THE MAGNITUDE AND REGULATION OF ANTIBODY RESPONSES TO ANTIGENS INJECTED INTO THE CENTRAL NERVOUS SYSTEM

A thesis submitted in accordance with the requirements for the Degree of Doctor in Philosophy in the Faculty of Medicine, University of London

by

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To my beloved sister

ALCIONE

A B S T R A C T

The study in this thesis is concerned with regulation of immune responses which are initiated within the central nervous system (CNS).

Injection of different antigens into the subarachnoid space (i. sas) and into conventional sites showed that the subarachnoid space is a highly efficient site for the induction of splenic antibody responses, in particular to low amounts of T-dependent antigens. Experiments in which the mononuclear phagocytic system (MPS) was blocked with colloidal carbon, indicated that the MPS of the CNS is highly efficient in presenting antigens.

The existence of a CSF-lymphatic outflow pathway was confirmed using radiolabelled erythrocytes (^{51} Cr-SRBC).

The failure of cyclophosphamide treatment to increase splenic antibody responses in i. sas immunised mice and also the inability to induce suppressor cell dependent tolerance by i. sas injections, suggested that antigenic challenge in the subarachnoid space leads to relatively ineffective activation of suppressor mechanisms and/or a correspondingly high helper T cell activity.

CNS injury markedly enhanced systemic antibody responses to intraperitoneally (i.p.) but not to i. sas injected antigens, and extracts of brain tissue were found to have adjuvant activity. Brain injury alone could initiate antibody production in tolerised animals, although higher levels were observed in tolerised mice which were challenged

with the antigen mixed with brain extract.

It was concluded that the high antibody levels observed after *i. sas.* injection is due to a combination of:

1) effective antigen presentation by the MPS in the CNS,
2) less effective activation of suppressor mechanisms,
3) generation of more vigorous T helper cell activity, and
4) release of substances with adjuvant activity from inflamed brain tissue.

These mechanisms may all have a common single pathway.

The clinical implications of these observations are discussed.

The work presented in this thesis was performed personally by the author, in the Department of Immunology, St. Mary's Hospital Medical School, during 1979-1982. It contains, to the best of my knowledge, no material which has been accepted for the award of any other degree or diploma, and also no material previously published, except where due reference is made in the text.

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C O N T E N T S

LIST OF TABLES

LIST OF FIGURES

A B B R E V I A T I O N S

Ab	antibody
AECM.Ficoll	aminoethyl carboxymethyl.Ficoll
AFC	antibody forming cell
APC	antigen presenting cell
BBB	blood-brain barrier
BCG	bacillus Calmette-Guerin
Be	brain extract
BGG	bovine gamma globulin
Bm	brain membrane
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
CFU	colony-forming unit
CNS	central nervous system
Con A	concanavalin A
^{51}Cr-SRBC	chromium labelled sheep red blood cells
CSF	cerebrospinal fluid
Cy	cyclophosphamide
d-BSA	de-aggregated bovine serum albumin
EAE	experimental allergic encephalomyelitis
FCS	foetal calf serum
FGF	fibroblast growth factor
GSF	glia cell stimulating factor
HC	hydrocortisone acetate
HZT	high zone tolerance
Ig	immunoglobulin
i.bp.	intra brain parenchyma
i.c.	intracerebrally
i.L.	intra liver parenchyma

i.p.	intraperitoneal
i.sas.	intra subarachnoid space
i.v.	intravenous
Le	liver extract
Lm	liver membrane
LMF	lymphocyte mitogenic factor
LN	lymph node
LZT	low zone tolerance
MBP	myelin basic protein
MPS	mononuclear phagocytic system
MS	multiple sclerosis
nBe	normal brain extract
PFC	plaque forming cell
PHA	phytohaemagglutinin
PLA	polyclonal lymphocyte activator
PRP	progressive rubella panencephalitis
PtnA	protein A
PWM	pokeweed mitogen
Sal	saline
SAS	subarachnoid space
sBe	stabbed brain extract
sBm	stabbed brain membrane
s.c.	subcutaneous
SRBC	sheep red blood cells
SSPE	subacute sclerosing panencephalitis
TD	thymus dependent antigen
TdR	thymidine radiolabelled
TI	thymus independent antigen
TNP	trinitrophenyl

TNP-AECM.Ficoll	trinitrophenylated aminoethyl carboxymethyl Ficoll
TNP-BSA	trinitrophenylated bovine serum albumin
TNP-SRBC	trinitrophenylated sheep red blood cells
TOL	tolerant

CHAPTER 1

GENERAL INTRODUCTION

1 INTRODUCTION

1.1 Anatomical considerations

The basic unit of the central nervous system (CNS) is the neuron, a highly specialised cell, consisting of a body (perikaryon) and processes (axon, dendrites). In addition to neurons, other non-neuronal cells (e.g. endothelial cells, neuroglial cells) are also present in the brain and spinal cord.

The neuroglial cells include astrocytes, oligodendrocytes and a heterogeneous group of cells including the ependymal cells, perivascular cells and microglia. Ependymal cells form the epithelial lining of the choroid plexuses of the ventricular system and the central canal of the spinal cord. Except for the microglia, the origin of which is still controversial, the other cells are all of neuroepithelial origin.

The brain and spinal cord, enclosed within the skull and the neural arches of the vertebrae, are further protected and sustained by the meninges and the cerebrospinal fluid (CSF).

The meninges include the dura mater which lies directly inside the skull, the arachnoid mater, a delicate non-vascular membrane and the pia mater, a fine highly vascular membrane which adheres to the contours of the brain. The arachnoid and pia mater are collectively known as leptomeninges and are separated from each other by a fluid-filled (CSF) subarachnoid space (SAS). The width of the SAS

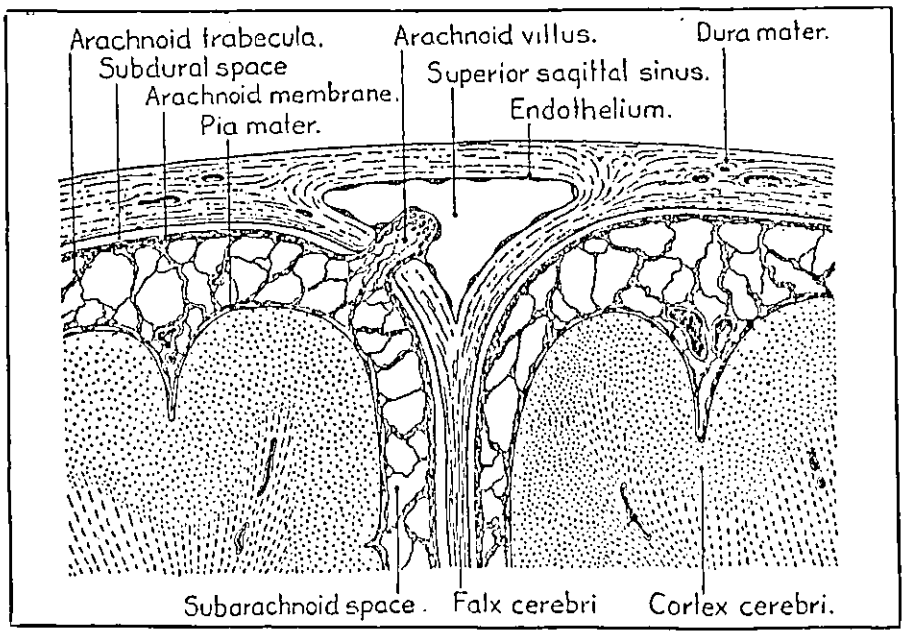
varies, because the arachnoid membrane rests on the dura mater whereas the pia mater adheres to the brain substance. Certain areas, named cisterns, hold large amounts of CSF (e.g. the cerebello-medullary cistern).

The blood vessels are surrounded by perivascular (Virchow-Robin) spaces which communicate freely with the SAS in which the CSF circulates. These spaces provide routes for the drainage of interstitial fluid from the brain parenchyma into the CSF (Cserr and Ostrach, 1974; Bradbury, 1981).

The arachnoid villi protrude into the subdural sinuses. They consist of a thin cellular layer, derived from the epithelium of the arachnoid and the endothelium of the sinus. The CSF is filtered through these villi (transcellular bulk transport) into the venous sinuses.

Fig. 1.1

Diagram illustrating the relationship between the various CNS spaces and meninges.



1.1.1 The formation and absorption of the CSF

The CSF is produced by both the choroid plexuses of the ventricles and by the subarachnoid pial surface of the brain (Bering, 1974). It enters the SAS through the inter-ventricular system (via the foramina of Luschka and Magendie), and is absorbed primarily into the cranial venous sinuses through the arachnoid villi. The CSF circulates freely into the extravascular spaces, thus acting as a "sink" for metabolites and proteins of the brain parenchyma (Cserr, 1974). It also provides protection and nourishment for the brain and spinal cord (Neuwelt and Clark, 1978).

The CSF in man is a clear fluid of low specific gravity (1.003-1.008) and with an electrolyte composition which is similar but not the same as plasma. Its protein content is very low and the few cells present are mainly lymphocytes. These vary in number from one to eight per cubic millimetre, and a count of over ten is usually indicative of disease. The difference in composition between plasma and CSF is mainly due to the presence of barriers between the blood and the various compartments of the CNS (Van Deurs, 1979).

1.1.2 The barriers of the central nervous system

The presence of a blood-brain barrier (BBB) was established by experiments with intravenous injection of acidic dyes, such as trypan blue, which stained most organs but not the brain, spinal cord and peripheral nerves (Wislocki and Leduc, 1952).

The capillaries within the brain substance and the spinal cord are enclosed by a continuous layer composed of astrocytic end-feet. This layer was once believed to represent the morphological basis of the BBB. However, the presence of channels and occasional gap junctions between the astrocytic lamellae has now been demonstrated. It is now known that the effectiveness of the BBB is dependent upon tight junctions between the capillary endothelial cells and also between the arachnoid and choroid epithelial cells. There is, furthermore, a relative absence of pinocytic vesicles within these cells (Jacobs, 1980).

A few specialised regions of the brain, which will be discussed later, lack the BBB.

The blood-CSF barrier consists of the choroid plexus epithelium. The ependymal cells have a variable morphology (cuboidal, columnar), but generally they have large numbers of microvilli at their luminal surfaces. Electron microscopically, the endothelium of blood vessels and the epithelium of the choroid plexus appear to be closely connected by rings of intercellular tight junctions. These cells therefore form a continuous layer, restricting the passage of macromolecules and cells. However, transport across this barrier is possible, because lipophilic solutes, ions, monosaccharides and perhaps aminoacids eventually equilibrate between the blood and the CSF (Pollay et al, 1974). Experimental studies have indicated that the blood-CSF barrier is not fully developed during foetal and perinatal life (Adinolfi et al, 1976).

The CSF-lymph barrier seems to present relatively little obstruction to the passage of particles. Following injections into the SAS, corpuscles between 0.5-20 μm diameter were found in the lymph nodes of the head and neck regions (Essick, 1920; Casley-Smith et al, 1976; Oehmichen, 1978). The functional relationship between the CSF and the lymphatics is further strengthened by the recent demonstration that, in several animal species, approximately 30% of the CSF is normally drained into the deep cervical lymphatic system (Bradbury and Cole, 1980; Bradbury, 1981).

The various barriers of the CNS act as selective filters and presumably protect the nervous system from potentially damaging substances present in the blood stream, and also help to maintain the special internal environment within the brain (Jacobs, 1980).

1.2 The mononuclear phagocytic system of the CNS

Recent studies based on cytokinetics, autoradiography and histochemistry have provided evidence supporting the haematogeneous origin of the CNS macrophages in both normal and inflammatory situations (Oehmichen, 1978; Wolinsky et al, 1981).

It can be demonstrated that blood monocytes can pass through the choroidal capillaries, enter the brain parenchyma and transform into microglia (Carpenter et al, 1970; Ling, 1979b). However, the presence of pluripotent haemopoietic stem cells in the brain suggests that under normal conditions the maintenance and renewal of the macrophage population in

the brain might also be dependent upon progenitor stem cells within the CNS (Bartlett, 1982). Furthermore, investigations using pulse labelling with ^{3}H-thymidine (^{3}H-TdR) demonstrated that in normal animals (rabbits) the macrophages exhibit a slight, but significant, proliferative capacity (Oehmichen, 1978).

The cells in the CNS which probably belong to the mononuclear phagocytic system (MPS) are: microglia (Fujita and Kitamura, 1975; Oehmichen, 1978; Ling, 1979a and 1979b); free subarachnoidal cells (Essick, 1920; Merchant and Low, 1977; Oehmichen, 1978); and intraventricular macrophages, including both the supra ependymal cells and the epiplexus cells (Carpenter et al, 1970; Merchant and Merchant, 1980; Albrecht and Bleier, 1979; Ling, 1979c). These cells may represent a relatively stable population, or more likely be constantly renewed from circulating monocytes as well as from the resident macrophages. They are phagocytic, express to a variable degree surface receptors for IgG (Fc portion) and complement (C3b) and show cytochemical patterns similar to those cells of the monocyte-macrophage series (Oehmichen, 1978).

The MPS of the brain is thought to play a critical role as a primary host defence mechanism against viral and bacterial invasion (Bleier et al, 1975; Albrecht and Bleier, 1979). The function of the brain MPS in the context of an immune response is still unclear, although the cells respond morphologically and functionally to antigenic challenge. It has been assumed that they function as antigen presenting

cells (McKeever and Balentine, 1978; Prineas, 1979; Prineas and Graham, 1981), and the presence of Ia positive dendritic cells in the choroid plexuses and meninges supports this possibility (Hart and Fabre, 1981).

In recent years it has become clear that macrophages play a major role in the development and expression of demyelinating diseases. Morphological studies have demonstrated that myelin basic protein (MBP) and, to a lesser extent, other proteins in the CNS are susceptible to neutral proteases secreted by activated macrophages (Cammer et al, 1978). This function of the macrophages is probably non-specific and has been called the "by-stander" effect. There is evidence that both perivascular macrophages and microglia are required for completing the digestion of myelin breakdown products (Prineas and Wright, 1978). Brosnan et al (1981) could protect animals against the development of experimental allergic encephalomyelitis (EAE) by inducing macrophage blockade with an intraperitoneal injection of silica dust, thus supporting the involvement of macrophages in the clinical and pathological expression of the disease.

The presence of receptors for the F_c portion of IgG has been described in the choroid plexuses, leptomeninges, arachnoid granulations and perivascular tissue of the neural parenchyma (Peress et al, 1981; Nyland, 1982). It was suggested that these receptors may help to transfer IgG from the blood into the CSF through the choroid plexuses and back from the SAS into the blood via the arachnoid granulations. It has also been suggested that the deposition of immune complexes in these

areas may play an important role in the development of the neuropsychiatric manifestations of systemic lupus erythematosus (SLE) (Atkins et al, 1972; Peress et al, 1981).

