Aspects of Macrophage and T Lymphocyte involvement in murine LCM

by

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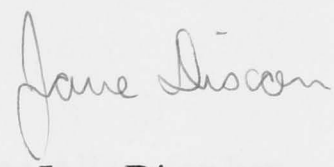
Statement.

Unless otherwise stated in the acknowledgements, the work described in this thesis was carried out by the candidate.



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Abstract

The work presented in this thesis examines the contribution of macrophages in the host response to LCMV and the capacity of class I MHC-restricted T cells to initiate macrophage recruitment to sites of LCMV-infection. Immunhistochemical staining for the macrophage marker, F4/80, and for the class II MHC glycoproteins that serve as a marker of macrophage activation, shows that there is a substantial increase in the number of activated macrophages in the liver and brain of LCMV-infected mice. Adoptive transfer studies indicate that the DTH response that recruits these macrophages to the brain is mediatied by $Lyt2^+$, class I MHC-resticted T cells.

In the liver, the increased content of activated macrophages following LCMV-infection, apparently results from the localisation of $F4/80^+$, Ia^+ monocytes, large numbers of which attach to the walls of the sinuses and the central and hepatic veins. The increased number of Ia^+ sinusoidal macrophages is preceded by an influx of lymphocytes which are frequently in close association with macrophages. Foci of $F4/80^-$, Ia^- mononuclear cells (probably T cells) develop both in the liver parenchyma and periportally. Only after there is evidence of cell death within the lesions, is there infiltration of these areas by $F4/80^+$, Ia^+ cells.

LCMV-infection of the brain is followed by a predominantly lymphocytic infiltration of the leptomeninges. However, 22% of the inflammatory cells are macrophages, approximately half of which are Ia^+. Phagocytic macrophages are generally Ia^+. Adoptive transfer studies in which immune spleen cells are injected intravenously into immunosuppressed, LCMV-infected mice indicate that a $Lyt2^+$, $L3T4^-$ cell population is responsibe for the localisation of $F4/80^+$ macrophages to the CNS. The macrophage accumulation in immunosuppressed, LCMV-infected mice given immune T cells differs from that found in immunocompetent mice, in that there is a much larger contibution by macrophages, but fewer of these macrophages are Ia^+.

Quantitation of the inflammatory response in the CSF of immunosuppressed, LCMV-infected mice receiving immune spleen cells, demonstrates that the DTH response in the CNS is initiated by $Lyt2^+$, $L3T4^-$ immune cells. Although Lyt2-depletion of the immune transfer population prevents the acute inflammatory response in the CNS, evidence of DTH may be found in the brain at later time points. This is apparently due to expansion of surviving $Lyt2^+$ precursors in the donor population. H-2 compatibility at the D locus between donor and recipients is sufficient to initiate a DTH response: these D-restricted T cells are also $Lyt2^+$, $L3T4^-$. T cell reactivity to LCMV in the spleens of recipient mice are of donor origin.

Thus, macrophage activation and recruitment to sites of LCMV-infection is initiated by class I MHC-resticted T cells. Close association between macrophages and lymphocytes in the early stages of infection, as well as the different localisation of these cell types in the liver, suggests a distinct funtional role for macrophages in the immune response to LCMV.

Publications.

Work presented in this thesis has been reported in the following publications:

Dixon, J. E., Allan, J. E., Doherty, P. C., Hume, D. A. 1986 Immunohistochemical analysis of the involvement of F4/80 and Ia-positive macrophages in mouse liver infected with lymphocytic choriomeningitis. J. Leuk. Biol., **40:** 617-628.

Dixon, J. E., Allan, J. E., Doherty, P. C. 1986 The acute inflammatory process in murine lymphocytic choriomeningitis is dependent on $Lyt2^+$ immune T cells. Cell. Immunol.,**104:** in press.

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Doherty, P. C., Allan, J. E., Dixon, J. E., Tabi, Z., Ceredig, R. Characteristics of the CSF inflammatory exudate in murine lymphocytic choriomeningitis. Proceedings of the "Workshop on Cellular and Humoral Components of CSF in Multiple Sclerosis", Henglehoef, Belgium, April, 1986. A. Lowenthal and J. Raus eds., Plenum Press. (In Press)

Allan, J. E., Dixon, J. E., Doherty, P. C. Nature of the inflammatory process in the central nervous system of mice infected with lymphocytic choriomeningitis. Current Top. Microbiol. Immunol. (In Press).

CONTENTS

Contents (cont.)

Abbreviations.

AA	acetaminophen
ADCC	antibody-dependent cell cytotoxicity
ALS	anti-lymphocyte serum
APC	antigen presenting cell
Arm	Armstrong strain of LCMV
B6.H-2^{bml}	C57BL/6.H-2^{bml}
B6Ka Thy1.1	C57BL/6Ka Thy1.1
B10.A	C57BL/10.A
B10.BYR	C57BL/10.BYR
B10.D2	C56BL/10.D2
BCG	*Bacillus Calmette-Guérin*
C	complement
CFU	colony-forming unit
CNS	central nervous system
Con A	concanavalin A
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
Cy	cyclophosphormide
d	day
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
EM	electron microscope
FCS	foetal calf serum
GM-CFU	granulocyte, macrophage colony-forming unit
GM-CSF	granulocyte, macrophage colony stimulating factor
GVHD	graft-versus-host disease
HSV	herpes simplex virus
i.c.	intracerebral
Ig	immunoglobulin
IL-1	interleukin 1
IL-2	interleukin 2
IL-3	interleukin 3
i.p.	intraperitoneal
Ir	immune response
i.v.	intravenous
LCM	lymphocytic choroidmeningitis

Abbreviations (continued).

LCMV	lymphocytic choroidmeningitis virus
LD_{50}	lethal dose for 50% of test animals
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAF	macrophage activating factor
MAgF	macrophage aggregation factor
MCF	macrophage chemotactic factor
MHC	major histocompatibility complex
MIF	migration inhibition factor
MLR	mixed lymphocyte reaction
NBS	newborn bovine serum
PBS	phosphate-buffered saline
pfu	plaque forming unit
PEC	peritoneal exudate cells
PLP	periodate-lysine-paraformaldehyde
PMN	polymorphonucleocyte
ROI	reactive oxygen intermediates
SEM	standard error from the mean
SPF	specific-pathogen-free
TCGF	T cell growth factor

Chapter 1

Introduction and Literature Review

This thesis is concerned with the involvement of macrophages in the immune response to lymphocytic choriomeningitis virus (LCMV), a response that is mediated by T lymphocytes. As an introduction, I will provide background information on the nature of the adaptive immune response effected by T lymphocytes, the role of macrophages in immunity and the viral disease which I have used as a model to study these aspects of immunity.

1.1 T LYMPHOCYTES.

1.1.1 Cell-Mediated Immunity - A Historical Perspective.

That cells are important in mediating a number of immunological phenomena was established by adoptive transfer studies in which viable lymphoid cells from donors exposed to an antigenic stimulus were infused into naive recipients. Landsteiner and Chase (1942) found that delayed-type hypersensitivity (DTH) to simple compounds could be transferred to naive guinea pigs by peritoneal cells from previously exposed animals. Tuberculin reactivity could also be transferred by immune cells from the spleen and lymph nodes (Chase, 1945; Bloom and Chase, 1967) but was not transferred with immune serum (Bloom and Chase, 1967).

Mitchison (1953, 1954, 1955) also used an adoptive transfer protocol to demonstrate cell-mediated resistance to an allogeneic lymphosarcoma. Immunity could be conferred to a naive recipient by the transfusion of cells (and not serum) from the draining lymph node of a syngeneic animal that was in the process of rejecting that tumor. Furthermore, survival of allogeneic cells in previously sensitised animals could be prolonged if they were transplanted in a Millipore filter, and therefore impervious to cellular infiltration (Weaver et al., 1955).

Small lymphocytes were thought to be the immunologically competent cells (Medawar, 1958; Billingham et al., 1962). On transfer into semiallogeneic rats, they changed rapidly into dividing cells with new morphological characteristics. Lymphocytes were implicated in the rejection of skin grafts (Gowans et al., 1962; Billingham et al., 1954; Billingham and Silvers, 1963), graft-versus-host disease (GVHD) (Billingham and Brent, 1959) and in providing immunological tolerance (Gowans et al., 1962). Lymphocytes were known to reside in the lymphoid system but different organs varied in their ability to provide adoptive immunity (Billingham and Brent, 1959), and model systems of immunity varied with respect to which lymphoid organs had reactive cells (Mitchison, 1954; Billingham and Brent, 1959).

Anti-lymphocyte serum (ALS) raised against thoracic duct lymphocytes depleted the levels of lymphocytes circulating in the blood and destroyed large areas of the spleen and lymph nodes (Gray et al., 1966; Monaco et al., 1966). Such treatment was accompanied by prolonged allograft survival in rats (Woodruff and Anderson, 1963) and mice (Monaco et al., 1966). Also, immunity to the facultative intracellular bacterium, *Listeria monocytogenes*, could be transferred by splenocytes or thoracic duct cells from immune mice and this

protection was abrogated by treatment of the transfer immune population with ALS (Mackaness, 1969; Mackaness and Hill, 1969; McGregor et al., 1970).

Lymphocytes are a heterogenous population. This fact was established by experiments in which the Bursa of Fabriscius in chickens (Graetzer et al., 1963; Szenberg and Warner, 1962; Aspinall et al., 1963) and the thymus in neonatal mice were removed. Lymphocytes were divided both functionally and on their site of differention into bursa-derived, immunoglobulin (Ig)-producing B cells and thymus-derived T cells.

The functional importance of thymus-derived lymphocytes, or T cells, was established by thymectomy of mammalian neonates. Such surgery decreased the ability of mice to reject grafts as adults (Miller, 1961, 1962; Martinez et al., 1962), to perform DTH responses (Arnason et al., 1962) and to mount a GVHD reaction to allogeneic thoracic duct cells (Miller and Mitchell, 1967). Neonatal thymectomy of mice resulted in severe depletion of lymphocytes from the lymph nodes and spleen (Miller, 1961), a lack of small lymphocytes in the blood and Peyer's patches and a severe wasting disease (Miller, 1962). These areas of lymphocyte depletion were found to correspond to those affected by treatment with ALS (Taub and Lance, 1968) and anti-thymocyte serum (Turk and Willoughby, 1967), and could be restored by intravenous (i.v.) transfer of thymus cell suspensions (Parrott et al., 1966).

Immunologically competent T cells are generated from resting precursors in the thymus (Stutman, 1978). These were thought to be phenotypically identical to peripheral T cells (Scollay et al., 1978; Scollay, 1982) but they probably do not lose the thymic cell surface marker, J11D, until they have left the thymus (McKinnon and Ceredig, 1986). Differentiation of lymphocytes within the thymus leads to the acquisition of a number of serologically detectably cell surface antigens (reviewed in Katz, 1977; McKenzie and Potter, 1979). In the mouse, the Thy-1 alloantigen, originally designated q, was the first to distinguish T from B lymphocytes and is now the standard T cell marker (Raff 1969, Lepault and Weissman, 1981; McKenzie and Potter, 1979). The ontogeny of the Thy-1 marker has been reviewed by Crawford and Barton (1986). Thymocytes also acquire Lyt antigens (Boyse et al., 1968; Mathieson et al., 1979, Scollay and Weissman, 1980; Lepault et al., 1983). All T cells express Lyt1 but with variable density, such that the most readily detectable levels are found on the $Lyt2^{-}/3^{-}$ subset of lymphocytes (Mathieson et al., 1979; Ledbetter et al., 1980). Traditionally, the helper T cell subset has been defined by expression of the Lyt1 but not the Lyt2/3 antigens, the latter being restricted to the cytotoxic/suppressor subset (Cantor and Boyse, 1975).

Function of T lymphocytes.

It had been shown that T cells do not produce Igs and thymectomised animals maintained intact antibody responses (Aspinall, et al., 1963; Graetzer et al., 1963) and Davies and coworkers (1967) had shown that T cells did not produce immunoglobulins, humoral responses to some antigens, such as sheep erythrocytes, were impaired (Miller and Mitchell,

1967). It became evident that two cell types were involved in effecting the antibody response. While the antibody response following neonatal thymectomy, could be restored by transfer of thymocytes, impaired responses due to sublethal irradiation required infusion of thymocytes and bone marrow cells together, and was not reconstituted with separate transfer of these cell populations (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Nossal et al., 1968; Martin and Miller, 1968). Therefore it was proposed that both antigen-reactive thymus-derived lymphocytes and bone marrow-derived immunoglobulin-producing cells interacted to effect the humoral antibody response (Claman et al., 1966; Davies et al., 1967; Mitchison, 1971).

Adoptive transfer studies established the role of T cells in eliciting the DTH response to both bacterial and viral antigens. Blanden and Langman (1972) found that the cellular response to listeria was abrogated in thymectomised, bone marrow-reconstituted mice, but could be restored by transfer of thymocytes. Also the DTH response and protection conferred on recipients by transfer of listeria-immune lymphocyte populations was abrogated by treatment of those populations with anti-Thy1 serum plus complement (Blanden and Langman, 1972; Lane and Unanue, 1972; Kaufmann et al., 1979). The cellular response to the mousepox virus, ectromelia, was also shown to be mediated by Thy1$^+$ cells (Blanden 1970, 1971a,b).

The importance of T cells in the recovery from viral infections has also been established from adoptive transfer studies (reviewed in Zinkernagel and Doherty, 1979). The transfer of protection against ectromelia by virus-immune spleen cells did not depend on the presence of antibody, interferon or macrophages in the transfer inoculum. Control of virus replication and recovery from infection was abrogated by T cell depletion of the transfer population (Blanden 1970, 1971a,b). Transfer of immune T cells to influenza- (Yap and Ada,1978; Wells et al., 1981) and cytomegalovirus- (Starr and Allison, 1977; Ho, 1980) infected mice has also been shown to result in reduced virus titres in organs. Other viral infections which are controlled by the T cell activity include Venezualan equine encephalomyelitis virus (Rabinowitz and Adler, 1973), herpes simplex virus, type 1 (Rager-Zisman and Allison, 1976) and flavivirus (Jacoby et al., 1980).

The in vitro cytotoxicty of immune lymphoid cells for target cells bearing the sensitising antigen, previously demonstrated by Rosenau and Moon (1961) and Koprowski and Fernandes (1962), and quantitated by the ^{51}Cr-release assay of Brunner and colleagues (1968) was shown to be mediated by T lymphocytes. Cerottini and coworkers (1970a,b) found that allo-reactive cytotoxic cells but not allo-antibody producing cells were generated in the thymus. They also showed that anti-Thy1 antibody and complement treatment prevented cytotoxicity but not antibody production by sensitised spleen cells. Cytotoxic immune cells reactive to viruses were also demonstrated to be T and not B cells (Gardner et al., 1974a,b; Yap and Ada, 1977). They were non-adherent, sensitive to complement treatment with anti-Thy1 serum but not anti-immunoglobulin serum, did not express Fc receptors on their cell membranes and required direct contact with the target cells to effect cytolysis. Graft destruction was thought

to be one in vivo correlate of T cell cytotoxicity. Sprent and Miller (1971) demonstrated that thoracic duct, Thy1$^+$ cells that were cytotoxic for allogeneic cells in vitro were also capable of initiating graft rejection and GVHD on transfer into allogeneic recipients.

In vitro studies of T cell function also established the importance of soluble mediators in both the regulation and the effector mechanisms of the immune response. Bloom and Bennett (1966) described the release, in culture, of a macrophage inhibition factor (MIF) from sensitised lymphocytes. Other soluble factors that affected leukocytes in vitro were also described: a mitogenic factor that triggered lymphocyte proliferation (Gordon and Maclean, 1965); chemotatic factors for macrophages (Ward et al., 1970), eosinophils (Cohen and Ward, 1971), basophils (Kay and Austyn, 1972) and neutrophils (Czuprynski et al., 1985; Lukacs et al., 1985); and colony stimulating factors for bone marrow cells (Metcalf and Johnson, 1978). The generic term of 'lymphokine' was given to such immunoregulatory soluble factors released from lymphocytes (Dumonde et al., 1969). These facors were apparently the in vitro correlate of the mediators of DTH reaction and Bennet and Bloom (1968) did find that injection of lymphocyte-derived factors into animals elicited a DTH response.

Lymphokine release by T cells could be stimulated in primary cultures with mitogens (Watson and Mochizuki, 1980) and alloantigen (Kirchner et al., 1979). They were also produced by cultured T cells obtained from mice infected with viruses (Ihle et al., 1981a).

Thus, a wide range of both in vivo and in vitro functions can be attributed to T lymphocytes.

1.1.2 MHC-Resriction of T Lymphocytes.

The Major Histocompatibility Complex (MHC).

Gorer and colleagues initially demonstrated that a blood group gene locus in mice controlled the rejection of tumors transplanted between different strains of mice. Their protein products were designated as antigen II. Seven to 14 genetic loci were found to control the fate of tumor grafts. These were designated H and numbered sequentially (Gorer et al., 1948; Snell, 1948). Tissue rejection across the H-2 locus was particularly rapid and consequently the murine H-2 became known as the major histocompatibility complex (MHC). All other H loci were designated as minor or non-H-2 antigens.

The protein products and organisation of the MHC gene region have been described in detail (reviewed by Klein et al., 1981; Hood et al., 1983; Klein et al., 1983). The H-2 complex of the mouse are divided into four regions named arbitrarily K, S, I and D. Each subregion contains a varying number of loci and these code for three structural classes of antigen, classes I-III.

The products of the K and D regions products display marked homology in sequence and structure and are designated class I MHC antigens. They are integral membrane proteins.

The K region of the MHC codes for 2 distinct molecules (Ivanyi and Demant, 1981; Tryphonas et al., 1983) and the D region codes for 4: D (Shimada and Nathenson, 1969; Reyes et al., 1982); L (Lemmonier et al., 1975); M (Ivanyi and Demant, 1979; Sears and Pollizi, 1980); and R (Hansen et al., 1981; Ivanyi and Demant, 1981). Not all these molecules are found in every mouse strain. The class I MHC antigens are ubiquitously distributed on mammalian cells and can be found to a some extent on all cell types. The lymphoid tissues have the highest levels of expression while in the brain, skeletal muscle and testes, class I molecules are barely detectable (Klein, 1975). Class I MHC antigens are the classical transplantation antigens that allow discrimination of self from non-self.

The class II molecules, or I region associated (Ia) antigens, are also integral membrane proteins. The molecules consist of 2 noncovalently associated polypeptide chains These α and β chains are encoded by subregions of the I region, I-A and I-E. The cell membrane I-A molecule is made up of the A_α and A_β polypeptides, whereas the I-E molecule is made up of E_α, encoded by the I-E subregion and $A_\varepsilon(E_\beta)$, encoded by the I-A subregion (Jones et al., 1978; Cook et al., 1979).

The distribution of Ia molecules is largely restricted to cells of the lymphomyeloid lineage. They are constituitively expressed by B cells and both lymphoid and interdigitating dendritic cells (Steinman et al., 1979; Tew et al., 1982). The level of expression of Ia on macrophages is less consistent and is probably, to a large extent, regulated by immune processes (Wong et al., 1983; Papiernik et al., 1986; Strassmann et al., 1986). While it is evident that activated human T cells synthesise and express class II MHC molecules (Charron et al., 1980; Brown et al., 1984), it is disputed whether murine T cells can synthesise Ia antigens, although they appear to absorb these molecules from the surrounding milieu (Lorber et al., 1982). Detectable levels of class II MHC antigens can be found on non-lymphomyeloid cells such as gut epithelia (Parr and McKenzie, 1979) and sperm (Hammerling, 1976) while a number of cell types are induced to express Ia during immune reactions (Mason et al., 1981; Schreiner et al., 1984; Pujol-Borrell et al., 1986). In the brain Ia expression appears to be restricted to reactive astrocytes (Traugott et al., 1985; Wong et al., 1984, 1985; Frank et al., 1986; Massa et al., 1986). I-A/E molecules are involved in the induction and regulation of immune responses.

The class III MHC molecules encoded at the S region are not histocompatibility antigens but, rather, components of the complement pathway (Atkinson et al., 1982).

MHC-Restriction of T Cell Reactivity.

The role of the MHC in controlling graft rejection was elaborated by in vitro studies. Mixed lymphocyte cultures of cells from 2, MHC-incompatible mouse strains resulted in a rapid proliferative response (Bain et al., 1964). It was further estimated that 1-3% of all T cells are reactive to cells bearing foreign MHC (Simonsen, 1967; Wilson et al., 1968). However, although H-2 was initially defined by graft rejection and a substantial proportion of

T cells are specific for allo-MHC antigens, subsequent studies indicated a much broader influence of the MHC on T lymphocyte function.

Class II MHC genes were found to control the induction and extent of the immune response to simple polypeptide antigens such as poly-L-lysine (McDevitt and Chinitz, 1969; McDevitt et al., 1972). For nude mice to mount an antibody response to sheep erythrocytes it was necessary to adoptively transfer thymocytes of the same H-2 haplotype (Kindred and Shreffler, 1972) and similar compatibility between T and B cells was necessary in the hapten-carrier system of antibody production (Katz et al., 1973a). MHC matching was also required for immune guinea pig T cells to proliferate in response to antigen-pulsed macrophages (Rosenthal and Shevach, 1973). Such observations led to the concept of immune response (Ir) genes which, in the cases cited above, were found to map to the I region of the MHC. It was hypothesised by Katz and colleagues (1973b) that a "physiological interaction" between both antigen-specific receptors and H-2 products on T cells, and the complementary recognition sites on somatic cells, was necessary for generation of the immune response.

The concept that immune responses are "restricted" by the MHC was elucidated by 2 independent studies on the cytotoxic activity of activated T cells. Zinkernagel and Doherty (1974a) demonstrated that virus-immune T cells killed infected target cells only if they shared the same H-2 antigens. Concurrently, Shearer (1974) found that the cytotoxicity of T lymphocytes for chemically modified cells was also MHC-restricted.

These papers initiated a cascade of work that further elucidated the requirement for MHC recognition and showed similar restriction for various immune responses. Cell lines that lacked detectable MHC antigens were not susceptible to lysis by either virus-immune (Zinkernagel and Oldstone, 1976; Doherty et al., 1977) or anti-minor (Bevan and Hyman, 1977) cytotoxic T cells. Furthermore, antibodies raised against class I MHC molecules were able to block anti-viral T cell cytotoxicity (Koszinowski and Ertl, 1975). It was shown that synthetic lysosomes only stimulated virus-immune T cells when viral and H-2 molecules were present in the same vesicle (Finberg et al., 1978; Loh et al., 1979).

MHC-restriction was demonstrated for the cytotoxic T cell response to minor histocompatibility antigens (Bevan, 1975 a,b), including the male H-Y antigen (Gordon et al., 1975) and to many different viruses (reviewed in Zinkernagel and Doherty, 1979). In vivo restriction of T cell activity has also been observed for a number of infectious diseases: listeria (Zinkernagel, 1974); ectromelia (Blanden et al., 1975; Kees and Blanden, 1976); influenza (Yap and Ada, 1978); and the intracellular protozoan *Theileria parva* (Eugui and Emery, 1981).

Compatibility at the K and/or D region of the MHC (but not the I region) was shown to be sufficient for recognition of virus-infected cells by cytotoxic immune cells (Blanden et al., 1975). Likewise, adoptively transferred ectromelia-immune T cells effected virus clearance in naive recipients only if K and/or D molecules were shared by donor and recipient mice (Kees

and Blanden, 1976). Also virus-specific memory responder cells are stimulated to divide in vitro by virus-infected cells that were similarly restricted (Pang et al., 1976).

The recognition of MHC molecules by T cells is very specific, even more so than that of antibody for its complementary antigen. A single point mutation at the K region is sufficient to abrogate the capacity of cytotoxic T cells to lyse virus-infected target cells (Blanden et al., 1976; Zinkernagel, 1976a) and to clear infectious virus in vivo (Kees and Blanden, 1976). This mutation was not serologically detectable (McKenzie et al., 1976). The most frequently observed mutations that affect target cell recognition occur in the second external domain of the class I MHC molecules (Klein et al.,1983).

An "altered-self" hypothesis for MHC restriction was proposed to explain MHC-restriction (Doherty and Zinkernagel, 1975c). Clonally expressed T cell receptors specifically recognised antigenic determinants that were generated by the association of "self" MHC molecules with "foreign" antigen on the target cells. In contrast, Katz and Benacerraf (1975) extended the "physiological interaction" hypothesis to suggest that a "dual-receptor" recognised foreign antigen independently of the MHC. The former hypothesis was favoured by the finding that antiviral effector T cells from F_1 hybrids consisted of 2 populations each recognising one or other of the parental strains. There was no evidence that individual F_1 effectors could recognise infected targets from both parental stains as predicted by the "physiological interaction" hypothesis (Zinkernagel and Doherty, 1974b). Further support for the concept of a single receptor was provided by the molecular biological examination of the T cell receptor (Hood et al., 1985).

Class I versus Class II MHC-Restriction.

MHC-restriction limits T cell function to cell-associated antigens. Recognition of soluble antigen only occurs after it has been processed by an antigen presenting cell (APC) and expressed on the surface of that cell. Examples of APC that are capable of endocytosing soluble antigen and re-expressing it on the plasma membrane are macrophages and dendritic cells. However, unlike that of macrophages, presentation of soluble antigen by dendritics is apparently restricted by its limitted phagocytic capacity and occurs without intracellular degradation (Kapsenberg et al., 1986).

Experiments with virus-immune T cells suggest that those antigens which need to be processed by an APC generate class II MHC restricted responses while antigens that are independently incorporated into the cell membrane are more likely to associate with the class I MHC products. Ertl (1981) examined the ability of different preparations of virus and APCs to restimulate T cells that had been primed in vivo with infectious Sendai virus. The resultant effector populations were tested for their ability to effect class I or class II MHC-restricted DTH. Class II restriction occurred only when the APCs were macrophages from the spleen or peritoneal cavity, and were therefore capable of endocytosing and processing the antigen. Class I MHC-restriction was not limited to APC that could process antigen, but the virus

preparation used had to be either infectious or capable of fusing with the cell membrane. Sugamurer and colleagues (1978) had previously shown that the ability of class I-restricted cytotoxic T lymphocytes (CTL) to lyse Sendai virus-infected target cells was dependent on the fusion activity of the virus envelope (i.e. the capacity of viral antigens to be directly incorporated into the target cell membrane).

The lipid envelope of Sendai virus has a particularly high fusion activity and no viral genome or protein synthesis is required to generate target cells that can be lysed by CTL reactive to this virus (Schrader and Edelmann, 1977). For other viruses such as ectromelia and herpes simplex virus, incorporation of viral antigens into target cells that can be lysed by class I-restricted T cells only occurs if the virus is infectious and if cellular protein synthesis is not impaired (Ada et al., 1976; Pfizenmaier et al., 1977). Restriction of influenza-reactive CTL to K and D molecules only occurs in mice that are infected with live virus (Braciale and Yap,1978). Priming with inactivated influenza virus, however, generates only a class II MHC-restricted response (Leung and Ada, 1980).

Further studies with influenza virus demonstrated that cytolosis of virus-infected cells by CTL that were restricted by either class I or class II MHC antigens, was determined also by the nature in which virus antigens were incorporated into the cell membrane. Class I MHC-restricted CTL, specific for the influenza hemagglutinin antigen, recognised only those target cells in which new hemagglutinin protein synthesis had occurred. Whereas, class II MHC-resticted CTL selectively recognised target cells that had been exposed to soluble hemagglutinin (Morrison et al., 1986).

The importance of glycoprotein metabolism in generating virus-infected targets capable of being lysed by class I-restricted CTL is suggested by the ability of glycosylation inhibitors to abrogate the lysis of targets infected with herpes simplex virus (HSV), (Lawman et al., 1980; Carter et al., 1981). However, this effect may have been due to inhibition of either virus metabolism or the synthesis of class I MHC molecules. That virus metabolism is probably the most important factor was suggested by experiments with temperature-sensitive mutants of HSV. Target cell sensitivity was decreased when infected with the mutants at their nonpermissive temperature, although cells infected with the wild-type virus were lysed (Lawman et al., 1980). The importance of viral protein synthesis was also implicated with experiments on temperature-sensitive mutants of vesicular stomatitis virus (Hale et al., 1978; Zinkernagel et al., 1978).

Although infectious virus is apparently required for generation of a class I MHC-restricted response, it can not be assumed that, in all cases, the viral antigen recognised has been incorporated into the plasma membrane as a native protein. Townsend and colleagues (1986) have demonstrated that newly synthesised proteins that lack the hydrophobic signal peptide required for insertion of the protein into lipid membranes, can still be presented to class I MHC-restricted CTL. They used a reconstructed hemagglutinin molecule of the influenza virus, lacking the signal peptide, and incorporated into a carrier vaccinia virus.

Hemagglutinin specific CTL were capable of lysing infected cells even though the protein could not be detected on the cell surface with specific antibodies. The authors have suggested that 2 pathways exist for the presentation of antigens to T cells, one dealing with recently synthesised proteins, the other with endocytosed soluble proteins. This hypothesis explains the ability of virus-reactive CTLs to recognise non-transmembrane viral antigens, such as the nucleoprotein. Proteins that are newly synthesised in the cytoplasm of an infected cell are presented at the cell surface independently of the processing mechanism used by such APCs as macrophages and dendritic cells. The 2 distinct presentation pathways for synthesised versus endocytosed proteins, as suggested by Townsend and his coworkers, may be the factor which determines by which class of MHC molecules T cells are restricted.

Thus, as a generalisation, the nature of a foreign substance is important in determining the class of MHC by which reactive T cells are restricted. Soluble antigens require processing by specialised APC and appear to generate a T cell response restricted to Ia molecules. K,D-restricted activity occurs when antigen is incorporated into the cell membrane independently of the processing pathway of the APC.

Activation of T Cells.

That antigen alone is not a sufficient stimulus to generate an immune response was demonstrated by Lafferty and coworkers (1974) and Schendel and Bach (1975). These workers found that pretreatment of the stimulator population in an MLR with either ultra-violet irradiation or a 1hr incubation at 45°C destroyed the proliferative response. The stimulating MHC antigens, however, remained serologically intact. Also, activation of resting T cells in an MLR or by mitogens requires cell-to-cell contact with metobolically active stimulator cells (Paetkau et al., 1976; Larsson and Coutinho, 1979; Lafferty et al., 1980).

Not all viable cell types, though, are capable of activating T cells. Stimulatory capacity is restricted to a few hemopoietic cell lines such as macrophages and dendritic cells (Sunshine et al., 1982; Minami et al., 1980). Most stimulator cells express class II MHC molecules. Even mitogens that activate T cells polyclonally are not able to do so in the absence of an Ia^+ cell population (Habu and Raff, 1977; Ahmann et al., 1978). Ia antigens, however, are not directly related to the stimulatory process. Some Ia^- tumor cell lines have the capacity to activate resting T cells (Lafferty et al, 1980) and Ia^+ B cells do not provide stimulation in an MLR (Glimcher et al., 1982) even though they are capable of processing antigen and re-expressing it in association with Ia molecules in such a way as to be recognised by T cells that are already activated (Lanzavecchia, 1985).

Cell types that are not normally capable of activating T cells acquire that capacity when cell-free supernatants from mitogen stimulated T cells are added to the culture (Talmage et al., 1977). One factor that is able to act as a costimulator is interleukin 1 (IL-1), a lymphokine that is released from macrophage-derived cell lines and macrophages that have been triggered by lipopolysaccharide (LPS) or activated T cells (Mizel et al., 1978; Farrar et al., 1980)

Thus, apart from foreign antigen it is apparent that a second signal is required to stimulate resting T cells. This soluble mediator is released by specialised hemopoietic cells that usually express class II MHC antigens.

1.1.3 Function of Class I and Class II MHC-Restricted T Cells.

Upon activation by APCs that present antigen and also release a costimulatory factor, T cells express receptors for interleukin-2 (IL-2). This lymphokine was initially termed T cell growth factor (TCGF) because it was required for the maintenance of primed T cells in vitro and for the generation of T cell clones (Gillis and Smith, 1977; Schreier et al., 1980). It received the less specific name of IL-2 (Aarden et al., 1980) because it was also capable of affecting the response of thymocytes to mitogen (Mills et al., 1976) and the differentiation and proliferation of B cells (Watson et al., 1979; Zubler at al., 1984). As well as being a proliferative signal, IL-2 is capable of inducing the differentiation of $Lyt2^+$ cells to the cytotoxic state (Yoshimoto et al., 1985).

Once primed and receptive for IL-2, T cells are capable of carrying out a number of functions including the lysis of specific target cells and the release of lymphokines. Such functions are triggered by interaction of the effector T cell with its specific cell-associated antigen. At this stage it is no longer necessary for the target cell to be metabolically active (Andrus and Lafferty, 1981). Also, lymphokine release from activated T cell clones can be elicited by mitogens in the absence of APCs (Ely et al., 1981). Antigen alone is sufficient stimulus for triggering effector T cell functions.

Class I and class II MHC-restricted T cells can be distinguished phenotypically by antigens that are acquired during development in the thymus (Swain, 1983). In the murine system, K/D-restriction correlates with expression of the Lyt2 marker defined by Cantor and Boyse (1975), while Ia-restricted T cells express the L3T4 antigen (Dialynas et al., 1983).

Traditionally, the two subsets of T cells were also distinguished on a functional basis. Class I MHC-restriction implied cytotoxic/suppressor function, while class II MHC-restricted T cells were the lymphokine-releasing, helper/DTH subset (Huber et al., 1976; Vadas et al., 1976; Zinkernagel and Doherty, 1979; Hollander, 1982). The restriction of cytotoxic activity to $Lyt2^+$ T cells, however, is not absolute: a number of instances in which killing is restricted by class II MHC antigens have been described (Wagner et al., 1975; Meuer et al., 1983; Spits et al., 1983; Kaplan et al., 1984; Yasukawa and Zarling, 1984; Jacobson et al., 1985; Morrison et al., 1986). Likewise, class I-restricted T cell clones can release lymphokines (Dennert et al., 1981; Morris et al., 1982; Kelso and Glasebrook, 1984).

T cell clones and hybridomas have proved to be powerful tools in the analysis of T cell function. Although some lines are constituitive producers of lymphokines (Nabel et al., 1981; Howard et al., 1979; Jones et al., 1981; Stull and Gillis, 1981), others are stimulated to

release them by mitogenic or antigenic stimuli (Kappler et al., 1981; Katz et al., 1980; Schrader et al., 1980; Nathan et al., 1981; Glasebrook et al., 1981). T cell clones (Prystkowski et al., 1982) and hybridomas (Zlotnik et al., 1983; Kelso and Glasebrook, 1984) are also capable of synthesising and releasing more than one lymphokine.

A number of authors have examined the MHC restriction of T cell function using cell lines whose effector capacity is triggered by antigens or mitogens. Kelso and coworkers (1982) found that the majority of T cell clones that are reactive to alloantigens, minor histocompatibility antigens or Moloney leukaemia virus, released macrophage activating factor (MAF), independently of the class of MHC molecules by which they were restricted. They also found that there was no correlation between the cytolytic capacity of the clones and their capacity to release such lymphokines as IL-2 and the colony stimulating factor for granulocytes and macrophages (GM-CSF). However, apart from some exceptions, most IL-2 releasing clones were not cytotoxic.

Kelso and MacDonald (1982) also looked at the precursor frequency of cytotoxic and lymphokine-releasing T cells from an MLR. They used limit dilution techniques to generate clones from $Lyt2^+$ or $Lyt2^-$ precursors and these were tested for both T cell functions. Production of MAF and GM-CSF was detected from both $Lyt2^+$ and $Lyt2^-$ lines. IL-2 release was found predominantly in the latter cell lines. CTL were only generated from Lyt2-bearing precursors, but in this study it would have been impossible to detect class II MHC-restricted killing because the Ia^- tumor cell line, P815, was the only target used. Similar results were obtained in a study looking at precursors from mitogen stimulated T cell populations (Guerne et al., 1984). These authors, however, found that about half of the $Lyt2^+$ clones could release IL-2.

The MHC-restriction pattern of T cells that clear influenza virus infections has also been examined. Specific class I-restricted cytotoxic clones can inhibit viral replication in vivo as well as inducing a DTH reaction (Lin and Askonas, 1981). Of 2 cytotoxic clones, Taylor and Askonas (1983) found that only the one that produced γ-interferon, in vitro, on contact with its appropriate target cell (Morris et al., 1982) was capable of clearing virus in vivo. Thus, in this case the cytotoxic capacity of T cells was not critical for in vivo function. However, the restriction pattern of the T cells is important. Leung and Ada (1982) demonstrated that class I, and not class II, MHC-restricted T cells could clear influenza virus infection in mice. On the other hand, class II MHC-restricted immune cells, which are generated by priming with inactivated virus (Leung and Ada, 1980), initiate immunopathology in the lungs of infected animals (Leung and Ada, 1980; Liew and Russell, 1983).

In summary, although class I and class II MHC-restricted T cells can be distinguished phenotypically, a clear functional distinction is not possible. Both subsets are capable of a range of effector activities. Which of these are important for T cell function, in vivo, is difficult to delineate.

1.2 MACROPHAGES

It was the Russian zoologist, Elie Metchnikoff, who first recognised the importance of macrophages. Working in Paris at the end of the nineteenth century with the fresh water flea, *Daphnia magma*, he demonstrated the capacity of phagocytes to engulf fungal spores. In later investigations of mammalian infections, he observed that macrophages from infected animals, compared to those from uninfected animals, had an enhanced capacity to ingest and kill bacteria. Since his time much information has been gleaned on the various macrophage cell types and their function.

1.2.1 Ontogeny and Heterogeneity of Macrophages.

All blood cell types are referred to as hematopoietic cells. In adult mammals they are usually generated from stem cells in the bone marrow and to a lesser extent in the spleen (Metcalf and Moore, 1971). Transfer of bone marrow cells to irradiated mice leads to the formation, in the spleen, of cell colonies containing differentiating erythroid, myeloid and lymphoid cells (reviewed in Metcalf and Moore, 1971; Till and McCulloch, 1980). The stem cells responsible for generating these colonies are referred to as colony-forming units (CFU)-spleen. Their clonal nature was established by use of bone marrow cells with chromosomal abnormalities or enzymic markers (Whang et al., 1963; Wu et al., 1968; Trentin et al., 1967; Adamson et al., 1976; Fialkow et al., 1977). Colonies can be either a single cell lineage or a mixed population of cell types. Stem cells that give rise to only granulocytes and macrophages are referred to as GM-CFU. While dividing, spleen colony stem cells either differentiate into precursors of particular blood types or are renewed as multipotential cells (Siminovitch et al., 1963)

The most immature cell of the macrophage lineage that can be identified in vivo is the promonocyte (van Furth et al., 1970), although in tissue culture a more primitive monoblast can be distinguished (Goud et al., 1975). Circulating monocytes are derived from rapidly proliferating promonocytes (van Furth and Diesseldorf-Dulk, 1970; van Furth et al., 1970) and ultimately differentiate into the tissue macrophages of the peritoneum, lungs, liver and skin (Volkman and Gowans, 1965a,b; Volkman, 1966; Virolainen, 1968; Howard, 1970).

A great deal of heterogeneity is observed in the macrophage population; both between, and within, different tissues. Resident macrophages from diverse anatomical sites differ extensively in their morphology and enzyme content, as well as such functional capacities as Fc-mediated phagocytosis, cytotoxicty for bacteria and tumor cells, and chemotaxis (Walker, 1974, 1976; Kastello and Canonico, 1981).

Within the same tissue, heterogeneity is observed because of the different stages of macrophage differentiation that are encountered. Different morphological (Hirsch and

Fedorko, 1970) and functional (Cohn and Benson, 1965) properties result from cell populations at various stages in the maturation from blood monocytes to resident macrophages, as well as the different states of activation of those macrophages (Walker, 1976).

F4/80 Antigen.

Although there is great heterogeneity in macrophage populations, they appear to be phenotypically distinguishable, in the mouse, by the expression on the cell surface of a specific antigenic marker, F4/80. This antigen was defined by Austyn and Gordon (1981) and is recognised by the non-cytotoxic, rat monoclonal antibody which is also designated F4/80. Immunoprecipitation after biosynthetic and lactoperoxidase labeling has shown that the antigenic determinant is part of a 160,000 daltons plasma membrane glycoprotein (Austyn and Gordon, 1981).

During differentiation of macrophages from bone marrow cells in culture, F4/80 is first expressed on preadherent progenitors. Its level of expression increases with maturation and it can be found on all macrophage colonies derived in vitro (Hirsch et al., 1981). Levels of F4/80, however, have been observed to decrease, along with those of Fc receptors, on macrophages from BCG-activated mice (Ezekowitz et al., 1982).

F4/80 is uniquely expressed on cells of the macrophage lineage. It is not expressed on other blood cell types such as B and T lymphocytes, polymorphonucleucytes and erythrocytes; nor is it present on the Steinman-Cohen dendritic cell found in mouse lymphoid tissue (Austyn and Gordon, 1981; Nussenzweig et al., 1981).

Immunohistochemical staining for the F4/80 marker has shown it to be present on all known tissue macrophage populations and absent from cells which are clearly not macrophages. In normal mice, $F4/80^{+}$ resident macrophages have been located in hematopoietic and lymphoid tissues (Hume et al., 1983b), kidney (Hume et al., 1984b), liver (Hume et al., 1984c), skin (Hume et al., 1983b), endocrine organs (Hume et al., 1984a), bone and connective tissues (Hume and Gordon, 1983). They are also associated with the epithelia of a number of organs (Hume et al., 1984c). F4/80 antigen has also been demonstrated on macrophages that migrate into the developing retina, that phagocytose dying neurones and that differentiate into microglial cells (Hume et al., 1983a). $F4/80^{+}$ microglia in the brain probably have a similar derivation (Perry et al., 1985).

Thus, the F4/80 marker has proved to be very useful in studying macrophage localisation in a wide variety of organs. Use of this marker in an absorption, indirect binding assay has also been made to estimate the total macrophage content of adult mouse tissues (Lee et al., 1985).

Macrophage Activation.

Macrophages are induced to differentiate in response to both immunological and

non-immunological stimuli. Traditionally, those induced by sterile agents such as thioglycollate and peptone mediators are referred to as 'elicited' macrophages (Cohn, 1978; Hume and Gordon, 1982). Sterile inflammatory macrophages can be differentiated from resting cells functionally by increased spreading on glass surfaces, pinocytosis and antibody-mediated phagocytosis; and enzymatically by increased plasminogen activator, collagenase and acid phosphatase, and decreased 5'-nucleotidase. Macrophages that have been stimulated by lymphokines from activated T cells or by bacterial products such as endotoxin are referred to as 'activated' macrophages. These activated macrophages are microbicidal, tumoricidal and have an enhanced capacity to release reactive oxygen products as well as the enzymes associated with the generation of and protection from these products (Cohn, 1978; Hume and Gordon, 1982).

Models of macropohage activation have, in the past, assumed a regular progression from monocytes to elicited macrophages and finally to activated macrophages (Cohn, 1978). More recently, however, Adams and Hamilton (1984) have postulated a more complex model of macrophage development based on the fact that more than 2 stages of activation can be detected and that macrophages from the various stages may or may not be able to progress from one to the other.

Macrophages are activated by various compounds. Bacteria and bacterial products such as the LPS endotoxins, N-formyl-methionone and the adjuvant muramyl dipeptide are potent stimulators of macrophage chemotoxis and activity. These effects are mediated independently from those of lymphokines and different mechanisms of activation are used by these 2 classes of stimulators (Onazaki and Hashimoto, 1985).

Activation of macrophages by microbial products has been reviewed by Murray (1984) and Nogueira and Cohn (1984) and will not be discussed further. Macrophage recruitment and activation during viral diseases is most likely effected by the activity of activated T cells, and the effect of lymphokines on macrophages will be dealt with in greater detail.

1.2.2 Lymphokine Regulation of Macrophages.

Approximately 100 lymphokine activities have been described (Waksman, 1980) but these do not result from an equal number of distinct molecules. Many lymphokines have multiple effects (Gresser et al., 1979). Those that regulate macrophage activity can be broadly classed into; - colony stimulating factors, migration inhibitors and chemoattractants, and activators of enhanced macrophage effector function. An example of the last category is the interferons. Although both α- and β- interferon are known to be macrophage activators (Schultz, 1980) and can enhance IL-1 secretion from human monocytes (Arenzana-Seisdedos and Virelizier, 1983), they are not lymphocyte products (Wilkinson and Morris, 1983) and therefore will not be discussed further.

Colony Stimulating Factors.

The regulation of the growth and maturation of macrophages from bone marrow stem cells by colony stimulating factors has been reviewed by Metcalf and Johnson (1978) and Metcalf (1986). In general, the colony stimulating factors that control the various stages of development are not derived from lymphocytes but are found in the conditioned medium of various non-hematepoietic tissues (Stanley et al., 1978; Burgess and Metcalf, 1980; Metcalf, 1986). Macrophages, themselves, exert control over their own hemopoiesis and are one of the primary producers of colony stimulating factor, especially when activated (Cline et al., 1974; Ruschetti and Chervenick,1974; Burgess and Metcalf,1980).

That the continual turnover of CFU-spleen is dependent on lymphokines, however, is indicated by the ability of products from activated T cells to increase the numbers of these multipotential stem cells and by the inability of bone marrow from thymectomised or nude mice to reconstitute irradiated hosts (Zipori and Trainin, 1973). The lymphokine responsible is probably interleukin 3 (IL-3), also known as multi-colony stimulating factor, burst promoting activity, P-cell stimulating factor and hemopoietic growth factor (reviewed in Ihle and Weinstein, 1985; Metcalf, 1986). IL-3 was originally defined by its ability to induce 20 α-hydroxysteroid dehydrogenase in spleens of nude mice (Ihle et al., 1981b). These authors suggested that the induced enzyme was a pre-T cell marker but it has since been found in nonlymphoid cell lines and long term bone marrow cultures (Hapel et al., 1985a).

Another activity of IL-3 that was defined in 1981 by Schrader was its capacity to support the growth, in vitro, of the mast cell-like, P cells. It has since been shown to be a growth factor for pluripotential stem cells and for multiple lineages of progenitor stem cells from the bone marrow (Bazill et al., 1983; Ihle et al., 1983; Schrader et al., 1983). Once cloned (Fung et al., 1984), the protein produced by the IL-3 gene was found to stimulate the growth of stem cells for granulocytes/macrophages, eosinophils, megakaryocytes, and erythrocytes as well as multi-potential colony forming cells from the fetal liver and adult bone marrow (Hapel et al., 1985b). Its stimulation of GM-CFU in the bone marrow (Koike et al., 1986), monocytes and peritoneal exudate macrophages (Chen and Clark, 1986) is synergised by colony stimulating factors that are released from macrophages.

IL-3 is released by alloantigen activated T cells (Hodgkin et al., 1985) and lymphocytes reactive to Moloney sarcoma virus (Ihle et al., 1981a) and murine cytomegalovirus (Sinikas et al., 1985). Its mechanism of action involves the redistribution of protein kinase C from the cytosolic cell compartment to the membrane of target cells (Farrar et al., 1985).

Lymphokines Affecting Macrophage Migration.

Migration inhibition factor (MIF) was described in early studies on the DTH reaction to tuberculosis. A capillary tube method for analysing the inhibition of macrophage migration was used by both David (1966) and Bloom and Bennett (1966) to demonstrate the activity of MIF, a soluble mediator derived from the interaction of lymphocytes with antigen. The

production of MIF in vitro has traditionally been associated with the in vivo DTH reaction (David and David, 1972; Pick, 1977). It is released by activated T cells triggered by their specific antigen (Yoshida et al., 1973; Landolfo et al., 1978) as well as a number of T cell clones (Jones et al., 1981). Biochemically, MIF has been characterised thoroughly (Remold and Mednis, 1979).

A less well characterised lymphokine affecting macrophage migration is the macrophage aggregation factor (MAgF). Although, Badenoch-Jones and coworkers (1981) have developed an accurate assay for this lymphokine, it is not well understood. However, it is produced by sensitised T cells (Lolekha et al., 1970) and its activity can be separated from that of MIF by the use of antisera (Postlethwaite and Kang, 1976).

A lymphokine that acts as a chemoattractant, macrophage chemotactic factor (MCF) has also been described but, again, is not well characterised. It has also been referred to as lymphocyte chemotatic factor and is released by antigen-reactive T cells (Boetcher and Meltzer, 1975; Ward and Volkman, 1975). The most obvious in vivo function for this lymphokine is in the recruitment of mononuclear phagocytes to sites of DTH (reviewed in Ewan and Yoshida, 1979).

Lymphokines that Activate Macrophages.

Macrophage activation, as measured by increased microbicidal activity was first observed for the development of immunity to tuberculosis (Lurie, 1942). These observations were extended by Mackaness (1960,1962,1964) for the staphylococal and listerial bacteria. It was also Mackaness who determined that lymphoid cells were involved in conferring increased bacterial resistance on macrophages (Mackaness, 1969, 1971), an observation that was confirmed by the adoptive transfer of T cells resulting in macrophage activation (Blanden and Langman, 1972; Lane and Unanue, 1972). The ability of activated macrophages to phagocytose antibody-coated bacteria and to present antigen to sensitised T cells is also dependent on the activity of splenic lymphocytes (Ron et al., 1981).

One lymphokine released by antigen-reactive T cells and capable of activating macrphages is MAF. Initially its activity was biochemically inseparable from that of MIF but they have been shown to be distinct molecules (Kniep et al., 1981). MAF enhances the resistance of macrophages to infection by intracellular bacteria (Krakenbuhl and Remington 1971; Simon and Sheagren, 1971). Other signs of macrophage activation by MAF include increased cytotoxicity for tumor cells and increased adherence to glass (Nathan et al., 1971; Hibbs et al., 1977). The activation of macrophages to cytotoxicity by MAF is distinct from the that induced by LPS (deWeger et al., 1986).

A lymphokine with similar activities to that of MAF is γ-interferon. High yields of this lymphokine can be derived from MLRs (Kirchner et al., 1979) and its activity is probably enhanced by the capacity of macrophages to internalise extracellular molecules, suggested by studies using liposome-incapsulated lymphokines (Kleinermann et al.,1985; Koff et al.,

1985).

Interferon induces a number of activities characteristic of activated macrophages. There is increased spreading on and adherence to glass (Shultz et al., 1978), enhanced levels of the cytoplasmic enzyme lactate dehydrogenase (Schultz, 1980), and the induction of transferrin receptor (Hamilton et al., 1984) and Ia (Wong et al., 1983; Gershon et al., 1985; Papiernik et al., 1986; Strassmann et al., 1986) expression on the cell membrane. Monoclonal antibodies raised to this lymphokine are able to inhibit the ability of supernatants from mitogen-stimulated murine spleen cells to induce the induction of both microbicidal activity and Ia expression on elicited macrophages (Schreiber et al., 1985). Moreover, these authors found that distinct topographical domains on the interferon molecules, identified by different monoclonals, were responsible for either anti-viral or MAF activity. The ability of γ-interferon that is released locally, to induce Ia expression on macrophages has been demonstrated for phagocytes in the thymic reticulum (Papiernik et al., 1986). Interferon- g is also capable of inducing class II MHC expression on a number of non-hematopoietic cell types but the receptor on these cells is different from that of mononuclear phagocytes (Orchansky et al., 1986).

The functional capacities of activated macrophages that are induced by γ-interferon include increased antibody-mediated phagocytosis (Donahoe and Huang, 1973; Hamberg et al., 1980), enhanced tumoricidal activity (Schultz et al.,1977; Schultz and Chirigos, 1978; Mannel and Falk, 1983), decreased support for the growth of intracellular bacteria (Schultz et al., 1978) and virus (Virelizer et al., 1977), increased cytotoxicity for virus infected cells (Koff et al., 1985), as well as the induction IL-1 activity (Newton, 1985). These multiltudenous effects indicate the significant role that interferon plays in potentiating the effector function of macrophages.

In recent years it has become apparent that MAF and γ-interferon are the same molecule (reviewed in Schreiber and Celada, 1985). Both the antiviral and macrophage activating properties of supernatants from T cell hybridomas were neutralised by antibodies to γ-interferon (Pace et al., 1983). Svedersky and coworkers (1984) have shown that any MAF activity in culture supernatants, that is not associated with anti-viral activity, can be blocked by an antibody to cloned γ-interferon. As well as the functional identity between the 2 lymphokines (Schulz and Kleinschmidt, 1983; Murray et al., 1985), they are also biosynthetically and physicochemically identical (Shreiber et al., 1983; Fukazawa et al., 1984).

