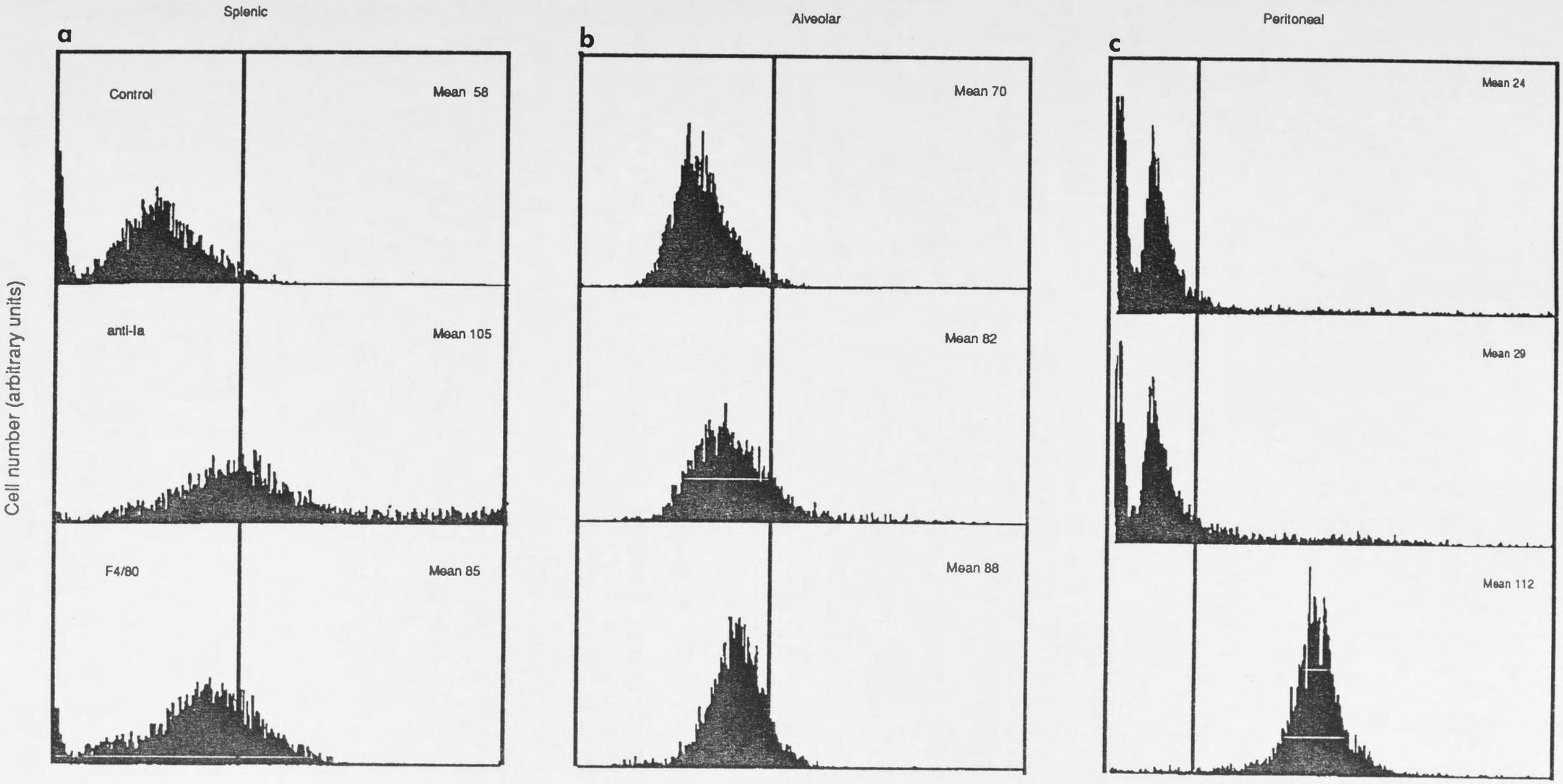


Figure 3.5

Flow cytometry of mesenteric lymph node responder cells. Mesenteric lymph node cells were incubated with TIB120 (anti-Class II MHC antibody), panned, then labelled with the specified antibodies, using the methods described in Figure 3.3. About 70% of the cells were L3T4$^+$ whilst 30% were Lyt2$^+$. The cells also expressed Pgp-1 as demonstrated by the shift of the curve to the right. The other curves were no different from the control (no primary antibody). Antibodies used included anti-Ia (TIB120), J11d (B cell) and F4/80 (macrophage).

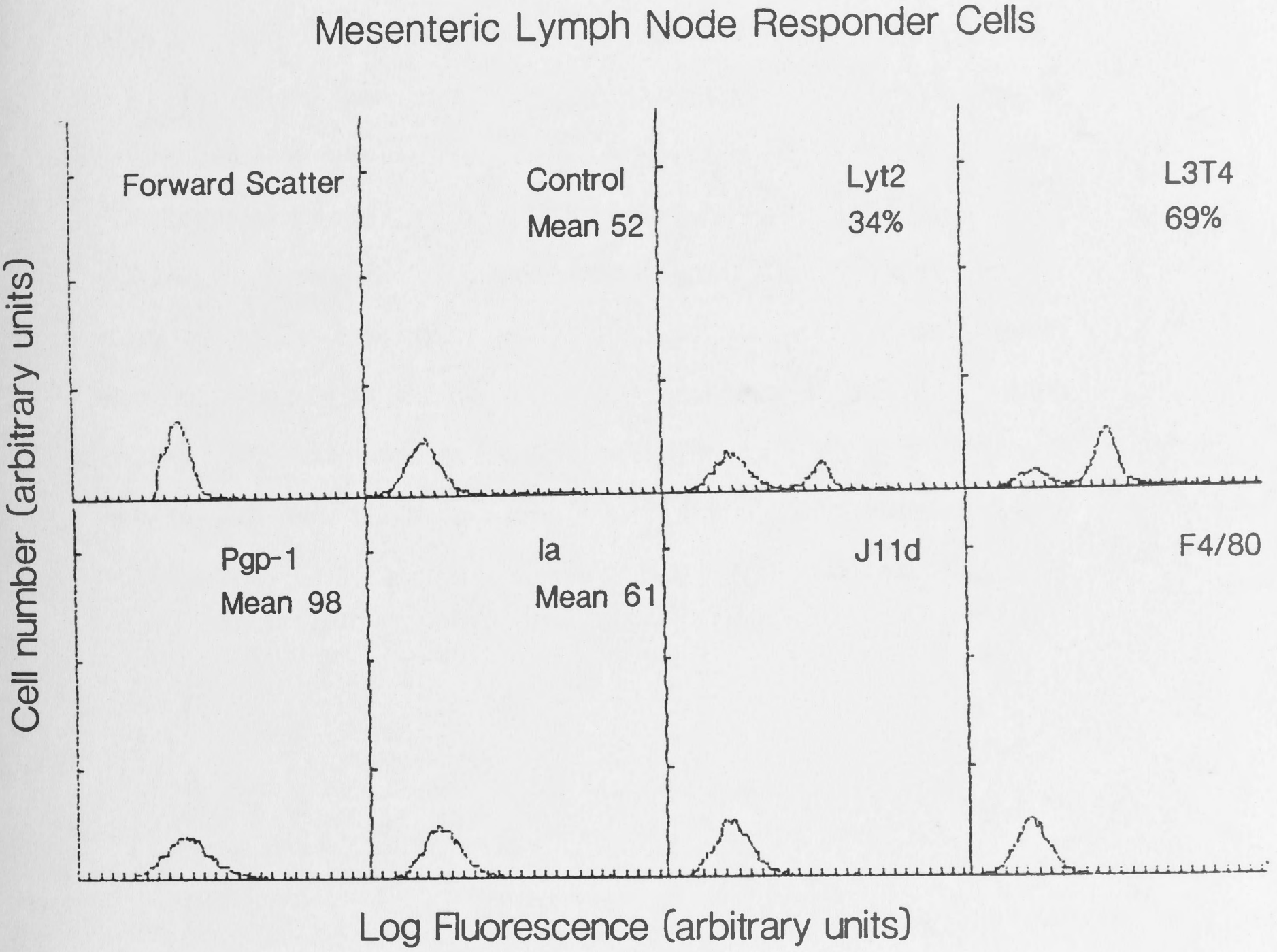

Mesenteric Lymph Node Responder Cells
Forward Scatter
Control
Mean 52
Lyt2
34%
L3T4
69%
Pgp-1
Mean 98
Ia
Mean 61
J11d
F4/80
Cell number (arbitrary units)
Log Fluorescence (arbitrary units)

Figure 3.6

MLR stimulation by lamina propria and Peyer's patch cells in the presence and absence of indomethacin. The abscissa represents increasing numbers of unfractionated small intestinal lamina propria cells (squares) or Peyer's patch cells (triangles) that were incubated in the presence (closed symbols) or absence (open symbols) of indomethacin (2 μg/ml). Lamina propria cells from C57BL/6 mice were added to $2x10^5$ mesenteric lymph node cells, depleted of Ia-bearing cells, from BALB/c mice. After 4 days, 1 μCi of [^{3}H]-thymidine was added to each microculture and the cells were freeze-thawed and harvested onto glass fibre discs after a further 16 h. Unstimulated responder cells and control stimulator cells incorporated $<$1000 cpm and $<$500 cpm respectively. The values are the means $\pm$ S.E. of triplicate assays.

Figure 3.7

MLR stimulatory activity: Comparison of unfractionated intestinal lamina propria cells, intestinal adherent cells and peritoneal macrophages in the presence and absence of indomethacin. The experiment was performed as described in Figure 3.6. Increasing numbers of lamina propria non-adherent cells (squares), adherent cells (diamonds) and peritoneal macrophages (inverted triangles) were added to $2x10^5$ MLN cells in the presence (closed symbols) and absence (open symbols) of indomethacin (2 μg/ml).

Figure 3.6

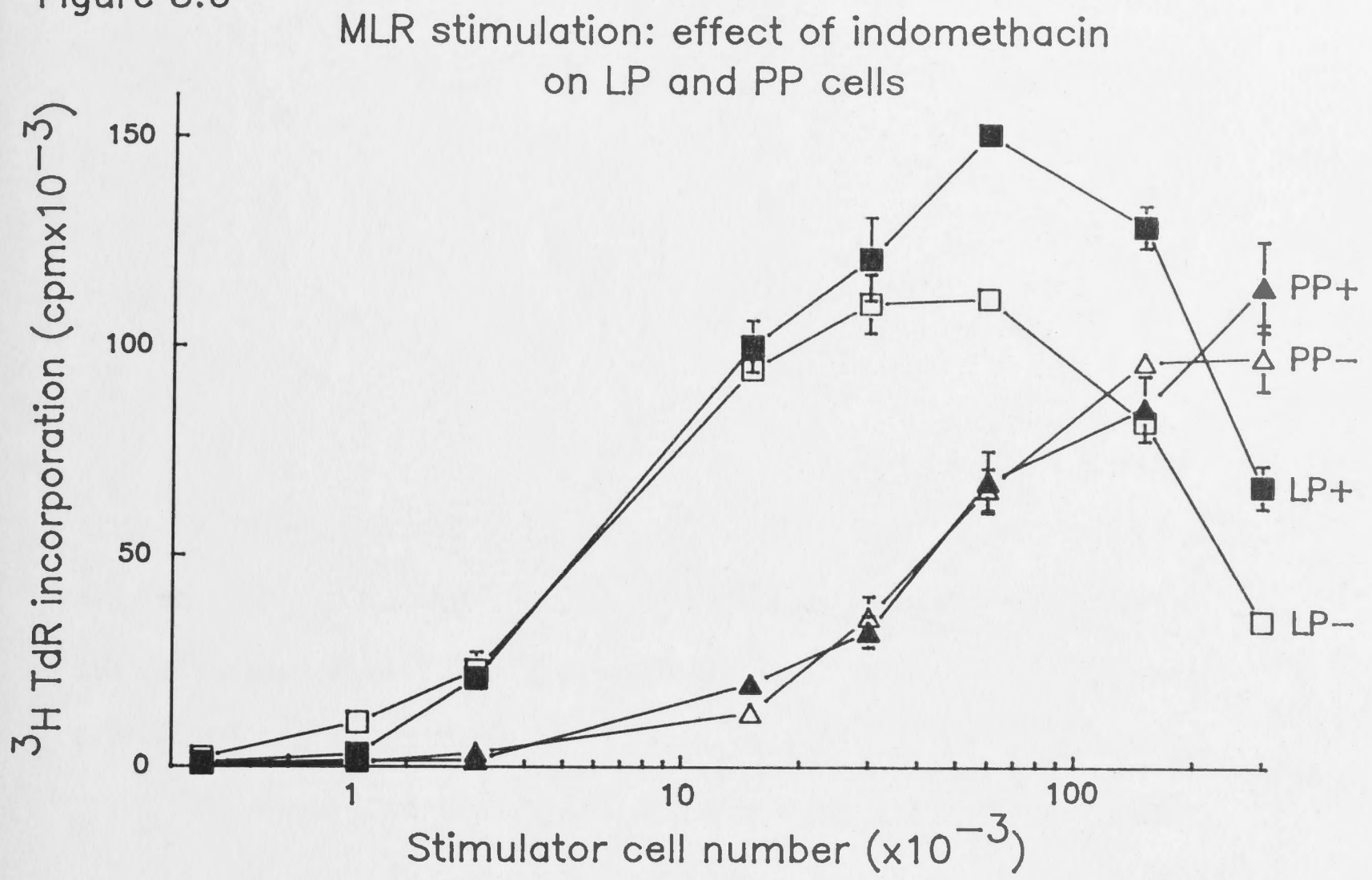


Figure 3.7

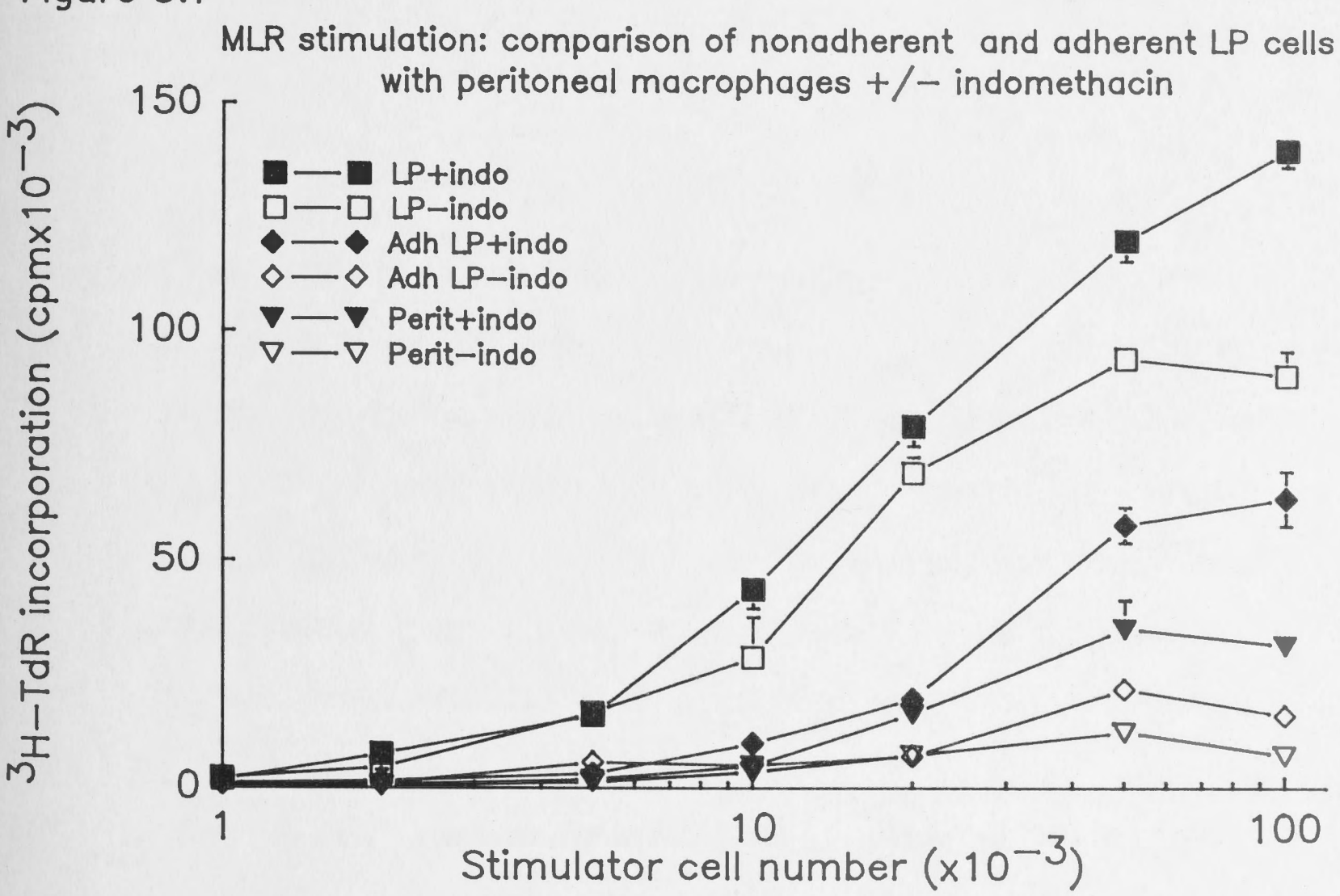


Figure 3.8

The effect of prostaglandin E2 (PGE_2) on MLR stimulation. The abscissa represents increasing amounts of PGE_2 added to microcultures containing $2x10^5$ MLN cells together with $2x10^4$ lamina propria cells (squares) or $2x10^5$ Peyer's patch cells (triangles). The MLR was performed as described in Figure 3.6.

Figure 3.9

Lamina propria macrophage-induced suppression of the MLR and the effects of indomethacin. The experiment was performed as described in Figure 3.6. Increasing numbers of lamina propria macrophages (enriched by fibronectin adherence) were added to $2x10^5$ purified mesenteric lymph node responder cells cultured with $1x10^5$ non-adherent lamina propria cells (squares) or $1x10^5$ Peyer's patch cells (triangles) in the presence (closed symbols) or absence (open symbols) of indomethacin (2 μg/ml).

Figure 3.8

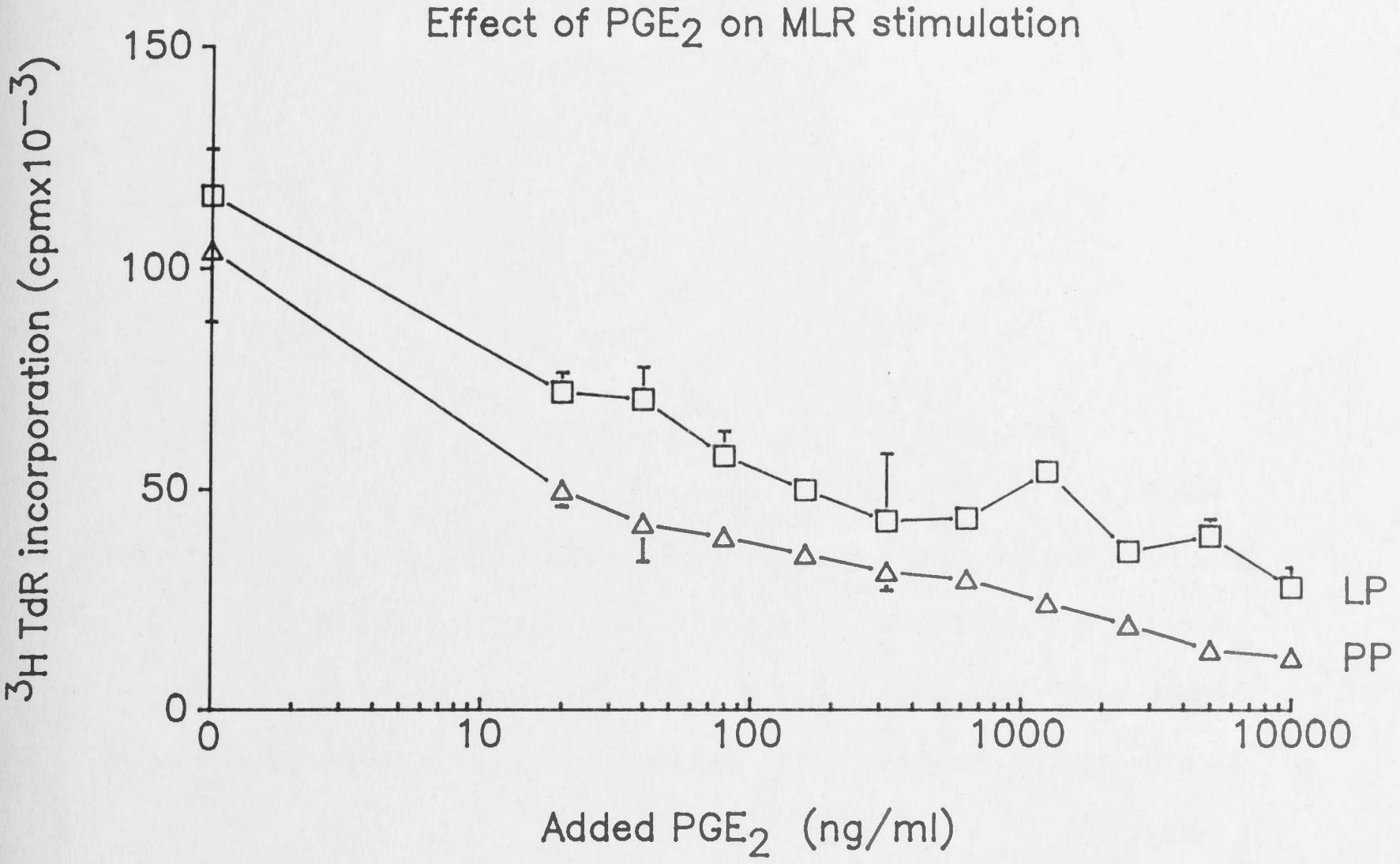


Figure 3.9

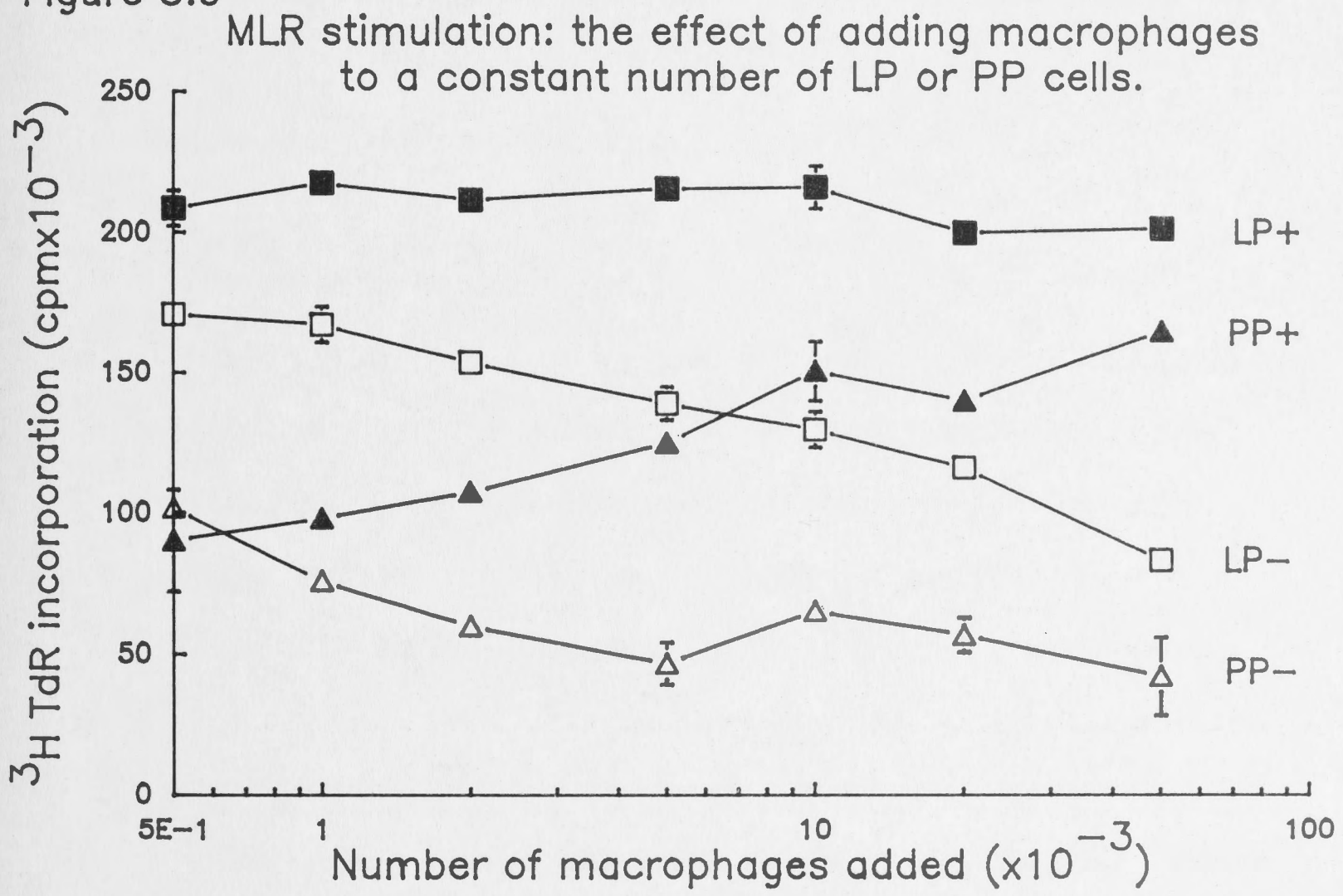


Figure 3.10

Lamina propria macrophage-induced suppression of the MLR and the effect of indomethacin. The experiment was performed as described in Figures 3.6 and 3.9 except that $5x10^4$ non-adherent lamina propria cells were used (squares). This number resulted in submaximal MLR stimulation.

Figure 3.11

Suppression of LPS-induced proliferation by lamina propria macrophages. The abscissa represents increasing amounts of LPS added to $5x10^4$ Peyer's patch (triangles) or lamina propria (squares) cells in the presence (closed symbols) or absence (open symbols) of $2x10^4$ intestinal macrophages.

Figure 3.10

MLR stimulation: the effect of adding macrophages to a constant number of nonadherent LP cells

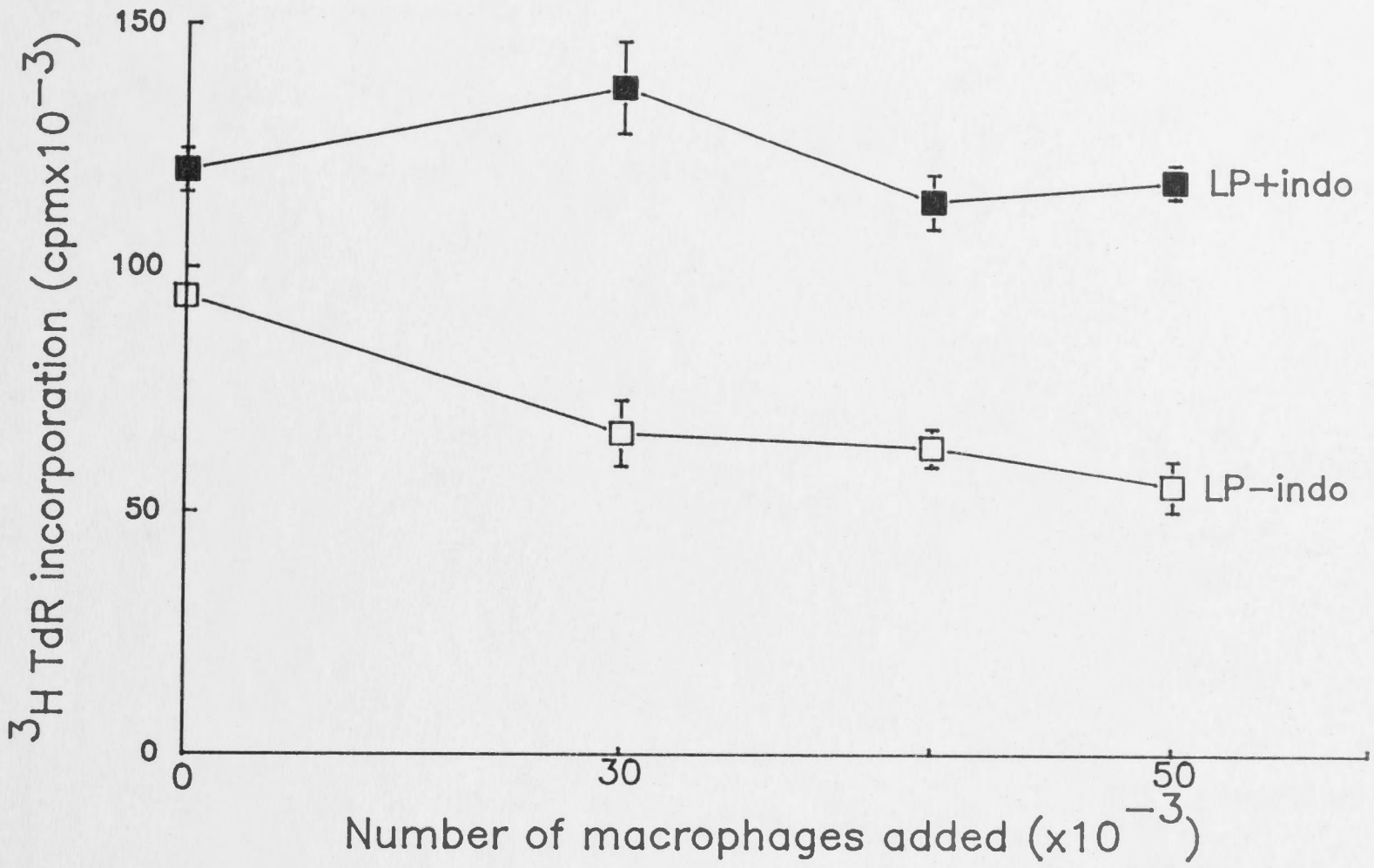


Figure 3.11

LPS−induced proliferation: effect of macrophages

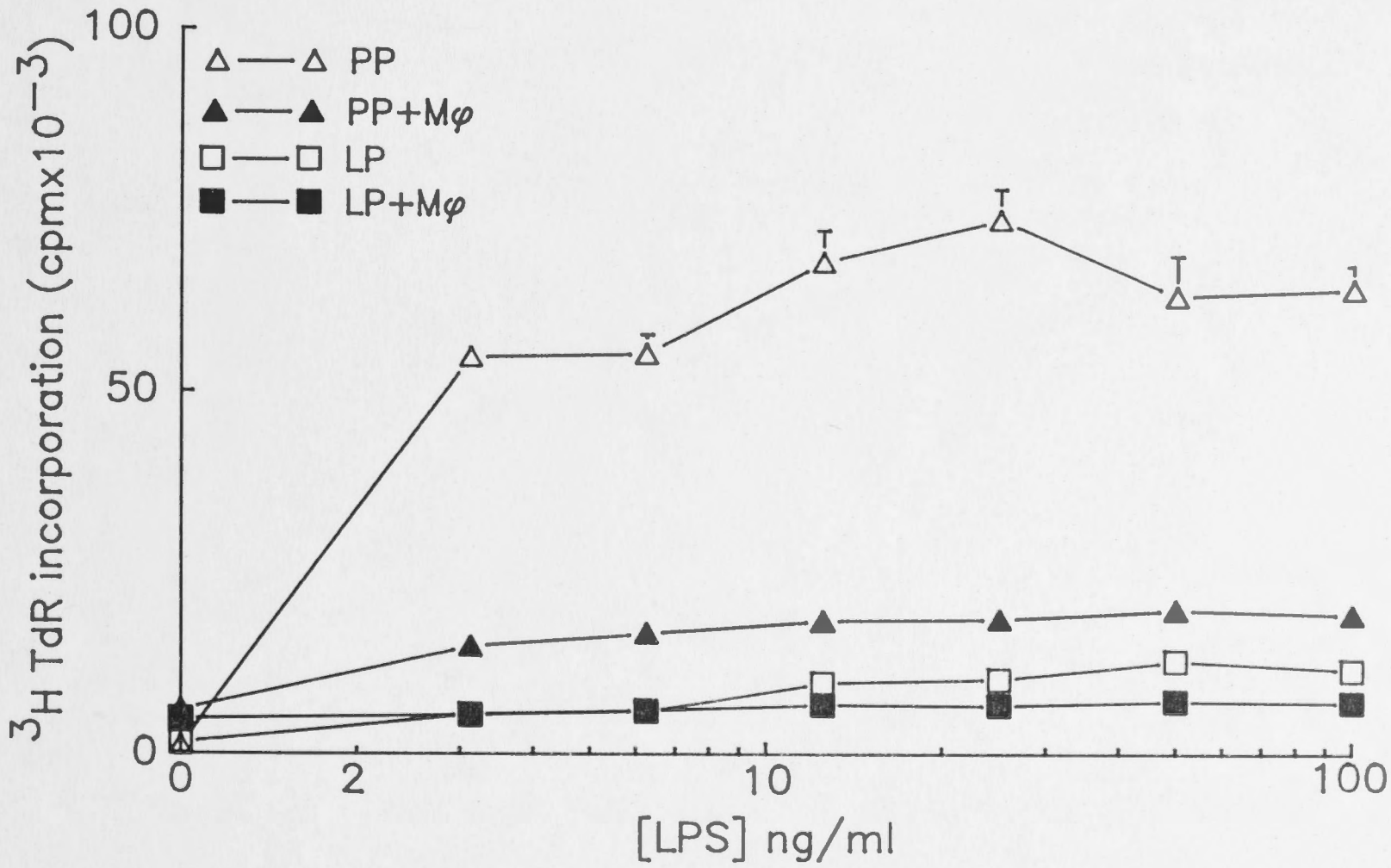


Figure 3.12

A comparison of MLR stimulatory activity of non-adherent and adherent lamina propria cells and the effect of indomethacin. The experiment was carried out as described in Figure 3.6. Increasing numbers of non-adherent (to fibronectin) (open squares) or fibronectin-adherent with (open diamonds) or without (closed diamonds) indomethacin (2 μg/ml) were added to purified mesenteric lymph node responder cells.