1.3 Evidence suggesting neuroendocrine regulation of immune responses

Although contradictory, available data indicate that neurotransmitters (e.g. noradrenaline) and hormones (e.g. growth hormone, thyroxine) do affect immune responsiveness as demonstrated by impaired delayed-type hypersensitivity (Jankovic and Isakovic, 1973), abrogation (Goldstein, 1978) or enhancement (Besedovsky et al, 1979; Miles et al, 1981) of antibody production, inhibition of blastogenesis (Galant and Remo, 1975; Keller et al, 1980) and enhancement of allograft rejection (Dann et al, 1979).

The presence of sympathetic innervation of the thymus, spleen (Williams and Felten, 1981) and lymph nodes (Giron et al, 1980) as well as the presence of catecholamine receptors on the lymphocyte membrane (Pochet et al, 1979), suggests a link between the CNS and the immune system. Furthermore, changes in blood hormone levels and in the electrical activity of neurons in certain areas of the hypothalamus have been observed immediately before the peak of antibody response to antigens (Besedovsky and Sorkin, 1977). Discrete lesions in certain areas of the CNS can modulate the development of antibody responses (Besedovsky et al, 1979; Cross et al, 1980). Furthermore, sympathectomy can markedly enhance the antibody responses to T-dependent and T-independent antigens

(Besedovsky et al, 1979; Miles et al, 1981).

These observations indicate that a balanced relationship may exist between the nervous and immune systems.

1.4 The brain as an immunologically privileged site

The brain is often referred to as an immunologically privileged site. This concept is based on the following observations:

(1) The CNS is anatomically isolated from the rest of the body by a closed compartment formed by the meninges and the skull. Except for the foramen magnum, this compartment is only perforated by small openings (foramina) through which blood vessels and nerves pass.

(2) The barriers between blood and extracellular fluid in the neuropil (BBB) and between blood and the ventricular system (Blood-CSF barrier).

(3) The absence of an intrinsic lymphatic drainage system and of organised lymphoid tissue similar to that found in peripheral organs.

(4) The relatively long survival of grafts within the brain.

Direct experimental evidence has been presented supporting the concept of the immunological privilege of the brain. Medawar (1948) showed that skin allografts transplanted within the brain parenchyma, away from the ventricular system, grew indefinitely whereas such grafts were rapidly rejected when transplanted subcutaneously. However, prior immunisation of the animals with an orthotopic skin graft, led to lymphocytic infiltration and rejection of the intracerebral

implant. It was therefore concluded that although the intracerebrally implanted allograft could not activate the afferent arc of the immune response, it could activate the efferent arc in a pre-sensitised host. Raju and Crogan (1977) studying allograft survival in rat brains, concluded that a quantitative deficiency in the efferent arc, combined with a delay in the activation of the afferent arc of the immune response, was largely responsible for the extended survival of the brain implants. Recent studies of allografts from the superior cervical ganglia in rat ventricles showed that the degree of differentiation of the allografted tissue is of critical importance for the survival of the graft (Rosenstein and Brightman, 1978).

1.4.1 Intracerebral graft rejection

Evidence suggesting that the immunological privilege of the brain may be incomplete came from a series of intracerebral grafting experiments. Thus Scheinberg *et al* (1965, 1966), Hašek *et al* (1977) and Lodin *et al* (1977) could demonstrate both first and second set rejections in the brains of allogeneic hosts by using skin allografts and transplantable tumours (C57 BL/6 ependymoblastoma and Rous sarcoma virus). The second set rejection could be induced by subcutaneous or intracerebral pre-immunisation with the tumour cells. Lodin *et al* (1977) could detect high titres of cytotoxic antibodies after intracerebral (*i.c.*) pre-immunisation, and recently Doherty *et al* (1981) reported that high numbers of cytotoxic lymphocytes were present in the CSF of mice rejecting an allogeneic mastocytoma (P 815) which had been transplanted *i.c.*

It was concluded that cytotoxic T lymphocytes were the cells responsible for the rejection.

These findings indicate that graft rejection mechanisms can operate in the brain.

1.4.2 Non-barrier sites and CSF-lymphatic connections in the brain

Dempsey and Wislocki (1955), using a tracer technique with silver nitrate in the drinking water of experimental animals, confirmed previous studies with intravenous injections of acidic dyes, that certain regions of the CNS lack a BBB. These regions include the circumventricular structures (median eminence, neurohypophysis, paraventricular organ, pineal body, area postrema) and the choroid plexuses. Morphological studies have shown that in these regions, the capillaries have fenestrations or pores, covered by a thin diaphragm, which allows rapid exchange of materials to and from the circulation (Weindl and Joynt, 1972; Jacobs, 1980).

Once intravenously injected tracers get access to the perivascular space by crossing the cerebral blood vessel walls, which lack the tight barrier junctions, there is no further barrier preventing their diffusion into the intercellular spaces of the neuropil (Blinzinger and Muller, 1971; Cserr, 1974).

Substances or particles injected into the SAS or brain parenchyma (i.bp.) flow rapidly out through CSF-lymphatic connections into the submaxillary and deep peritracheal lymph

nodes where they can be detected in very high quantities (Oehmichen, 1978; Bradbury and Cole, 1980; Bradbury, 1981).

Essick (1920) demonstrated that following injection of particulate material into the SAS there was a migration of macrophages towards the lymphatics of the head and neck regions. McKeever and Balentine (1978) suggested that this process was slow and possibly dependent on the amoeboid activity of the cells and/or the presence of specific surface receptors. The movement of antigenic material free or bound to brain macrophages from the CNS directly into the lymphatic system, might provide the basis for a positive activation of the afferent arc of the immune response.

Czernjawska (1970) demonstrated that communications between the CSF and the lymphatic system might provide a route of access for viral and bacterial infections of the brain, even against the direction of the CSF flow.

1.4.3 Organised lymphoid accumulations in the brain

Recent studies in mice, rats and birds, demonstrated the presence of organised lymphoid tissue in the pineal region, choroid plexuses and meninges of normal adult animals (Abe *et al*, 1971; Uede *et al*, 1981; Cogburn and Glick, 1981). These lymphoid accumulations increase in size and numbers with age and then gradually recede until only scattered nodules remain. Thus Cogburn and Glick (1981) could detect no fewer than 15 germinal centres in the pineal body of 32 week old chicken. In the mouse and rat the majority of pineal lymphocytes are T cells, because they react with anti-

T cell serum, and the ability to respond to antigenic stimulation suggests that they are immunocompetent (Cogburn and Glick, 1979; Uede et al, 1981).

The pineal body lacks a BBB and has fenestrated blood vessels which receive autonomic innervation from the periphery. These features suggest that the lymphocytes in this region may be derived from the blood stream. However, the extirpation of the central lymphoid organs (thymus and bursa of Fabricius) followed by whole body irradiation (700 R), only temporarily inhibited the formation of germinal centres in the pineal body, suggesting that the lymphocyte population in this region may, at least in part, arise in situ (Cogburn and Glick, 1981).

Recently Bartlett (1982), using a spleen colony-forming unit (CFU) assay, reported the presence of pluripotent stem cells in the brains of adult mice. These cells were capable of reconstituting lethally irradiated recipients and it was suggested that haemopoietic stem cells, which had migrated into the brain during foetal life, were prevented from leaving after the development of the brain barrier systems.

Stensaas and Horsley (1975) could induce formation of lymphoid accumulations by implanting micropipettes containing phytohaemagglutinin (PHA) into the brain parenchyma of adult rats. These accumulations, which were not observed after implantation of micropipettes containing pokeweed mitogen (PWM), consisted of lymphocytes, plasma cells and macrophages, and they persisted at least up to the age of ten

weeks. Lastly, it should be pointed out that organised lymphoid tissue and production of thin walled channels similar to lymphatic capillaries, and containing lymphocytes, have been observed in the CNS of patients with certain neurological diseases, notably multiple sclerosis (Prineas, 1979).

It can thus be concluded that, even with respect to transplantation immunity, the brain is not a privileged site in an absolute sense.

CHAPTER 2

AIMS AND OBJECTIVES OF THE STUDY

2 Aims and objectives of the study

Little is known about the distribution and traffic of B and T lymphocytes in the normal CNS. The presence of perivascular infiltrates ("cuffs") of lymphocytes and plasma cells and the rapid local production of immunoglobulins, with restricted heterogeneity, in the CSF and brain tissue are striking features of certain diseases affecting the CNS. These include multiple sclerosis (Paterson and Whitacre, 1981), subacute sclerosing panencephalitis (SSPE) (Valdimarsson et al, 1979; Tourtellotte et al, 1981) and progressive rubella panencephalitis (PRP) (Vandvik et al, 1978; Wolinsky et al, 1982).

Several studies have demonstrated that viral and bacterial infections of the CNS are associated with production of large amounts of specific antibodies. This has been shown for measles in SSPE (Mehta et al, 1982), rubella in PRP (Wolinsky et al, 1982), treponema in neurosyphilis (Pedersen et al, 1982) and Mycobacterium tuberculosis in tuberculous meningitis (Kinnman et al, 1981). These findings suggest a relative hyperactivity of the humoral immune system when antigens are presented within the CNS. The presence of T cells with receptors for IgG (T_γ cells) which may represent suppressor cells, are found in increased proportions intra and extrathecally in MS patients (Manconi et al, 1978; Goust et al, 1980). Couch et al (1978) described the presence of hyperactive suppressor T cells in the CSF of patients with histoplasma meningitis. It has been suggested that the extremely high antibody titres in the serum of SSPE patients

might be due to defective T suppressor cell function (Valdimarsson et al, 1974). The available evidence suggests that the persistent production of oligoclonal immunoglobulin in the CSF is due to an imbalance between T cell subsets involved in immunoregulation.

The vigorous humoral immune responses in these diseases contrast with the relatively weak cell-mediated reactions reported in relation to the brain allograft rejection. With the exception of a few reports that antigens injected into the SAS evoke a vigorous systemic antibody response (Jankovic et al, 1961; Panda et al, 1965; Šterzl and Lodin, 1978), the humoral immune responses to defined antigens has not been extensively investigated in relation to the CNS. It was therefore decided to carry out a systematic study of antibody responses to CNS injections of T-dependent and T-independent antigens.

CHAPTER 3

GENERAL METHODS

3 General Methods

3.1 Animals

(CBA x C57BL/6) F1 hybrid mice were obtained from the breeding colonies at St. Mary's Hospital Medical School. All animals were male and generally between 3-4 months old.

3.2 Preparation of antigens

3.2.1 Thymus independent soluble antigen (TNP-AECM.Ficoll)

This was prepared according to Inman (1975).

Recrystallisation of NaTNBS (Picryl sulphonic acid, sodium salt, Aldrich Chemical Co. Ltd., England) was performed by heating and stirring the NaTNBS with ethanol-acid (0.1N HCl) and 3g Charcoal (Darco G60) until reflux. The crystals were collected by vacuum filtration and allowed to dry at room temperature. The above procedure was continued until the material was considered free from charcoal.

Carboxymethylation of Ficoll (CM-Ficoll) 13.3g of Ficoll (Pharmacia Chemicals, Uppsala, Sweden) were dissolved in 85ml of 1.35M sodium chloro acetate (Fisher Sc. Co., Silver Spring, Md) and 10N NaOH added at time zero. The reaction was allowed to proceed for two hours at room temperature under constant stirring, then stopped with 2M NaH_2PO_4, the pH being adjusted to 7.0 with 10N HCl. The solution was then dialysed for five days with two daily changes against distilled water saturated with toluene.

AECM.Ficoll The volume of the CM.Ficoll was measured and amino ethyl groups introduced by adding an excess (12.5mg/ml) of carbodiimide (EDC-HCl, Calbiochem, La Jolla, Calif.) in aqueous solution. The mixture was kept under constant stirring for four hours at room temperature, the pH monitored throughout and kept at 4.7. The solution was then dialysed against 0.5M NaCl for two days and for a further four days against distilled water. After dialysis the material was lyophilised and maintained in a dessicator over $CaCl_2$.

Conjugation of NaTNBS to AECM.Ficoll The procedure of Lewis and Goodman (1977) was followed. The hapten was conjugated to the activated carrier (AECM.Ficoll) by mixing 1.5g of AECM.Ficoll dissolved in 0.28M cacodylate buffer pH 11.0, with 900mg of NaTNBS previously dissolved in cacodylate buffer. The reaction was allowed to proceed under constant stirring at room temperature for six hours. All procedures, including dialysis, were carried out in a foil-wrapped container to prevent photodecomposition. The deep yellow solution was then dialysed in the cold-room ($4^{o}C$) for two days against 0.2M NaCl and for three more days against distilled water. After dialysis the material was lyophilised and kept in a dessicator ($4^{o}C$) protected from light. The molar ratio TNP:AECM.Ficoll determined spectrophotometrically (Fields, 1972; Inman, 1975) was 14:1.

3.2.2 Thymus dependent soluble antigen (TNP-BSA)

This was prepared (Lewis and Goodman, 1977) by reacting 300mg of BSA (Bovine albumin-Fraction V, Miles Lab. Ltd.),

dissolved in 15ml of 0.28M cacodylate buffer pH 11.0, with 180mg of recrystallised NaTNBS previously dissolved in 15ml of cacodylate buffer. The reaction was allowed to proceed under constant stirring for six hours at room temperature, followed by exhaustive dialysis against water and lyophilisation. All procedures were done in a foil-wrapped container to prevent photodecomposition. The molar ratio of hapten:carrier was measured spectrophotometrically (Fields, 1972) and found to be 12:1.

3.2.3 Thymus dependent particulate antigens

3.2.3.1 Sheep Red Blood Cells

SRBC (TCS, Slough, England) in Alsever's were washed three times with normal sterile saline and used as the T-dependent particulate antigen for both *in vivo* and *in vitro* tests.

3.2.3.2 TNP-SRBC

TNP-SRBC was used only for the *in vitro* tests. SRBC were coupled to NaTNBS by the method of Rittenberg and Pratt (1969). SRBC in Alsever's were first washed three times with normal sterile saline. Recrystallised NaTNBS (40mg), dissolved in 5ml of cacodylate buffer pH 6.9, was mixed with 4ml of packed SRBC resuspended in 15ml of cacodylate buffer pH 6.9. After constant stirring for 45 minutes at room temperature, the reaction was stopped with 220mg/ml of glycyl glycine (Sigma Chem. Co., England) in cacodylate buffer pH

6.9 and the preparation was washed many times with saline. Spectrophotometrical analysis of TNP-SRBC (Fields, 1972) showed that 10^9 TNP molecules were bound to each SRBC.

3.2.4 Preparation of de-aggregated BSA (d-BSA)

This was prepared (Hudson and Hay, 1980) by centrifuging 10mg/ml of BSA (Bovine albumin-Fraction V, Miles Lab. Ltd., England) dissolved in sterile normal saline in a MSE automatic super speed 40TC (angle rotor 10 x 10ml) at 100,000 x g for three hours at 4°C.

3.3 Immunisation routes

3.3.1 Subcutaneous injection (s.c.)

The animals were held by the tail and injected (200µl) into the dorsal back region with a 1ml disposable syringe and 25G $\frac{5}{8}$ inch needle.