Thus, a variety of lymphokines facilitate the involvement of macrophages in the immune response to infection. They effect recruitment of mononuclear phagocytes by stimulating the proliferation of bone marrow stem cells as well as inhibiting migration of macrophages away from the site of inflammation. The macrophage activating lymphokine, γ-interferon, is vital in inducing the full effector capactiy of macrophages. This results in the induction of Ia expression on the macrophage cell surface; a convenient marker of activation.

1.2.3 Macrophage Function.

Macrophages are important both in the initiation of the immune response and in its execution. Because of their ability to process antigen, as well as producing the costimulatory factor IL-1, macrophages are capable of activating T cells. Once activated, T cells can be triggered to release lymphokines by any cell bearing antigen and MHC molecules, but macrophages are one of a few cell types capable of processing foreign antigen to present in association with Ia molecules. Recruitment and activation of inflammatory macrophages by lymphokines then initiates a plethora of macrophage effector functions.

Antigen Presentation and T Cell Activation.

The role of macrophages in initiating the immune response has been reviewed extensively (Persson et al., 1978; Unanue, 1978, 1981, 1984; Grey and Chesnut, 1985). The ability of macrophages to present soluble antigen is dependent on an active internal processing step that is blocked by chloroquine (Ziegler and Unanue, 1981). Macrophage-like accessory cells are required to present antigen to virus-immune T cells (Pang and Blanden, 1976; Blanden et al., 1977; Leung et al., 1981) and to T cells that 'help' antibody responses (Erb and Feldmann, 1975 a, b, c). Different classes of macrophage are able to act as APCs, including human monocytes (Bjerke and Gaudernack, 1985). Presentation of soluble antigen on the cell surface, following internalisation and processing by macrophages, results in a tight coupling between the antigen and Ia molecules (Puri et al., 1985; Unanue and Allen, 1986).

To activate resting T cells it is necessary that foreign antigen is presented on the cell membrane of macrophages that also bear the class II MHC molecules (Rosenthal, 1978; Unanue, 1981). Macrophages are heterogenous for Ia antigen expression and it is only those that express class II MHC molecules that are capable of activating resting T cells (Cowing et al., 1978; Beller and Unanue, 1980, 1981; Beller et al., 1980; Unanue, 1984). The requirement for Ia molecules on the cell surface of stimulatory macrophages does not necessarily imply that recognition of foreign antigen and Ia is obligatory, since, in this case, only class II MHC-restricted T cell responses would be generated. The necessity for Ia expression probably reflects the fact that only activated macrophages can stimulate resting T cells. Macrophage activation results in both Ia expression and the ability to release the costimulatory factor necessary for T cell stimulation, IL-1.

IL-1, initially termed lymphocyte activating factor (LAF), was first described by Gery and Waksman (1972) for its mitogenic effects on thymocytes and its ability to costimulate the proliferative response of T cells. IL-1 promotes the production by T cells of their own growth factor, IL-2 (Smith et al., 1980). It is released by macrophages triggered with both bacterial products and activated T cells (Farrar et al., 1980; Mizell et al., 1978; Oppenheim et al., 1979) and from macrophage cell lines (Lachman et al, 1977; Cowing et al., 1978; Mizel et al., 1978; Mizel and Rosentreitch, 1979; Booth et al., 1983). It is also produced by other cell types

thought to be involved in activating T cells, such as dendritic and Langerhan cells (Steinman, 1981; Fisher et al., 1983; Sauder et al., 1984).

As well as its costimulatory activity, IL-1 has many other capacities including that of endogenous pyrogen (Bernheim et al., 1980; Murphy et al., 1980; Sztein et al., 1981), stimulation of B cell activation along with anti-immunoglobulin antibodies (Howard et al., 1983) and stimulation of fibroblast proliferation (Schmidt et al., 1982). Differentiation, in vivo, of the costimulatory function of IL-1 from its other functions may result from its location on the surface of the macrophage rather than its release into the blood stream. A membrane-associated form of IL-1 has been identified (Kurt-Jones et al., 1985) and has been shown to be necessary for stimulating T cell lines and clones (Unanue and Allen, 1986).

Ia expression and IL-1 production by macrophages are not necessarily linked functionally. This is suggested by the finding that the Ia^- cell line, P815, is able to stimulate alloreactive T cells (Lafferty et al., 1980) while Ia^+ aveolar macrophages have a limited capacity to release IL-1 (Wewers et al., 1984) and are poor stimulators of the MLR (Lipscomb et al., 1986). Contrary evidence, however, is the finding that antibodies to the human class II MHC molecules are able to induce the release of IL-1 from monocytes (Palacios, 1985) or block its release from murine macrophages (Durum et al., 1984).

Macrophages are also capable of releasing an inhibitor of IL-1, and it is apparent that some immunosuppressive viruses, such as the respiratory syncitial virus, are potent inducers of this inhibitor (Roberts et al., 1986).

The role of IL-1 as an immunological mediator has been reviewed by Durum and colleagues (1985) and it is apparent that the ability of macrophages to initiate T cell activation is dependent on their capacity to release this interleukin.

Cytotoxic Function of Macrophages.

The microbicidal and tumoricidal activity of macrophages is probably due to the release of toxic substances, (reviewed by Cohn and Scott, 1982). These include enzymes such as lysozymes, neutral proteases and esterases. Bacteria and fungi containing peptidoglycans that are catabolised by lysosymes are killed by macrophages releasing this enzyme (Brumfitt and Glynn, 1961; Collins and Pappagianis, 1974; Gadebusch and Johnson, 1966). Neutral proteases and esterases have been implicated in the killing of tumor cells by macrophages (Adams et al., 1980; Piessens and Sharma, 1980) and the secretion of arginase by phagocytes is capable of inhibiting the growth of arginine-dependent tumors (Currie and Basham, 1978). The myeloperoxidase-mediated system of phagocytes also generates potent cytotoxicity for bacteria (Klebanoff, 1968), fungi (Howard, 1973), and viruses (Belding et al., 1970).

Macrophages are also able to generate potent oxidising agents by a metabolic event known as the 'respiratory burst'. Products generated include hydrogen peroxide, the hydroxyl radicals, superoxide anions and singlet oxygen (reviewed in Davies and Bonney, 1979). Activation of macrophages results in their increased respiratory burst response (Pabst et al.,

1980; Bryant et al., 1982). In many instances enhanced microbicidal activity can be correlated with their capacity to release reactive oxygen products (Johnston et al., 1978; Nathan and Root, 1977; Wilson et al., 1980). The oxidative metabolism of macrophages is also capable of restricting infection of vesicular stomatitis virus (Rager-Zisman et al., 1982). Because of the high toxicity and non-specific nature of the oxygen products, phagocytes have developed a protective system of enzymes that can reduce the reactive species (Voetman and Roos, 1980).

Other secretory products that are capable of cytotoxic activity are thymidine which blocks deoxyribonuclic acid (DNA) synthesis of certain tumors (Stadecker et al., 1977), a labile cytotoxine (Macfarlan and White, 1980), a listericidal substance (Bast et al., 1974) and a tumor necrotising factor (Mannel et al., 1980).

Another killing mechanism used by macrphages is antibody-dependent cell cytoxicity (ADCC). Through their Fc receptors, macrophages can lyse cells that are coated with immunoglobulin in the absence of complement (Sissons and Oldstone, 1980). Examples of virus infected cells that are lysed by the ADCC mechanism include HSV (Ramshaw, 1975, Kohl et al., 1979), influenza (Greenberg et al., 1977), measles and vaccinia (Perrin et al., 1977a,b) viruses.

Role of Macrophages in Protection from Viral Infections.

Although activated macrophages are able to kill or inhibit the growth of a wide range of organisms both in vitro and in vivo, this discussion will be restricted to the role they play in viral infections.

The importance of macrophages in resistance to viral infections has been inferred by their recruitment to sites of infection and by studies using cytotoxic agents to remove macrophages in vivo (reviewed in Mogensen, 1979). Blanden (1974) noted that mononuclear phagocytes, identified by injecting animals with carbon ink, accumulated at sites of ectromelia infection. Regression of infectious foci followed this accumulation (Blanden 1971b). When recruitment of macrophages is enhanced with the use of such immunomodulators as *Cornyebacterium parvum*, there is an increased resistance to virus infections (Larson et al., 1972; Mak et al., 1983). The in vivo treatment of animals with certain agents such as silica (Allison et al., 1966) can severely deplete their macrophage population. Use of such agents has implied the importance of this cell type in resistance to viral infections (Zisman et al., 1970; du Buy, 1975; Haller et al., 1976; Turner and Ballard, 1976).

Although the implication is often that macrophages resolve infections that are already established, they are also capable of forming a barrier to infection by intrinsically restricting viral growth. This capacity varies between different strains of animals and virus, but there is a distinct correlation between the ability of a virus to grow in macrophages, in vitro, and the pathogenicity fo the infection, in vivo. This has been demonstrated for lymphocytic choriomeningitis virus (LCMV) (Tosilini and Mims, 1971), HSV-type 2 (Mogensen, 1977), Wesselsbron virus (Olson et al., 1975) and mouse hepatitis virus (Virelizier, 1981).

Macrophages from older animals are often a more efficient barrier than those from young animals. Peritoneal macrophages from both suckling and adult mice are infected in vitro by HSV but only those from young mice supported the spread of infection to other cells (Johnson, 1964). When transferred to young mice, macrophages from adults confer resistance to subsequent infection (Mogensen, 1978). The intrinsic antiviral activity of macrophages is probably related to their ability to either phagocytose and degrade the virus as occurs in influenza virus infection of mouse macrophages (Rodgers and Mims, 1981), or to the restriction of viral replication in the cytoplasm (reviewed in Stohlman et al., 1982).

Activation of macrophages during viral infection increases their intrinsic antiviral activity. Activated macrophages can be recovered from the lungs of mice infected with influenza virus (Mak et al., 1983) and these have an increased resistance to in vitro infection with influenza virus (Rodgers and Mims, 1981) that is associated with the release of lysozyme and hydrogen peroxide (Rodgers and Mims, 1982). Activation of peritoneal macrophages by *C. parvum* results in the abortive infection with ectromelia in which virus attaches to the cell membranes but does not replicate in the cytosol (Cohen et al., 1984).

Macrophages also display extrinsic antiviral activity in that they can either inhibit viral replication in normally permissive cells or kill virus-infected cells (Stohlman et al., 1982). Viral plaque formation and replication in fibroblast cultures can be inhibited by macrophages from animals infected with herpes simplex virus (Morahan et al., 1977). Also, both resident and elicited peritoneal macrophages are capable of restricting the in vitro replication of mouse hepatitis virus in permissive cells (Stohlman et al., 1982) and elicited macrophages are cytostatic for cells infected with Sendai and influenza viruses (Golman and Hogg, 1978).

Activated macrophages can selectively destroy virus-infected but not uninfected cells, by a mechanism independent of ADCC. Systems in which such killing has been shown include infection of mice with influenza (Watanabe and McKenzie, 1982; Mak et al., 1983) and reoviruses (Letvin et al., 1982), herpes and vaccinia infection of hamsters (Chapes and Tompkins, 1979) and herpes infection of humans (Stanwick et al., 1980, 1982).

The process by which macrophages are able to kill virus-infected cells is not understood. More information is available on the method used to inhibit viral growth in co-cultured cells but even this mechanism remains an enigma in most cases. For the multiplicaion of HSV, Wildy and colleagues (1982) showed that arginase released by macrophages depleted the quantity of arginine that is required for the replication of this virus. These authors have also suggested that the antiviral activity of macrophages in many systems may be due to the release of interferons.

1.3 LYMPHOCYTIC CHORIOMENINGITIS VIRUS.

LCMV is an arenavirus which is a natural pathogen of mice. It is, in general, a nonlytic virus and its propagation does not cause obvious damage to the host cell. LCMV can persist in immunocompromised mice with continual production of large amounts of virus, but with only minor effects on the health of the animals. In immunocompetent mice, however, the lymphocytic response to the virus can result in resolution of the infection, or immunopathological damage. Many excellent reviews on LCMV have been published (Hotchin, 1971; Lehmann-Grube, 1971, 1984; Cole and Nathanson, 1974; Doherty and Zinkernagel, 1974; Gilden, 1975; Bro-Jorgensen, 1978; Buchmeier et al., 1980; Oldstone et al; 1985). This section will consider the evidence that indicates a role for class I MHC-restricted T cells in the immune response and also the immunopathogenesis of the acute disease of the disease following LCMV infection. Data that indicate the involvement of macrophages in lymphocytic choriomeningitis (LCM) will also be discussed.

1.3.1 Class I MHC-Restriction of the T Cell Response to LCMV.

It was Rowe (1956) who suggested that inflammatory cells played a crucial role in the induction of LCM. He found that protection from the disease was conferred on adult mice that were pretreated with x-irradiation. This protection was associated with leukopenia and decreased inflammation of the chorio-meninges, lungs and liver. LCM was thus due to the reactivity of the host to the virus. However, because survivors were no longer susceptible to the disease on intracerebral (i.c.) rechallenge with the virus, Rowe believed that irradiation had not interferred with immunity.

Hotchin and coworkers confirmed that protection from LCM was associated with leukopenia and decreased meningeal infiltration, but found no effect on virus replication. They proposed that the decreased immune response resulting from irradiation conferred on adult mice a tolerance to the virus, similar to that seen in neonatally infected animals (Collins et al., 1961; Hotchin and Weigand, 1961). Thus, the lymphocytic response to LCMV was thought to be responsible both for viral clearance and the pathological consequences of infection (Hotchin, 1962).

By infecting adult mice that had been thymectomised as neonates, Rowe and colleagues (1963) were able to show that both LCM disease and viral clearance were effected by T cells. This finding was confirmed by East and coworkers (1964) and Hotchin and Sikora (1964). The ability of anti-thymocyte serum (Hirsch et al., 1967) and anti-lymphocyte serum (Gledhill, 1967; Lundstedt and Volkert, 1967) to protect mice from LCM, also supported the view that T cells were responsible for the induction of the disease.

Hotchin (1962) proposed that LCMV-infected cells produced a surface antigen that was

recognised as foreign by the immune system. He suggested that sensitised lymphocytes eradicate infected cells in a manner similar to that of graft rejection. This hypothesis was borne out by the finding that skin and adenocarcinoma cells from LCMV-carrier mice were rejected by syngeneic uninfected hosts in the same time span as that of allografts (Holterman and Majde, 1971). Also Lundstedt (1969) showed cytotoxic activity of LCMV-immune spleen cells for LCMV-infected L-cells. This was the first in vitro demonstration of anti-viral cytotoxicity and confirmed the view that it was LCMV-induced modifications on the cell surface that were recognised by immune cells.

More substantial evidence that T cells were responsible for inducing LCM came with the use of antibodies specific for T cells, immunogenetics and LCMV-reactive T cell clones, in combination with an adoptive transfer model of the disease.

Immunosuppressive agents, such as cortisone and methotrexate, had been used to induce a virus-carrier state in adult mice infected with LCMV (Hannover Larson, 1969), and Gilden and coworkers (1972a) demonstrated that cyclophosphamide (Cy) was an equally potent inducer of the carrier state. Transfer of LCMV-immune spleen cells into such mice resulted in the development of symptoms typical of LCM (Gilden et al., 1972b). That T cells were resposible for inducing the disease was demonstrated when the capacity of immune cells to transfer LCM was abolished by treatment with anti-Thy1 serum plus complement (Cole et al., 1972; Doherty and Zinkernagel, 1975a). Zinkernagel and Doherty (1973) also showed that T cells which had been sequested to the cerebrospinal fluid (CSF) after i.c. inoculation of the virus, were cytotoxic for LCMV-infected target cells and that this cytotoxicity was depleted by treatment of CSF cells with anti-Thy1 and complement.

As discussed in Section 1.1, studies on the killing of infected target cells by LCMV-immune spleen cells were instrumental in showing the MHC-restriction of T cell responses (Zinkernagel and Doherty, 1974a). This restriction was for the K and D regions only; class II MHC molecules were not restriction elements for LCMV-reactive CTL (Blanden et al., 1975; Doherty and Zinkernagel, 1975b; Marker and Andersen, 1976). Not only is the in vitro cytotoxic response to LCMV-infected cells MHC-restricted, but induction of LCM disease by the transfer of immune cells into immunosuppressed, infected mice only occurs if the donors and recipients are compatible at the class I region of the MHC (Doherty et al., 1976b; Doherty and Allan, 1985). Furthermore in congeneic C57BL/10 mice, susceptibility to cerebral symptoms has been mapped to the D region of the MHC (Zinkernagel et al., 1985). Virus clearance from spleens of mice receiving adoptively transferred immune spleen cells is also restricted by the K and D region of the MHC. Recognition of the I region is neither sufficient nor necessary for in vivo function of LCMV-reactive T cells (Zinkernagel and Welsh, 1976).

With the development of LCMV-reactive T cell clones, it became possible to show definitively that single cloned lines of Class I MHC-restricted T cells are capable of both inducing the immunopathological symptoms of LCM, as well as effecting viral clearance.

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Baenziger and colleagues (1986) demonstrated that cloned Lyt2$^+$/L3T4$^-$, LCMV-reactive T cell lines were able to induce cerebral disease in immunosuppressed, infected recipients when injected intracerebrally. When inoculated peripherally, these cells effected a severe respiratory and wasting syndrome, presumably due to lodgement of injected cells in the lungs of recipient mice. In immunocompetent mice LCM disease could be prevented if cloned T cells were admixed with the virus inoculum before i.c. injection, indicating the capacity to clear virus infection before the immunological consequences of the disease were manifest. Cloned CTL were also able to clear virus from the spleens (to which they migrated) of acutely infected mice within 20-30hr of cell injection (Byrne and Oldstone, 1984, 1986).

Thus, it is well established that the immune response to acute infection with LCMV, both in inducing disease and in clearing viral infection, is restricted by class I MHC molecules.

1.3.2 Involvment of Macrophages in the Host Response to LCMV.

Involvement of macrophages during the response to the acute infection of mice with LCMV was first recorded in the histological descriptions of Lillie and Armstrong (1945) and subsequently by many authors, including Lerner and Haas (1958), Gilden et al., (1972b), Cole and Nathenson (1974), Walker et al., (1975) and Schwendemann et al., (1983). Macrophages and monocytes were observed amongst inflammatory cells infiltrating the central nervous system (CNS).

Activation of macrophages during LCMV infection, as measured by increased resistance to listeria infection, was reported by Blanden and Mims in 1973. They found that activation correlated with peak T cell activity. Buchmeier and coworkers (1980) also observed an increase in the size, number and vacuolation of macrophages from mice infected by the intraperitoneal route. Peak activation occurred 2-3 days after that of T cell cytotoxicity. Activation was not seen in nude mice and is therefore likely to depend on T cell function. In the adoptive transfer model used by Lehmann-Grube and coworkers (1986), however, there was no evidence that macrophages were activated by transferred LCMV-immune spleen cells to resist listeria infection, even though such resistance was observed in mice acutely infected with the LCMV.

Only a few papers have questioned the role played by macrophages in LCMV infection. Tosilini and Mims (1971) noted that LCMV could grow in peritoneal exudate macrophages, but that this ability varied between different strains of mice. Mouse strains that were most resistant to infection had macrophages that did not support viral replication. Also, resident macrophages were apparently the first cells to be infected (Tosilini and Mims, 1971; Lohler and Lehmann-Grube, 1981). These observations led to the postulate that macrophages were an initial barrier to LCMV infection. Thomsen and coworkers (1983b) supported this view with the finding that treatment of mice with carrageenan or silica 2hr before virus infection

resulted in increased blood titres of LCMV. Further examination of this phenomenon by Thomsen and Volkert (1983) showed that an irradiation-insensitive, carrageenan-sensitive cell population (presumably resident macrophages) was important in restricting viral replication in the early stages of infection.

A role for non-resident macrophages in the immune response to LCMV has been implicated in adoptive transfer experiments in which a radio-sensitive population was necessary for effective viral clearance. When transferred into mice on the same day of infection, primary immune T cells cleared virus from the animals within 5 days. This ability was abrogated by irradiation of the recipients prior to T cell transfer. Therefore, transferred cells need a radiosensitive cell population to remove virus. This population is not T cells, since adoptively transferred cells can clear virus in nude mice, and is most likely to be monocytes (Thomsen and Volkert, 1983). To accept that macrophages are involved, however, it would be necessary to use a more definitive macrophages marker such as F4/80.

Most of the inference for a role for macrophages in acute LCMV infection derives from the DTH reaction observed to accompany infection with this virus. Tosilini and Mims (1971), who first tested peripherally-infected mice for their ability to mount a DTH response to LCMV injected into the footpad, found that it had a similar time course in its development to that of T cell cytotoxicity in the spleen. They also found that the DTH response in the footpad could be transferred to recipients with i.v. injection of immune spleen cells. This adoptive transfer of DTH (footpad swelling) to LCMV by immune spleen cells is, like the transfer of disease symptoms, restricted by the K and D regions of the MHC (Zinkernagel, 1976b).

However, because the neurological disease, LCM, could be induced rapidly in recipients that had increased susceptibility to listeria infection, due to Cy or cortisone treatment, Zinkernagel and Doherty (1975) proposed that macrophages were not important in the induction of fatal LCM. This view has been challenged recently (Thomsen and Volkert, 1983; Thomsen et al., 1983a,b; Marker et al., 1985). Pretreatment of mice with Cy, in contrast to treatment 3-5 days after virus injection (Gilden et al., 1972a), did not prevent the development of T cell cytotoxicity in the spleens of infected mice but did decrease the footpad swelling that follows challenge with infected cells. Drug pretreatment afforded protection from mortality 7-8 days after i.c. inoculation, as well as footpad swelling 7-8 days after primary intraplantar infection (Thomsen et al., 1983a). In the same study, it was found that spleen cells from drug-treated infected animals that were adoptively transferred to naive recipients, were able to confer the capacity to mount a DTH response to LCMV. Thus, the authors suggested that the capacity of the immune lymphoid cells to elicit DTH or to lyse virus-infected cells was not impaired by Cy pretreatment, but the capacity of ancillary cells such as macrophages to respond to these T cells was inhibited. In a subsequent paper, it was shown that the differential effect of Cy pretreatment on the cytotoxic and DTH response was dose-dependent with doses as low as 80mg/kg of the drug being able to inhibit footpad swelling after intraplantar infection, while up to 150 mg/kg could be tolerated before an effect

on cytotoxicity of spleen cells was detected. Using a regime in which Cy treatment inhibits footpad swelling but not T cell cytotoxicity, the authors found that virus clearance from the blood was impaired for the same length of time that the DTH response was impaired. Because treatment of mice with agents that are cytotoxic for macrophages also prevented virus clearance from infected mice, the authors concluded that the ability of T cells to elict macrophage effector function, rather than their ability to lyse infected cells, was crucial for resolving the infection (Thomsen et al., 1983b).

In both of the papers discussed above, Thomsen and his coworkers inferred that the same T cell population that was cytotoxic for infected cells in vitro was capable of eliciting the DTH response in the footpad. The effect of pretreatment with Cy on the DTH reaction was due to suppression of ancillary cells and not T cells. After finding that the impairment of the DTH response in mice responding to a high i.c. dose of LCMV correlated with the ability to survive infection and because lymphoid cells from high dose responders were still unable to elicit DTH when transferred into naive recipients, this group altered their stance and suggested that there were 2 distinct subsets of class I MHC-restricted T cells, one that was cytotoxic and the other mediating DTH responses (Marker et al., 1985). However, the fact that these authors found no difference between high and low dose responders in the cytotoxic capacity of inflammatory cells from the CNS suggests, in itself, that the DTH response in the brain had remained intact. If there had been no DTH in the CNS with the consequent recruitment of non-cytotoxic ancillary cells, there should be a higher concentration of cells with cytotoxicity activity and the dose response to infected targets would be enhanced. Thus, although immune cells from high-dose responders had a reduced capacity to induce footpad swelling on adoptive transfer to naive animals, it would seem that they are still capable of initiating an inflammatory response in the CNS.

Determination of DTH response by measurement of footpad swelling in the experiments discussed above is open to criticism. Although statistically significant results are reported within each experiment the range in measurements between different experimental systems is large. When footpad swelling is measured after challenge with live virus, positive results are in the range of 4-37 x 10^{-2} mm increase in footpad thickness. Positive swelling measured after challenge with virus-infected cells ranges between 47 and 84 x 10^{-2} mm, while the negative control values from uninfected mice vary from 16 to 26 x 10^{-2} mm and this is not statistically different from the other negative values from Cy-pretreated infected animals (20-42 x 10^{-2} mm). These latter negative control values vary non-significantly by the same amount as the total positive values reported after challenge with virus alone (Thomsen et al., 1983a). A similar difference between these two systems of measuring footpad swelling can also be seen in the experiments alleging that mice responding to a high dose of LCMV generate T cells that are defective in mediating DTH, and in some cases significant differences between high and low dose responders could only be measured on one of three days following challenge (Marker et al., 1985). Thus, these observations and the fact that very small increases in the

width of the footpad are measured (sometimes only 20 x 10^{-2} mm), leads one to question the value of using footpad swelling, in these experiments, as an accurate measure of the DTH response.

A more accurate measurement of DTH, and a more relevant one, is the number of inflammatory cells in the CSF. Recruitment of cells to the CNS during infection, reflects the capacity of class I-restricted T cells to initiate an inflammatory response (Doherty et al., 1976b). Doherty and Allan (1985) have shown that pretreatment of mice with Cy has little effect on either the cytotoxic capacity of LCMV-reactive spleen cells, or their capacity to induce an inflammatory response in the CNS. Furthermore, unlike the DTH response in the footpad, the inflammatory response to LCMV in the CSF was not inhibited by Cy pretreatment of the recipients (Doherty and Allan, 1985). Thus, although the DTH response in the footpad is class I MHC-restricted (Zinkernagel, 1976b), footpad swelling probably does not accurately reflect the inflammatory response occurring in the relevant target organ, the brain.

The DTH response in the CNS of LCMV-infected mice involves the nonspecific recruitment of cells that express the glycoprotein marker, Pgp. Macrophages are among the cell types that express this marker (Trowbridge et al., 1982) and by flowcytometric analysis of morphology are in CSF during LCM. Immune spleen cells transferred into bone marrow reconstituted radiation chimeras recruit cells of bone marrow origin to the CNS, even though these are of a different Pgp allele to that of the donor T cells (Doherty, Allan and Ceredig; manuscript in preparation). That is, although the T cells that accumulate in LCMV-infected brain are restricted by the MHC haplotype of the radiation-resistant, virus-infected cell population (Doherty and Allan, 1986), the recruited cells are derived from the radiation-sensitive bone marrow compartment.

In contrast to the conclusions of Thomsen, Marker and their coworkers that were described above, it has been suggested by Baenziger and colleagues (1986) that the induction of LCM disease is dependent on the cytotoxic capacity of virus-immune T cells rather than their ability to recruit bone marrow-derived cells to the CNS. With their work on LCMV-reactive T cell clones, these authors found that the induction of cerebral LCM disease, in contrast to DTH in the footpad, was independent of radiosensitive bone marrow cells. They found that the ability of clones to induce footpad swelling in pre-irradiated, infected recipients was enhanced by bone marrow reconstitution, whereas mortality due to i.c. inoculation of T cells did not require such reconstitution. However, there are a number of variables between the 2 disease systems that were not taken into account. Footpad swelling in non-reconstitiuted mice was not absent but reduced compared with those transplanted with bone marrow. Because inflammation in the CNS is lethal, it would be difficult to detect an enhanced response following reconstitution. In any case no experiments on cerebral LCM in which irradiated mice received bone marrow reconstitution were reported. A decrease in the time to mortality following reconstitution may have been detected, if such an experiment had been performed. Also, the time after irradiation at which footpad swelling and LCM mortality were measured

differed by 1-3 days. Because the footpad response is measured earlier the contribution of cells derived from reconstituted bone marrow may be more significant.

The capacity of LCMV-reactive T cells to release lymphokines involved in the recruitment and activation of macrophages has not been reported extensively. Each of the four class I MHC-restricted, LCMV-specific T cell clones tested by Oldstone's group released γ-interferon when co-incubated with syngeneic, virus-infected target cells (Andersson et al., 1985). Presumably other factors are also produced.

In summary, macrophages are observed in the cell infiltrates of LCMV-infected organs. They can be infected by this virus and they are activated during viral infection. Class I MHC-restricted T cells are responsible for mediating DTH during LCMV infection and are capable of releasing at least one of the lymphokines responsible for activating macrophages, suggesting that this cell type may be functioning to recruit mononuclear cells to sites of LCMV infection.

1.4 SCOPE OF THIS THESIS.

Acute LCM disease is mediated and resolved by class I MHC-restricted, $Lyt2^+$ T cells. A role for class II MHC-restricted, $L3T4^+$ T cells during LCMV infection has not been defined. However, the DTH response which is traditionally associated with the latter cell type, is conspicuous in this disease. The extent to which macrophages, a major component of the DTH response, are involved in the immune response to LCMV is not well understood. It is also not known whether their recruitment during infection is mediated by class I or class II MHC-restricted T cells.

To investigate these problems, the experiments reported in this thesis aimed to determine the in situ localisation of macrophages during acute LCMV infection and, in addition, to investigate whether $Lyt2^+$ T cells are responsible for the recruitment of macrophages to sites of virus infection.

Chapter 2

Materials and Methods

2.1 Mice.

The C57BL/6J, C57BL/10.A (B10.A), C57BL/6.H-2^{bm1} (B6.H-2^{bm1}), C57BL/6Ka Thy1.1 (B6Ka Thy1.1), C57BL/10.D2 (B10.D2) and C57BL/10.BYR (B10.BYR) mice were all bred at the John Curtin School of Medical Research (JCSMR) and used at 7-12 weeks of age. All mice, except those referred to in Chapter 3, were bred under specific-pathogen-free (SPF) conditions. The experiments described in Chapter 3 were done before SPF conditions were re-established in the animal breeding unit. The H-2 haplotypes of these mouse strains are given in the table 2.1.

TABLE 2.1 H-2 COMPOSITION OF MOUSE STRAINS USED.

Strains	K	I		D	$L^{\dagger}$
		I-A	I-E/C		
C57BL/6J	b	b	b	b	-
B6.H2^{bml}	bml	b	b	b	-
B6Ka Thy1.1	b	b	b	b	-
B10.BYR	q	k	k	b	-
B10.A	k	k	k	d	d
B10.D2	d	d	d	d	d

2.2 Viruses.

LCMV strains were originally obtained from Dr F. Lehmann-Grube (University of Hamburg, Germany) and have been maintained at the JCSMR since 1974. The viscerotropic WE3 strain of LCMV was grown in BHK-21 cells, for inoculation into mice, and in L929 fibroblasts, for infection of tissue culture. The neurotropic Armstrong E350 (Arm) virus was grown in suckling mouse brain, and prepared as a 10% brain homogenate.

For the Arm strain and the WE3 strain grown in BHK-21 cells, the minimal dose that was lethal for 50% of inoculated mice (LD_{50}) was determined following intracerebal (i.c.) injection into adult mice. This in vivo titration system is considered to be approximately 10-fold more sensitive than existing in vitro techniques (Varho et al., 1981). For the WE3 strain grown in L929 cells, plaque forming units (p.f.u.) were determined by titration on Vero cell monolayers.

Virus stocks were diluted in 0.2M borate-buffered gelatine saline (pH 7.2-7.4).

2.3 Media.

Dulbecco's Modified Eagle's Medium (DMEM) was prepared by dissolving 10g of medium powder (GIBCO, Grand Island, NY, USA; product code 430-2100) per litre of double distilled deionized water and was supplemented with 2g/l of sodium bicarbonate, 6mg/l of folic acid, 36mg/l of L-asparagine, 116mg/l of L-arginine and 4g/l of glucose.

RPMI 1640 was prepared according to manufacturers instruction, by dissolving 10.4g of medium powder (GIBCO, Grand Island, NY, USA; product code 430-1800) per litre of double distilled deionized water containing 2g sodium bicarbonate.

DMEM and RPMI were sterilised by filtration through a 0.22µm membrane and were supplemented with 100 i.u. penicillin/ml, 100µg streptomycin/ml and 50µg neomycin/ml.

Heat-inactivated newborn bovine serum (NBS) and foetal calf serum (FCS) were prepared by incubation at 56°C for 30min. Serum batches from the Flow Laboratories (North Ryde, N.S.W., Australia) were selected on their ability to support the growth of mitogen-stimulated lymphocytes.

2.4 Monoclonal antibodies.

<u>Antibodies used for immunohistochemical staining.</u>

Both primary antibodies were rat immunoglobulins of the IgG_{2b} subclass. The optimum working concentration of tissue culture supernatants containing antibody was

determined by titration in the staining procedure described in section 2.8. The sensitivities of the two antibodies for their antigen on fixed tissue was judged to be similar by the ability to stain areas in the spleen known to express these antigens.

F4/80: The hybridoma cell line F4/80 secretes antibody to the F4/80 antigen, a mouse plasma membrane glycoprotein specific for cells of the monocyte/macrophage lineage (Austyn and Gordon, 1981).

Anti-Ia: The hybridoma M5/114.15.2 (TIB 120, American Type Culture Collection) produces an anti-Ia antibody which binds to common antigenic determinants present on molecules encoded at the I-$A^{b,d,q}$ and I-$E^{d,k}$ regions of the mouse MHC (Bhattachrya et al. 1981).

Antibodies used for complement-dependent depletion of spleen cell populations.

The optimum concentration of the antibody preparations was determined by titration in a complement-dependent depletion assay on normal thymocytes.

Anti-Lyt2: The hybridoma 3.168.8 secretes a rat immunoglobulin of the IgM subclass recognising a determinant on the Lyt2 molecule of mouse T lymphocytes (Sarmiento et al., 1980).

Anti-L3T4: The hybridoma LICR.LAU.RL172.4 (RL172) secretes a rat IgM recognising a determinant on the L3T4 molecule (Ceredig et al., 1985).

Anti-Thy1.1: Mouse IgM antibody from the T11D7e hybridoma was obtained from Olac Ltd., Blackthorne, Oxon, England (Lake et al., 1979).

Anti-Thy1.2: Mouse IgM antibody from the F7D5 hybridoma was also obtained from Olac Ltd., Blackthorne, Oxon, England (Lake et al., 1979).

Hybridoma cell lines were grown in supplemented DMEM containing 10% FCS.

2.5 Fixative.

Periodate-lysine-paraformaldehyde (PLP) was prepared according to the method of McLean and Nakane (1974). The final composition of the fixative was; 0.01M sodium M-periodate, 0.075M L-lysine, 0.037M sodiom phosphate buffer and 2% paraformaldehyde.

Stock solutions of lysine-phosphate buffer were stored at -20°C. L-lysine HCl (BDH, Poole, England) at a concentration of 0.2M was adjusted to pH 7.4 with 0.1M Na_2HPO_4 and the volume was adjusted to give 0.1M L-lysine HCl with 0.1M sodiom phosphate buffer (4:1 ratio of 0.1M Na_2HPO_4 and 0.1M NaH_2PO_4).

Eight percent weight/volume paraformaldehyde solution was prepared on the day of use. Paraformaldehyde was dissolved in distilled water by heating to approximately 60°C

and adding sufficient 10N NaOH to clear the solution.

Paraformaldehyde was combined with the lysine-phosphate buffer in a ratio of 3:1 and sodium m-periodate powder (AJAX, Sydney, Australia) was added to give a final periodate concentration of 0.01M. The PLP fixative was adjusted to pH 7.4 using 10N NaOH.

2.6 Perfusion of mice for immunohistochemistry.

Mice were anaesthetized with ether and perfused by inserting a plastic cannula (pp50) into the left ventricle. When liver samples were required the inferior vena cava was cut above the renal veins and below the liver. This avoided perfusion of the lungs. When perfusing the brain, the circulation to the lower body was stopped by clamping the descending aorta and vena cava above the diaphram, and outward flow of perfusate was obtained by cutting the right atrium.

Blood was cleared by perfusion for 2-3 min with phosphate-buffered saline (PBS) containing heparin (2 units/ml heparin (CSL, Melbourne, Australia)). The animals were then perfused with 100-200ml PLP fixative and the fixative was cleared with a further 10-15 min perfusion with heparinised PBS. All perfusions were carried out at a pressure of approximately 120mm Hg, by maintaining the height of the perfusion fluid above the animal at about 160cm.

2.7 Tissue preparation for immunohistochemistry.

The right lobe of the liver, or the brain, was removed from perfused mice and stored in 1% sucrose in 0.05M sodium phosphate buffer (pH 7.4), before being processed for embedding in paraffin. Tissue was dehydrated and embedded in low temperature melting point (42-44°C) paraffin RAL wax (Stansen, Sydney, Australia), by 20 min immersions in the following solutions: once in 70% ethanol, twice in 90% ethanol, twice in absolute ethanol, twice in chloroform and twice under vacuum in RAL wax melted at approximately 50°C. After embedding in Ral wax, sections were cut and placed on multispot microscope slides (Hendley-Essex, Loughton, England). Liver sections (Chapter 3) were cut at 4µm thickness, brain sections from experiments described in Chapter 4.2.1 and 4.2.2 were 8µm thick and those from Chapter 4.2.3 were 6µm thick.

2.8 Immunohistochemistry.

Immunoperoxidase staining was carried out according to the method based on that of Hsu et al. (1981) and modified by Hume and Gordon (1983), using reagents (Vectastain Kit, PK4004) supplied by Vector Laboritories, Birmingame, CA, USA.

Sections were dewaxed with three 20sec immersions in xylenes, rehydrated with 10sec each in 2 x 90% ethanol, 2 x 70% ethanol, 1 x 50% ethanol and rinsed in PBS.

The staining procedure involved the following steps:

-blocking reagent: 30min incubation with diluted rabbit serum (Vectastain Kit) plus 1% goat anti-mouse IgG absorbed against rat IgG (Cappel, West Chester, PA, USA).

-blocking reagent flicked off.

-primary antibody: 2-3hr incubation with monoclonal antibodies described in section 2.4.

-PBS wash.

-endogenous peroxidase block: 30min immersion in 0.3% H_2O_2 (Pacific, Rozelle, Australia, 30% weight/weight) in methanol.

-PBS wash.

secondary antibody: 1-2hr incubation with biotinylated rabbit anti-rat IgG (Vectastain Kit).

-PBS wash.

-avidin-biotin complex: 1-2hr incubation with avidin-biotin-peroxidase complex (Vectastain Kit).

-PBS wash.

- peroxidase substrate: 1-15min incubation with excess diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride, grade II, Sigma, St Louis, MO, USA) in PBS plus 10mM imidizole (pH 7.4, Sigma, St Louis, MO, USA), plus 0.0225% H_2O_2.

-counterstain: 1min in Mayer's Haematoxylin (GURR BDH, Poole, England).

-mounting: slides were dehydrated through graded alcohols, cleared in xylenes and mounted under coverslips with Histoclad (Clay Adams, Parsippany, NJ, USA).

All incubations were carried out at room temperature in a humidified atmosphere. PBS washes involved two 5min incubations in saline. The edges of the slides and the areas between sections were wiped dry so that incubation solutions did not run between sections.

Antibody against mouse IgG was added to the blocking reagent to prevent any cross-reaction of the biotinylated secondary antibody-reagent with mouse IgG as well as possible non-specific cytoplasmic staining (Hume and Gordon, 1983). The optimal concentration of primary antibodies was determined by titration in this staining procedure. Imidizole was added to the peroxidase susbstrate to intensify the colour of the reaction product as suggested by Strauss (1982).

2.9 Classification of cells on immunohistochemical sections.

Kupffer cells lined the sinusoids of the liver and had a ruffled cytoplasmic membrane that stained for the F4/80 antigen.

Resident macrophages of the brain were F4/80$^+$ cells in uninfected brain that spread along the meningeal membranes or along the capillary walls of the choroid villi.

Monocytes were identified as mononuclear inflammatory cells with crescent-shaped nuclei.

Rounded macrophages in brain sections were inflammatory F4/80$^+$ cells that were not obviously attached to, or spread along, the meningeal membranes. They appeared loose and rounded within the meningeal spaces.

Stretched macrophages in brain sections were those F4/80$^+$ cells that were attached to, or spread along, the meningeal membranes of LCMV-infected mice. They resembled the resident macrophages seen in uninfected animals.

Lymphocytes were small inflammatory mononuclear cells that had dense, darkly-stained nuclei and a low cytoplasm to nucleus ratios. Some obvious lymphocytes in virus-infected tissue were also Ia$^+$ and were probably B cells. Larger F4/80$^-$ mononuclear cells with paler nuclei and a higher percentage of cytoplasm were probably lymphoblasts (blasts) and for the purpose of this thesis were classified as such.

2.10 Numerical analysis of immunohistochemical sections.

The % liver involvement in inflammation was determined by examination of tissue sections under a grid at 100x magnification. The tissue under 100 intersecting grid points per field was scored as positive or negative and 4 fields of view were examined for each section. This point-count method of planimetry provides an accurate determination of the volume fraction of a randomly distributed component within a specimen (Elias and Hyde, 1983).

The occurrence of F4/80$^+$ and Ia$^+$ cells, lymphocytes, and interactions between these cell types in the liver sinuses, was quantitated using a Zeiss projection microscope. Counts were made for 4 fields of view, which did not include large blood vessels or foci of inflammation. The images were projected on to a 605 cm^2 area, representing a final magnification of 850x.

For brain tissue, numerical analysis was performed on inflammatory cells in sections from those mice in which a sufficiently heavy infiltration allowed statistically significant examination. This occurred in only two groups of mice, after 7 days of infection (section 4.2.1) and 3 days after immune spleen cell transfer (section 4.2.2). Five hundred to eight hundred inflammatory cells from approximately 10 randomly chosen areas within the

meninges were counted. One tissue section from each animal was used for each of the antigens examined. Cells scored were monocytes, resident tissue macrophages, rounded macrophages, mononuclear cells that were unstained for antigen (lymphocytes and presumptive blasts), cells that were unstained for antigen and possessed an irregularly shaped nucleus, and pyknotic nuclei of dead cells. The numbers of pyknotic nuclei engulfed by stained cells were counted independently. Data were expressed as the percentage of the total number of cells scored.

2.11 Adoptive transfer model for the induction of LCM.

Donor mice were injected in the tail vein with 1,000 LD_{50} of WE3 LCMV. Immune spleen cells from these mice were used 8d later as a source of primary cytotoxic T lymphocytes (CTL). Single cell suspensions were prepared as described in section 2.12 and in some experiments were treated with antibodies and complement as described in section 2.13. Cells were finally washed 3 times in serum-free DMEM or RPMI and resuspended at the appropriate concentration to allow the transfer of the stated cell dose in 0.5ml of serum-free medium.

Recipients were injected i.c. with 1,000 LD_{50} of Arm LCMV. They were immunosuppressed 5 days later by a single i.p. injection of Cy (Endoxan, Asta-Werke A-G, Bielefeld, FRG) at a dose of 200mg/kg body weight. On the following day, spleen cells were transferred to recipient mice i.v.. This adoptive transfer model is outlined in Fig 2.1.

2.12 Preparation of single cell suspensions of spleen or lymph node.

Organs were removed asceptically from mice that had been killed by cervical dislocation. Single cell suspensions were prepared by mincing the tissue finely and pressing it gently through a fine stainless steel mesh sieve into DMEM or RPMI supplemented with 10% FCS. Large clumps of tissue were removed by rapidly passing the suspension through a cotton wool plug in the base of a glass funnel. After rinsing the cotton wool thoroughly with medium, the suspension was centrifuged at 400g for 7min at 4°C and resuspended in fresh medium. A viable cell count was performed on cells mixed with an equal volume of 0.1% trypan blue dye dissolved in PBS. Viable cells excluded dye.

2.13 Antibody and complement-dependent depletion of immune cells.

Single cell suspensions, at a concentration of 1-2 x 10^7 cells/ml of DMEM or RPMI supplemented with 10% FCS, were incubated with the appropriate concentration of cytotoxic antibody for 15-30min in a 37°C water bath. Low-toxicity rabbit complement was then added and the cells incubated for a further 45-60min. Cells were shaken each 10-15min during the incubation periods. After complement treatment, cells were centrifuged at 400g for 7min and resuspended in fresh medium.

Complement was prepared from rabbit serum which had low toxicity for mouse thymocytes. Serum was absorbed with agarose at 0°C (Type 1, Sigma, St Louis, MO, USA) according to the method of Cohen and Schlesinger (1970) and stored at -70°C. The optimum concentration for use was determined by titration with anti-Thy1 antibody on normal thymocytes.

2.14 Quantitation of meningitis.

Mice were anaesthetized with 0.3-0.5ml of the anaesthetic, avertin. Avertin was prepared by dissolving 1.0g 2,2,2-tribromoethanol (Fluka AG, Switzerland) in 1ml 2-methyl-2-butanol (Fluka AG, Swizerland). The solution was diluted to a final volume of 50ml with hot tap water (50°C) and shaken vigorously to mix. Mice were exanguinated by bleeding from the axilla and heart. Samples of CSF were then obtained from the cisterna magna by a method adapted from Carp et al. (1971) and described by Doherty (1973). Skin and muscle were reflected from the atlanto-occipital region to expose the dura mater covering the cisterna magna. A small hole was made in this membrane with a 26 gauge needle. Using a 20µl pipette (Microcaps, Drummond, Philadelphia, Pa, USA) with its holder attached to a 1ml syringe, slight negative pressure was applied to aspirate the CSF.

Using this method 10-15µl of clear fluid can be obtained from healthy mice. Brain swelling in mice with severe LCM, however, reduces the space within the cisterna magna and less CSF can be obtained.

CSF samples were diluted in 0.1% trypan blue dye and counted in a haemocytometer. Cell counts are expressed as mean ± standard error from the mean (SEM) $\log_{10}$ cells per µl of CSF.

2.15 Cytotoxicity assay.

The cytotoxic assay utilized MC57G fibroblasts (H-2^b) as target cells. Monolayers were grown on 75cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in RPMI

supplemented with 10% NBS and passaged with a split ratio of approximately 1/3, using 0.25% trypsin in PBS. Monolayers were washed 3 times with sterile PBS, 44-48 hrs before use, and were infected with WE3 LCMV at approximately five infectious units of virus per target cell. During the infection period, fibroblast growth was limited by using media containing only 2% NBS. On the day of use, infected and uninfected monolayers were treated with trypsin, pelleted and resuspended at a concentration of approximately 3 x 10^6/ml in medium containing 10% FCS. Cells were incubated with 100μCi/ml $Na^{51}CrO_4$ (Amersham Int. Ltd., Amersham, U.K.) for 1-2hr at 37°C. After washing with 3 changes of medium, target cells were resusupended at 10^6 viable cells per ml.

Effector cell populations to be tested for cytotoxic activity were prepared in DMEM or RPMI containing 10% FCS and a number of 2-fold dilutions in triplicate were prepared in round bottom 96-well microtitre trays (Linbro, Flow Laboratories, McClean, Virginia, USA) in a final volume of 0.1ml. Each well received 0.1ml ^{51}Cr- labelled target cell suspension. Spontaneous ^{51}Cr release was determined by mixing 0.1ml medium and 0.1ml target cell suspension. Total releasable chromium was determined by mixing 0.1ml target cell suspension with 0.1ml Triton X detergent. Duplicate assays were set up for infected and uninfected target cells.

Cell mixtures were incubated for 7-8hr at 37°C in a humidified atmosphere of 10% CO_2 in air. At the end of this period, 0.1ml supernatant was collected from each well without disturbing the cells. Supernatants were counted for 1min in a gamma scintillation counter.

Percentage lysis of target cells was calculated by the formula:

$$\frac{\text{\% release by immune cells - \% release in medium}}{\text{\% total release - \% release in medium}}$$

Percentage specific lysis was determined by subtracting the % lysis of uninfected targets from the % lysis of infected targets for each dilution of effector cells.

2.16 Statisitical analysis.

For the numerical evaluation of liver histological sections (chapter 3), one way analysis of variance was used to determine the significance of the changes occurring during infection, compared with the variance within groups of animals. For each quantitation, the f value obtained was <0.0001 (i.e. the probability that the variation that occured during infection was due to the variation between animals was <0.0001).

The significance in the difference between inflammatory cell counts from CSF of mice with adoptively transferred LCM was determined using the Wilcoxan rank analysis test.

2.17 Electronmicroscopy

Specimens were fixed in 3% gluteraldehyde and 0.1M cacodylate buffer (pH 7.4) for 3 hours, then post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) for one and half hours. Samples were then 'En-bloc' stained with 1% aqueous uranyl acetate for one and a half hours, dehydrated and embedded in 'Spurrs' resin, sections were cut on a LKB ultramicrotome III and stained in 'Reynolds' lead citrate. Micrographs were taken on a Philips 301 electronmicroscope.

2.18 Light Photomicrography.

Histological sections were photographed with a Leitz Vario-Orthomat camera attached to a Leitz Orthoplan microscope and using a Leitz CB16.5 blue filter.

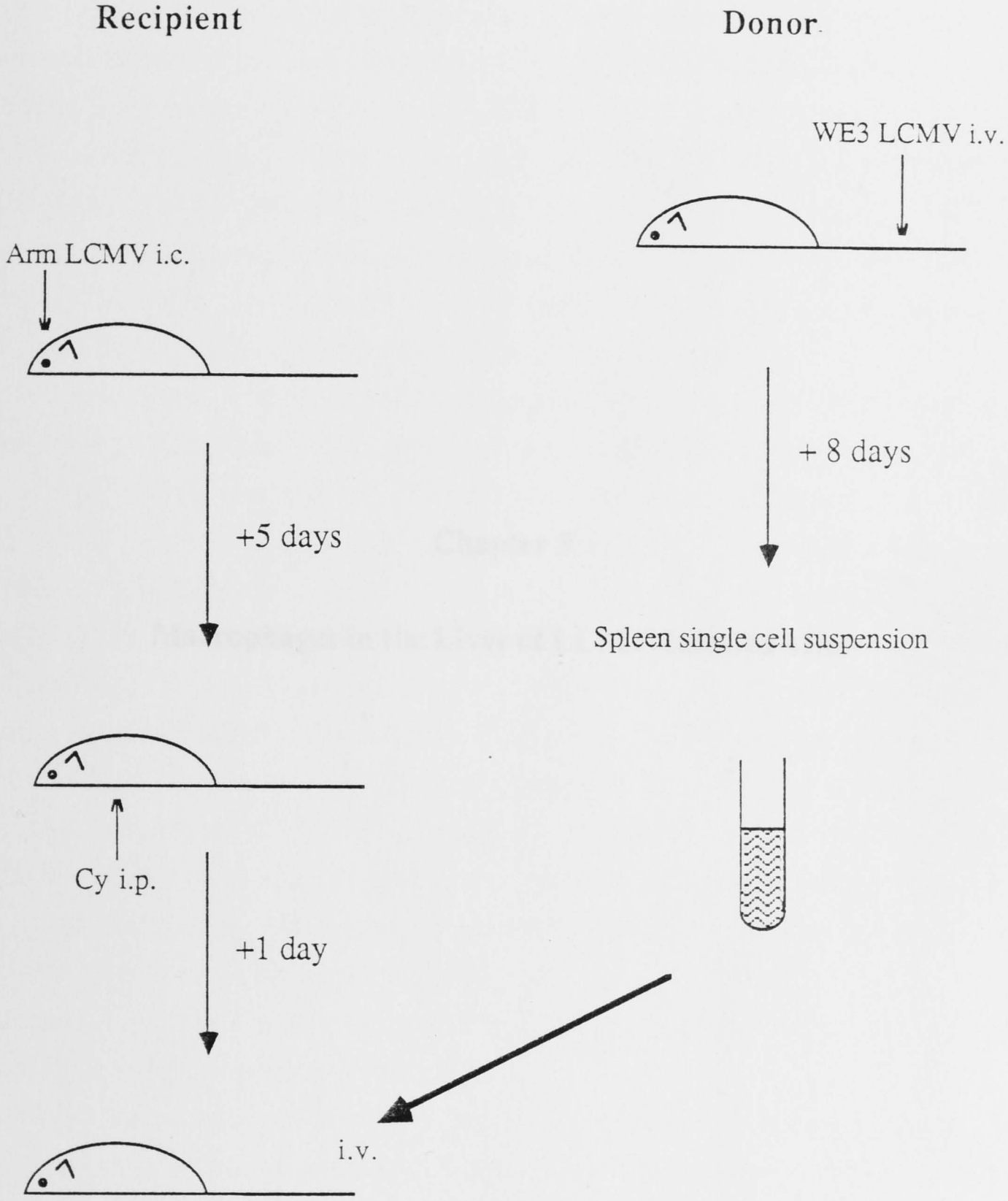


Figure 2.1: Diagramatic representation of the adoptive transfer model used to induce acute cerebral LCM

Chapter 3

Macrophages in the Liver of LCMV-Infected Mice

3.1 INTRODUCTION

Experimental infection with the viscerotropic (WE3) strain of LCMV produces a generalised disease in adult mice which is non-fatal for most mouse strains (Tosilini and Mims, 1971). After i.v. injection the virus causes marked infection and pathological changes in the liver and spleen, as detected by viral titrations, and both immunofluorescent staining for viral antigens and routine histological studies (Tosilini, 1970; Tosilini and Mims, 1971; Lehmann-Grube et al., 1985). Virus titres peak in the blood d4-9 after inoculation, fall dramatically on d9-12 and are virtually absent by d16 (Marker and Volkert, 1973). The cellular immune response to the virus, as measured by both DTH footpad swelling (Tosilini and Mims, 1971) and in vitro cytotoxicity of spleen and lymph node cells for infected targets (Marker and Volkert, 1973), is maximal 8-10d after inoculation.