Figure 3.12

MLR Stimulation: LP nonadherent and adherent cells.

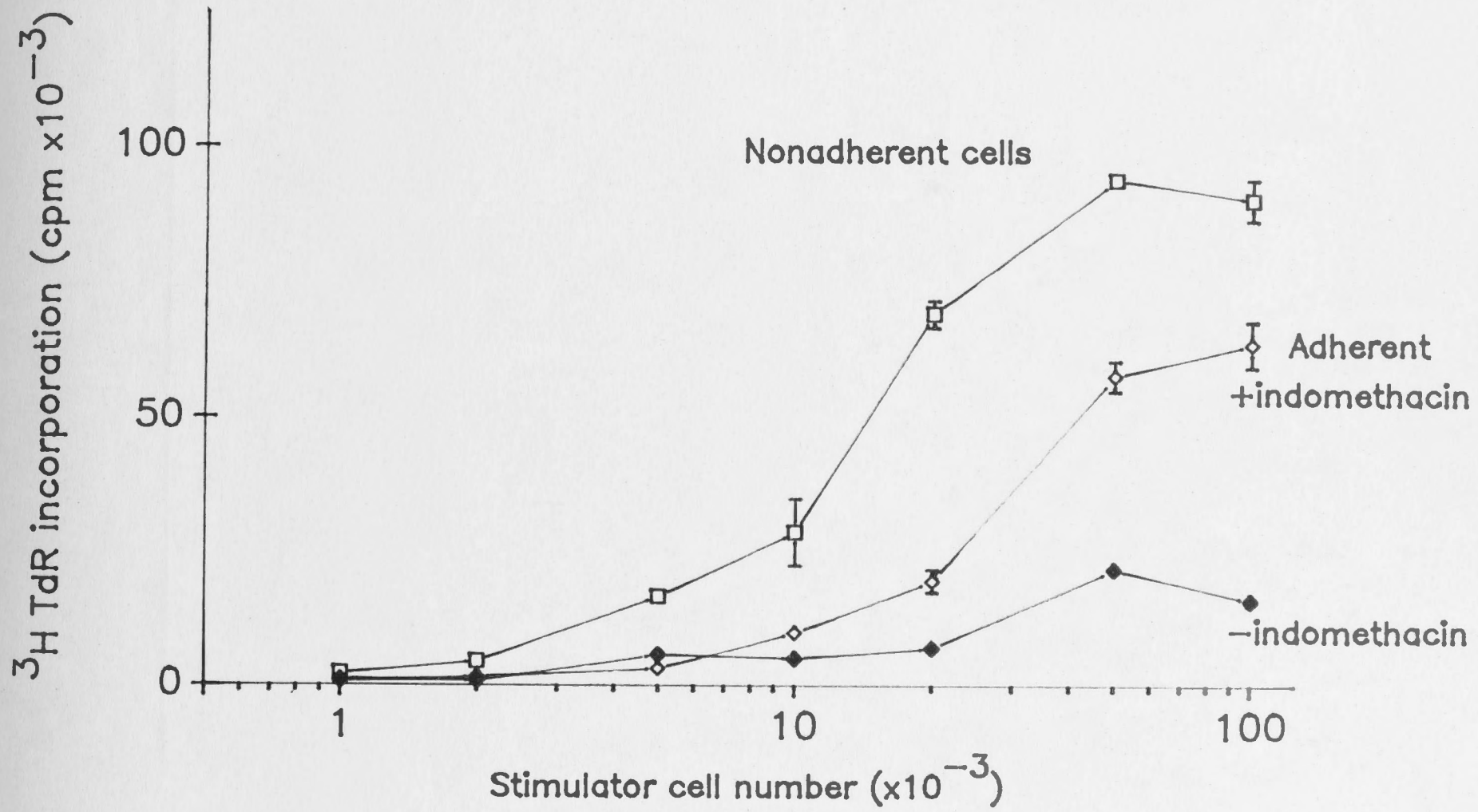
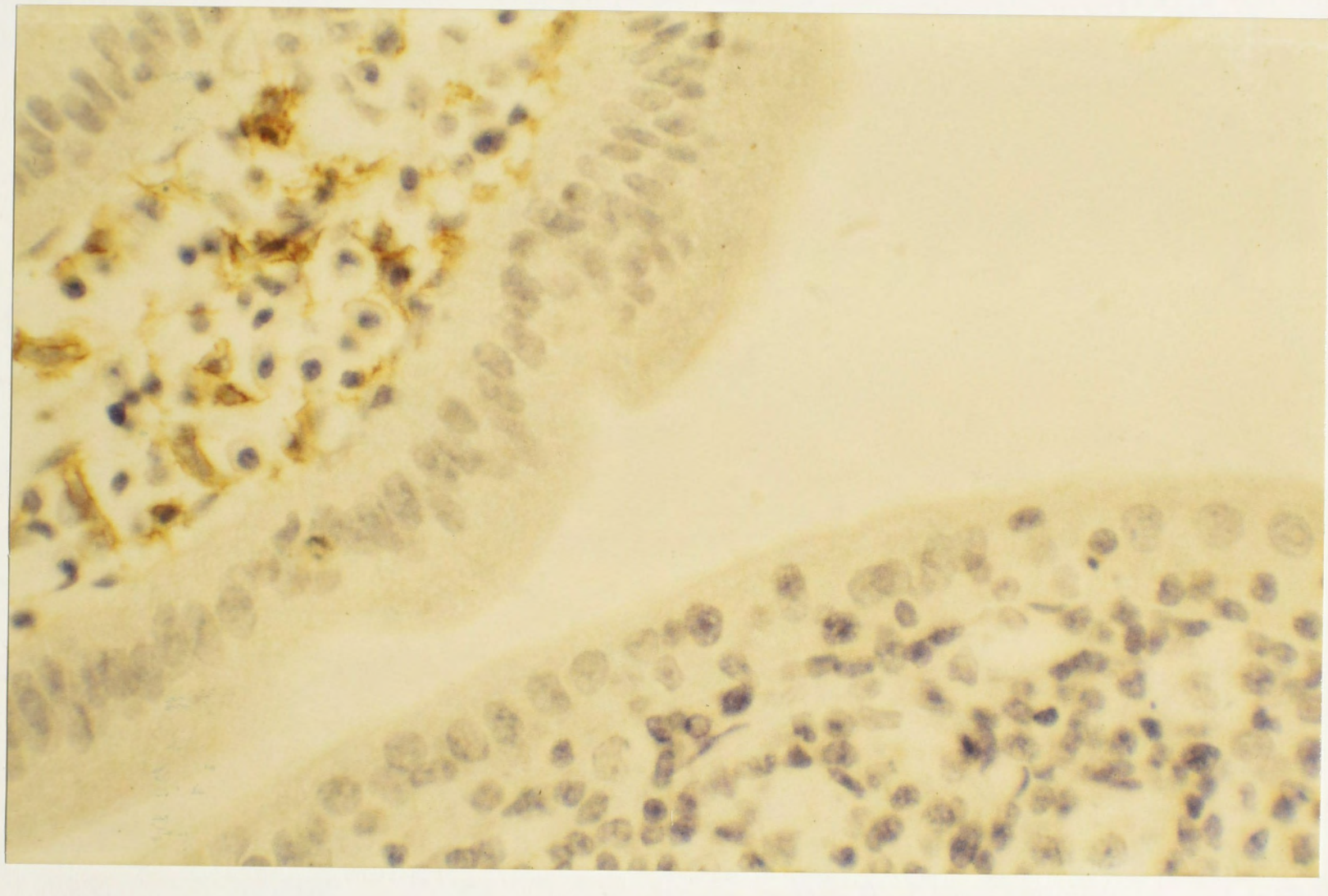


Figure 3.13

Immunoperoxidase staining of a section through a murine small intestinal villus (upper left) and a Peyer's patch (lower right) using the anti-macrophage antibody, F4/80. The methods are described in references 169, 172 and 173, and are summarized in the legend to Figure 1.2. The slides were prepared by Dr DA Hume. $F4/80^+$ cells can be identified only in the small intestinal villus.



CHAPTER 4

INTESTINAL LAMINA PROPRIA DENDRITIC CELLS

"seek, and ye shall find;"

St Matthew

4.1 INTRODUCTION

Cells with the morphology and phenotype of veiled cells have been described in Peyer's patches from mouse [433], rat, guinea pig and pig [509]. Their presence in non-Peyer's patch lamina propria was also demonstrated in the guinea pig and pig but not in the rat [509]. The differences between species is not explained. Human intestinal dendritic cells will be considered in Chapter 5.

Functional studies on dendritic cell-enriched populations from Peyer's patches showed that these cells were low-density, non-adherent and potent accessory cells in the oxidative mitogenesis model [433]. A particularly interesting observation is that dendritic cell-T cell clusters originating from Peyer's patches preferentially induced splenic or Peyer's patch B cells to secrete IgA. [434, 435]. The mechanism is as yet unexplained.

In this chapter, a method for obtaining populations of MLR stimulatory dendritic cells from murine intestinal lamina propria and Peyer's patches is described. These cells were as potent as splenic dendritic cells in their ability to stimulate the MLR.

4.2 METHODS

The materials and methods used for obtaining adherent cell-depleted single cell suspensions from the intestinal lamina propria or Peyer's patches were as described in Chapters 2 and 3 unless otherwise stated.

4.2.1 Cell Separation

Disaggregated lamina propria or Peyer's patch cells at concentrations of 10-20x10^6/ml were layered onto 2-3 ml Nycodenz (Nyegaard, Oslo, Norway) and centrifuged at 600 g for 15 min at 20^{o}C. Cells at the interface or in the pellet were washed and resuspended in RPMI for further experiments.

4.2.2 Splenic dendritic cells

Splenic dendritic cells were obtained by a modification of a previously described technique [447]. Spleens from C57BL/6 mice were minced by pushing through a fine wire grid, washed and resuspended in serum-free media. The cells were seeded at 3x10^6/ml in 185 cm^2 tissue flasks (Nunclon, Roskilde, Denmark) at 50 ml per flask, and allowed to adhere for 2 h at 37^{o}C. Non-adherent cells were removed by washing with HBSS and remaining adherent cells recultured in complete medium (F15 with 5% FCS and 10^{-5}M 2-mercaptoethanol) overnight. Non-adherent or loosely adherent cells were harvested by gentle pipetting and treated with anti-Thy 1.2 antibody (Serotec, Oxford, UK) and rabbit complement (Low Tox, Cedarlane, Ontario, Canada) with DNase 30 μg/ml. Dead cells were removed by separation on Isopaque-Ficoll (Pharmacia). To remove surface immunoglobulin- and F_C-receptor-bearing cells, the cell suspension was then rosetted with sheep erythrocytes coupled by chromium chloride ($CrCl_3$) to hyperimmune sheep anti-mouse immunoglobulin (Division of Cell Biology, JCSMR). Following further separation on Ficoll, splenic dendritic cells were obtained. Yields from the spleen were 0.5-1% [368]. The cells were >85% positive for expression of Class II MHC antigens by flow cytometry (Fig. 4.1).

4.2.3 Electron microscopy

Cells were fixed in 3% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) and processed for scanning and transmission electron microscopy as described by Bucana et al, (1983) [70]. The cells were examined with a JEOL 1200X scanning electron microscope or a JEOL 1200EX transmission electron microscope.

4.3 RESULTS

4.3.1 Enrichment of MLR stimulator cells

The lamina propria and Peyer's patch cells that had been depleted of fibronectin-adherent macrophages could be further enriched for MLR-stimulatory activity by density gradient centrifugation. The yield of cells harvested at the Nycodenz interface (density <1.068 g/ml) was 2.7% of the starting cell number from both sites (see Table 4.1). These cells had 20-50x greater MLR-stimulatory activity than the initial, unfractionated lamina propria cell suspensions (Fig. 4.2).

4.3.2 MLR stimulator cells in the colon

Because of initial concern over the problem of eliminating bacterial contamination, fewer studies of lamina propria cells from the large intestine were performed. As noted above, the yield of cells from the small and large intestinal lamina propria was comparable. In experiments in which cells from small and large intestine of the same animals were compared the MLR-stimulating activity was not distinguishable (see below).

4.3.3 Comparison of lamina propria, Peyer's patch and splenic dendritic cells

Isolated dendritic cells from the lamina propria or Peyer's patches were not distinguishable in terms of the number of cells required to induce a maximal MLR (Fig. 4.3). Morphologically, they are large cells which have oval, irregular or pleiomorphic nuclei and a basophilic cytoplasm. Characterization is best done by phase-contrast microscopy, immunofluorescence or by immunocytochemistry, where they can be identified by their morphology and the expression of Class II MHC antigens (Fig. 4.4). Like splenic dendritic cells, they expressed very high levels of Class II MHC proteins with low, but detectable, levels of F4/80, Pgp-1 (Figs. 4.1 and 4.5), and the dendritic cell specific marker, 33D1 (not shown). Other markers, in particular, surface immunoglobulin, were negative. The majority of the remaining cells in the intestinal low density fractions (20-

35% of the total cell number at cytofluorometry) were Thy-1$^+$. The low density cells from the lamina propria of either the small or large intestine were greatly enriched for MLR-stimulatory activity (Fig. 4.6).

When studied by electron microscopy, the cells exhibited the cytological features described previously for lymphoid dendritic cells. The nucleus was irregular with a peripheral rim of heterochromatin and small nucleoli. The cytoplasm contained well-developed mitochondria and scattered smooth vesicles but no evidence of phagolysosomes. The surface of the cells observed with the scanning electron microscope showed characteristic cytoplasmic processes often observed in cells from afferent lymphatics and referred to as veils [233] (Micrographs 4.1a and 4.1b).

The function of dendritic cell-enriched intestinal cell populations was compared with that of splenic dendritic cells (Fig. 4.7). MLR stimulatory activity was almost identical. When equal numbers of splenic and lamina propria dendritic cells were mixed together, there was no evidence of either enhanced or suppressed activity. Treatment of splenic dendritic cells with the enzyme cocktail used for disaggregation of the lamina propria for 2-3 h did not alter their MLR stimulatory activity (not shown).

4.4 DISCUSSION

This study demonstrates the presence of MLR stimulatory cells in both the Peyer's patches, as described previously [433], and in the non-Peyer's patch lamina propria. These cells resemble the lymphoid dendritic cell first described by Steinman and Cohn (1973) [443], in their surface phenotype (Ia-positive, absence of typical macrophage, T cell and B cell markers), their physical properties (low density, weakly or non-adherent, non-phagocytic) and their cytological and ultrastructural features (absence of secondary lysosomes, pleiomorphic nucleus).

Intestinal macrophages could be differentiated from dendritic cells on the basis of morphology, function (macrophages are phagocytic, adherent overnight in culture, and suppress MLR stimulation), and the expression of Class II MHC antigens (dendritic cells express very high levels, Fig. 4.8). The MLR stimulator cells differed further from macrophages in failing to adhere to fibronectin.

Other studies have demonstrated opposing actions of dendritic cells and macrophages on T cell responses in vitro and in vivo. For example, hapten-specific tolerance was observed when haptenated, Ia^+-macrophages were injected intravenously. This macrophage-induced tolerance could be overcome by subsequent injection of haptenated dendritic cells [64]. (See also Chapter 3).

Cells resembling dendritic cells have been isolated from afferent lymph draining the mesenteric nodes of the rat [290, 300, 311, 378] and other species. These so-called veiled cells, or non-lymphoid cells, express high levels of Class II MHC antigens and are potent antigen-presenting cells in vitro and in vivo [264, 290, 300, 311, 378]. Like lamina propria dendritic cells, they lack most defineable macrophage markers [290]. Veiled cells in the rat and dendritic cells in the mouse are bone marrow-derived and have high rates of turnover [378, 449]. If

lamina propria dendritic cells are the precursors of similar cells in mouse afferent lymph, their turnover would be expected to be similarly rapid.

There was consistently 5-10 times greater MLR stimulatory activity in the unfractionated lamina propria cell suspensions when compared to Peyer's patches. However, the yield of dendritic cells from the two sources was similar (Table 4.1) and maximal MLR stimulation occurred with similar numbers of purified stimulator cells from both the lamina propria and Peyer's patches (Fig. 4.3). In the unfractionated cell suspensions, the difference may represent a genuine decrease in Peyer's patch dendritic cell activity due to a deficiency of macrophage-derived factors. This possibility is supported by the data (Fig. 3.9) showing that macrophages can increase the sensitivity of the MLR to Peyer's patch cells. This effect may be mediated by macrophage production of IL-1 which amplifies dendritic cell function [241].

When compared to splenic dendritic cells, lamina propria dendritic cells had similar MLR stimulatory activity (Fig. 4.7). These observations are consistent with those of Spalding et al, (1983) [433] who demonstrated that splenic and Peyer's patch dendritic cells had equivalent T cell stimulatory ability in the oxidative mitogenesis assay. In contrast, dendritic or Langerhans cells of the epidermis are weak stimulators of the MLR upon initial isolation [181] but in the presence of granulocyte/ macrophage-colony stimulating factor (GM-CSF) and IL-1, in vitro rapidly mature into potent MLR stimulators [156, 515]. Tissue variations in cytokine production may account for the differences in maturity of dendritic cells at these sites. GM-CSF is produced constitutively by cells of the human intestinal lamina propria [380], but levels were not compared with those of the epidermis. An alternative explanation is that the isolation process produced different effects on cell maturation.

Table 4.1 Cell yields (% starting cell number) (range)

	Whole intestine	LP	PP
Fibronectin-adherent	3-5	10 (6-13)	<0.1
Non-adherent	41 (32-57)	30 (22-44)	55 (22-90)
High density* (>1.068 g/ml)	22 (15-31)	15 (9-20)	27 (12-37)
Low density (<1.068 g/ml)	3 (2-6)	2 (1-3)	2 (1-3)

* The cells were predominantly small lymphocytes.

Table 4.1 details the yields at different stages of the isolation procedure as a percentage of the starting cell number. LP refers to the yields from lamina propria from which Peyer's patches have been removed and PP indicates the yields from Peyer's patches alone.

LEGENDS TO FIGURES

Figure 4.1.

Flow cytometric analysis of antigen expression by splenic dendritic cells. Splenic dendritic cells from C57BL/6 mice were incubated with no antibody (control), anti-Ia (TIB 120), F4/80 or Pgp-1 (PGP); then washed and labelled with FITC-conjugated $(Fab')_2$ goat anti-rat IgG and analysed by flow cytometry. The abscissa represents fluorescence intensity (logarithmic scale, arbitrary units) and the y axis, cell number (linear scale, arbitrary units). The mean level of fluorescence intensity for each population of cells is indicated. The proportion of cells positive for each marker is also given. (The level of fluorescence at which a cell was considered to be positive was arbitrarily defined as that level which was not exceeded by >5% of the control population.) Over 90% of the cells expressed high levels of Ia. The cells expressed intermediate levels of the Pgp-1 antigen, and low levels of the F4/80 antigen.

Splenic dendritic cells

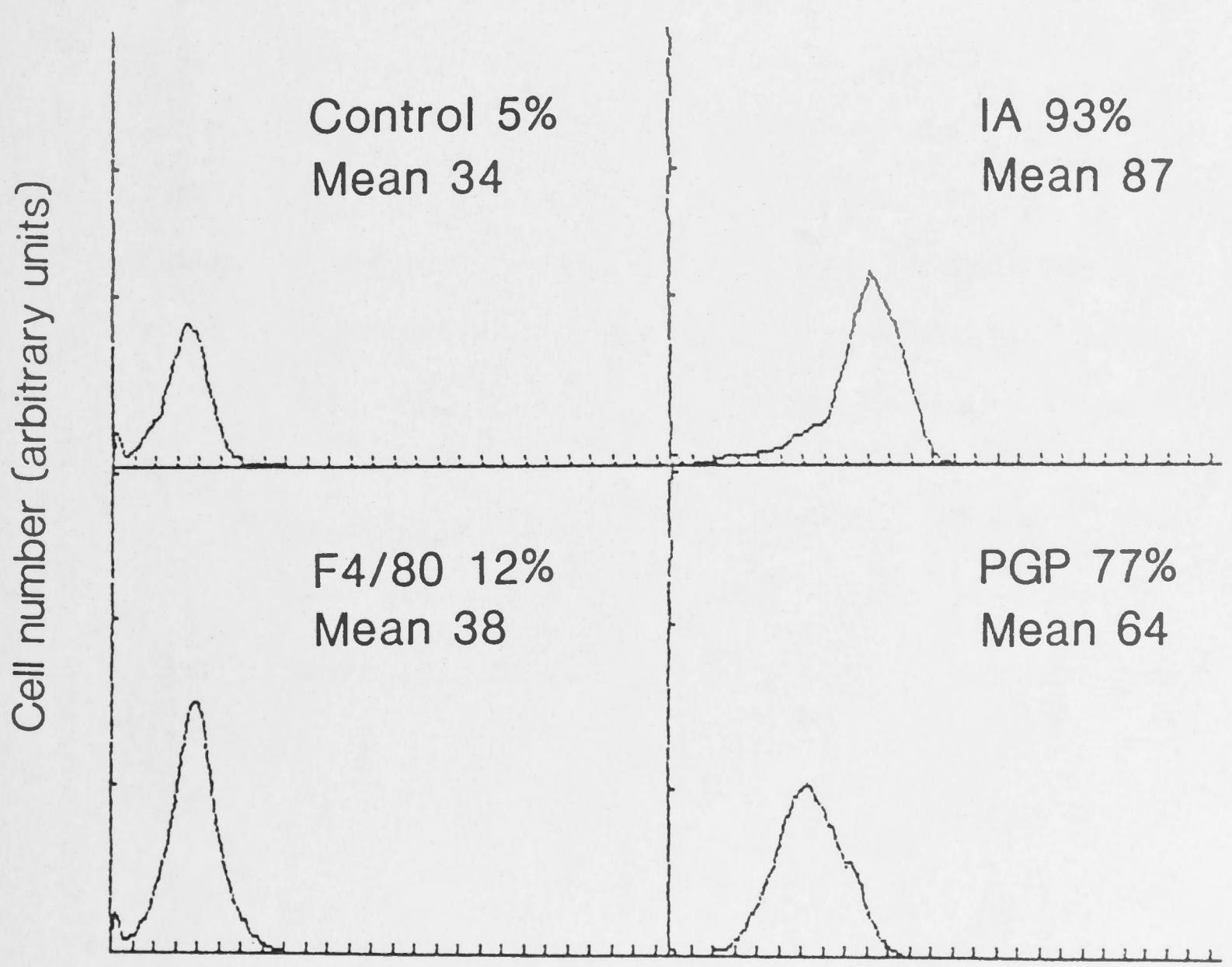


Figure 4.2.

MLR stimulation by lamina propria dendritic cells and unfractionated cells. The abscissa represents increasing numbers of unfractionated small intestinal lamina propria cells (open squares), lamina propria cells that were fibronectin-non-adherent and low density (closed squares) and splenic dendritic cells (closed diamonds). Lamina propria cells from C57BL/6 mice were added to $2x10^5$ mesenteric lymph node cells, depleted of Ia-bearing cells, from BALB/c mice. After 4 days, 1 μCi of tritiated thymidine was added to each microculture and the cells were freeze-thawed and harvested onto glass fibre discs after a further 16 h. Unstimulated responder cells incorporated <1000 cpm and irradiated stimulator cells <200 cpm.

Figure 4.3.

Comparison of MLR stimulation by dendritic cell-enriched populations from Peyer's patches and intestinal lamina propria. The experiment was performed as described in Figure 4.2. Closed triangles, Peyer's patch dendritic cell-enriched populations; closed squares, lamina propria dendritic cell-enriched populations.