3.3.2 Intravenous injection (i.v.)

The animals were kept in a mouse holder, the tail cleaned, warmed with distilled water (40°C) and then dried. The injection (200µl) was given into the lateral tail vein, using a 1ml disposable syringe and 25G $\frac{5}{8}$ inch needle.

3.3.3 Intra peritoneal injection (i.p.)

The animals were held firmly and the injection (100-500µl) performed just below the umbilicus with a 1ml disposable

syringe and 25G $\frac{5}{8}$ inch needle.

3.3.4 Material used for surgery

- Sagatal (Pentobarbotine sodium - May and Baker Ltd., Dagenham, England) 10mg/kg given i.p.
- Electric shaver
- Solution of 1% glycerol-70% ethanol
- Filmated swabs (5 x 5cm, Johnson and Johnson)
- Sterile scissors, tweezers, forceps and a scalpel with a sharp blade
- Autoclips 9mm (stainless steel No. 7631, Clay and Adams)
- Catgut 5/0 BP, 60 in ETHICON
- Needles: Lane's cleft palate curved or curved needles for the eye
- Normal sterile saline

3.3.5 Intra liver parenchyma injection (i.L.)

The i.L. injections were performed with the animals under deep anaesthesia. The overlying skin was shaved, cleaned with glycerol-ethanol and a sagittal incision made through both skin and peritoneum, just below the right costal margin. One hundred microlitres of antigen preparation was injected slowly with a microsyringe (Hamilton micromeasure BV-710 LT) and a small needle (Microlance 26G, $\frac{3}{8}$ inch, Ind. Brasileira). The peritoneum was then sutured with catgut, the skin wound closed with small animal clips and the surface finally irrigated with

a solution of glycerol-ethanol. Mice which bled profusely after the injection were withdrawn from the experiment.

3.3.6 Intra brain parenchyma injection (i.bp.)

The mice were deeply anaesthetised and the overlying skin of the head and neck regions shaved and cleaned with glycerol-ethanol. A parasagittal incision was made through all soft tissue with a sharp-pointed scalpel blade and a posterior parietal 2mm diameter hole drilled with a fine burr, attached to a variable drilling motor ($\frac{1}{8}$, 1,200-14,000 rpm). Two to ten microlitres of the antigen preparation in sterile saline was injected slowly with a 10µl microsyringe (Hamilton 701-N). The wound was then irrigated with sterile saline and dried with a filmated swab. The hole was then closed with zinc cement No. 1 (SS White, Ind. Brasileira) and the incision with small clips.

3.3.7 Intra subarachnoid space injection (i.sas.)

These were performed through the right temporo-parietal region, with the animals under anaesthesia. The injections were done slowly with a microsyringe (Hamilton micromeasure BV-710 LT) and a small needle (microlance B-G 10 x 4.5 26G $\frac{3}{8}$, Ind. Brasileira) penetrating no more than 2mm. Mice which bled after the injection were withdrawn from the experiment.

Following the i.L., i.bp. and i.sas. injections, the animals were kept in a microflow incubator (37°C) to speed recovery, and the drinking water was sterile and contained antibiotics (neomycin sulphate 100mg/L and polymyxin B 10mg/L).

3.4 Preparation of brain and liver membranes (Mallet et al, 1979)

The mice were anaesthetised with ether and exsanguinated by sectioning the heart. Liver and brains (including cerebrum, cerebellum and meninges) were removed immediately and placed in a small plastic Petri dish containing 10ml of an ice-cold sterile solution of 0.32M sucrose in 3mM $MgCl_2$ and 10mM Tris-HCl, pH 7.3. The tissues were pushed through a plastic medium mesh sieve, and then through a fine mesh wire sieve with the plunger of a 5ml disposable syringe. The suspension was then transferred to a 30ml plastic container and homogenised with 25 up and down strokes. The homogenate was centrifuged at 800 x g for 10 minutes in a MSE Coolspin centrifuge and the pellet washed twice with sucrose medium. The supernatants were pooled, diluted three times with sterile distilled water and centrifuged for 45 minutes at 100,000 x g in a MSE automatic super speed 40 TC at 4°C. The pellet was collected, the protein content determined and the preparations stored at -20°C.

3.5 Preparation of extracts

3.5.1 Homogenates of brain and liver

Mice were anaesthetised with ether and exsanguinated by sectioning the heart. Brain and liver from four mice were removed and placed into a plastic Petri dish containing an ice cold solution of 0.15M NaCl. The organs were sliced with scissors and rinsed with cold 0.15M NaCl. The tissues were pushed through a nylon sieve following dissociation by twenty

passages through a Pasteur pipette, then filtered through a fine mesh stainless steel sieve, clipped on to a 30ml Sterilin Universal container (128 A-Sterilin, England). The preparation was centrifuged twice at 2,200 x g for 30 minutes on a MSE Coolspin. The pellet was resuspended in sterile 0.15M NaCl, stored at -20°C and used as brain or liver homogenate.

3.5.2 Crude saline brain extract (Rostrom et al, 1981)

Mice were anaesthetised by ether and exsanguinated by sectioning the heart. The brains (cerebrum, cerebellum and meninges) were removed and placed into a plastic Petri dish containing an ice cold solution of 0.15M NaCl. The tissues were minced with scissors and rinsed with cold 0.15M NaCl to remove contaminating peripheral blood cells. The tissues were then strained through sieves of graded mesh (60, 80 inch). The homogenisation of the resultant cell suspension was carried out in an Ultrasonic disintegrator, with four cycles of 20 minutes each. The homogenate was centrifuged at 100,000 x g for 30 minutes at 4°C and the supernatant collected. The pellet was resuspended in 0.15M NaCl (1:2 weight/vol) and then sonicated again. This procedure was repeated twice. The supernatants were collected, frozen at -80°C and lyophilised after measuring the protein content. The preparation was stored at -20°C and used as a crude saline extract from mouse brain (Extract I).

3.5.3 Brain lipid-proteolipid and ganglioside extract

The pellet remaining after the crude saline extraction (see 3.5.2) was mixed with 5 volumes of chloroform-methanol (1:1 vol/vol) and 0.5 volumes of aqueous 2M KCl. This procedure was repeated three times. The collected extracts were submitted to a Folchi partition, into a lower chloroform phase (Extract II) and an upper water methanol phase (Extract III). The lower chloroform phase (Extract II), which has been shown to contain tissue lipid-proteolipids, and the ganglioside rich upper phase (Extract III), were both evaporated under nitrogen, followed by lyophilisation to remove chloroform and methanol. The resulting preparation was resuspended in a 20ml 0.15M NaCl and then dialysed against distilled water to remove most of the KCl. After measuring the protein content, the extracts were lyophilised and stored at -20°C.

3.6 Determination of the protein content

This was performed by using the Lowry protein assay (Lowry et al, 1951).

3.7 Preparation of cell suspensions

3.7.1 Spleen and lymph node cell suspensions

Mice were killed by ether and dipped into 1% glycerol-70% ethanol. Spleen and lymph nodes (axillary, cervical, brachial, mesenteric, and inguinal) were aseptically removed

and placed into small plastic Petri dishes (5 x 5cm Sterilin, England), containing 3ml of medium (Hank's BSS with 0.35g/L sodium bicarbonate, Flow Lab., U.K.). The organs were dissected free of connective tissue and fat. The tissues were then transferred to fine (80 inch) mesh stainless steel sieves (Young Archibald and Son Ltd., Scotland) clipped on to a 30ml Sterilin Universal container (128 A Sterilin, England) and teased apart with forceps and mashed gently with the plunger of a 2ml disposable syringe. During this procedure, the tissues were rinsed frequently with cold medium. Cell clumps were allowed to sediment for a few minutes. The supernatant was then transferred to a new Universal container and spun for 5 minutes at 200 x g in a bench centrifuge. The cells were washed twice more with medium and resuspended in 3ml of medium (Joklik Modification of MEM for cultures, Flow Lab., U.K.) with 20% foetal calf serum (FCS - SeraLab, U.K.). Cell viability was assayed by dye exclusion (trypan blue 0.12% in saline).

3.7.2 Brain cell suspension

Mice were anaesthetised with ether, the body cleaned with glycerol-ethanol and then exsanguinated. The brains (cerebrum, cerebellum and meninges) were aseptically removed and placed in a small Petri dish containing medium (Hank's BSS) with 50µg/ml of DNase (Deoxyribonuclease I DN 25, Sigma Chem. Corp., U.K.) to reduce cell clumping. The tissues were transferred to a medium mesh nylon sieve, teased apart with forceps and mashed with the plunger of a 5ml disposable

syringe. Any particulate material trapped on the nylon was discarded. The cell suspension was then washed with cold medium. The resultant cell suspension was then dissociated into a single cell suspension by several passages through needles of graded gauges (19G, 21G, 23G). It should be mentioned that the animals were not submitted to any perfusion procedure prior to the removal of the brains.

3.7.3 Isolation of CNS lymphocytes (Lublin and Maurer, 1980)

The resultant brain cell suspension was first layered over a modified Ficoll-Paque gradient (6 parts of Ficollpaque with 1 part of medium-DNase) and centrifuged at 2,000 x g for 30 minutes at 4°C in a MSE Coolspin. The pellet was resuspended in a medium-DNase, layered again over Ficoll-Paque gradient and centrifuged at 2,000 x g for 30 minutes at 4°C. The interface was then removed, washed twice and resuspended in Joklik Modification of MEM, containing sodium bicarbonate and supplemented with 10% FCS. The viability was assessed by the trypan blue dye exclusion test.

3.8 Measurement of the antibody responses

3.8.1 Haemagglutination assay

The Takatsy microtitre system was used. Antisera were inactivated by heating at 56°C for 30 minutes in a water bath and double dilutions made with normal saline in a microtitre plate (M 24AR - Sterilin Products, England). SRBC or TNP-SRBC

at a concentration of 2% were then added, and the plates incubated for one hour at 37°C or overnight at 4°C. Haemagglutination was read from the macroscopic patterns of red cell deposition at the bottom of the wells. The results are expressed as mean $\log_2 \pm$ SE and geometric mean in parenthesis or as reciprocal antibody titres.

3.8.2 Assays for antibody forming cells

3.8.2.1 Monolayer (Cunningham) technique

The numbers of specific antibody forming cells (AFC) in the spleen, lymph nodes and central nervous system were estimated for individual mice by the haemolytic plaque method (Cunningham and Szenberg, 1968) usually four to five days after antigen challenge. Direct plaques, developed without anti-globulin serum, were considered to represent IgM antibodies. Indirect plaques developed by adding 1:100 dilution of anti-IgG were considered to reflect IgG antibody production.

3.8.2.2 Localised haemolysis in gel (LHG)

Lymphocytes isolated from the brains of four to five mice were assayed by the Petri dish assay (Dresser, 1978) for the presence of specific antibody forming cells. Indirect plaques were developed by adding 20µl of 1:100 dilution of anti-IgG serum. SRBC or TNP-SRBC were used at a concentration of 20% in medium.

Guinea pig serum absorbed with both SRBC and TNP-SRBC was

used as the source of complement, either undiluted for the Cunningham technique or as 1ml of 10% dilution in medium for LHG. The results are expressed as $\log_{10} \pm$ SE and geometric mean in parenthesis.

3.9 Collection of mouse cerebrospinal fluid (CSF)

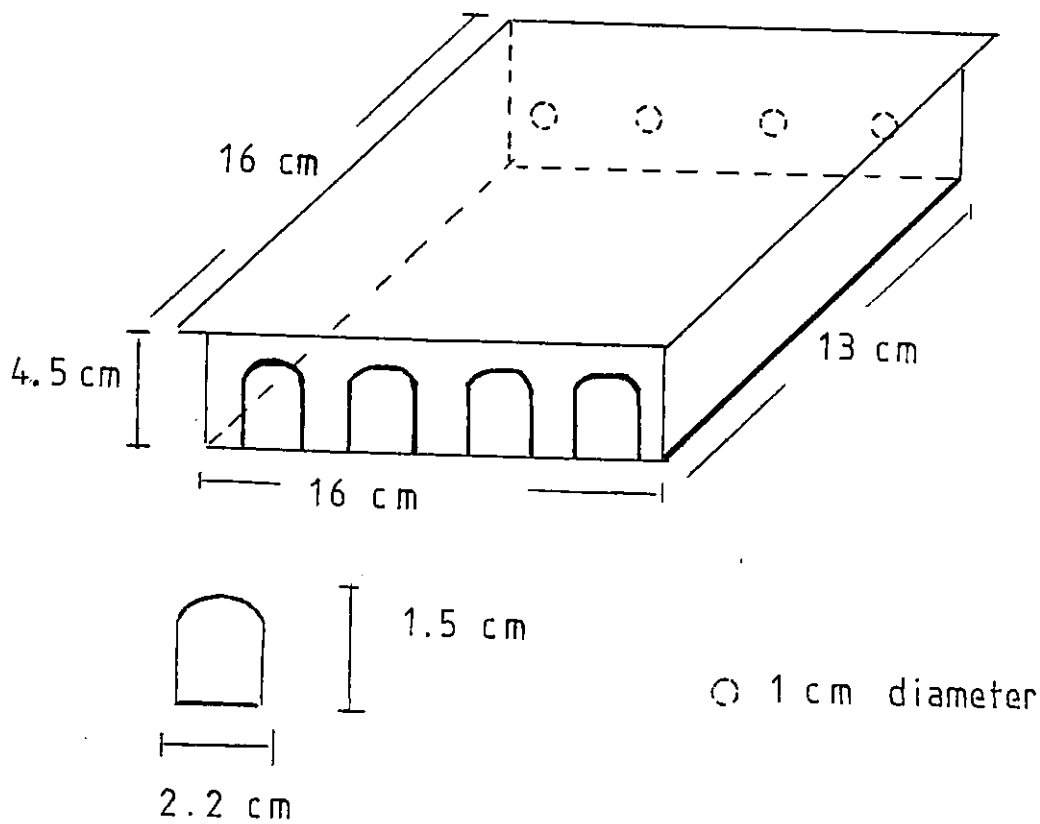
This was performed using the technique described by Carp et al (1971). Mice were first anaesthetised with Sagatal (10mg/kg, i.p.), the body cleaned with glycerol-ethanol and then exsanguinated by sectioning the heart. The overlying skin of the head and neck regions was removed. The animals were then held by the head with forceps. The dura mater over the foramen magnum was pierced with a very fine needle attached to a microsyringe (Hamilton 701 N). With this technique, 2-10µl of transparent CSF was obtained from each mouse. The CSF samples were pooled, but those demonstrating macroscopical contamination with blood were discarded.