The immunological response and histological consequences of LCMV infection are due to the activation of class I MHC-restricted T lymphocytes (Doherty and Zinkernagel, 1974; Doherty et al., 1976b; Zinkernagel and Welsh, 1976; Byrne and Oldstone, 1984; Allan and Doherty, 1985a; chapters 5). Although the effector function of this subset of T cells was traditionally thought to be restricted to cytotoxicity of infected targets (Hollander, 1982), there is increasing evidence that $Lyt2^+$ effectors can induce severe inflammatory process in murine LCM (Zinkernagel, 1976b; Allan and Doherty, 1985a, chapter 6). It is not clear whether T cells acting alone resolve viral infection (Blanden, 1974): mononuclear phagocytes may also play a role through the clearance of virus as suggested by Thomsen and Volkert (1983).

To gain an understanding of the role that macrophages play in the immune response to LCMV, their distribution in infected liver was determined using immunohistochemical analysis. Activated macrophages were recognised by their expression of the class II MHC antigens, as well as the macrophage marker, F4/80.

Reference will be made to various anatomical features of the liver during this chapter. The following is a brief account of these areas.

Hepatocytes are arranged in interconnecting plates that are distributed radially with respect to terminal branches of the hepatic veins. These branches have been designated central veins because of their location at the centres of hexagonally shaped units of the liver parenchyma that constitute the liver lobules. There is no layer of connective tissue between these lobules in the mouse (as there is in the adult pig), the boundaries being defined by the regularly distributed portal tracts. The portal tracts consist of branches of the portal vein, the hepatic artery and a bile ductule, enclosed in a common investment of connective tissue. Bile ducts can be distinguished histologically by their cuboidal epithelium. The cross sectional appearance of these vessels is often referred to as the portal triad.

The plates of liver cells are exposed on either side to the blood that flows in interconnecting parallel channels or sinusoids. The sinusoids are lined with both endothelial cells and resident macrophages, the Kupffer cells. Blood enters the hepatic sinusoids from

small branches of the hepatic artery and portal vein, flows through the lobule centripetally, and leaves via the central vein (Fig 3.1).

A more functional unit of the liver than that defined by the lobule is the acinus. Rappaport (1958) defined the simple liver acinus as a small parenchymal mass arranged around the axis of the portal tract, and lying between two or more central veins. The acinus is divided into three zones, the former being nearest the portal tract and the incoming blood supply, the last located close to the central veins (Fig 3.2).

Both the parenchymal and vascular distribution of macrophages during LCMV-infection of the liver has been investigated.

3.2 RESULTS.

Eight to twelve week old C57BL/6J mice were inoculated i.v. with 300 i.c. mouse LD_{50} of WE3 LCMV. Groups of 2-4 animals were sampled from d1 to d8 and on d10, 12, 14, 17, and 28 post-infection (p.i.). Four uninfected control mice were given gelatine saline dilutent only. The right caudate lobe of the liver was removed and processed as described in section 2.7. Serial sagittal sections were prepared for immunohistochemical analysis. The staining procedure is described in section 2.8 and the primary antibodies used were F4/80, anti-Ia or, as a control, normal horse serum. No immunohistochemical staining was observed in sections with control serum.

The cellular infiltrate in the livers of mice infected with LCMV was essentially mononuclear in nature. Large numbers of infiltrating monocytes and lymphocytes were first detected at 5 or 6 days after infection, reaching a peak from d7 to d10 and resolving by d17. Foci of inflammatory cells developed both perivascularly and in the liver parenchyma. The percentage of the liver involved in these foci was determined by planimetric analysis, (Section 2.10). Much inflammatory activity was also observed outside these foci, throughout the liver lobule. This activity was quantitated by counting particular cell types in areas of the liver which did not contain focal lesions (Section 2.10).

The following is a detailed analysis of the kinetic and qualitative aspects of the inflammatory process at various anatomical sites.

3.2.1 Inflammatory Cells within The Liver Vasculature.

In uninfected liver, the expression of F4/80 antigen was restricted to the resident sinusoidal macrophage population, the Kupffer cells, 7% of which express Ia antigens. Vascular endothelial cells which also line the sinuses were $F4/80^-$, Ia^-.

During LCMV infection there was an increase in the number of $F4/80^+$, Ia^+ cells lining the sinuses. Fig 3.3 depicts quantitatively the change in the number of macrophages lining the sinuses during the course of infection. There was an initial increase in Ia^-, $F4/80^+$ macrophages (d1-5) and maximum numbers of sinusoidal macrophages were seen at d8-14. A proportion of the $F4/80^+$ sinusoidal macrophages remained Ia^-, as indicated by the differences in frequency between intra-sinusoidal $F4/80^+$ and Ia^+ cells, throughout the course of the disease process (Fig 3.3). At the time of maximum sinusoidal macrophage numbers, d8-14, approximately 50-75% were Ia^+. The relative differences in the intensity of staining for F4/80 and Ia are not simply the result of a difference in the sensitivity of the antibodies, since staining for both antigens was strong in normal spleen tissue. In general, $F4/80^+$ and Ia^+ cells in the sinusoids showed the morphology of Kupffer cells.

The kinetics of monocyte localization and attachment to the central and hepatic veins

was similar to that of Ia^+ cells in the sinuses. Monocytes were identified as $F4/80^+$, Ia^+ cells with cresent-shaped nuclei (Fig 3.4). They were observed attached to blood vessel walls from d5-17, with large numbers seen d7-12. Histological sections were scored from 0-4 for increasing numbers of monocytes and the results are summarised in Fig 3.5. When associated with larger vessels, most monocytes were seen in central/hepatic veins (Fig 3.6), with a few attached to portal veins on d8 only. Monocytes were also observed in the sinuses of the liver with varying degrees of plasma membrane extensions along the sinusoidal walls. Thus, they appeared to take on the morphology and location of Kupffer cells. Here, monocytes have been defined as those cells with cresent-shaped nuclei and $F4/80^+$ membranes. However, there was also a discreet population of cells with similar morphology but which were $F4/80^-$. These cells appeared earlier (d4 p.i.) in central veins and sinuses and continued as a minority of the monocyte population (<5%) till d14 p.i. (Fig 3.4).

Attachment of a smaller number of lymphocytes to blood vessel walls was also observed from d5-12 but, in contrast to monocytes, they were often seen in portal veins. Cells with typical lympohocyte morphology were counted in sections stained for F4/80 or Ia (Fig 3.7). They included both small, densely nucleated lymphocytes and larger lymphoblasts with paler nuclei. These cells were $F4/80^-$ but 8-30% were found to be Ia^+. In both cases significant numbers of lymphocytes were present in sinuses from d6, peaked on d7 and then declined steadily until d17 (Fig 3.7). This preceded the localisation of maximal numbers of intravascular Ia^+ macrophages by one to two days (compare Fig 3.3 and Fig 3.7). Small lymphocytes were more predominant early in infection (d4-6) with blasts becoming more dominant later (d7-14).

Many of the lymphocytes were intimately associated with $F4/80^+$ macrophages in the sinuses (Fig 3.8). Again, the numbers of such interactions were assessed quantitatively in sections stained with either the F4/80 or the anti-Ia reagent (Fig 3.9). Close association between macrophages and lymphocytes was seen early in infection (d1-4) at which time the macrophages involved were Ia^-. A sharp increase in the number of lymphocytes and macrophages in contact with each other was observed to peak at d8. There was a corresponding increase in the number of these macrophages which were Ia^+. However, peak contact between lymphocytes and Ia^+ macrophages occurred 2 days later than that observed with the total macrophage population. A comparison of the data in Fig 3.7 and Fig 3.9 indicates that as many as one in four of these intravascular lymphocytes were associated with $F4/80^+$ cells on d8, or with Ia^+ cells on d10.

Some cells in the walls of blood vessel expressed Ia as early as d5, at a time when they were $F4/80^-$ (Fig 3.10). By d14 most vessel walls were strongly Ia^+ but were also positive for F4/80. Ia expression, however, was much stronger with a band of cells under the vessel walls expressing the Class II MHC antigens (Fig 3.11). Close examination of Ia^+ vessels revealed that the majority of the class II MHC antigen was expressed on perivascular capillaries early in infection (for example on d5). Such capillaries were probably part of the

peribilary capillary plexus formed by branches of the hepatic artery (Hansen, 1978). Later in infection, the band of Ia staining around vessels was apparently due to staining of infiltrating cells (Fig 3.12).

3.2.2 Focal Inflammatory Lesions.

Inflammatory lesions developed within the liver as the disease progressed and Fig 3.12 depicts, quantitatively, the extent of the liver involved. A few small lymphocyte aggregates were seen in uninfected animals but, with LCMV infection, the extent of inflammation rose dramatically. The liver was highly inflamed from d6-14 with a peak at d8, and returning to normal on d17-28. Inflammatory lesions were seen perivascularly, especially around the portal triad (Fig 3.6), zone 1 of Rappaport's liver acinus as well as in zone 2 of the liver parenchyma. Foci were rarely seen in zone 3 close to the central veins.

Initially, on d1-2, the few foci present generally contained polymorphonuclear leukocytes (PMN) associated with F4/80$^+$ macrophages many of which were also Ia$^+$, especially those in perivascular foci (Fig 3.13). PMNs were seen in the liver until d4 p.i. From this stage onward lesions generally consisted of large pale cells (presumptive lymphoblasts) arranged in sheets to form perivascular cuffs, or in fairly tight spheres within the liver lobule (Fig 3.14). Until d10 these foci also contained smaller densely-nucleated lymphocytes.

Initially, the perivascular foci were predominantly lymphocytic, being comprised of mononuclear cells which were essentially F4/80$^-$, Ia$^-$, while the F4/80$^+$, Ia$^+$, monocytes were associated mainly with the sinusoids and the central hepatic veins (Fig 3.6). By d7, some evidence of cell death, presumably of lymphocytes, was seen in these perivascular foci, though most sites were still devoid of F4/80$^+$ cells. Numerous pyknotic nuclei were evident in the foci from infected livers taken on d10 or d12 when macrophage infiltration from the edge of the lesion was observed (Fig 3.15). Within a further 2 days (d14), the parenchymal (Fig 3.16a) and perivascular (Fig 3.16b) foci became heavily infiltrated with F4/80$^+$, Ia$^+$ cells, and many of the lymphocytes now appeared to be Ia$^+$ (Fig 3.16c). Thus, the intimate association of lymphocytes and macrophages in these perivascular inflammatory sites was a late process, which seems to have been involved with resolution of the lesion.

3.3 DISCUSSION.

From the above results, the inflammatory response to LCMV in the liver can be divided into 3 distinct stages. At the earliest stage, d1-4, when virus would be present in the liver (Tosilini, 1970), there is a minor inflammatory response involving PMN (Fig 3.13) and an increase in the number of Ia^- sinusoidal macrophages (Fig 3.3). In the second stage of the inflammatory response, d5-8, there is extensive infiltration of the liver with lymphocytes and Ia^+, $F4/80^+$ monocytes. The former cells are destined particularly for inflammatory foci, that develop primarily around the portal triad but also in Rappaport's zone 2 of the liver acinus. The monocytes take up the normal sinusoidal location of Kupffer cells. The final stage of the disease process on d10-17 involves inflammatory cell death within the foci, and infiltration by activated macrophages resulting in the resolution of the inflammatory lesions. There is an accompanying decline in the sinusoidal inflammation.

The following discussion will consider the localisation and role of both macrophages and lymphoid cells during LCMV infection of the liver, as well as the early PMN and Kupffer cell response seen in the first 4 days after virus inoculation.

Localisation of Macrophages in LCMV-Infected Liver.

Monocytes and macrophages localised to the sinusoids, and the central and hepatic veins of LCMV-infected livers. They infiltrated perivascular and lobular foci of inflammation after there was evidence of cell death in these lesions.

The initial localisation of $F4/80^+$ cells to efferent blood vessels may result from the accumulation, at these sites, of factors which can induce macrophage migration. Pilaro et al., (1985) have also observed a pericentral accumulation of macrophages in the livers of rats treated with the analgesic, acetaminophen (AA). The medium from isolated hepatocytes treated with AA was chemoattractive for isolated Kupffer cells. Thus, it is likely that the pericentral localisation of phagocytes observed by those authors results from the drainage of chemotactic factors from AA-damaged hepatocytes into the central veins. Likewise, macrophage migration factors which are released from stimulated, LCMV-reactive T cells in the sinusoids and inflammatory foci may accumulate in the central and hepatic veins. Cell debris, which is also seen in the lumina of veins in LCMV-infected liver (Tosilini, 1970), would provide added attractant for macrophages. Localisation of phagocytes to the draining regions of the liver provides an ideal mechanism for the clearance of cell debris and viral products from the inflamed organ.

Infiltration of inflammatory foci by activated macrophages, occurring after the appearance of pyknotic nuclei, was probably a response to cell death in these lesions. A similar response to cell death was observed in periportal inflammatory foci of mice and rats during GVHD (Takacs et al., 1985).

Ia Expression on Macrophages in LCMV-Infected Livers.

In the results reported here, 7% of Kupffer cells in uninfected controls were found to express class II MHC antigens. This value is not consistent with the findings of some other workers. Richman et al., (1979) found that 50-80% of Kupffer cells isolated from mouse liver were Ia^+. Also, staining of frozen liver sections from mice that had been injected i.v. with colloidal carbon, revealed that all phagocytic sinusoidal cells were $F4/80^+$, Ia^+ and Fc $receptor^+$ (Lepay et al., 1985b). In contrast, Momburg et al., (1986) found no expression of class II MHC antigens on Kupffer cells in frozen sections of mouse liver. Intravenous interferon-γ treatment, however, induced Ia expression on these cells. The authors also found that, although Kupffer cells were Ia^-, they did express the invariant chain that is noncovalently associated with the α- and β- chains of the class II MHC antigens. Antisera raised against Ia antigens would be likely to recognise the invariant chain on Kupffer cells. Thus the finding of Ia expression by resident macrophages of the liver is equivocal and would depend on the specificity of the used. The 7% positive value found in this study may represent the true level of class II MHC expression in the liver or, alternatively, the procedure used may not have been sensitive enough to detect cells bearing low levels of Ia antigens.

$F4/80^+$ monocytes entering the liver during LCMV infection expressed class II MHC molecules. These cells appeared to adopt the typical Kupffer cell position in the liver and between 50-70% of sinusoidal $F4/80^+$ cells from d8-14 of infection were also Ia^+ (Fig 3.3). The sinusoidal macrophages that do not express class II MHC antigens may be resident Kupffer cells which are refractory to activation, or may represent inflammatory macrophages on which Ia antigen expression is transient (Beller et al., 1980; Scher et al., 1980; Steeg et al.,1980; Steinman et al., 1980; Ezekowitz et al.,1982).

Macrophage-activating lymphokines released from stimulated LCMV-reactive T cells may act to induce class II MHC molecules on macrophages or may maintain expression on cells which are already Ia^+. Precedents for each of these possibilities can be found in the literature. Momburg et al., (1986) and Lepay et al., (1985a) have shown that i.v. administration of recombinant interferon-γ is capable of inducing Ia expression on Kupffer cells of otherwise untreated mice.

Ia expression on cells in inflammatory foci generally parallelled that of F4/80, indicating that infiltrating macrophages are activated. However, on d14, when foci are heavily infiltrated, cells other than macrophages had acquired Ia antigens (Fig 3.16). This may reflect the capacity of T cells to absorb class II MHC antigens (Lorber et al., 1982).

Role of Macrophages in LCMV-Infected Liver.

The first histological evidence of macrophage function in LCMV-infected liver was increased adherence between Kupffer cells and lymphocytes. An initial increase, on d1-4, involved $F4/80^+$, Ia^- sinusoidal macrophages while a later, more pronounced increase involved both Ia^+ and Ia^- macrophages (Fig 3.9). Because of the time necessary to activate T

cells, it is unlikely that macrophage activity occurring on d1-4 is due to lymphocyte activity, and the increased adherence to Kupffer cells seen during this period may be a function of the macrophages. McCuskey et al., (1982), using in situ high resolution light microscopy, have shown increased adherence of lymphocytes to Kupffer cells within 15min of endotoxin treatment. Early uptake of virus inoculum and infection of Kupffer cells with LCMV (Tosilini, 1970) may also stimulate increased contact between these cells and lymphocytes.

Once lymphocyte activation has occurred, infected sinusoidal macrophages may act as presenting cells to stimulate T cell effector function. Isolated mouse Kupffer cells are capable of presenting antigen to both myoglobin- and mitogen-reactive T cells (Richman et al., 1979). Rogoff and Lipski (1980) have shown that guinea pig Kupffer cells take up and present antigen to primed T cells and support mitogen-induced T cell proliferative responses. However, since reactive T cells in LCMV-infected mice are likely to be class I MHC-restricted, any infected cells expressing these MHC molecules and viral antigen would be able to act as antigen presenting cells. Thus, adherence of lymphocytes to Kupffer cells need not reflect the ability of the phagocytic cells to present particulate antigen, but is probably a consequence of the high rate of infectivity of Kupffer cells compared with, for example, endothelial cells (Tosilini, 1970).

It is possible that the major effector functions performed by macrophages are carried out by cells which localise to the liver during the response to LCMV infection; the resident Kupffer cells being terminally differentiated, may be refractory to activation and unable to mount a response specific for viral infection. The activation to express respiratory burst activity by liver macrophages is apparently restricted to newly recruited macrophages. Matsuo et al., (1985) found increased release of reactive oxygen intermediates (ROI) by Kupffer cells isolated fom BCG-treated rats and attributed this activity to the increased yield of Kupffer cells. Lepay et al., (1985a) found that Kupffer cells isolated from normal mice were deficient in ROI releasing ability even after particulate stimulation with zymosan or with phorbol ester treatment. They found that this deficiency correlated with impaired toxicity for toxoplasma and leishmania. In a later study, Lepay et al., (1985b) showed that cell-mediated immunity in murine listeriosis was correlated with an influx into the liver of macrophages capable of generating ROI. One might conclude that Kupffer cells represent an end stage in the differentiation of macrophages and are refractory to activation signals. Such a possibility is supported by the analysis of LCMV-infected liver. An incomplete overlap in the Ia and F4/80 antigen staining profiles of sinusoidal macrophages, as well as the observation that inflammatory monocytes are also Ia^+, suggests that it is the resident Kupffer cell population which remains Ia^-.

However, there is also evidence in the literature indicating that Kupffer cells are able to react to inflammatory stimuli. Lepay et al., (1985a), themselves, showed increased Ia expression on isolated Kupffer cells that were treated with interferon-γ. Such enhanced expression of the class II MHC antigens is associated with macrophage activation (Beller et

al., 1980; Scher et al.,1980; Steeg et al.,1980; Ezekowitz et al.,1982). Pilaro et al., (1985) found that isolated Kupffer cells were stimulated to increase their phagocytic activity and ROI release following treatment with hepatocyte degradation products of analgesics. Isolated Kupffer cells also respond to MAF with increased tumoricidity (Stukart et al., 1985). More significantly (as far as the anti-viral response is concerned), isolated Kupffer cells have an increased capacity to inhibit virus replication in infected cells, following treatment with LPS (Keller et al., 1985). Thus, it would appear that Kupffer cells are not refractory to stimulation by inflammatory process. The incomplete overlap of F4/80 and Ia antigen profiles of sinusoidal macrophages, in LCMV-infected liver, must therefore be attributed to the transient nature of Ia expression.

Because infiltration of inflammatory foci by activated macrophages appears to occur after there are signs of cell death in these foci, it is probable that these phagocytes are important in the clearance of cell debris. Localisation of monocytes and macrophages to the draining regions of the liver is also suggestive of a phagocytic function for $F4/80^+$ cells during LCMV infection. Such localisation would optimise the clearance of viral and dead cell products from the blood before it passes out of the liver.

Apart from their phagocytic and antigen-presenting functions, activated macrophages are probably more directly involved in virus elimination. The restriction of viral replication in macrophages that are activated has been implicated for several different classes of viruses, including vesicular stomatitis (Rager-Zisman et al., 1982; Belardelli et al., 1984), influenza (Rogers and Mims, 1982; Mak and Ada, 1984) and ectromelia (Cohen and Bubel, 1983; Cohen et al.,1984) viruses. Activated macrophages have been shown to lyse cells infected with herpes simplex (Koff et al., 1983), influenza (Mak and Ada, 1984) and vaccinia (Chapes and Tompkins, 1979) viruses. More importantly, activated macrophages have an extrinsic anti-viral activity that inhibits viral growth in target cells with which they are co-cultivated (Hayashi et al., 1980; Morse and Morahan, 1981; Stohlman et al., 1982; Wildy et al., 1982; Keller et al., 1985). Wildy et al., (1982) found that arginase activity released by activated macrophages can restrict several arginine-requiring viruses. In the case of other viruses such as herpes simplex, it is likely that activated macrophages release a factor which alters host cell metabolism (Morse and Morahan, 1981). Hayashi et al., (1980) also found that the anti-viral activity of PEC did not require phagocytosis but was, rather, attributable to the cytostatic effects of macrophages on target cells. That such extrinsic anti-viral activity can also occur in the liver has been demonstrated by Keller et al., (1985), who showed that LPS treatment of isolated Kupffer cells conferred on these cells the ability to restrict vaccinia replication in co-cultivated target cells.

Localisation of Lymphocytes and Blasts.

Lymphocytes and blasts accumulated in the liver sinusoids and aggregated into inflammatory foci around the portal tract and, less frequently, in zone 2 of the liver acinus.

Small lymphocytes predominated early (d4-6), giving way to blasts later in the disease. In previous studies of LCMV-infected liver, which did not extend past 7 days p.i., lymphocytic infiltration began on d4 (Tosilini, 1970) and d5 (Wilsnack and Rowe, 1964). Tosilini and Mims (1971) also observed marked infiltration of the portal tracts and sinusoids of infected liver.

This accumulation did not correspond in location to previous descriptions of viral antigen distribution in infected liver. Following i.p. injection of a viscerotropic strain of LCMV, Wilsnack and Rowe (1964) first detected viral antigen 3d post inoculation and the liver was still heavily infected on d7. Antigen in the liver was largely confined to the parenchymal cells and Kupffer cells were rarely infected. Involved hepatocytes were usually found in foci of 10 to 20 cells, and infrequently as single infected cells. The distribution of infected cells was not restricted to either periportal or lobular regions of the liver (Wilsnack and Rowe, 1964).

Tosilini (1970) gave a more detailed analysis of the distribution of LCMV antigen in infected liver. He used the same virus strain and route of inoculation as used in this chapter, but the amount of virus injected was much higher ($10^7 LD_{50}$ compared with $300 LD_{50}$). Five minutes after inoculation, viral antigen was detected in the Kupffer cells. It had disappeared within an hour, reappearing after 11hr, presumably following an infectious cycle in the Kupffer cells. By d4-6, 50-60% of these cells were infected. Increasing numbers of hepatocytes were also infected from d2-6. Infected endothelial cells were present in some hepatic and portal veins by 18hrs p.i. In contrast to the findings reported in this chapter, Tosilini found necrotic foci of infected hepatocytes. Only rarely were pyknotic nuclei of parenchymal cells seen in the experiment reported in this chapter. This discrepancy may be due to the different doses of LCMV inoculated or, more likely, to the use of different mouse strains. Tosilini (1970) used WEHI mice which he later found to be particularly susceptible to LCMV infection compared with C57Bl/6 mice (Tosilini and Mims, 1971). Necrotic lesions tended to be mid-zonal in distribution and hepatic cells around portal tracts and central veins were least affected (Tosilini, 1970).

Although the two studies showed differences in the distribution of viral antigen with respect to Kupffer cells, they found no indication of a predominance of virus in periportal cells and, in fact, there appeared to be a preference for lobular cells. Periportal accumulation of inflammatory cells has also been reported in GVHD in rats and mice (Takacs et al., 1985), and in hepatitis A infection of the owl monkey (Keenan et al., 1984). There is also no correlation between lymphocyte accumulation and antigen distribution in these cases. In GVHD, where there is selective destruction of bile duct epithelium, the antigen responsible for stimulating the inflammatory response is distributed throughout the liver. The majority of infiltrating cells are $Lyt2^+$ and would react with allo-class I MHC antigens which are most strongly expressed on sinusoidal endothelial cells (Lautenschlager et al., 1984). Likewise, hepatitis A virus which, like LCMV is noncytopathic, is found in Kupffer cells throughout the liver (Huang et al.,

1979; Shimizu et al., 1982). The possibility exists that the level of expression of H-2 on lobular cells is down-regulated during viral infection, so that, although viral antigen is present, the T cell restricting element may be absent. Such regulation, however would not explain the localisation of lymphocytes during GVHD. Thus, periportal accumulation of lymphocytes is not a function of the distribution of reactive antigen. It appears that the architecture of the liver, itself, influences the distribution of inflammatory cells.

Role of Lymphocytes in LCMV-Infected Liver.

It has not been possible to study the nature of the inflammatory lymphocytes using cell surface markers. Of the lymphocytes in the sinusoids 8-30% are Ia^+ and are probably B cells. However, absence of Ia staining in the discrete inflammatory foci suggests that few B lymphocytes are present in these lesions. Therefore, there must be some selection of lymphocytes that move from the sinusoids to the foci. It is likely that the majority of these cells are $Lyt2^+$, class I MHC-restricted T cells. The correlation of findings from functional analysis in vitro and the adoptive transfer of immune spleen cells and cloned cytotoxic T lymphocyte lines has indicated that such cells mediate the elimination of LCMV (Doherty et al., 1976b; Zinkernagel and Welsh, 1976; Byrne and Oldstone, 1984; Allan and Doherty, 1985a). There is also specific recruitment of class I MHC-restricted T cells to the liver during infection with viscerotropic LCMV (Zinkernagel et al., 1986). In addition, recruitment of $Lyt2^+$ T cells in the mouse and $OX8^+$ T cells in the rat to periportal inflammatory foci has been demonstrated for GVHD (Takacs et al., 1985).

From the results reported in this chapter, there is no evidence that it is the cytotoxic function of $Lyt2^+$ T cells which is responsible for virus elimination from the liver. Pyknotic nuclei, although numerous among the lymphoid cells of inflammatory foci, are rare in the parenchyma. T cell-mediated virus clearance therefore probably depends on the release of lymphokines from activated lymphocytes. Such lymphokines as γ-interferon may act directly on infected cells to inhibit viral replication. More importantly, lymphokines which effect macrophage proliferation, migration and activation would contribute to the anti-viral nature of the inflammatory response. This postulate is supported by the observation that a sharp peak in the number of lymphocytes infiltrating the liver precedes a more prolonged influx of $F4/80^+$, Ia^+ cells (compare Fig 3.3 and Fig 3.7).

Thus, it is likely that T cells and macrophages co-operate in the resolution of LCMV infection in the liver.

Initial PMN and Kupffer Cell Response.

The initial PMN inflammatory response and increase in Ia^- Kupffer cells occurred before there was time for lymphocyte activation and presumably was related more directly to the virus inoculated. The PMNs observed did not have eosinophilic cytoplasm when stained with haematoxylin and eosin, and were presumably neutrophils and not eosinophils.

Activation of the neutrophil response possibly occurs in response to connective tissue injury (Ogawa et al., 1985). Infection with the non-lytic LCMV is unlikely to generate products of tissue damage but the virus inoculum itself, homogenised guinea pig lung, would contain such products. This possibility could be tested by inoculating a homogenate of normal guinea pig lung. Within 5 mins of i.v. LCMV inoculation, 90% of the virus had been cleared from the blood with accompanying localisation of viral antigen to the Kupffer cells (Tosilini, 1970). Thus, the liver is likely to contain a some of the inoculum tissue debris which would provide a chemoattractive source for polymorphs. It should be stressed that the PMN inflammatory response is minor especially when compared with the later massive influx of lymphocytes and macrophages. A minor invasion of PMN was also observed in LCMV infection of the CNS (Chapter 4) but occurred at the final stages of the disease when there was abundant inflammatory cell death.

There was also an increase in the number of Kupffer cells during the first 5 days of infection, distinct from that seen on d8-14, in that it was not accompanied by a similar increase in Ia^+ sinusoidal macrophages (Fig 3.3). These separate increases in the number of Kupffer cells may represent the 2 sides of the controversy over the origin of Kupffer cells that is epitomised by the papers of van Furth (1980) and Wisse (1980). The former author expounded the view that Kupffer cells are derived from blood monocytes quoting evidence provided by experimental work on chimeras, ^{3}H-thymidine-labelled cells and cytochemical analysis; while the latter believed they were generated by mitosis of Kupffer cells.

Wisse has more recently modified his stand and conceded that monocyte-derived phagocytes are found in the liver, at least in response to zymosan injection, although these macrophages maintain ultrastructural and cytochemical characteristics that are distinct from those of the resident Kupffer cells (Bouwens and Wisse, 1985). Also, irradiation of either the bone marrow or the liver has demonstrated that 38% of the increased number of Kupffer cells occurring, in response to zymosan, is derived from the former site and 61% from the latter (Bouwens et al., 1986). Thus it would seem that sinusoidal macrophages can arise from either local proliferation of Kupffer cells or recruitment from the blood monocyte pool.

In the experiment described in this chapter, it is possible that the increased numbers of sinusoidal macrophages seen on d1-5 resulted from the division of local Kupffer cells. This division may be stimulated by the presence of effete PMNs, such an effect of dead neutrophils having been demonstrated in vitro on resident peritoneal macrophages of mice (Yui and Yamazaki, 1986). The rise in the number of sinusoidal macrophages seen on d8-14, however, was probably derived from the blood, since influx of monocytes into the liver preceded this increase and because there was a concomittant rise in the number of Ia^+ macrophages (the class II MHC antigens also being expressed on monocytes).

Bouwens and Wisse (1985) suggest that newly arrived sinusoidal macrophages, derived from blood monocytes, remain distinct from the resident Kupffer cells. They found that, following i.v. injection of zymosan into rats, monocytes adhered to sinusoids and central

veins but not to the larger draining hepatic veins. The authors suggested that monocytes developed into macrophages which moved slowly through the liver and left via the central veins. They were no longer adherent to the vessel walls by the time blood had drained into the hepatic veins. In support of this view they quoted unpublished evidence of McCuskey who used in vivo microscopy of living rat liver to show small mononuclear phagocytes slowly migrating through the sinusoids before disappearing in the central veins. In LCMV-infected liver, however, increased attachment of Ia^+ monocytes to the liver vasculature precedes the rise in Ia^+ sinusoidal macrophages (Fig 3.3). Thus, observed monocytes are likely to be entering rather than leaving the liver. Also, unlike the observations of Bouwens and Wisse (1985), monocyte attachment to hepatic veins was common (Fig 3.6). This would not be expected of cells leaving the liver, which would presumably detach from the vasculature walls before the blood had drained from the central veins into the hepatic veins.

Thus, the initial response to LCMV-infection of the liver involves a minor invasion by PMN, death of which may stimulate the division of Kupffer cells. Increased numbers of sinusoidal macrophages that occur later in infection probably derive from invading monocytes.

In summary, the histological evidence suggests that both lymphocytes and macrophages play a significant role in the pathogenesis of LCMV infection of the liver. These 2 cell types were observed together in the sinusoids during virus infection, where they appeared to be closely associated. However, the acccumulation of lymphocytes into periportal inflammatory foci, and the localisation of macrophages and monocytes to the central and hepatic veins, suggest that these cell types play distinct functional roles that necessitate different anatomical localisation. It is also apparent that the effector function of macrophages during LCMV infection is associated with their activation and expression of class II MHC antigens.

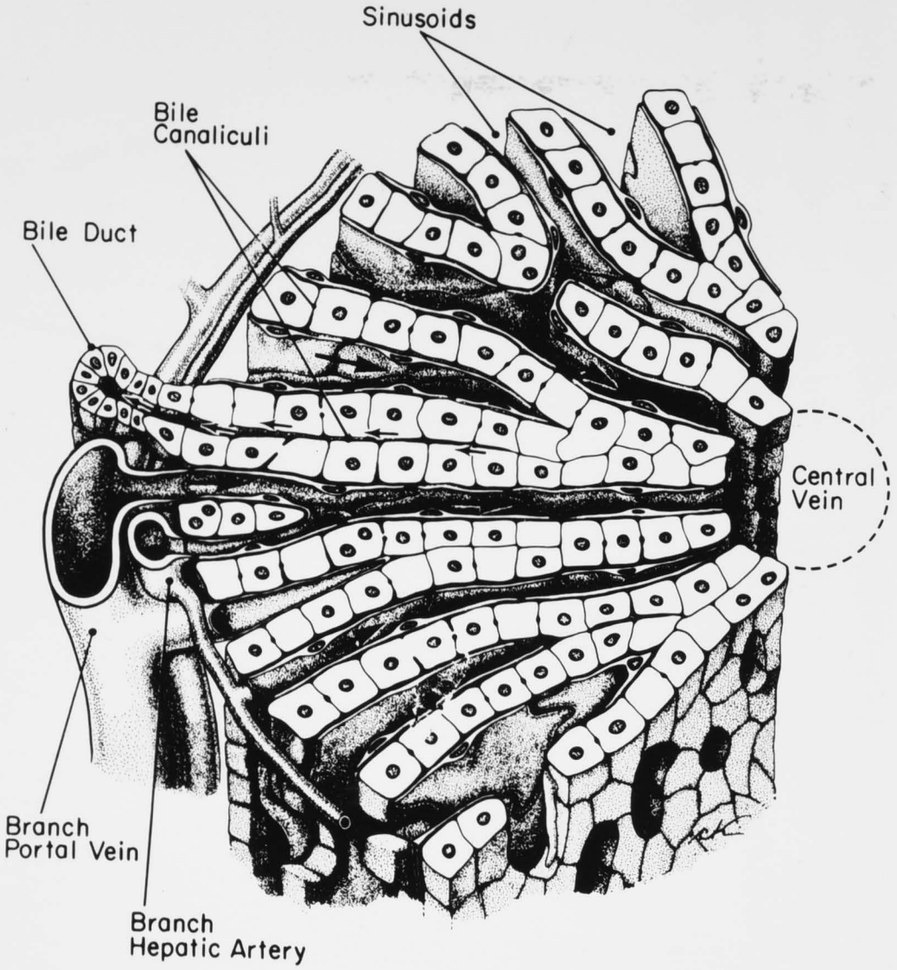


Figure 3.1. Diagrammatic representation of the radial disposition of the liver cell plates and sinusoids around the terminal hepatic venule or central vein, showing the centripetal flow of blood (large arrows) from branches of the hepatic artery and portal vein, and the centrifugal flow of bile (small arrows) to the small bile duct in the portal space. (Copied from Bloom and Fawcett (1975), p694).

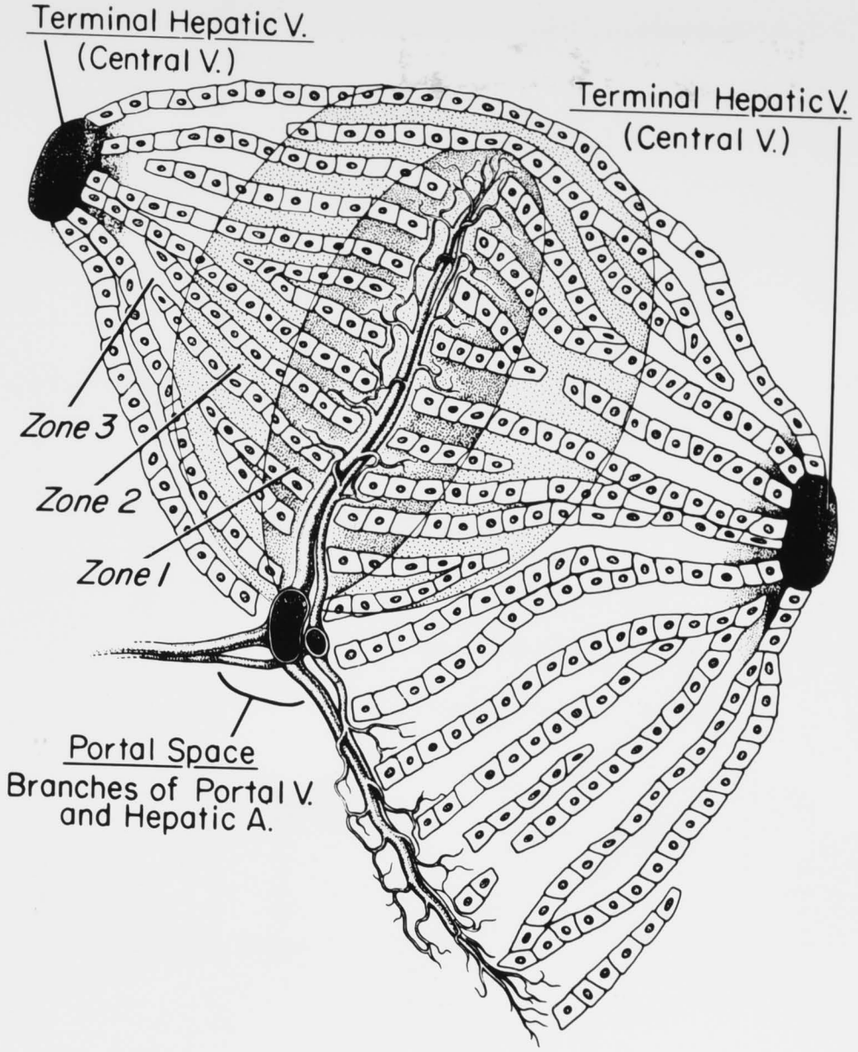


Figure 3.2. Diagram illustrating the functional unit of liver parenchyma (the acinus) according to Rappaport (1958). It consists of the parenchyma centered around the terminal branches of the hepatic artery and portal vein. Cells in Zone 1 nearest these vessels have first access to the incoming blood, while the cells of Zone 2 are less favored and those of Zone 3 near the central veins are least favorably situated. (Copied from Bloom and Fawcett (1975), p695).

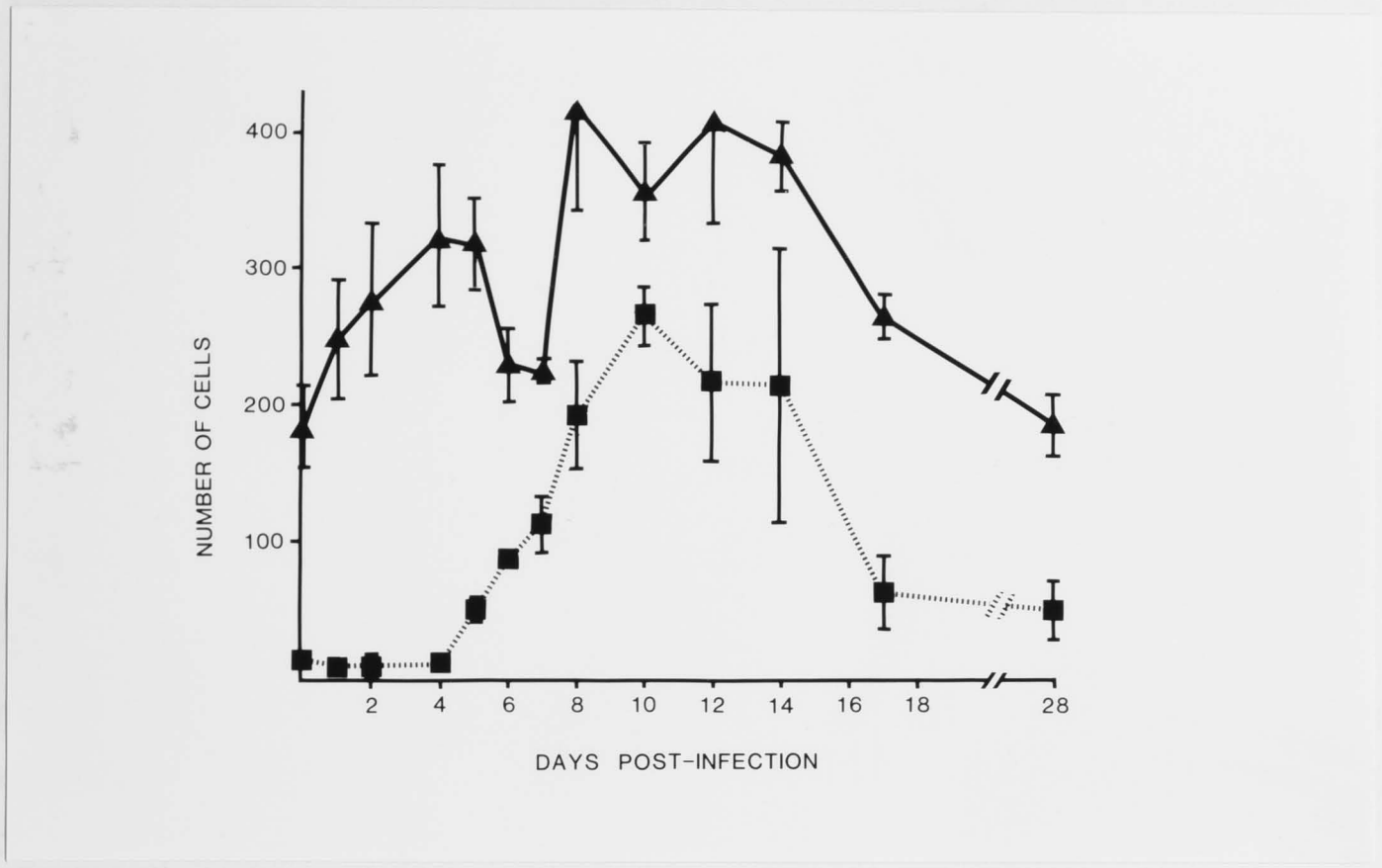


Figure 3.3. The number of F4/80$^+$ (▲) and Ia$^+$ (■) cells lining liver sinuses during the course of LCMV infection was assessed using a projection microscope at 850x magnification. These cells include both resident KC and recruited macrophages. The vertical axis represents the number of cells counted in four fields of view. Points represent the mean ± standard error for 2-4 mice. Error bars not marked when they fall within the area symbol.

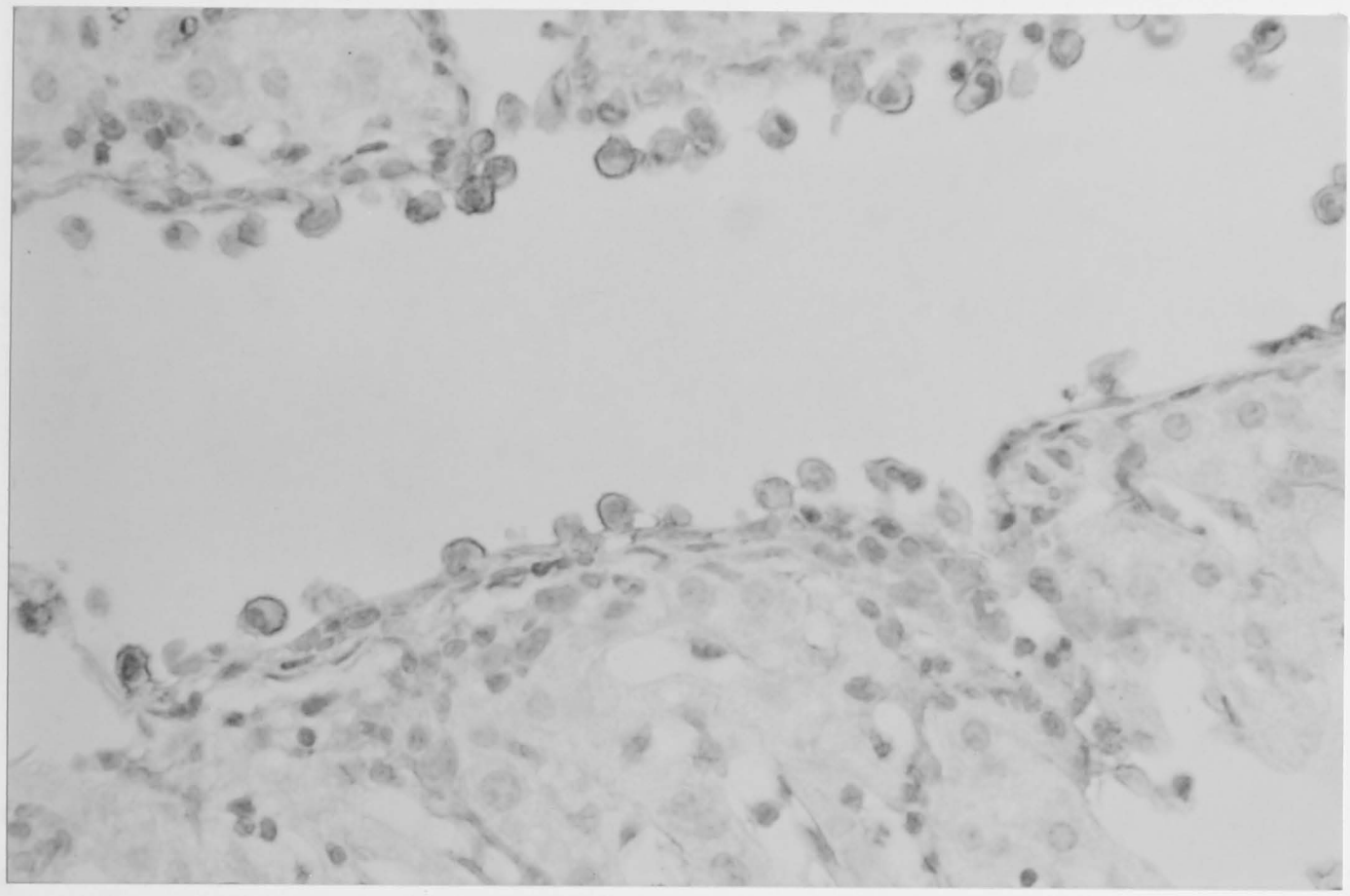


Figure 3.4. Day 8-infected liver, F4/80: $F4/80^{+}$ monocytes with cresent-shaped nuclei adhere to the walls of a central vein. One $F4/80^{-}$ cell with a cresent-shaped nucleus can be seen on the lower left-hand side of the vessel. Magnification x500.

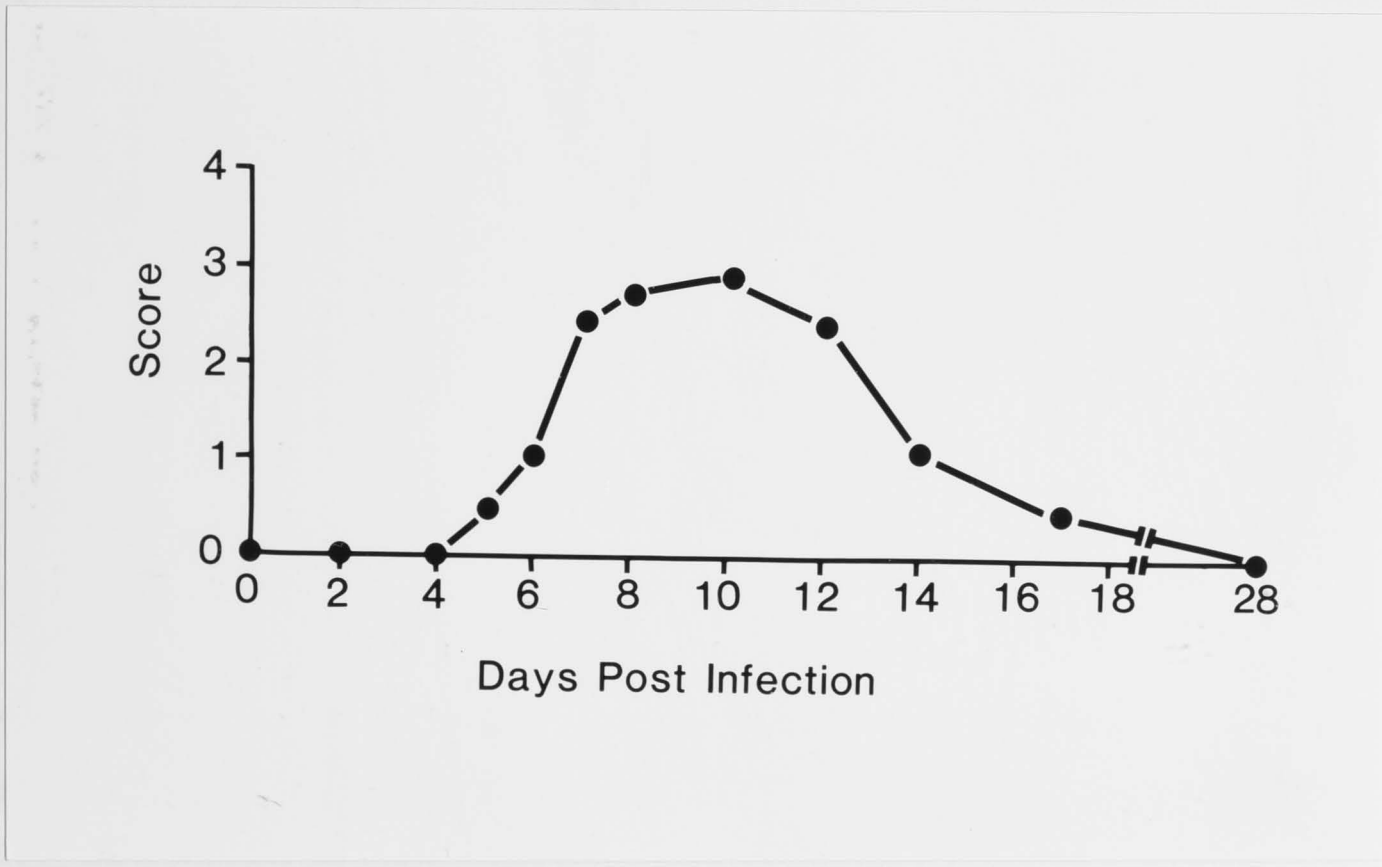


Figure 3.5. The extent of monocyte infiltration into the liver of each animal was histologically scored from O (no infiltration) to 4 (maximal infiltration). Points represent the mean for 2-4 mice.