Figure 4.2

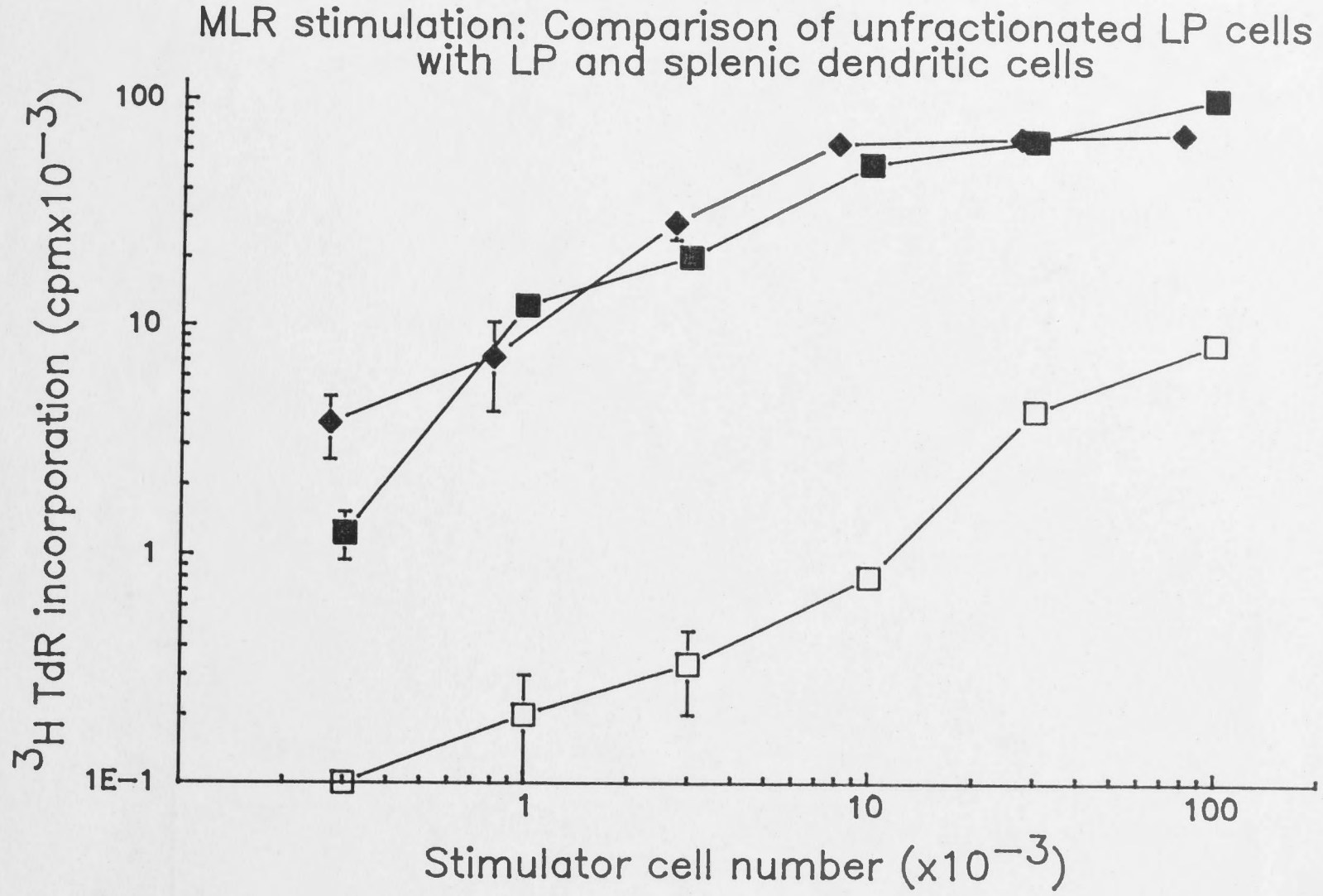


Figure 4.3

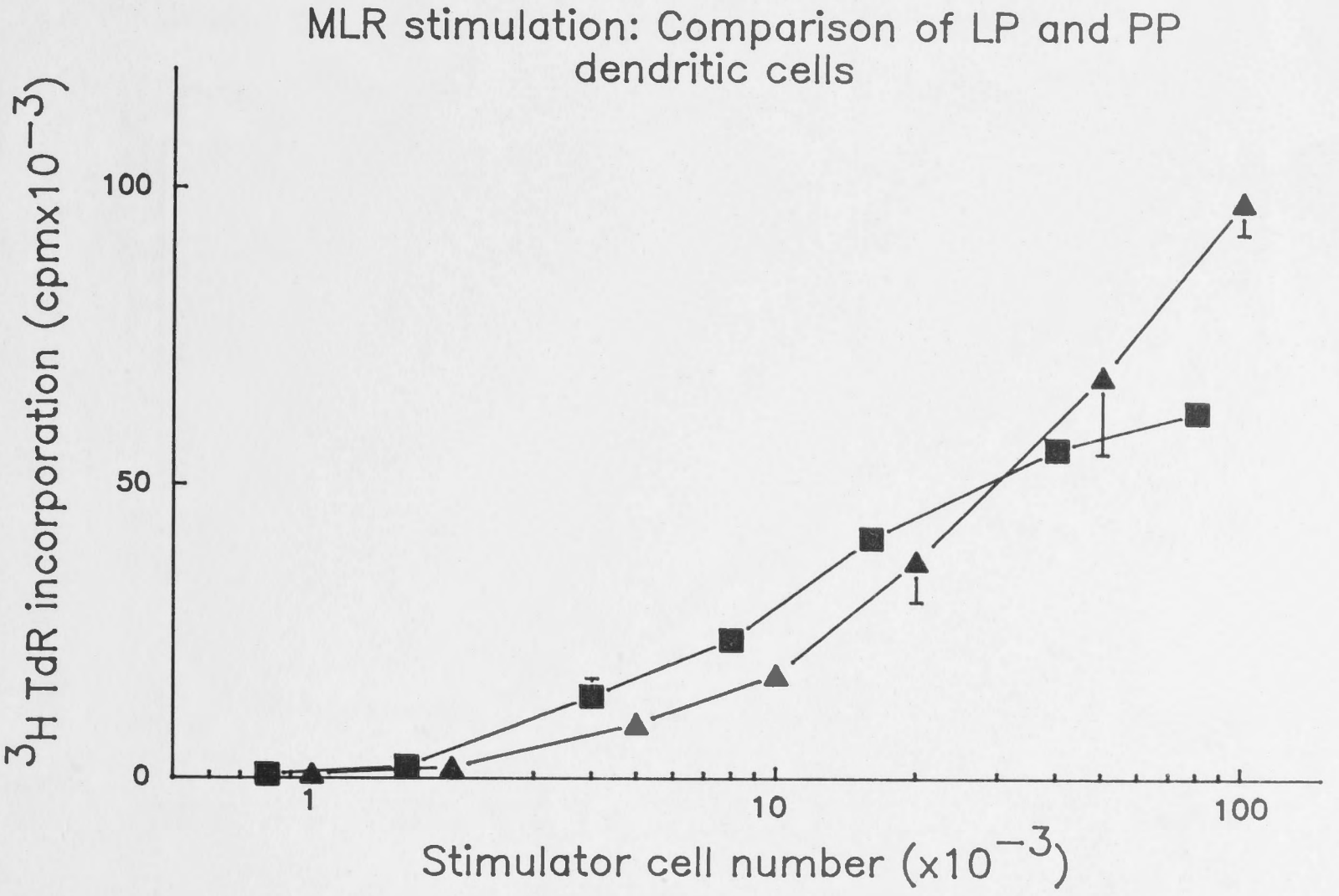
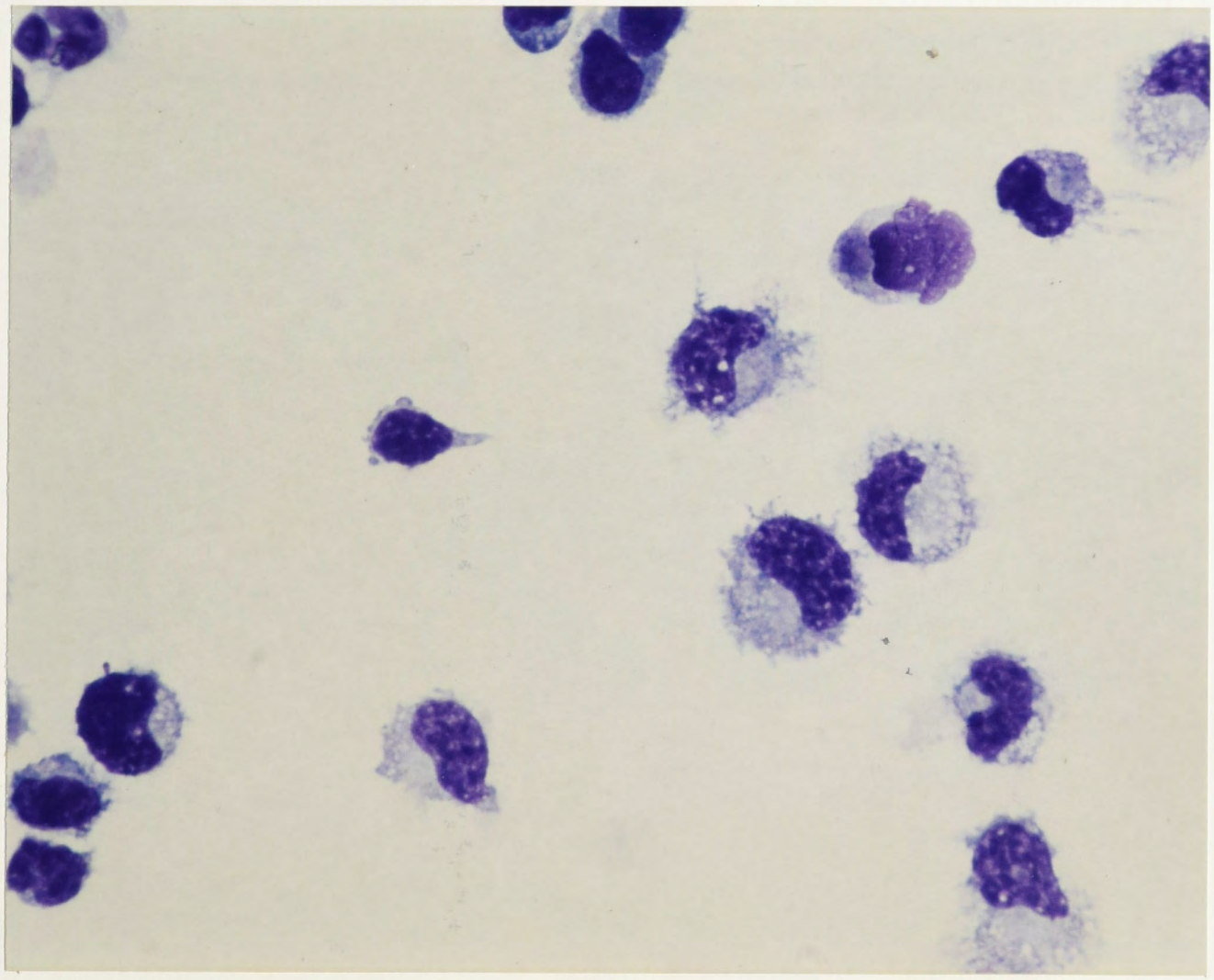


Figure 4.4.

Immunocytochemistry of lamina propria dendritic cell-enriched populations. Cytospin slides of dendritic cell-enriched populations from the lamina propria were (a) stained with a modified Giemsa stain (Diff-Quik); or (b) incubated sequentially with TIB120 (anti-Class II MHC) antibody, biotinylated sheep anti-rat immunoglobulin and avidin-biotin-peroxidase complex, developed with diaminobenzidine and counterstained. Dendritic cells were large irregular cells with oval or pleiomorphic nuclei.

a



b

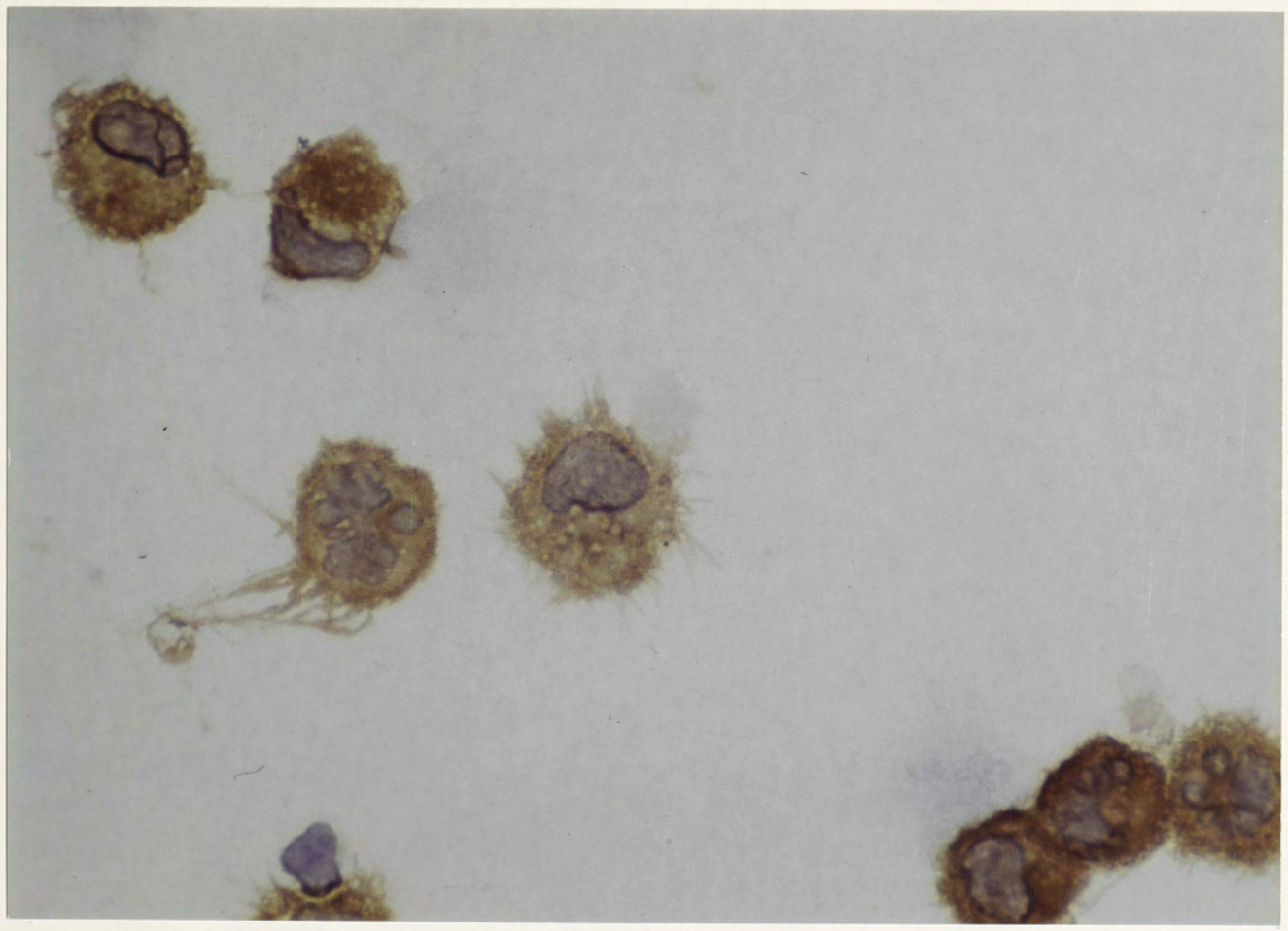


Figure 4.5.

Flow cytometric analysis of antigen expression by fibronectin-non-adherent low density cells. The experiment was performed as in Figure 4.1 using dendritic cell-enriched populations from C57BL/6 mice. Over 70% of the cells expressed high levels of Class II MHC antigens. The cells were negative for surface immunoglobulin (using FITC-conjugated goat anti-mouse IgG) (not shown).

Lamina propria dendritic cells

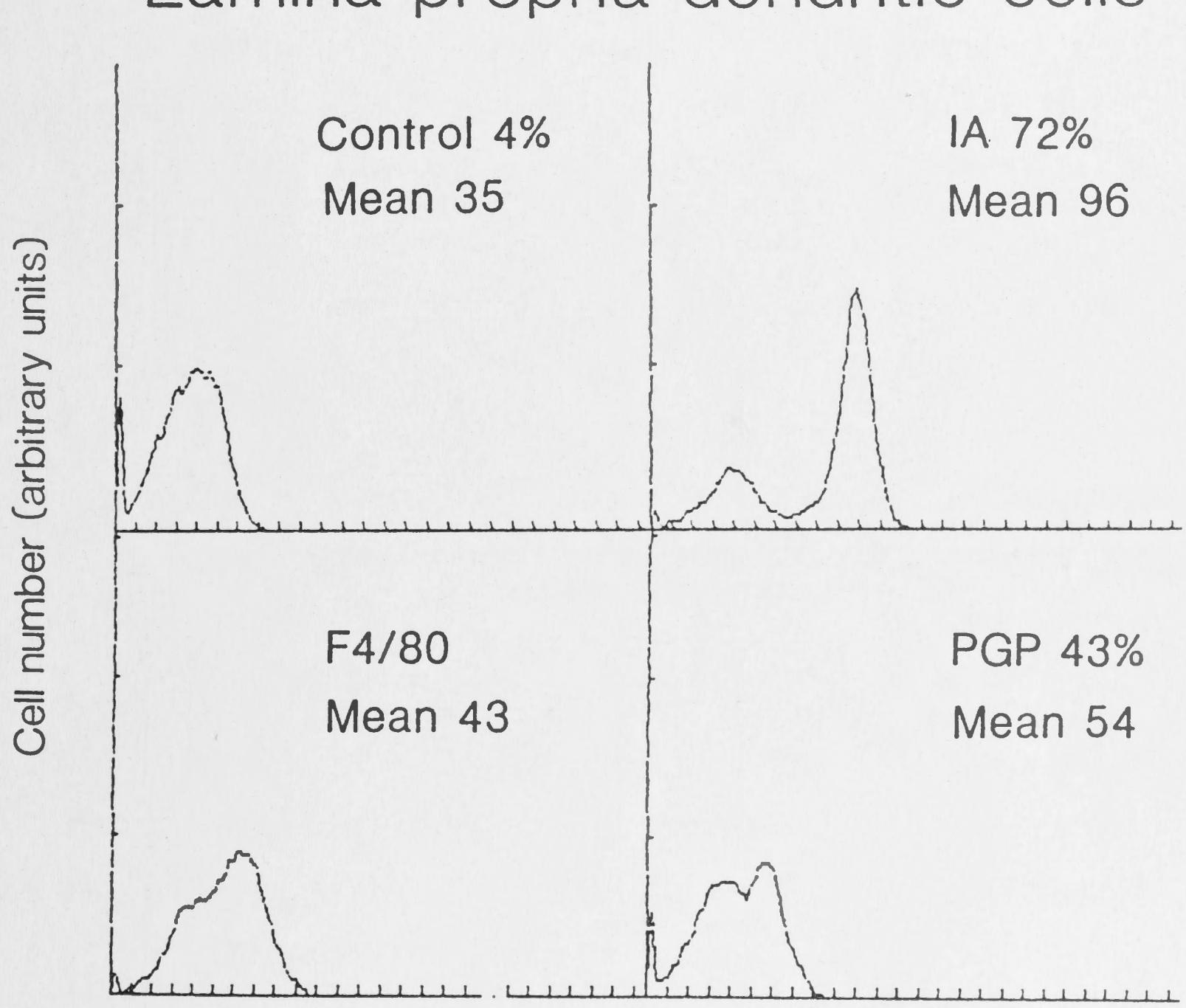


Figure 4.6.

Fractionation of lamina propria dendritic cells from small or large intestine. Fibronectin-non-adherent cells from the small intestinal- (SI, squares) or large intestinal- (colon, inverted triangles) lamina propria were separated into low density (<1.068 g/ml, open symbols) or high density (>1.068 g/ml, closed symbols) fractions by centrifugation over a Nycodenz density gradient. Increasing numbers of cells were added to $2x10^5$ purified mesenteric lymph node responder cells and the experiment was performed as described in Figure 4.2.

Figure 4.7.

Comparison of MLR stimulation by lamina propria and splenic dendritic cells. The experiment was performed as described in Figure 4.2. Closed squares, lamina propria dendritic cell-enriched populations (60-80% Ia^+); closed diamonds, splenic dendritic cells; closed inverted triangles, equal numbers of splenic and lamina propria dendritic cells.

Figure 4.6

MLR Stimulation: Small intestinal and colonic low and high density lamina propria cells

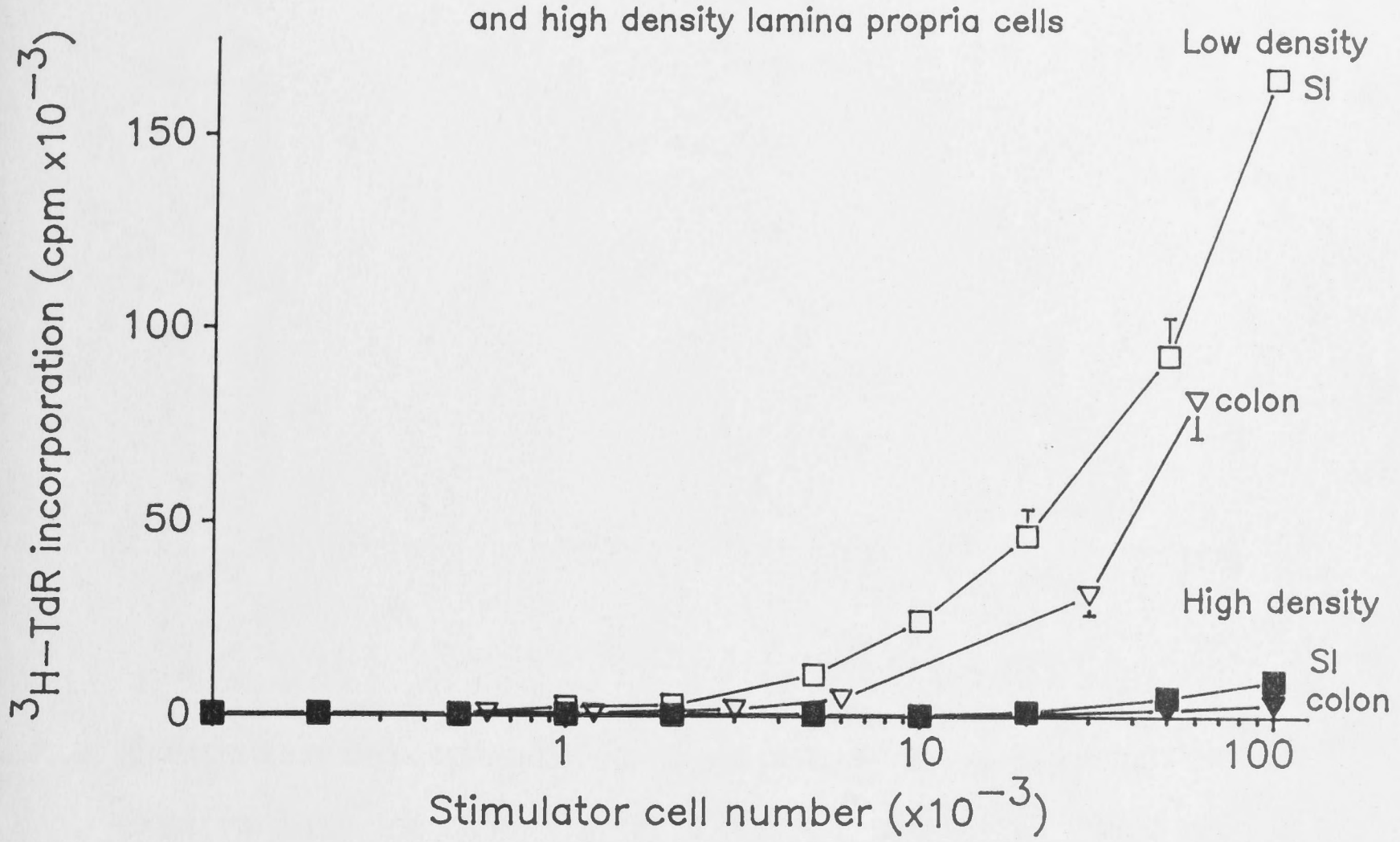


Figure 4.7

MLR stimulation: Comparison of intestinal and splenic dendritic cells

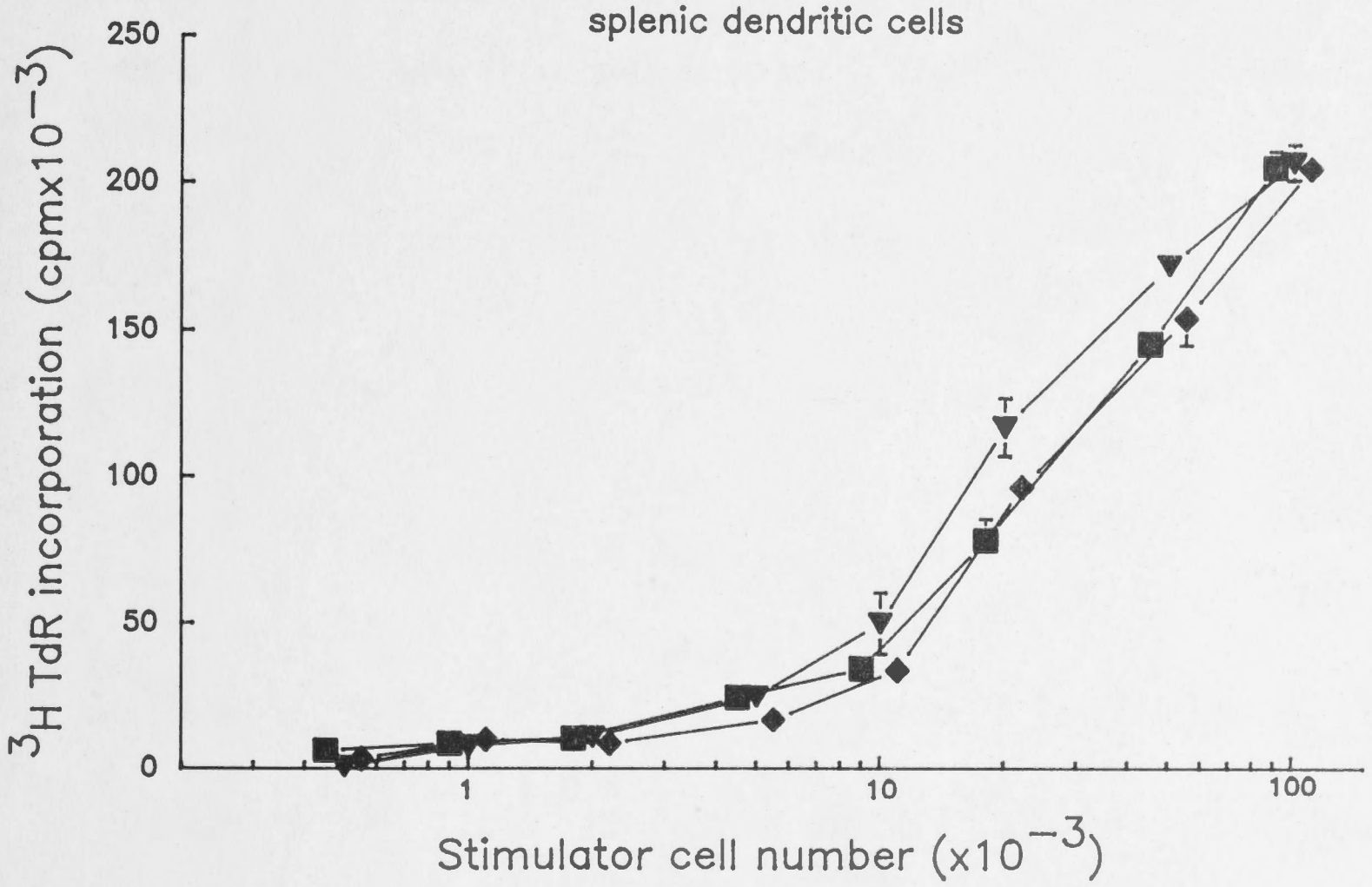
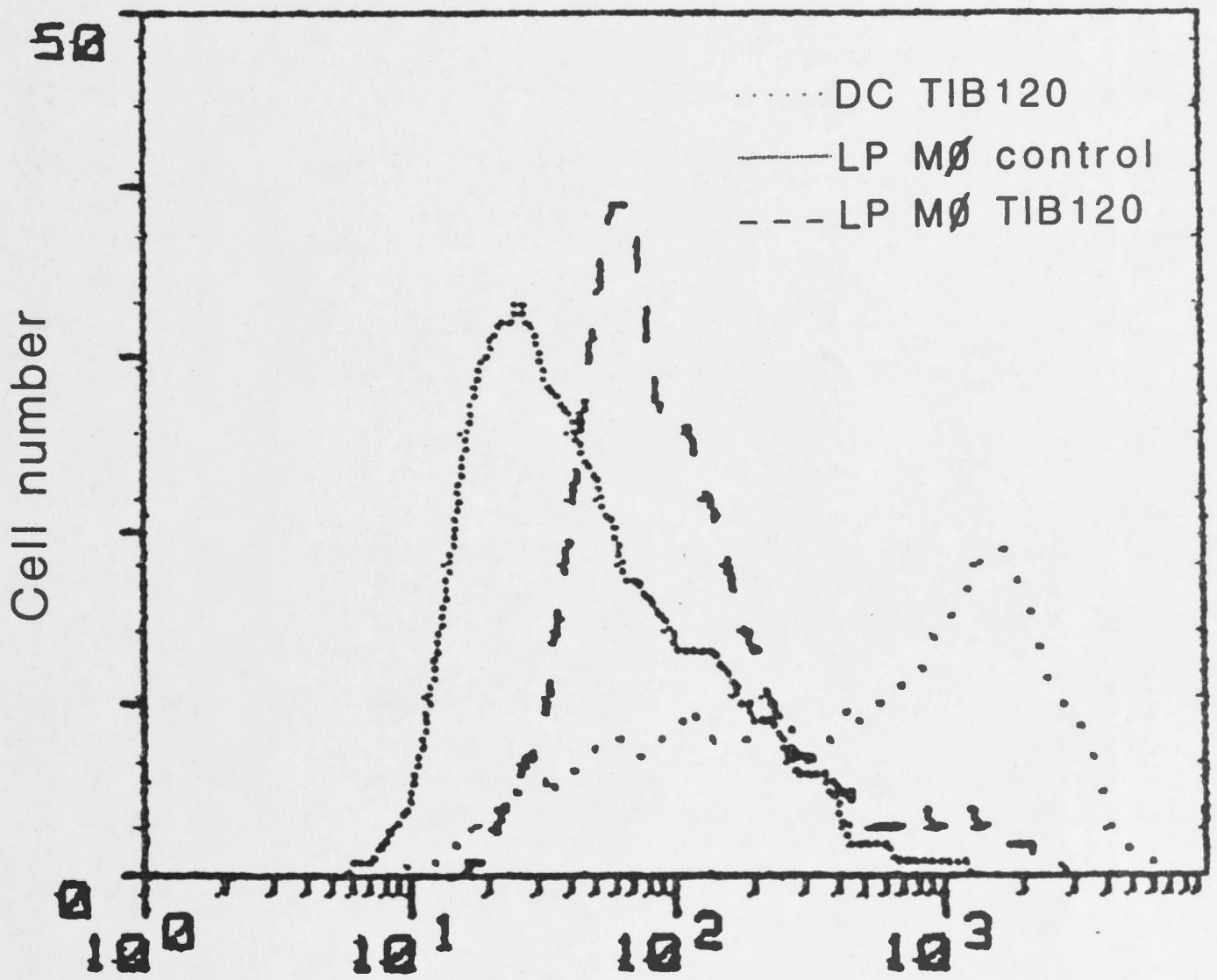


Figure 4.8.

Flow cytometry of intestinal lamina propria macrophages and dendritic cells. Intestinal lamina propria fibronectin-adherent cells and fibronectin-non-adherent, low density cells were incubated with an isotype-matched control (LP Mϕ control) or with TIB120 (anti-Class II MHC antibody) (LP Mϕ TIB 120 and DC TIB 120 respectively). The experiment was performed as described in Figure 4.1. The macrophages had low, but definite expression of Class II MHC antigens. The level of dendritic cell Class II MHC expression was at least one order of magnitude greater than the macrophages.


DC TIB120
LP MØ control
LP MØ TIB120
Cell number
50
0
10^0
10^1
10^2
10^3
Log Fluorescence (arbitrary units)

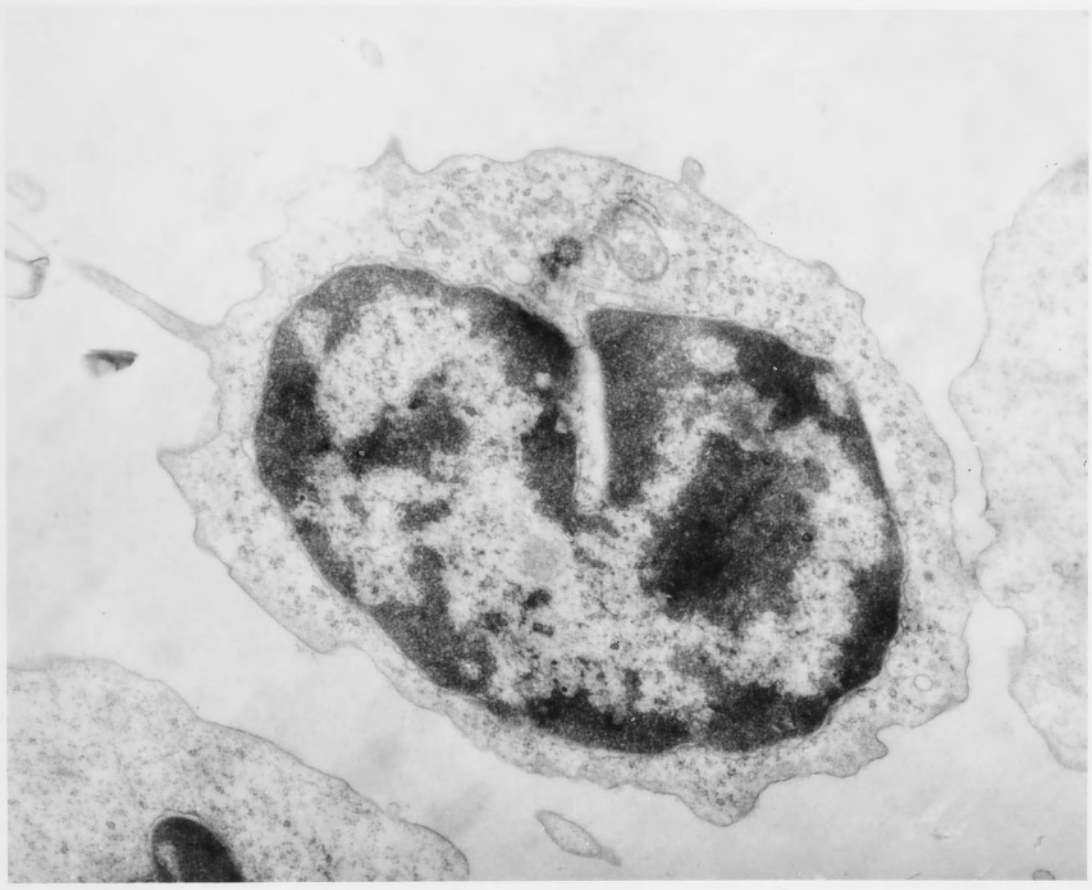
Micrograph 4.1

Transmission electron micrograph of a murine intestinal lamina propria dendritic cell. The nucleus is irregular with a peripheral rim of heterochromatin and a small nucleolus. The cytoplasm contains scattered smooth vesicles with no evidence of phagolysosomes. Final magnification x 7,500.

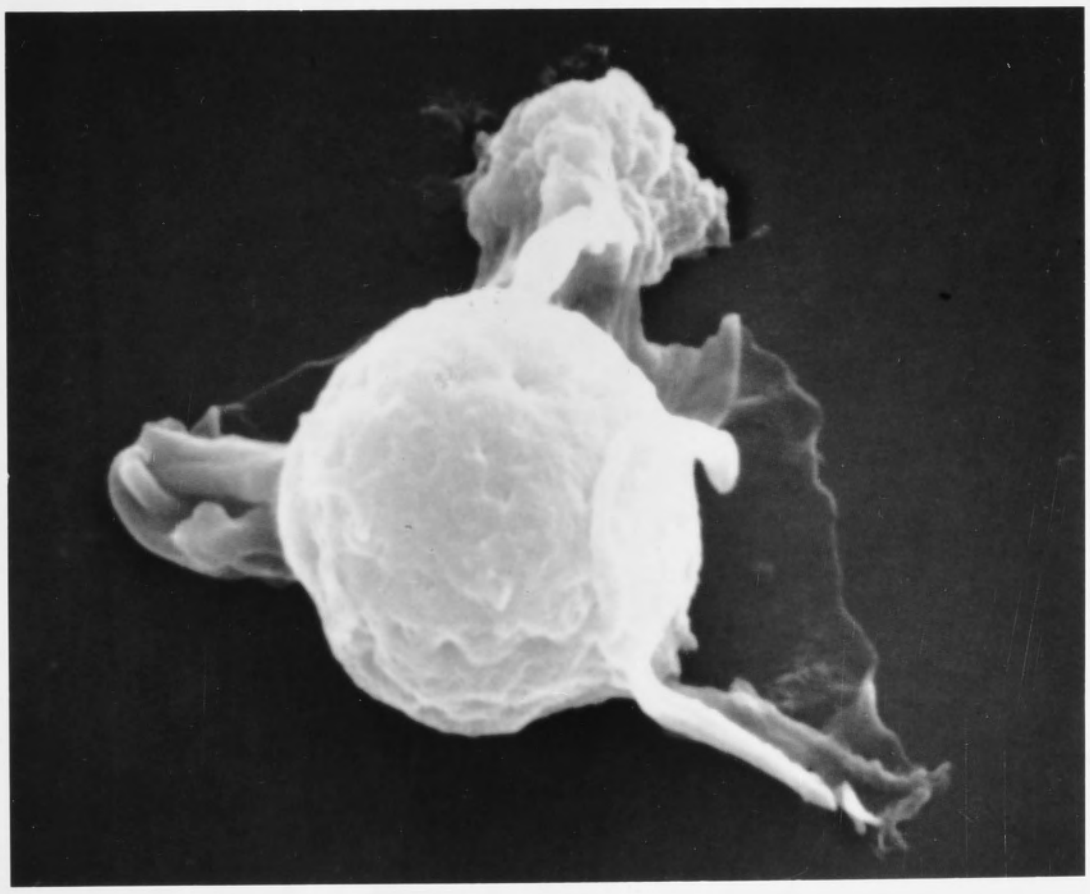
Micrograph 4.2

Scanning electron micrograph of a murine intesinal lamina propria dendritic cell showing the characteristic cytoplasmic processes or veils. Final magnification x 6,700.