3.10 X-irradiation

Mice were anaesthetised with Sagatal (10mg/kg, i.p.). The X-ray machine (Stabilipan X-Ray machine) was operated at 240 KV constant potential and 10 mA, with or without 0.5mm copper and 10mm aluminium filters. The irradiation field was uniform within 3% over 15cm. A lead shielding box was used to shield the body with the exception of the head and conversely to shield only the head. Groups of four mice were submitted to the following exposure rates and f.s.d.:

Distance from the source (f.s.d.)	Radiation/min	Total	Filters
20 cm	322.5	2000R	-
60 cm	30.3	200R	+
60 cm	37.9	400R	+

Specifications of the lead-shielding box: 2.5mm lead sheet



3.11 Foetal calf serum (FCS)

This was supplied by SeraLab, Flow Lab., U.K. It was inactivated by heating 56°C for 30 minutes, distributed in 30ml plastic containers (128 A Universal containers) and stored at -20°C until used. Just before use the FCS was thawed and sterilised by filtration through a Sartorius membrane filter, pore size 0.22 and used at 10% in the media.

3.12 Culture media

The media were obtained in powder form and dissolved in double distilled water. The pH was adjusted to 7.2 with either 1N HCl or 1N NaOH. The medium was sterilised by filtration through 293mm diameter Sartorius membrane sterilisation filters, and stored at 4°C.

3.13 Statistical analysis

Transformed data ($\log_{10}$, $\log_2$) were analysed for significance using Student's t-test. Only values of p less than 0.05 are considered significant.

C H A P T E R 4

COMPARATIVE STUDY OF THE MAGNITUDE OF THE ANTIBODY RESPONSE IN ADULT MICE IMMUNISED INTO VARIOUS SITES

4.1 Introduction

Humoral immune responses originating within the CNS have been little analysed in terms of cellular interactions and kinetics. There are only a few reports indicating that injections of xenogeneic red blood cells into the SAS of adult animals lead to higher systemic antibody responses than injections into conventional sites (Jankovič *et al*, 1961; Panda *et al*, 1965; Šterzl and Lodin, 1978). Injections into the brain parenchyma itself resulted in antibody levels similar to those achieved by subcutaneous immunisation (Panda *et al*, 1965). It was suggested that the high antibody titres were due to a prolonged stimulation of the immune system by the slow removal of SRBC from the CSF (Panda *et al*, 1965).

Most immunological studies of diseases involving the CNS have focussed on lymphocytes from extra-neural sites (peripheral blood, spleen, lymph nodes). Only a few studies of immune responses by lymphocytes from the CNS have been reported.

Šterzl and Lodin (1978) used frozen tissue sections of brains from mice immunised intracerebrally (*i.c.*) with SRBC; Lublin and Maurer (1980) using a modified Ficoll technique, isolated brain lymphocytes from animals with EAE and Gerhard and Koprowski (1977), using a discontinuous BSA gradient, detected a much higher ratio of virus specific B cells in the brains of mice than in their spleen or peripheral blood. They also demonstrated the persistence of these cells in the brains for at least 4-7 months after *i.c.* inoculation.

The purpose of the study reported in this chapter was to compare the magnitude of systemic antibody responses to different doses of T-dependent and T-independent antigens injected into the CNS and into conventional sites. Furthermore, brain lymphocytes were isolated and assayed for the presence of specific B memory cells.

A classification of antigens into thymus dependent (TD) and thymus independent (TI) types has proved very useful. TD antigens are only able to activate B lymphocytes effectively in the presence of macrophages and T lymphocytes or T-derived factors, whereas TI antigens appear to be minimally dependent upon the presence of lymphocytes and macrophages to stimulate B cells (Mond et al, 1980).

In the present study, SRBC and TNP-BSA were used as TD particulate and soluble antigens respectively, and TNP-Ficoll as a TI soluble antigen.

4.2 Materials and methods

4.2.1 Animals

Male (CBA x C57BL) F1 mice, 3-4 months old. Each experimental group consisted of at least 4 mice.

4.2.2 Antigens

Sheep Red Blood Cells (SRBC - TCS, Slough, England) in Alsever's solution were washed three times with normal sterile saline and used as the TD particulate antigen in

various doses.

TNP-BSA was prepared according to Lewis and Goodman (1977), as described in General Methods (3.2.2) and used as the TD soluble antigen at 10 and 100µg.

TNP-Ficoll was prepared according to Inman (1975), as described in General Methods (3.2.1) and used as the TI soluble antigen at 10µg.

TNP-SRBC was prepared according to Rittenberg and Pratt (1969) as described in General Methods (3.2.3) and used for *in vitro* tests.

4.2.3. Immunisation

All antigens were suspended in normal sterile saline. For injections into the subarachnoid space (*i.sas.*) and liver parenchyma (*i.L.*) 10 or 100µl of antigen were used. For subcutaneous (*s.c.*), intravenous (*i.v.*) and intraperitoneal (*i.p.*) injections, 200µl were used and 2 or 10µl for injections into the brain parenchyma (*i.bp.*). Immunisation into the subarachnoid space and intra brain parenchyma were performed as described in General Methods (3.3).

4.2.4 Preparation of cell suspensions

Spleen and lymph node cell suspensions were prepared as described in chapter 3.7 and used for *in vitro* experiments.

Isolation of CNS lymphocytes: These were prepared as described in chapter 3.7 according to Lublin and Maurer (1980)

and used for *in vitro* experiments.

4.2.5 Measurement of antibody responses

Serum from each individual mouse was collected and antibody levels titrated in a direct haemagglutination assay (as in 3.8.1).

Primary responses were tested 7 days after priming and secondary responses 5, 7, 10, 20 and 30 days after rechallenge. The results are expressed as mean $\log_2 \pm$ SE or as the reciprocal of antibody titres.

The numbers of antibody forming cells (PFC) were determined for each mouse either 4 or 5 days after priming or 4-7 days after rechallenge, by the direct and indirect haemolytic plaque technique (Cunningham and Szenberg, 1968) (as in 3.8.2.1). For the development of indirect PFC, a rabbit anti-mouse IgG serum was used at 1:100 dilution.*

The numbers of PFC in the CNS were determined by localised haemolysis in gel (LHG) (Dresser, 1978) (as in 3.8.2.2), and by the Cunningham and Szenberg (1968) technique.

Results are expressed as mean $\log_{10} \pm$ SE and the geometric mean in parenthesis.

* (a kind gift from Dr. M. Salaman).

4.3 Results

4.3.1 The influence of the route of immunisation on the humoral antibody responses

In a preliminary study, groups of four mice were primed and rechallenged either with SRBC (2×10^6) or TNP-Ficoll (10µg) i.v., i.sas., i.L. and s.c. In this study, the animals received volumes of 100µl (i.sas. and i.L.) or 200µl (i.v. and s.c.). The mice were bled seven days after the priming and five days after the rechallenge for primary and secondary responses respectively.

The results shown in Table 4.3.1 represent the mean of three experiments. Injections of SRBC into the subarachnoid space (i.sas.) induced significantly higher ($p < 0.01$) systemic antibody levels during the secondary response than immunisation by any other route. In contrast, the T-independent antigen did not show enhanced immunogenicity in the SAS, neither in the primary nor in the secondary response.

Table 4.3.1

Antigen	Route of immunisation	Antibody Titres ⁎	
		Primary response	Secondary response
SRBC	s.c.	15 ± 9	29 ± 17
	i.v.	46 ± 11	205 ± 31
	i.L.	37 ± 14	131 ± 72
	i.sas.	59 ± 20	614 ± 102
TNP-Ficoll	s.c.	32 ± 16	19 ± 7
	i.v.	64 ± 13	64 ± 13
	i.L.	53 ± 11	35 ± 16
	i.sas.	96 ± 16	66 ± 17

⁎ Log_2 ± SEM

4.3.2 The effect of different volumes of antigen injected i.sas. on the antibody responses

It has been demonstrated that injections of large volumes into the SAS can cause permanent damage to the blood brain barrier (BBB) systems (Cairns, 1950). Additional experiments were therefore carried out to verify whether the same degree of systemic antibody response could be achieved by i.sas. injections of 10 and 100µl of the antigens.

Groups of four mice were primed and rechallenged seven days later, either with SRBC (2×10^6) or TNP-Ficoll (10µg) i.sas. The animals were bled on day 7 for the primary

response and on days 3, 5, 10, 20, 24 after rechallenge for the secondary response. The results represent the mean of reciprocal antibody titres of four pooled sera. Fig. 4.3.1 shows that the magnitude and time course of antibody responses were similar in both groups, regardless of the volumes injected (10 or 100µl) into the SAS.

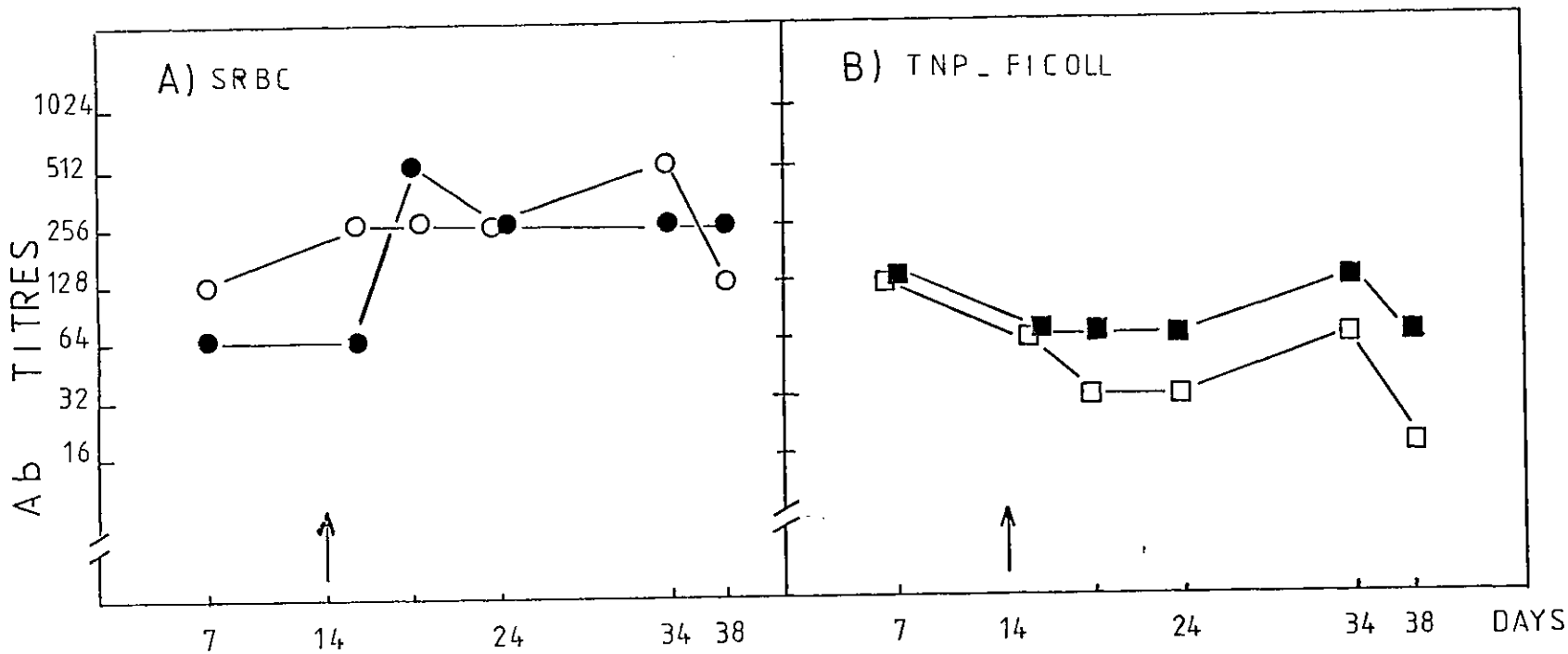
4.3.3 Time course of the systemic antibody responses

In these experiments, groups of four mice were primed and rechallenged with SRBC (2×10^5), TNP-BSA (100µg) or TNP-Ficoll (10µg) either i.v. or i.sas. The mice were bled seven days after the priming and 3, 5, 10 and 20 days after the rechallenge for primary and secondary responses respectively. The results represent the reciprocal mean of the antibody titres.

The antibody responses for the three antigens had a similar time course whether injected i.v. or i.sas. (Fig. 4.3.2).

With SRBC the difference in antibody levels between i.v. and i.sas. was maximal ten days after rechallenge, while a four-fold difference in antibody titres was observed with TNP-BSA during both the primary and secondary responses (Fic. 4.3.2a). In contrast, TNP-Ficoll induced identical antibody titres and time courses in the i.v. and i.sas. immunised animals (Fig. 4.3.2b).

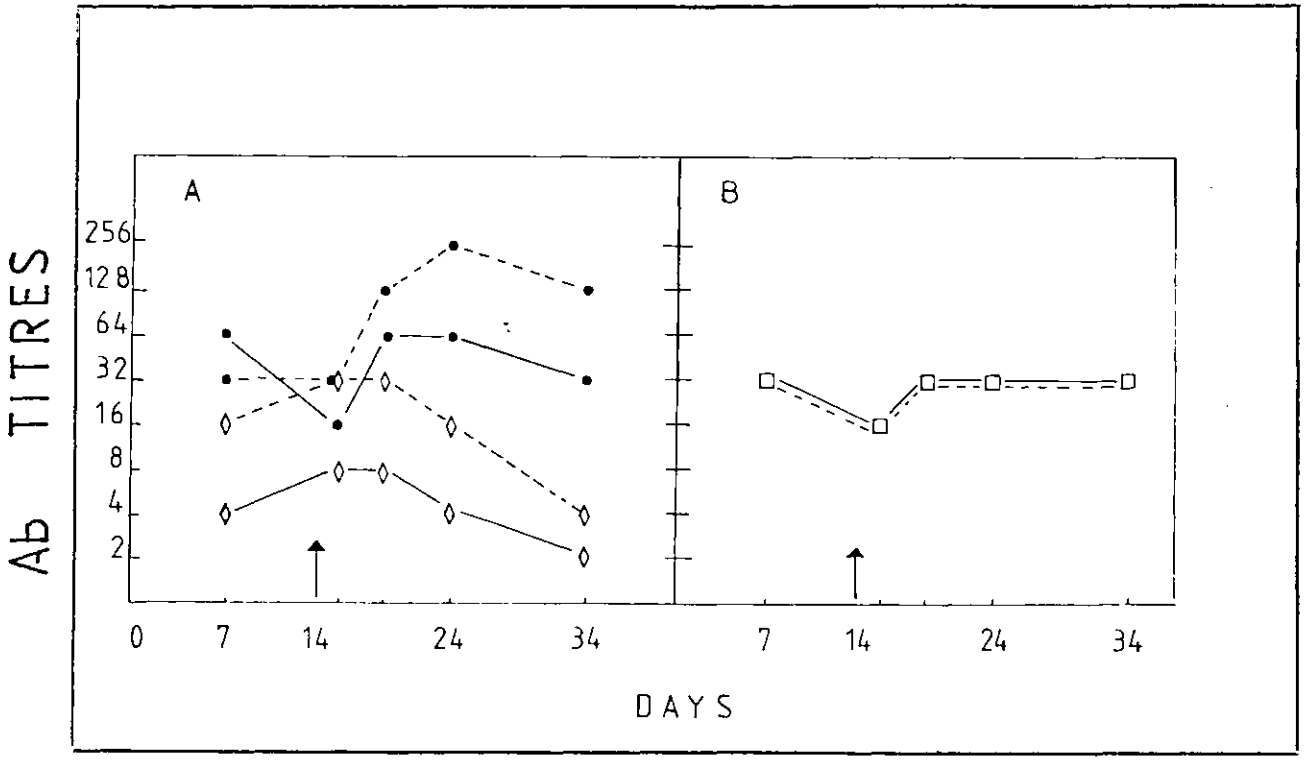
Fig. 4.3.1 The effect of different volumes of antigen injected i.sas. on the antibody response.