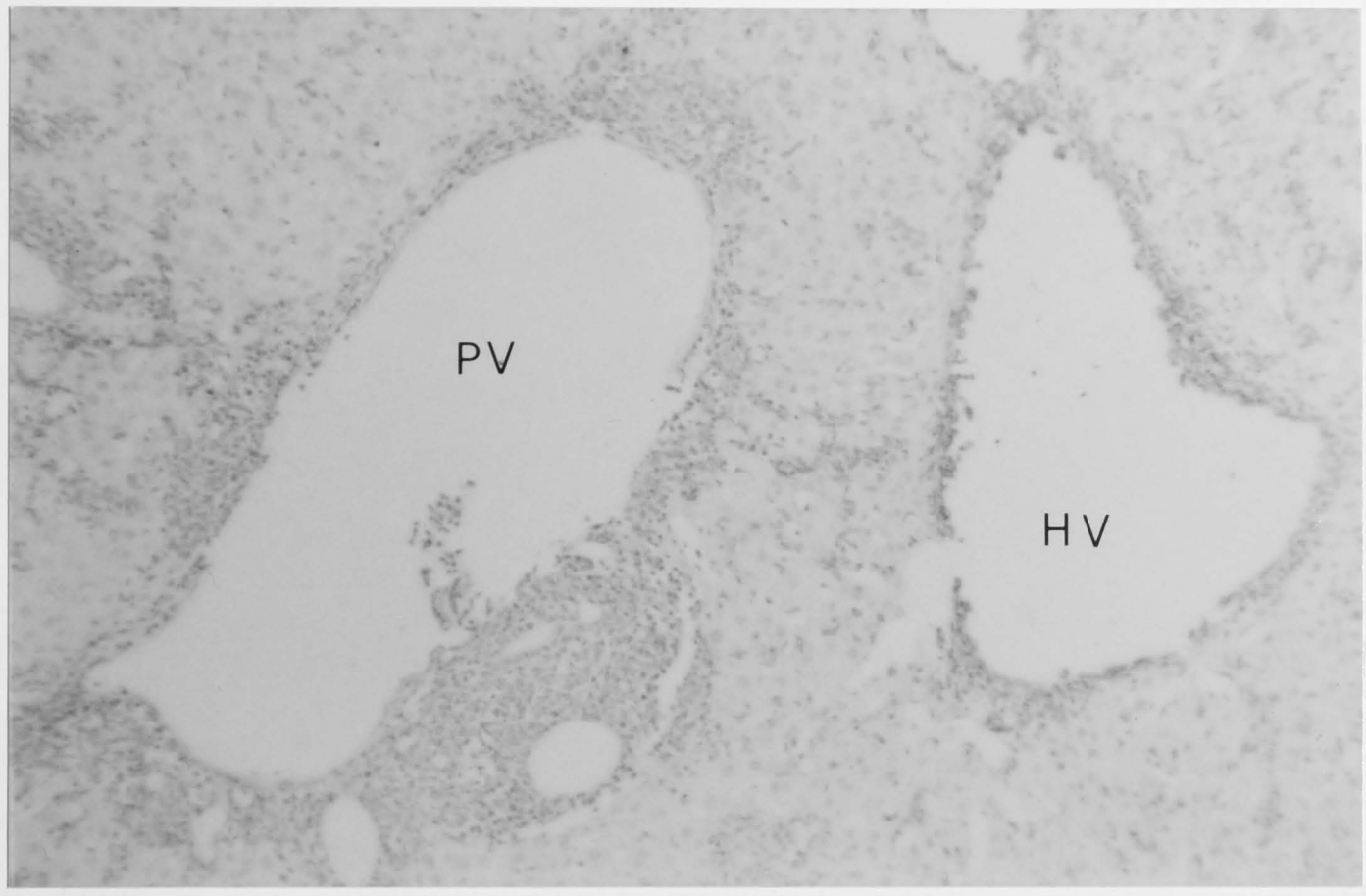


Figure 3.6 Day 12-infected liver, F4/80: the portal triad of a large potal vein (PV), hepatic artery and bile duct is seen on the left, and a hepatic vein (HV) is located to the right of it. There is extensive perivascular cuffing around the portal triad, particularly in the region of the bile duct. Adherence of monocytes is restricted to the walls of the hepatic vein. Magnification x125.

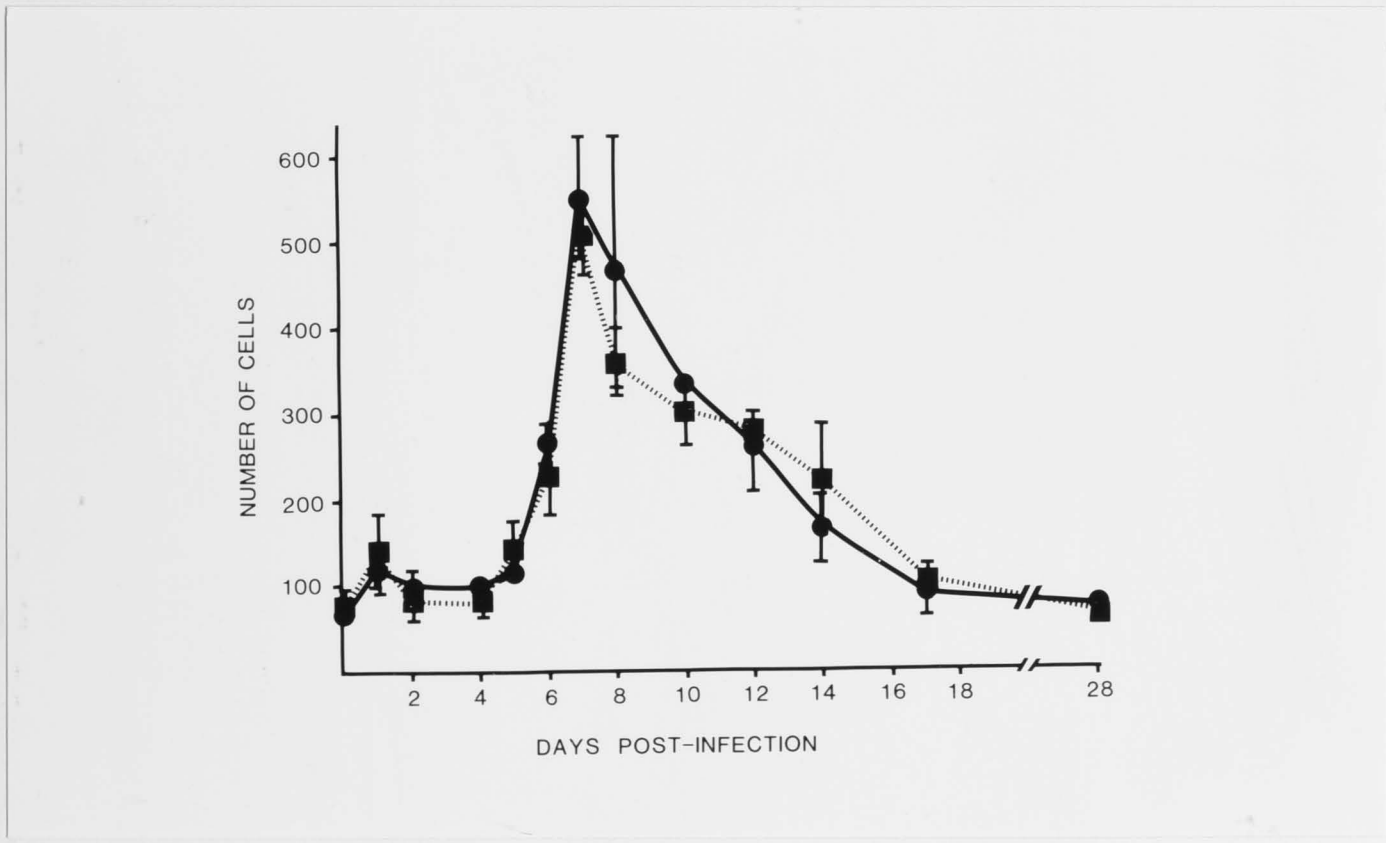


Figure 3.7. The number of lymphocytes in the liver sinuses of mice infected with LCMV was assessed using a projection microscope at 850x magnification. The total numbers of lymphocytes from four fields of view were counted using liver sections stained either for F4/80 (●) or Ia (■). Lymphocytes were identified by morphology only on the section stained for Ia. On sections stained for F4/80 the criteria included that lymphocytes were F4/80$^-$. Essentially similar results were found in each case. Points represent the mean ± standard error for 2-4 mice.

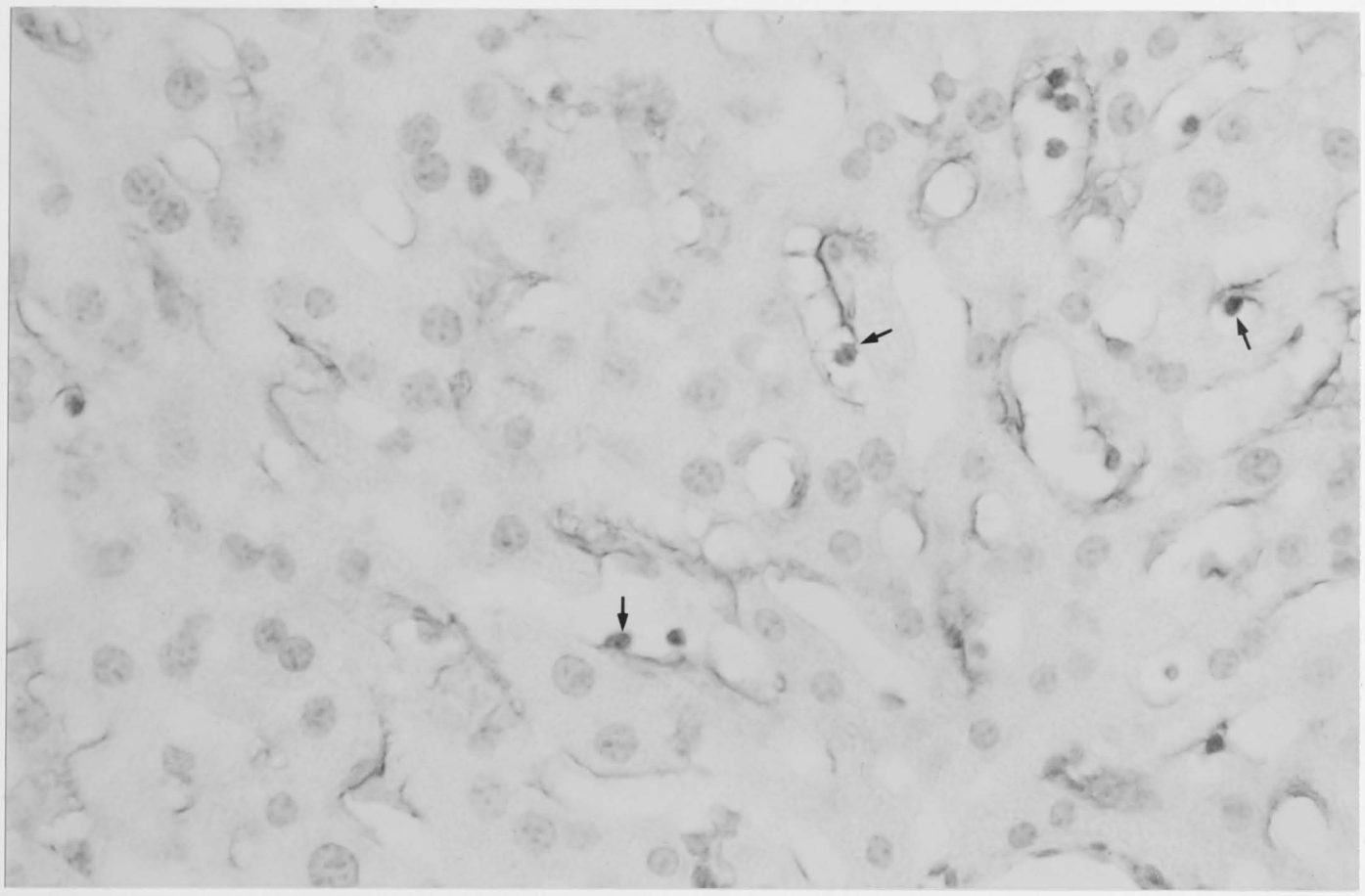


Figure 3.8. Day 4-infected liver, F4/80: examples of the close association between lymphocytes and sinusoidal macrophages are indicated by the arrows. Magnification x500.

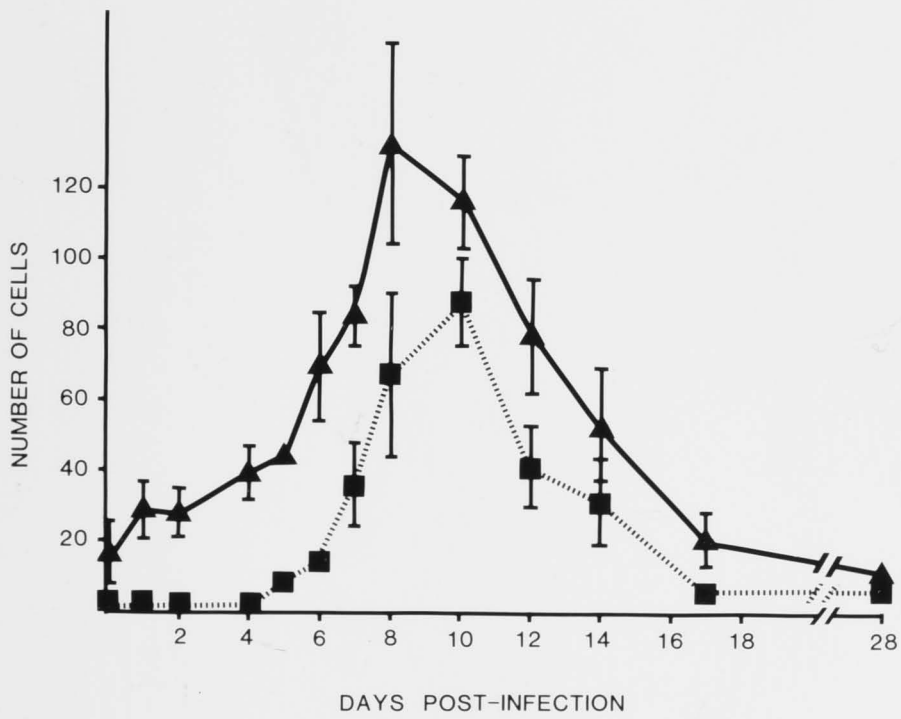


Figure 3.9. The number of lymphocytes within the sinuses which were attached to $F4/80^+$ (▲) or Ia^+ (■) sinusoidal macrophages (as seen in Figure 9) were counted in four fields of view at 850x magnification using a projection microscope. Points represent the mean ± standard error for 2-4 mice.

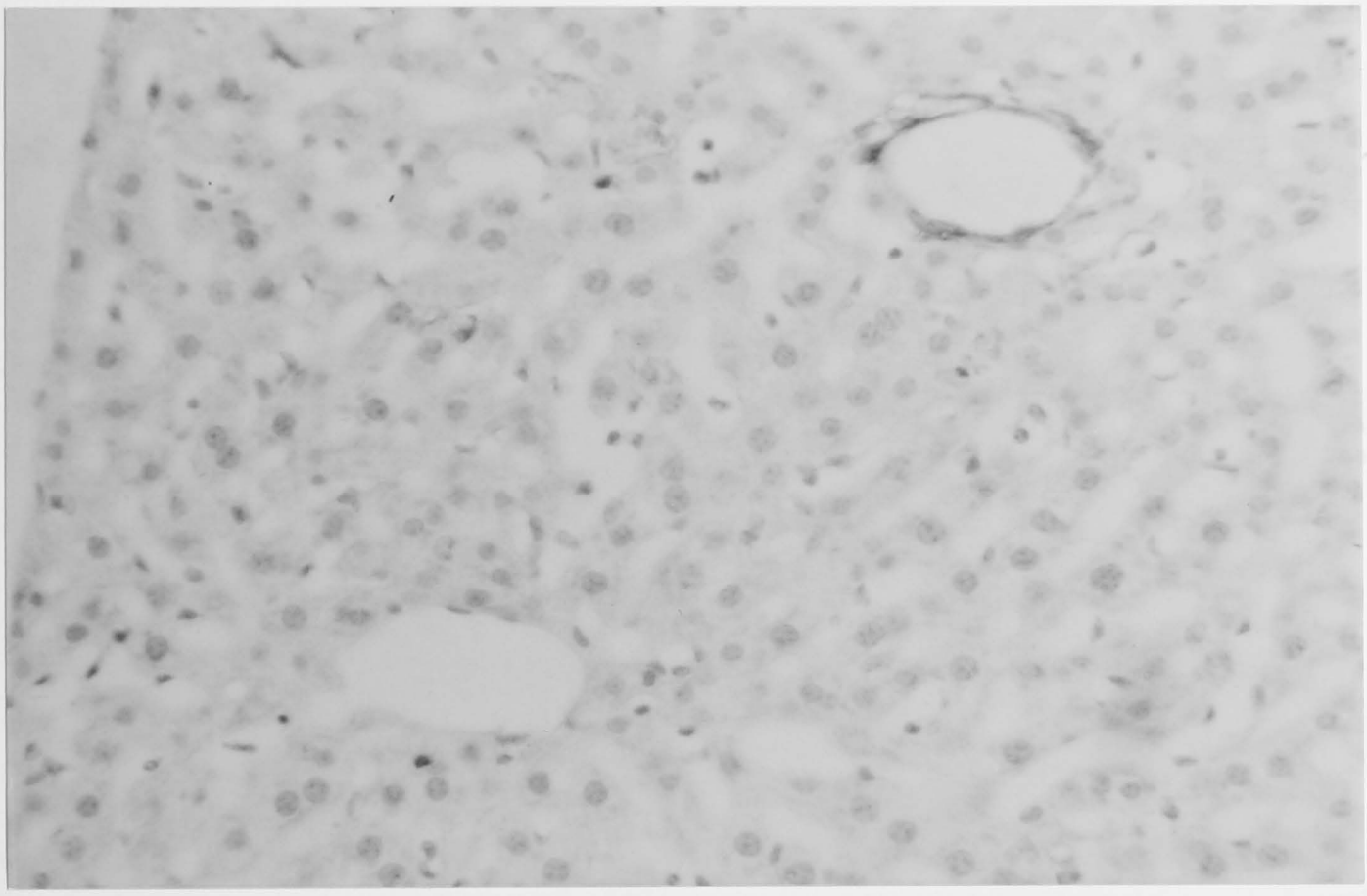


Figure 3.10. Day 5-infected liver, Ia: two blood vessels can be seen, one of which stains positively for Ia antigens. Magnification x312.

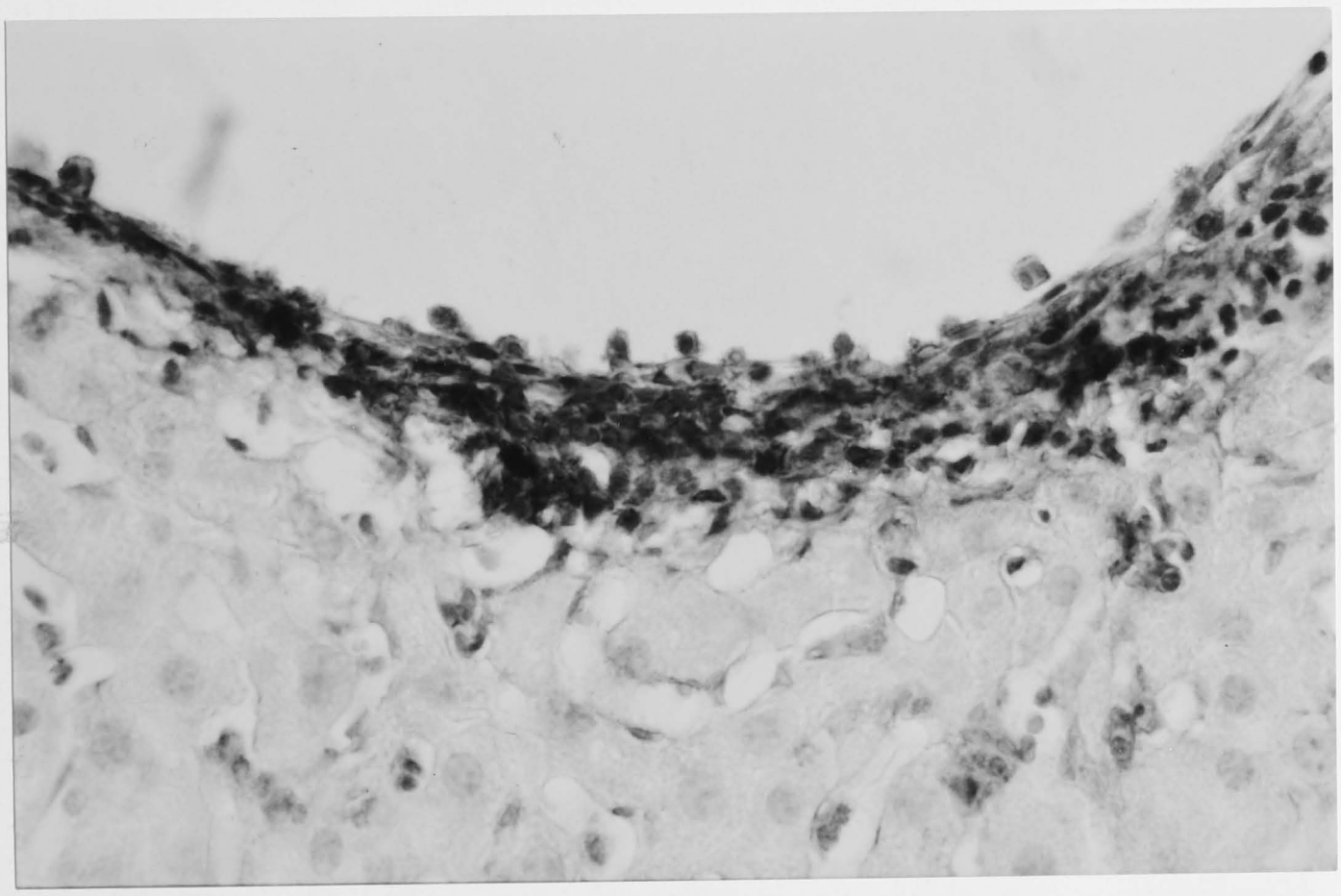


Figure 3.11. Day 14-infected liver, Ia: a band of cells below the blood vessel stain positively for Ia antigens. Positively stained sinusoidal macrophages can also be seen. Magnification x500.

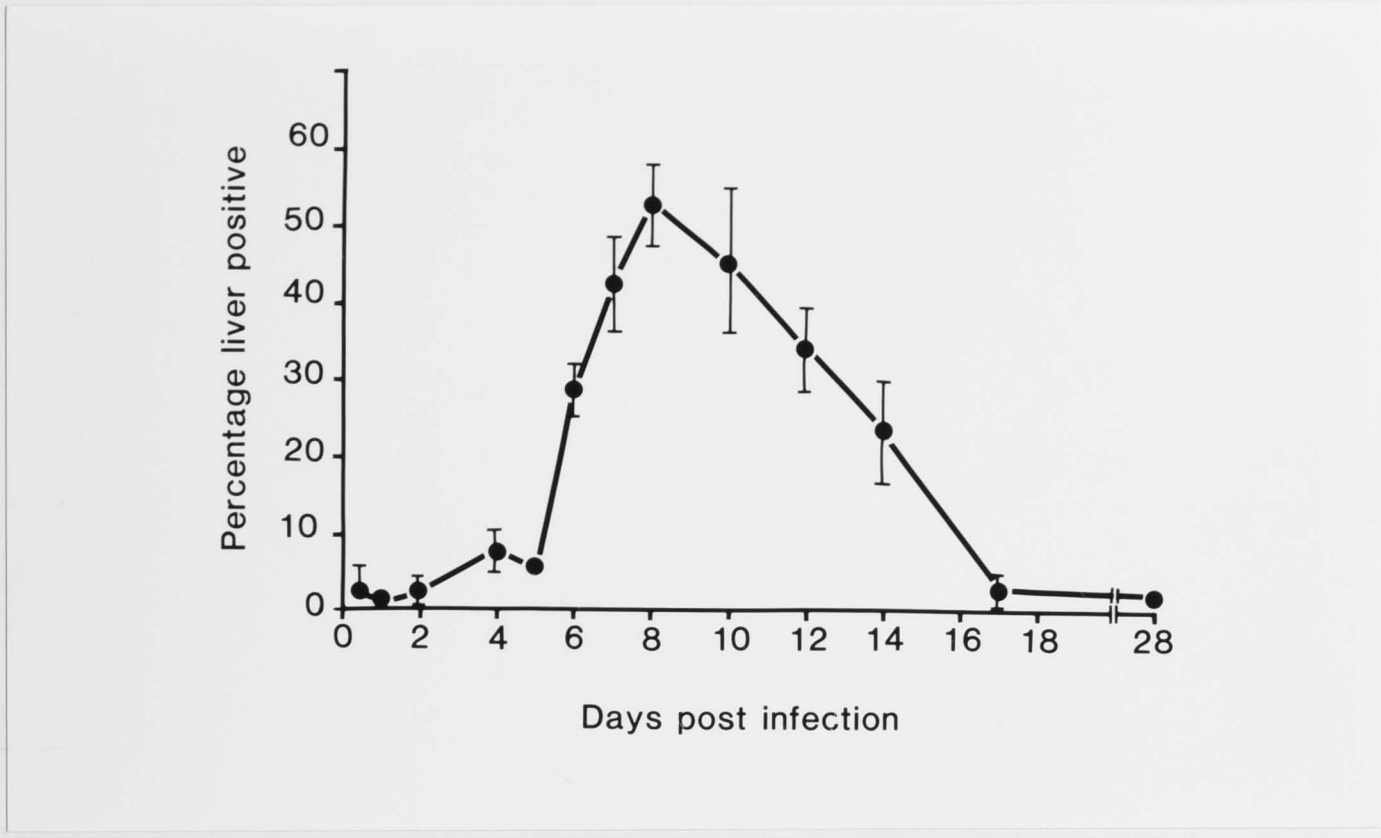


Figure 3.12. The severity of inflammation was determined by scoring tissue for the presence of inflammatory cells at 100 intersecting points of a grid at 100x magnification. The results were expressed as a percentage of those points that were positive. Four fields of view were examined for each liver section, and the points represent the mean ± standard error for 2-4 mice. The point dot analysis is described in Elias and Hyde (1983).

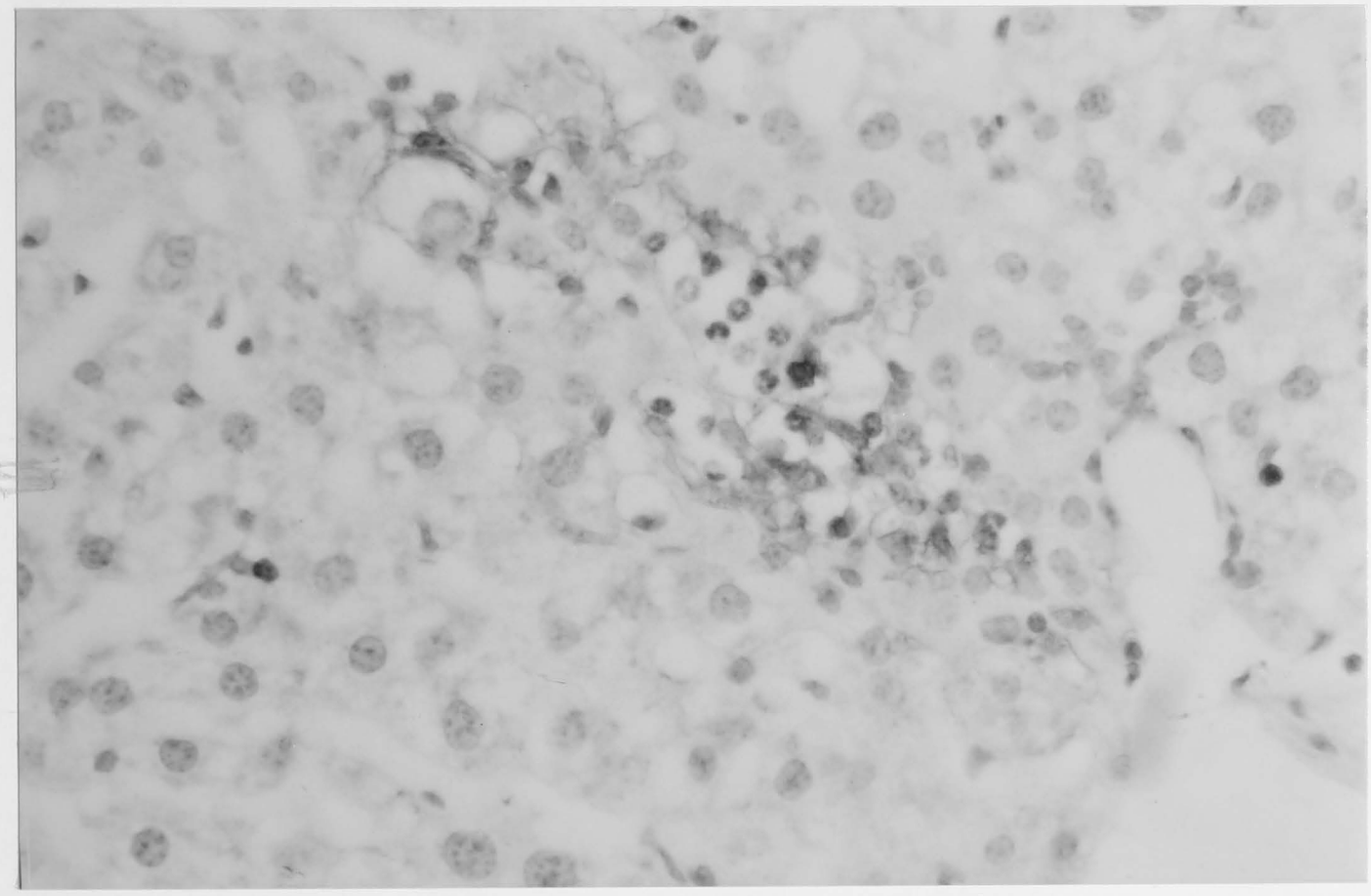


Figure 3.13. Day 2-infected liver, Ia: a foci of polymorphonuclear cells interwined by Ia^+ cells can be seen. Magnification x500.

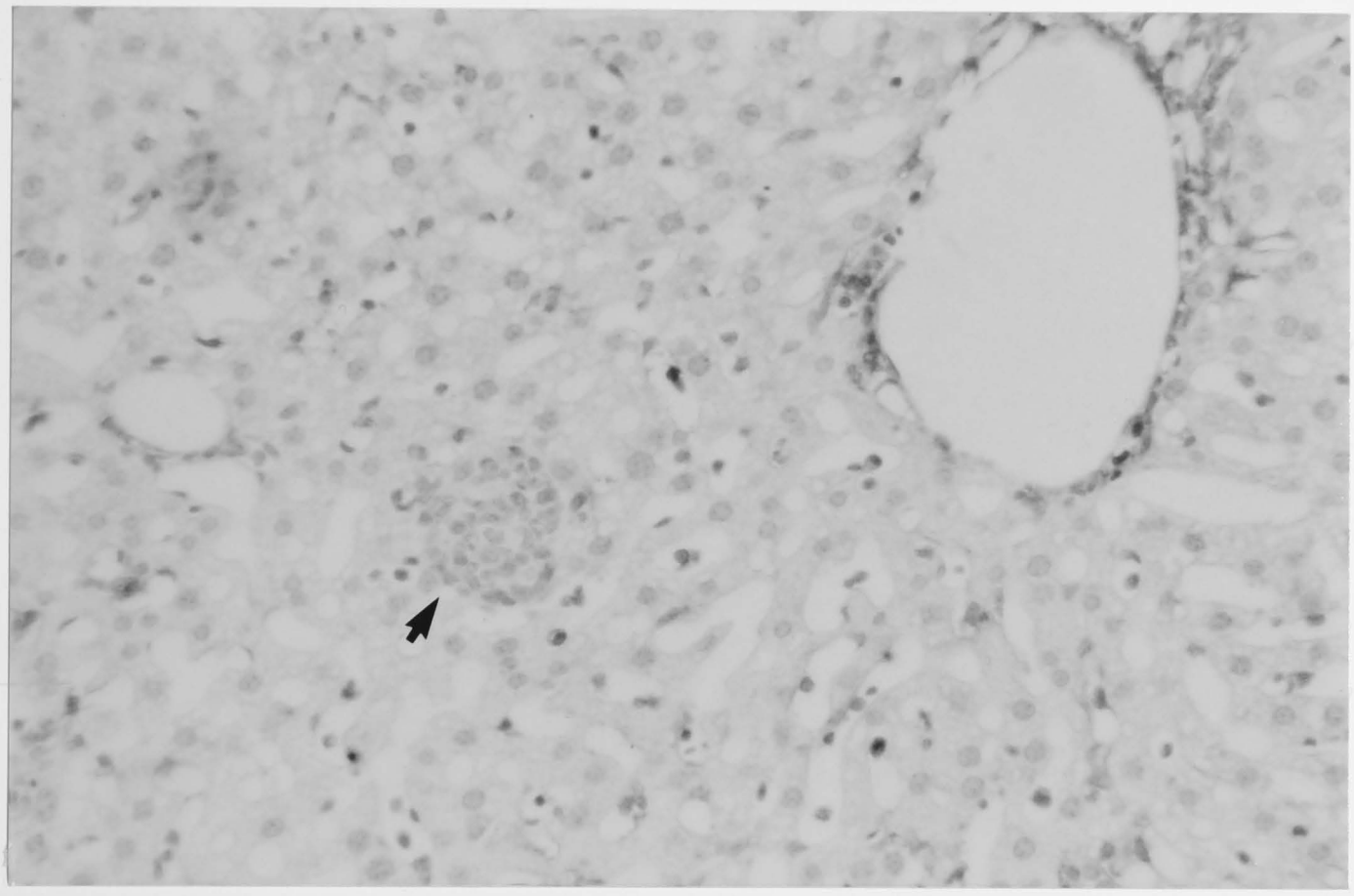


Figure 3.14. Day 10-infected liver, Ia: the parenchymal focus (arrow) consists of a compact sphere of lymphoid cells. Magnification x312.

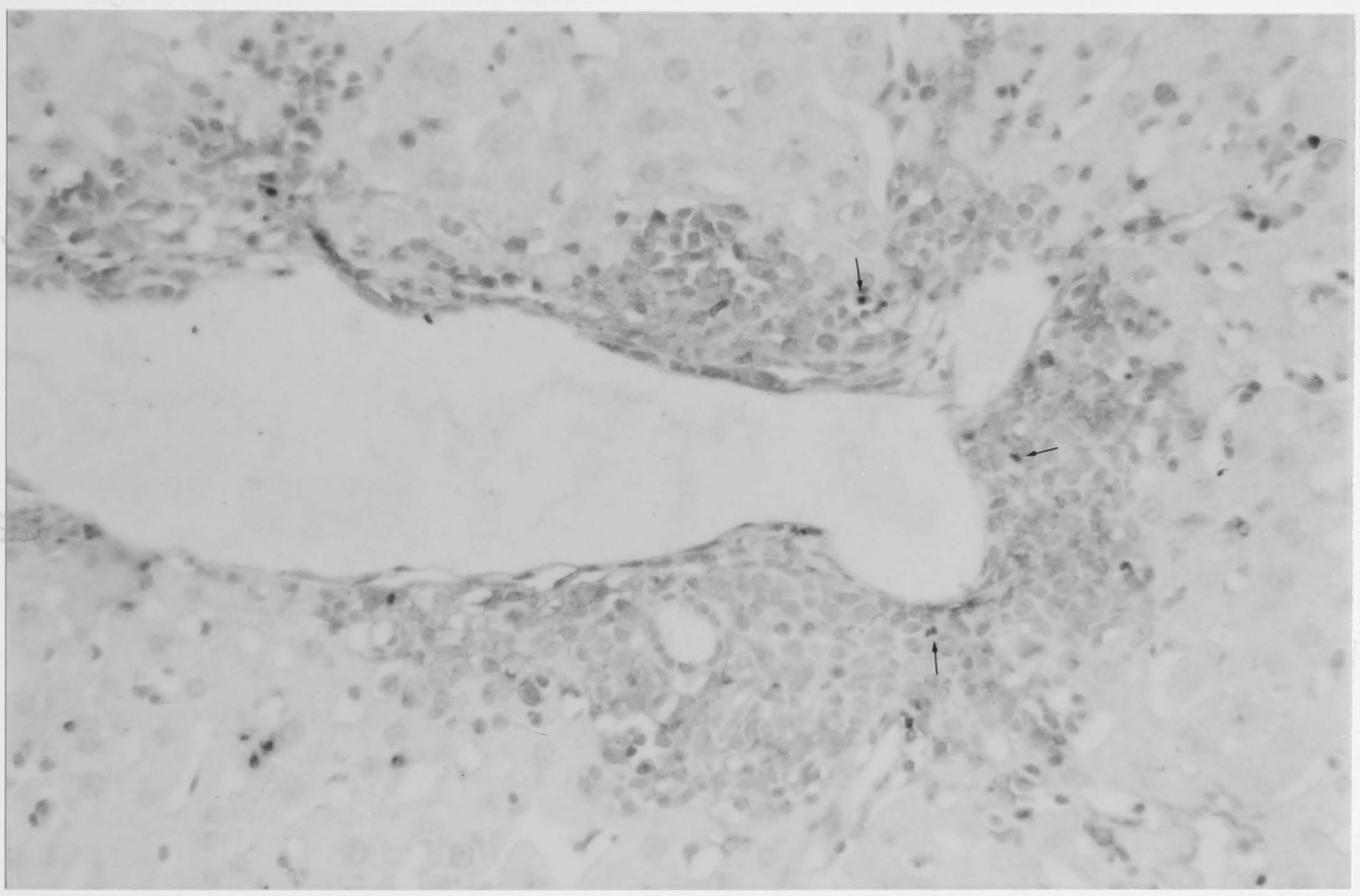


Figure 3.15. Day 12-infected liver, Ia: pyknotic nuclei (arrows) can be seen in this perivascular cuff of a portal triad. Magnification x312.

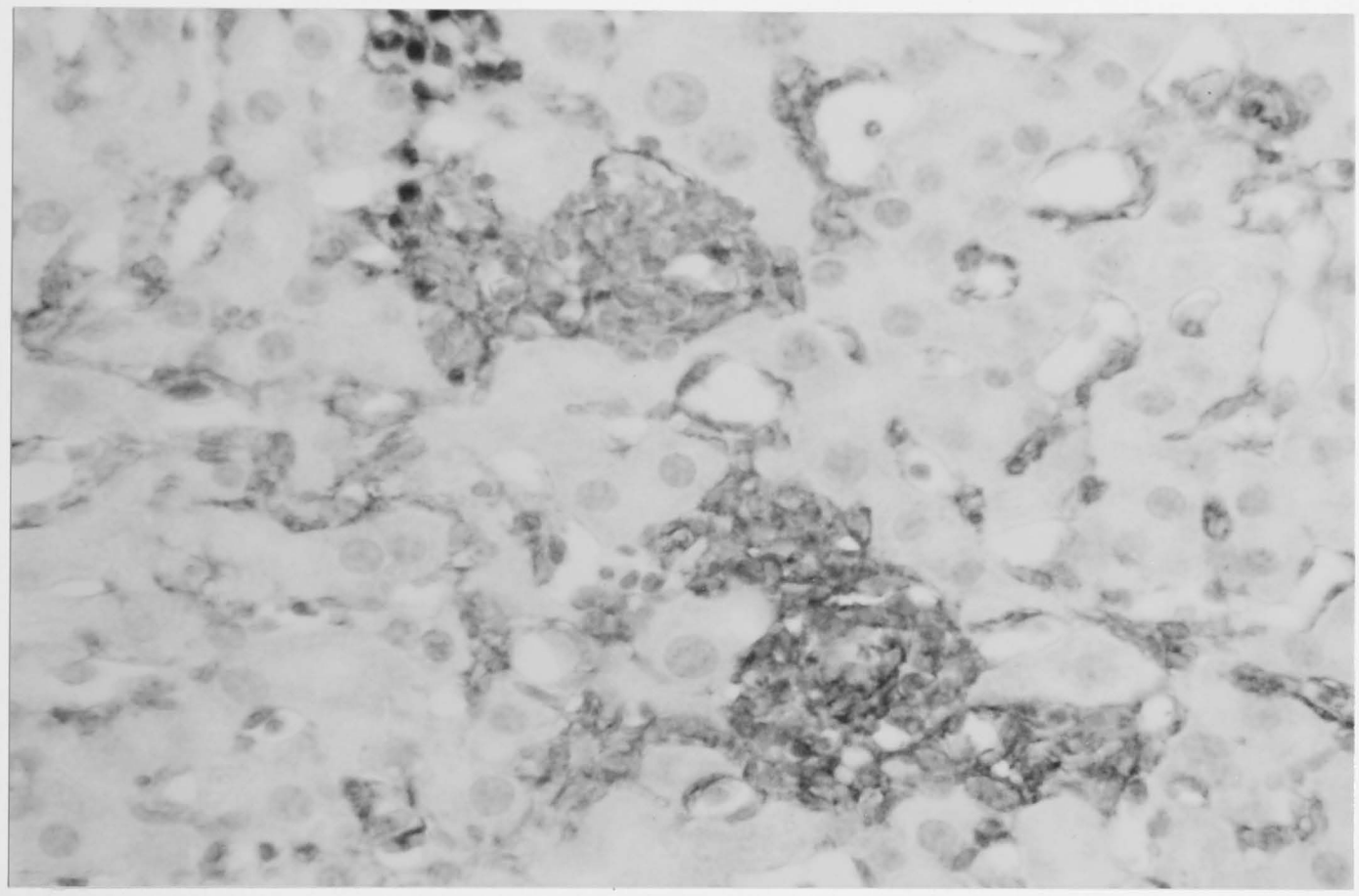
Figure 3.16.

A. Day 14-infected liver, F4/80: infiltration of parenchymal foci with $F4/80^+$ cells can be seen. Most sinusus are lined with $F4/80^+$ cells at this stage of infection. Magnification x500.

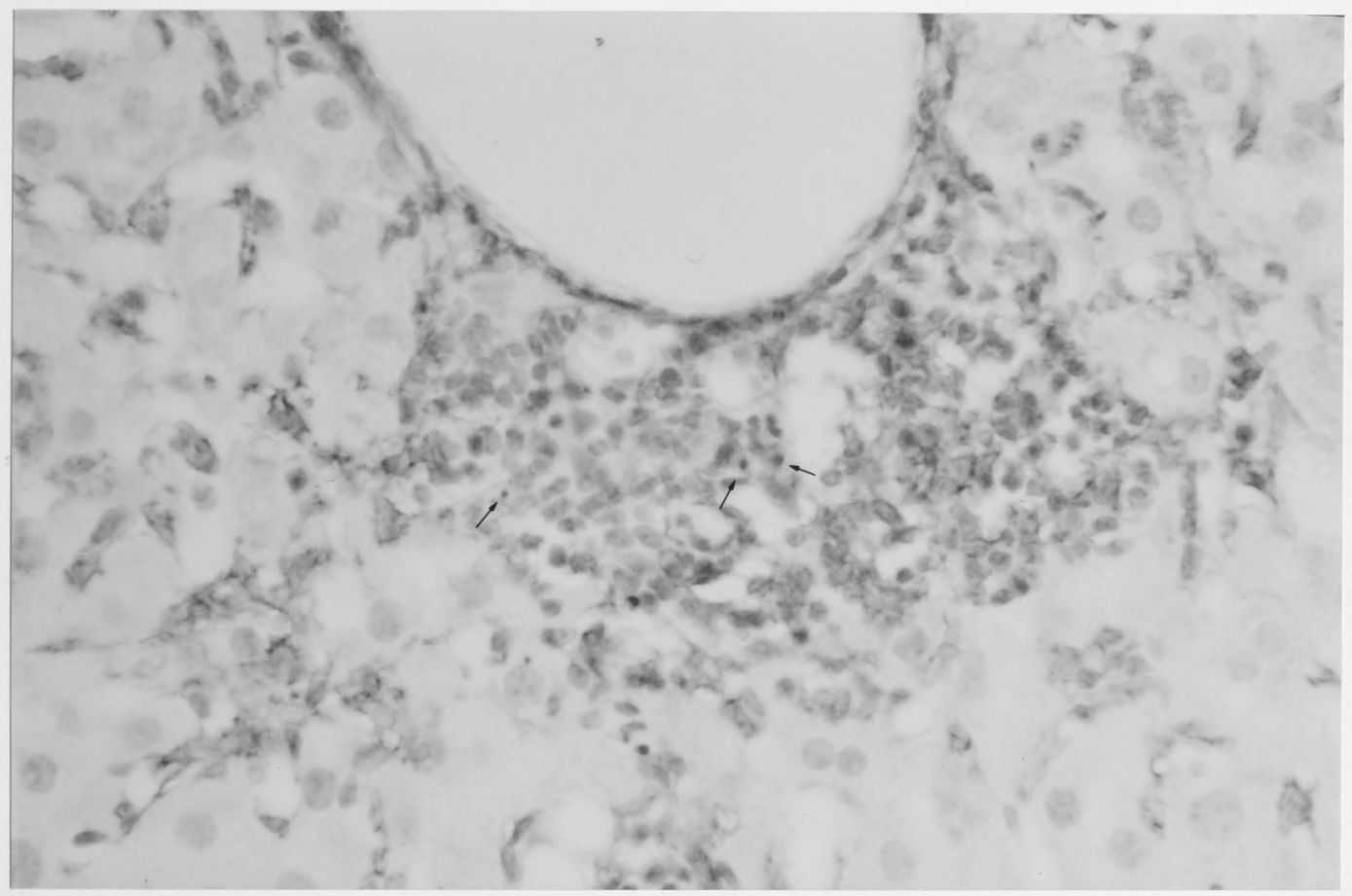
B. Day 14-infected liver, F4/80: infiltration of a perivascular foci can be seen. Pyknotic nuclei are marked by arrows. Magnification x500.

C. Day 14-infected liver, Ia: the same inflammatory focus as shown in Fig 3.16b; staining for Ia is much more intense than F4/80 because the antigen is apparently expressed not only on incoming macrophages but on many of the lymphoid cells in the focus. Magnification x500.

A



B



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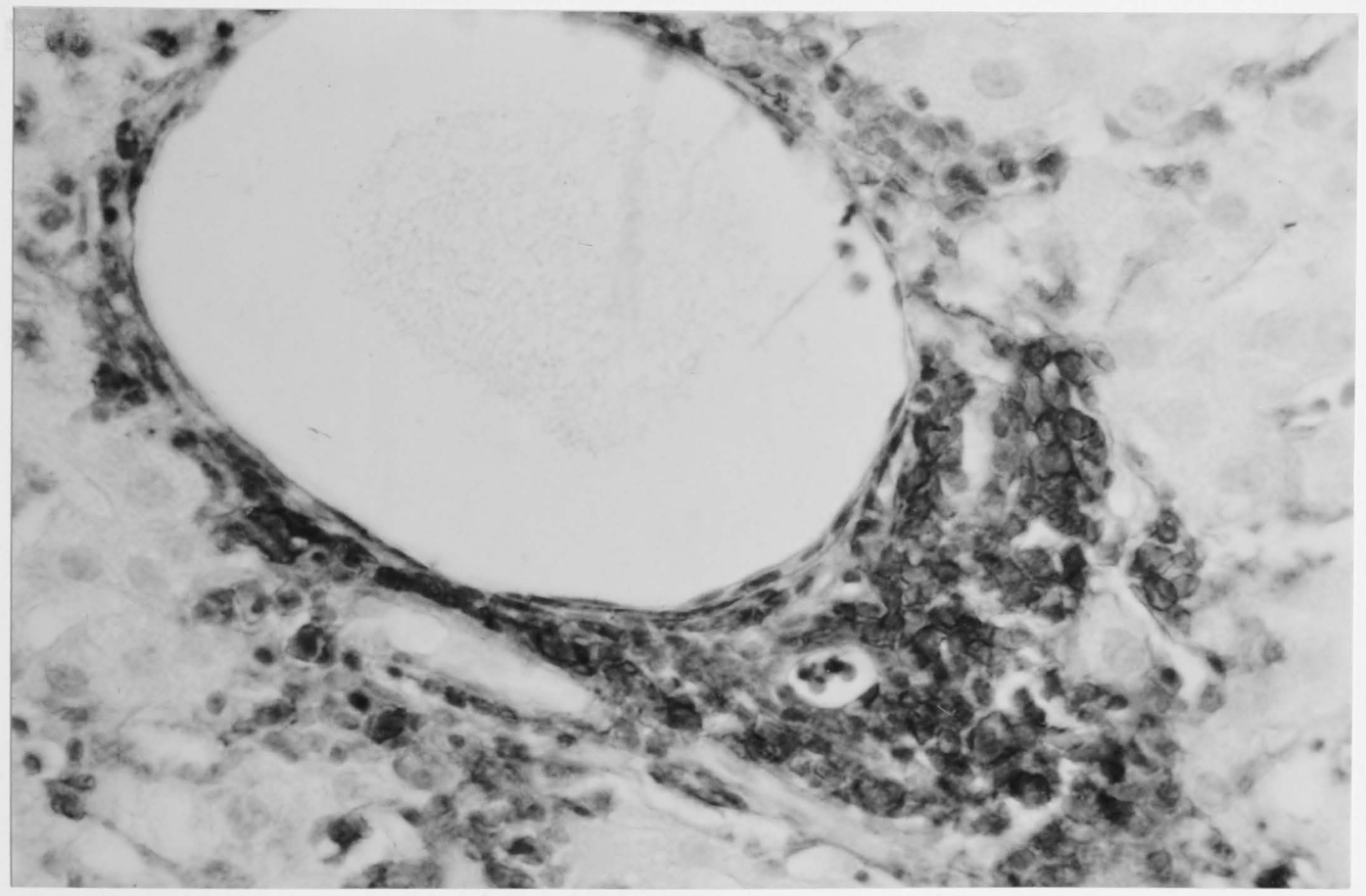


Chapter 4

Macrophages in the Brain of LCMV-Infected Mice

4.1 INTRODUCTION.

Intracerebral inoculation of LCMV into adult mice results in the classical disease of the CNS that was first described by Rivers and Scott (1936) and has been reviewed by a number of authors (Lehmann-Grube, 1971; Cole and Nathanson, 1974; Doherty and Zinkernagel, 1974). Five days after infection, mice develop a nondescript illness with ruffled fur, hunched posture and inactivity. They show signs of neurological impairment on d6-8, with increased irritability and the development of clonic convulsions. The latter can be induced when the animals are disturbed, particularly when they are spun by the tail. Convulsions often terminate in extensor spasms of the hind legs and death.

Clinical symptoms coincide with severe inflammatory changes in the CNS. Acute LCM is, essentially, a disease of the CNS membranes and choroid plexus. Little effect on the brain parenchyma is observed. Viral distribution within the brains of acutely infected mice is restricted to the meninges, the epithelium of the choroid plexus, and the ventricular ependyma (Wilsnack and Rowe, 1964; Tosolini and Mims, 1971; Gilden et al., 1972a; Gilden, 1975; Walker et al., 1975; Camenga et al., 1977; Schwendemann et al., 1983; Marker et al., 1985) and viral antigen has also been observed in the Virchow-Robin spaces (Tosolini and Mims, 1971). Infiltration of mononuclear cells into the leptomeninges, the choroid plexus and the ventricles of i.c. infected mice is well documented (reviewed in Lehmann-Grube, 1971). The contribution of cells of the macrophage lineage to the development of disease is not clear. As described in this chapter, results obtained from immunohistochemical staining for activation (Ia) antigens and the macrophage marker, F4/80, indicate that activated macrophages form a substantial proportion of the infiltrating cells.

Since meningitis is triggered by $Lyt2^+$, class I MHC-restricted T cells (reviewed in Zinkernagel and Doherty, 1979; chapter 5) further experiments were designed to determine whether $Lyt2^+$ T cells also recruit the macrophages. Although $Lyt2^-$, class II MHC-restricted T cells have been traditionally associated with DTH function (Huber et al., 1976; Vadas et al., 1976; Zinkernagel and Doherty, 1979; Hollander, 1982) it has been reported that DTH to LCMV, measured by foot pad swelling, is mediated by class I MHC-restricted T cells (Zinkernagel, 1976b).

The role of T cell subsets in recruiting macrophages was investigated by transfer of immune cell populations to immunosuppressed, LCMV-infected recipients based on the protocol of Gilden and colleagues (1972a,b). Recipient mice infected intracerebally with Arm LCMV were immunosuppressed 5 days later with 150mg/kg Cy, a procedure which prevents the acute disease usually observed 6 to 8 days after infection and abrogates the development of cytotoxic T cell activity (Gilden et al., 1972a, Allan and Doherty, 1985b). One day after Cy treatment recipients were given immune spleen cells depleted of $Lyt2^+$ or $L3T4^+$ cells by monoclonal antibody and complement treatment (section 2.13). Brains were examined by immunohistology.