4.1



4.2



CHAPTER 5

HUMAN INTESTINAL DENDRITIC CELLS

"and he that seeketh findeth."

St Matthew

5.1 INTRODUCTION

The experiments reported in the preceding chapters demonstrate the presence of MLR stimulatory dendritic cells in the Peyer's patches and lamina propria of the murine small intestine, and, with more limited data, in the murine colon. In contrast, murine intestinal macrophages were shown to inhibit the function of the dendritic cells in vitro.

The following chapter describes a method for the partial enrichment and characterization of human colonic MLR stimulatory cells. These cells have the morphological, phenotypic and ultrastructural features of dendritic cells.

5.2 MATERIALS AND METHODS

5.2.1 Mucosal specimens

Specimens of intestine were obtained from patients undergoing surgery for colorectal cancer (30 patients), Crohn's disease (2) and non-malignant/non-inflammatory conditions (3), including diverticular disease (2) and ischaemic colitis (1). The results of histological examination of adjacent tissue were recorded. Mucosa was taken at least 5 cm from tumours.

5.2.2 Disaggregation of intestinal mucosa

Full thickness specimens of mucosa were obtained and transported to the laboratory in ice cold HBSS supplemented with penicillin (100 IU/ml) and gentamicin (50 mg/ml). Disaggregation of the tissue was initiated in all cases within 1 h of resection. Tissue was disaggregated using a method based on modifications [134] of Bull and Bookman's (1977) [56, 73] original method. Strips of mucosa (2-3 cm x 0.5 cm) were dissected free from the muscularis and incubated with continuous stirring in Wheaton flasks in calcium-magnesium free HBSS (CMF-HBSS) containing 20 mM HEPES (pH 7.4), penicillin, gentamicin and 0.75 mM EDTA at 37^{o}C for 60 min. The tissue was washed in CMF-HBSS with EDTA for 30 min periods until there was no increase in particulate matter between washes (4-5 washes). The strips of tissue were then washed once in CMF-HBSS without EDTA. The tissue was minced finely (2 x 2 mm) and incubated overnight with gentle stirring in RPMI 1640 (Flow Labs, Australia) containing 10% heat-inactivated FCS (CSL Melbourne), 2 U/ml purified collagenase (CLSPA type, Worthington Biochemical Corp, Freehold, NJ.), 5 U/ml DNase II (Calbiochem, San Diego, CA.), 100 IU/ml penicillin, 50 mg/ml gentamycin, 100 U/ml nystatin, 20 mM HEPES and 2 mM glutamine. The undigested tissue fragments were then allowed to settle and the digest filtered through four layers of surgical gauze supported in a sterile Buchner funnel. The cells were washed (400 g, 10 min at 4^{o}C) and resuspended in RPMI 1640 with

10% FCS. For isolation of lamina propria mononuclear cells and removal of red cells, neutrophils and debris, the cells were layered on to a Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) density gradient (1.077 g/ml) and spun at 400 g for 30 min at 4^{o}C. Interface cells were harvested and washed twice. Viability was assessed by the exclusion of 0.1% Trypan blue. Cytospin slides were prepared using 1x10^{5} cells in a Shandon cytocentrifuge (500 rpm, 5 min) followed by fixation in methanol and staining in Diff-Quik.

5.2.3 Fibronectin adherence and binding to human γ-globulin

The method of fibronectin adherence was as described in Section 3.2.1.

Bacteriological grade plastic Petri dishes were prepared with normal human γ-globulin (CSL, Melbourne) using a modification of the technique of Young and Steinman (1988) [521]. Pooled human γ-globulin (5 ml; 10 mg/ml) was added to 100 mm dishes for 30 min at 20^{o}C. The plates were washed and the lamina propria cell suspension added at a final concentration of 5-10 x10^{6}/ml. After incubation at 37^{o}C for between 30 min and 4 h, the non-adherent cells were removed and the plates washed twice with warm medium. Adherent cells were harvested using EDTA in RPMI as described in Section 3.2.1.

5.2.4 Carbonyl iron phagocytosis

The lamina propria cell suspension at 2-3x10^{7}/ml was added to 4 mg carbonyl iron powder (Sigma, St Louis, MO) and mixed thoroughly. The cells were incubated at 37^{o}C for 30 min with occasional mixing. The test-tube containing the cells was placed in a magnet (Dynal AS, Oslo, Norway) for 10 min at 4^{o}C. The cells in suspension were transferred to a second tube which was placed in the magnet for a further 10 min at 4^{o}C. The remaining cells were removed, washed and counted.

5.2.5 AET-Sheep red cell rosetting of T lymphocytes

A 4% solution of 2-aminoethylisothiouronium bromide (AET) (pH 9.0) was mixed with packed sheep red blood cells (1-10 days old) in a 4:1 ratio (vol/vol)

for 20 min at 37°C. The mixture was resuspended at a final concentration of 4% sheep red cells and used fresh.

Colonic cell suspensions at a concentration of 10^7/ml were mixed with equal volumes of AET-sheep red cells at 4% and RPMI/FCS (final concentration 15%). The cells were centrifuged at 300 g at 20°C for 10 min and left on ice for a minimum of 1 h. The cells then were resuspended gently, layered over Ficoll-Paque and centrifuged at 600 g at 20°C for 25 min. The cells at the interface (T cell-depleted) were washed and used in subsequent experiments. When required, the rosetted T cells in the pellet were obtained after lysing the sheep red cells with 0.5 ml sterile water and washing three times in complete medium.

5.2.6 Density gradient centrifugation

Density gradients used included Ficoll-Paque (Pharmacia, Uppsala, Sweden), Nycodenz Monocytes (Nyegaard, Oslo, Norway), Lymphoprep (Nyegaard) and Percoll (Pharmacia) both as continuous and discontinuous gradients. Continuous gradients were generated by centrifuging isotonic solutions of 40%, 45% and 50% Percoll at 30,000 g for 15 min in a Beckman L5-65B ultracentrifuge using 6.5 cm cellulose nitrate tubes (Beckman).

5.2.7 Immunocytochemistry

The methods used were as described in Section 3.2.5, using monoclonal antibodies directed against human cell surface antigens (Table 5.1) and biotinylated sheep anti-mouse immunoglobulin (Amersham, Amersham, UK). To inactivate endogenous peroxidase activity, some air-dried slides were treated with 0.1% (vol/vol) hydrogen peroxide in methanol for 30 min at 20°C. The slides were rehydrated with decreasing concentrations of ethanol in water (90%, 70%, 50%) before blocking. The methods were otherwise unchanged.

5.2.8 Flow cytometry

The methods were as described in Section 3.2.6 using monoclonal antibodies directed against human cell surface antigens (Table 5.1) and FITC-con-

jugated, affinity-purified anti-mouse immunoglobulin (Silenus, Hawthorn, Victoria) at a dilution of 1:200.

5.2.9 Electron microscopy

Cells were fixed in 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 for 2 h and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4 for 90 min. For scanning electron microscopy, samples were then dehydrated, critical point dried and gold coated, with micrographs taken on a Hitachi 7000 scanning attachment. For transmission electron microscopy, samples were "en bloc" stained in 1% aqueous uranyl acetate for 1 h, dehydrated and embedded in "Spurrs" resin. Micrographs were taken on a Hitachi 7000 electron microscope.

5.2.10 Mixed leucocyte reactions

Responder cells were derived from buffy coats prepared from normal blood donors (Red Cross Blood Transfusion Service, ACT).

The buffy coat was diluted 1:2 with HBSS and underlaid with Ficoll-Paque. The cells were centrifuged at 600 g for 30 min at 4^{o}C and the interface harvested and washed twice. The cells were resuspended at 10-20x10^{6}/ml in the supernatant of the L243 hybridoma cell line (anti-Class II MHC) for 30 min at 4^{o}C, washed three times and the antibody-labelled cells removed by panning or complement lysis. The technique of panning was as described in Section 2.2.4 except that the plates were treated with affinity-purified rabbit anti-mouse immunoglobulin G (Cappel, Cochranville, PA). For complement lysis, the peripheral blood mononuclear cells prelabelled with primary antibody were resuspended at a final concentration of 10^{7}/ml and complement (Pel-Freez, Rogers, AR or Cedar Lane, Hornby, Ontario) was added at a final dilution of 1:5. The cells were incubated for 30 min at 37^{o}C, washed, layered over Ficoll-Paque to remove dead cells (600 g at 20^{o}C for 20 min) and then washed three times. The same method was used for lamina propria cells.

Responder peripheral blood mononuclear cells were also prepared from buffy coats using the method of Warren (1981) [498]. Peripheral blood mononuclear cells were cultured in a preparation of lymphokine (see below) for 3 days to expand the population of lymphokine-reactive cells. Centrifugation of the cultured cells on discontinuous Percoll gradients yielded a low density fraction (<1.0623 g/ml) that contained monocytes, blast cells and medium-sized lymphocytes, and high density fractions (<1.0659 and <1.0689 g/ml) that contained only small lymphocytes.

The lymphokine preparation was prepared by stimulation of human tonsil lymphocytes ($2x10^7$/ml) with PHA (25-50 mg/ml) in serum-free medium [499]. After a 2 h incubation, the cells were washed free of unbound mitogen and incubated for a further 17 h. The supernatant was harvested, concentrated, and kept at -20°C for further use.

Responder cells were frozen in RPMI containing 20% FCS and 20% DMSO (Malinckrodt, Paris, KY) and kept in liquid nitrogen until use. They were thawed on the day of use.

Cultures were performed in triplicate in round-bottom 96-well plates (Linbro Flow Labs, McLean, VA). A constant number of responder cells (generally $2x10^5$/well) was incubated with varying numbers of irradiated stimulators from the colon (2,500 Rads). The reaction was carried out in a total volume of 0.2 ml of medium supplemented with 5% AB serum. After 4 days, 1 μCi tritiated thymidine (Amersham, Surry Hills, NSW) was added to each well and the incubation continued for a further 16 h. The plates were freeze-thawed and the cells harvested automatically onto glass-fibre discs (Whatman, Maidstone, UK) using a cell harvester (Dynatech CH-103). Non-aqueous scintillant (0.5% 2,5-diphenyloxazole in xylene) (5 ml/vial) was added and the assays performed on a Packard Tri-Carb K60 counter.

5.2.11 Method for enrichment of colonic dendritic cells

The most effective depletion of macrophages and maximum enrichment of dendritic cells was obtained using the following method:

The colonic cell suspension was incubated on human γ- globulin coated plastic Petri dishes for 2-4 h at 37^{o}C. The non-adherent cells were removed, washed and then treated with carbonyl iron (4 mg/10^{7} cells) as described above. The remaining cells were incubated in siliconized dishes at 5-10x10^{6} cells/ml in RPMI containing 5% heat-inactivated pooled human AB serum overnight. The cells were removed, washed, resuspended at 5-10x10^{6} cells/ml, underlaid with Nycodenz Monocytes (Nyegaard) (density 1.068 g/ml) and centrifuged at 600 g for 20 min at 20^{o}C. The low density (dendritic cell-enriched) cells and high density (T cell-enriched) cells were washed twice, counted and the viability was assessed.

5.3 RESULTS

5.3.1 Cell yields and the development of the methods

Cell yields after Ficoll-Paque were $15\pm12x10^6$/g tissue. Recovery represented $69\pm15\%$ of the number applied. The amount of tissue obtained from surgical specimens ranged from 5-21 g. The final yield of dendritic cell-enriched populations was always $<2\%$.

Initial attempts to enrich for MLR stimulatory cells involved overnight adherence to fibronectin-coated gelatinized flasks and density gradient centrifugation (Nycodenz Monocytes and Percoll at various densities). It was evident that this method did not allow the effective separation of human colonic macrophages from dendritic cells. Examination of the cytospin preparations and electron micrographs (Micrograph 5.1) showed that the low density, non-adherent cells contained low, but significant (5-10%), numbers of macrophages. Similarly, the fibronectin-adherent cell fraction contained dendritic cells.

Each of the techniques described in the Methods Section was used in different sequences and combinations to try to obtain populations of cells maximally enriched for dendritic cells with minimal macrophage contamination. The procedures were monitored for their effectiveness in removing macrophages and enriching for dendritic cells mainly by examining cytospin preparations and immunocytochemical slides (assessing the proportion of Class II MHC$^+$ or 25F9$^+$ cells, see below). In later experiments, flow cytometry was also used. To date, the best isolation technique is as described in Section 5.2.11.

Effective removal of macrophages required at least two procedures. The use of human γ-globulin to immobilize the macrophages permitted their harvesting and use in subsequent experiments. Adherence to plastic did not remove as great a proportion of macrophages, whilst adherence to fibronectin resulted in populations of macrophages containing significant numbers of dendritic cells. Macrophages not adherent to γ-globulin-coated dishes were

depleted by carbonyl iron phagocytosis. The use of carbonyl iron phagocytosis as the initial step precluded comparative studies of MLR stimulation. If performed on the third day of the isolation, this procedure was not as effective in depleting the cell suspension of macrophages. This may be because the macrophages had exhausted their phagocytic capabilities by ingesting the cellular debris during the isolation procedure.

5.3.2 Immunocytochemistry

5.3.2.1 Adherent cells (macrophage-enriched)

The majority (>90%) of fibronectin- or plastic-adherent cells with the morphology of classical macrophages had detectable Class II MHC antigens when studied with immunocytochemistry (Fig. 5.2a). The mature macrophage marker, 25F9, also labelled virtually all the macrophages (Fig. 5.2b). The other macrophage markers used, OKM1 and Leu-M5 were either not detectable on intestinal macrophages or present on small numbers (<5%) only. Immunohistochemical studies of intestinal macrophages [170] and immunocytochemistry of populations of macrophages isolated from the colon [134] demonstrated similar findings.

5.3.2.2 Low density non-phagocytic non-adherent cells (dendritic cell-enriched)

Cells with dendritic cell morphology were labelled with the antibody to Class II MHC antigens (Fig. 5.3). The monoclonal antibody, Leu-M5, directed against the p150,95 antigen (CD11c) weakly labelled dendritic cells in three of eight experiments in which it was used (Fig. 5.4). None of the other antibodies used stained dendritic cells.

5.3.2.3 Inactivation of endogenous peroxidase activity

Some cytospin slides (particularly the plastic-adherent and high density fractions) contained significant numbers of eosinophils with endogenous peroxidase activity. The use of hydrogen peroxide in methanol effectively inactivated

this activity and produced preparations with better preservation of cellular morphology. However, the use of this step was associated with a loss of staining intensity with the ABC technique.

5.3.3 Flow cytometry

5.3.3.1 Adherent cells

When the human γ-globulin-adherent cells were analysed using forward and side scatter, two populations were demonstrated (Figs. 5.5, 5.6). The larger cells expressed high levels of Class II MHC antigens but did not have detectable macrophage, T or B cell antigens (Fig. 5.5). These cells had much higher levels of background fluorescence (nonspecific primary and secondary antibody staining and autofluorescence) than the smaller cells (Fig. 5.6). The population of smaller cells consisted of mainly T and B cells and comprised <25% of the total cell number.

5.3.3.2 Low density non-phagocytic non-adherent cells

The low density non-adherent non-phagocytic cells also consisted of two populations when analysed using forward and side scatter characteristics (Figs. 5.7, 5.8). When fluorescence was plotted against size or forward scatter (Fig. 5.9), it was apparent that the highly fluorescent cells were spread over a wide size range. In addition, almost all the remaining small cells expressed intermediate levels of Class II MHC determinants.

Analysis of a gated population of large cells (Fig. 5.7) demonstrated high level expression of Class II MHC antigens, with no detectable macrophage, T or B cell marker expression. This population comprised 40% of the total cell number.

When the smaller population was analysed (Fig. 5.8), the cells expressed T cell markers (OKT3 and OKT4) with intermediate and high levels of Class II MHC antigens. Markers expressed on macrophages and B cells were undetectable or expressed only on small numbers of cells.

5.3.3.3 AET-SRC rosetting cells

The cells obtained by AET-rosetting consisted of a homogenous population of lymphocytes (mainly $CD4^+$) expressing low-intermediate levels of Class II MHC antigens (Fig. 5.10).

5.3.3.4 MLR responder cells

The peripheral blood responder lymphocytes consisted of a homogenous population of T cells with a $CD4^+$:$CD8^+$ ratio = 70:30 (Fig. 5.11). There was no detectable expression of Class II MHC antigens on the cells treated with L243 and either complement lysis or panning.

5.3.4 MLR stimulation

In the initial experiments using fibronectin-adherence, greater MLR stimulatory activity was always found in the low density, non-adherent cells (Fig. 5.12). There was, however, significant activity in the adherent fraction. This suggested either that there was significant contamination with dendritic cells (as suggested by their presence in cytospin and immunocytochemical slides) and/or that macrophages had MLR stimulatory activity. In order to differentiate between these two possibilities, the effect of adding macrophages to dendritic cell-enriched populations was studied and the activities of the separate populations was directly compared.

When added to dendritic cell-enriched populations, human colonic macrophages neither inhibited nor potentiated MLR stimulation, either in the presence or absence of indomethacin (Fig. 5.13).

The direct comparison of dendritic cell-enriched populations (40% dendritic cells) with macrophage-enriched populations (>75% macrophages), non-adherent colonic lamina propria cells (<1% dendritic cells) (Fig. 5.14) and high density cells (<5% dendritic cells) (Fig. 5.15) showed greatly enhanced MLR stimulatory activity in the dendritic cell population. A small cross-contami-

nation with dendritic cells can explain the weak activity of the macrophage-enriched population but the converse does not hold true.

5.4 DISCUSSION

Cells with the morphology of veiled cells were first described in the human intestinal lamina propria in tissue obtained from patients suffering from inflammatory bowel diseases [507, 508]. These cells, which were neither phenotyped nor functionally characterized, may have been tissue dendritic cells, and were found in greater numbers in inflammed bowel than in normal controls.

Dendritic cells have been obtained from human tissues including peripheral blood, synovial fluid, tonsils, thymus and the lung (See Section 1.2.3). The isolation of human dendritic cells was associated with many of the problems encountered in the mouse eg. low cell numbers and the absence of specific cell surface markers. Particular problems have been encountered using human tissues. For example, human dendritic cells are fibronectin-adherent, at least in the short-term [129], so this property did not permit their separation from macrophages. In addition, the function and viability of human peripheral blood dendritic cells appears to be sensitive to the toxic effects of complement [521]. Colonic dendritic cell function was affected by complement so this method could not be used for enrichment. The purest populations of human dendritic cells reported to date were obtained by highly sophisticated techniques, including cell sorting by negative selection using a broad range of monoclonal antibodies [149, 317].

Particular importance was placed on the development of a method to effectively deplete the colonic cell suspensions of mononuclear phagocytes. This was because of the demonstration of a suppressive effect of mouse intestinal lamina propria macrophages (Chapter 3), and the observation that cell populations need to be depleted of monocytes to see the rapid development of large cell aggregates in the human MLR [118]. In addition, there is still some controversy about the antigen-presenting cell function of the human intestinal macrophage. Mahida et al (1988) [292] reported the association of antigen-

presenting cell activity with intestinal macrophages. These cells were obtained using fibronectin-adherence so that it is likely that there was a significant proportion of "contaminating" dendritic cells. These investigators used cell depletion experiments (panning with a monoclonal antibody directed against human monocytes, 3C10) to demonstrate that MLR stimulatory activity was diminished. The data (Table 2, [292]) show that panning with 3C10 reduced the MLR stimulatory activity of the treated population to 55% of an unfractionated cell population. Although statistically significant, these results are higher than would be expected if the MLR stimulatory cell was 3C10 positive. The omission of important data make the interpretation of the cell-depletion experiments difficult. For example, an assessment of the effectiveness of the macrophage depletion was not included in the results. In addition, the appropriate control (colonic cells not incubated with monoclonal antibody and then panned) was not available for comparison.

In these experiments, the differentiation of dendritic cells from intestinal macrophages was difficult because of their shared properties. These included fibronectin-adherence (dendritic cells weakly), low density and the expression of Class II MHC antigens (dendritic cells > macrophages). This distinction could be made, however, using several criteria. Firstly, macrophages had a characteristic morphology with oval or round nuclei and basophilic cytoplasm which contained varying numbers and sizes of phagolysosomes (Micrographs 5.1, 5.2); the nuclei of dendritic cells were oval or pleiomorphic whilst the cytoplasm contained, if any inclusions, only small vesicles (Micrographs 5.2, 5.3). Secondly, when cytospin preparations were examined, the majority of the macrophages expressed the antigen labelled by the antibody, 25F9. (This marker was not readily detectable at flow cytometry because it labels a predominantly intracellular, rather than a cell surface, antigen [170].) This antigen was not detectable on dendritic cells using either method. Thirdly,

macrophages could be distinguished from dendritic cells by their electron microscopic features (Micrographs 5.1-3 and Frontispiece); and finally, dendritic cells were not adherent to human γ-globulin and were not phagocytic.

Our results showed that low density, fibronectin-adherent cells were potent stimulators of the MLR (Fig. 5.12), but that there were, on morphological grounds, low, but functionally significant, numbers of dendritic cells. The addition of macrophage-enriched cells to a constant number of low density dendritic cell-enriched population, resulted in neither suppression nor enhancement of MLR stimulatory activity, either in the presence of, or the absence of indomethacin. This suggested that human intestinal macrophages played no role in MLR stimulation in spite of their high level expression of Class II MHC antigens.

When direct comparisons were made between macrophage-depleted, low density cells and human γ-globulin-binding macrophages, the greater MLR stimulatory activity was associated with the former. The levels of maximal proliferation and stimulator:response curves of the dendritic cell-enriched populations are comparable to those using purified populations of human tonsillar dendritic cells [149].

Examination of the data presented reveals variations in the absolute levels of MLR-induced stimulation. There are at least two explanations to account for these differences. Firstly, the stimulation depended on the degree of mismatching between different pairs of allogeneic stimulators and responders, and secondly, in the initial experiments (Figs. 5.12, 5.13), a foreign antigen (FCS) was introduced into the assay system.

In summary, dendritic cells are the major stimulators of the MLR in the human colonic lamina propria. Intestinal macrophages, in spite of expressing high levels of Class II MHC antigens, have no effect on MLR stimulation in vitro.

TABLE 5.1

Monoclonal Antibodies Used in Human Experiments

Name	Determinant recognized	Class	Reference
L243	Class II MHC, monomorphic	IgG_{2a}	J Immunol 1980; 125: 293
25F9	Monocyte, macrophage antigen	IgG_1	J Immunol 1985; 134: 1487
OKT1	T cells	IgG_1	Proc Natl Acad Sci USA 1980; 77: 4914
OKT3	CD3	IgG_{2a}	Proc Natl Acad Sci USA 1980; 77: 4914
OKT4	CD4	IgG_{2b}	Proc Natl Acad Sci USA 1980; 77: 4914
OKT8	CD8	IgG_{2a}	Proc Natl Acad Sci USA 1980; 77: 4914
OKM1	CD11b	IgG_{2b}	Proc Natl Acad Sci USA 1980; 77: 4914
Leu M5	CD11c	IgG_{2b}	Blood 1985; 65: 974
Leu11b	CD16	IgG_1	Proc Natl Acad Sci USA 1985; 82: 1766

All antibodies were mouse-derived

LEGENDS TO FIGURES

Figure 5.1

Human colonic lamina propria macrophages. Cytocentrifuge slide of human colonic γ-globulin-adherent cells stained with Diff-Quik (modified Giemsa stain) (x 630).

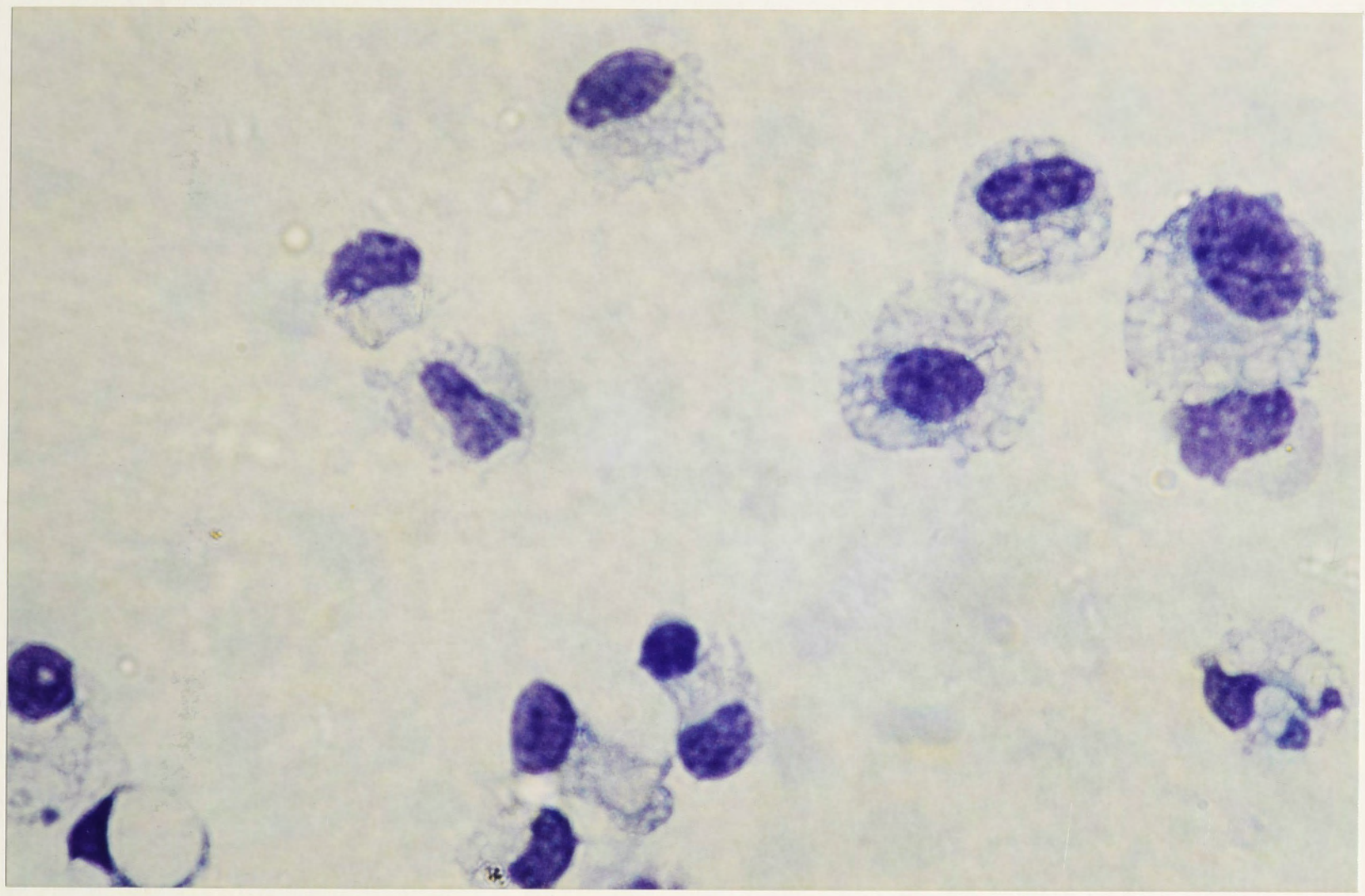
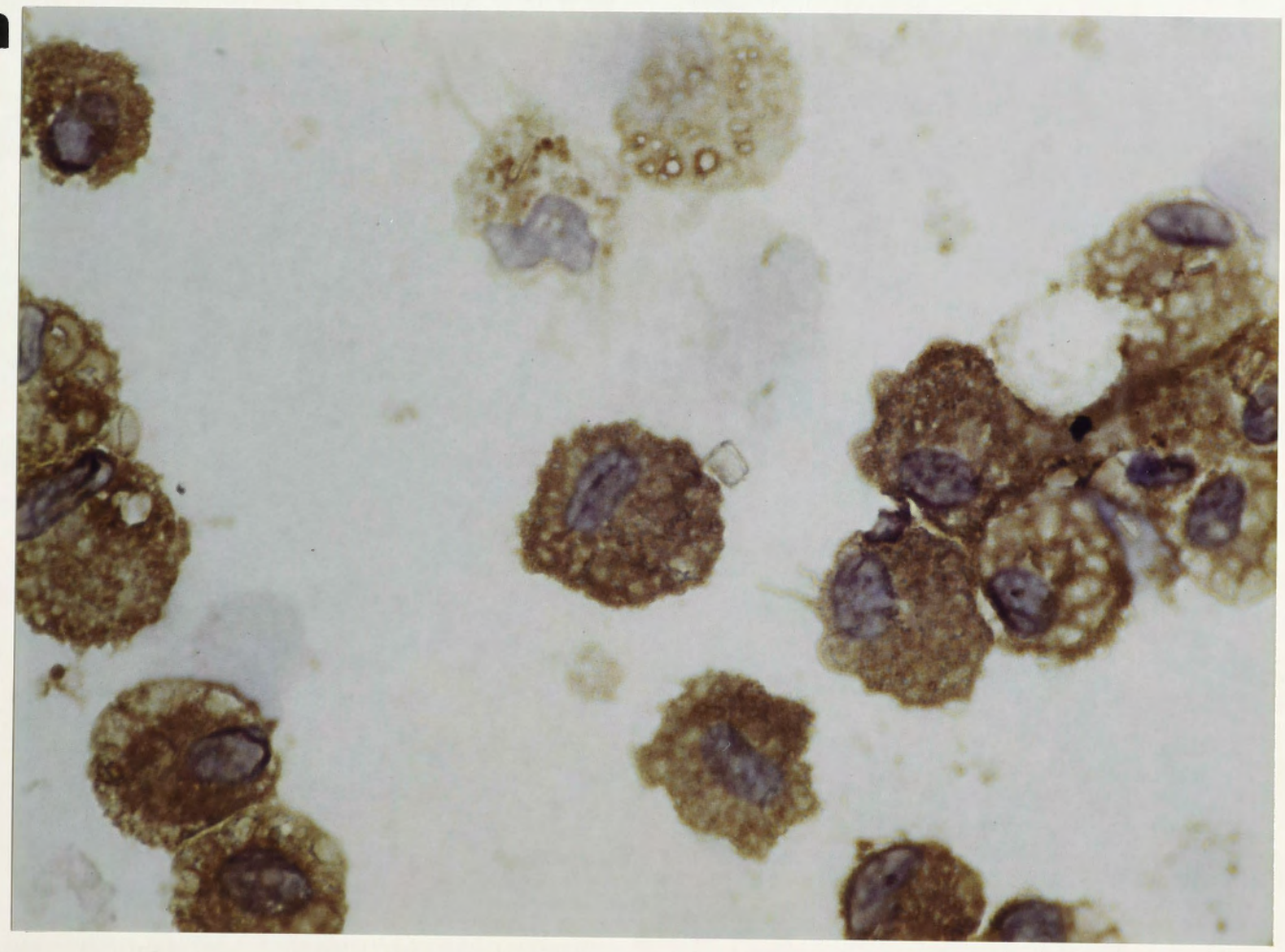