Groups of mice were primed on day 0 and rechallenged on day 14 (arrows) with the same antigen dose i.sas.

(A) SRBC (2×10^6) ●— ● 100ul, o — o 10ul. (B) TNP-Ficoll (10ug) ■— ■ 100ul, □ — □ 10ul.

Fig. 4.3.2 Time course of systemic antibody responses in groups of mice immunised i.v. or i.sas. Priming on day 0, rechallenge on day 14 (arrows). The same antigen dose was used for priming and rechallenge.



(A) ●—● SRBC i.v., ●---● SRBC i.sas. ◊——◊ TNP-BSA i.v., ◊----◊ TNP-BSA i.sas.

(B) □—□ TNP-Ficoll i.v., □----□ TNP-Ficoll i.sas.

4.3.4 The effects of varying doses of TD antigens on antibody responses

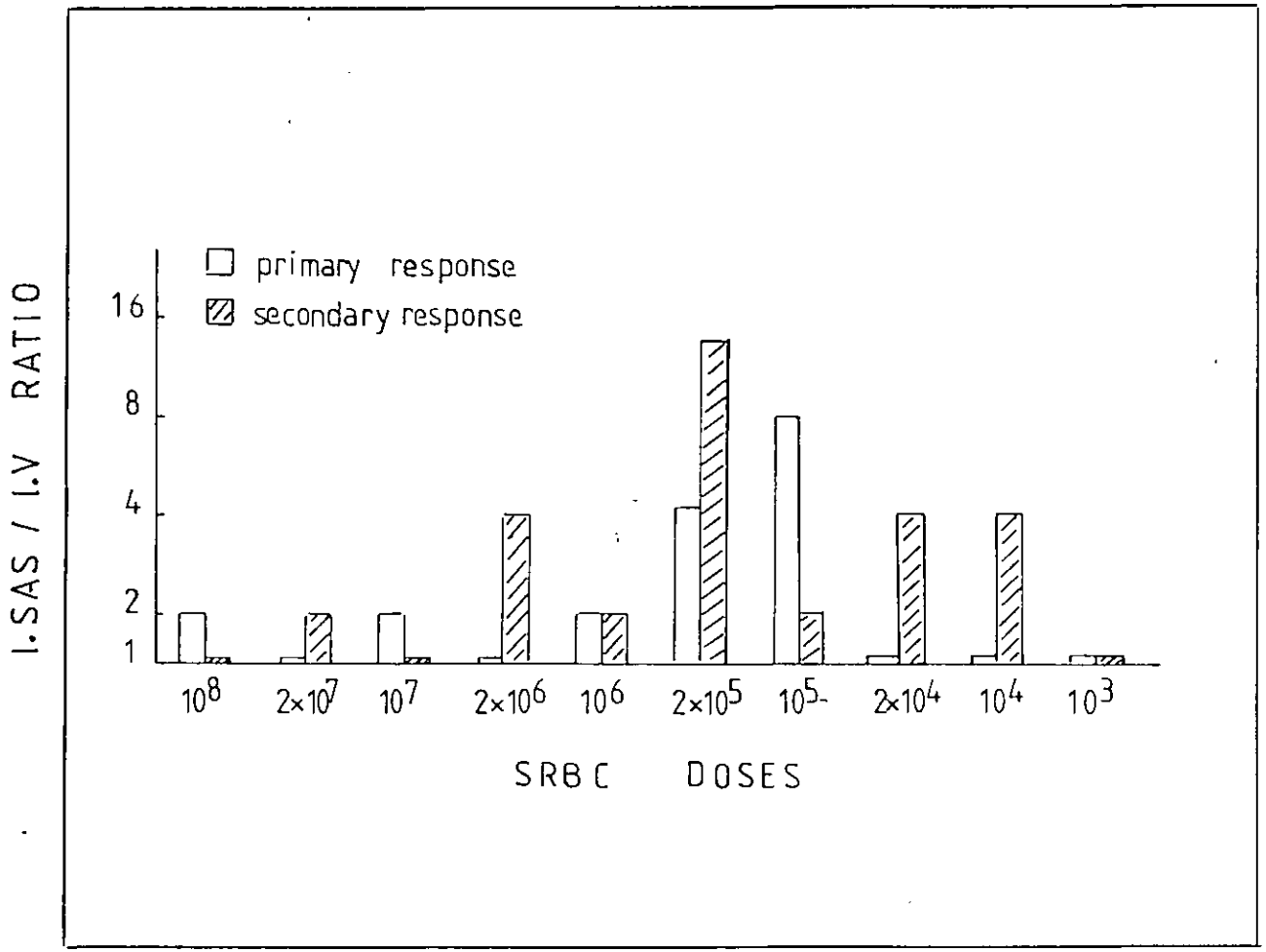
Ten groups of four mice were injected i.v. and i.sas. with different numbers of SRBC ranging from 10^8 to 10^3 per animal. Primary responses were measured seven days after the priming and secondary responses 5 days after the rechallenge. The results (Fig. 4.3.3) are presented as a ratio of the mean antibody titre in each group of i.sas. injected animals over the mean titre of the corresponding i.v. injected group.

There was a tendency towards higher primary and secondary antibody responses in the i.sas. injected group throughout the dose range, although this was only consistently observed for secondary responses in animals receiving less than 10^7 SRBC.

As the secondary antibody responses were measured only five days after rechallenge, it is possible that higher i.sas./i.v. ratios would have been observed for the secondary responses in the low dose range, if the antibody levels had been measured later (Fig. 4.3.2).

Using the same protocol the i.sas./i.v. ratios were higher than 1 in both primary and secondary responses against both 10 and 100 µg of TNP-BSA (Table 4.3.2). In contrast, TNP-Ficoll induced the same antibody titres in both primary and secondary responses regardless of the route of immunisation.

Fig. 4.3.3 The effects of varying doses of SRBC antigen on antibody responses.



Each column represents a ratio of the mean antibody titre of four i.sas. injected mice over the mean titre of the corresponding i.v. injected group.

Table 4.3.2

Effect of the dose and route of antigen on the antibody responses.

Antigen	Dose	Route	Rec. Ab Titres		i.sas./i.v. Ratios	
			Prim.	Sec.	Prim.	Sec.
TNP-BSA	100 µg	i.v.	4	32	4	2
		i.sas.	16	64		
TNP-BSA	10 µg	i.v.	2	4	4	4
		i.sas.	8	16		
TNP-Ficoll	10 µg	i.v.	32	32	1	1
		i.sas.	32	32		

4.3.5 Antibody responses at the cellular level in mice immunised by different routes

A series of experiments was carried out to compare antibody levels and numbers of PFC in the spleen, lymph nodes and CNS of mice immunised by different routes. Each set of data is derived from 2-3 experiments.

Groups of four mice were immunised i.v., i.p., or i.sas. with SRBC (2×10^7) and TNP-BSA (100µg). The animals were killed four days later and the numbers of PFC determined in the spleen and lymph nodes from each mouse.

The results (Table 4.3.3) show that mice immunised with SRBC by the i.sas. route had consistently higher ($p < 0.05$ to

$p < 0.01$) numbers of PFC in their spleens and lymph nodes than the mice receiving i.p. or i.v. injections.

Table 4.3.3

The influence of the route of immunisation on the numbers of PFC in the spleen and lymph nodes.

Groups	Spleen PFC		Lymph Node PFC	
	/spleen	/10^7 cells	/LN	/10^7 cells
i.v.	4.66 ± 0.07 (46,089) $p < 0.05$	3.85 ± 0.05 (7,206) NS	ND*	ND*
i.p.	4.62 ± 0.04 (42,576) $p < 0.01$	3.77 ± 0.05 (5,899) NS	3.32 ± 0.23 (2,109) $p < 0.01$	3.46 ± 0.20 (2,940) $p < 0.01$
i.sas.	4.88 ± 0.07 (76,988)	3.91 ± 0.05 (8,282)	4.06 ± 0.08 (11,526)	4.12 ± 0.06 (13,415)

* ND: not done.
All p values refer to the i.sas. immunised group.

Similar patterns of response were observed in mice immunised with 100µg of TNP-BSA (Table 4.3.4). However, with this antigen no increase was observed in the numbers of PFC in the lymph nodes of the i.sas. immunised mice. This is most likely due to the characteristic of the antigen, since the particulate antigen, SRBC, was able to induce increased numbers of PFC in the draining lymph nodes after a single i.sas. injection. Soluble antigens like TNP-BSA might need rechallenge(s) for this effect, but this was not tested.

Table 4.3.4

The influence of the route of immunisation of TNP-BSA on the numbers of PFC in the spleen and lymph nodes.

Groups	Spleen PFC		Lymph Node PFC	
	/spleen	/10^7 cells	/LN	/10^7 cells
Control*	2.59 ± 0.07 (390)	1.71 ± 0.03 (52)	1.93 ± 0.07 (86)	2.26 ± 0.05 (184)
i.p.	3.18 ± 0.08 (1,534) $p < 0.01$	2.29 ± 0.09 (196) $p < 0.05$	2.04 ± 0.09 (112) NS	1.91 ± 0.12 (83) NS
i.sas.	3.52 ± 0.07 (3,323)	2.70 ± 0.17 (507)	2.20 ± 0.05 (161)	2.08 ± 0.03 (122)

* The skulls of the mice were perforated with a 26G needle at the site of the i.sas. injection.

All p values refer to the i.sas. immunised group.

NS: not significant.

Table 4.3.5 gives the results of one experiment in which groups of five mice were primed and rechallenged with TNP-Ficoll (10µg). The animals were killed five days after the rechallenge and the numbers of PFC determined in the spleens and lymph nodes from each mouse.

The results show that despite rechallenge, no increase in spleen or lymph node PFC numbers was observed in mice receiving the T-independent antigen TNP-Ficoll into the SAS, over those immunised i.p.

Groups of 4-5 mice were immunised and rechallenged seven days later with SRBC (2×10^6) by various routes: s.c., i.p.,

Table 4.3.5

Influence of the route of immunisation with TNP-Ficoll on the numbers of PFC.

Groups	Spleen PFC		Lymph Node PFC	
	Direct	Indirect	Direct	Indirect
Control[*]	2.17 ± 0.20 (151)	2.27 ± 0.24 (186)	ND	ND
i.p.	4.34 ± 0.06 (22,327)	4.34 ± 0.09 (22,086)	2.43 ± 0.07 (272)	2.51 ± 0.14 (327)
i.sas.	4.36 ± 0.12 (23,379)	4.31 ± 0.10 (20,633)	2.56 ± 0.17 (371)	2.66 ± 0.03 (460)

[*] Skull was perforated with a 26G needle on the site of i.sas. injection.

ND: not done.

i.sas., and i.bp. Immunisation into the brain parenchyma was also performed with a very small inoculum (2µl) to avoid extensive damage to the brain architecture and barrier systems, and also to compare the degree of antibody response induced with small (10µl) and very small (2µl) i.bp. volumes of antigen preparations.

Table 4.3.6 shows that the difference between the numbers of spleen PFC in i.sas. versus i.p. or s.c. was always significant ($p < 0.02$ to $p < 0.001$). This also applied for the low volumes (2 and 10µl) of antigenic preparations injected i.bp. The total number of PFC isolated from each brain was only about 0.2% of the PFC isolated from each spleen. Thus the results indicate that the high antibody levels achieved after

Table 4.3.6

Detection of PFC in the spleen and brain from mice immunised with SRBC.

Groups	PFC/spleen		PFC/CNS	
	Direct	Indirect	Direct	Indirect
s.c.	1.86 ± 0.13 (73) $p < 0.001$	1.90 ± 0 (80) $p < 0.001$	ND	ND
i.p.	3.98 ± 0.07 (9,727) $p < 0.001$	4.28 ± 0.13 (19,287) $p < 0.02$	Ø	Ø
i.sas.	4.60 ± 0.04 (40,127)	4.76 ± 0.07 (58,325)	420	540
stabbed i.bp. (sham)	1.75 ± 0.08 (56)	1.60 ± 0 (40)	Ø	Ø
i.bp.*	4.30 ± 0.13 (20,072) $p < 0.05$	4.38 ± 0.21 (24,122)	300	480
i.bp.**	4.23 ± 0.08 (17,169) $p < 0.01$	4.34 ± 0.11 (22,213)	480	480

All p values refer to differences compared to the i.sas. values.

ND: not done; Ø: no PFC: * 2µl and ** 10µl injected i.bp.

PFC/CNS: the results refer to five pooled brains.

i.sas. or i.bp. injections were largely due to antibody production outside the CNS.

It should be pointed out that the presence of antigen specific B cells in the brain was only detected after rechallenge with SRBC i.sas. or i.bp.

Groups of mice were immunised and rechallenged six days later with TNP-BSA (100µg) by various routes: s.c., i.p., and i.sas. The animals were killed five days after rechallenge and the numbers of PFC determined in the spleen and brains.

The results (Table 4.3.7) show that injections of TD soluble antigen i.sas. induced significantly higher ($p < 0.02$ to $p < 0.001$) numbers of spleen PFC than did s.c. and i.p. injections. Again the presence of antibody producing cells in the brain (five pooled brains) could only be detected in mice receiving the antigen i.sas. and only after rechallenge.

These results thus indicate that injections of T-dependent antigens (particulate or soluble) are capable of inducing high numbers of antibody producing cells in the spleen compared with i.p. immunised animals, and also in the brain. It is unlikely that the PFC detected in the brain were due to contamination with blood during preparation, because the animals were first exsanguinated (see General Methods, 3.7.2). Moreover i.p. and s.c. immunised mice had no detectable PFC in the brain, thus ruling out the possibility that contamination with blood was responsible for the presence of PFC in the CNS of mice immunised i.sas. and i.bp.

Table 4.3.7

Detection of PFC in the spleen and brain from mice immunised with TNP-BSA.