The histological observations are described in relation to the meninges, the Virchow-Robin spaces, the choroid plexus and the ventricles. Two of the membranes lining the surface of the brain, the pia mater and the arachnoid together form the leptomeninges. Apart from the fact that the arachnoid is avascular, the two membranes are structurally similar and are probably derived from a single layer. They are mainly composed of interlacing collagenous bundles surrounded by fine elastic networks. Among the cells of the membranes are fixed macrophages. These macrophages have been described in ultrastructural (Dobrovol'skii, 1984) and immunohistochemical (Perry et al., 1985; Esiri and McGee, 1986) studies.

The pia and arachnoid membranes are separated by the subarachnoid space, which is filled with CSF. They remain connected by trabeculae that traverse this space. Larger blood vessels entering and leaving the brain are surrounded by pia mater and a continuation of the subarachnoid space. These perivascular spaces are termed Virchow-Robin spaces and extend in increasingly attenuated form as far as the arterioles and venules.

The choroid plexuses are found in each of the ventricles where they are a major site of formation of CSF. They are formed by invaginations of vascular mater, containing a core of connective tissue with many wide capillaries overlayed by simple cuboidal epithelium. CSF passes through the ventricles to the cisterna magna, where it is possible to sample the fluid, and then circulates in all directions through the subarachnoid space surrounding the brain. CSF leaves the CNS through the arachnoid villi which project into the dural sinuses.

Immunohistochemical examination was performed on the brains of mice infected with LCMV with the intent of determining the extent of infiltration of the brain by activated macrophages and the ability of $Lyt2^+$ T cells to mediate this recruitment. Particular attention was paid to the meninges, and the fourth ventricle, including its choroid plexus. The results described in this chapter encompass three different experiments: 1) LCM disease initiated by i.c. inoculation of Arm strain LCM virus; 2) LCM disease transferred into immunosuppressed, infected mice with immune spleen cells; and 3) LCM disease transferred with immune spleen cells that were depleted of either $Lyt2^+$ or $L3T4^+$ cells.

4.2 RESULTS.

4.2.1 LCM DISEASE FOLLOWING INTRACEREBRAL INOCULATION.

Following i.c. inoculation of C57BL/6J mice with 10^3 LD_{50} of Arm strain LCM virus, clinical signs of LCM appeared first on d6 p.i.. The mice were hunched, their fur was ruffled, and trembling could be detected in their tails. By d7 p.i. the mice were moribund.

Mice were sampled for immunohistochemical analysis on d4, d5, d6 and d7 p.i. and, as a control, on d4, d5 and d6 following i.c. injection of gelatine saline. A description of F4/80 and Ia antigen distribution in the brains of gelatine saline injected controls will be given first before proceeding with descriptive and numerical analyses of infected mice.

UNINFECTED MICE.

Resident macrophages of the meninges were located primarily on the pial membrane. They were observered, usually, as dense $F4/80^+$ staining stretched along the surface of the brain (Fig 4.1) but were also present on the lining of some pial blood vessels. Occasional $F4/80^+$ membrane staining was observed on the outside of larger blood vessels traversing the subarachnoid space and on the inner surface of the arachnoid membrane. Some of these leptomeningeal macrophages stained strongly for Ia antigens. More rounded and apparently loosely attached macrophages were occasionally observed in the subarachnoid space, as were small dense lymphocytes. Monocytes that were $F4/80^+$ and Ia^+ were observed more rarely.

Within the choroid plexus, $F4/80^+$ cells were found in the stroma of the choroid villi (Fig 4.2). They lined the blood capillaries that supply the epithelium and their morphology resembled that of Kupffer cells in the liver, having a ruffled cytoplasm. Small dense lymphocytes were occasionally seen within the capillary lumen. In general, about 30% of the resident macrophages of the choroid plexus also stained for Ia antigens. Endothelial cells of the capillary walls and other cells of the plexus were Ia^-.

F4/80 staining was not observed in the ependyma of the ventricles, but in two of nine mice examined, $F4/80^+$ cells were attached to the ventricle ependyma or the ventricular side of the choroid plexus (Fig 4.2).

Macrophages were also associated with the vasculature of the CNS. Although not observed frequently, both perivascular (Fig 4.3) and luminal macrophages were detected in capillaries of the brain parenchyma. More predominant was the perivascular location of macrophages around the large blood vessels that invaginate the brain along the various fissures of both the cortex and the cerebellum. The location of F4/80 staining in these Virchow-Robin spaces probably reflects the presence of resident macrophages in the accompanying pial lining.

Some cells with similar morphology and location within the Virchow-Robin spaces of the fissures also stained for Ia antigens, as was seen with some of the pial macrophages. Capillary macrophages, however, did not stain for the class II MHC antigens, suggesting differences in behavior, origin or activation state of these macrophages.

Another major perivascular site of macrophages within the CNS was the area postrema located at the pointed, caudal end of the floor of the fourth ventricle (Fig 4.4). This is a highly vascular area with large capillaries resembling blood sinusoids. Its endothelium is fenestrated like that of the choroid plexus and is permeable to compounds which do not normally penetrate the walls of other vessels in the CNS (Zeman and Innes, 1963; Netsky and Shuangshoti, 1975; Leslie, 1986). These cells were never observed to express Ia antigens.

Although microglia within the brain parenchyma have been shown previously to stain for the F4/80 antigen (Hume et al., 1983a; Perry et al., 1985), they did not in these sections. Ia expression on parenchymal cells was observed occasionally. The majority of these positive cells were isolated cells with star-like processes, but sometimes groups of such cells were observed (Fig 4.5). Positive parenchymal cells did not resemble, morphologically, the microglia seen by Hume et al. (1983a) and Perry et al. (1985), their processes being more dense. Their star-like appearance suggests that they are astrocytes.

In summary, macrophages are located along the leptomeninges, and perivascularly in the choroid plexus and Virchow-Robin spaces. Some of these macrophages express Ia antigens.

LCMV-INFECTED MICE.

The most notable histological outcome of LCMV infection was the influx of inflammatory cells into the subarachnoid space of the meninges with involvement of the choroid plexus occurring later. A slight inflammation was already present in mice on d4 after i.c. infection with LCMV, the first day of observation. The severity of inflammation was similar on the following day, with extensive infiltration occurring on d6 and d7. Fifty percent of the infected mice were dead on d7, and the remaining 4 animals, which were perfused for immunohistochemical analysis, were moribund. In this experiment, there was no clinical evidence of recovery from infection. The observations will be discussed by anatomical site: meninges, choroid plexus, ventricles and parenchyma.

a. Meningeal inflammation.

In general, inflammation within the meninges consisted of a loose accumulation of infiltrating cells within the subarachnoid space. At the later stages of infection, on d6 and d7, more compact regions of inflammatory cells formed. They occurred in areas where the arachnoid membrane lies very close to the pial membrane and no trabeculae are seen traversing

the subarachnoid space.

During the course of infection, there was no apparent increase in the number of 'resident', F4/80$^+$ macrophages lining the meningeal membranes. These macrophages, lining the meningeal membranes, during LCM disease will be referred to as 'stretched macrophages'. In contrast, rounded macrophages, (those that had not stretched along the meningeal membranes), approximately half of which expressed Ia antigens, increased in number during LCMV infection. They were apparent on d4, d5 and d6 p.i. but were most numerous on d7 (Fig 4.6). Likewise monocytes, though fewer in number, were present on d4, d5 and d6 with more substantial numbers observed on d7. Cells that were morphologically identified as monocytes in this location were generally negative for Ia expression and a minority (<5%) were also negative for the F4/80 antigen (Fig 4.6).

Although macrophages constituted a significant proportion of the infiltrating cells, most cells were negative for both F4/80 and Ia staining. Many of these were obviously lymphocytes, having small dense nuclei and a low cytoplasm to nucleus ratio. The morphology of other cells (larger, irregularly-shaped with a higher proportion of cytoplasm and a pale nucleus) was consistent with their being activated lymphocytes or blasts. A significant number of small lymphocytes were seen in the meninges early in infection, a stage at which the level of inflammation was fairly minor and they were very numerous on d6 before a slight decline on d7 p.i.. A minor subpopulation of these (<10%) stained for Ia antigens and were probably B lymphocytes. Blast cells also appeared in large numbers on d6, but again fewer were seen on the following day (Fig 4.6).

Although the number of lymphocytes and blasts had fallen by d7, the overall level of inflammation remained very high, the lymphocytes and blasts being replaced in number by a cell type which had a very indented, irregularly shaped nucleus (Fig 4.6). These cells were not PMNs since they did not have granular cytoplasms. However, a minor neutophil infiltration into the meninges was observed on d7 after infection. Cell death, as determined by the presence of pyknotic nuclei, was another obvious feature of the inflammatory exudate on d7 p.i. (Fig 4.6). It is possible that those cells with irregularly shaped nuclei were also dead or dying cells. Because this could not be determined by light microscopy, CSF samples were taken from mice infected i.c. for 7 days with LCMV and examined under the transmission electron microscope (EM). Forty mice were needed to obtain sufficient inflammatory cells from the cisterna magna to yield a pellet large enough for EM processing.

Dead cells, with obviously disrupted cytoplasm and plasma membrane, were observed (Fig 4.7A). There were also many cells at a less advanced stage of cell death (Fig 4.7E). These cells retained a largely intact plasma membrane, but the nuclear envelope had degenerated and coarse granularity of the chromatin was observed. Condensation of the cytoplasm was also obvious. Those cells with irregularly shaped nuclei were not readily identified under EM, possibly because of the thinness of the sections. However, because these cells, seen under the light microscope, appeared to replace the large numbers of

lymphocytes and blasts present on d6 p.i. (Fig 4.8B), and because the EM study showed there were many dying cells that still retained their plasma membrane, it is likely that cells with irregularly-shaped nuclei were in the process of dying. These cells were possibly dying lymphocytes and/or blasts, cell types which appeared in very large numbers on d6 of infection. Examples of pyknotic nuclei of presumptive lymphocytes were also seen (Fig 4.7B lower right hand corner, and Fig 4.7F).

Activated macrophages were obviously involved in the phagocytosis of these dead cells. Numerous rounded F4/80$^+$, Ia$^+$ cells (Fig 4.6), and, more rarely, monocytes and stretched 'resident' macrophages were observed to contain engulfed pyknotic nuclei. Macrophages and monocytes from CSF samples examined under EM were also seen with engulfed material (Fig 4.7C,D).

There was also evidence that meningeal cells were dying during the later stages of infection (d6,7 p.i.). In many cases it was difficult to clearly distinguish such dead cells from those in the overlying infiltrate, but on numerous occasions pyknotic nuclei of pial cells were seen and the clear spaces surrounding them indicated that the cytoplasm of these cells had been in the brain parenchyma (Fig 4.8a,b).

b. Inflammation in the choroid plexus.

There appeared to be no change with time or in comparison to controls, in the number of macrophages lining the blood capillaries of the choroid plexus or the extent of Ia staining of these cells. Inflammation within the vasculature of the villi of the choroid plexus was a relatively late and unusual event, with extensive lymphocytic infiltration being observed in only one of the d7 infected animals (Fig 4.9 - compare with Fig 4.10).

Much more notable was the inflammation under the choroid plexus in the space between the brain parenchyma and the villi, and in the stalk of the plexus (Fig 4.10). These spaces surround the blood vessels supplying the plexus and, as such, may be considered Virchow-Robin spaces. First to appear at this location were elongated, 'stretched', F4/80$^+$, Ia$^+$ cells. These macrophages were seen in the non-inflamed d4 infected animals, were most plentiful on d5 p.i. (Fig 4.11) and were still obvious on d6 and d7. A few lymphocytes were closely associated with the macrophages (an indication of this association is given in Fig 4.12). By d6 and d7, they were no longer the predominant inflammatory cell type, being superceded by a large influx of lymphocytes and blasts. Also present in d7 infected animals were pyknotic nuclei and the irregularly nucleated cells described previously. Rounded F4/80$^+$, Ia$^+$ macrophages were less prominent.

c. Inflammation in the ventricles.

As with inflammation in the choroid plexus, the appearance of infiltrating cells in the ventricles of infected mice was a relatively late event compared with that in the meninges. By d7 p.i. considerable numbers of macrophages, lymphocytes and blasts were attached to the

ventricle walls and in one case there was substantial damage to this wall, judged by the presence of pyknotic nuclei and disruption of the ependymal layer. Macrophages within the ventricles were sometimes located on the choroidal epithelium and damage to this layer was observed in the one mouse from the d7 infected group whose plexus was heavily infiltrated.

d. Inflammation in the parenchyma.

Relatively little inflammation was observed in the brain parenchyma. Occasionally, tissue sections passed through the needle tract left from the virus inoculation. In the brain parenchyma of the needle track area, F4/80$^+$ and Ia$^+$ cells, some of which contained pyknotic nuclei, could be observed.

Perivascular cuffing with lymphocytes or blasts was observed only very occasionally and tended to be around larger vessels which were probably not capillaries but arterioles with surrounding leptomeninges. Inflammatory cells were observed within some parenchymal vessels and the neuropil surrounding such vessels sometimes contained pyknotic nuclei (Fig 4.13).

In general, pyknotic nuclei in the brain parenchyma were scarce, though significant considering their relative absence in gelatine saline injected controls. They were most frequently observed in the nasal lobes, especially in mice infected for 7 days, where there was also a slight, concomitant increase in the number of perivascular macrophages.

e. Numerical evaluation.

A numerical analysis of the area with the most intense inflammation, the meninges, was made on mice infected for 7 days with LCMV (Chapter 2.10). The areas within the meninges from which cells were counted were chosen randomly. The accuracy of these counts is indicated by the similar values obtained for cell types which were scored independently in sections stained with either F4/80 or anti-Ia antibodies (Table 4.1), for example pyknotic nuclei (8.0 ± 2.0 and 10.1 ± 2.2 respectively) and cells with irregularly shaped nuclei (20.9 ± 0.9 and 23.0 ± 3.7 repectively).

Approximately 20% of inflammatory cells were macrophages (Table 4.1), the majority being rounded rather than resident cells or monocytes. A minor subpopulation of these contained engulfed pyknotic nuclei with similar numbers being counted in sections stained independently for F4/80 and class II MHC antigens. Fewer monocytes stained for Ia. When the total number of macrophages staining for F4/80 was compared with those staining for Ia from the same animal, about 50% were found to be activated to express class II MHC antigens (Table 4.1).

In summary, a predominantly lymphocytic infiltration of the leptomeninges followed the i.c. inoculation of LCMV. Twenty-two percent of the inflammatory cells were of the macrophage lineage and approximately half of these expressed the class II MHC antigens.

Large numbers of dying inflammatory cells were present on the final day of examination. An early accumulation of stretched macrophages occurred in the Virchow-Robin space beneath the choroid plexus. These macrophages were often closely associated with lymphocytes. Inflammation of choroid villi and ventricles was a late event.

4.2.2 TRANSFERRED DISEASE.

Acute LCM disease can also be induced by transfer of immune spleen cells into immunosuppressed mice infected with the virus (Chapter 2.11, Fig. 2.1). Both the clinical manifestations (Gilden et al., 1972b, reviewed in Allan et al., in press) and inflammatory response, as measured by CSF cell counts, are very similar to that induced by i.c. inoculation of the virus. In this study, C57BL/6J mice were injected i.c. with Arm strain LCMV at the same time as those injected for the i.c. study described above and the same stocks of virus and mice were used. The two studies are therefore internally controlled and allow comparisons for the same time points after virus inoculation. Recipients received 10^7 immune spleen cells from primed donor mice. A control group of immunosuppressed mice, which did not develop acute LCM disease, received normal spleen cells. Cy-treated mice were sampled for immunohistological analysis on the day of spleen cell transfer (d0) and from both immune and normal cell transfer groups on the following 3 days. On the final day of sampling, one animal from the immune transfer group was dead and the remaining 4 mice were moribund.

a. The effect of immunosuppression on the histology of cerebral LCM.

The mice taken on the day of cell transfer corresponded to those described earlier which were taken 6 days after i.c. infection, except that they had received Cy treatment on d5. A marked decrease in the level of inflammation was observed following immunosuppression, and the compact areas of lymphocytes and blasts observed in immunocompetent mice (see 4.2.1a) did not develop.

In the control groups of mice that received normal spleen cells on the day after Cy treatment, there was a marked decrease in the level of Ia expression on resident macrophages of the choroid plexus, the membranes of the Virchow-Robin spaces and the meninges.

b. Meningeal inflammation.

The extent of inflammation in the meninges was slight on the first two days following immune cell transfer and it was only on the third day after transfer that large numbers of infiltrating cells were observed.

On the 2 days following transfer, however, there was induction of class II MHC expression on some resident macrophages. In contrast, resident macrophages remained Ia^- in mice receiving normal spleen cells. The entry of some lymphocytes on the first day,

accompanied by a few blasts and monocytes on the second day, following transfer of immune cells, was observed. Also apparent was the appearance of pyknotic nuclei in both inflammatory and meningeal cells (Fig 4.14). Such cell death was observed in the previous study only when the meninges was highly inflamed.

The severity of inflammation on the third day after transfer of activated T cells was similar to that of immunocompetent mice infected for 6 or 7 days. Most predominant were $F4/80^+$ cells, but only a few were positive for class II MHC antigens (compare Fig 4.15, A and B). Both rounded macrophages and monocytes were numerous. Lymphocytes and blasts, though also numerous, were not the major cell type as seen previously in the menigitis of i.c. infected animals. Also in striking contrast, was the scarcity of cells with irregularly shaped nuclei. Pyknotic nuclei, though, were equally abundant, and some were engulfed by $F4/80^+$ cells (Fig 4.16).

c. Inflammation in the choroid plexus.

Ia staining of the resident macrophages lining the capillaries of the choroid plexus villi was similar to that of the gelatine saline-injected controls in the d0 Cy-control group. On subsequent days following transfer of normal spleen cells or, on d1 and d2 following transfer of immune spleen cells, no class II MHC antigen could be detected in the choroid plexus. Small numbers of Ia^+ were observed in the choroid plexuses of the d3 immune transfer group. This decrease in Ia expression was dramatic. Ia expression on other resident macrophages (such as those of the Virchow-Robin space) and the meningeal membranes was also reduced in the d0 Cy-control group, and was virtually absent in the Cy-treated groups that received normal spleen cells.

The area under the plexus showed no inflammation until the third day after immune cell transfer. At this stage, there were large numbers of rounded macrophages and lymphocytes. Monocytes, $F4/80^+$ and occasionally Ia^+, were seen, as well as some cells with irregularly shaped nuclei. Pyknotic nuclei, some engulfed by macrophages, were notable. In general, the villi of the choroid plexus remained uninvolved in the inflammatory response.

d. Inflammation in the ventricles.

Inflammation within the ventricles was much more severe in this form of LCM disease than that described earlier, and was accompanied by disruption of the ventricle wall and ependymal cell death (Fig 4.17a,b). There were numerous round macrophages, some containing pyknotic nuclei and, even more numerous, were $F4/80^+$ monocytes. Few of these cells stained for Ia. Lymphocytes and blasts were also abundant.

e. Numerical evaluation.

Numerical evaluation confirmed the differences observed between the direct and transferred forms of LCM disease (Table 4.1). A greater proportion of the inflammatory cells

were F4/80$^+$ (45.3% compared with 21.9%). All macrophage types, rounded and stretched, as well as monocytes, were increased in number. However, there was no increase in the number of phagocytosing macrophages. A significant decrease in the level of Ia staining was confirmed. Less than 20% of the total macrophage population expressed class II MHC antigens compared with approximately 50% of those in the normal infection. The other significant difference was the scarcity of cells with irregularly shaped nuclei in mice with the transferred disease.

In summary, although the clinical development of LCM disease following transfer of immune cells was similar to that following i.c. inoculation of the virus, there were considerable differences in the nature of the inflammatory exudate in the meninges. Most pronounced was a much larger contribution by cells of the macrophage lineage in the transferred disease. There was also a relative absence of cells with irregularly-shaped nuclei and the level of Ia staining of both inflammatory and resident macrophages was much lower. This probably reflects the use of a high dose of Cy in the recipients. Inflammation in the ventricles and destruction of the ependymal layer was more severe in this form of the disease.

4.2.3 LYT2 AND L3T4 DEPLETION OF THE TRANSFERRED IMMUNE CELLS.

To determine the cell type that was responsible for the recruitment of macrophages observed in the study described above, antibody and complement depletion of the immune cell population prior to transfer,was performed to remove either Lyt2 or L3T4 bearing cells (Chapter 2.13).

Treatment with the anti-Lyt2 or anti-L3T4 antibodies plus complement removed 45.5% and 9.5% of the immune spleen cells respectively. Complement treatment alone had no effect on the number of live cells or on the cytotoxic activity of the immune spleen cells. Removal of L3T4 bearing cells also had no effect on the cytotoxicity, whereas it was substantially reduced by anti-Lyt2 treatment. Cytotoxic activity was determined by the method described in section 2.15 and the percent specific lysis at the effector to target ratio of 25:1 was as follows: untreated immune spleen cells, 38%; spleen cells treated with C only, 36%; L3T4-depleted spleen cells, 30%; and Lyt2-depleted spleen cells, 8%.

The same protocol as that described above for inducing transferred LCM disease (section 4.2.2) was used, except that 1.75×10^7 spleen cells were given to recipient mice. The groups of transferred cells were; immune spleen cells which were treated with 1) complement only, 2) anti-Lyt2 mAb plus complement, 3) anti-L3T4 mAb plus complement and 4) a control group of normal spleen cells.

The development of LCM disease in this experiment differed somewhat from that observed in the previous experiment in that the severity of inflammation at 3 days after transfer of immune cells was much reduced. This reflects the variation between experiments which is found when using this protocol of disease induction. Inflammation, as severe as that found in the previous experiment, was seen at later time points when activation of naive cells in the transfer inoculum could be expected. The nature of the disease which develops in mice that survive longer than 3 days after immune cell transfer will be discussed in chapter 5. For the purpose of this chapter, only the acute disease which develops within the first few days of transfer will be considered.

The meninges of mice receiving normal or Lyt2-depleted cells showed little evidence of inflammation on either d2 or d3 after cell transfer.

There was essentially no qualitative difference in the nature of the inflammatory response for the immune and the L3T4-depleted transfer groups, although the extent of inflammation in the latter was slightly less. In the following description they will be referred to, together, as the group receiving immune spleen cells.

Minor inflammation was observed in the groups receiving immune cells on d2, but on d3 a moderate level of infiltration was observed. The major infiltrating cell type, $F4/80^-$ and Ia^-, contained obvious lymphocytes and many blasts. $F4/80^+$ monocytes were present. Ia^+ rounded macrophages and fewer Ia^+ monocytes were seen. Cell death, determined by the presence of pyknotic nuclei, was also observed.

In the villi of the choroid plexus, there was an absence of Ia expression by resident macrophages until the third day after transfer, when the immune cell group had relatively high levels of class II MHC expression.

Again, the more significant inflammation of the choroid plexus was not in the villi, but in the space between the plexus and the brain parenchyma. On the second day after transfer of immune cells there was a localisation of stretched Ia^+ macrophages and a few lymphocytes to this area. By the third day following transfer more extensive inflammation was seen in this area in the immune transfer groups with an influx of lymphocytes and some blasts.

Ventricular inflammation in the immune group on d3 was much more severe than any seen in the previous experiment, even though meningeal inflammation was comparatively moderate (Fig 4.18). Also in contrast to the previous experiment, most of the macrophages at this site expressed Ia antigens. Lymphoblasts were present and deterioration of the ependymal wall was apparent (Fig 4.19).

In summary, this experiment determined that reduction of $Lyt2^+$ cells in the transfer population reduced the extent of meningitis and the recruitment and activation of macrophages. Depletion of L3T4 bearing cells, however, had little effect on these parameters.

4.3 DISCUSSION.

Immunohistochemical staining for the F4/80 antigen has determined the localisation of macrophages during LCM (Sections 4.2.1 and 4.2.2). Macrophages constitute about 20% of the inflammatory cells in LCM induced by i.c. inoculation and 45% in LCM resulting from adoptive transfer of immune cells (Table 4.1). Macrophages are recruited to the CNS by $Lyt2^+$ cells (Section 4.2.3).

Macrophage localisation.

Monocyte entry into the meninges was most prominent in the disease transferred by immune cells. In both forms of LCM induction, however, the expression of class II MHC antigens on monocytes was very low in comparison to the level on other types of macrophages (Table 4.1). This suggests that activation to express Ia antigens occurred in the brain in response to locally released lymphokines, and was not a feature of circulating monocytes.

Although Ia expression by monocytes remained low throughout infection, a slight increase was observed in that few Ia^- monocytes were observed in very inflamed meninges at the end of the disease. At this stage, also, $F4/80^-$ monocytes were far less obvious than earlier in the disease. The levels of F4/80 antigen has previously been shown to decrease on some stimulated macrophages and appears to correlate with increased plasma membrane fluidity in conjunction with increased adherence or spreading (Austyn and Gordon, 1981; Ezekowitz et al., 1982). The decreased expression of this antigen on monocytes that was observed in the early stages of the disease may reflect increased plasma membrane spreading and motility associated with the recruitment of the monocytes into this locality.

Monocytes presumably differentiate into fully functional macrophages within the CNS, although they are, themselves, capable of phagocytosis. $F4/80^+$ and Ia^+ cells with cresent shaped nuclei, containing phagocytosed pyknotic nuclei, were observed and EM analysis has also revealed that monocytes contain phagocytosed material (Fig 4.7D). Schwendemann et al. (1983) have also observed phagocytic monocytes during EM examination of brain tissue from mice inoculated i.c. with LCMV. The ability of monocytes to contribute to an inflammatory response without further differentiation into more mature macrophages has been demonstrated in a number of other systems (Feinman et al., 1986; Tweardy et al., 1986; Wright et al., 1986) and it is likely that the same is true in LCM disease. However, monocytes contributed only a minor portion of the total $F4/80^+$ population (Table 4.1) and their functional importance was probably outweighed to some extent by other more numerous macrophages.

Many monocytes were found in the ventricles of mice 3 days after immune cell transfer. This was accompanied by severe meningitis which also contained numerous monocytes, but there were far fewer monocytes in the equally severe inflammation under the choroid plexus or within the plexus villi. Therefore, it seems unlikely that monocytes infiltrate the ventricles via the choroidal epithelium. It is possible that infiltration into the ventricles was retrograde from

the subarachnoid space, where there was an abundant supply of monocytes, via the cisterna magnum back to the ventricles, and occurred against the flow of CSF. Walker and colleagues (1975), however, found ultrastructural evidence of leukocyte migration from the choroid capillaries, through the epithelial linings into the ventricles. In this case, though, the villi of the choroid plexus were infiltrated heavily.

The most predominant of the F4/80$^+$ cells in the inflammatory infiltrate were those that were apparently detached from the meningeal membranes and were round in shape. More than half these macrophages, in the disease following i.c. inoculation of LCMV, expressed the Ia activation antigens, while only 20% of those in the disease induced by adoptive transfer were positive for class II MHC (Table 4.1). This reflects the generally low level of Ia expression in mice treated with Cy. Is Ia expression necessary for efficient macrophage function? In animals infected for 7 days with LCMV the same percentage of Ia$^+$ cells as F4/80$^+$ cells contained engulfed pyknotic nuclei, suggesting that phagocytic activity is restricted to activated macrophages. Engulfed nuclei probably indicate a constant proportion of the macrophages with phagocytic activity in the two forms of disease in which there was a similar level of dead cells. In this case, it would appear that, although there were many more macrophages in the transferred form of the disease, there was not a similar increase in the number showing evidence of phagocytoses. This observation supports the view that it is activated macrophages, expressing the class II MHC antigens, which are functional in phagocytosis.

There was no apparent increase in the number of resident macrophages associated with the general inflammation within the meninges. However, there was specific localisation of stretched F4/80$^+$, Ia$^+$ cells in the Virchow-Robin space under the choroid plexus. Since these macrophages appeared at around the same time as the first infiltrating lymphocytes, they are probably not mobilised by local T cell activation. Lymphocytes were often seen in close contact with these macrophages which were possibly acting as a targeting population for other incoming inflammatory cells

The relative absence of Ia expression on resident macrophages in the choroid plexus of Cy-treated mice, with no observable effect on F4/80 expression, suggests that Ia expression is regulated by a Cy sensitive mechanism. Immunosuppression may decrease the level of circulating or local lymphokines which maintain Ia expression on resident macrophages. The fact that class II MHC antigen is again expressed in the choroid plexus within three days after transfer of immune spleen cells but not after transfer of normal cells, also suggests that resident macrophages can be activated by local inflammation. Thus, it would appear that resident macrophages can be activated by an immunological stimulus.

Although the resident macrophages of the brain parenchyma, the microglia, have previously been shown to stain for the F4/80 antigen (Hume et al., 1983a; Perry et al., 1985), F4/80$^+$ microglial cells were not observed in the studies reported here. Lack of F4/80 antigen expression probably reflects the greater sensitivity of this antigen to denaturation with PLP fixation as compared with the O.5% gluteraldehyde as used by Hume et al., (1983a) and Perry

et al., (1985). This suggests that the density of the macrophage marker on microglia is somewhat lower than that on circulating macrophages, and other resident macrophages such as those of the choroid plexus and meninges.

Difference in the macrophage populations between the two forms of LCM disease.

One of the most obvious differences in disease resulting from the two forms of induction of LCM was the difference in the relative numbers of F4/80$^+$ cells in the inflammatory exudate - a higher proportion of macrophages being observed in the transferred disease. This did not seem to affect the outcome of the infection with respect to the mortality rate, but it is still an interesting phenomenon. One explanation is that activated macrophages which were present in the transferred cell population were infiltrating the brain along with immune T cells. However, Ceredig et al (manuscript in preparation), using the same tranfer model have shown that the cells infiltrating the CNS are of both host and donor origin. They made use of the two different alleles of the Pgp (Trowbridge et al., 1982) and Thy-1 antigens, and flowcytometric analysis of CSF exudate cells to differentiate between transferred and recipient inflammatory cells that infiltrate the CNS. T cells in the CSF were of donor origin and also expressed the donor Pgp allele, while non-T cells were all of recipient origin. Thus, it seems unlikely that macrophages that originate from the donor immune population contribute to the meningeal inflammation.

Macrophage localisation to the CNS would most likely occur in response to lymphokines released from activated immune T cells recognising virus-infected brain cells. It is possible that the distribution of immune T cells given i.v. in transferred LCM recruit macrophages more effectively than T cells localised after i.c. inoculation of the virus. Alternatively, priming of donors with the viscerotropic WE3 strain of LCMV may activate T cells which produce a range of lymphokines more conducive to macrophage recruitment than that of lymphocytes primed with the neurotropic Arm strain of virus.

An alternative explanation for the relative abundance of macrophages in LCM resulting from the adoptive transfer of T cells, is that the immune response which had already been initiated in the mice by d5 of infection, before immunosuppression, was sufficient to initiate the mobilisation of macrophages from precursors in the bone marrow. Four days later (3 days after transfer) many more macrophages would then be available to respond to a new wave of infiltration initiated by the transferred immune spleen cells. Lymphocyte activation had, indeed, occurred by d5 after i.c. inoculation, as demonstrated by the appearance of this cell type within a mildly inflamed meninges. Stimulation of these lymphocytes by virus infected cells in the brain would therefore result in the release of lymphokines that would effect mobilisation and activation of macrophages.

A preferential sparing effect on macrophages by Cy may then have allowed the proliferation of this cell type to continue uninterrupted by the drug treatment. Macrophages

and monocytes, in general, appear to be more resistant to the immunosuppressive effects of Cy than lymphoid cells, although administration of the drug results in an initial monocytopenia (d3-5) followed by extreme monocytosis (Buhles and Shifrine, 1977). The drug, however appears to have no direct effect on the survival or viability of monocytes in vitro (Balow et al., 1977) while, in vivo, the radiosensitive influx of macrophages from blood monocytes into peritoneal tumors continues after Cy treatment (Dye and North, 1980).

In many instances, Cy appears to enhance macrophage activity. More macrophage granulomas develop in the peritoneum of BCG-inoculated mice, if they are pretreated with Cy (Birman and Mariano, 1981). Receptor-mediated phagocytosis and incorporation of ^{3}H-thymidine of macrophages accumulating in the DTH response to SRBC is enhanced following drug treatment (Birman and Mariano, 1981). Such an increase in specific phagocytosis of peritoneal exudate cells has been demonstrated to peak on d1 following Cy administration and returning to normal levels by d13 (Giordano and Isturiz, 1983). Other workers, however, have found no change in the phagocytic capacity of peritoneal exudate macrophages following drug treatment (Luster et al., 1981). The sparing effect for macrophages by Cy appears to extend to their precursors in the bone marrow in that, although general spleen colony forming units are greatly reduced by in vitro treatment with Cy metabolites, granulocyte-macrophage colony forming units are relatively spared both in vitro (de Jong et al., 1985) and in vivo (Buhles and Shifrine, 1978).

It is not known why the macrophage lineage is relatively unaffected by this drug. They do not proliferate less than other cell types whose functions are suppressed, and their response to growth factors is unlikely to differ from those of other cells to keep them in a less susceptible stage of the cell-cycle. The possibility that their DNA repair mechanisms are more capable of overcoming with Cy alkylation seems unlikely since macrophage responses which are sensitive to DNA damage by γ-rays are not susceptible to the drug (Dye and North, 1980). It is more likely that this cell lineage is capable of inactivating Cy metabolites before they initiate DNA damage. It is also conceivable that the strongly electophilic nature of alkylating agents (Connors, 1975) makes them more susceptible to the antioxidant machinery of the macrophage. Evidence supporting this possibility is the finding that resistance of pluripotent hematopoietic cells to Cy is abrogated by inhibitors of the enzyme aldehyde dehydrogenase (Kohn and Sladek, 1985).

Thus, Cy treatment of LCMV-infected mice may suppress the T cell response to the virus without affecting the proliferation and differentiation of monocytes and macrophages. The fact that macrophages did not accumulate in the CNS in the first few days following drug administration probably resulted from the absence of migration inhibition factors that would normally be released from activated T cells. It would be interesting to determine the relative contribution of macrophages to the inflammation in mice suppressed by γ-irradiation. This form of immunosuppression is known to inhibit the replication of mononuclear phagocytes.

There may, also, be a negative feedback loop on macrophage recruitment which is

dependent on the presence of activated macrophages such as those that express Ia antigens. Thus, in the disease induced by transferred immune cells, where the ratio of Ia^+ to Ia^- macrophages was lower, there may have been a continual recruitment of macrophages from the blood stream to supplement the number which were activated. Circulating monocytes may have matured into end stage macrophages without the influence of lymphokines from activated T cells during the first 3 days after Cy administration, and consequently became refractory to these lymphokines. Dye and North (1980) found a similar effect of Cy on the tumoricidal activity of peritoneal cells which could not be activated by endotoxin 6 days after treatment with the drug even though large numbers of macrophages had accumulated in the peritoneum of the tumor bearing mice. The authors suggested that Cy had no direct effect on macrophage function, but decreased the levels of sensitised lymphocytes which were providing stimulation for tumoricidal activation.

Thus, a number of factors possibly contribute to the observed predominance of macrophages in LCM induced by the transfer of immune cells. Sparing of mononuclear phagocytes, in contrast to lymphoid cells, by Cy is probably a significant factor in this phenomenon.

Brain Surface Pathology.

Pathological destruction of cells lining the surfaces of the brain has not, in the past, been reported for infection with the Arm strain of LCMV. This includes the leptomeninges as well as the ependyma and choroid plexus of the ventricles, all of which show widespread infection with the virus (Tosilini and Mims, 1971, Walker et al., 1975). Some deterioration of epithelial cells in the choroid plexus was observed in mice whose LCM disease was extended with the use of anticonvulsants (Walker et al., 1975). Schwendemann et al. (1983), however, did find evidence of cell death in the meninges and choroidal epithelia of mice 6 days after i.c. infection with the WE strain of LCMV.

In the study presented in this chapter, apparent destruction of meningeal cells was observed at the latter stages of LCM disease. Because extensive cell death within the inflammatory exudate was also occurring, it is difficult to establish the full degree of this destruction, as pyknotic inflammatory cells lying close to the pial and arachnoid membranes may have been mistaken for meningeal cells. However, the occasional observance of indentations in the brain surface containing pyknotic nuclei strongly suggested that at least some leptomeningeal deterioration was occurring.

Much more obvious was the destruction of cells lining the walls of the ventricles. Here, pyknotic, necrotic and absence of easily defined, ependymal cells were observed. Such deterioration of the ventricle walls was seen frequently in mice with transferred LCM, but observed in only one mouse on d7 after i.c. inoculation. It generally occurred in the vicinity of small clumps of inflammatory cells which consisted of lymphocytes, blasts, monocytes or macrophages. Although minor ependymitis has been reported by other workers (Walker et al.,

1977; Schwendemann et al., 1983) pathological changes in the lining of the ventricles was not observed (Tosilini and Mims, 1971; Walker et al.,1975), even when LCM disease was extended with the use of anticonvulsants (Walker et al., 1977). Destruction of ependymal cells probably plays no role in the development of clinical LCM disease. Mims and Murphy (1973) observed a complete stripping of the ependymal lining, and Schwendemann and Lohler (1979) also found pathological alterations to the ventricle walls, in mice that had recovered from the neurological complications of Sendai virus infection.

No pathological disturbance of the choroid plexus was detected in this study, an observation which is in agreement with that of Tosilini and Mims (1971). Ultrastructural studies of the plexus of LCMV infected mice, however, has shown that minor loss of microvilli from some epithelial cells does occur (Walker et al., 1977). The authors suggest that this loss is insufficient to markedly affect CSF production. Infection with the WE strain of LCMV, however, does result in death of cells in the choroidal epithelium (Schwendemann et al., 1983).

Presumably cell death in the ependyma and leptomeninges results from the inflammatory response to infected cells. One might assume that, since this response is mediated by $Lyt2^+$ T cells, cell death is effected by the cytotoxic activity of these cells which recognise virus antigen in association with class 1 MHC molecules on the surface of infected cells. Such cytotoxicity, although readily demonstrated against target cells in vitro, has not been clearly shown in vivo. Studies in other systems in which tissue damage is mediated by $Lyt2^+$ T cells suggest that it is not the cytotoxic activity of these cells which is responsible. In these studies cyclosporine A, a drug which inhibits lymphokine release from activated lymphocytes without affecting their cytotoxic potential, was found to inhibit the ability of transferred T cells to, 1) effect class I MHC restricted GVHR (Hodgkin et al., 1984), 2) initiate an immune response to influenza in the lungs of infected mice (Schiltknecht and Ada, 1985a,b) or 3) effect rejection of pancreatic islet allografts (Hodgkin et al., 1985). Since the cytotoxic potential of the transferred cells remained intact, the authors concluded that it was not this arm of the T cell defense which was responsible for tissue damage. It is possible that the lymphokine release inhibited by cyclosporine was required for the recirculation of activated T cells rather than for the initiation of tissue damage. However, the drug prevented graft rejection when T cells were transplanted together with the graft (Hodgkin et al., 1985) and footpad swelling was inhibited in allogeneic mice injected directly into the footpad with activated T cells (Hodgkin et al., 1984). Under both of these conditions lymphocyte recirculation is not required. Cyclosporin A treatment also spares the majority of mice acutely infected with LCMV (Saron et al., 1984), supporting the view that mortality is not due to T cell cytotoxicity.

It also seems likely that the $Lyt2^+$ T cell response to LCMV does not evoke strong cytotoxicity activity in vivo. Certainly viral clearance from the brain parenchyma of carrier mice can be achieved by cloned $Lyt2^+$ T cells without cell death or even close contact between

lymphocytes and infected brain cells (Oldstone et al., 1986). However, Schwendemann et al. (1983), using electron microscopy, claim that occasional contact between lymphoblasts and potential target cells in the leptomeninges and choroid plexus as well as the close spatial association between blasts and some dead cells, is indicative of T cell mediated cytolysis. However, close contact between the virus-MHC complex on the target cells and the T cell receptor on the effector cells must occur for both lymphokine release and cytotoxic activity. Furthermore, Pfau and colleagues (1985), working with an 'aggressive' and a 'docile' strain of LCMV, found that the cytotoxic capacity of T cells within the meningeal infiltrate was similar for i.c. infection with both viruses, even though only the 'aggressive' strain caused cerebral disease. Thus, the possibility that cell damage in the brains of infected mice results from the DTH response, rather than the cytotoxic activity of class I MHC-reactive T cells , can not be excluded. Such damage may be mediated by macrophages.

In summary, histological examination of the brains of mice infected with LCMV indicates that both lymphocytes and macrophages are prominent in the meningeal infiltrate. Macrophages that localised at the base of the choroid plexus early in the disease, and those that were obviously phagocytic at later stages of infection, were activated to express the class II MHC antigens. Infiltrating monocytes, however, were generally Ia^-. This suggests that activation of macrophages occurs at the site of inflammation.

The proportion of macrophages in the disease induced by transfer of immune cells into immunosuppressed mice was considerably higher than that in the disease following i.c. inoculation. However, at the time when the disease was clinically manifest, the overall level of meningeal inflammation was similar in both forms of LCM induction. This suggests that the relative concentrations of lymphocytes and macrophages is not crucial in determining the pathology of the disease. The predominance of macrophages in the disease induced by transfer of immune spleen cells possibly resulted from a sparing of this cell type by the immunosuppressive drug, Cy.

Removal of $L3T4^+$ cells from the transfer immune population had no effect on macrophage or lymphocyte recruitment to the brain, whereas Lyt2-depletion severely reduced inflammation. Thus, it is likely that class II MHC-restricted T cells are not important in the DTH response in LCM. Class I MHC-restricted T cells are responsible for initiating the infiltration of leukocytes into the CNS.

Immunosuppression with Cy reduced the level of Ia expression on both resident and inflammatory macrophages. Therefore, maintenance and induction of class II MHC on macrophages results from the activity of a Cy-sensitive cell type, probably T lymphocytes.

Cell death in both the inflammatory exudate and the lining of the brain parenchyma was a predominant feature in mice with LCM. However, there appears to be no correlation between the extent of this cell death and the clinical consequences of the disease.

Table 4.1 Numerical Analysis of Inflammatory Cells in the Meninges of Mice with LCM.

	DAY 7 LCMV INFECTION		DAY 3 TRANSFER	
	F4/80	Ia	F4/80	Ia
Monocytes (cresent-shaped nuclei)	2.8 ± 0.8	0.6 ±0.46	6.1 ± 1.0	0.03±0.05
Rounded macrophages	16.0 ± 3.6	9.0 ± 2.0	31.9 ± 1.8	6.2 ± 0.9
Stretched resident macrophages	3.2 ± 0.6	1.9 ± 1.2	7.4 ± 2.8	2.3 ± 0.6
Total percentage of cells stained	21.9 ± 4.6	11.5 ± 3.0	45.3 ± 1.0	8.5 ± 1.2
Rounded macrophages containing pyknotic nuclei	1.5 ±0.8	1.4 ±0.8	1.7 ± 0.8	0.8 ± 0.2
Stretched resident macrophages containing pyknotic nuclei	0	0.07±0.1	0.5 ± 0.45	0.1 ± 0
Unstained mononuclear * cells	48.3 ± 6.4	56.7 ± 11.4	46.2 ± 3.4	82.5 ± 1.8
Cells with irregularly * shaped nuclei	20.0 ± 0.9	23.0 ± 3.7	0.3 ± 0.36	0.6 ± 0.26
Pyknotic nuclei *	8.0 ± 2.0	10.1 ± 2.2	10.4 ± 2.5	9.3 ± 1.0

Numerical analysis (Chapter 2.10) was performed on inflammatory cells in brain sections from mice seven days after inoculation with LCMV and 3 days after the adoptive transfer of immune cells into infected, immunosuppressed recipients. Data for each cell type are expressed as the percentage ± S.E. of the total number of cells counted.

*Although counts were made on sections stained for F4/80 or Ia, these figures refer to cells that did not stain for either antibody.

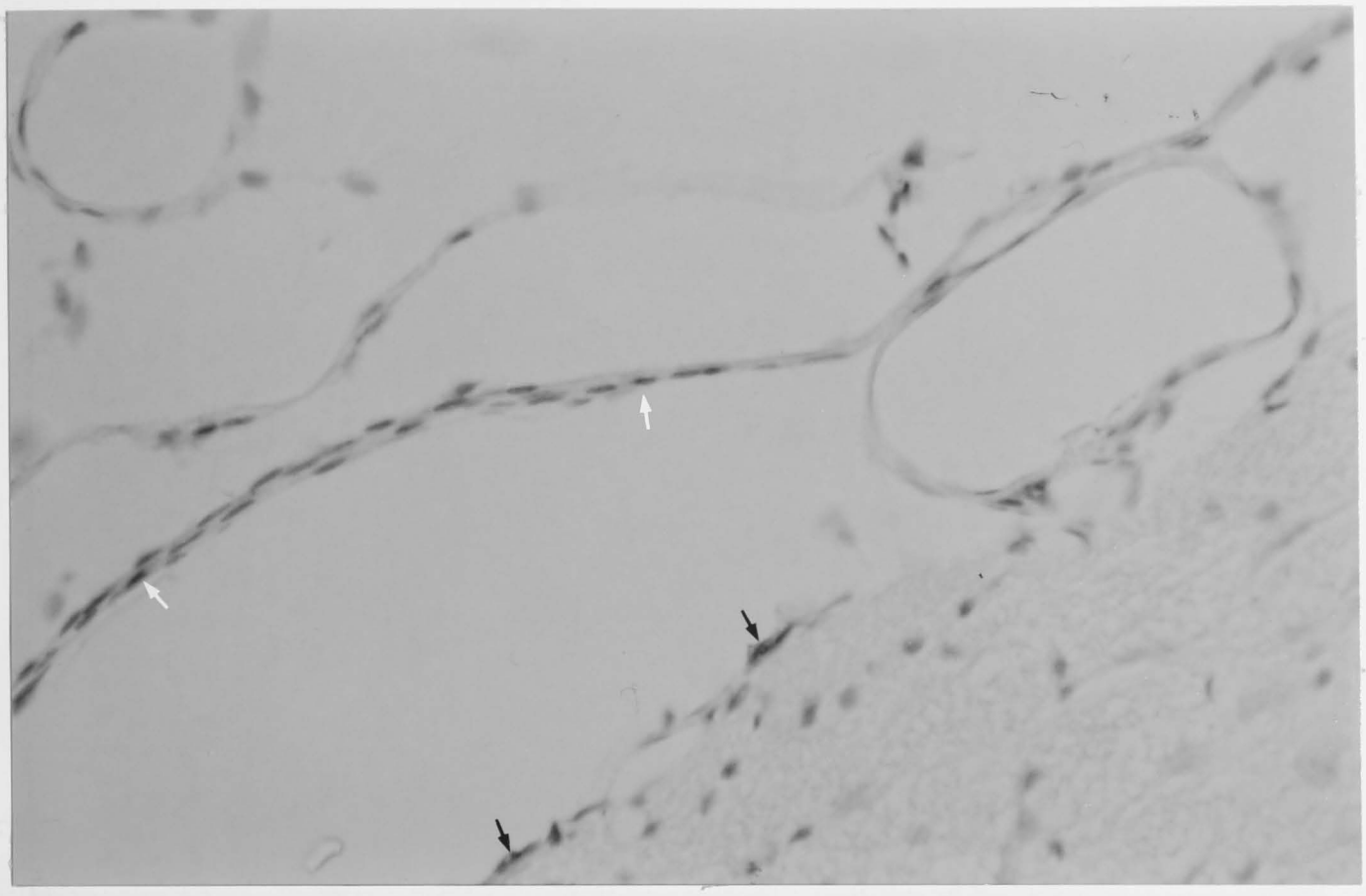


Figure 4.1 Gelatine saline-injected control brain, F4/80: examples of F4/80$^+$ cells on the pial membrane at the surface of the brain are indicated by black arrows and those on the arachnoid membrane are indicated by white arrows. Because two brain sections were side by side, there are two arachnoid membranes seen in this photogragh. The dura mater rarely remained intact during removal of the brain from the skull. Magnification x300

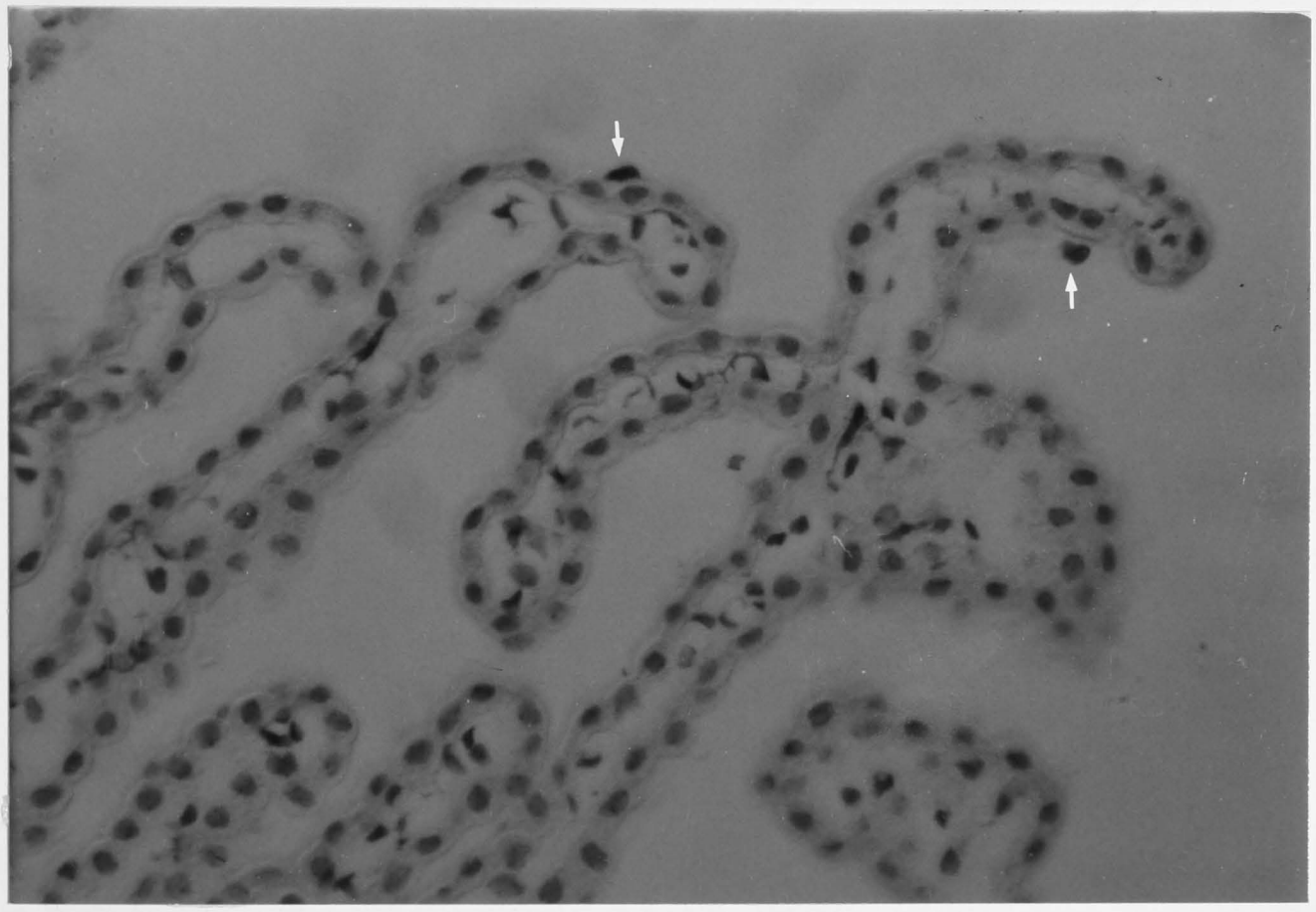


Figure 4.2 Gelatine saline-injected control brain, F4/80: in the choroid plexus of the 4th ventricle, F4/80$^+$ cells line capillaries of the villi and some are also present on the venticular surface of the choroidal epithelium (arrows). Magnification x370