Figure 5.2

Immunocytochemistry of colonic macrophages. Cytocentrifuge slides of γ-globulin-adherent cells were air-dried overnight and stained with (a) L243 (anti-Class II MHC); and (b) 25F9 (anti-macrophage), using the method described in Section 3.2.5. Inactivation of endogenous peroxidase using 0.1% H_2O_2 in methanol was not performed (x 630).

a



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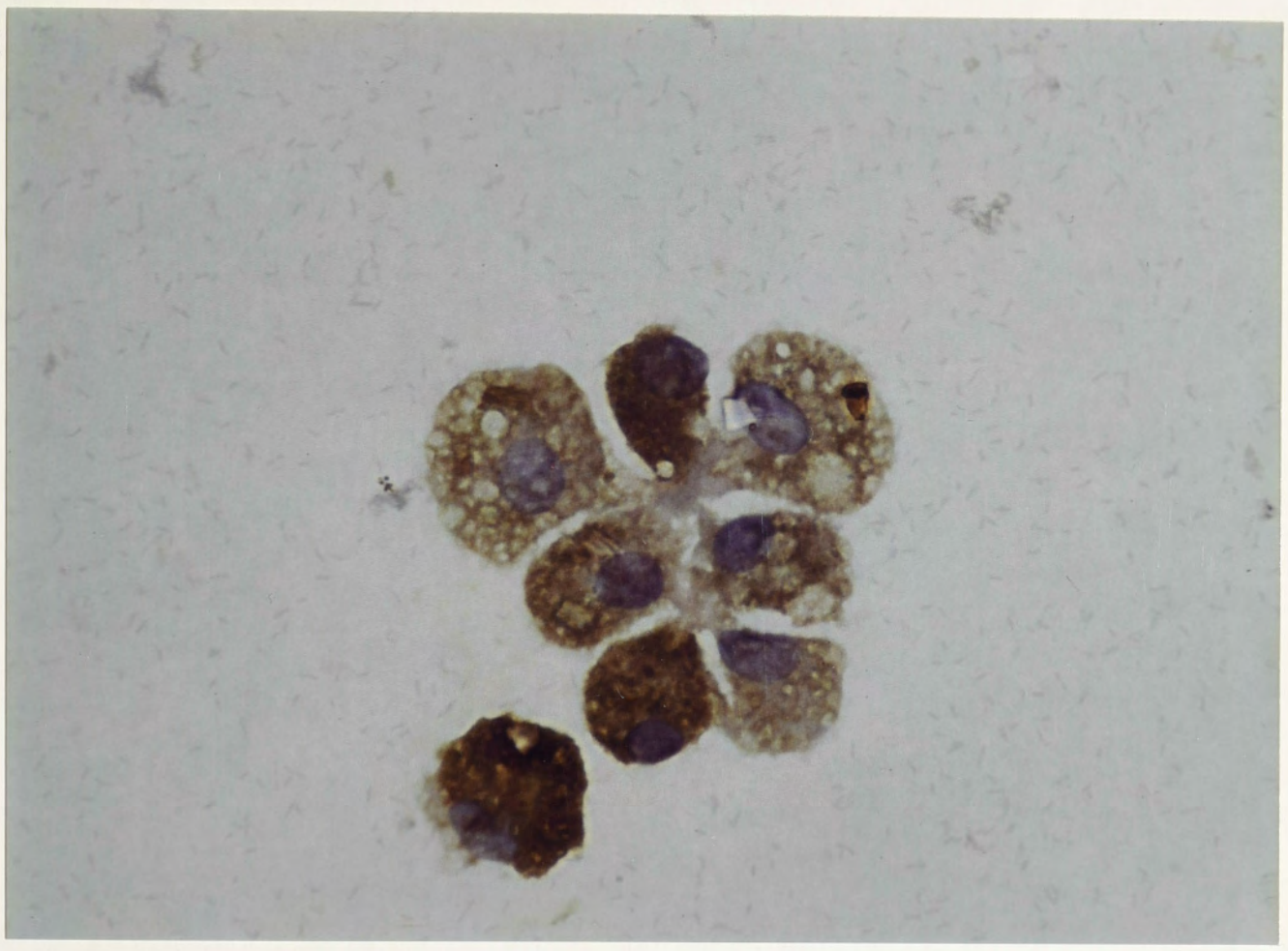
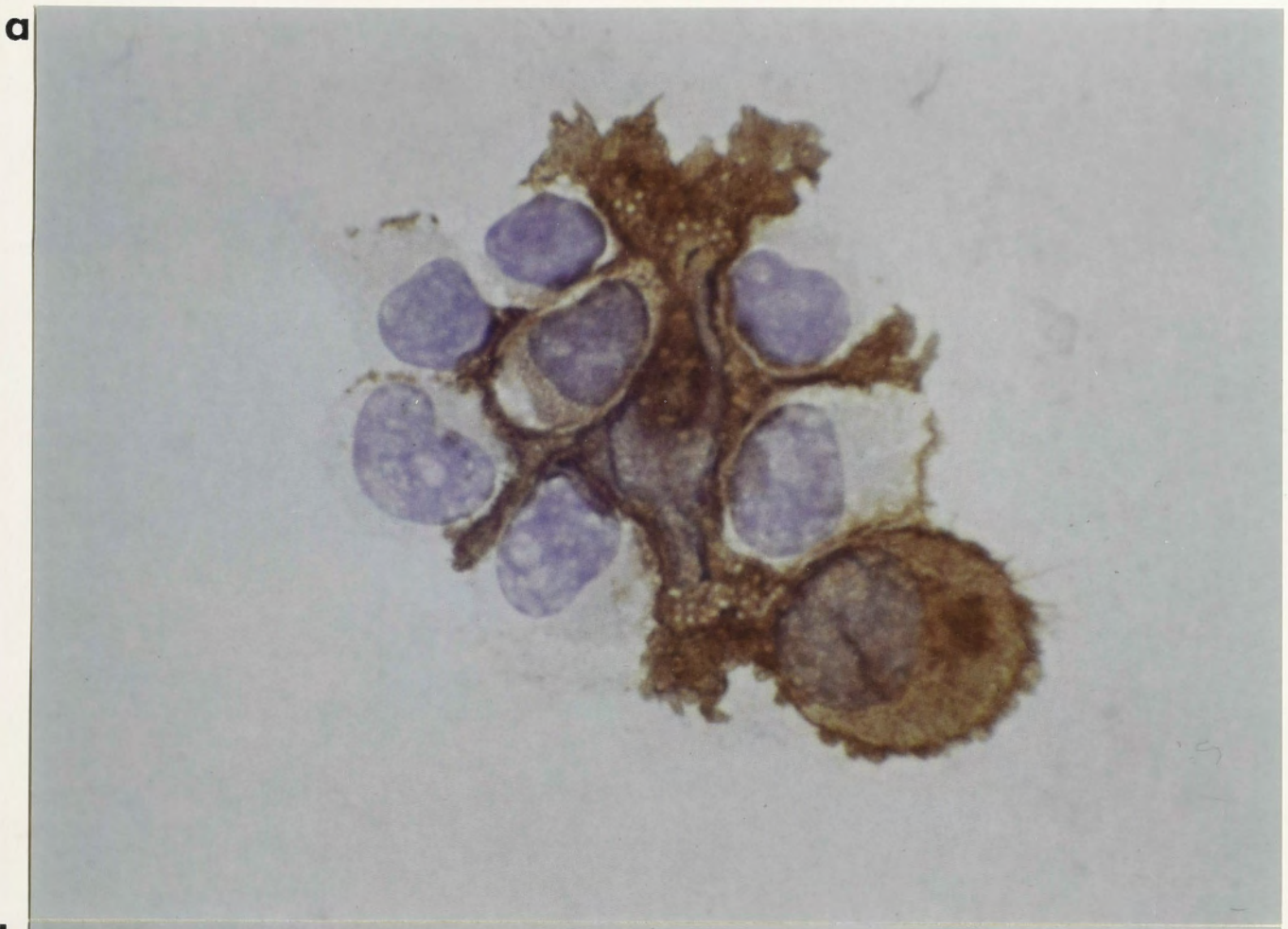


Figure 5.3

Immunocytochemistry of low density, non-phagocytic, non-adherent colonic lamina propria cells. The technique was performed as described for Figure 5.2 . The monoclonal antibodies used included (a) and (b) L243 (anti-Class II MHC) (x 1000); and (c) Leu-M5 (anti-CD11c) (x 400). Note the irregular nuclear morphology and the tendency to form clusters with lymphocytes.

a



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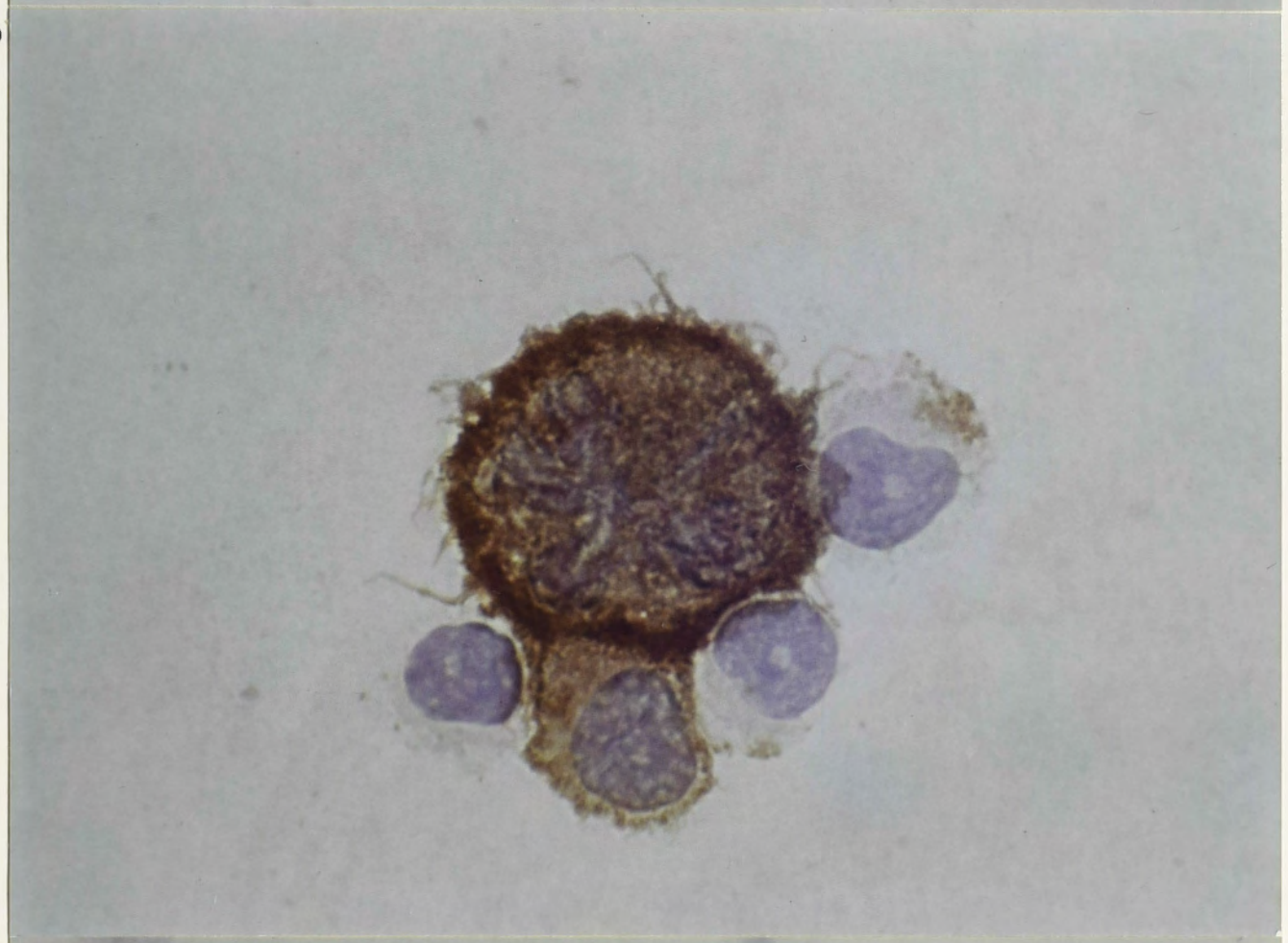


c

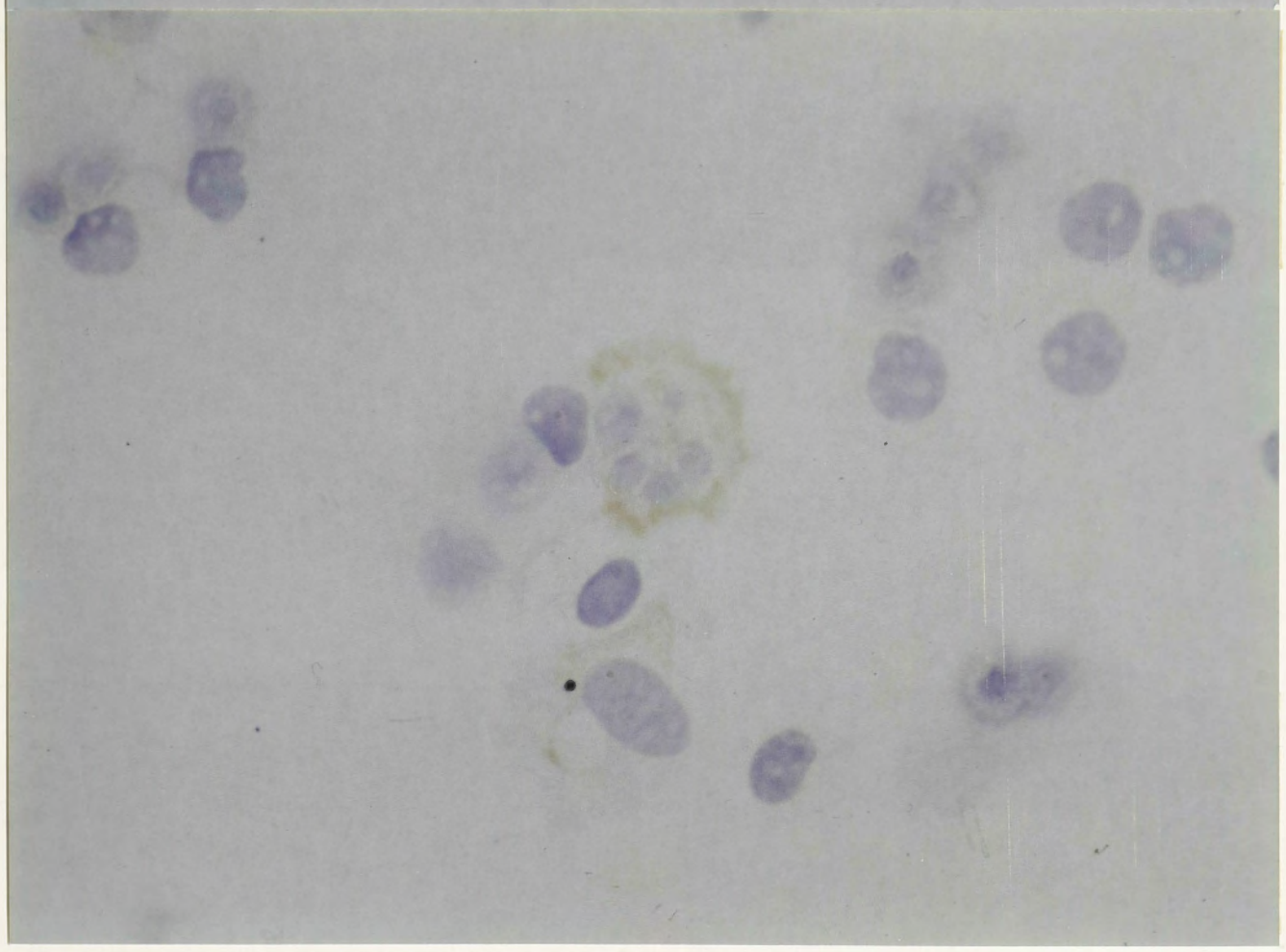


Figure 5.4

Immunocytochemistry of low-density, fibronectin-non-adherent cells. The methods are as described in Figure 5.2 using 25F9 (anti-macrophage). The $25F9^{+}$ macrophages are readily discernable in the cell preparations. The antibody did not stain the cells with the irregular nuclear morphology and was used to assess the degree of macrophage contamination. (x 400).

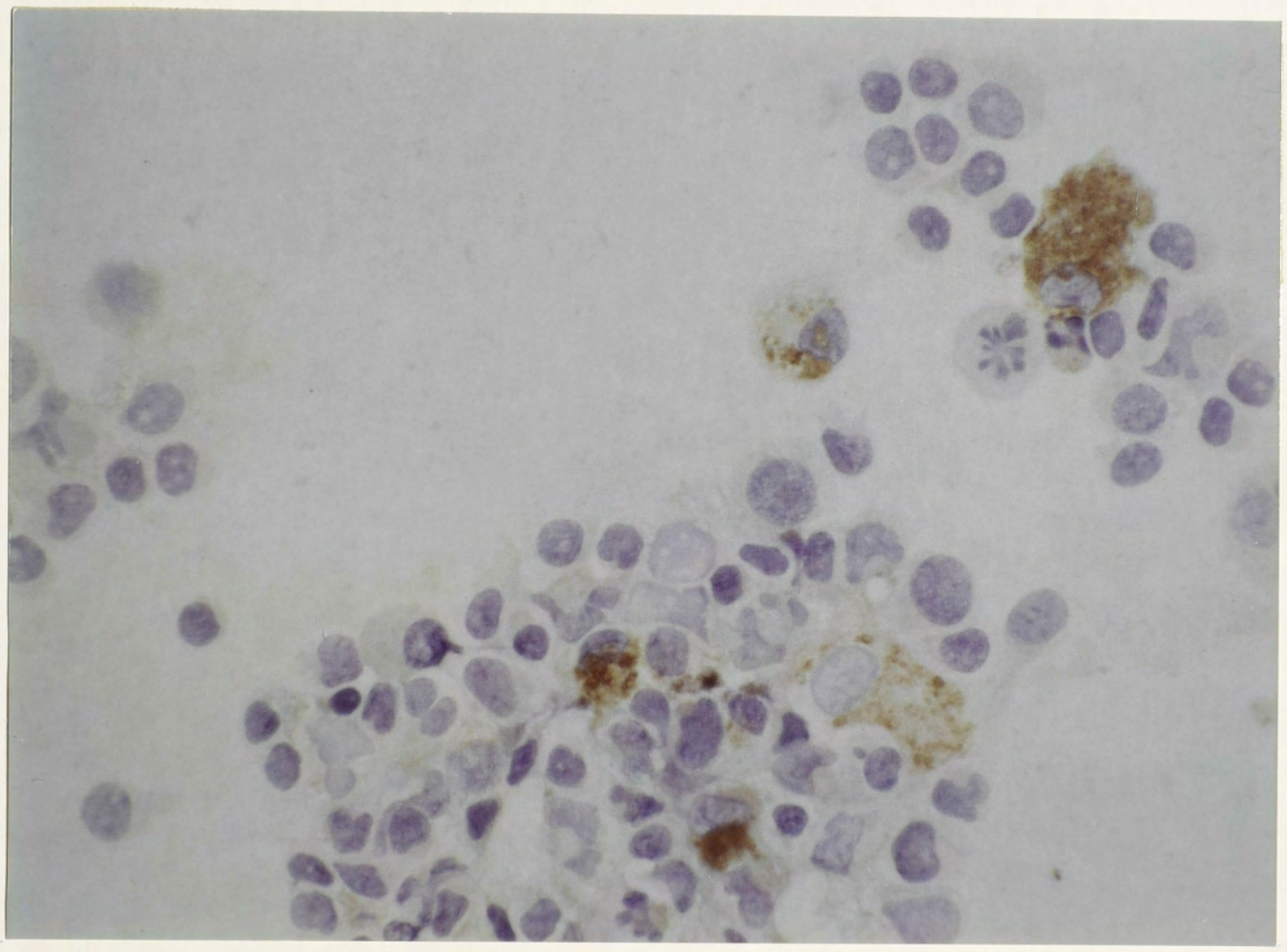
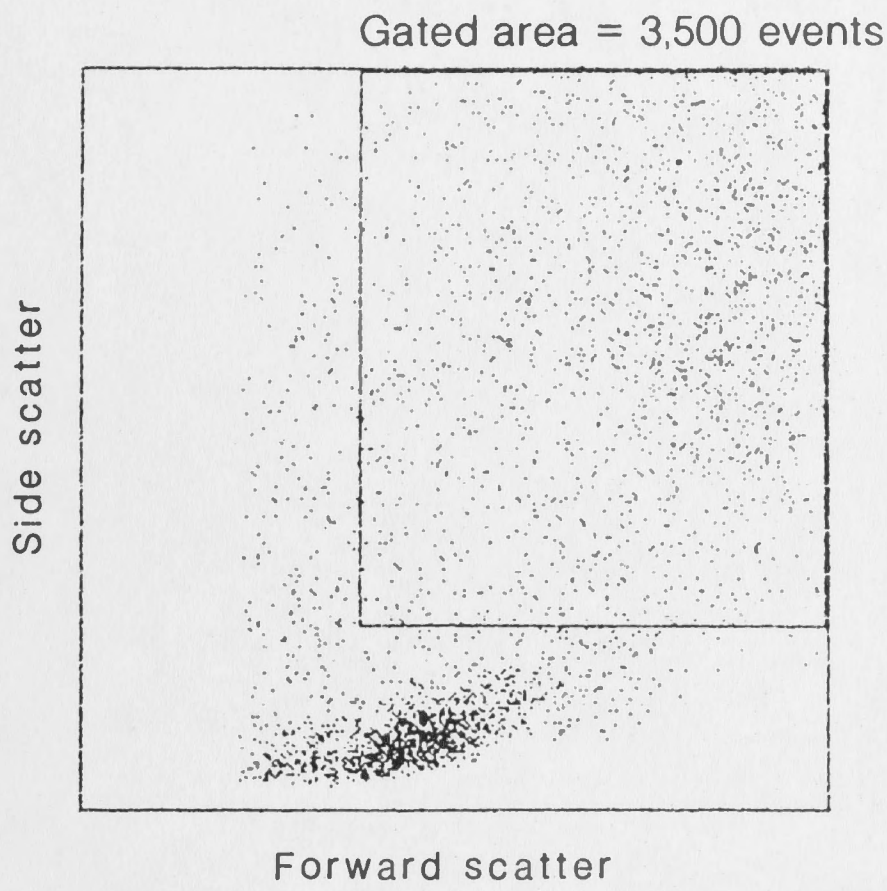
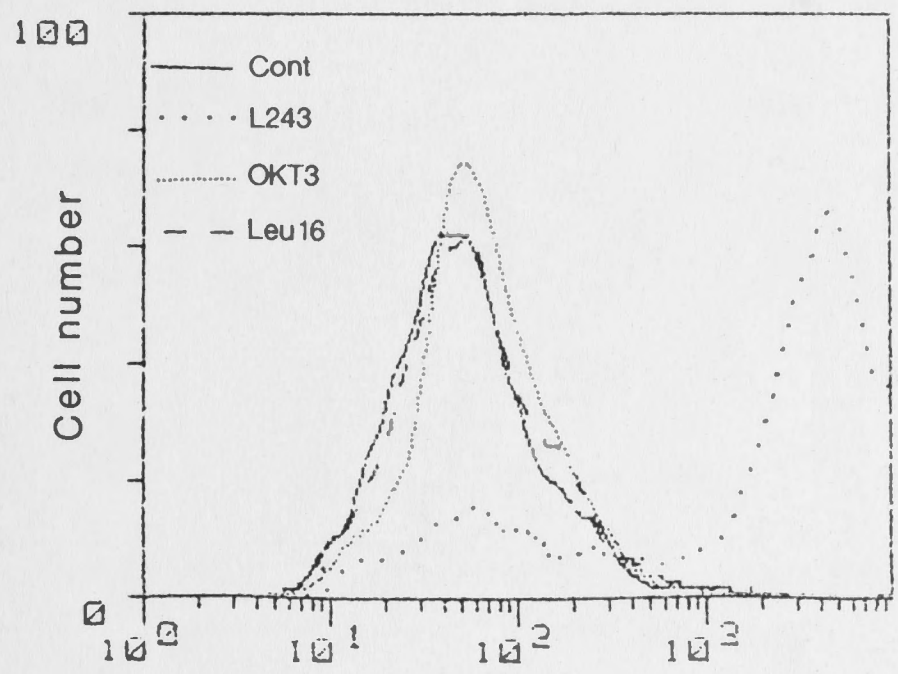


Figure 5.5

Flow cytometry of human colonic γ-globulin-adherent cells: Analysis of a gated population of large cells. Adherent cells were incubated with no antibody (control) or the specified antibody, then washed and labelled with FITC-conjugated sheep anti-mouse IgG and analysed by flow cytometry (5000 events). The abscissae represent forward scatter (linear scale, arbitrary units) or fluorescence intensity (logarithmic scale, arbitrary units) as indicated. The ordinate axes represent side scatter (linear scale, arbitrary units) and cell number (linear scale, arbitrary units). The gated population comprised >75% of the total numbers analysed. These cells expressed high levels of Class II MHC antigens but did not express any other markers. These are the same cell populations as illustrated in Figures 5.1 and 5.2.

Human colonic adherent cells





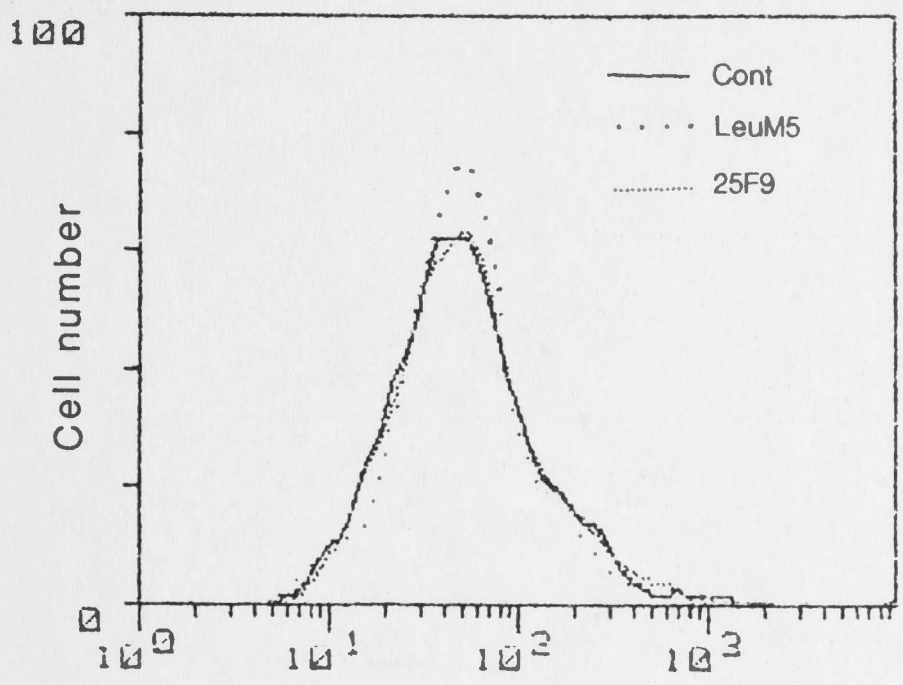
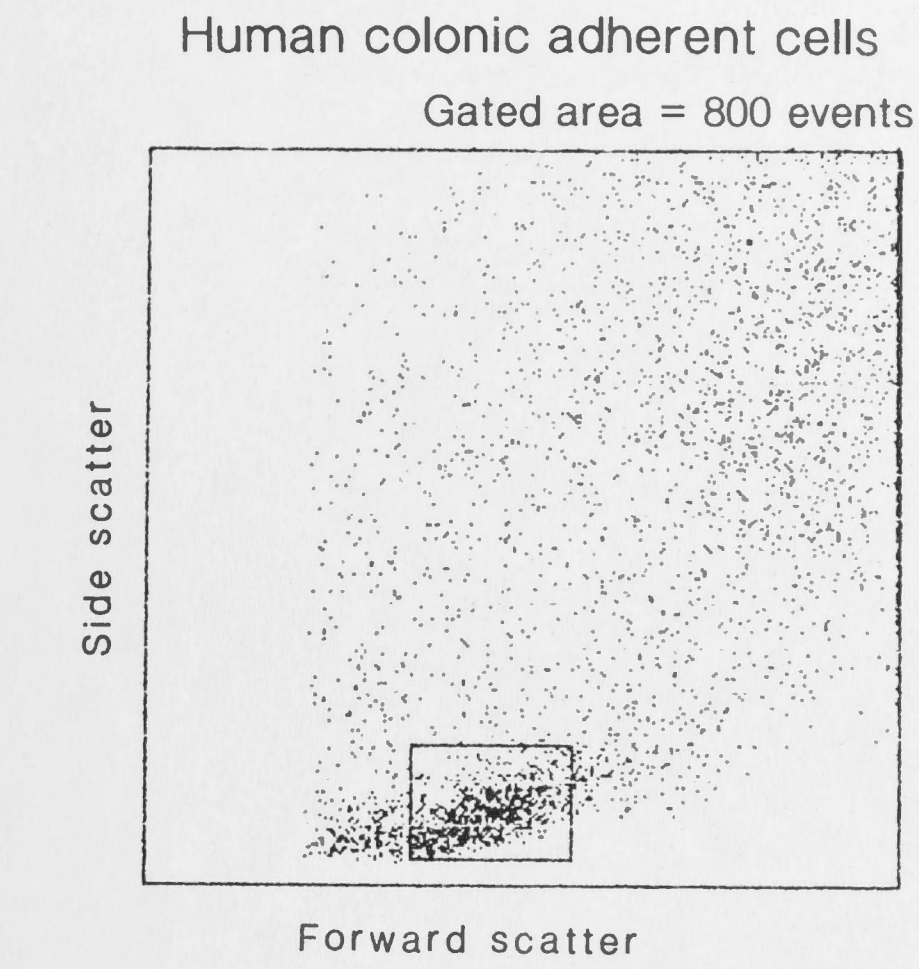
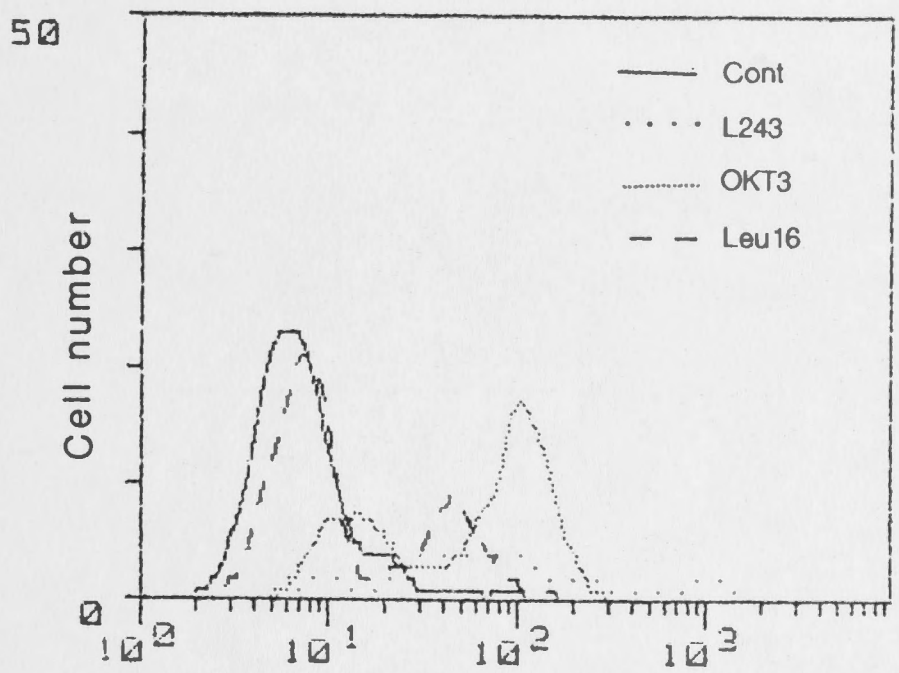


Figure 5.6

Flow cytometry of human colonic γ-globulin-adherent cells: Analysis of a gated population of small cells. The experiment was performed as described in Figure 5.5. The gated population comprised 16% of the total cell number analysed. These cells were mainly T cells ($OKT3^+$) with a subpopulation of B cells ($Leu\ 16^+$).