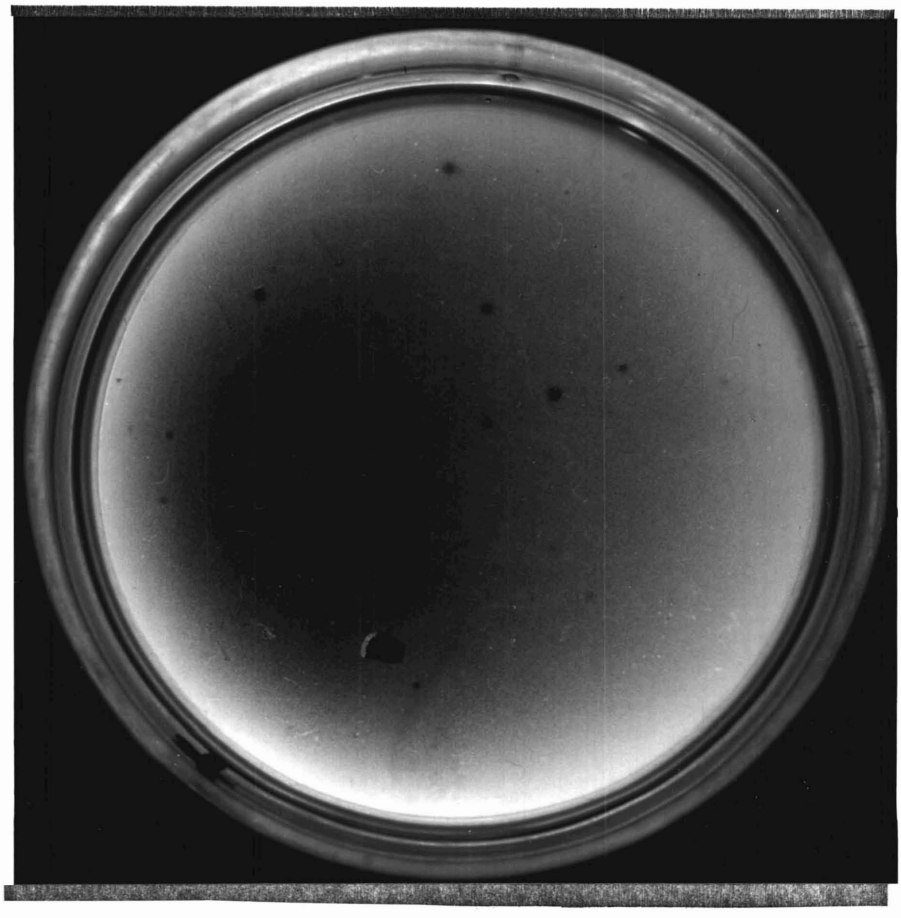
Groups	PFC/spleen		PFC/CNS		Reciprocal
	Direct	Indirect	Direct	Indirect	Ab Titres
Control*	1.90 ± 0.05 (70)	2.90 ± 0.86 (88)	ø	ø	< 1
s.c.	2.31 ± 0.07 (206) p < 0.001	3.31 ± 0.17 (2,054) p < 0.001	ø	ø	16
i.p.	2.80 ± 0.08 (635) p < 0.001	3.80 ± 0.14 (6,401) p < 0.02	ø	ø	16
i.sas.	3.39 ± 0.06 (2,498)	4.29 ± 0.08 (19,721)	40	200	32

* The skulls were perforated with a 26G needle in the site of i.sas. injection.

ø No detectable PFC.

Detection of PFC in the CNS

(LHG)



The numbers of PFC in the brains of mice were detected by localised haemolysis in gel (LHG), five days after rechallenge.

4.4 Discussion

Injection of TD particulate or soluble antigens into the SAS of adult mice induced more vigorous systemic antibody responses than injections into other sites commonly used for immunisation. In contrast, the TI antigen (TNP-Ficoll) did not have this effect, even after rechallenge.

It has been reported that injections of dog red blood cells (Panda *et al*, 1965) and SRBC (Šterzl and Lodin, 1978; Jankovic *et al*, 1961) into the SAS result in high systemic antibody responses, but to my knowledge this is the first demonstration that only TD and not TI antigens show enhanced immunogenicity when injected into the subarachnoid space. Furthermore, the results also indicate that *i.sas.* injections of TD but not TI antigens are capable of inducing significantly higher numbers of antibody producing PFC in the spleen than immunisation by other routes.

Panda *et al* (1965) have demonstrated that immunisation into the brain parenchyma induced systemic antibody responses which were similar in magnitude to those observed after subcutaneous immunisation. However, in the present study, injections of 2 or 10µl of TD antigens into the brain parenchyma resulted in antibody responses which were significantly higher than those observed after subcutaneous injections. Although it is difficult to exclude minor leakage into the SAS, in animals receiving 10µl *i.bp.*, this seems less likely with 2µl injections. Bradbury (1981) reported that radio labelled albumin followed the same route

into the deep cervical lymph nodes regardless of whether it was injected i.sas. or into the brain parenchyma (caudate nucleus).

The results indicate that the high systemic antibody level achieved following i.sas. or i.bp. injections is largely due to antibody production outside the CNS.

Koch et al (1982) have reported the spleen as being the principal site for antibody production only during the first week after challenge and beyond that period the bone marrow accounted for the majority of all PFC. It should be pointed out that in the present experiments, the numbers of spleen PFC were assayed at the peak of antibody production in the spleen, 4 to 5 days after rechallenge.

Since the elevated antibody responses were only observed with TD antigens, regulatory T cells and/or macrophages are likely to be involved. Available data indicate that suppressor T cells may regulate the antibody production to TD antigens by acting directly on B cells and also by interfering with the activity of specific T helper cells (Benacerraf and Unanue, 1981b). Assuming that TI responses are less affected by suppressor cells than are T-dependent responses, the high antibody responses to i.sas. introduced antigens could be due to a relatively ineffective induction of suppressor cell activity. Alternatively, the i.sas. route may be associated with the generation of more vigorous T helper cell function than immunisation by other routes. A combination of relatively low suppressor and vigorous helper cell activities

is certainly also possible. Furthermore, it is conceivable that the subarachnoid macrophage population might contain relatively few cells capable of rapid antigen degradation and a correspondingly high proportion of antigen presenting cells. This possibility is further supported by the observation that the difference in antibody titres was more pronounced when small amounts of TD antigens were used. It should be pointed out in this context that TI antigens are slowly metabolised and therefore tend to persist in the host tissues for prolonged periods of time (Baker et al, 1974).

Lastly, although trauma alone did not influence the background numbers of PFC (Tables 4.3.4, 4.3.5, 4.3.7), it is conceivable that the inflammatory reaction resulting from the injection of antigen into the CNS may cause the release from the brain of substances with adjuvant activity or disturb regulatory functions which may be exerted by the CNS on antibody production (Besedovsky and Sorkin, 1977).

Chapters 5, 6 and 7 of this thesis are concerned with experiments which were carried out in order to study to what extent the above-mentioned mechanisms might be responsible for the enhanced immunogenicity of T-dependent antigens when injected into the CNS.

C H A P T E R 5

STUDIES ON THE EFFECTS OF MONONUCLEAR PHAGOCYTIC SYSTEM BLOCKADE

5.1 Introduction

Since the observation by Mosier (1967) that the *in vitro* primary antibody response to a TD antigen (SRBC) requires the participation of accessory cells in addition to T and B lymphocytes, a general consensus has now been reached that antigen presenting accessory cells (APC) play a fundamental role in the development of an immune response.

Reports from several laboratories have indicated that the APCs for T-dependent antibody responses can be separated into at least two subsets depending on surface markers, phagocytic activity, and morphology. The population which predominates in most resting tissues shows avid Fc receptor binding and phagocytic activities (Beller and Unanue, 1980). These cells have low amounts of Ia antigens but are able to respond to macrophage Ia recruiting factors (MIRF) produced by sensitised T cells (Lu *et al*, 1981). The biosynthesis by macrophages of Ia antigens is increased in the presence of lymphokines (Beller and Unanue, 1981).

The minority population is characterised by a stellate dendritic morphology (Steinman, 1981), expression of large amounts of Ia antigens and limited phagocytic capcity (Beller and Unanue, 1980).

The function of the brain MPS in the context of an immune response is still unclear, although it has been demonstrated that it responds morphologically and functionally to antigenic stimulation (Essick, 1920; Merchant and Low, 1977).

The presence of Ia positive dendritic cells in the meninges and choroid plexuses (Hart and Fabre, 1981) strongly suggests that antigens can be presented within the CNS.

It was suggested (Chapter 4) that the macrophages in the subarachnoid space of adult mice, may contain relatively few cells capable of rapid antigen degradation and a correspondingly high proportion of antigen presenting cells. The proportion of the two macrophage subsets in the CNS remains to be determined.

The standard method for investigating the activity of mononuclear phagocytes in experimental animals has been to determine the rate of clearance, and the organ uptake, of intravenously injected particles from the blood stream (Stuart et al, 1978; Bradfield, 1980). The major limitation of this approach is that it does not provide information about the phagocytic ability of macrophages which do not line blood vessels and it does not necessarily measure the antigen presenting function of the MPS.

The experiments described in this chapter are concerned with the effects of MPS blocking agents (colloidal gold and colloidal carbon) on the organ distribution of i.sas. injected SRBC and the antibody responses to TD antigen injected into different sites, including the SAS.

5.2 Materials and Methods

5.2.1 Animals

Male (CBA x C57BL) F1 mice, 3-4 months old. Each experimental group consisted of 5-8 mice.

5.2.2 Blocking Agents

Colloidal carbon (C 11/1431 a, Gunther Wagner, Pelikan Works, Hanover, Germany) containing approximately 90-100 mg/ml of carbon was diluted with 1% sterile gelatin, and used for both i.p. and i.sas. injections.

Myocrisin (sodium aurothiomalate, May and Baker Ltd., Dagenham, England) containing preservative was used for i.p. injections only. For i.sas. injections, myocrisin without preservative (donated by May and Baker) was used.

5.2.3 Injections

For i.p. injections the mice received 4mg of myocrisin or 10mg of colloidal carbon. For i.sas. injections, the animals were first anaesthetised with Sagatal (10 mg/kg, i.p.) and then injected either with 4µg of myocrisin or 10µg of colloidal carbon.

5.2.4 Protocol

To induce blockade of the MPS the mice received the blocking agent on days -4,-1; -3,-1 or 3 hours before the

injection of the SRBC. The mice were killed 4 days later for measuring antibody responses.

5.2.5 Chromium labelling of sheep erythrocytes (^{51} Cr-SRBC)

SRBC in Alsever's solution were washed three times in sterile saline. 50µCi of ^{51} Cr (sodium chromate B.P., Radiochemical Centre, Amersham, England) were incubated with 1ml of packed SRBC at 37°C for 45 minutes on a roller at low speed. The cells were then washed three times with sterile saline and adjusted to the desired concentration.

5.2.6 Immunisation and organ uptake of ^{51} Cr-SRBC

Groups of mice, either untreated controls or treated -3,-1 days previously with colloidal carbon, were injected i.sas. with 2 x 10^6 ^{51} Cr-SRBC and the mice killed 30 minutes, 20 and 96 hours after injection. Blood, upper lymph nodes (cervical, axillary, brachial, deep peritracheal), lung, liver, spleen, brain and skull were removed. The radioactivity of each organ was measured in a well-type scintillation counter (Packard Autogamma Scintillation Spectrophotometer) calibrated for use with ^{51}Cr and the results calculated as the percentage of the injected radioactivity.

5.2.7 Measurement of antibody responses

Antibody titration: Blood was obtained from each individual mouse by heart puncture (as in 3.8.1).

Detection of antibody forming cells: This was performed

as described in General Methods (3.8.2.1).

5.3 Results

5.3.1 The immediate effect of myocrisin on spleen PFC

In a preliminary study, groups of four mice were injected with myocrisin either i.p. (4mg) or i.sas. (4µg) three hours before immunisation with SRBC (2×10^5). The animals were killed four days later and their spleens removed to determine the numbers of direct PFC.

The injection of myocrisin three hours before challenge with SRBC resulted in a 28-fold ($p < 0.01$) increase in the numbers of direct PFC (Table 5.3.1).

Similar results have been reported with colloidal carbon (Souhami, 1972).

Stiffel et al (1970) have demonstrated that the kinetics of phagocytosis of colloidal carbon and colloidal gold are identical. Assuming that the particle diameter of colloidal gold and colloidal carbon are similar, the enhancement of the antibody response induced by myocrisin or colloidal gold given three hours before antigen challenge, is most likely to be due to a redistribution of the antigen to the spleen, as a result of depressed hepatic phagocytosis.

According to Allner et al (1974) the dose of myocrisin is of critical importance, due to its toxicity. However, the dose of myocrisin used throughout (4mg i.p.) has been

Table 5.3.1

The immediate effect of myocrisin on spleen PFC.

Route of immunisation	Myocrisin injection	Direct PFC /10^7	Direct PFC /spleen	p <
i.p.	-	1.52 ± 0.19 (34)	2.34 ± 0.14 (235)	
i.p.	i.p.	2.98 ± 0.19 (968)	3.79 ± 0.14 (6,272)	0.01
i.sas.	-	2.89 ± 0.17 (782)	3.70 ± 0.18 (5,114)	
i.sas.	i.sas.*	ND	ND	

* All the animals died shortly after injection of myocrisin i.sas., possibly due to contaminating preservative.

ND: Not done.

demonstrated to exhibit similar toxicity as colloidal carbon when tested in different strains of mice.

Because commercial preparations of myocrisin contained preservative (Phenyl mercuric nitrate), it was dialysed for 24 hours against distilled water in a cold room (4°C) with a 24 Å diameter pore size tubing. Despite this precaution, all animals died shortly after myocrisin was injected into their SAS. This was probably due to some remaining preservative, because in later experiments the mice survived injections of preservative-free myocrisin into the SAS.

5.3.2 The effect of i.p. myocrisin treatment on spleen PFC

In this experiment, groups of four mice received a total dose of 4mg of myocrisin i.p. on days -4 and -1 before immunisation with SRBC (2×10^6). The numbers of PFC in the spleens were determined four days later. The results represent the mean of two experiments.

Table 5.3.2

The effect of i.p. myocrisin treatment on spleen PFC.

Route of immunisation	Myocrisin treatment	Direct PFC /10^7	p <
i.p.	-	3.54 ± 0.13 (3,545)	
i.p.	+	2.21 ± 0.16 (166)	0.001
i.sas.	-	3.81 ± 0.05 (6,657)	
i.sas.	+	3.12 ± 0.09 (1,340)	0.001

There is a decrease in the numbers of PFC in the myocrisin treated mice ($p < 0.001$).

As shown in Table 5.3.2, the myocrisin treatment practically abolished the spleen PFC response of i.p. immunised mice, but had much less effect on the spleen PFC responses of those mice which received the antigen i.sas. It was concluded from these experiments that the MPS of the CNS can process SRBC for effective activation of B cells in the spleen.

Cairns (1950) and Mims (1960) have demonstrated that the majority of a 30µl i.sas. inoculum spills over directly into the blood stream due to breakdown of the brain barrier systems. Thus it is not surprising that the blockade of the MPS outside the brain, diminished the antibody response to an antigen injected into the SAS.

It should be noted that myocrisin is capable of inducing enhancement (Table 5.3.1) or suppression (Table 5.3.2) of the antibody response, depending on the time between the injection of the drug and the antigenic challenge.