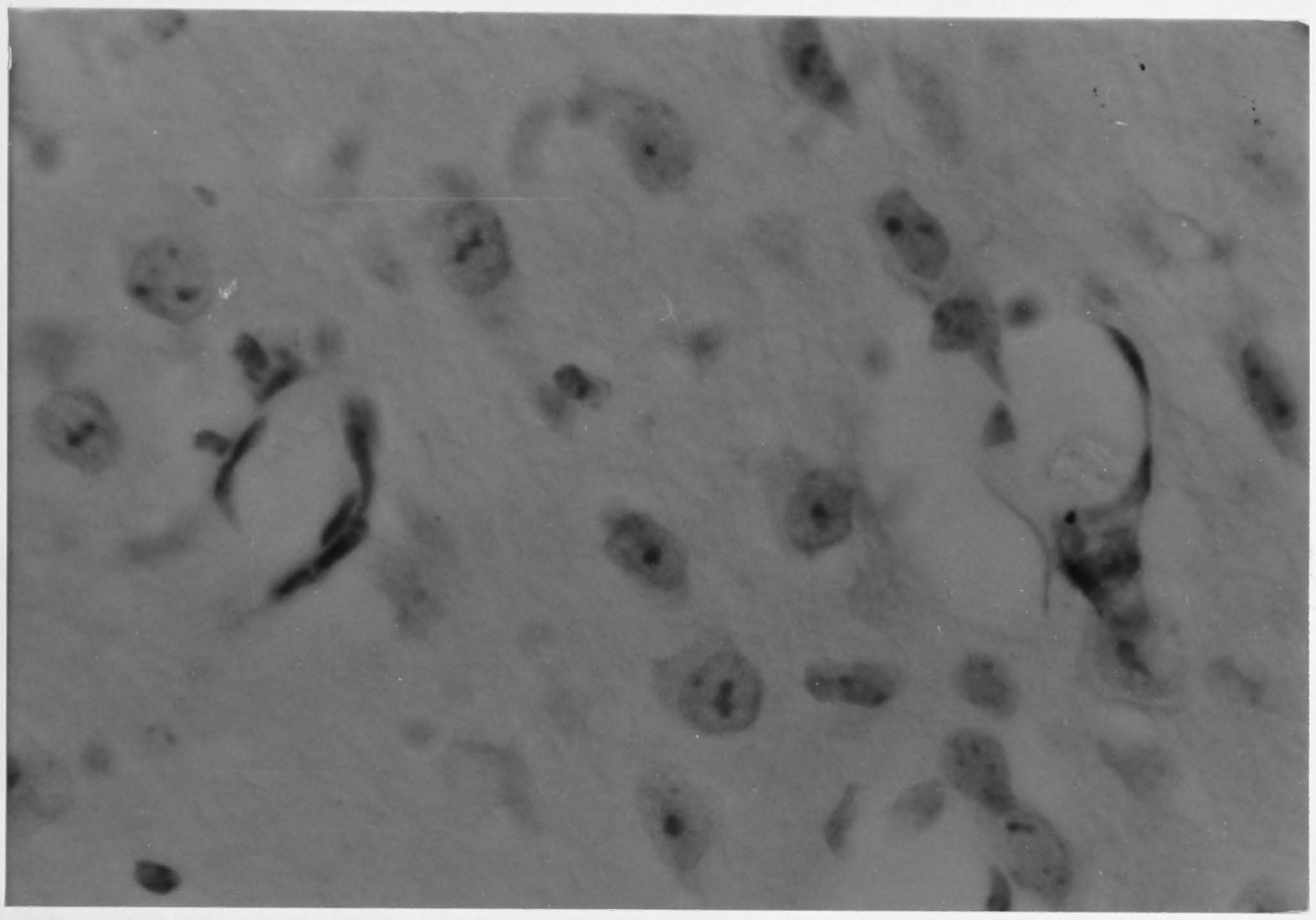


Figure 4.3 Gelatine saline-injected control brain, F4/80: perivascular F4/80^{+} cells are seen in the brain parenchyma. Magnification x730

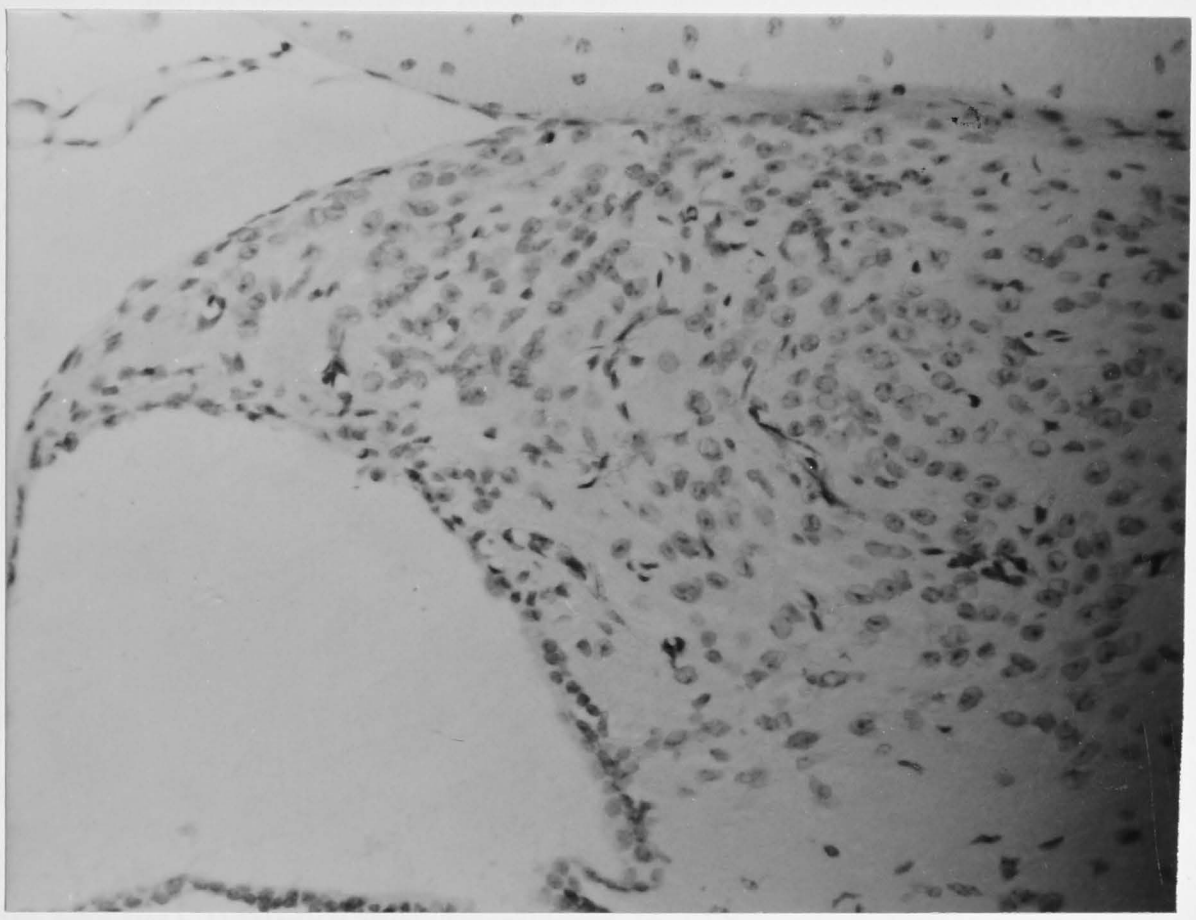


Figure 4.4 Gelatine saline-injected control brain, F4/80: F4/80^{+} cells line the sinusoids of the area postrema at the caudal end of the 4th ventricle. Magnification x230

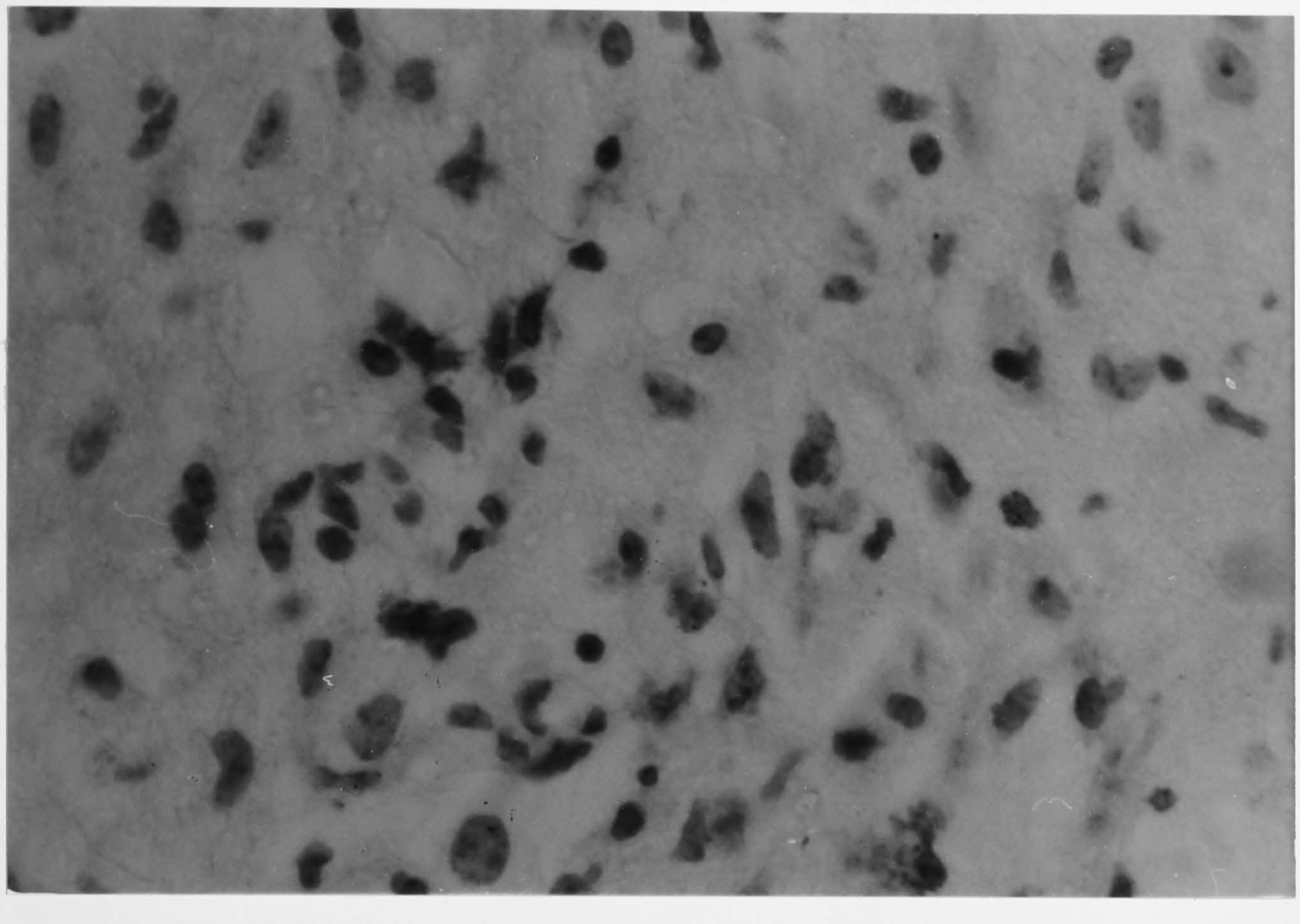


Figure 4.5 Gelatine saline-injected control brain, Ia: Ia^+ cells with star-like processes are seen in the brain parenchyma. Magnification x460

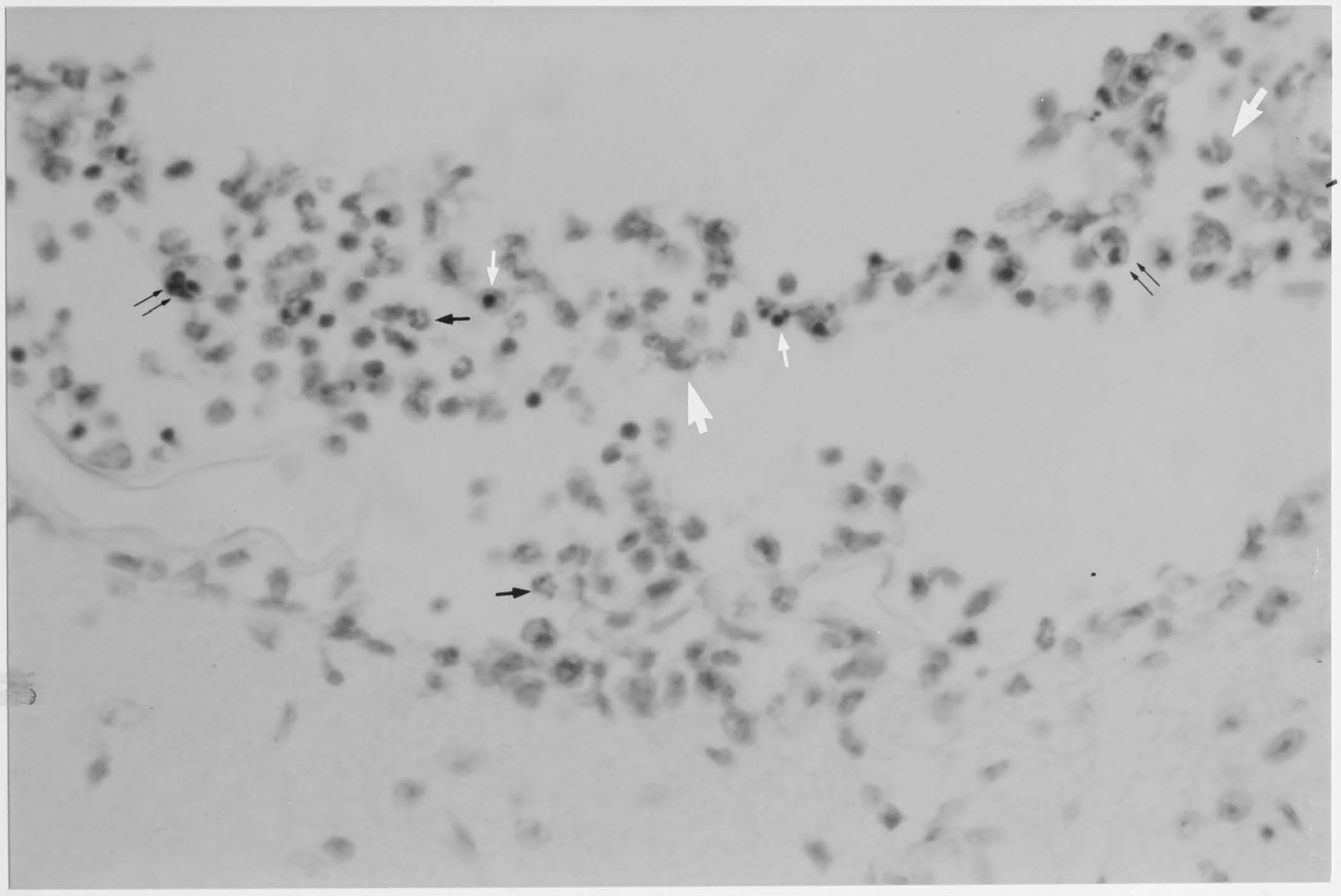


Figure 4.6 Day 7-infected brain, F4/80: meningeal inflammation consists of $F4/80^+$ macrophages, monocytes with cresent-shaped nuclei (large white arrows), pyknotic nuclei (small white arrows), lymphocytes, blasts and cells with irregularly shaped nuclei (black arrows). $F4/80^+$ cells containing phagocytosed pyknotic nuclei can be seen (double arrows) and in the cell indicated on the right, both the nucleus of the macrophage and the pyknotic nuclei can be distinguished. Variation in the extent of F4/80 staining of moncytes can be seen on the two examples indicated. Magnification x600

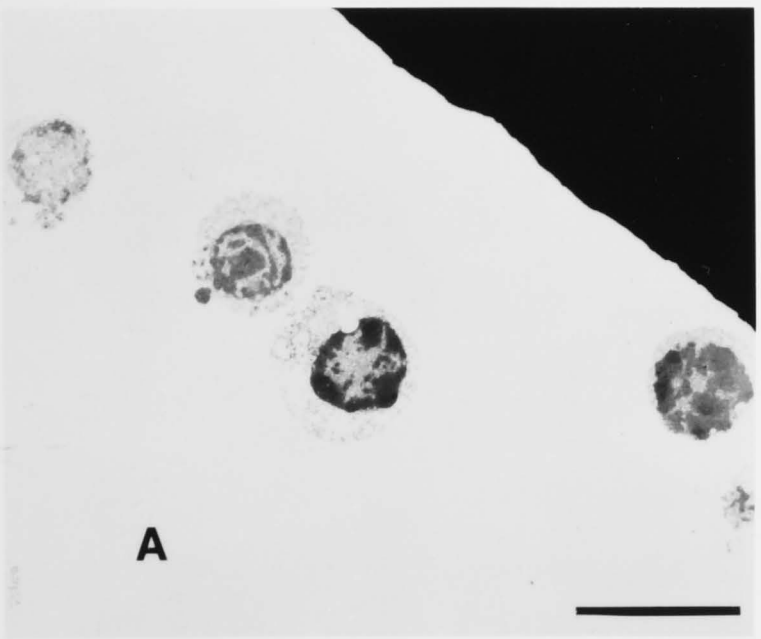
Figure 4.7 Electronmicrographs of CSF cells taken from mice on d7 after LCMV inoculation

A. Dead cells with disrupted cytoplasm and cell membrane. Variation can be seen in the extent of nuclear degeneration. Bar=10μm.

B. Healthy presumptive lymphoblasts (centre, left and upper edge) and two dead cells (lower middle and lower right). Bar=1μm.

C. Activated macrophage with extended pseudopods and phagocytic vessicle containing engulfed material (arrow). Bar=1μm.

D. Monocyte with cresent-shaped nucleus and phagocytic vessicle containing engulfed material (arrow). Bar=1μm.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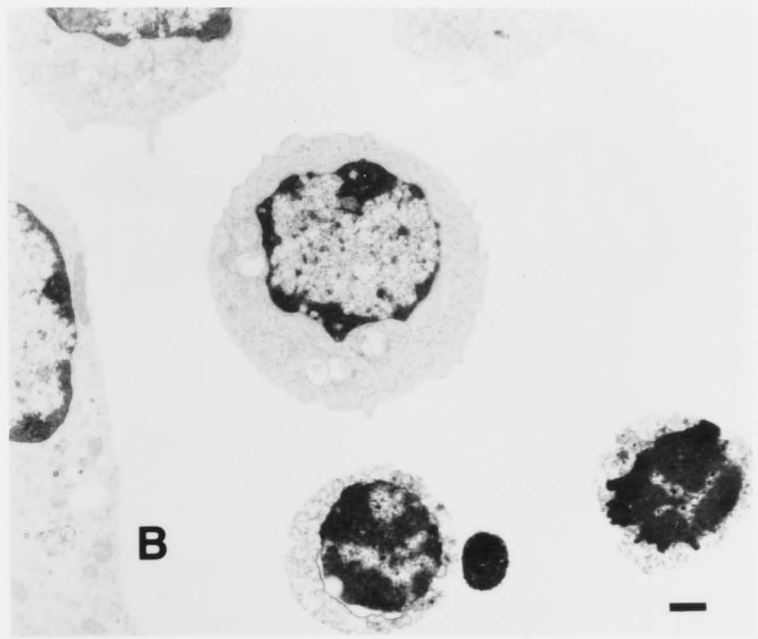
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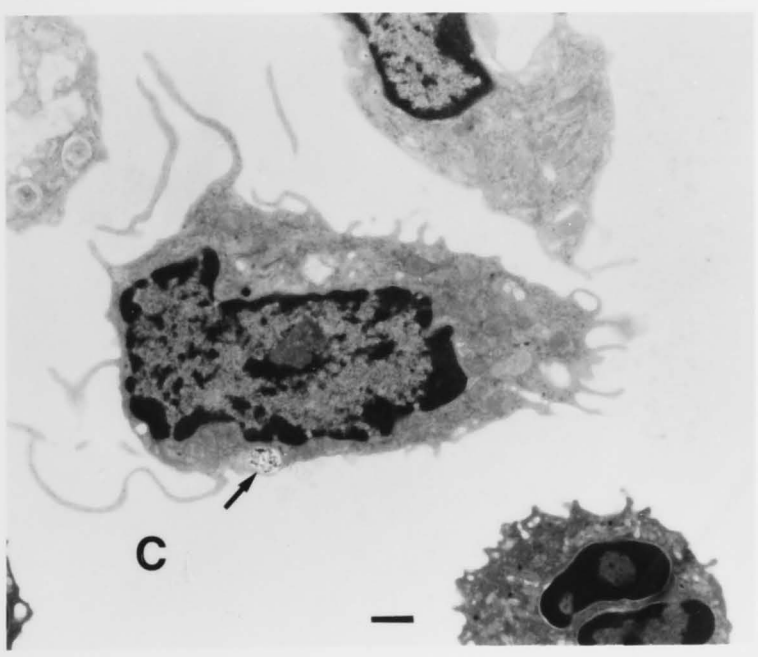
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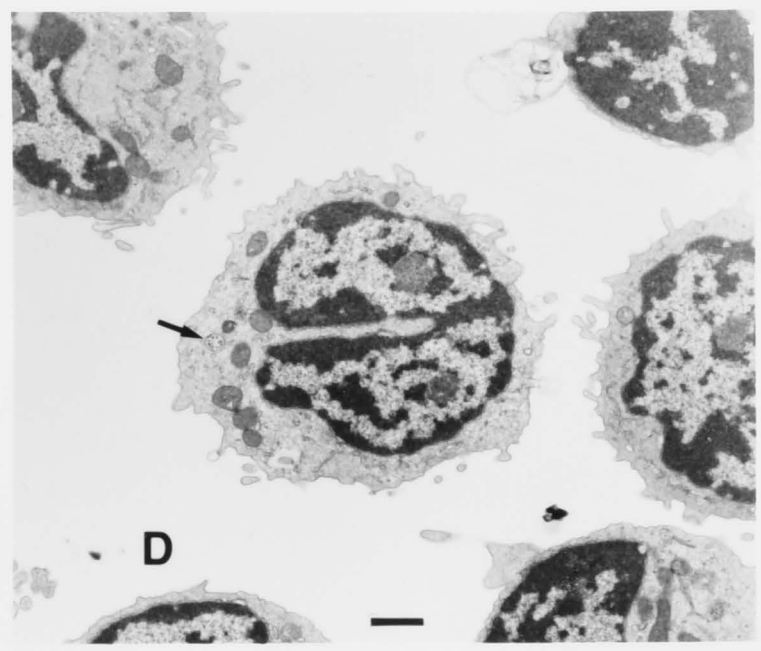
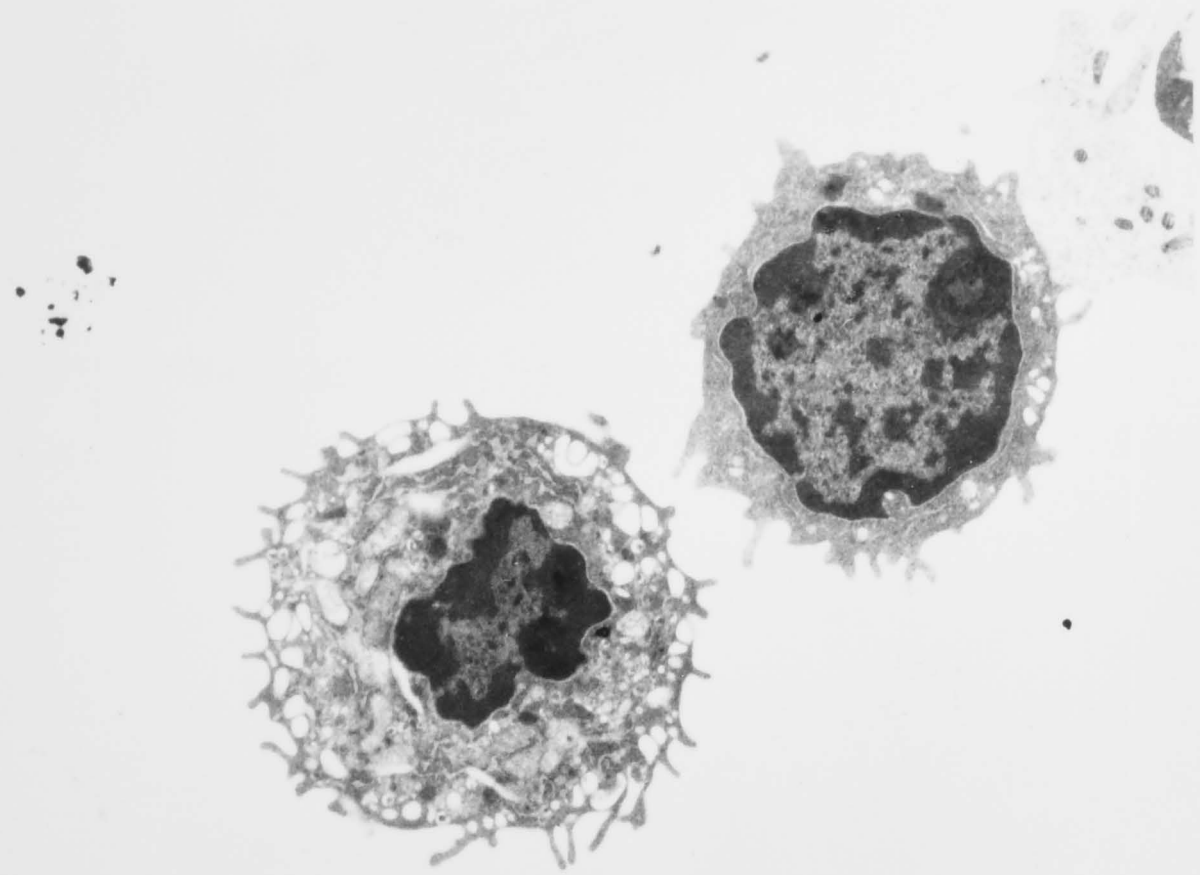

D

Figure 4.7 continued.

E. Examples of dying cells at a less advanced stage of cell death than those seen in A, B and F. The nuclear membrane has degenerated, condensation of the cytoplasm is observed and, at higher magnification (not shown), course granularity of the chromatin was seen. Bar=1μm.

F. Condensed chromatin in the pyknotic nucleus of a lymphocyte. Bar=1μm.



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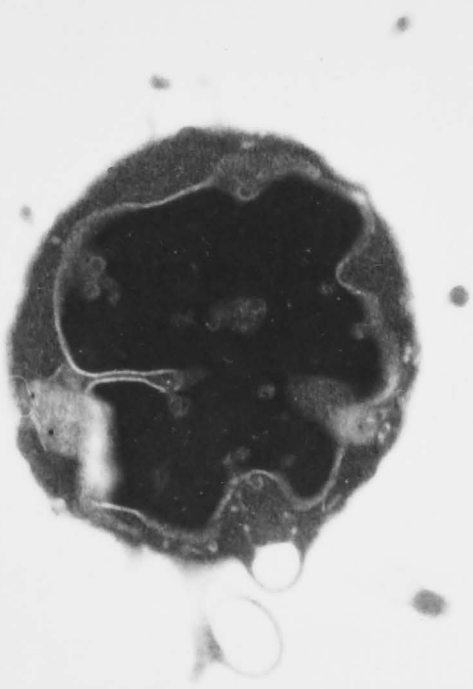
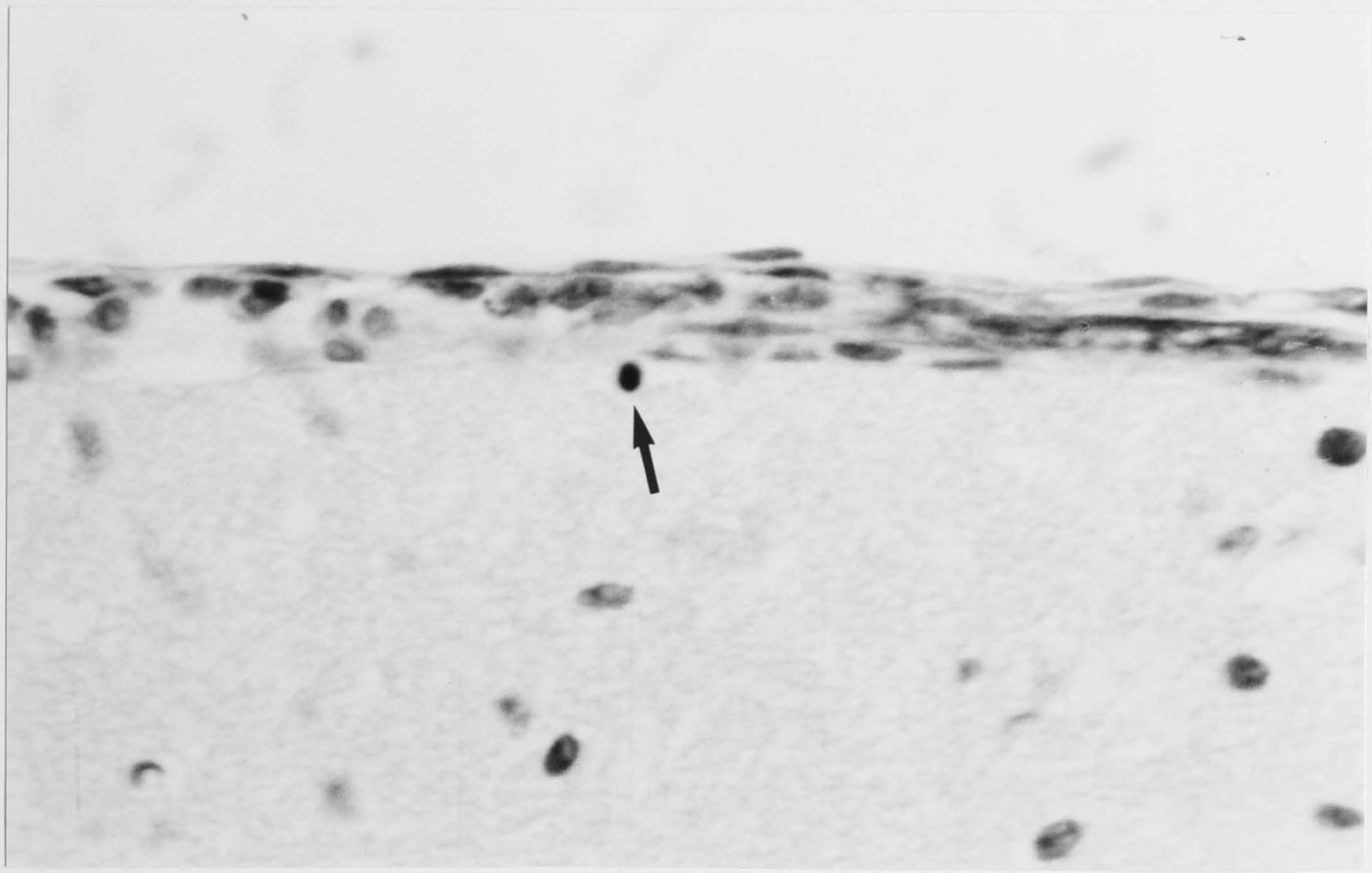


F

A



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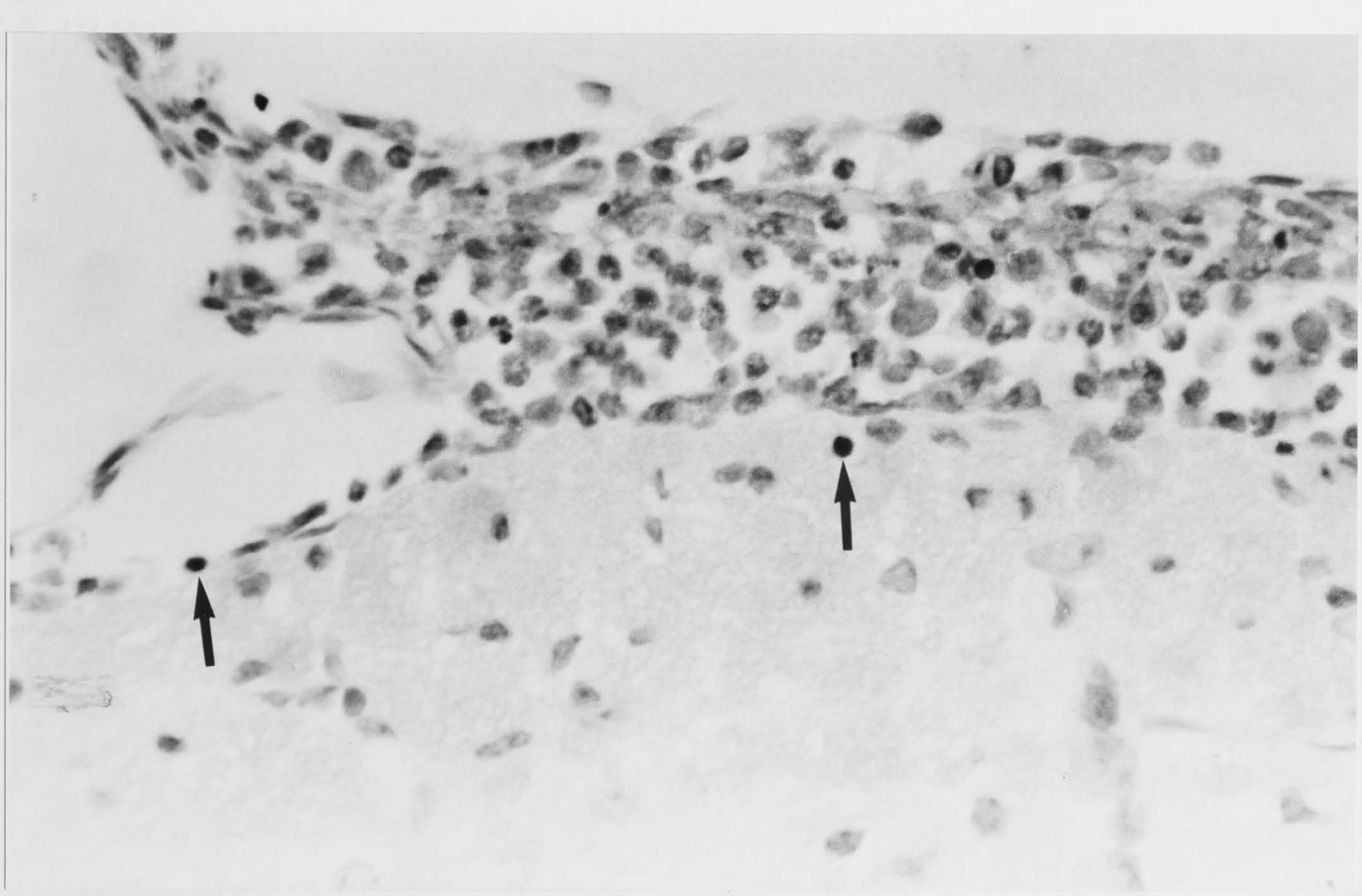


Figure 4.8 Pyknotic nuclei in the pial membrane.

A. Day 7-infected brain: F4/80. A pyknotic nucleus on the brain surface is indicated by an arrow. The area immediately surrounding this nucleus in the brain parenchyma is clear, indicating disruption of the cytoplasm. Magnification x970

B. Day 7-infected brain: Ia. Pyknotic nuclei on the brain surface are indicated by arrrows. A large number of inflammatory cells with irregularly shaped nuclei can be seen in the meningeal infiltration. Magnification x600

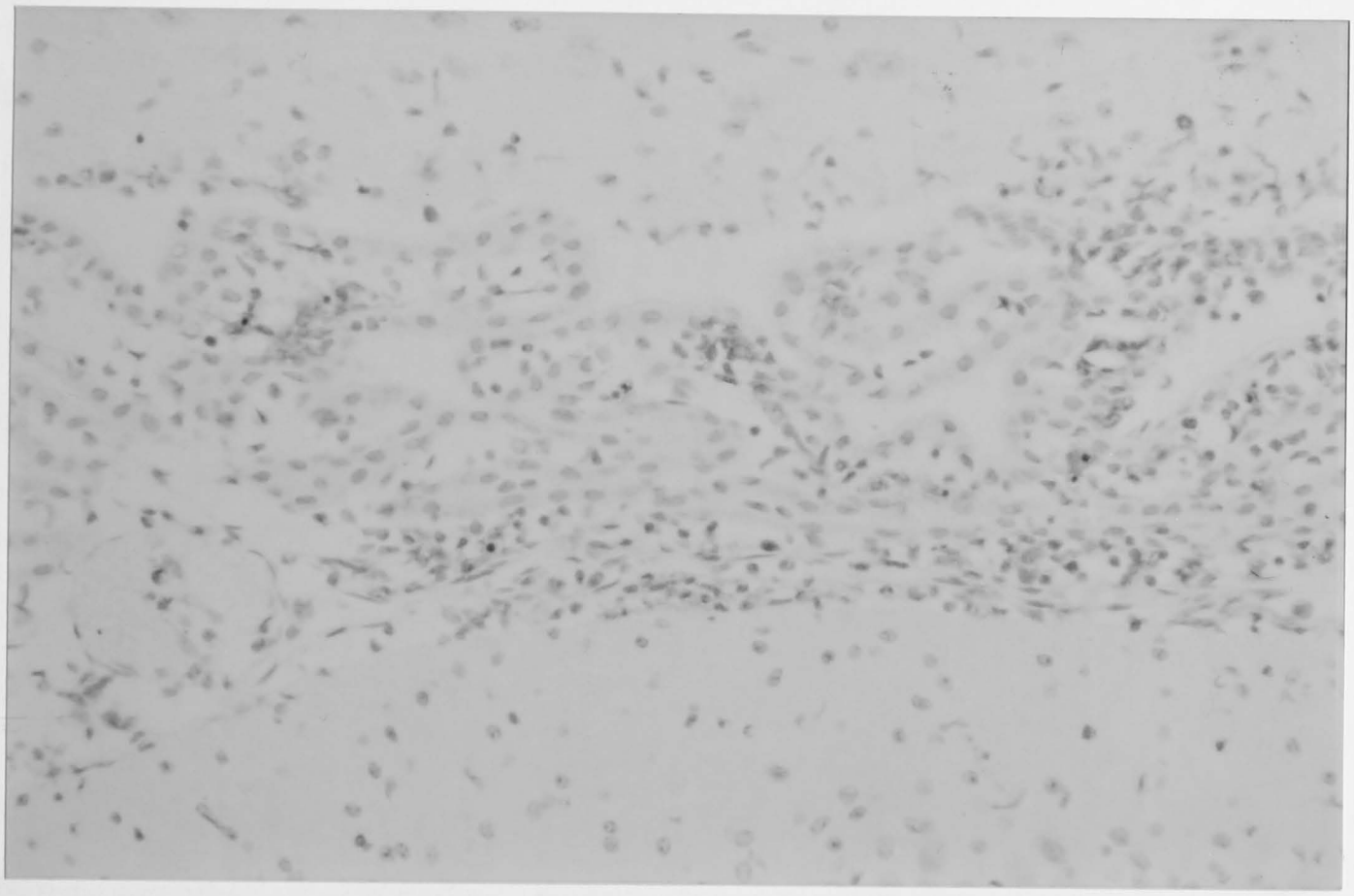


Figure 4.9 Day 7-infected brain, F4/80: the villi of the choroid plexus are infiltrated with both F4/80$^+$ and F4/80$^-$ cells. Inflammation of the choroidal villi was seen in only 1 of 4 mice that had been infected with LCMV for 7 days. Magnification x300.

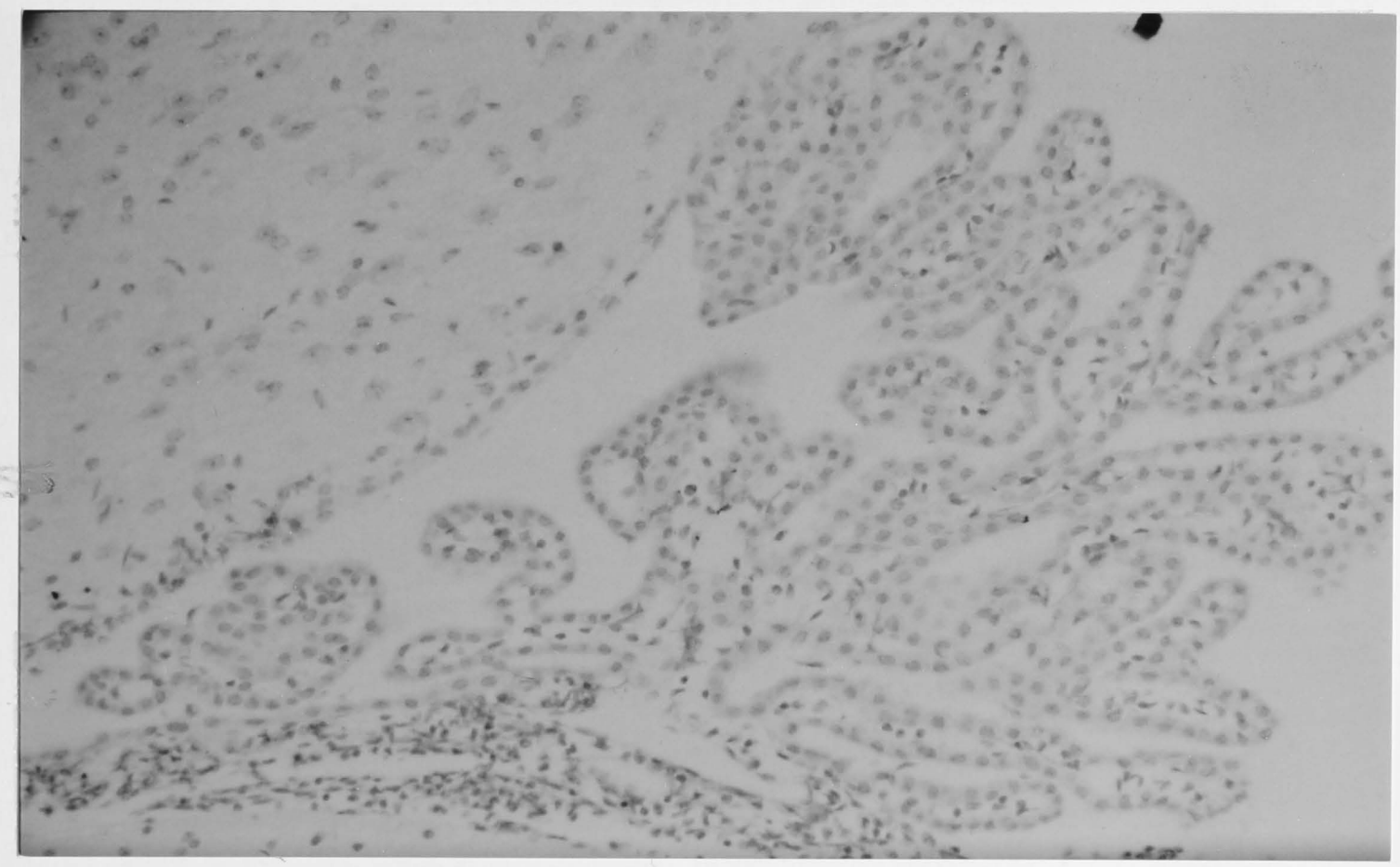


Figure 4.10 Day 7-infected brain, F4/80: in general, the villi of the choroid plexus were not inflamed and infiltrating cells were restricted to the base of the plexus. Magnification x200.

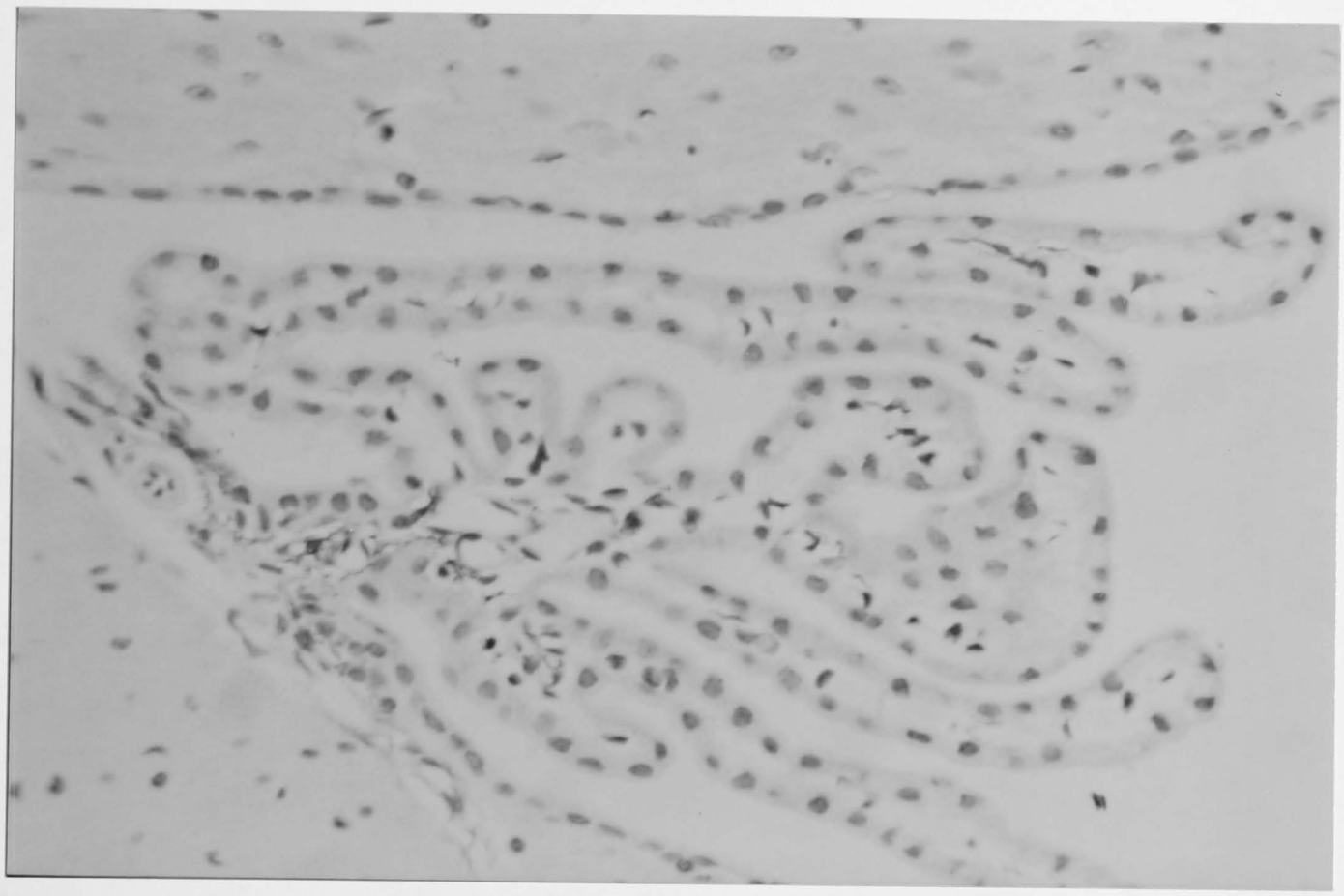


Figure 4.11 Day 5-infected brain, F4/80: an accumulation of F4/80^{+} macrophages are stretched along the base and in the stalk of the choroid plexus. Magnification x380.

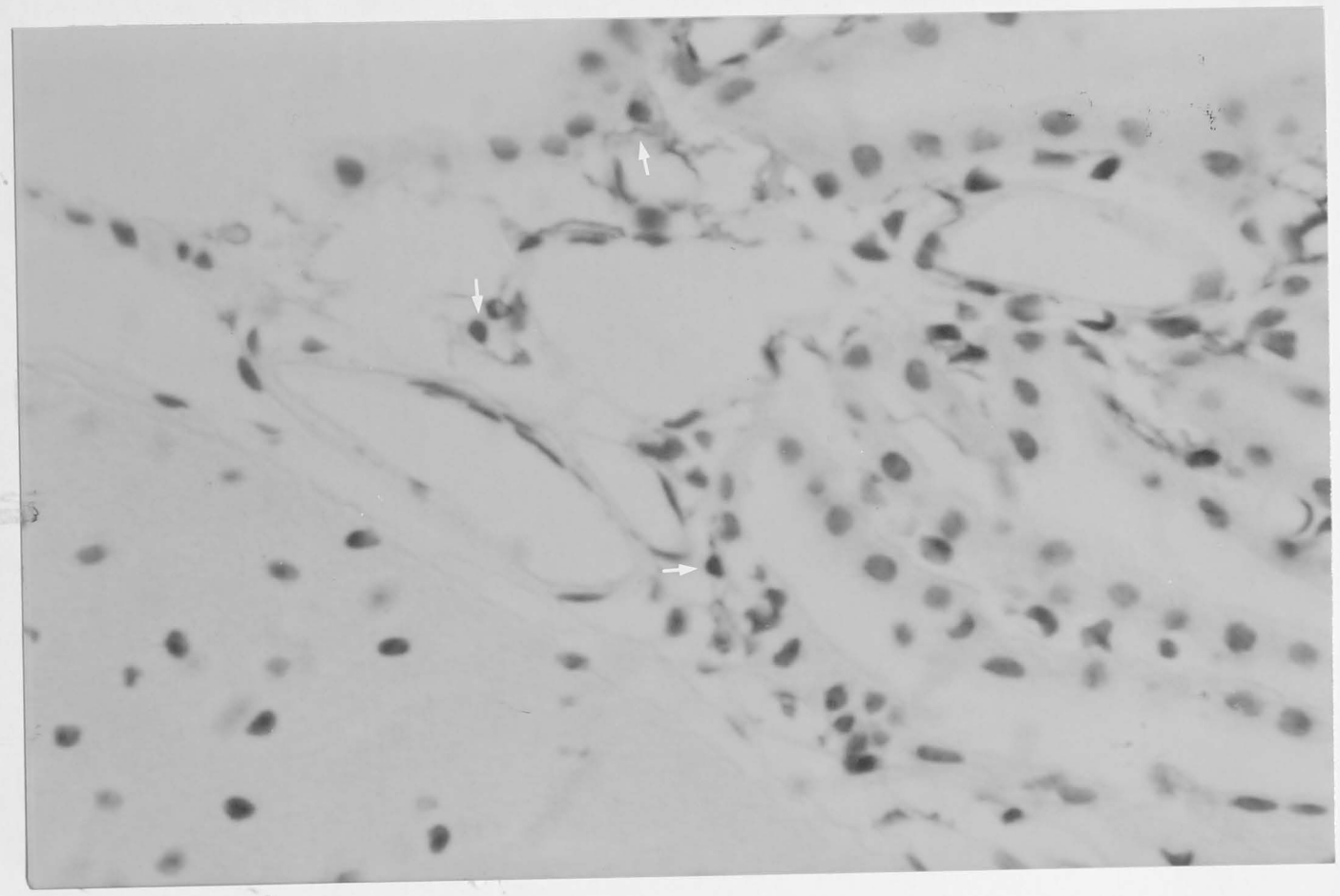


Figure 4.12 Day 5-infected brain, F4/80: close association between lymphocytes and F4/80^{+} macrophages at the base of the choroid plexus are indicated by arrows. Magnification x750.