Human colonic adherent cells
Gated area = 800 events
Side scatter
Forward scatter


50
Cont
L243
OKT3
Leu16
Cell number
0
10^0
10^1
10^2
10^3

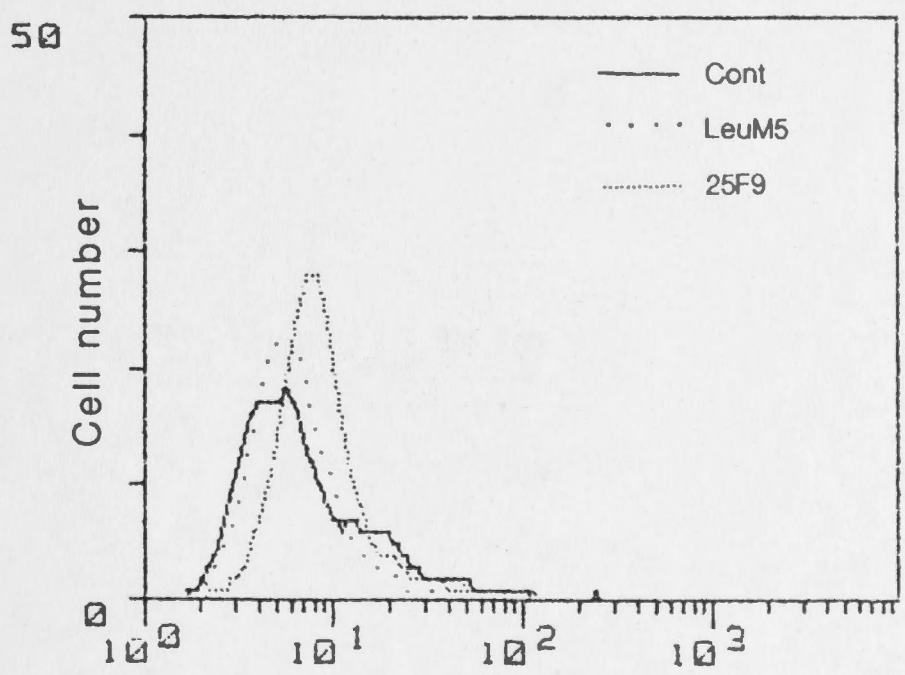
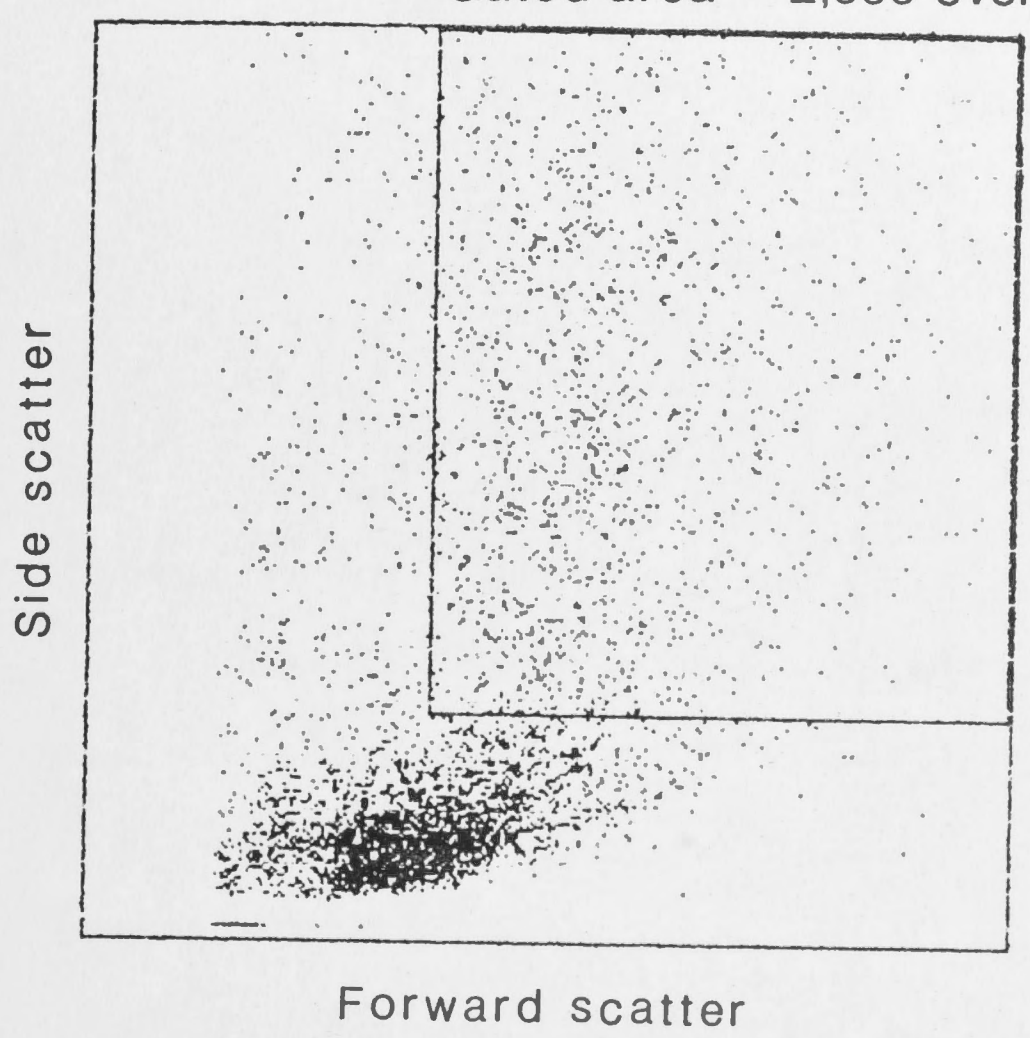

50
Cont
LeuM5
25F9
Cell number
0
10^0
10^1
10^2
10^3
Fluorescence (arbitrary units)

Figure 5.7

Flow cytometry of human colonic low density non-adherent cells: Analysis of a gated population of large cells. Low density non-phagocytic non-adherent human colonic lamina propria cells were incubated with no antibody (control) or the specified antibody. Fluorescence labelling was performed as described in Figure 5.5. The gated population comprised 40% of the total cell number analysed. All these cells expressed high levels of Class II MHC antigens.

Human colonic low density non-adherent cells



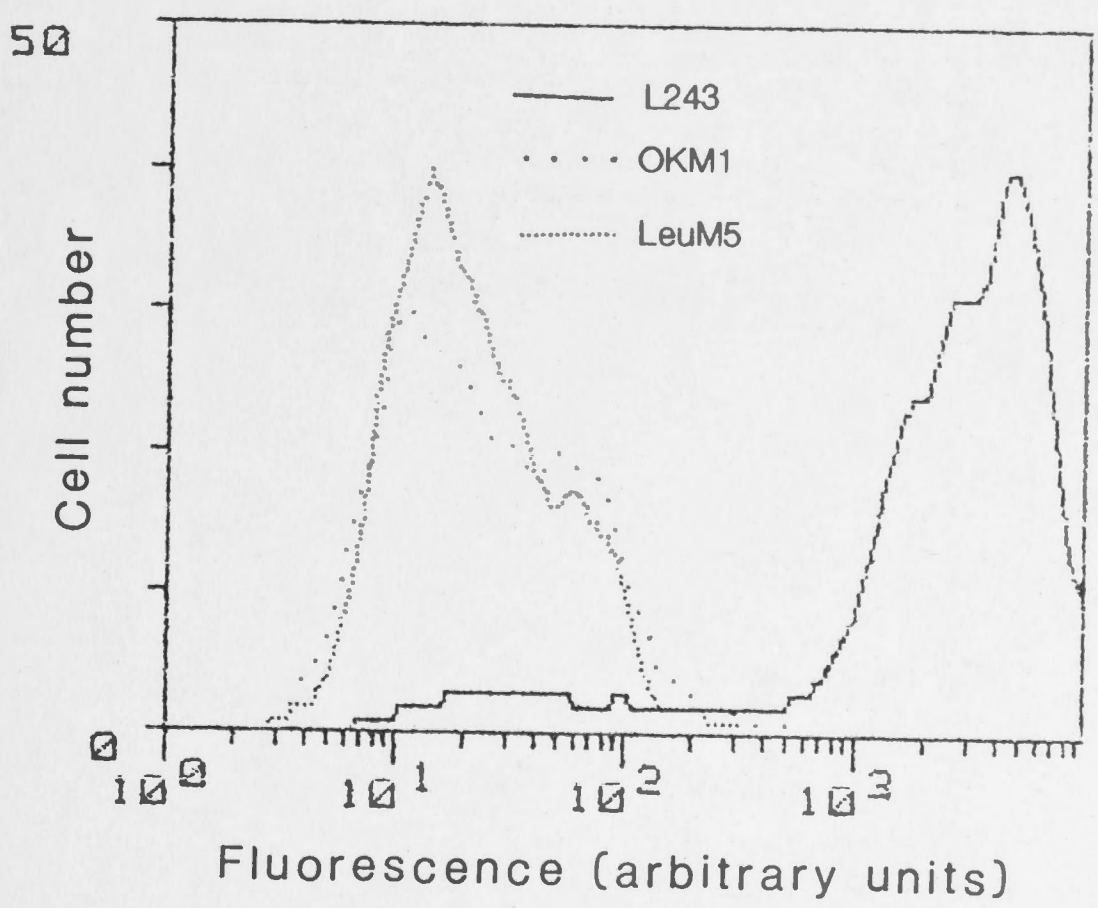


Figure 5.8

Flow cytometry of human colonic low density non-adherent cells: Analysis of a gated population of small cells. The experiment was performed as described in Figure 5.7. The gated population comprised 40% of the total cell number analysed. A significant subpopulation of these cells were OKT3$^+$ and OKT4$^+$. Double staining was not performed, but it is likely that these cells also expressed intermediate levels of Class II MHC antigen (see Figure 5.10). The T3$^-$ cells may be expressing high levels of Class II MHC antigens and may be small dendritic cells (see also Figure 5.9). The cells were not Leu16$^+$ (B cells).

Human colonic low density non-adherent cells

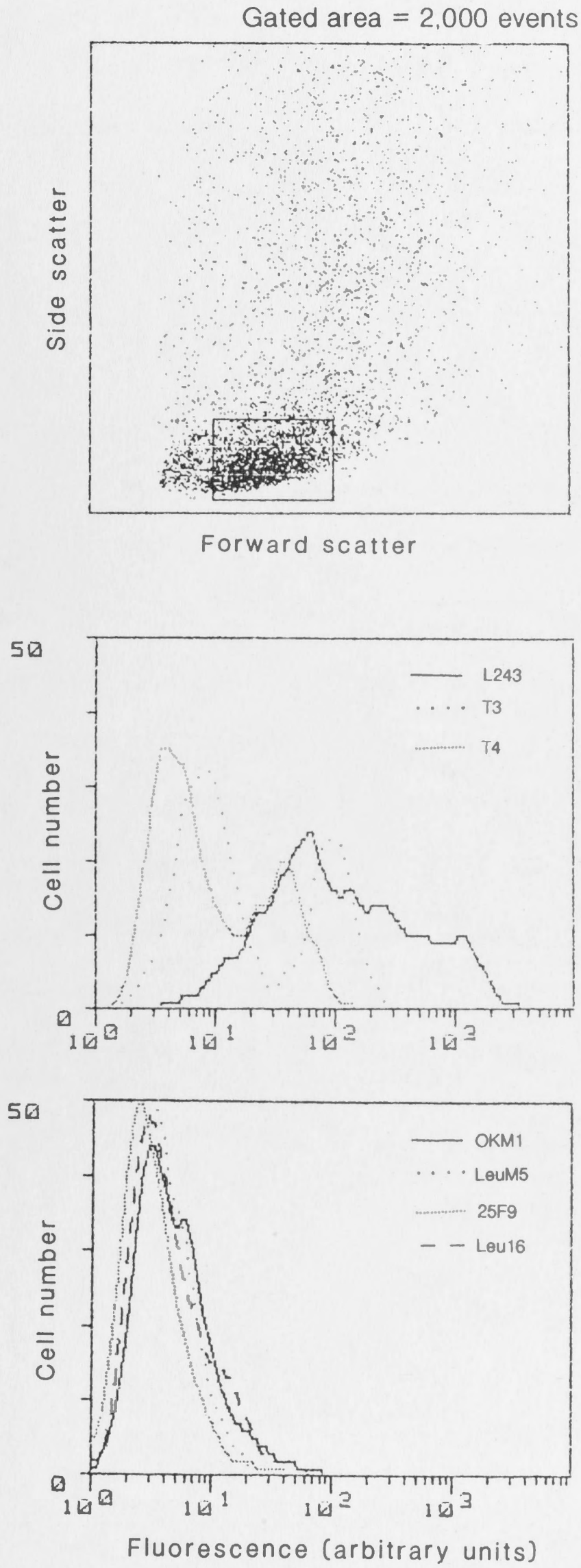


Figure 5.9

Flow cytometry of human colonic low density non-adherent cells: Dot-plot of fluorescence against forward scatter (cell size). The experiment was performed as described in Figure 5.7. The abscissae represent fluorescence intensity (logarithmic scale, arbitrary units) and the ordinate axes forward scatter (linear scale, arbitrary units). In the top graph, the cells were labelled with an isotype-matched control as described in Figure 5.7, and 5,000 events analysed. The bottom graph represents labelling with L243 (anti-Class II MHC). The events with high fluorescence intensity spanned a wide range of sizes from very large (which may represent cell clusters) to a size approximating that of a lymphocyte. The smaller cells (lymphocyte size) expressed intermediate levels of L243 when compared to the isotype-matched control.

Human colonic low density non-adherent cells

Isotype control

L243

Figure 5.10

Flow cytometry of human colonic lamina propria cells obtained by rosetting with AET-treated sheep red blood cells. The experiment was performed as described in Figure 5.5. The cells are a homogenous population of $CD3^+$ cells expressing low-intermediate levels of Class II MHC antigens. The expression of all other markers was similar to that of the control (no primary antibody).

AET–SRC rosetting intestinal lamina propria cells

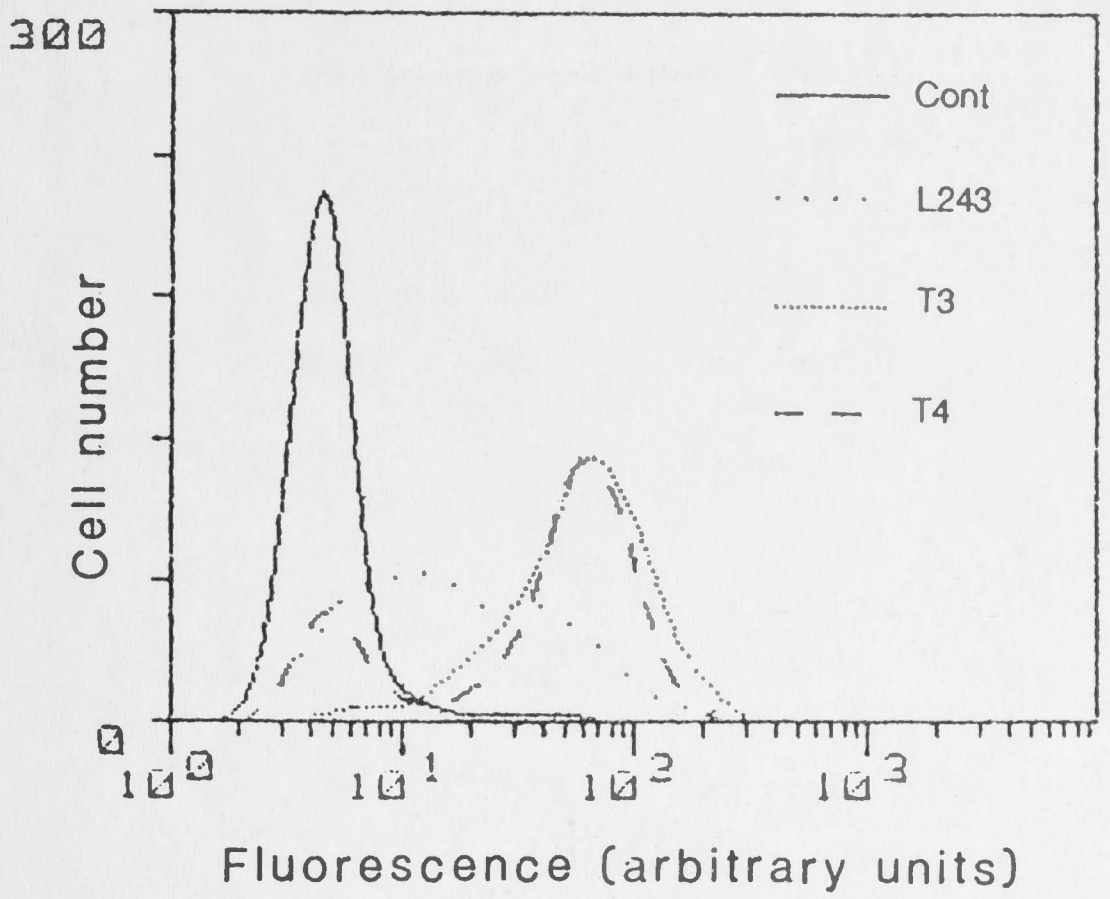


Figure 5.11

Flow cytometry of responder human peripheral blood lymphocytes. Peripheral blood lymphocytes were incubated with L243 (anti-Class II MHC), washed and then "panned" to remove cells expressing Class II MHC antigens. The remaining cells were incubated with no antibody (control) or the specified antibody, washed and then labelled with FITC-conjugated sheep anti-mouse immunoglobulin. The abscissae represent fluorescence intensity (logarithmic scale, arbitrary units) and the ordinate axes, cell number (linear scale, arbitrary units). The proportion of cells expressing each marker is indicated. (The cut-off was chosen by selecting a level of fluorescence intensity at which less than 5% of the control cells were positive.)

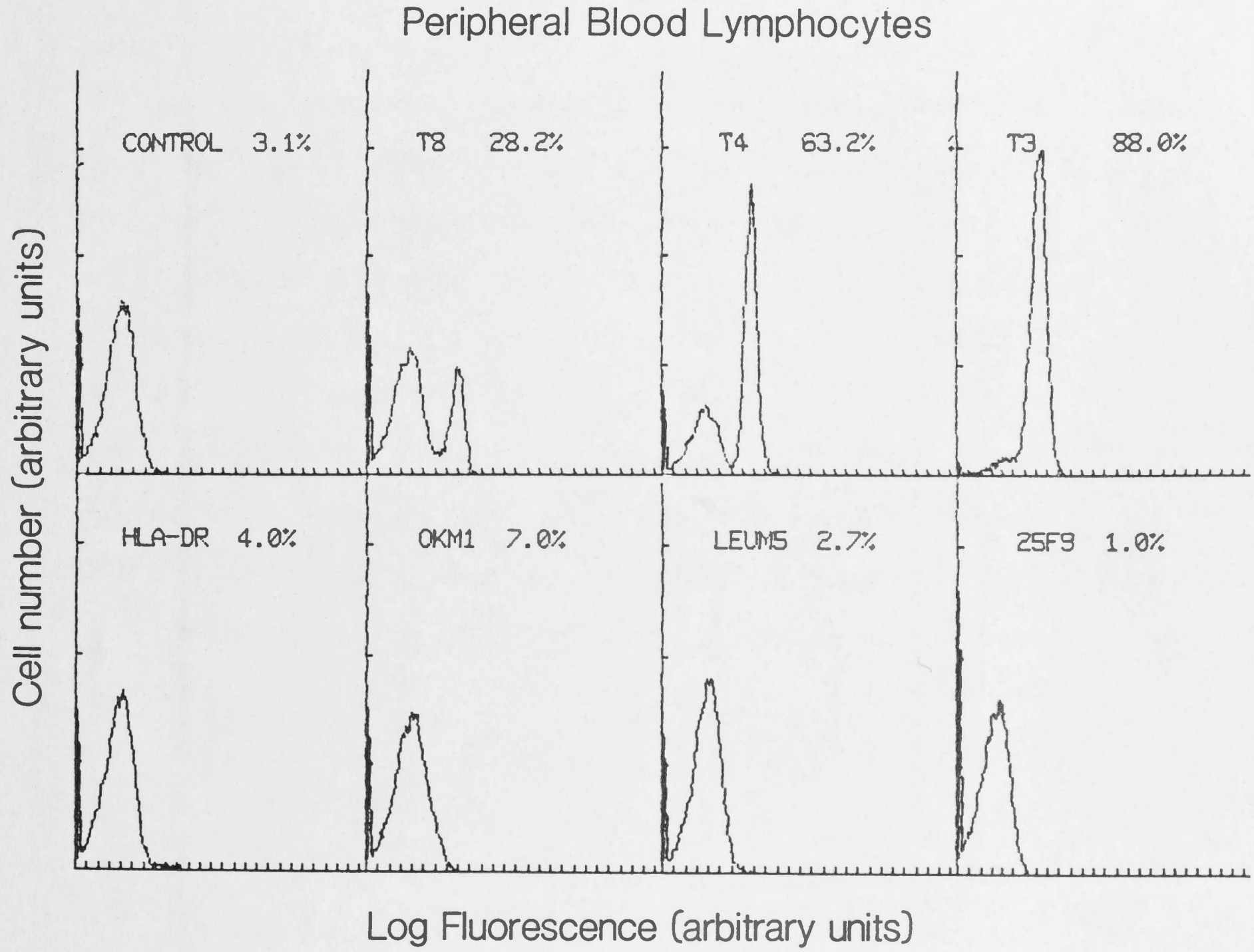

Peripheral Blood Lymphocytes
CONTROL 3.1%
T8 28.2%
T4 63.2%
T3 88.0%
HLA-DR 4.0%
OKM1 7.0%
LEUM5 2.7%
25F9 1.0%
Cell number (arbitrary units)
Log Fluorescence (arbitrary units)

Figure 5.12

MLR stimulation: Comparison of high- and low-density non-adherent intestinal lamina propria cells with fibronectin-adherent cells in the presence and absence of indomethacin. The abscissa represents increasing numbers of low-density (squares) and high-density (circles) non-adherent cells and fibronectin-adherent cells (triangles) that were incubated in the presence (closed symbols) and absence (open symbols) of indomethacin (2 μg/ml). Intestinal lamina propria cells were added to $2x10^5$ peripheral blood cells from an unrelated donor. After 4 days, 1 μCi of [^{3}H]-thymidine was added to each microculture and the cells freeze-thawed and harvested onto glass-fibre discs after a further 16 h. Unstimulated responder cells and control stimulator cells incorporated <1000 cpm. The values are the means $\pm$ SEM of triplicate assays. The absolute yields of high-density, adherent and low density cells in this experiment were $50x10^6$, $2x10^6$ and $2.5x10^6$ respectively. Assuming relative activities of 1:10:50 from the graph, this indicates that 26%, 10% and 64% of the total activity was present in the respective fractions.

Figure 5.13

MLR stimulation: The effects of adding intestinal macrophages. The experiment was performed as described in Figure 5.12. Increasing numbers of human colonic macrophages (enriched by fibronectin-adherence) were added to $2x10^5$ peripheral blood responder cells cultured with $5x10^4$ low-density non-adherent lamina propria cells in the presence (closed symbols) or absence (open symbols) of indomethacin (2 μg/ml).

Figure 5.12

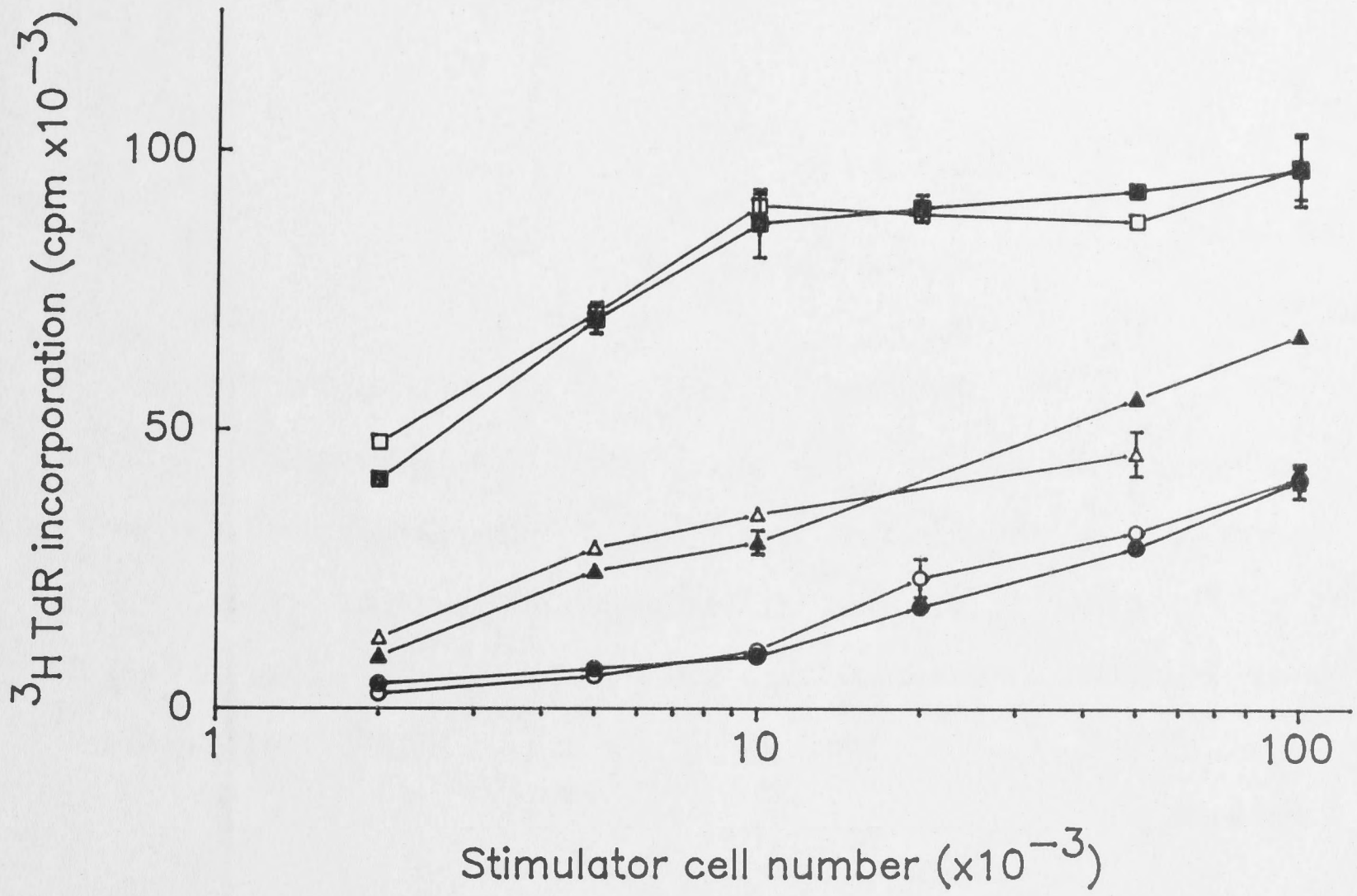


Figure 5.13

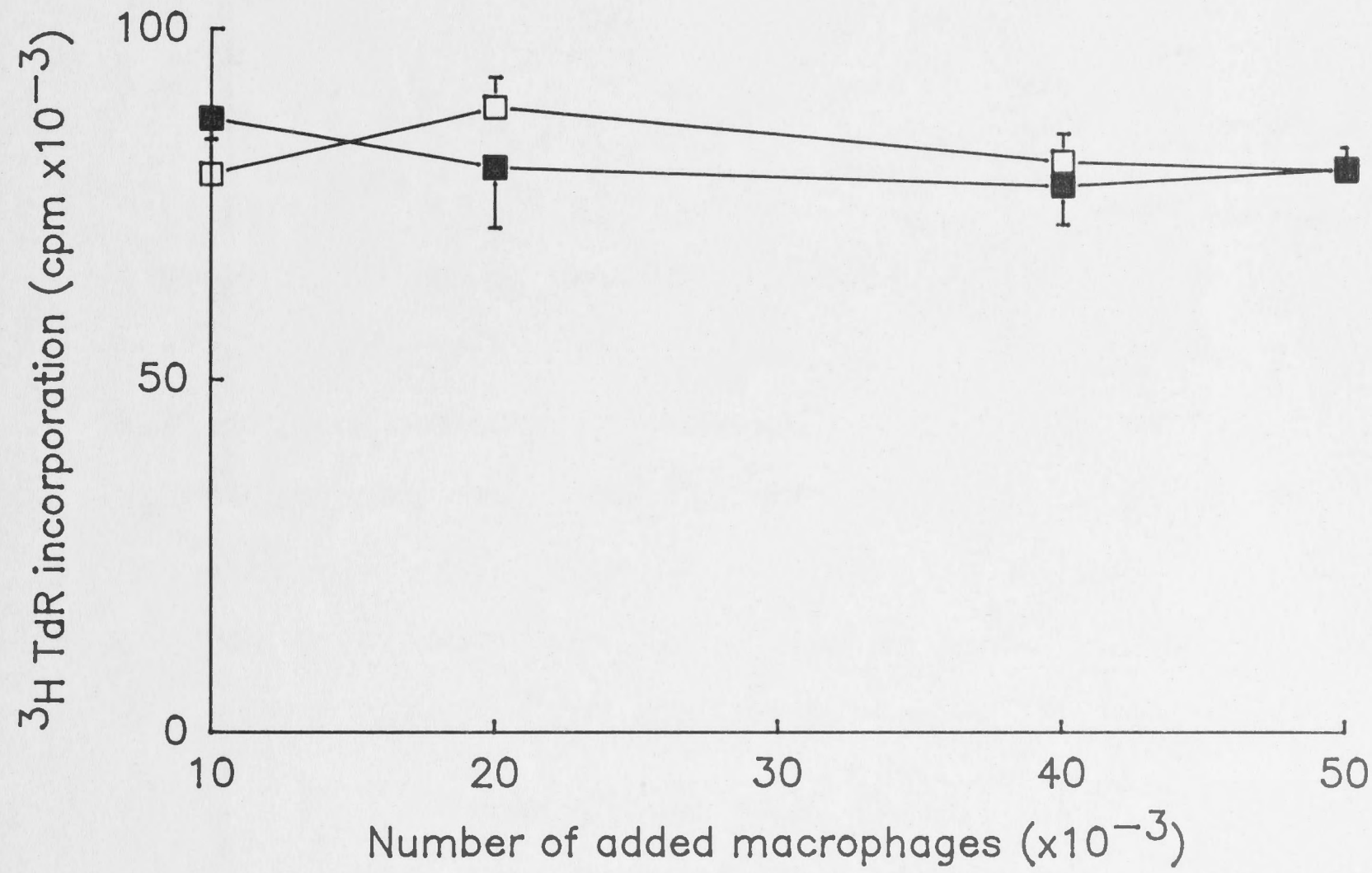


Figure 5.14

MLR stimulation: Comparison of high- and low-density lamina propria cells using two different responder cell populations. The experiment was performed as described in Figure 5.12. Increasing numbers of high-density (circles) and low-density (squares) cells were added to $2x10^5$ peripheral blood cells obtained by panning with L243 (anti-Class II MHC) (open symbols) or using the method of Warren (1981) [498] (closed symbols).