5.3.3 Effect of myocrisin treatment i.p. or i.sas. on the antibody response

Groups of 5-11 mice were injected with myocrisin i.p. (4mg) or i.sas. (4µg) on days -3,-1 before immunisation with SRBC (2×10^6) i.p. or i.sas. Four days after antigenic challenge the animals were killed. The blood from each individual mouse was collected by cardiac puncture, the serum heat inactivated (56°C/30min) and tested by a direct micro haemagglutination assay. The spleens were also removed and the numbers of direct PFC determined. The results are expressed as PFC/10^7 spleen cells, and represent the mean of 2 and 3 experiments respectively for i.sas. and i.p.

The results (Table 5.3.3) confirmed that myocrisin given on days -3,-1 before antigenic challenge cause a decrease in the numbers of direct PFC in the spleens. The degree of suppression was more marked when both drug and antigen were injected i.p. (37 fold) compared with 21 fold decrease when

the drug was injected i.p. and the antigen i.sas. The injection of both drug and antigen i.sas. caused only 2 fold ($p < 0.05$) suppression when compared with the untreated i.sas. immunised animals.

Table 5.3.3

Effect of myocrisin treatment i.p. or i.sas. on the antibody response.

Route of immunisation	Myocrisin treatment	Direct PFC /10^7	p <	Ab titres Prim. resp.	p <
i.p.	-	3.45 ± 0.09 (2,845)		4.72 ± 0.19 (26)	
i.p.	i.p.	1.88 ± 0.17 (76)	0.001	1.37 ± 0.37 (2)	0.001
i.sas.	-	3.93 ± 0.08 (8,569)		6.60 ± 0.27 (97)	
i.sas.	i.p.	2.61 ± 0.32 (413)	0.001	2.00 ± 0.50 (3)	0.001
i.sas.	i.sas.	3.65 ± 0.10 (4,527)	0.05	5.25 ± 0.25 (38)	NS

The degree of suppression induced by myocrisin was more marked in the i.p. treated mice.

NS: not significant.

These results confirm the findings presented in Table 5.3.2, namely that myocrisin injected i.p. suppresses the numbers of spleen PFC to SRBC given i.p. or i.sas. Furthermore it supports the conclusion that the MPS of the CNS is able to process antigens and initiate a systemic antibody

response to SRBC injected i.sas.

It should be pointed out that the changes in the antibody titres correlated closely to the changes in spleen PFC numbers.

The decrease in antibody titres following the myocrisin treatment and i.sas. antigen injection makes unlikely the possibility that the antigen was diverted to other sites. However, it may be possible that this treatment did not effectively induce MPS blockade in the CNS.

The animals receiving myocrisin free of preservative i.sas. appeared to behave normally and did not show any signs of illness, although neither histological nor behavioural tests were performed.

5.3.4 Effect of colloidal carbon treatment i.p. and/or i.sas. on the spleen PFC and humoral antibody responses

In view of the possibility that the i.sas. myocrisin treatment may have failed to produce an effective blockade of the MPS in the CNS, and also because myocrisin has been reported to affect lymphocytes (Lipsky and Ziff, 1976), further studies were carried out with colloidal carbon. This agent is known to have minimal effects on lymphocytes and it has also been more widely used and better standardised than myocrisin for selective blocking of the MPS.

Groups of 5-15 mice were treated with colloidal carbon i.p. (10mg) and/or i.sas. (10µg) on days -3,-1 before

immunisation with SRBC (2×10^6) i.p. or i.sas. The animals were killed four days after antigenic challenge. The antibody titres and the numbers of direct spleen PFC were determined for each mouse. The results represent the mean of at least three experiments.

The findings in Table 5.3.4 confirmed the principal observation of the myocrisin experiments. Thus while i.p. injection of colloidal carbon nearly abolished PFC responses in the spleens of i.p. immunised mice, it did not have any effect on the PFC responses in the spleens of mice which received the SRBC into the SAS. Indeed, the response in the i.p. colloidal carbon treated mice was slightly higher than in the untreated i.sas. control group, suggesting that the decrease observed in the corresponding myocrisin treated group (Tables 5.3.2, 5.3.3) may have been due to the effect of myocrisin on lymphocytes.

Assuming that the MPS of the CNS was unaffected by the i.p. injected colloidal carbon, the observations recorded in Table 5.3.4 clearly indicate that SRBC can be effectively processed within the CNS for induction of antibody responses in MPS blocked spleens. However, attempts to block antigen processing within the CNS by injecting colloidal carbon into the SAS did not cause significant reduction of PFC in the spleens. It is unlikely that this is due to overspill of the i.sas. injected SRBC into the blood stream, because the injection of colloidal carbon both i.p. and i.sas. did not cause reduction of PFC in the spleens. It therefore probably reflects an increased persistence of antigen in the brains of these animals (Table 5.3.5).

Table 5.3.4

Effect of colloidal carbon treatment i.p. and/or i.sas. on the spleen PFC and humoral antibody responses.

Route of immunisation	C. carbon treatment	PFC Direct /10^7	p <	Ab titres prim. resp.	p <
i.p.	-	3.45 ± 0.09 (2,845)		4.72 ± 0.19 (26)	
i.p.	i.p.	2.41 ± 0.14 (236)	0.001	2.00 ± 0.77 (4)	0.001
i.sas.	-	3.93 ± 0.08 (8,569)		6.60 ± 0.27 (97)	
i.sas.	i.p.	3.94 ± 0.06 (8,865)	NS	5.75 ± 0.25 (54)	NS
i.sas.	i.sas.	3.84 ± 0.14 (6,926)	NS	5.83 ± 0.47 (57)	NS
i.sas.	i.p/i.sas	4.00 ± 0.07 (10,005)	NS	5.16 ± 0.54 (36)	NS

Colloidal carbon suppressed the antibody response (PFC and Ab titres) only in the i.p.-i.p. treated group.

NS: not significant.

However, it cannot be ruled out that 10µg of colloidal carbon injected into the SAS was insufficient to block antigen processing by the MPS in the CNS. It is also possible that the antigen processing activity of the MPS in the CNS is relatively resistant to colloidal carbon treatment.

5.3.5 Organ distribution of ^{51}Cr-SRBC in normal and carbon treated mice

A group of control mice received a single injection of ^{51}Cr-SRBC (2×10^6) into the SAS. The animals were killed at different times (30min, 20h, 96h) after the injection and the radioactivity of brain, skull, lymph nodes (cervical, axillary, deep peritracheal, brachial), liver, spleen, lung and blood was measured in a well-type scintillation gamma counter.

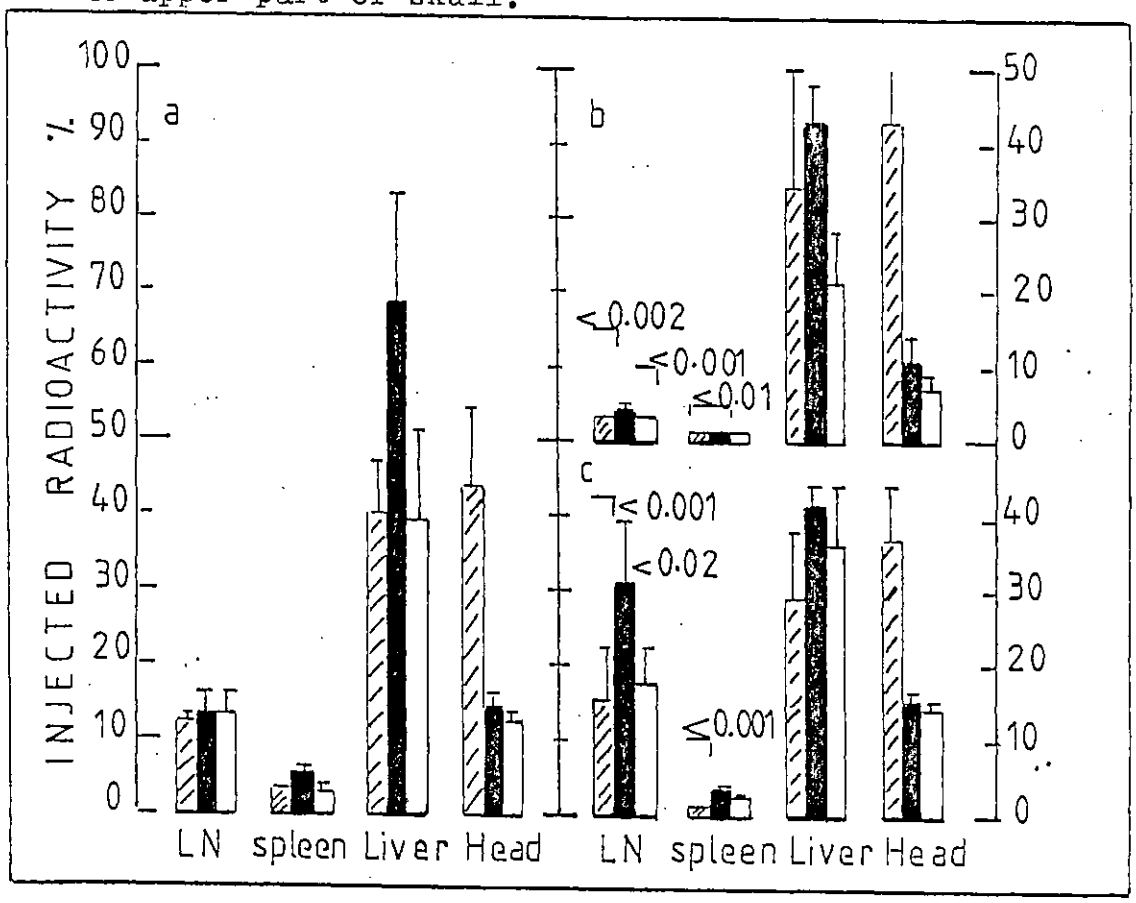
The results (Fig. 5.3.1a) show that in the control group the liver was the main site of clearance followed by the upper lymph nodes, while the spleen accumulated less than 6% of the injected radioactivity. Minimal radioactivity was found at any time in the blood and lungs. It should be noted that the radioactivity in the lymph nodes remained constant (12-13%) over the period of the experiment.

The high quantities of radioactivity found in the liver after i.sas. injection, might be explained by acute breakdown of the brain barrier systems. Although the injection was performed slowly and using a small volume (20µl) the very delicate structures on the arachnoid villi may have been temporarily affected. Cairns (1950) and Mims (1960), using phage label and indian ink respectively, demonstrated that the liver of a mouse was labelled/blackened within a few seconds after a 30µl intracerebral injection.

Groups of mice were treated with 10µg of colloidal carbon i.sas. on days -3,-1 before i.sas. injection of labelled SRBC

Fig. 5.3.1

The distribution of i.sas. ^{51}Cr-SRBC (2×10^6) in the organs of normal (a), carbon-treated mice i.sas. (b), and i.p. and i.sas. (c) on days -3,-1 before SRBC. The animals were killed at 30min (▨), 20 hours (■) and 96 hours (□) after immunisation. Radioactivity is expressed as percentage of the injected dose. Results show mean ± SE. Head includes brain and the upper part of skull.



(2×10^6). Fig. 5.3.1b shows that compared to the control group, there was much reduced radioactivity in the organs, although the liver was still the main site of clearance.

The i.sas. carbon treatment significantly reduced SRBC uptake by the spleen ($p < 0.01$) and also in the upper lymph nodes ($p < 0.002$ to $p < 0.001$). The brain-skull compartment was not greatly affected by the i.sas. carbon treatment, except that at 96 hours there was less radioactivity in the brain.

5.3.6 Persistence of i.sas. injected ^{51}Cr-SRBC in the brain and skull of normal and carbon-treated mice

Further groups of mice were injected with colloidal carbon both i.p. and i.sas. on days -3,-1 before 2×10^6 labelled SRBC were injected into the SAS. Fig. 5.3.1c shows that the organ uptake was generally reduced, but otherwise showed a pattern which was largely similar to that observed in the control group (Fig. 5.3.1a). However, the amount of radioactivity in the upper lymph nodes of these animals was significantly greater ($p < 0.02$ to $p < 0.001$) than in those mice receiving colloidal carbon only i.sas. (Fig. 5.3.1b).

Moreover, there was much higher radioactivity (Table 5.3.5) ($p < 0.002$ to $p < 0.001$) at 20 and 96 hours in the brains of mice treated with colloidal carbon both i.p. and i.sas. compared with those receiving colloidal carbon only i.sas.

Visual inspection demonstrated that the liver, spleen,

Table 5.3.5

Persistence of i.sas. injected ^{51}Cr-SRBC in the brain and skull of normal and carbon-treated mice.

°C. carbon treatment	Brain			Skull		
	30min	20h	96h	30min	20h	96h
Control	34 ± 6	7 ± 0.7	7 ± 1 p < 0.02	10 ± 4	7 ± 2	5 ± 0.2
i.sas.	29 ± 6	7 ± 1 p < 0.001	3 ± 0.7 p < 0.002	14 ± 7	4 ± 2	4 ± 2
i.p/ i.sas.	28 ± 3	13 ± 1	11 ± 1	9 ± 4	2 ± 0.8	3 ± 0.9

The proportion of labelled cells in the brains of i.p./i.sas. carbon treated mice increased with time, compared to i.sas. carbon treated group.

cervical and deep peritracheal lymph nodes were heavily loaded with colloidal carbon. The brain and skull were also blackened but not as heavily as liver and spleen. The comparison of organ distribution (Fig. 5.3.1c) and the development of antibody responses (Table 5.3.4) shows that the blockade of the MPS inside and outside the brain by i.p. and i.sas. injections of colloidal carbon, increased the numbers of PFC in the spleen. As previously mentioned, this might be explained by a persistence of antigen in the brain and its slow release to the draining cervical lymph nodes.

5.4 Discussion

The experiments with colloidal carbon show that the MPS of the CNS can process SRBC for effective activation of B cells, because the antibody response to SRBC injected into the subarachnoid space was not affected by i.p. blockade with colloidal carbon. This indicates that the MPS of the brain is able to participate in the development of antibody responses to T-dependent antigens injected into the SAS.

It is likely that this population is relatively resistant to blocking with colloidal carbon, because vigorous spleen PFC responses were observed in i.sas. immunised animals whose spleen and brain MPS were loaded with colloidal carbon. However, the possibility that the protocol was ineffective and/or the quantities used to induce blockade of the brain MPS were insufficient cannot be ruled out.

It has recently been reported that Ia bearing macrophages are present in the choroid plexus and meninges of adult rats (Hart and Fabre, 1981). As the i.sas. colloidal carbon treatment did not inhibit antibody responses, it is suggested that the macrophages in the CNS are relatively more effective in antigen presentation than in phagocytic function.

The studies of the organ distribution provide an additional evidence for the existence of a CSF-lymphatic outflow pathway for tracer (colloidal carbon) and cells (SRBC) injected into the SAS.