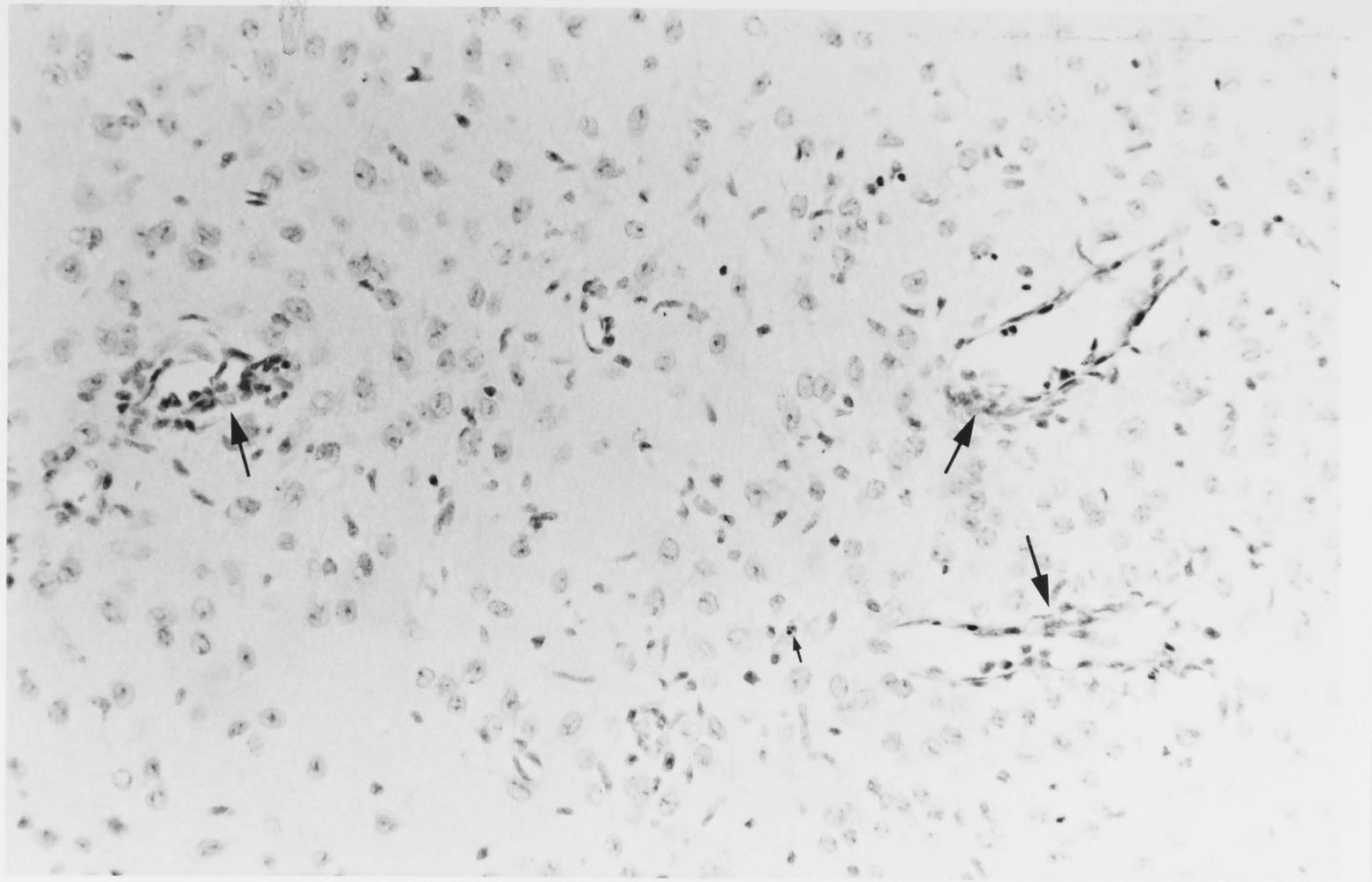


Figure 4.13 Day 7-infected brain, Ia: inflammation can be seen in parenchymal vessels of the brain (large arrows). Pyknotic nuclei can also be seen in the brain parenchyma (small arrow). Magnification x340

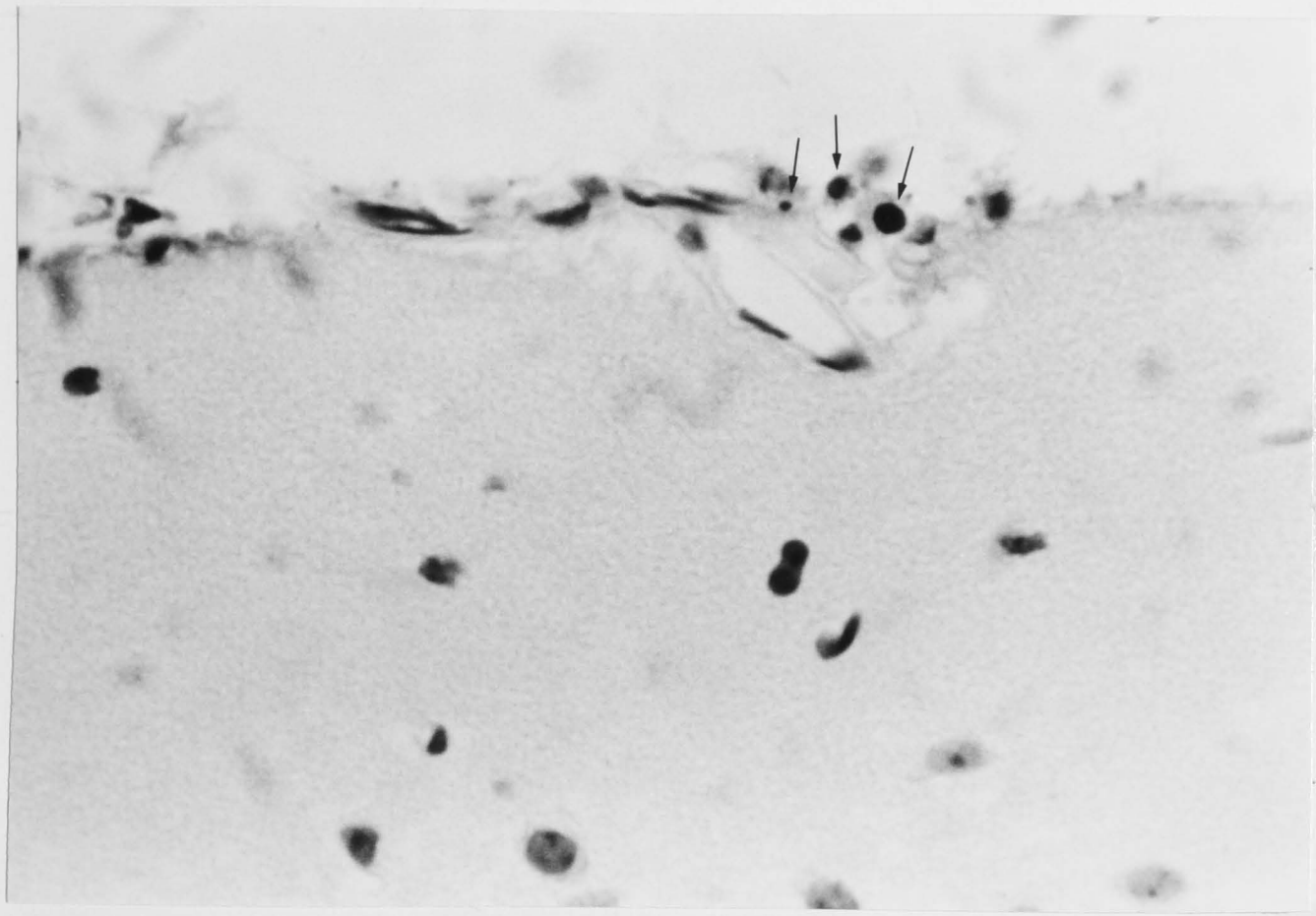
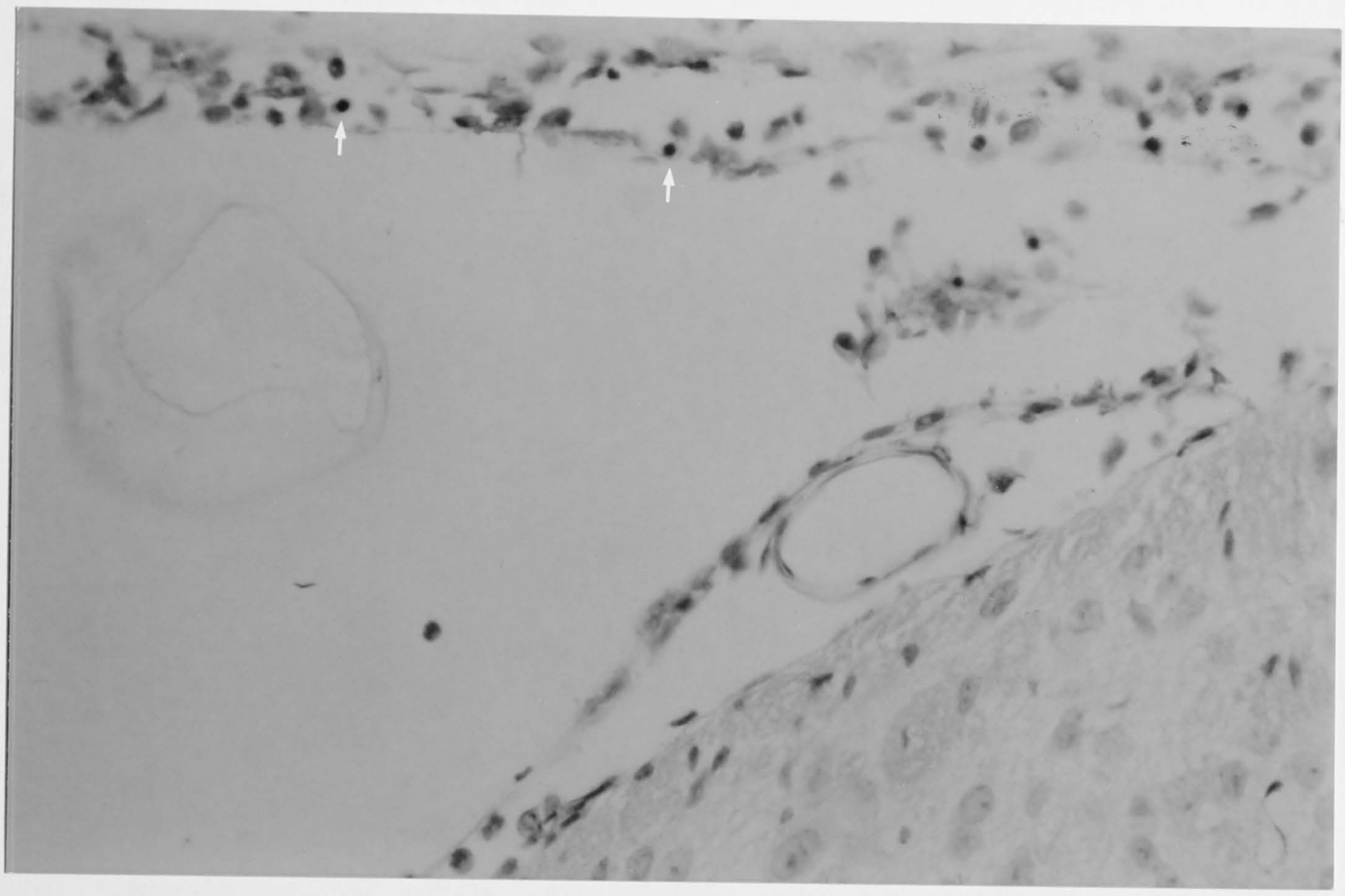


Figure 4.14 Day 2 after immune transfer, Ia: the level of meningeal inflammation on d2 after transfer of immune spleen cells is low. Pyknotic nuclei in the pial membrane are indicated by arrows. Magnification x970.

A



B

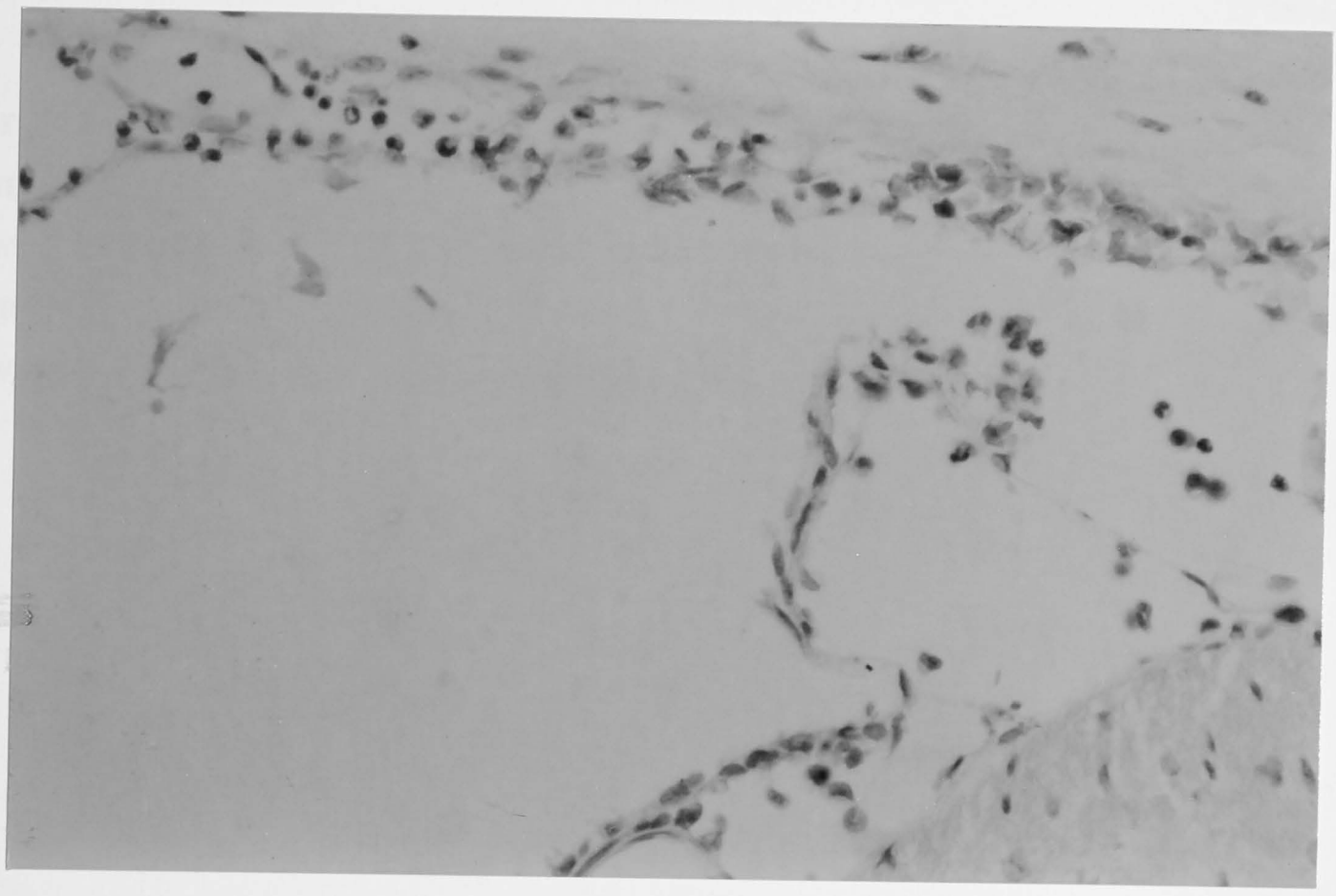


Figure 4.15 **A.** Brain taken on d3 after transfer of immune cells, F4/80: $F4/80^+$ and $F4/80^-$ cells, and pyknotic nuclei (arrows) can be seen in the meningeal infiltrate. Magnification x300. **B.** Brain taken on d3 after transfer of immune cells, Ia: a serial section of the brain shown in A is stained for Ia here. Only a few of the $F4/80^+$ cells appear to be Ia^+. Magnification x300.

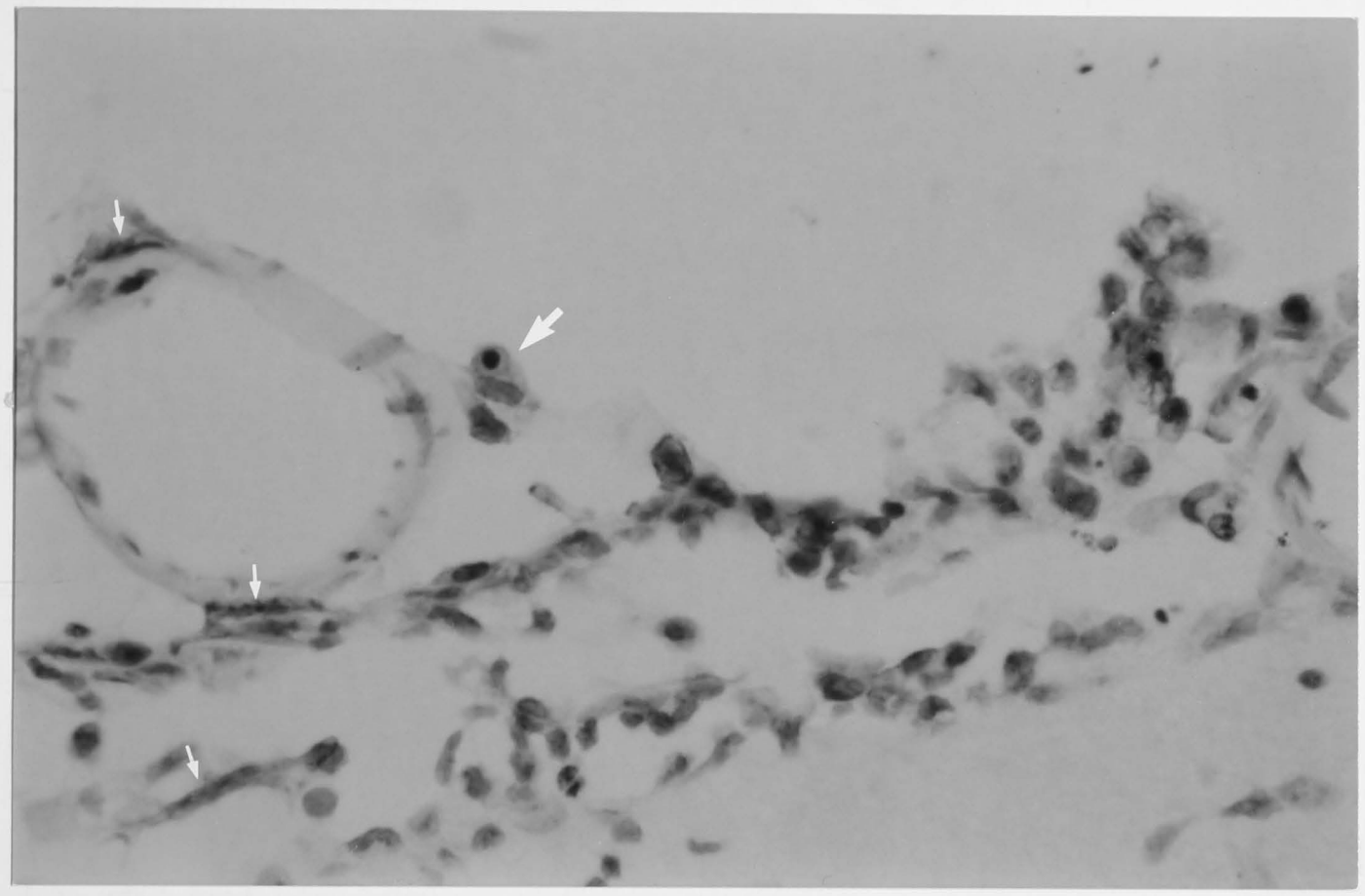
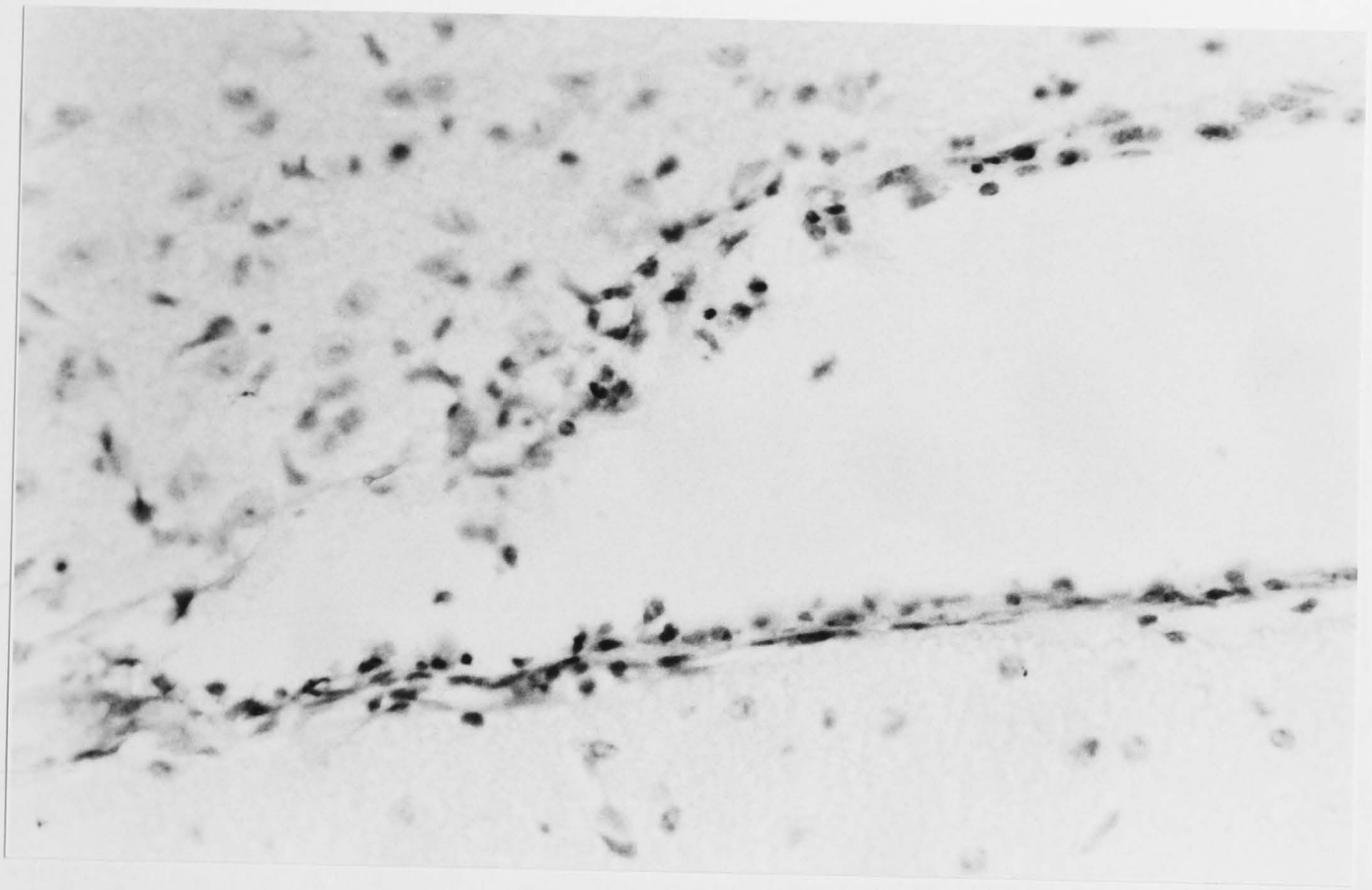


Figure 4.16 Brain taken on d3 after transfer of immune cells, F4/80: meningeal inflammation on d3 after transfer of immune spleen cells is high. An example of an $F4/80^+$ cell with an engulfed pyknotic nucleus, in which the macrophage nucleus is also apparent, is indicated by the large arrow. Examples of 'stretched' macrophages are indicated by the small arrows. Magnification x480.

A



B

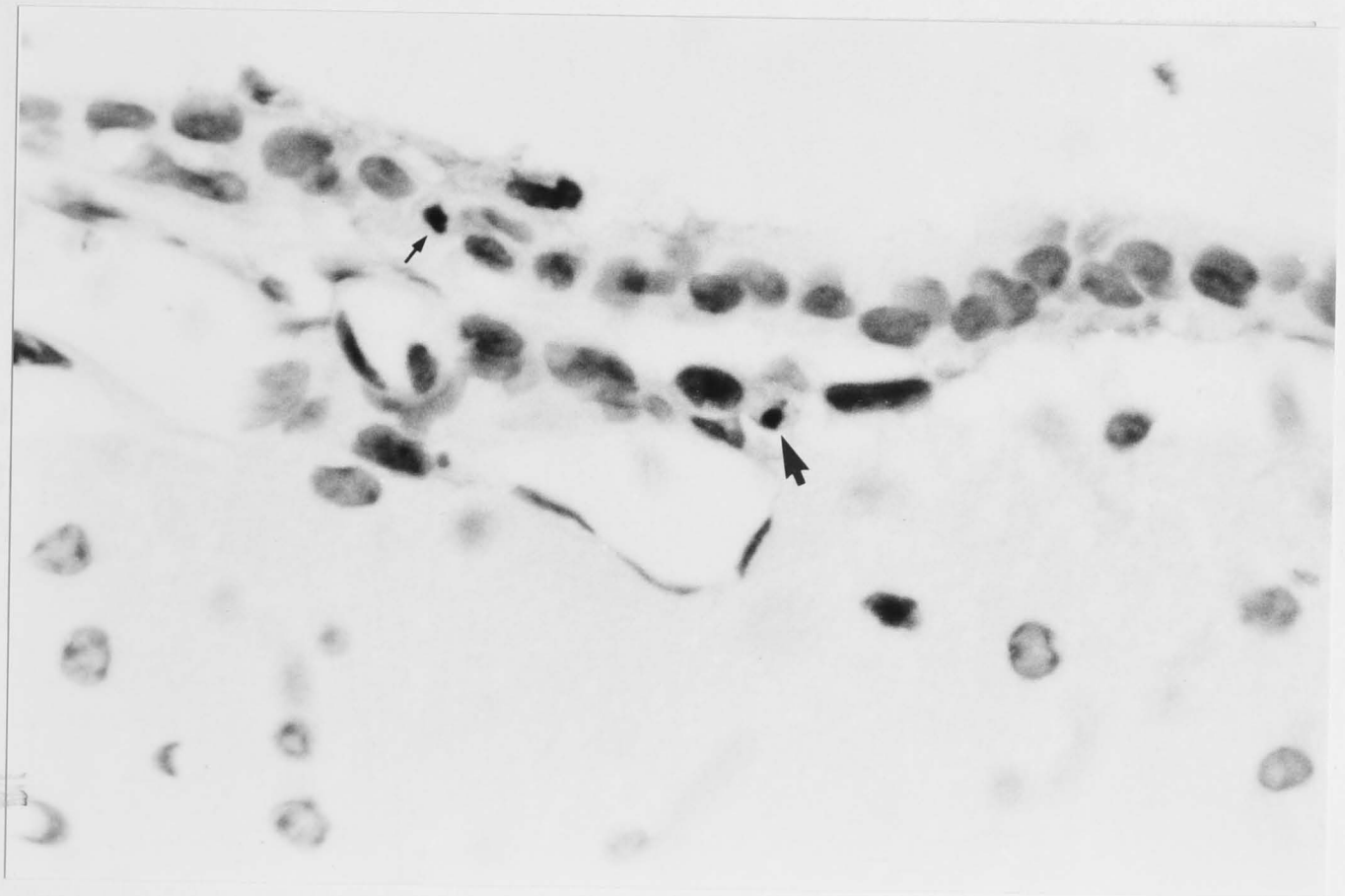


Figure 4.17 Damage to the ependymal wall of the fourth ventricle on d3 after transfer of immune spleen cells into immunosuppressed, infected mice. F4/80:

A. Inflammation within the ventricle and disruption of the ependymal wall can be seen. Magnification x390.

B. A pyknotic nucleus of a cuboidal cell in the ependymal lining is indicated by the small arrow. There is a macrophage ($F4/80^{+}$) adherent to this section of the ventricle wall. A pyknotic nuclei in the underlying vasculature (large arrow) can also be seen. Magnification x970.

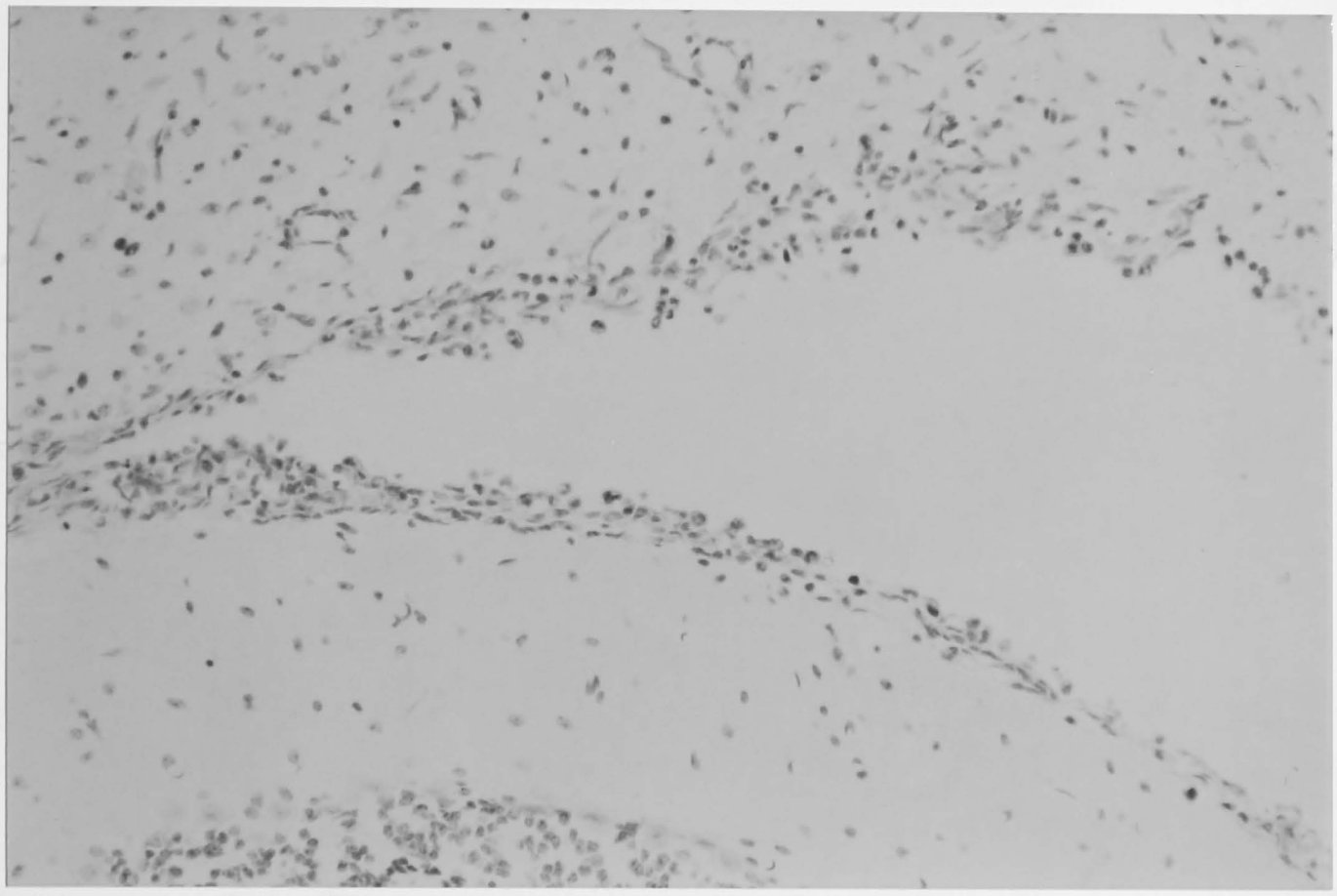


Figure 4.18 Brain taken on d3 after transfer of C-treated immune cells, Ia: substantial infiltraion of both Ia^+ and Ia^- inflammatory cells into the ventricle can be seen. Magnification x240.

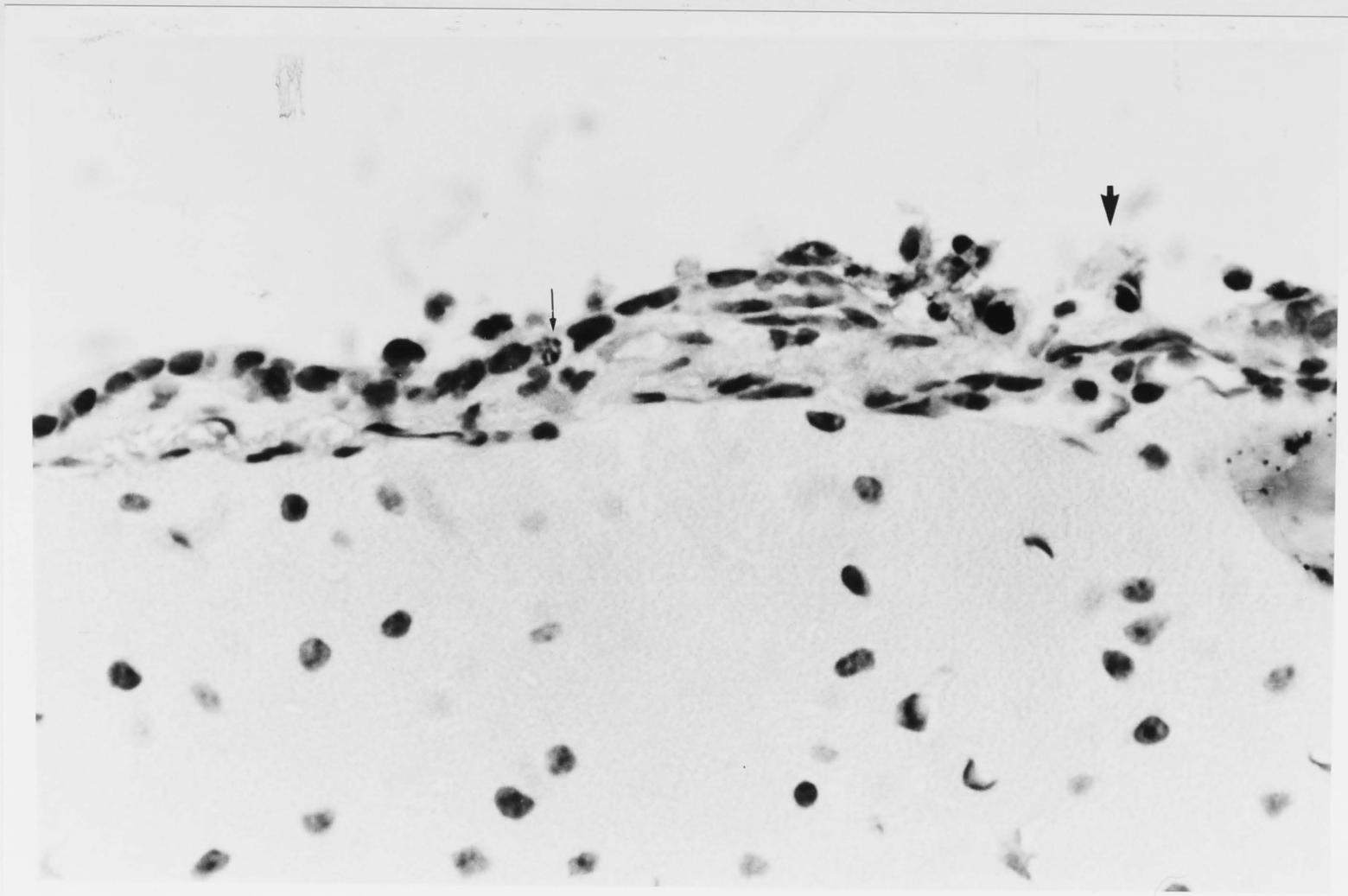


Figure 4.19 Brain taken on d3 after transfer of C-treated immune cells into immunosuppressed infected mice, Ia: damage to the ependymal wall of the fourth ventricle can be seen. A pyknotic nucleus of a cuboidal cell in the ependymal lining is indicated by the small arrow and disruption of the ventricle wall is indicated by the large arrow. Magnification x930.

Chapter 5

Lyt2$^+$, L3T4$^-$ T Cells Induce DTH in the Brain of LCMV-Infected Mice

5.1 INTRODUCTION

Results discussed in the preceding chapters have demonstrated that the inflammatory response to LCMV infection involves the localisation of both lymphocytes and macrophages to sites of infection. Immunohistochemical staining for F4/80 and Ia antigens indicate a role for, and the activation of, mononuclear phagocytes during LCM. However, the protocol used for the preparation of tissue for histological examination, while providing good morphology and preservation of these markers, did not preserve such T lymphocyte antigens as Thy1, Lyt2 or L3T4. Thus, the important question of the role played by various T cell subsets during LCM could not be approached using the immunohistochemical technique that had yielded substantial information on the distribution of activated macrophages.

To establish the relative importance of $Lyt2^+$ and $L3T4^+$ T cells in LCM, an adoptive transfer protocol was used to induce the disease. This protocol has been described in Chapter 2.11 and is outlined in Fig. 2.1. Briefly, infected immunosuppressed recipients received immune spleen cells which had been depleted of the test cell population. Using a similar protocol, Cole et al., (1972) and Doherty and Zinkernagel (1975a) have previously shown that a $Thy\text{-}1^+$ population in the transferred immune cells is required for the induction of LCM.

It is likely that the immune response to LCMV infection is mediated by the class 1 MHC-restricted subset of T cells (Zinkernagel and Doherty,1979). Such T cells are likely to bear the Lyt2 marker (Swain, 1983). LCMV-reactive CTL only lyse target cells which are compatible at either the K or D regions of the MHC (Blanden et al., 1975; Doherty and Zinkernagel, 1975b; Marker and Andersen, 1976; Zinkernagel and Doherty, 1979). Complete reduction of this cytotoxic response is achieved by treatment of the immune cell population with anti-Lyt2 antibody and C, but not with anti-Lyt1 antibody and C, (Varho et al., 1981; Allan and Doherty, 1985a). Likewise, in the adoptive transfer model of LCM induction, acute disease only occurs when the donor and recipient are compatible for at least one class I MHC allele (Doherty et al., 1976b; Doherty and Allan, 1985).

Though the transfer of Lyt2-depleted virus-immune T cells into Cy-suppressed, virus-infected recipients resulted in a dimunition of both mortality and virus clearance from the brain, the onset and magnitude of the inflammatory response was not obviously modified (Allan and Doherty, 1985a). However, the immunosuppressive regime used in this study, administration of Cy 2d before virus inoculation, was probably suboptimal. When demonstrating that transferred cells are $Thy1^+$ and class I MHC-restricted, Cole and coworkers, (1972) and Doherty and colleagues, (1976b) administered the drug 2-3 days after infection of the recipients. Gilden et al., (1972a) had previously demonstrated that maximum protection from LCM is achieved if mice are immunosuppressed 3-5d after virus inoculation. Also Allan and Doherty (1985b) have since shown that complete abrogation of the CTL response is only seen if the drug is administered 4 or 5 days after virus exposure. Thus, although Allan and Doherty (1985a) found that Lyt2 depletion of the transfer cells reduced

their capacity to clear virus and induce LCM death, a reduced ability to cause inflammation may have been masked by residual host T cell activity in the recipients.

In the experiments reported in this chapter, adoptive transfer experiments with Lyt2- and L3T4- depleted immune cells were performed to study the relative importance of these T cell subsets in induction of LCM. Long term studies were performed to see whether $Lyt2^+$ immune cells can emerge in Cy-treated mice and whether the CTL are of donor or host origin. An immunosuppressive regime that minimised recipient CTL activity was used.

5.2 RESULTS

5.2.1 The acute inflammatory response to LCMV in the CNS is mediated by $Lyt2^+$ T cells.

To investigate the relative contributions of $L3T4^+$ and $Lyt2^+$ cells in the induction of LCM, spleen cells were adoptively transferred from C57BL/6J mice to infected, immunosuppressed recipients of the same strain. Before transfer, immune cells were treated with mAb plus C (section 2.13) to remove $L3T4^+$ or $Lyt2^+$ cell populations. Anti-L3T4 treatment removed 20% of the spleen cells and had no effect on the CTL activity for LCMV-infected targets. Anti-Lyt2 treatment removed 40% of the cells and was associated with a marked reduction in capacity to mediate cytotoxicity (Fig. 5.1A). At a dose of 1.8×10^7, spleen cells were transferred to 6 mice in each of the following groups: mice receiving 1) immune cells treated with C only; 2) cells treated with anti-L3T4 antibody plus C; and 3) cells treated with anti-Lyt2 antibody plus C. A control group of recipients received no cells.

Three days after transfer, recipient mice were analysed for the extent of inflammation in CSF (Fig 5.1B) and for the cytotoxic activity in spleen (Fig 5.1C) and lymph nodes (Fig 5.1D). Mice that had received cells treated with either anti-L3T4 antibody + C or with C alone, had high levels of cytotoxicity in their spleens and a high CSF inflammatory response. Those mice injected with the anti-Lyt2 plus C-treated population had a much reduced level of cytotoxicity and CSF inflammation. The latter was not significantly different from animals that received no cells. The cytotoxicity measured in the lymph nodes of recipient mice reflected that measured in the spleens, but was at a lower level.

There was an apparent enhancement of the ability of transferred cells to cause inflammation in the CSF, following the removal of $L3T4^+$ cells. This was probably due to enrichment of the $Lyt2^+$ population. This was tested in the following experiment where the dose of cells transferred reflected the number remaining after depletion of the T cell subsets.

C57BL/6J donors and recipients were again used. Anti-L3T4 plus C treatment removed 20% of the donor spleen cell population and anti-Lyt2 treatment removed 31%. Cells were transferred at a dose of 2×10^7 from each of the following groups: 1) C-treated immune cells; 2) $L3T4^-$ cells; 3) $Lyt2^-$ cells; and as a control 4) spleen cells from normal, untreated donors. In addition, groups of mice received spleen cell inocula from antibody and C treated populations that were equivalent to the C only treated group with respect to the undepleted cells, i.e., 1.6×10^7 anti-L3T4 and 1.38×10^7 anti-Lyt2 treated cells were transferred.

Three days after transfer, there was no cytotoxic activity in the spleens and low levels of inflammation in the CSF, of mice receiving normal spleen cells or Lyt2-depleted immune cells (Fig 5.2). Cytotoxic activity and CSF inflammation were present in the recipients of immune and L3T4 depleted populations and there was no increase in the number of cells in the CSF of recipients receiving the $L3T4^-$ dose of cells that reflected the cell number remaining after

treatment (Fig 5.2).

Thus, the cell population that initiates LCM following adoptive transfer of immune cells is $Lyt2^+$, $L3T4^-$.

5.2.2 H-2 compatibility at the D locus between donor T cells and the recipient is sufficient to induce LCM.

To see whether class I MHC restriction is sufficient for the induction of LCM, donor spleen cells (B10.BYR) were transferred into B6.H-2^{bml} recipients that were H-2 compatible only at the D locus of the MHC for H-$2D^b$. The H-2 haplotype of the mouse strains used are given in Table 2.1. $L3T4^+$ and $Lyt2^+$ cell depletions were performed on the transfer immune populations, depleting 10% and 35% of the cells, respectively. Recipients received 1.5×10^7 immune cells treated with 1) C only ; 2) anti-L3T4 plus C; 3) anti-Lyt2 plus C; or 4) 0.75×10^7 cells from the L3T4-depleted population. Two control groups were mice that received no cells and mice that received immune cells from H-2 incompatible donors (B10.D2). The latter immune population was capable of inducing CSF inflammation in B10.A recipients which are compatible at the D and L loci (3.3 ± 0.1 $\log_{10}$cells/µl CSF).

Animals compatible only at H-2D which received immune cells treated with C only or anti-L3T4 plus C developed high levels of cytotoxicity in their spleens and high cell counts in their CSF. Removal of $Lyt2^+$ cells from the transfer population reduced the spleen cytotoxicity and CSF inflammation to the level of the two control groups (Fig 5.3).

Thus, H-2 compatibility at the D locus between donor and recipient, is sufficient for the transfer of LCM. D-restricted immune T cells are $Lyt2^+$, $L3T4^-$.

5.2.3 Cytotoxic T cells in the spleen of mice with transferred LCM are of donor origin.

To establish whether the cytotoxic T cells in the spleens of mice with transferred LCM originate from the donor or recipient, Thy 1.1 donors (B6Ka Thy1.1) and Thy 1.2 recipients (C57BL/6J) were used. Donor spleen cells were treated with either C only or with anti-Lyt2 + C. The latter treatment abrogated their capacity to lyse LCMV-infected MC57G target cells (Fig5.4A). Recipient mice received 1.0×10^7 C-treated or $Lyt2^-$ immune cells. Four days after transfer, CSF inflammation in mice that received C-treated immune cells (Fig 5.4B) was again correlated with high cytotoxicity in the spleen (Fig 5.4C). Anti Thy1.1 plus C treatment removed 50% of the recipient spleen cell population and reduced cytotoxic levels as effectively as treatment with anti-Lyt2 plus C. Depletion of Thy 1.2^+ cells reduced the cell numbers by 43% but had no effect on cytotoxicity (Fig 5.4C). Meningitis was significantly reduced in

mice receiving Lyt2$^-$ immune cells (Fig 5.4B) and cytotoxicity of recipient spleen was low (Fig 5.4D). However, residual cytotoxic activity of donor Thy1.1$^+$ cells was suggested by the apparent enhancement of lysis following anti-Thy1.2 treatment (Fig 5.4D).

Thus, T cell reactivity to LCMV in the spleens of mice with transferred LCM is derived from the donor spleen cells.

5.2.4 Depletion of Lyt2$^+$ cells from the transfer population does not prevent LCM at 7 days post transfer.

On some occasions, C57BL/6J mice survived the CNS inflammatory response that occurred 3 days after transfer. In the two passive transfer experiments described in Chapter 4, one had a much higher level of histological and clinical evidence of CNS inflammation than the other. In the first experiment, mice receiving immune spleen cells were moribund, and one was dead, on d3 post transfer. In the second experiment, the equivalent clinical state was not reached until 1-2 days later. The CNS inflammation of animals on d5 post transfer was histologically equivalent to that seen in the previous experiment on d3. Thus, the clinical symptoms of LCM are closely correlated with the histological development of the disease.

The latter experiment, in which there was late development of LCM, will now be considered. Histologically, the brains of mice receiving Lyt2-depleted cells resembled those receiving normal spleen cells. When compared to those receiving C only or anti-L3T4 plus C treated immune cells, there was little evidence of CNS inflammation on d3 (Chapter 4). On d5 post transfer, 2 mice that had received C-treated immune cells, one that had received L3T4-depleted cells and 2 that had received Lyt2-depleted cells were examined histologically. Meningeal inflammation in all these mice was extensive, equivalent in severity and extent of macrophage infiltration to that seen 3d after transfer in the previous experiment. Depletion of Lyt2$^+$ cells from the immune transfer population abolished their capacity to induce histologically detectable meningitis on d3 post transfer (Chapter 4), but by d5 there was no detectable difference in the extent of inflammation between mice receiving depleted or undepleted populations. Clinically, mice in all groups were huddled on d5 post transfer and one animal in the group receiving normal speen cells died from LCM on d6.

On d7, inflammatory cells in the CSF were counted and the cytotoxic activity in the spleen measured in the remaining mice. The numbers of mice were: 1) 4 receiving cells treated with C only; 2) 7 receiving L3T4$^-$ cells; 3) 7 receiving Lyt2$^-$cells; and 4) 9 receiving normal cells. The inflammatory cell count was not performed on mice receiving normal spleen cells but was high in all other groups (Fig 5.5A). Spleen cell cytotoxicity was high for all groups of mice (Fig 5.5B), including those receiving normal spleen cells.

Thus, although Lyt2-depleted immune spleen cells and normal spleen cells are unable to induce LCM at 3d post transfer, LCM reactivity as measured by spleen cell cytotoxicty and CSF inflammation can develop at a later date.

5.3 DISCUSSION

It is now accepted that the Lyt phenotype generally defines the MHC-restriction pattern, rather than the functional status, of T cell subsets (Swain,1983). The experiments reported here demonstrate that the depletion of $Lyt2^+$, but not $L3T4^+$, cells from LCMV immune spleen cells reduces their capacity to lyse virus infected target cells and their ability to induce acute inflammation (d3-4 post transfer) in the CNS of immunosuppressed, infected recipient mice. These results confirm the notion that the immunopathological consequences of LCMV infection are mediated by class I MHC-restricted T cells, as previously shown by the necessity for the donor and recipient to share at least one class I MHC allele (Doherty et al., 1976a,b; Doherty and Allan, 1985). The fact that compatibility at the D locus of the MHC is sufficient for induction of CSF inflammation has been confirmed, and the effector cells were shown to be $Lyt2^+$ (Fig 5.3A).

Removal of the L3T4 subpopulation from immune cells sometimes (Fig 5.1B), though not always (Fig 5.3A), enhances their capacity to induce acute cerebral LCM. This probably results from the enrichment for $Lyt2^+$ effector cells in the transfer population. When the number of transferred cells is not adjusted for the decreased cell number after antibody and C treatment, no enhancement is observed (Fig 5.2A, column C). Doherty et al., (1976b) found that the capacity of immune cells to induce inflammation was dose responsive, 2×10^7 immune cells having a greater effect on recipient mortality than 1×10^7 cells. However, Doherty and Zinkernagel (1975a) found that 10^8, 5×10^7, and 2.5×10^7 cells were equally effective in causing recipient mortality. Likewise, Allan and Doherty (1985b) found that 1.5×10^7 and 2×10^7 transferred immune cells induced the same level of CSF inflammation and mortality. Thus, the dose responsiveness of LCM induction by transferred immune cells must have a plateau which begins around $1\text{-}2 \times 10^7$ cells . This was the range of cell doses used in this study. The capacity of transferred cells to induce LCM would be determined by a number of factors, including the potency of the immunisation used to generate the cells. The number of cells transferred might not, therefore, be the sole factor in determining the efficiency of the inoculum to induce LCM. Thus, the variation in the enhancing effect following depletion of the $L3T4^+$ subpopulation may be due to variation in the potency of the transferred inoculum.

Use of the B6Ka Thy1.1 mice as donors of immune cells for syngeneic $Thy1.2^+$ recipients, confirmed that LCMV-reactive CTL in recipient mice, 4 days after transfer, were of donor origin (Fig 5.4C). Within this time span, there is no evidence of any residual recipient CTL activity. In fact, removal of recipient T cells ($Thy1.2^+$) had an enhancing effect on spleen cell cytotoxicity which probably resulted from the enrichment of $Thy1.1^+$ effector cells. This enhancement is most prominent when examining the CTL reactivity of mice receiving Lyt2-depleted $Thy1.1^+$ cells. These mice have very low levels of both meningitis and spleen CTL activity (Figs 5.4 B,D). However, depletion of the spleen cell population of $Thy1.2^+$, but not $Thy1.1^+$ or $Lyt2^+$ cells, appears to unmask a low level of cytotoxicity in the recipients.

Thus, there exists the possibilty that, 4d after transfer of Lyt2-depleted immune cells, some reactivity to LCMV is emerging.

This cellular response in mice receiving Lyt2-depleted cells is also seen histologically on d5 after transfer (section 5.2.4), and is apparent on d7 when CSF cell counts and spleen CTL activity are high (Fig 5.5). Mice receiving normal spleen cells also suffer clinically from LCM from d5-7 post transfer and have high levels of spleen cell cytotoxicity on d7 (Fig 5.5B).

One possible explanation for the late development of LCM is that Cy treatment only delays, rather than abolishes, the CTL response to LCMV. This is unlikely in view of the effectiveness of Cy immunosuppression in preventing the onset of LCM disease and in establishing a virus carrier state. Administration of 150mg/kg of Cy to BALB/c mice 3d after i.c. inoculation of LCMV resulted in only 10% mortality from LCM, 1-2 weeks later (Gilden et al., 1972a). While Allan and Doherty (1985b) found a 100% mortality rate in C57Bl/6 mice at d14 p.i. for the same immunosuppressive regime, there was less than 20% mortality if the drug was administered 5d after inoculation (the time point used in this study). Therefore, it is likely that the delayed development of LCM in mice receiving normal or Lyt2-depleted immune transfer populations results from the activity of donor cells.

Doherty and Zinkernagel (1975a) and Zinkernagel et al., (1975) also found, in a number of experiments, that mice receiving normal spleen cells died 6-9 days later. Doherty et al., (1976b) found a similar delayed mortality but in only 38% of recipients. The time span between the transfer of normal cells and the development of LCM disease seen in the experiments reported here (6-7 days), is compatible with the stimulation and proliferation of naive LCMV-specific precursors within the transfer population.