Figure 5.15

MLR stimulation: Comparison of non-adherent lamina propria cells with γ-globulin adherent cells and low-density, non-phagocytic, non-adherent cells. The experiment was performed as described in Figure 5.12. Increasing numbers of non-adherent lamina propria cells (inverted triangles), γ-globulin adherent cells (triangles) and low-density, non-phagocytic, non-adherent cells (squares) were added to $1x10^5$ peripheral blood responder cells (obtained using the method of Warren, 1981 [498]).

Figure 5.14

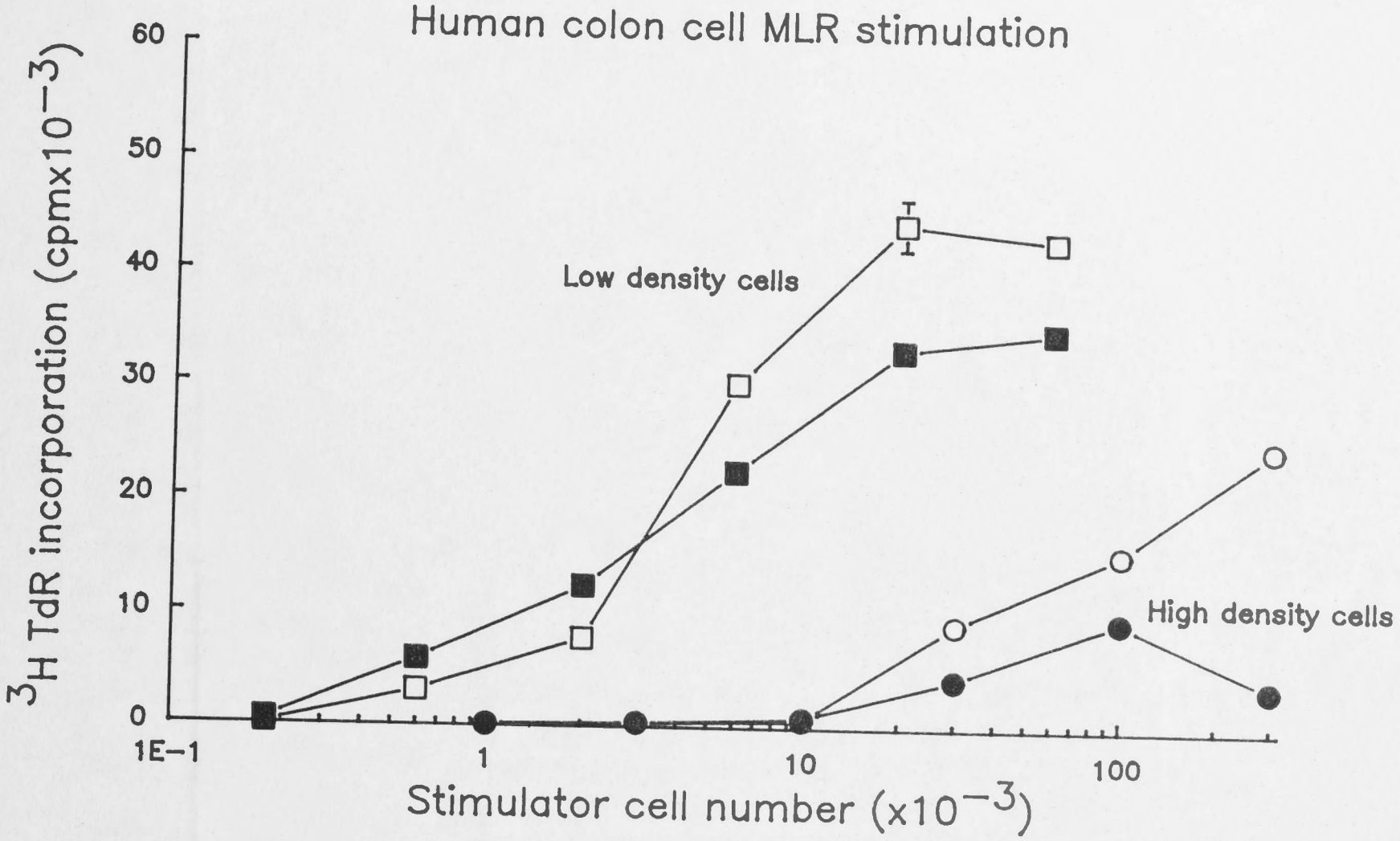
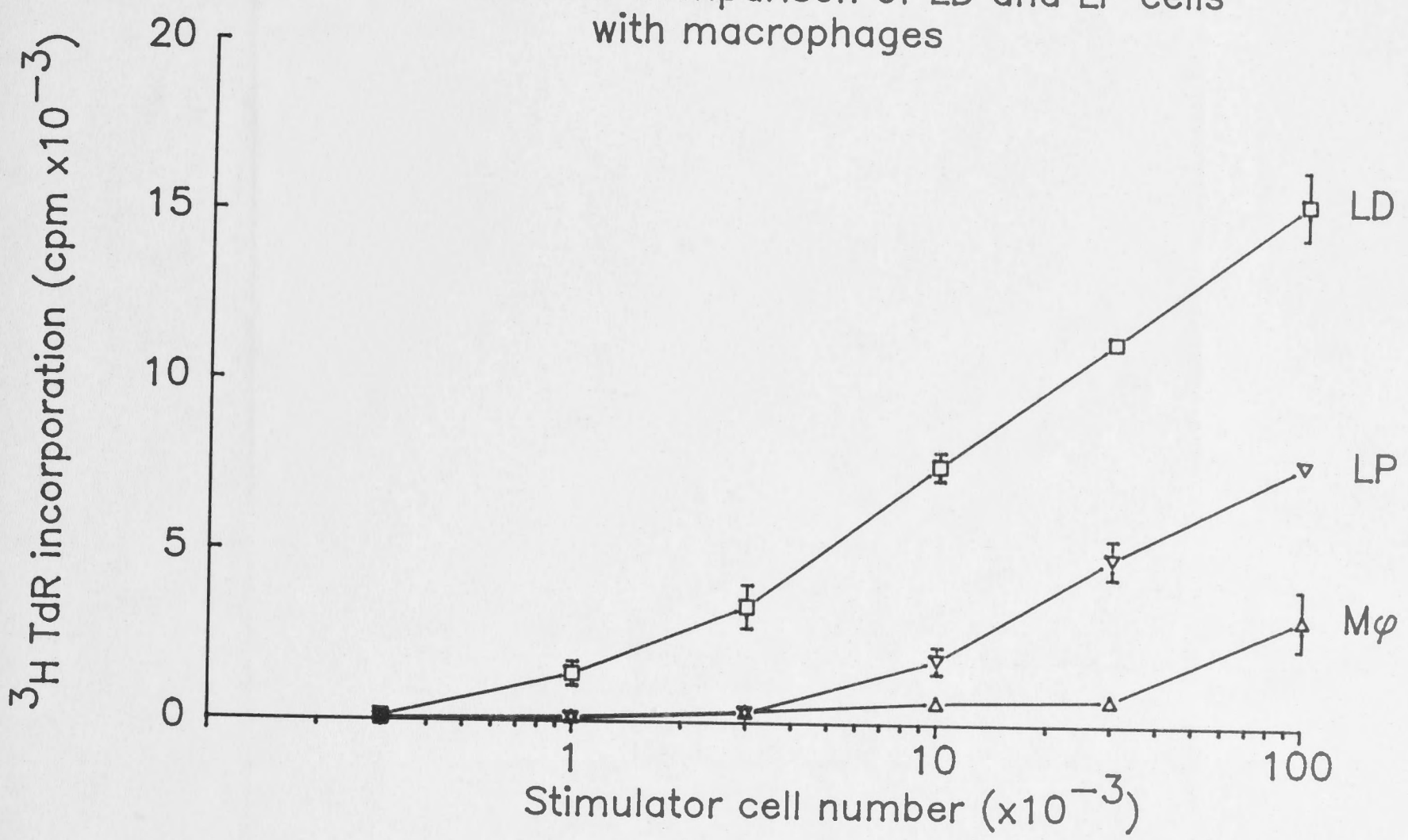


Figure 5.15



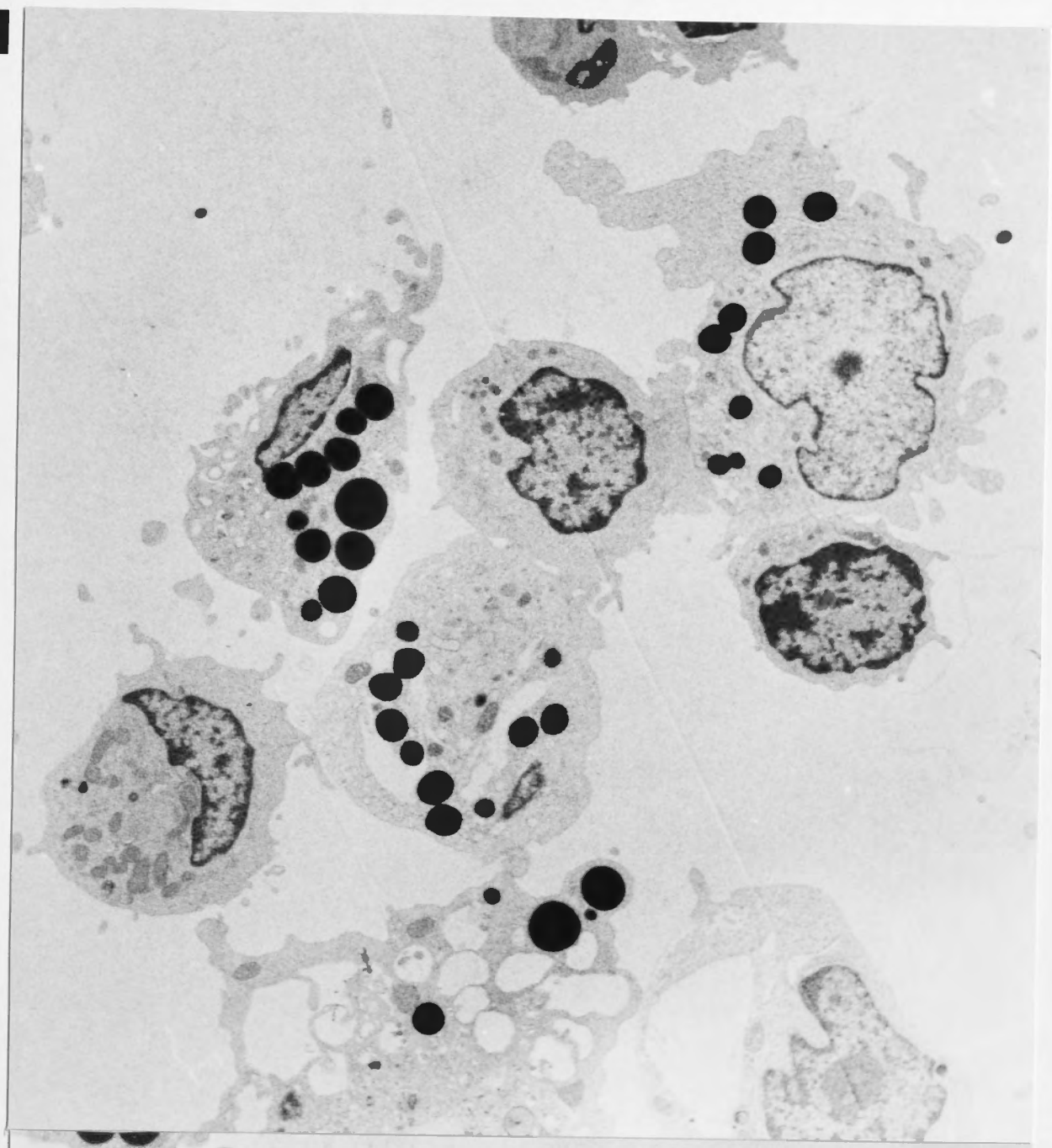
Micrograph 5.1

Transmission electron micrograph of the low density cells prepared by density gradient centrifugation (Nycodenz Monocytes, density 1.068 g/ml) of a single cell suspension of human colonic lamina propria cells (from which macrophages had not been removed). In the low density fraction there are populations of both macrophages, which contain numerous phagolysosomes (electron-dense and electron-lucent vesicles) of various sizes, and dendritic cells which are smaller, have an irregular nucleus with a peripheral rim of heterochromatin and do not contain phagolysomes. Final magnification x 3,600.

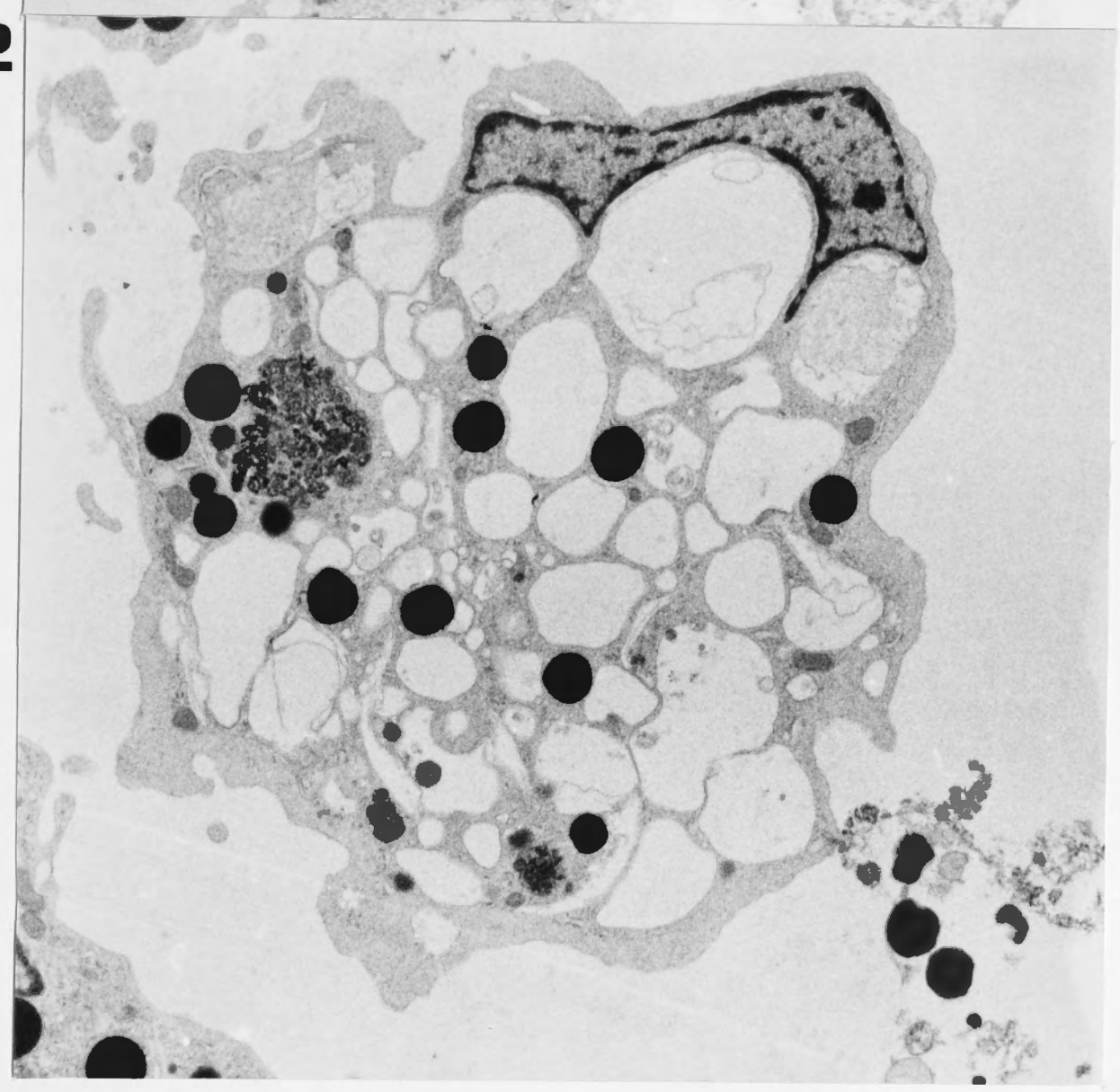
Micrograph 5.2

Transmission electron micrograph of an intestinal lamina propria macrophage. Final magnification x 5,900.

5.1

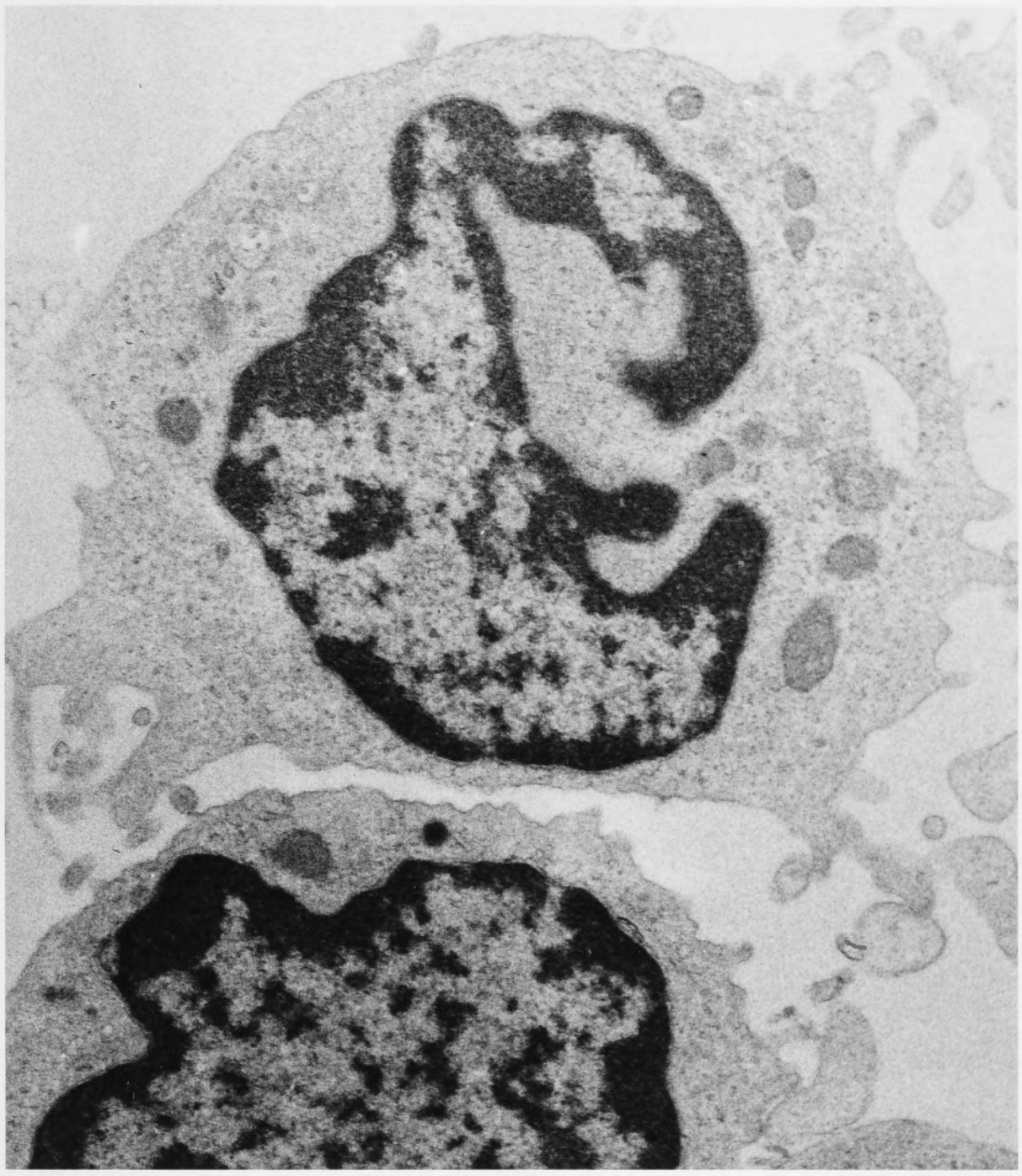


5.2



Micrograph 5.3

Transmission electron micrograph of human colonic lamina propria dendritic cells demonstrating irregular nuclei, the absence of phagolysosomes and the irregular cytoplasmic processes. Final magnification x 13,000.



CHAPTER 6

DISCUSSION

"The more we study, we the more discover our ignorance."

Scenes from *"The Magico Prodigioso of Calderon"*

Percy Bysshe Shelley (1792-1822)

6.1 INTRODUCTION

The study of the host response to foreign antigens has, for many years, bypassed the mucosal immune system. This has been both in the physical sense, by the direct inoculation of antigen into host tissues, and in terms of research interest, although there has been greater interest in the last two decades.

Most environmental antigens are encountered during or after their passage through an epithelial barrier eg. the epidermis and dermis of the skin and the laminae propriae of the respiratory, gastrointestinal or genitourinary tracts. Each of these sites is a unique microenvironment. For example, the intestinal lamina propria surrounds a lumen containing a wide array of ingested food antigens proximally, while distally, there is passive diffusion of bacterial breakdown products, including LPS, through the lamina propria [385]. In spite of these differences, there are many similarities between the mucosa-associated lymphoid tissues (MALT), which are often classified as a unified system. Two responses, in particular, are characteristic of the mucosal route of exposure to most antigens - the preferential induction of T cell tolerance and/or IgA antibody production. These responses result in minimal inflammation and the ability of the epithelial barrier to subserve specific functions, eg. gas transfer, the absorption of nutrients, electrolytes and water, etc. When these responses are deranged, "hypersensitivity" diseases (eg. gluten-sensitive enteropathy, hypersensitivity pneumonitis, inflammatory bowel disease, etc.) may result.

The main conclusions drawn from the experiments described in this thesis are that:

(a) the intestinal lamina propria contains small, but significant numbers of antigen-presenting dendritic cells; and that

(b) intestinal macrophages are found predominantly in the non-Peyer's patch lamina propria and may oppose the effects of dendritic cells on T cell activation.

In addition, the migration of monocytes into the intestinal lamina propria may result in differentiation into macrophages with a characteristic phenotype, and possibly, function. Other workers have suggested that dendritic cell functions are also affected by their environment (see below).

This discussion will focus on the role of dendritic cells and macrophages in the induction of T cell activation and show how they may participate in the regulation of mucosal immune responses in the gut. Particular points to be addressed include the unique properties of these cells in the intestinal lamina propria; their role in antigen handling; and mechanisms by which they may control T cell-mediated immune responses, with the consequent induction of either oral tolerance, specific mucosal protection or, rarely, chronic inflammation.

Several excellent and more comprehensive reviews of intestinal immune responses have been published [36, 61, 105, 310, 350, 518] and the reader is referred to these for consideration of other aspects of intestinal immunity.

6.2 INTESTINAL MACROPHAGES - EFFECTS OF THE MICROENVIRONMENT

Intestinal macrophages, like other tissue macrophages, are a heterogeneous population of cells [482]. Studies of their turnover have shown that peripheral blood monocytes migrate into tissues where they differentiate into tissue macrophages [482]. In the intestinal lamina propria, this process presumably occurs in response to antigenic stimuli (including bacterial lipopolysaccharide {LPS}) and the components of the immune system, particularly the cytokines (eg. IFN-γ and GM-CSF). As a result of these complex interactions, macrophages can secrete any number of a wide range of secretory products [346], including substances which enhance immune responses eg. interleukin 1 (IL-1), or which are immunosuppressive eg. prostaglandin E_2 (PGE_2). Although E-type prostaglandins are often regarded as being pro-inflammatory [eg. 284], analysis of many of their effects suggests a suppressive function, eg. the inhibition of cytokine-inducible Class II MHC expression [474, 488], the inhibition of T cell activation and down-regulation of the transferrin receptor by effects on protein kinase [87], the suppression of macrophage IL-1 activity [326], the inhibition of NK cell activity [127] and its effects on suppressor T cell induction [reviewed in Section 3.4]. Thus, at any point in time, the intestinal lamina propria contains macrophages, at varying stages of differentiation, presumably attempting to eliminate absorbed antigens or invasive microorganisms. In contrast to this postulated role of macrophages, the observations reported above suggest that activated intestinal macrophages suppress T cell responses. Are these two roles compatible?

Before considering this point, another problem should be addressed, namely, can in vitro findings be extrapolated to the in vivo situation? Attempts to do so should always be made with great caution, since there are many

confounding factors, eg. the activation or selection of subsets of cells, or the disturbance of cellular function by the disaggregation process.

In the lung, a situation similar to that of the intestine pertains. Alveolar macrophages in vitro down-regulate responses in an indomethacin-sensitive prostaglandin-mediated manner [116, 117, 163, 326]. Evidence that alveolar macrophages suppress pulmonary immune responses in vivo was provided by Thepen et al (1989) [470]. Alveolar (but not interstitial) macrophages were effectively removed from the lungs of mice by the intratracheal instillation of liposomes containing dichloro-methylene-diphosphonate. Subsequent intratracheal administration of antigen resulted in enhanced pulmonary immune responses (plaque-forming cell assays) suggesting that elimination of alveolar macrophages abrogated their normally suppressive role.

It is reasonable to assume, therefore, that intestinal macrophages also play a predominantly suppressive role in vivo. The question again arises, can intestinal macrophages effectively eliminate foreign antigens and have a moderating effect on T cell responses? In teleological terms, it would be in the best interests of the host to minimize the adverse effects on absorptive function that may arise from the amplification of immunological responses to commonly encountered luminal antigens, eg. LPS.

Some insights into this problem are provided by studies examining intestinal mononuclear cell responses to, and production of, various cytokines.

Both GM-CSF and IL-1 are produced constitutively by the cells of the human colonic lamina propria [293, 380]. The basal low-level production of IL-1_β by control colonic intestinal mononuclear cells was unaffected by exposure to LPS [293]. Mononuclear cells obtained from specimens resected from patients with inflammatory bowel disease, however, responded to LPS with increased IL-1_β production. This observation was interpreted as suggesting that the IL-1_β-producing activated macrophages were derived from circulating monocytes and

implies that monocytes become refractory to the effects of LPS as they differentiate into intestinal macrophages. Another interpretation is that refractory macrophages regain their sensitivity to LPS in response to cytokines produced by the lamina propria cells in inflammatory bowel disease. In both cases, it can be argued that intestinal macrophages are refractory to the effects of LPS in the normal colon.

The use of in situ hybridization to detect IL-1α and IL-1$_\beta$ m-RNA producing cells in the mouse intestine demonstrated only scattered positive cells in the stroma of intestinal villi and in the interfollicular areas of Peyer's patches [466]. These observations indicate either that the sensitivity of the assay was not great enough to detect low level production of IL-1 m-RNA and/or that there is a small subpopulation of intestinal macrophages producing the background levels of IL-1. These cells may be newly-arrived monocytes which respond to the environmental stimuli (eg. LPS, cytokines, etc), differentiate and become activated before they lose responsiveness to these signals and produce net negative effects on immune responses. In any case, there is evidence that intestinal macrophages have blunted responses to agents that normally result in activation.

The mechanisms by which the responses are down-regulated are unknown, but a parallel may be drawn with the responses of murine peritoneal macrophages to GM-CSF [154]. Short-term incubation with GM-CSF primed macrophages for IFN-γ+LPS-stimulated TNF$_\alpha$ release. In contrast, prolonged incubation with GM-CSF resulted in a markedly reduced responsiveness to IFN-γ+LPS, an effect which was probably PGE$_2$-mediated. Thus, incubation of macrophages with GM-CSF resulted in the sequential generation of two products mediating activation and then suppression of immune responses. In this autoregulatory circuit, PGE$_2$ limited GM-CSF-induced macrophage activa-

tion. LPS also primes macrophages for prostaglandin production [5], indicating the presence of multiple mechanisms of negative feedback control.

These processes are presumably affected by many other factors, but do argue for the presence of mechanisms to explain the apparently contradictory properties of the intestinal macrophage in vivo, namely, the effective elimination of invasive or particulate antigens, and the maintenance of a suppressive effect on the recruitment of other effector arms of the immune response.

6.3 DENDRITIC CELL FUNCTION IN THE INTESTINAL LAMINA PROPRIA

In contrast to the postulated inhibitory effect of intestinal macrophages in vivo, the experimental data reported above suggest that dendritic cells stimulate T cell responses. In vitro, this was measured by the induction of T cell proliferation in the allogeneic MLR. Mesenteric lymph node cells were chosen as responder cells in order to simulate the conditions in the lamina propria as closely as possible, bearing in mind the known organ-specific trafficking of T lymphocytes [74, 199, 344, 455]. In terms of MLR stimulatory function, no differences could be demonstrated between enriched populations of dendritic cells obtained from the spleen, from the lamina propria and from Peyer's patches. This is in contrast to the potentiation of MLR stimulatory ability observed following the culture of Langerhans cells with IL-1 and GM-CSF (See Discussion Chapter 4). It is likely that these disparities are a reflection of the factors affecting differentiation at varying sites, and suggests that intestinal dendritic cells are constantly exposed to antigen, IL-1 and GM-CSF.

The demonstration that intestinal dendritic cells preferentially induce IgA secretion indicates that intestinal dendritic cells have other properties related to their environment. Spalding et al (1984) [434, 435] showed that successful induction of polyclonal IgA secretion by either Peyer's patch or splenic B cells was dependent upon their interaction with Peyer's patch-derived (but not spleen-derived) dendritic cell-T cell mixtures. These observations raised the possibility that Peyer's patch dendritic cells had special properties leading to the preferential induction of IgA secretion. Subsequent work [432] supported the hypothesis that the isotype of antibody secreted and the extent of pre-B cell differentiation was dependent on the lymphoid tissue source of dendritic cells (but not T cells). (These results need confirmation using purified populations of dendritic cells. In the experiments reported above, the degree of Peyer's patch

dendritic cell enrichment was not reported, but was presumably around 60% [433]. It is possible that the small numbers of intestinal T cells present in the "Peyer's patch dendritic cell" populations directed isotype switching to IgA. This result is possible because of the potent activity of transforming growth factor-β (TGF_β) in inducing IgA isotype secretion by LPS-stimulated B lymphocytes [89]).

On the other hand, immunoglobulin subclass and isotype secretion is also known to be regulated by subsets of T cells [218] with characteristic profiles of cytokine production [153, 328]. The final arbiters of the differential regulation of antibody production [eg. 89, 429, reviewed in 364, 452] are likely to be these T cell-derived products.

Spalding and Griffin (1986) [432] suggested possible mechanisms to explain the observed interactions between dendritic, T and B cells. Firstly, Peyer's patch dendritic cells may selectively bind, from a heterogeneous population, subsets of T cells that would preferentially induce IgA secretion. Alternatively, dendritic cells may bind T cells nonselectively, but would then induce activation and differentiation of T cells into isotype-specific helper cells capable of providing the signals for antibody production.

In summary, Peyer's patch dendritic cells appear to regulate B cell differentiation and preferentially induce IgA secretion. The final steps in this regulatory pathway involve the production of specific cytokines by T cell subsets. The early stages (the differentiation of dendritic cells in the intestinal lamina propria) may be the consequence of the interaction of dendritic cells with tissue-specific receptors and/or the effects of locally generated cytokines. The result is dendritic cells with properties determined by their environment - in the case of the intestine, fully immunocompetent dendritic cells.

Having considered the unique properties of intestinal macrophages and dendritic cells, I will now consider the role of these cells in intestinal antigen handling.

6.4 INTESTINAL LAMINA PROPRIA ANTIGEN UPTAKE

Intact particles, macromolecules and soluble antigens are taken up from the intestinal lumen and transported into the lamina propria [31, 500, Section 1.1.3, reviewed in 310, 350, 517]. The fate of such antigen and the subsequent generation of an immune response probably depends on many factors including the nature of the antigen itself, a history of previous exposure, the amount and frequency of antigen administration and various host factors. Inert particles can gain access to the systemic circulation and can be gradually excreted without initiating any immune responses at all [reviewed 350]. Most antigens are presumably taken up by lamina propria macrophages and dendritic cells - the resultant immune response is considered below.

The various means by which antigens gain access to the lamina propria include:

(a) non-specific uptake of antigens between epithelial cells, presumably associated with leaking intercellular tight junctions;

(b) persorption, ie. the passage of macromolecules or small particulate antigens into the lamina propria by kneading between epithelial cells - a consequence of normal intestinal motility;

(c) villus uptake of antigen via the exclusion zone. The villi undergo cycles of contraction and relaxation which are associated with the extrusion of cells from the villous tip. Uptake of antigen through gaps between epithelial cells occurs during the relaxation phase;

(d) active uptake of antigen through the epithelial cell by non-specific endocytosis or by receptor-mediated uptake. Intracellular processing may also result; and

(e) transportation through the membranous epithelial (M) cell into the underlying Peyer's patch.

6.4.1 M cells in antigen handling

[reviewed in 430, 517]

The M cell acts as a conduit for the transportation of luminal antigens, including viruses and bacteria, to the extracellular space. These cells form part of the epithelial layer overlying intestinal follicles and can be distinguished from absorptive cells by their lack of a well-developed brush border, their decreased height and their proximity to the underlying lymphoid cells (which are often found in an indentation of the M cell cytoplasm called the "central hollow").

Macromolecules and micro-organisms shown to be transported to the underlying lamina propria include ferritin, horseradish peroxidase, wheat germ and other agglutinins, cholera toxin, reovirus types 1 and 3, mycobacteria, chlamydiae, Vibrio cholera and Cryptosporidium [517]. There is some evidence to suggest that the virulence of microorganisms is determined by the ability of M cells to take up and transport organisms to the subepithelial lymphoid tissue [430]. For example, for organisms that need to pentrate the mucosa to initiate infection (reovirus, poliovirus, Mycobacterium tuberculosis, salmonella) translocation to the lamina propria is a positive virulence factor. On the other hand, for non-invasive organisms which colonize the mucosal surface, eg. the RDEC-1 strain of enteroadherent E. coli, M cell transportation may result in the initiation of immune responses and prevention of such colonization and/or elimination of the pathogenic organism. For these organisms, virulence requires a mechanism of adherence to the absorptive or M cell in the absence of pathways for translocation.

Because of the proximity of the M cell to the underlying lymphoid tissue, immunological processing of luminal antigens and microorganisms is facilitated. The factors governing the adherence and transportation of antigens to the M cell is not known, nor is it known whether there is one or a number of pathways, nor, indeed whether antigens undergo processing within the M cell. It is conceivable

that these factors may influence the nature of the subsequent immune response. Alternatively, the M cell may play a purely passive role with the underlying lymphoid cells determining the outcome.

6.4.2 Antigen handling by intestinal macrophages and dendritic cells

What is the fate of the absorbed antigens? At least four possibilities exist. Firstly, the antigens may be completely degraded in situ eg. by the enzyme systems of resident or elicited macrophages. Another possibility is that macrophages sequester antigen in an inactive form and are either extruded into the gut lumen or migrate to, and become resident in an inactive site, eg. the draining lymph nodes or subserosal area. A third possible consequence is the release of either undegraded or partly degraded antigens into the surrounding tissue and subsequent uptake and presentation to T cells by dendritic cells. Finally, dendritic cells may directly take up antigens which are presented to T cells, either in situ or after migration to the draining nodes.

This discussion is concerned primarily with the role of macrophages and dendritic cells in the regulation of T cell responses so their role in the degradation of absorbed antigens will not be considered. This and other important aspects of intestinal and other tissue macrophage function (eg. secretion of oxygen free radicals, neutral proteases and lysosomal enzymes) have been reviewed extensively elsewhere [155, 171, 262, 310, 391, 468, 480].

Both murine and human intestinal macrophages had evidence of previous phagocytic activity (Chapters 3 and 5). Large vacuoles (presumed phagolysosomes) were present and the expression of the monoclonal antibody, 25F9, on human macrophages strongly suggested previous endocytic activity [170].

They also had features of "activation", a process which is associated with enhanced phagocytosis. These included the expression of Class II MHC antigens and the down-regulation of F_C and complement receptors in both species, and the down-regulation of the F4/80 antigen in the mouse (Chapters 3 and 5). Although not examined in this work, others have shown that F_C receptors are not normally expressed on macrophages in the human colon in vivo [291] and that expression increased with time in culture [513].

Absorbed antigens are more likely to be taken up by macrophages than by other cells because of their numerical superiority (10% of disaggregated lamina propria cells vs <1% dendritic cells). What then is the fate of these phagocytosed antigens? A potential mechanism for their removal may be by the shedding of antigen-laden macrophages into the intestinal lumen. The presence at immunohistochemistry of large numbers of macrophages with evidence of prior phagocytosis in the subepithelial regions of the colonic mucosa [170] is consistent with such a mechanism. Other supportive evidence is reviewed by Le Fevre et al (1979) [262] and includes the migration of latex-containing macrophages from Peyer's patch to the tips of nearby villi and their possible extrusion. This process is analogous to the migration of waste-laden macrophages from the lungs via the mucociliary elevator system.

Another possible destination of antigen-laden macrophages is the draining lymph nodes. This pathway has been described in the intestine [263, 303] and the lung [148]. In a guinea pig model of melanosis coli, apoptotic bodies derived from surface intestinal epithelial cells were phagocytosed by intraepithelial macrophages and transported into the lamina propria [497]. These macrophages passed through fenestrations in the surface epithelial basement membrane, accumulated progressively in the lamina propria, entered the submucosa and finally migrated to the regional lymph nodes. This migration pathway is analogous to the translocation of inert particles from alveolar spaces to

draining nodes in the lung [148] and need not be associated with active inflammation.

The presence of bacterial antigens in association with veiled cells in the intestinal afferent lymph [311] suggests that dendritic cells transport antigen from the intestinal lamina propria in the same way that Langerhans cells migrate from the skin to its draining nodes. It is not clear whether these antigens are taken up by dendritic cells directly or indirectly after processing by macrophages. These and other aspects of dendritic cell-macrophage interaction need to be examined further.

In summary, antigen once present in the lamina propria is taken up by macrophages and/or dendritic cells. It may be degraded in situ, eliminated by passing into the intestinal lumen within macrophages, or carried to the draining nodes by dendritic cells or macrophages. The subsequent reactions may be an active immune response, a null response or immunosuppression. The relative proportion of antigen eliminated from the intestinal lamina propria by each mechanism and the significance of each in the regulation of immune responses has yet to be determined.

Having considered the properties of macrophages and dendritic cells in the unique environment of the intestinal lamina propria and their possible participation in lamina propria antigen handling, I will now review their roles in the regulation of immune responses following antigen exposure. In particular, I will try to relate their properties to the possible outcomes in the gut, namely, the induction of oral tolerance, the generation of T cell-mediated or B cell-mediated (predominantly IgA) responses or the development of hypersensitivity reactions.

6.5 ORAL TOLERANCE

"Oral tolerance" is a state of specific immunological unresponsiveness induced by prior oral administration of antigen [330, reviewed in 81, 85]. In 1829, Dakin reported that South American Indians ate poison ivy leaves in an attempt to prevent contact hypersensitivity reactions to the plant [cited in 330]. This observation raised the possibility that the oral ingestion of an antigen could modify subsequent systemic immune responses. This phenomenon was further explored this century following the studies of Beredska, Wells, Osborne and Chase which showed that feeding proteins to experimental animals could inhibit systemic responses (anaphylaxis and contact hypersensitivity) to the same antigens [reviewed in 330].

Oral tolerance induction affects various immune parameters including helper T cell function, T cell proliferation, delayed-type hypersensitivity and T-dependent specific antibody production [85]. B cells themselves are potentially reactive as shown by studies bypassing the requirement for helper activity eg. by removing T cells and stimulating B cells with antigen plus LPS [335] or by the in vivo administration of T-independent antigens [471].

Several lines of evidence suggest that there are different mechanisms by which oral tolerance can be induced. In particular, the time course of induction of oral tolerance and the responses of different arms of the immune response vary. For example, feeding the antigens, ovalbumin [85] and gliadin [475], resulted in the dose-dependent suppression of both cell-mediated and humoral immune responses. In contrast, feeding cholera and related toxins, inhibited delayed-type hypersensitivity responses whilst having no effect on the generation of specific antibody [220]. Even when only one protein antigen is studied, for example, ovalbumin, variations in the duration of tolerance for cell-mediated and humoral immunity [457] and differences in the doses required to induce

tolerance in the effector arms of the immune response [334] imply that there are different means of generating systemic hyporesponsiveness.

Mechanisms which have been suggested include antigen-processing [68], the generation of circulating antibodies [205], antigen-antibody complexes [eg. 14] or suppressive serum factors [304], and both T and B suppressor cells [reviewed in 220, 330]. Both MHC-influenced [331] and non-MHC-linked [205, 257, 304] mechanisms have been described.

Intestinal handling of antigen plays an important role. In the early phase of oral tolerance induction, a serum factor, which passively transfers antigen-specific oral tolerance for T cell- (but not B cell-) mediated immune responses to syngeneic recipients, can be identified one hour after feeding [67, 68, 220, 458]. Studies of the nature of this factor using ovalbumin suggest that the protein undergoes some form of subtle immunochemical modification resulting in antigenic material similar in size to native ovalbumin and reactive with anti-ovalbumin antibodies [68]. Although removal of these immunoreactive ovalbumin antigens abrogated oral tolerance induction [68], modifications of the protein structure are likely to be more important than the quantity of unchanged antigen absorbed since: (i) serum levels of ovalbumin were unrelated to the development of tolerance [69] and; (ii) the parenteral administration of ovalbumin in a range of doses did not induce immunological hyporesponsiveness [458]. Other evidence suggesting some form of antigen-processing or modification is provided by studies using cholera toxin [219]. Formaldehyde-modification of the protein conformation of cholera toxin before feeding did not affect the ability of the two antigenic forms to induce totally cross-reactive oral tolerance. This suggests that the putative suppressive serum protein has undergone some form of antigen-processing rather that being absorbed unchanged in its tertiary configuration. Another factor which may be important in the generation of oral tolerance may be the rate of delivery of antigen to the intestinal

lamina propria - antigen concentration is an important factor in the development of other forms of tolerance (see below).

The nature of the cell responsible for the generation of such tolerogenic proteins is not known - possibilities include the M cell of the Peyer's patch, the enterocyte or the intestinal lamina propria macrophage. The generation of immune responses by bypassing the epithelium (eg. the injection of antigen directly into Peyer's patches [37]) implies that the former cells play a significant role. Other evidence, however, suggests that the epithelial cell is not as important as a lymphoid cell in the generation of oral tolerance. Cyclophosphamide-treated [458] or irradiated [69] mice lose the capacity to generate the tolerogenic form of protein antigens in a manner which appears to be unrelated to the effects of the treatment on the donor epithelial cell. This ability may be reconstituted by the adoptive transfer of splenic lymphoid cells [69]. As discussed in Section 1.5.6, if the epithelial cell does play a role in oral tolerance induction, this function is unlikely to be a property of the expression of Class II MHC molecules on the small intestinal epithelial cell.

Another mechanism for the suppression of delayed-type hypersensitivity is the generation of suppressor T cells which can be identified in Peyer's patches, mesenteric lymph nodes, thymus and spleen after feeding [305]. Transfer of "fed" serum to syngeneic recipients resulted in the induction of antigen-specific suppressor cells and was abrogated by treatment of the recipient (but not donor) animals with cyclophosphamide [220, 458]. These cells appeared to preferentially regulate the afferent limb of the immune response, since feeding the protein antigen, cholera toxin, in tolerogenic doses did not suppress the activity of mature delayed-type hypersensitivity effector cells. Other mechanisms are also involved since oral tolerance induction can occur in the absence of demonstrable splenic suppressor cells [256].

The importance of the macrophage and its products in oral tolerance induction is illustrated by the abrogation of this response by agents which activate the "reticulo-endothelial system", eg. oestrogen, muramyl dipeptide, LPS and the graft versus host reaction [332, 333, 456, 459]. In addition, treatment with the anti-macrophage agent, carageenan, partially relieves the suppression of T cell responses induced by feeding protein antigens [305], whilst inhibitors of macrophage production of prostaglandins (eg. indomethacin) interfere with the induction of systemic tolerance in vivo [415].

6.5.1 Possible roles of macrophages and dendritic cells in oral tolerance induction

Although oral tolerance is generally considered separately from systemic immunological tolerance, they may have similar mechanisms of induction, namely: (i) antigen sequestration; (ii) clonal deletion; (iii) generation of suppressor cells; and (iv) clonal anergy [reviewed in 351]. Immunoregulatory mechanisms which may be involved in the intestine include:

(i) **antigen sequestration** in the lumen by physical agents and antibody ("antigen exclusion" [61]) will not be considered here.

(ii) **defective antigen presentation**

The initial failure to demonstrate antigen-presenting cell activity in the intestine [37, 206] was the result of the inability of the physical disaggregation techniques to release antigen-presenting cells from the extracellular tissue matrix (See Discussion Chapter 2). Subsequent work demonstrated that potent antigen-presenting cells (dendritic cells) were present in murine Peyer's patches [433] and that both murine and human intestinal lamina propria cells were capable of stimulating the MLR.

(ii) **generation of suppressor T cells**

Adoptive transfer of oral tolerance by T cells is well recognized [305, 392, reviewed in 330] but the mechanisms of suppressor cell generation have not been elucidated. Possible mechanisms were described in Section 4.4 and include prostaglandin-mediated $CD8^+$ cell induction or the interaction with $I\text{-}J^+$ antigen-presenting cells [332]. Complex immunoregulatory suppressor and contrasuppressor circuits have also been proposed [141, 228, 269, 465].

As discussed above, immunoreactive protein antigens, implicated in the generation of suppressor T cells, are observed in serum after feeding. Intestinal macrophages may play a role by modifying absorbed antigens to produce these tolerogenic forms.

(iii) **clonal anergy**

Feeding protein antigens tolerizes both cellular and humoral immunity, with the former generally being more consistently affected (see above). The development of clonal anergy, at least in B cells, depends on both the concentration of the tolerogen, and the affinity of the cell to the antigen involved [351]. One conceivable function of the enterocyte or the M cell is the delivery of antigen in concentrations critical for tolerance induction.

The induction of clonal anergy may involve intestinal macrophages either directly, by the release of prostaglandins or other cytokines, or indirectly by the generation of antigen-specific suppressor T cells.

The presentation of antigen to T cell clones in the absence of IL-1 or other accessory cell-derived factors [271, 382] results in the transmission of a tolerogenic signal and the induction of a long-lived state of proliferative non-responsiveness. The compartmentalization of macrophages between the lamina propria and the Peyer's patch may explain the differences between the antigen-presenting cell activity of the unfractionated cell suspensions from these distinct sites (ie. the lesser activity of the Peyer's patch cells may be due to the absence

of macrophage-induced synergistic factors) and suggests a possible mechanism of clonal anergy - that is, the presentation of antigen to T cells by dendritic cells in the Peyer's patch in the absence of macrophage-derived factors, eg. IL-1.

However, these observations in vitro are obviously artefactual. In situ, macrophages far outnumber dendritic cells and are present in greater numbers than can be supported by culture medium in vitro (ie. $>1\text{-}2\times10^6$/ml). These facts, in the context of the experimental data reported above (Chapter 3), suggest that the net effect of these forces in the lamina propria is suppressive. Oral tolerance would therefore be the result of the balance between "weak" immunostimulatory and "stronger" immunosuppressive factors present in the intestinal lamina propria. The generation of intestinal immune responses would depend on the ability of invasive antigens to override these suppressive influences in the lamina propria or to be selectively taken up by the M cells of the Peyer's patch. In the Peyer's patch, the presence of immunostimulatory cells without suppressive macrophages enables the Peyer's patch to subserve its known functions of luminal antigen sampling and the initiation of mucosal immune responses.

(iv) **clonal deletion**

Clonal selection - the maturation of the lymphocyte through a stage when any contact with a recognizable antigen leads to the death of that cell - is an important mechanism for the induction of T cell self-tolerance. Nossal (1989) [351] distinguishes between clonal abortion - the elimination of a cell before it has become immunocompetent - and clonal deletion - the elimination of a previously functional lymphocyte. Clonal abortion within the thymus [6] may be the main mechanism of self-tolerance in the T cell compartment. This process requires antigen-presenting dendritic cells, but the end result (activation or elimination) is dependent on the maturational stage of the T cell [306] and not on the

properties of the dendritic cells. For example, splenic dendritic cells are potent activators of mature T cells, but were the most potent inactivators of young developing thymocytes [306].

A similar process may occur in draining lymph nodes, where T cells proliferate in response to the entry of antigens [360]. In athymic nude mice, small numbers of T cells which have undergone extrathymic differentiation can be found. These cells are tolerant of self, as well as being capable of allogeneic responses [306]. These observations are consistent with the conclusion that the outcome of antigen-recognition is dependent on the maturational stage of the lymphocyte. The site of such extrathymic differentiation is not known, and may be the secondary lymphoid structures such as lymph nodes.

The factors affecting the possible outcomes (proliferation and differentiation into effector, helper or suppressor cells, ie. clonal "expansion", clonal anergy, clonal deletion, and perhaps even clonal abortion) remain to be elucidated, but may include such parameters as antigen concentration, and cytokine production, amongst many others.

6.6 ROLE OF DENDRITIC CELLS AND MACROPHAGES IN NORMAL RESPONSES - IMMUNOHISTOCHEMICAL STUDIES

The anatomical distribution of human intestinal macrophages and dendritic cells in normal and diseased tissues has been examined using enzyme and immunohistochemical techniques [10, 131, 170, 291, 422]. In normal tissue, macrophages were found to be concentrated towards the luminal surface and expressed the following phenotype: acid phosphatase$^+$, non-specific esterase$^+$, HLA-DR$^+$, CD4$^+$, CD11b+/-, macrophage-specific markers$^+$ (25F9, EBM/11, 3C10, RFD7, Y1/82A).

Dendritic cells (RFD1$^+$, HLA-DR$^+$, Heca-452$^+$ {specific for an adhesion molecule on high endothelial venules}, acid phosphatase$^-$) were found mainly in highly organised tissue present at the deeper layers in the gut wall, around the broad zone of scavenger macrophages at the bottom of ulcers and fissures in inflammatory bowel disease, in lymphoid aggregates adjacent to granulomas in Crohn's disease [422] and, in smaller numbers, in the subepithelial regions [291], and in the epithelium itself [497]. Localization of these cells in rodents also demonstrated the presence of macrophages at the site of antigen entry, eg. in the peri-epithelial region of the small intestine [169], and in the subepithelial dome of Peyer's patch [361, 514], whilst putative dendritic cells were identified in the subepithelial regions of the villi and Peyer's patch [312, 510] and in the epithelium [312] and the interfollicular area of Peyer's patch [510].

This pattern of distribution is in keeping with the proposed roles of intestinal macrophages as phagocytic, degradative cells and dendritic cells as a "second line" defence involved in the presentation of persisting or evasive antigens to other effector arms of the immune response.

6.7 THE ROLE OF DENDRITIC CELLS AND MACROPHAGES IN INTESTINAL INFLAMMATION

In tissue samples resected from patients suffering from chronic inflammatory bowel disease, "scavenger" macrophages, arranged in band-like zones forming the bases of ulcers or fissures, were present in greater numbers than in control tissue [291, 422].

The granuloma of Crohn's disease was often localized along the draining lymphatics of the intestinal wall and in the mesenteric lymph nodes. These were composed of "epithelioid" cells expressing acid phosphatase, non-specific esterase, HLA-DR, CD11b, CD71, and the macrophage markers, EBM11, RFD9, 3C10, Y1/82A [131, 291, 422]. They were negative for the dendritic cell-associated marker, RFD1 [422], although not all would agree [291]. There is also evidence for the presence of a population of recently elicited cells in the inflammatory infiltrate as suggested by the presence of $CD11b^{+}$, $25F9^{-}$ monocytes [170]. These may be the cells which are able to respond to LPS and activating cytokines by producing detectable IL-1 mRNA [466] and IL-1 itself [293]. Thus, in the state of chronic low grade inflammation which is regarded as normal in the intestinal mucosa, and in areas of acute and chronic inflammation, there are heterogeneous populations of macrophages which are derived from recently elicited macrophages. These may differentiate under the influence of the predominant regulatory factors into activated phagocytic tissue macrophages or epithelioid cells, and effectively eliminate or transport the putative pathogen through the lymphatics to draining nodes.

Similarly there is considerable evidence pointing towards the dendritic cell playing a prominent role in chronic inflammatory diseases, eg. rheumatoid arthritis [reviewed 376] and infections [eg. 222]. In studies of ongoing inflammation, dendritic cells can be identified around blood vessels before the arrival of lymphocytes. When T cells do arrive, a significant proportion can be shown to

express activation markers. Furthermore, in the rheumatoid synovium there appears to be a definite spatial organization of lymphocytes around dendritic cells [376] (possibly representing in vitro cluster formation). These observations suggest that the dendritic cell is involved in the recruitment, activation and organization of T lymphocytes in the inflammatory response.

In immunohistochemical studies of dendritic cells in inflammatory bowel disease, increased numbers of dendritic cells were identified as part of organized lymphoid cell accumulations (predominantly T cells). These aggregates were found surrounding the bands of "scavenger" macrophages or were adjacent to granulomas [422] in Crohn's disease, whilst in ulcerative colitis, dendritic cells were identified in large numbers in association with lymphoid infiltrates in the lamina propria [10].

Disaggregated mucosal tissue resected from patients suffering from inflammatory bowel disease contained larger numbers of veiled cells than tissue from controls [508]. These veiled cells were the targets of a process termed peripolesis, in which lymphocytes migrate around other cells, and which was sometimes followed by target cell lysis [507].

Thus, in inflammatory bowel disease, the presence of increased numbers of dendritic cells, their anatomical distribution and their known function as activators of T cell responses, suggests that they play a role in the initiation and continuation of the inflammatory response. The macrophage may subserve two functions - firstly, as an effector cell, and later as a regulatory (suppressive) cell. The net result would therefore depend on the putative effects of the predominant population - either the recently elicited "activated" monocyte or the "resident" suppressive tissue macrophage.

6.8 CONCLUSIONS

6.8.1 Putative mechanisms of induction of intestinal T cell responses

After consideration of the above information, it is possible to arrive at a reasonably cogent theory of intestinal antigen handling and the role of intestinal dendritic cells and macrophages in the induction of T cell responses:

Luminal antigen is endocytosed by (Class II MHC positive or negative) epithelial cells and secreted from the basolateral membrane into the intestinal lamina propria via the basement membrane or via intraepithelial macrophages. The rate of antigen secretion from epithelial cells may be an important criterion in the development of clonal anergy, ie. the nature of the responses may depend on the local antigen concentration - which may in turn be regulated by the epithelial cell.

The antigen may undergo some process (eg. attachment to a particular receptor or transport molecule) which renders it less immunogenic, and then travel into the systemic circulation in this form. Alternatively, the antigen may be taken up by macrophages or dendritic cells. The numerical preponderance of the former make this the more likely result. Successful destruction of the antigen (or microorganism) would presumably result in a net negative signal mediated, for example, by immunosuppressive molecules, such as PGE_2. The tolerogenic protein antigen and prostaglandin-mediated inhibition of lymphocyte proliferation or induction of antigen-specific suppressor T cells would ensure systemic hypo-responsiveness.

Failure of the macrophage to successfully eliminate the antigen, even after activation by ambient LPS and cytokines (eg. GM-CSF and IFN-γ) may cause release of the antigen together with dendritic cell-potentiating agents, eg. IL-1. Subsequent uptake and transportation of antigen to draining nodes by dendritic cells would result in induction of T cell-mediated responses with

recruitment of antigen-specific effector T cells to the site of inflammation or infection. IFN-γ-induced Class II MHC expression on any cell may help focus this response. In addition, the intestinal dendritic cells would effect the preferential differentiation of B cells into IgA-producing plasma cells.

Eventual eradication of the antigenic load would see a shift towards the net negative signals and the attraction of fewer monocytes to the focus of inflammation. The biphasic responses of elicited macrophages (secretion of "activating" cytokines initially and then subsequent generation of immuno-suppressive signals) would help minimize the period of active inflammation in the gut.

6.8.2 Further characterization of mucosal immune responses

6.8.2.1 Intestinal lamina propria antigen handling

The above discussion opens up many exciting avenues of research. Perhaps the most important experiments would be aimed at understanding the mechanisms by which oral tolerance and intestinal immune responses are generated, eg. by determining the nature of antigen handling in the lamina propria. One approach would be to observe the traffic of cells following oral administration of a labelled antigen eg. ovalbumin, in studies similar to those performed by Macatonia et al (1987) [282]. Initially, the time course of antigen trafficking would be determined by identifying the antigen using immunohisto-chemistry or immunofluorescence. The nature of the antigen-bearing cell(s) could also be ascertained using double-labelling techniques. The appearance of antigen-bearing cells at different sites (eg. the lamina propria, Peyer's patches, mesenteric lymph nodes, spleen or other sites such as popliteal or inguinal lymph nodes) would indicate the route of antigen trafficking. Removal of recirculating cells (eg. by mesenteric lymphadenectomy and cannulation of the thoracic duct) would help clarify the importance of local (eg. the production of

macrophage suppressive agents) versus systemic factors (eg. suppressor T cells) in the generation of local immune responses.

Subsequent work could be aimed at isolating antigen-bearing cells to verify the information supplied by the immunohistochemistry described above. The phenotype of the antigen-bearing cell would be determined using flow cytometry and a panel of monoclonal antibodies. If necessary, double labelling could be performed using FITC-conjugated antigen and phenotyping using monoclonal antibodies and an alternative chromogen, eg. rhodamine.

Adoptive transfer experiments using cells exposed to antigen in vivo or in vitro would determine which cells were responsible for the induction of tolerance or sensitization [64]. Mixing experiments may also help to determine the nature of the interaction between the candidate antigen-bearing cells (macrophages and dendritic cells) and the effects of pharmacological intervention assessed (eg. inhibitors of prostaglandin synthetase or macrophage activating agents). Finally, and of major importance, methods of subverting oral tolerance induction could be explored with the ultimate goal being the development of successful oral vaccines.

6.8.2.2 Regulation of macrophage and dendritic cell differentiation

Another area of interest is the understanding of the mechanisms controlling macrophage and dendritic cell migration, differentiation and activation in the intestinal lamina propria. As discussed in Section 6.2 monocytes recently arrived in the lamina propria become down-regulated in their responses to LPS (in terms of IL-1 production). By minimizing immune activity in the lamina propria, the normal absorptive functions of the epithelial layer remain operative whilst "hypersensitivity" diseases may impair epithelial function and result in the failure of the host to survive. Factors controlling macrophage differentiation and activation could be undertaken using disaggregated cell suspensions in order to examine basal and stimulated cytokine production or the expression of m-RNA

for the cytokine of interest. Correlation of the findings with in vivo studies using in situ hybridization would also be required.

Similarly, the factors controlling dendritic cell function need further investigation. One particular point to be addressed is whether the preferential induction of IgA by intestinal dendritic cells is truly a property of the dendritic cell (either directly or indirectly by the induction of certain patterns of cytokine production in T cells) or whether the T cell production of cytokines is dependent on the signals present in the mucosal microenvironment and independent of the dendritic cell. Such studies would necessitate the use of pure populations of dendritic cells and T cells in mixing experiments using recombinant cytokines and/or the appropriate inhibitory anti-cytokine antibodies.

6.8.2.3 The role of intestinal macrophages and dendritic cells in disease

Ulcerative colitis and Crohn's disease are two conditions of unkown aetiology afflicting the human gastrointestinal tract [the immunological disturbances are reviewed in 106, 284]. Although commonly grouped together as "idiopathic inflammatory bowel disease", there is increasing evidence to suggest that the two conditions reflect two distinct pathogenic pathways.

Ulcerative colitis is a diffuse condition which arises in the rectum, spreads proximally through the colon in continuity, and affects the superficial layers of the mucosa. A number of observations support the hypothesis that the epithelial cell is the target of a predominantly antibody-mediated immune response. Firstly, abnormal survival of the epithelial cell has been documented, even in quiescent disease [133]. Secondly, epithelial cell-associated antigens have been identified and characterized [94, 340] and are the putative targets of an autoimmune process. In addition, studies on the pattern of immunoglobulin production in ulcerative colitis show greatly enhanced production of IgG_1 and to a

lesser degree, IgG_3 (in contrast to the increase of all IgG isotypes seen in Crohn's disease [reviewed in 284]) - a pattern which may be associated with the activation of subsets of T cells producing the cytokine, IL-4 [452, reviewed in 364]. Increased production of IL-4 in the lamina propria of patients suffering from ulcerative colitis may also account for the observed impairment of lymphokine-activated killer cell activity [254] if the generation of these cells is inhibited by IL-4 as proposed by Kanoff and Strober (1989) [207].

Crohn's disease is a condition characterized by focal, transmural inflammation occurring anywhere in the gastrointestinal tract, with relative sparing of the epithelial cell layer. The appearances histologically and at immunohistochemistry are those of a delayed-type hypersensitivity reaction with the granuloma being a significant feature. Studies on the cytokine production of mononuclear cells obtained from specimens resected from patients suffering from Crohn's disease have shown that they generate greater amounts of IL-1 than those from controls and from patients with ulcerative colitis [380]. This factor participates in the induction of granulomata [214]. Similarly, the delayed-type hypersensitivity reaction and granuloma formation may also reflect activation of a subset of T cells producing IL-2 and IFN-γ [4, 153, 328].

Although possibly an incidental observation, the pattern of histological damage in Crohn's disease (focal, transmural, with granulomata in submucosal lymphoid tissue and draining lymph nodes) parallels the known migratory pathways of macrophages and dendritic cells. Increased numbers of both these cell types are seen in Crohn's disease (see Section 6.7) but no consistent functional abnormality has yet been detected. This may due to our inability to identify the defect if it exists. Ways in which a macrophage abnormality may contribute to the pathogenesis of Crohn's disease include:

(i) a deficiency of the degradative pathways resulting in the inability of the host to eliminate a normal commensal gut microorganism or an "infective agent" which would not cause symptoms in a normal host;

(ii) a heightened response to normal or abnormal gut flora resulting, for example, in the "overproduction" of cytokines and other inflammatory mediators, recruitment and amplification of intestinal immune responses and disordered regulation; or

(iii) a diminished host response. McElrath et al (1988) [315] hypothesized that the effectiveness of a granulomatous response required the destruction of parasitized host cells in a lymphokine-rich environment. A defect in this process may see persistence of an invasive microorganism and ongoing inflammation.

The ability to isolate and enrich populations of cells, including macrophages, dendritic cells and T cells from the intestinal lamina propria and to examine them using complementary techniques (such as functional assays including cytokine production, immunohistochemistry, and molecular biological techniques such as in situ hybridization), should contribute to our understanding of their roles in the pathogenesis of inflammatory bowel disease and other conditions. The ultimate objective will be to modulate the mechanisms governing regulation of intestinal immune responses in order to treat such conditions and to develop strategies for effective oral immunization. That is to:

"Venienti occurite morbo"

(Confront disease at its onset)

Satires iii 64

Persius (A.D. 34-62).

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"A man should keep his little brain attic stocked with all the furniture that he is likely to use, and the rest he can put away in the lumber-room of his library, where can get it if he wants it."

From *"The Adventures of Sherlock Holmes"*,

"Five Orange Pips"

Arthur Conan Doyle (1859-1930)

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