In the case of delayed development of LCM following the transfer of Lyt2-depleted immune cells, it is less likely that naive LCMV-reactive cells are being stimulated after transfer, since these would have been stimulated to proliferate during the initial immunising procedure. Anti-Lyt2 plus C treatment would then have removed these reactive CTL. Perhaps the difference in virus strain between priming and challenge has resulted in the delayed development of LCM. The immune donor cells were derived from mice primed with the WE3 strain of LCMV and recipients were inoculated with the neurotropic Armstrong strain. Therefore, there is possibly a sub-population of CTL that respond to the latter strain and, if not cross-reactive to the immunising strain, would remain naive at the stage of transfer. Although all strains of LCMV are serologically cross-reactive (Rowe et al., 1970; Matthews, 1982), Dutko and Oldstone (1983) have demonstrated that the two strains of virus used in this study differ extensively on the basis of their RNase T_1 fingerprint patterns. The authors have suggested that these strain differences account for their varying biological properties. Parekh and Buchmeier (1986) also demonstrated that distinct antigenic sites could be distinguished on different strains of LCMV by monoclonal antibodies. In particular, they found a neutralising site on the Armstrong CA-1371 virus that was specific for this strain and was absent in the WE strain. Studies by Oldstone's group established that strains of LCMV could also be

distinguished on the capacity of primary CTL to kill a panel of target cells infected with the various virus strains (Ahmed et al., 1984). Moreover, the authors found that the degree of cross-reactive immunity was dependent not only on the virus strain but also on the major histocompatibility haplotype of the infected animals. Thus, within the group of LCM viruses, the CTL response is not completely cross-reactive. However, Ahmed and coworkers (1984) did not find that the Arm and WE virus strains could be distinguished in C57BL/6J mice. Even if there were determinants of the Arm strain that were not recognised by WE3 immune CTL and could stimulate a cytotoxic response in the transfer cell population, one would expect that these naive $Lyt2^+$ cells would also be depleted during the antibody treatment. Such cells , however, might express lower levels of Lyt2 than the larger activated blasts. Such a situation occurs for expression of class I MHC glycoproteins during the cell cycle of Con A-treated spleen cells (Muller et al., 1986).

A more likely explanation for the delayed onset of LCM in mice receiving Lyt2 depleted immune cells, is that the antibody treatment, though greatly reducing the cytotoxicity of the transfer population, did not remove all activated CTL. The possibilty that some cytotoxic cells remain is indicated by the presence of low levels of activity in the depleted populations at the higher E:T ratios (for example, Fig 5.1A). Such residual cells would require time to multiply in the host before they could effect LCM disease.

The possibility that the later appearence of LCM is due to the activity of the L3T4 population is unlikely, in view of the fact that mice receiving Thy1-depleted immune cells also show a delayed onset of mortality (Doherty and Zinkernagel, 1975a).

In conclusion, the results reported in this chapter demonstrate that acute inflammation in the CSF of immunosuppressed, infected mice is mediated by donor $Lyt2^+$ T cells. Immunohistological analysis has shown that a large proportion (45%) of the inflammatory cells infiltrating the meninges are macrophages. Therefore, in LCMV infection, class I MHC-restricted T cells have the capacity to elicit a DTH response that involves the recruitment of mononuclear phagocytes. Depletion of the $L3T4^+$ T cell subset does not qualitatively change the nature of the inflammatory response (Chapter 4), but can, in some circumstances, influence the extent of inflammation through enrichment of the $Lyt2^+$ cell population.

Figure 5.1 The acute inflammatory response to LCMV in the CNS is mediated by Lyt2$^+$ T cells.

A: Cytotoxic activity of the transfer cell populations. Single cell suspensions of immune spleen cells from C57BL/6 mice that had been primed 8d previously with 1000 LD_{50} of WE3 LCMV, were 1) untreated, 2) treated with C only, 3) treated with anti-Lyt2 + C, or 4) treated with anti-L3T4 + C. Cytotoxicity was measured as % specific lysis of ^{51}Cr-labelled MC57G LCMV-infected target cells.

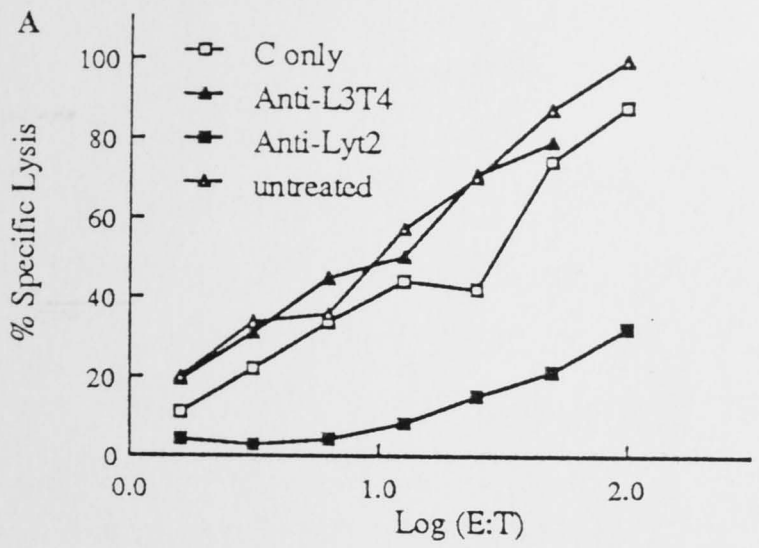
B: CSF cell counts of recipient C57BL/6 mice 3d after cell transfer. Syngeneic recipients had been inoculated i.c. with 1000 LD_{50} of Arm LCMV, given 200 mg/kg of Cy i.p. 5d later and immune spleen cells after a further 24hr. Mice received: A) C-treated immune cells; B) L3T4-depleted immune cells; C) Lyt2-depleted immune cells; or D) normal spleen cells. The cell counts were compared using Wilcoxan rank analysis. .The values for C and D were significantly less than those for A and B ($p<0.01$). There was no significant difference between A and B or between C and D.

C: Cytotoxic activity in the spleens of mice receiving 1) C-treated immune cells, 2) L3T4-depleted immune cells, 3) Lyt2-depleted immune cells, or 4) normal spleen cells.

D: Cytotoxic activity of lymph node cells of mice receiving 1) C-treated immune cells, 2) L3T4-depleted immune cells, 3) Lyt2-depleted immune cells, or 4) normal spleen cells. Cell suspensions were derived from a pool of the cervical, axillary and mesenteric lymph nodes of recipient mice.

All log values given are to the base 10.

A



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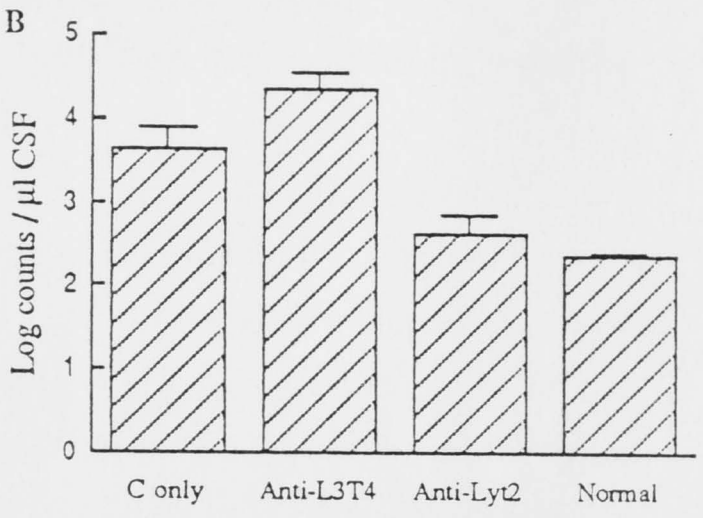
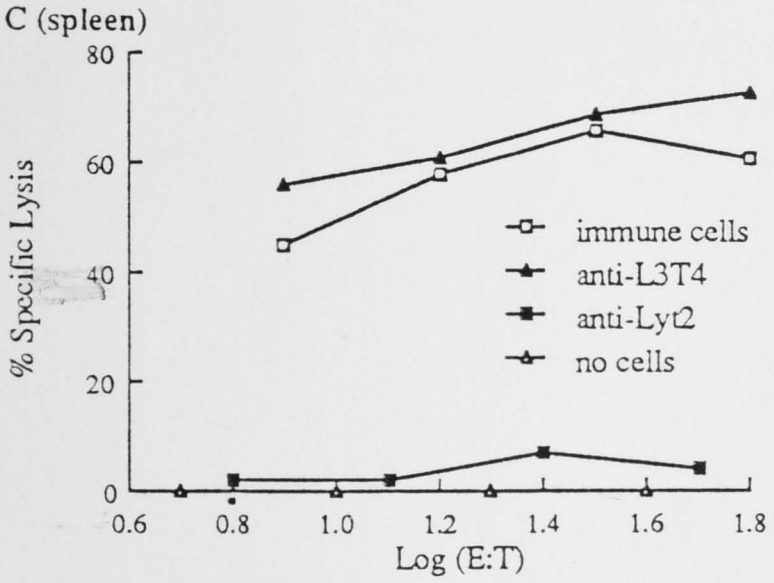


C (spleen)



D (lymph node)

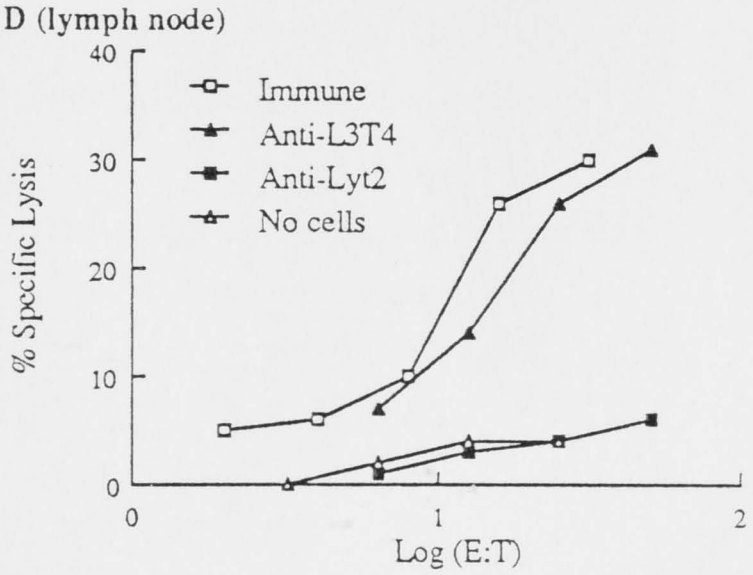


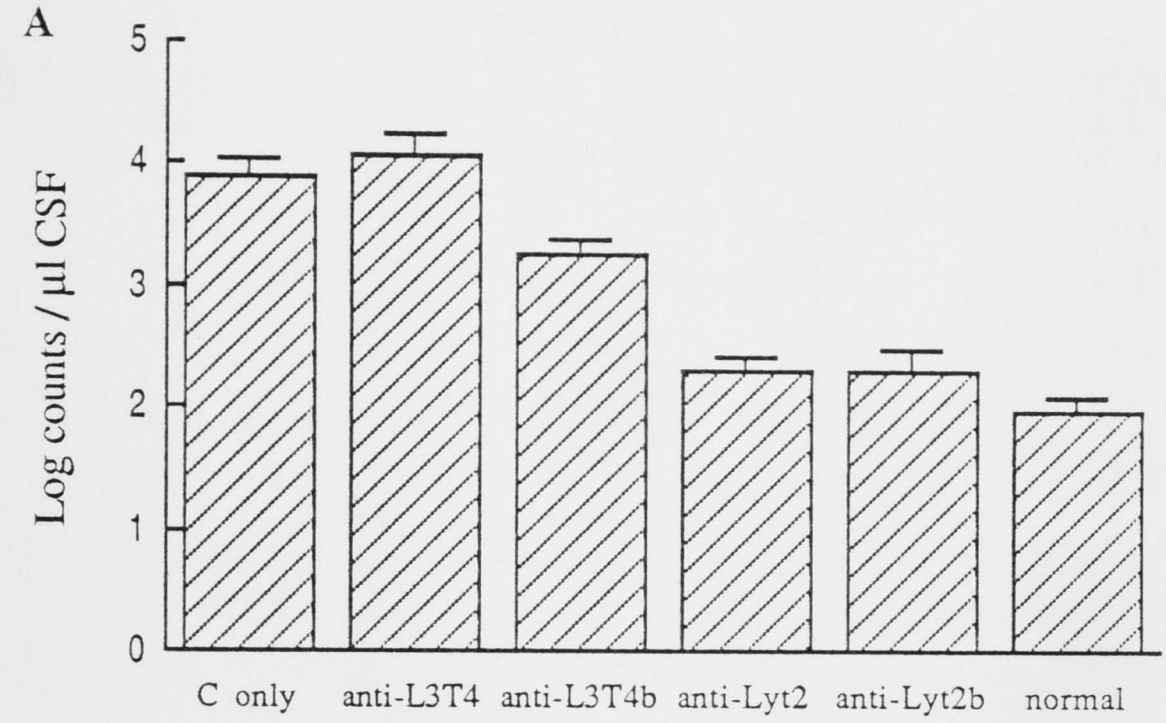
Figure 5.2 Adjustment of the dose of cells transferred to reflect the number remaining after antibody and complement treatment.

A: CSF cell counts of recipient C57BL/6 mice 3d after cell transfer. Recipients had been inoculated i.c. with 1000 LD_{50} of Arm LCMV, given 200 mg/kg of Cy i.p. 5d later and immune spleen cells after a further 24hr. Donor spleen cells were from C57BL/6 mice that had been primed 8d previously with 1000 LD_{50} of WE3 LCMV. Mice received: A) 2×10^7 C-treated immune cells; B) 2×10^7 L3T4-depleted immune cells; C) 1.6×10^7 L3T4-depleted immune cells; D) 2×10^7 Lyt2-depleted immune cells; E) 1.3×10^7 Lyt2-depleted immune cells; or F) 2×10^7 normal spleen cells. The cell counts were compared using Wicoxan rank analysis. The values for C, D, E and F were significantly less than those for A and B ($p<0.02$). The values for D, E and F were significantly less than that for C ($p<0.02$).

B: Cytotoxic activity in the spleens of mice receiving 1) 2×10^7 Lyt2-depleted immune cells, 2) 2×10^7 C-treated immune cells, 3) 2×10^7 L3T4-depleted immune cells, or 4) 1.6×10^7 L3T4-depleted immune cells. Cytotoxicity was measured as % specific lysis of ^{51}Cr-labelled MC57G LCMV-infected target cells.

All log values given are to the base 10.

A


Log counts / µl CSF
5
4
3
2
1
0
C only
anti-L3T4
anti-L3T4b
anti-Lyt2
anti-Lyt2b
normal

B

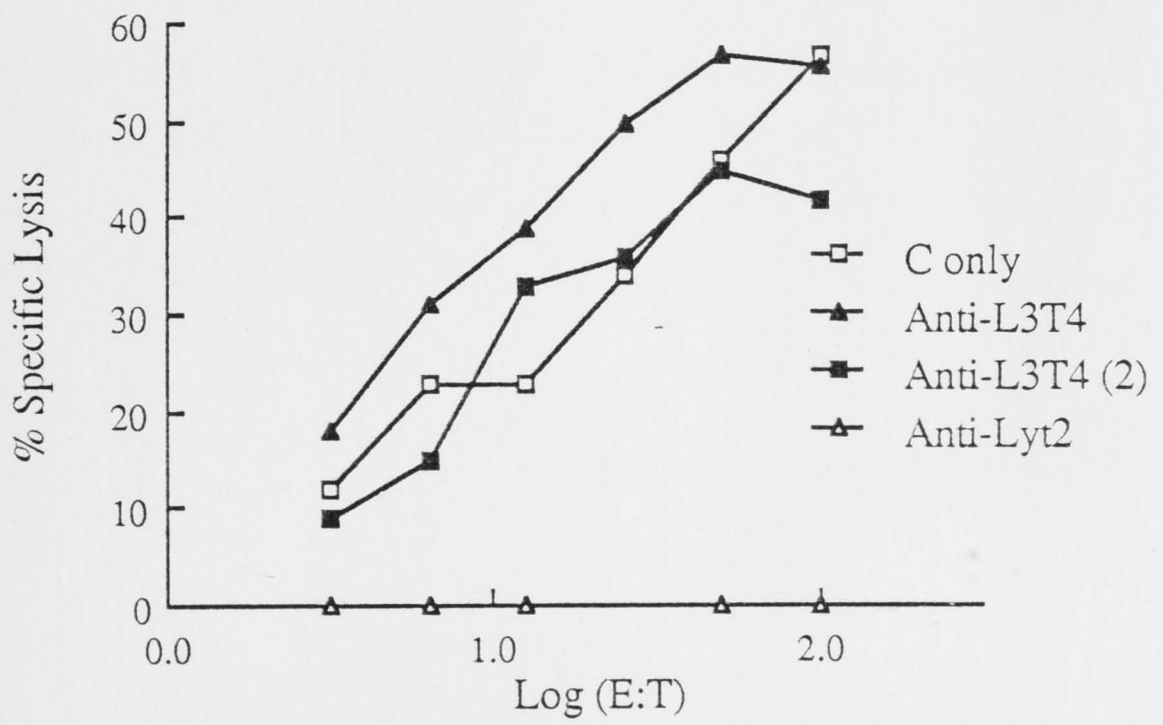

% Specific Lysis
60
50
40
30
20
10
0
0.0
1.0
2.0
Log (E:T)
C only
Anti-L3T4
Anti-L3T4 (2)
Anti-Lyt2

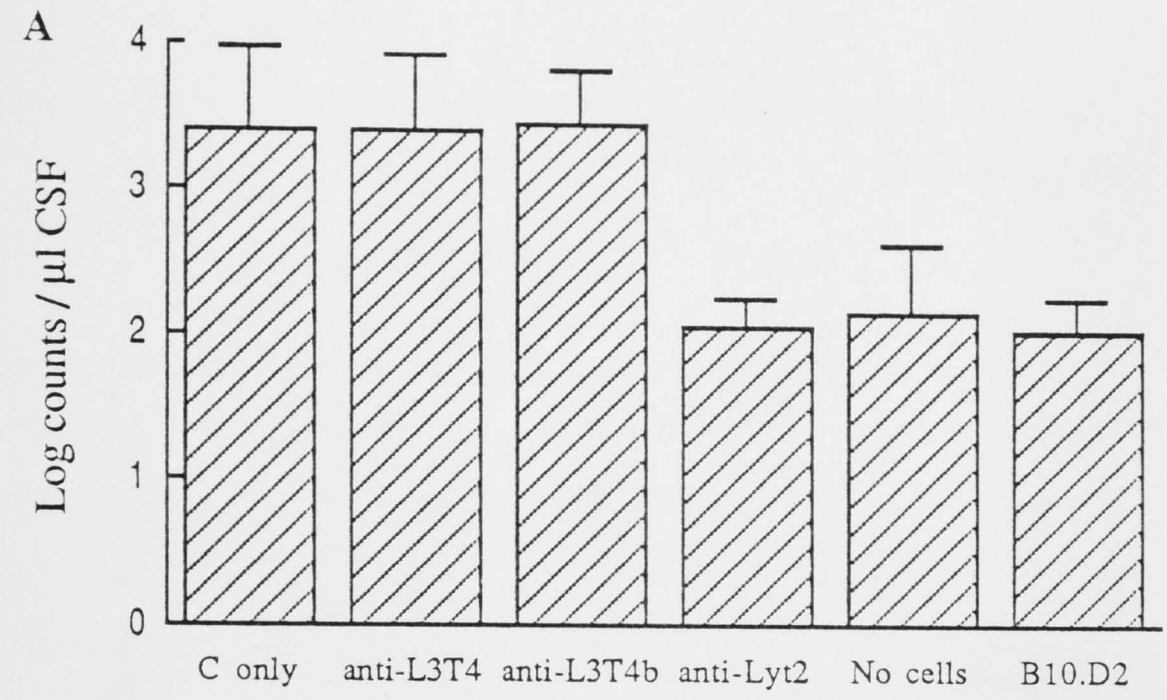
Figure 5.3 H-2 compatibility at the D locus between donor T cells and the recipient is sufficient to induce LCM.

A: CSF cell counts of recipient B6.H-2^{bm1} mice 4d after cell transfer. Recipients had been inoculated i.c. with 1000 LD_{50} of Arm LCMV, given 200 mg/kg of Cy i.p. 5d later and immune spleen cells after a further 24hr. Donor spleen cells were from mice that had been primed 8d previously with 1000 LD_{50} of WE3 LCMV. Mice received: A) 1.5×10^7 C-treated B10.BYR immune cells; B) 1.5×10^7 L3T4-depleted B10.BYR immune cells; C) 0.75×10^7 L3T4-depleted B10.BYR immune cells; D) 1.5×10^7 Lyt2-depleted B10.BYR immune cells; E) no cells; or F) 1.5×10^7 immune B10.D2 cells. The cell counts were compared using Wicoxan rank analysis. The values for D, E and F were significantly less than those for A, B and C ($p<0.02$). The values for D, E and F were significantly less than that for C ($p<0.02$).

B: Cytotoxic activity in the spleens of mice receiving 1) 1.5×10^7 C-treated B10.BYR immune cells, 2) 1.5×10^7 L3T4-depleted B10.BYR immune cells, 3) 1.5×10^7 Lyt2-depleted B10.BYR immune cells, 4) no cells, or 5) 1.5×10^7 immune B10.D2 cells. Cytotoxicity was measured as % specific lysis of ^{51}Cr-labelled MC57G LCMV-infected target cells.

All log values given are to the base 10.

A


Log counts / µl CSF
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3
2
1
0
C only
anti-L3T4
anti-L3T4b
anti-Lyt2
No cells
B10.D2

B

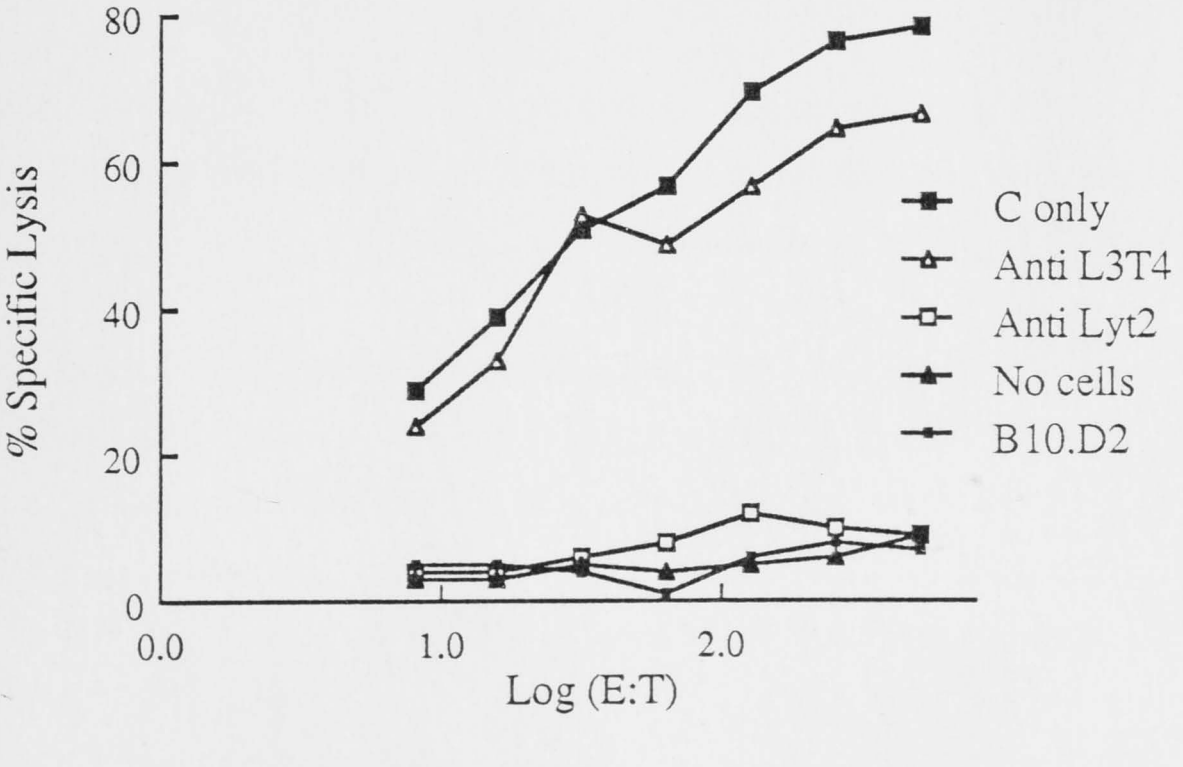

% Specific Lysis
80
60
40
20
0
0.0
1.0
2.0
Log (E:T)
C only
Anti L3T4
Anti Lyt2
No cells
B10.D2

Figure 5.4 Cytotoxic T cells in the spleen of mice with transferred LCM are of donor origin.

A: Cytotoxic activity of the transfer cell populations. Single cell suspensions of immune spleen cells from B6Ka Thy1.1 mice that had been primed 8d previously with 1000 LD_{50} of WE3 LCMV, were 1) untreated, 2) treated with complement only, or 3) treated with anti-Lyt2 + C. Cytotoxicity was measured as % specific lysis of ^{51}Cr-labelled MC57G LCMV-infected target cells.

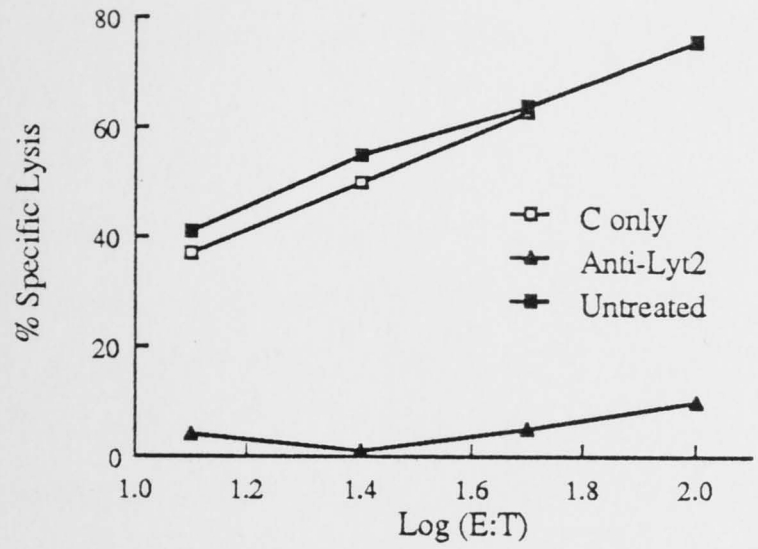
B: CSF cell counts of recipient C57BL/6 mice 4d after cell transfer. Recipients had been inoculated i.c. with 1000 LD_{50} of Arm LCMV, given 200 mg/kg of Cy i.p. 5d later and immune spleen cells after a further 24hr. Mice received: A) C-treated immune cells; or B) Lyt2-depleted immune cells. The cell counts were significantly different when compared using Wilcoxan rank analysis ($p<0.01$).

C: Cytotoxic activity in the spleens of mice receiving C-treated immune cells. Recipient spleen cells were 1) untreated, 2) treated with anti-thy1.2 + C, 3) treated with anti-thy1.1 + C, or 4) treated with anti-Lyt2 + C.

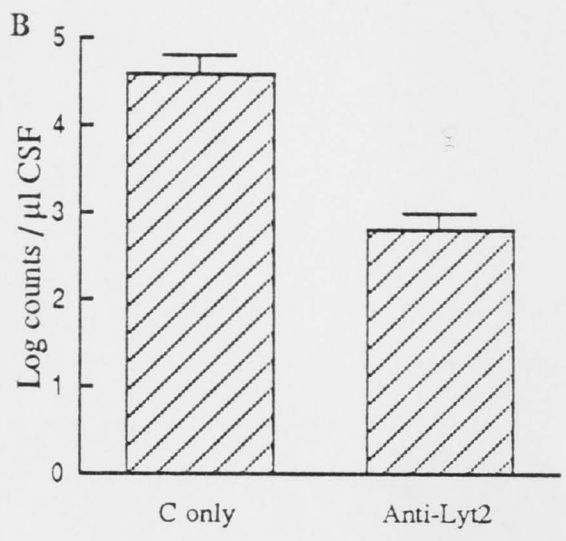
D: Cytotoxic activity in the spleens of mice receiving immune cells treated with anti-Lyt2 + C. Recipient spleen cells were 1) untreated, 2) treated with anti-thy1.2 + C, 3) treated with anti-thy1.1 + C, or 4) treated with anti-Lyt2 + C.

All log values given are to the base 10.

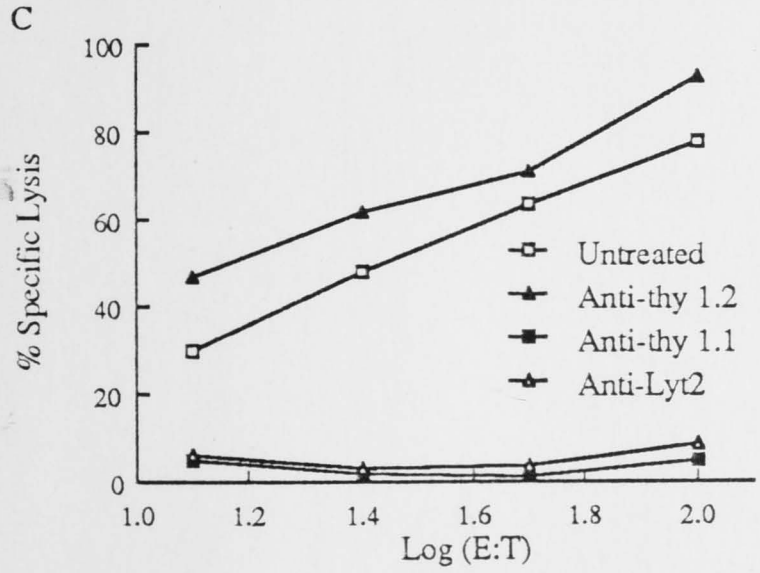
A


% Specific Lysis
80
60
40
20
0
1.0
1.2
1.4
1.6
1.8
2.0
Log (E:T)
C only
Anti-Lyt2
Untreated

B


Log counts / µl CSF
5
4
3
2
1
0
C only
Anti-Lyt2

C


% Specific Lysis
100
80
60
40
20
0
1.0
1.2
1.4
1.6
1.8
2.0
Log (E:T)
Untreated
Anti-thy 1.2
Anti-thy 1.1
Anti-Lyt2

D

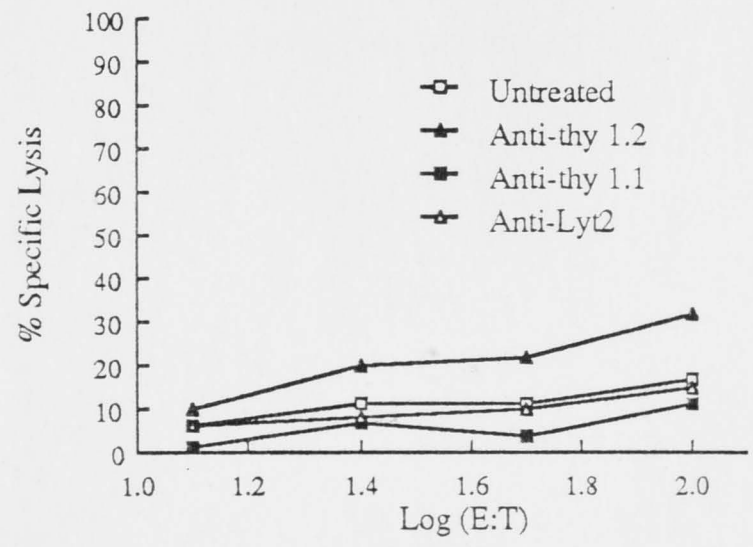

% Specific Lysis
100
90
80
70
60
50
40
30
20
10
0
1.0
1.2
1.4
1.6
1.8
2.0
Log (E:T)
Untreated
Anti-thy 1.2
Anti-thy 1.1
Anti-Lyt2

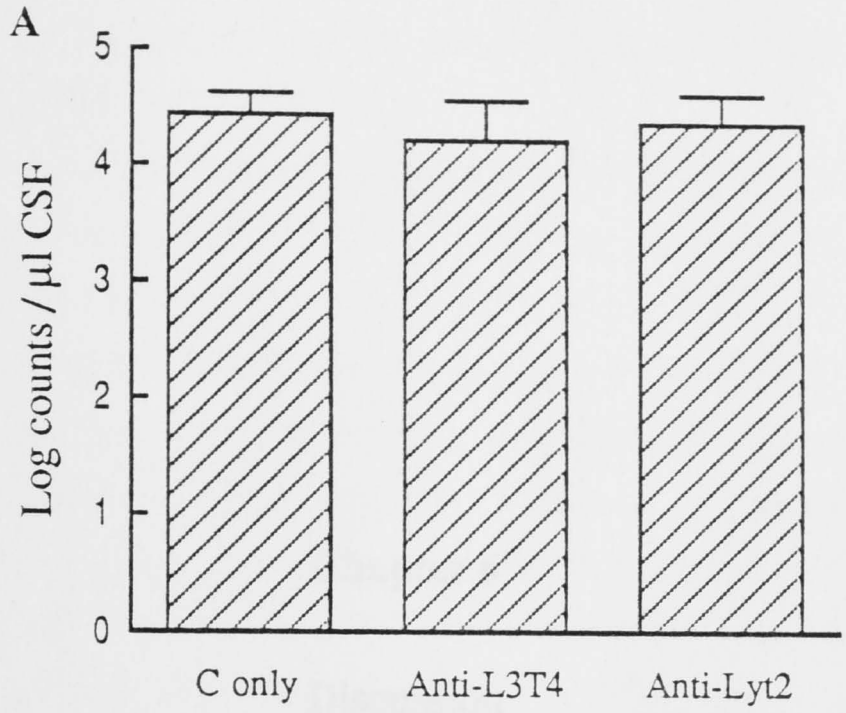
Figure 5.5 Depletion of Lyt2$^+$ cells from the transfer population does not prevent LCM at 7 days post transfer.

A: CSF cell counts of recipient C57BL/6 mice 7d after cell transfer. Recipients had been inoculated i.c. with 1000 LD_{50} of Arm LCMV, given 200 mg/kg of Cy i.p. 5d later and immune spleen cells after a further 24hr. Donor spleen cells were from C57BL/6 mice that had been primed 8d previously with 1000 LD_{50} of WE3 LCMV. Mice received: A) 1.75 x 10^7 C-treated immune cells; B) 1.75 x 10^7 L3T4-depleted immune cells; or C) 1.75 x 10^7 Lyt2-depleted immune cells. The cell counts were not significantly different when compared using Wicoxan rank analysis.

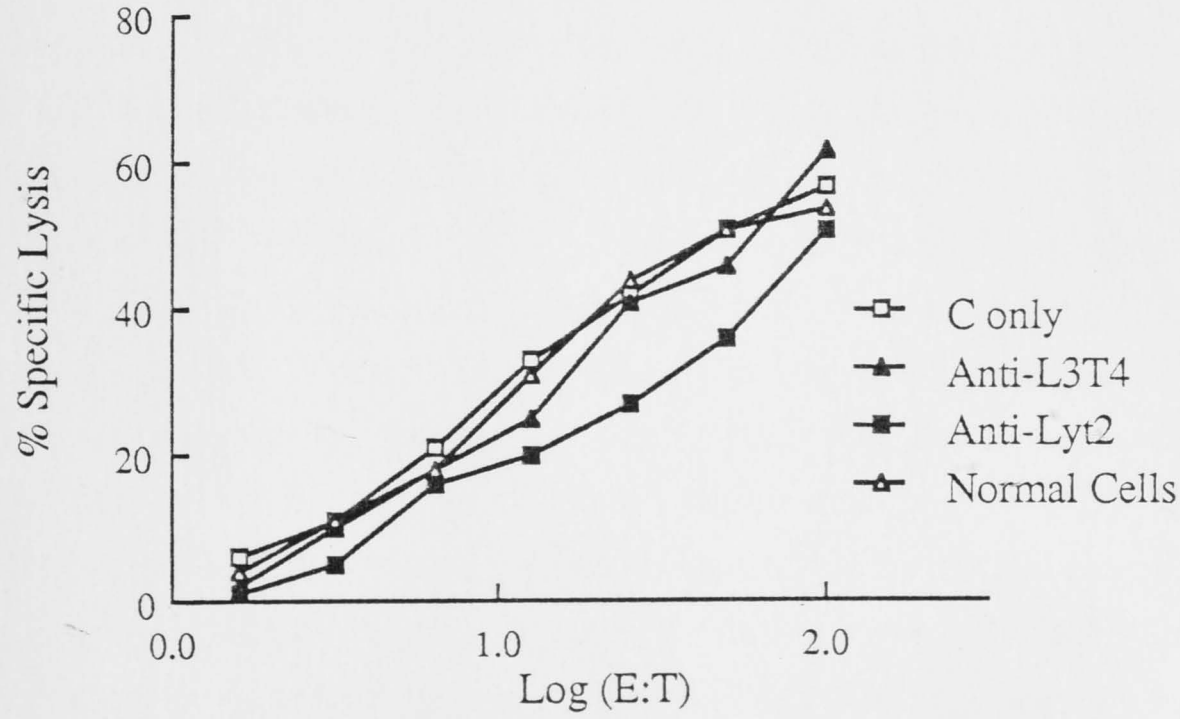
B: Cytotoxic activity in the spleens of mice receiving 1) 1.75 x 10^7 C-treated immune cells, 2) 1.75 x 10^7 L3T4-depleted immune cells, 3) 1.75 x 10^7 Lyt2-depleted immune cells, or 4) 1.75 x 10^7 normal cells. Cytotoxicity was measured as % specific lysis of ^{51}Cr-labelled MC57G LCMV-infected target cells.

All log values given are to the base 10.

A


Log counts / µl CSF
5
4
3
2
1
0
C only
Anti-L3T4
Anti-Lyt2

B


% Specific Lysis
80
60
40
20
0
0.0
1.0
2.0
Log (E:T)
C only
Anti-L3T4
Anti-Lyt2
Normal Cells

Chapter 6

Discussion

In this thesis, the extent to which macrophages are involved in the host response to LCMV has been investigated. Their distribution in infected liver and brain was determined by immunohistochemical analyses. Activated macrophages were recognised by expression of class II MHC antigens, as well as the macrophage marker, F4/80. The ability of $Lyt2^+$ T cells to mediate this recruitment in the CNS was determined using an adoptive transfer model for inducing LCM disease. The results obtained showed that there was both recruitment and specialised localisation of macrophages to sites of virus infection. This recruitment was effected by class I MHC-restricted T cells.

That lymphocytes are responsible for recruiting macrophages to LCMV-infected liver was suggested by the fact that peak levels of infiltration by lymphocytes (Fig 3.7) preceded that of monocytes (Fig 3.5) and sinusoidal macrophages (Fig 3.3). In the CNS, use of the adoptive transfer model enabled identification of the T cell sub-population that recruits macrophages during LCM. Immunohistochemically defined, $F4/80^+$, macrophages infiltrated the leptomeninges of virus-infected, immunosuppressed mice after transfer of immune spleen cells (section 4.2.2). The transfer studies demonstrated that the responsible subset was class I MHC-restricted and $Lyt2^+$. When the transfer population was depleted of Lyt2-bearing cells, but not when it was depleted of $L3T4^+$ cells, macrophage infiltration was abrogated (section 4.2.3). Similarly, Lyt2-depletion, but not L3T4-depletion, inhibited the capacity of immune spleen cells to initiate a DTH response as determined by measuring the number of inflammatory cells in the CSF (section 5.2.1). Macrophages constitute a significant proportion of these CSF cells, as judged by flowcytometric analysis of their 90° light scatter properties and distribution of the Pgp marker (Ceredig, Doherty, Allan; manuscript in preparation). Further indication that class 1 MHC-restricted T cells are responsible for effecting the DTH response in the brain, was the observation that cells adoptively transferred to mice compatible only at the D region of the MHC were able to initiate CSF inflammation, and this inflammation was abrogated following removal of $Lyt2^+$ cells from the transfer population (section 5.2.2).

A role for class II MHC-restricted, $L3T4^+$ T cells in initiation of the influx of macrophages into the CNS also seems unlikely because of the failure to observe such cells in the CSF inflammatory exudate. Work of Ceredig and colleagues (1987) has clearly demonstrated that, in both the transferred disease and that following i.c. inoculation of the virus, most $Thy\text{-}1^+$ cells in the CSF are $Lyt\text{-}2^+$. It is possible, though, that $L3T4^+$ cells have been retained in the neural tissue. However, a predominance of class I MHC-restricted T cells was detected, by immunohistochemical staining, in perivascular cuffs in the brains of multiple sclerosis patients (Hauser et al., 1986). Whereas, in experimental allergic encephalomyelitis, a disease known to be mediated by class II MHC-restricted T cells (Hauser et al.,1984; Brostoff and Mason, 1986), the majority of lymphocytes in the perivascular cuffs were class II MHC-restricted; $Lyt1^+$, $Lyt2^-$ in the mouse (Sriram et al., 1982) and $W3/25^+$ in the rat (Polman et at., 1986). Therefore, in diseases of the CNS that are mediated by a particular T cell subset,

there appears to be little contribution by lymphocytes of the other subset.

Thus, class 1 MHC-restricted T cells are responsible for the DTH response to LCMV in the CNS of infected mice, a response which involves the recruitment of macrophages to the brain. Lymphokines likely to be involved in macrophage recruitment are γ-interferon, MIF, MAF, IL3 and GM-CSF (reviewed in Schrader et al., 1984; Gemsa et al., 1985; Waksman, 1985; Metcalf, 1986). It is not known what the spectrum of lymphokines produced by LCMV-reactive T cells is, but release of these lymphokines from class I MHC restricted lymphocytes is known to occur (Dennert et al., 1981; Kelso et al., 1982; Kelso and MacDonald, 1982; Morris et al., 1982; Guerne et al., 1984; Kelso and Glasebrook, 1984). LCMV-specific, class I MHC-resticted T cells are also capable of lysing virus-infected target cells in vitro (Zinkernagel and Doherty, 1974a; Blanden et al., 1975; Marker and Andersen,1976; Varho et al., 1981; Doherty and Allan,1986), and inducing clinical symptoms of LCM (Doherty et al., 1976b; Doherty and Allan, 1985; Zinkernagel et al., 1985). Whether the same $Lyt2^+$ T cells are responsible for all these effector functions is unknown. The development of LCMV-reactive clones with different capacities to mediate these functions would determine whether they are coexpressed and their relationship to each other. Such variation in function between T cell lines has been demonstrated in other non-viral systems (Kelso et al., 1982; Guerne et al., 1984; Mosmann et al., 1986). However, the analysis of in vitro capacities of clones would not necessarily determine the functions of particular T cell subsets that were stimulated and functional during the in vivo immune response.

During LCMV infection, in the experiments reported in this thesis, macrophages localised to areas of infected tissue which were, in some cases, distinct from those areas infiltrated by lymphocytes. This was particular noticeable in infected liver where the anatomical features are uniformly dispersed and readily identifiable. Macrophages were initially absent from periportal and zonal lymphocytic foci and incoming monocytes accumulated in efferent rather than afferent blood vessels, suggesting a preferential localisation in areas that drained the lymphocytic foci (Chapter 3). This accumulation maybe determined by the concentration of MIF released from lymphocytes in both the sinusoids and the inflammatory foci. Equivalent drainage sites were not readily distinguished in the CNS sections described in Chapter 4. They would be expected to occur where CSF drains through the arachnoid villi into the major dural sinuses and along the olfactory nerves. Meticulous sectioning of the brain to locate these areas would be useful in determining whether macrophages accumulate at drainage sites in the CNS.

In general, an increase in the number of macrophages in LCMV-infected organs was accompanied by an increase in the number of cells that expressed Ia antigens. The infiltration of infected organs by activated macrophages was preceded by (chapter 3), or concomitant with (chapter 4), an influx of lymphocytes. Release of γ-interferon from activated T cells on interaction with virus-infected target cells may have been responsible for the activation of macrophages to express class II MHC antigens.

There was variation in the extent of macrophage activation, as measured by the levels of Ia expression, in liver and brain and with the different infection procedures used. Most monocytes entering the liver expressed class II MHC antigens (Chapter 3). Activation of monocytes to express Ia during viscerotropic infection with the WE3 strain of LCMV may have resulted from extensive release of γ-interferon from activated T cells in many organs including the bone marrow and spleen. Thus, monocytes entering the liver may already have been subjected to the influence of lymphokines in their organ of origin. In the CNS, fewer of the monocytes infiltrating the meninges were activated, especially on the initial days of examination and in mice that had been immunosuppressed (Chapter 4). Intracerebral inoculation of adult mice with the neurotropic, Arm LCMV does not result in the infection of viscerotropic organs (Tosilini and Mims, 1971). Thus, in the experimental system used here, the first exposure of monocytes to activating lymphokines may not occur until they have reached the brain.

Local release of γ-interferon is possibly important in inducing class II MHC expression on resident macrophages as well as infiltrating monocytes. Immunosuppression with Cy virtually abolished Ia expression from all resident macrophages in the choroid plexus, meninges and Virchow-Robin spaces of the brain (Chapter 4). This effect probably resulted from the depletion of T cell factors that were necessary for maintaining Ia levels. The possibility that γ-interferon is important in such maintenance could be tested by the in vivo administration of this lympohokine during Cy immunosuppression. Re-expression of Ia antigens on some macrophages followed the transfer of immune, but not normal, spleen cells. Such Ia^+ macrophages, however, may have been derived from the influx of macrophages into the brain, stimulated by the transferred immune cells. Although there was no observable difference in the number of $F4/80^+$ cells before or after Cy treatment to suggest that this was the case, it would be necessary to use the appropriate morphometric techniques to accurately measure the extent of expression of the two antigens on inflammatory cells that have localised to the surfaces of the brain.

The functional importance of macrophages in the immune response to LCMV is difficult to determine. A carrageenan-sensitive, irradiation-insensitive cell population, most likely macrophages, has been implicated in the control of LCMV replication early in infection, and it has been suggested that resident macrophages are an initial barrier to viral spread (Thomsen and Volkert, 1983; Lehmann-Grube et al., 1986). The role of macrophages as a first line of defense in viral infections has been reviewed by Mogensen (1979). It seems unlikely, however, that resident macrophages are able to resist infection by LCMV. This virus grows readily in peritoneal macrophages (Tosilini and Mims, 1971) and infection in the liver is preceded by an apparent replicative cycle of the virus in Kupffer cells (Tosilini, 1970).

The role of resident macrophages early in infection may be in stimulating T cell activity, i.e. the carrageenan-sensitive cells are responsible for initiating the immune response, rather than acting as a physical barrier to the virus. The initial increase in the number of Kupffer cells

within 5 days of viscerotropic inoculation (Fig 3.3), suggests the possibility of such a role for resident macrophages. However, these sinusoidal macrophages did not express class II MHC antigens. Although such expression and stimulatory capacity are not strictly linked (Lafferty et al., 1980; Glimcher et al., 1982), macrophage populations that are capable of initiating immune responses are generally Ia^+ (Unanue, 1984). Furthermore, although carrageenan treatment implicates the importance of resident macrophages early in the response to LCMV, it has no effect on the ability of mice to mount a cytotoxic T cell response to LCMV (Marker and Volkert, 1973; Thomsen and Volkert, 1983). Thus the ability of resident macrophages to restrict viral spread must be dependent on functional capacities other than that of T cell activation.

One functional role played by macrophages recruited to sites of LCMV infection, which was discerned by immunohistochemical analysis, was that of phagocytosis of pyknotic nuclei. Death of inflammatory cells during LCMV infection was evident during the later stages of the infection. Pyknotic nuclei were observed in inflammatory foci in the liver (Chapter 3) and among cells of the meningeal infiltrate (Chapter 4). Such cell death, in the liver, preceded the infiltration of foci by activated macrophages and, in the CNS, was accompanied by the appearance of phagocytosing macrophages. Thus, a 'mopping up' role can be defined for mononuclear phagocytes during the resolution of LCMV infection. Macrophages that perform this function were activated to express class II MHC antigens.

What other functions are performed by activated macrophages that are recruited to areas of virus infection, remains unknown. A role in virus clearance has been implicated in adoptive transfer experiments. An irradiation-sensitive, carrageenan-insensitive effector population was required for elimination of virus from the spleens of LCMV-infected mice which were examined 4 days after transfer of immune cells (Thomsen and Volkert, 1983). Lehmann-Grube and colleagues (1985), however, found that such a population did not play an auxillary function in the reduction of spleen viral titres. Virus reduction was mediated within 2 days of transfer of, as few as, 200 immune T cells. Such clearance could be effected within 6 hours if a larger number of CTL were transferred - hardly sufficient time to allow for the recruitment and activation of macrophages. McIntyre and coworkers (1985), also, have suggested that non-specific mediators of virus clearance are unlikely to be operational within the first 2 days of immune cell transfer. In mice co-infected with LCMV and another arenavirus, Pichindie virus, only the virus for which immune spleen cells were specific was cleared from spleens after cell transfer. If macrophages were responsible for virus clearance in this case, one would expect an equal effect on the the two viruses which were shown to infect cells lying in close proximity in the spleen (McIntyre et al., 1985).

The discrepancy in the results and conclusions found by these 2 groups of workers may reflect the time points at which viral elimination was measured. Undoubtedly, macrophages are not involved in the initial restriction of viral replication occurring early after the transfer of small numbers of immune cells (Lehmann-Grube et al., 1985). However, measurments of

reduced viral titres after a longer time span (Thomsen and Volkert, 1983) may be indicative of the importance of macrophages in clearing virions from organs in which viral replication has been contained. The localisation of macrophages to sites which drain areas of infection, as was seen in the liver (Chapter 3), may reflect their capacity to remove virus particles that have been released into the blood stream.

The functional importance of macrophages in the pathology of LCM has not been elucidated. Thomsen and coworkers (1983a) have suggested that the major contribution to the development of LCM disease is the DTH response to the virus, with macrophages playing a pivotal role. This conclusion is based on the fact that protection from the disease in Cy-pretreated mice correlated with decreased footpad DTH responses. Certainly, a major DTH response does occur in the brains of infected mice as judged by an extensive influx of inflammatory cells into the CSF (Chapter 4). However, to determine definitively a pathological role for macrophages, it would be necessary to show protection from LCM with the in vivo removal of this cell type, followed by re-expression of the disease after macrophage replacement.

The mechanism by which the immune response to viral antigen in the CNS causes the neurological symptoms and death associated with LCMV has not been elucidated. Symptoms are analogous to those of tentorial coning, a condition that results from the build up of pressure above the sheet of dura mater separating the cerebrum from the cerebellum (the tentorium) (Plum and Posner, 1972; Jennett, 1970). What causes supratentorial pressure in mice with LCM is speculative.

It is unlikely that the breakdown of the blood-brain-barrier contributes to increased intracranial pressure in LCM, since no edema can be detected by measuring the difference between infected and uninfected brains in their ratio of wet to dry weight (Camenga et al., 1977) or density on percoll gradients (Thumwood, Doherty and Allan; pers. comm.). The possibility that clinical symptoms result from the breakdown of the CSF-blood barrier (Doherty and Zinkernagel, 1974) could be supported by the observation of damage to the ependyma and meninges in mice suffering from LCM (Chapter 4). However, extensive damage to both the ependyma and choroidal epithelium which occurs during the latter stages of Sendai virus infection is not associated with neurological symptoms. (Mims and Murphy, 1973).

Increased intracranial pressure could result from impairment of CSF resorption across the arachnoid villi or along the olfactory nerves. Such impairment may result from increased viscosity of CSF (Welch, 1975) which, in LCM, could result from the extensive death of inflammatory cells in the subarachnoid space (Chapter 4) with the consequent accumulation of cell debris. The inflammatory exudate itself may alter the fluid viscosity. Doherty and Zinkernagel (1974) have, however demonstrated that neurological symptoms similar to those of LCM do not accompany massive infiltration of cells into the CSF during either tumor growth and rejection or severe meningitis following toga- and herpes- virus infections.

What may be important in effecting the putative blockage of CSF resorption, however, is the anatomical localisation of inflammatory cells. If, in the meninges as in the liver, there is specific accumulation of macrophages at sites that drain areas of LCMV-reactive T cells, CSF resorption through arachnoid villi may be impaired. To investigate this hypothesis, it would be important to determine whether there is specific accumulation of macrophages at CSF outflow sites.

In summary, the work presented in this thesis has shown that $Lyt2^+$, class I MHC-restricted T cells generated during the immune response to LCMV are capable of recruiting activated macrophages to sites of virus infection. In the liver, at least, the localisation of macrophages to anatomical sites distinct from that of lymphocytes suggests that mononuclear phagocytes play a distinct functional role during viral infection.

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