The results of MPS blockade and organ distribution experiments suggest that the brain is able to trap antigens

for prolonged periods. It is possible that antigens trapped in this way may then be slowly released into the lymphoid system of the head and neck regions.

Further studies of the MPS of the CNS, using monoclonal antibodies and a wide dose range of selective stimulants and blocking agents, might help to define better its role in the development of an immune response to an antigen in the CNS.

CHAPTER 6

COMPARISON OF HELPER AND SUPPRESSOR CELL ACTIVITIES AFTER I.P. AND I.SAS. ANTIGENIC CHALLENGE

6.1 Introduction

It was argued (Chapter 4) that the high antibody levels observed after i.sas. injection of antigens could be due to relatively vigorous induction of helper cells, ineffective activation of suppressor cells or a combination of both. It was decided to test these possibilities by methods that selectively eliminate suppressor cells and by studying suppressor cell dependent tolerance.

The T lymphocyte pool is very heterogeneous and plays an important role in the regulation of the immune response. Suppressor T cells (Lyt 1^-, 2 3^+; I-J $^+$) are generated following injection of an optimal dose of antigen (Gershon and Kondo, 1971; Yoshikai et al, 1981). Specific Lyt 1^+ helper T cells generated in such circumstances stimulate Lyt 1^+, 2 3^+ precursor T cells to differentiate into Lyt 1^-, 2 3^+ suppressor T cells which then turn off the specific immune response (Benacerraf and Unanue, 1981b). Any procedure (e.g. drug, irradiation) which interferes with the pool of precursor Lyt 1^+, 2 3^+ T cells, will affect the T cell suppressor systems.

Suppressor cells can inhibit antibody production at different levels, but precursor cells are sensitive to cyclophosphamide (Cy) treatment (Asherson et al, 1980), and the administration of this drug in low doses before antigenic challenge eliminates suppressor cell mediated mechanisms fairly selectively, with consequent augmentation of both humoral and cell mediated reactivities (Turk and Parker, 1979). Cy has

therefore been widely used to analyse the role of suppressor cells in the regulation of immune responses.

Successful induction of tolerance depends on the physical characteristics of the tolerogen, the immunological state of the animal and also on the rate of antigen processing by the macrophages (Weigle, 1973; Basten et al, 1975; Yoshikai et al, 1981).

Tolerance can be induced to soluble T-dependent antigens in adult animals by either very low doses injected repeatedly or amounts far above the immunogenic dose, thus giving rise to low and high zone tolerance respectively (Weigle, 1973; Basten et al, 1975).

Low zone tolerance (LZT) is a phenomenon in which T cells activated by low doses of antigen inhibit antibody responses to subsequent challenges with that antigen. LZT has been extensively studied with protein antigens that require co-operation between helper T cells and B cells (Chiller and Weigle, 1971; Gershon and Kondo, 1971; Weigle, 1973; Basten et al, 1975; Brüner and Kölsch, 1981). It has been demonstrated that LZT to haptenated BSA is accompanied by activation of suppressor T cells which probably act on the carrier specific helper T cells. This tolerance is sensitive to Cy treatment and is stable in adoptive transfer experiments (Brüner and Kölsch, 1981).

For induction of LZT, a TNP-BSA carrier system was selected because it has already been carefully analysed in relation to carrier specific suppressor T cell function

(Brüner and Kölsch, 1981). Moreover, BSA is a soluble protein antigen which after injection into any site equilibrates rapidly between extravascular spaces (Weigle, 1973) and in principle it is able to reach all antigen reactive cells, regardless of the route of injection (i.p; i.sas).

6.2 Materials and Methods

6.2.1 Animals

Male (CBA x C57BL) F1 mice, 3-4 months old. Each experimental group consisted of 4-15 mice.

6.2.2 Chemicals

Cyclophosphamide (Endoxana W B - Pharmaceuticals Ltd., London) was used either at 10mg/kg i.p. or 1mg/kg i.sas. 48 hours before antigenic challenge unless otherwise stated.

Hydrocortisone acetate (The Boots Co. Ltd., England) was given i.p. 48 hours before induction of unresponsiveness.

6.2.3 Antigens

Sheep Red Blood Cells (SRBC - TCS, Slough, England) in Alsever's solution were washed three times with normal sterile saline and used at various doses (2×10^4; 2×10^7; 2×10^8) either i.p. or i.sas.

BSA (Bovine Albumin Fraction V, Miles Lab. Ltd., England) dissolved in sterile saline was used as protein antigen (100µg) or as a carrier for haptens (TNP).

TNP-BSA was prepared according to Lewis and Goodman (1977) (see 3.2.2). The mice were injected with 1mg of the conjugate in sterile saline either i.p. or i.sas.

TNP-SRBC were prepared according to Rittenberg and Pratt (1969) (see 3.2.3.2) and used for in vitro determination of the numbers of anti-TNP antibody forming cells in the spleen.

6.2.4 Preparation of the tolerogen

De-aggregated BSA (d-BSA) was prepared according to Hudson and Hay (1980) by centrifuging 10mg/ml of BSA dissolved in sterile saline in a MSE automatic super speed 40 TC centrifuge (angle rotor 10 x 10ml) at 100,000 x g for 3 hours at 4°C. The upper 0.5ml was removed carefully and diluted 1:10 for i.p. injections.

D-BSA is referred to as a tolerogen, i.e. a form of the antigen that can induce an unresponsive state (tolerance), in contrast with immunogen, which refers to a different form of the same antigen capable of inducing an immune response. D-BSA obtained by ultracentrifugation readily induces an unresponsive state of relatively long duration in adult mice (Weigle, 1973; Basten et al, 1975; Brüner and Kölsch, 1981).

6.2.5 Induction of low zone tolerance (LZT)

Low zone tolerance (LZT) was induced in adult mice by an injection of 2.5mg Hydrocortisone acetate (HC-acetate) i.p. 48 hours before tolerogenic treatment. The animals then received seven consecutive daily injections of 10µg d-BSA

either i.p. or i.sas. Cy was given i.p. (10mg/kg) 48 hours before TNP-BSA challenge. Groups of mice without Cy treatment were also used. Control group was primed with 100µg BSA (i.p. or i.sas.) and treated with HC-acetate (i.p.), saline (i.p. or i.sas.) and Cy (i.p. or i.sas.). On day 12 all groups were challenged with 1mg TNP-BSA either i.p. or i.sas., and the IgG anti-TNP spleen PFC responses were determined six days later.

6.2.6 Measurement of antibody responses

Antibody titration: as described in General Methods (see 3.8.1).

Detection of antibody forming cell: as described in General Methods (3.8.2.1).

6.3 Results

6.3.1 The effect of Cy treatment on antibody responses to i.p. and i.sas. injected antigen

In a preliminary experiment mice were pre-treated with Cy either i.p. (10mg/kg) or i.sas. (1mg/kg) 48 hours before immunisation with SRBC (2×10^7) i.p. or i.sas.

Table 6.3.1 shows that the Cy treatment i.p. increased the spleen PFC responses in the i.p. immunised mice. In contrast, the same treatment caused a 2-5 fold decrease in the numbers of spleen PFC in the i.sas. immunised mice. The numbers of spleen PFC were further reduced in the group

receiving both Cy and antigen i.sas.

Table 6.3.1

Groups	PFC-Direct		PFC-Indirect	
	/10^7	/spleen	/10^7	/spleen
i.p.	1.69 ± 0.27 (49)	2.70 ± 0.15 (504)	2.14 ± 0.26 (138)	3.14 ± 0.15 (1,399)
i.p.(a)	2.37 ± 0.35 (238)	3.37 ± 0.17 (2,349)	2.53 ± 0.38 (345)	3.53 ± 0.20 (3,404)
i.sas.	2.67 ± 0.15 (471)	3.50 ± 0.11 (3,179)	2.93 ± 0.16 (852)	3.75 ± 0.12 (5,748)
i.sas.(a)	2.28 ± 0.12 (191)	3.10 ± 0.13 (1,281)	2.50 ± 0.05 (322)	3.33 ± 0.06 (2,159)
i.sas.(b)	2.06 ± 0.10 (116)	2.90 ± 0.08 (808)	2.42 ± 0.07 (265)	3.26 ± 0.11 (1,837)

Cy 10mg/kg i.p. (a); 1mg/kg i.sas. (b).

The following protocol was used to test the effect of Cy treatment on later stages of suppressor T cell induction.

Groups of 5 mice were primed with SRBC (2×10^8) and rechallenged six days later with SRBC (2×10^4) either i.p. or i.sas. Cy was given either i.p. or i.sas. 48 hours before the antigen rechallenge. The secondary antibody responses were determined in the spleens of mice six days after rechallenge.

The Cy treatment increased the numbers of spleen PFC

($p < 0.05$ to $p < 0.001$) in those mice receiving both antigen and Cy i.p. (Table 6.3.2).

6.3.2 Effect of Cy treatment i.p. on the antibody response

Table 6.3.2

Groups	PFC-Direct		PFC-Indirect	
	$/10^7$	/spleen	$/10^7$	/spleen
i.p.	2.64 ± 0.09 (439) $p < 0.05$	3.61 ± 0.04 (4,087) $p < 0.05$	3.01 ± 0.04 (1,025) $p < 0.001$	3.97 ± 0.03 (9,550) $p < 0.001$
i.p.-Cy	3.01 ± 0.08 (1,036)	3.86 ± 0.08 (7,274)	3.39 ± 0.01 (2,487)	4.24 ± 0.02 (17,464)

Cy (10mg/kg) was given i.p. 48 hours before rechallenge.

There is an increase in the PFC responses in Cy-treated mice ($p < 0.05$ to $p < 0.001$).

Thus the i.p. Cy treatment decreased the activity of suppressor cells involved in the regulation of secondary antibody responses.

In contrast, the same protocol caused a marked suppression of spleen PFC responses when the antigen was injected i.sas. (Table 6.3.3) and this effect was more evident in the indirect PFC responses ($p < 0.002$). Cy injected i.sas. also decreased the numbers of spleen PFC in mice challenged i.sas. with SRBC ($p < 0.001$).

6.3.3 The effect of Cy treatment on the antibody response to i.sas. injected SRBC

Table 6.3.3

Groups	PFC-Direct		PFC-Indirect	
	/10^7	/spleen	/10^7	/spleen
i.sas.	2.83 ± 0.16 (676)	3.79 ± 0.12 (6,210)	3.28 ± 0.13 (1,946) $p < 0.02$	4.25 ± 0.08 (17,866) $p < 0.002$
i.sas.(a)	2.68 ± 0.10 (488)	3.57 ± 0.10 (3,787) $p < 0.02$	2.75 ± 0.08 (568)	3.64 ± 0.08 (4,405)
i.sas.(b)	2.84 ± 0.12 (697)	3.37 ± 0.03 (2,346)	3.13 ± 0.12 (1,363)	3.66 ± 0.05 (4,589)

Cy was given i.p. (a) or i.sas. (b) 48 hours before i.sas. injection of SRBC (2×10^4).

6.3.4 Induction of unresponsiveness in adult mice

The experiments described here are derived from a TNP-BSA hapten carrier system. This system allows characterisation of the cell populations involved in the regulation of TNP antibody production.

Groups of 5-15 mice were injected either i.p. or i.sas. with d-BSA. The following groups were used in these experiments:

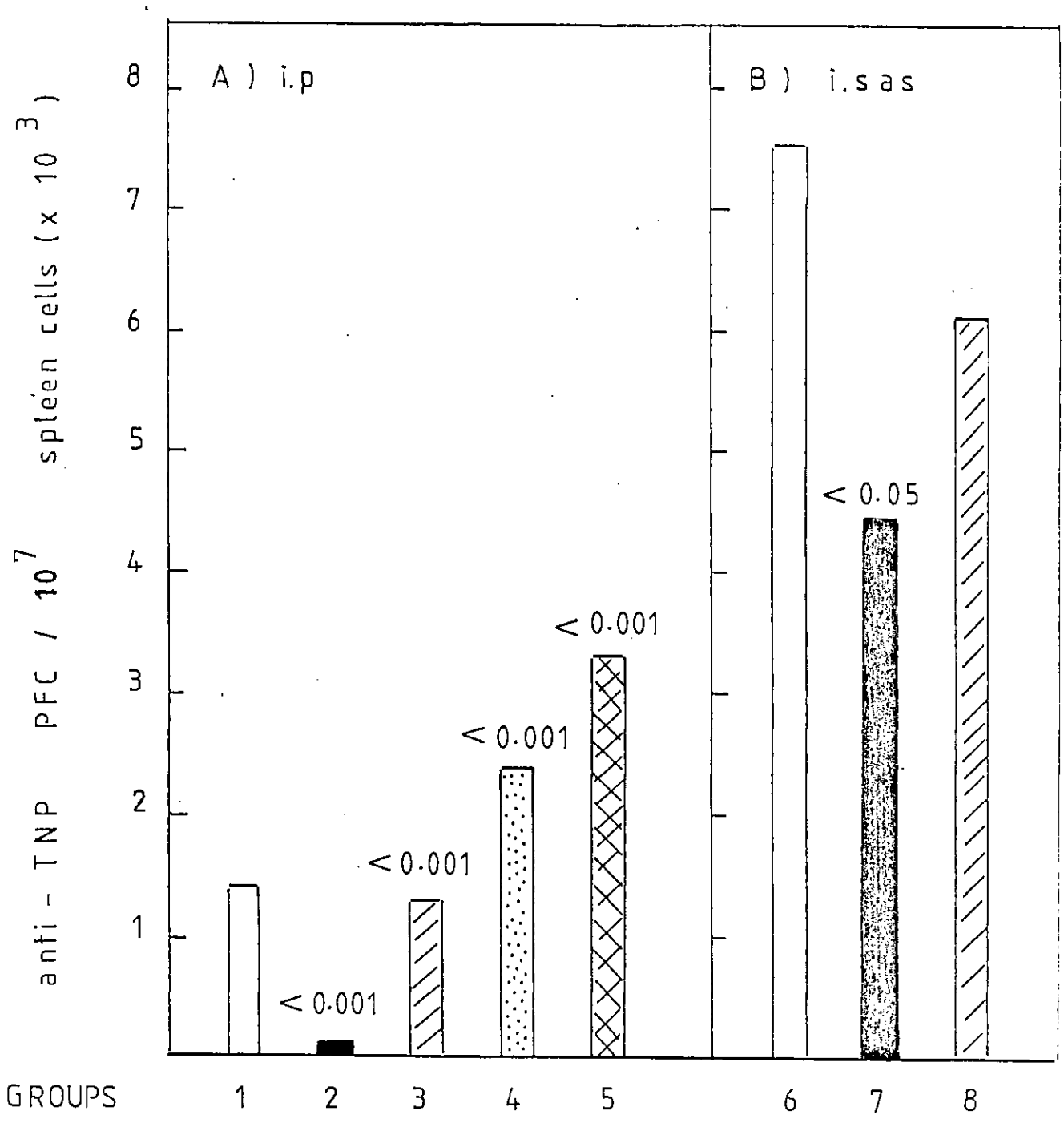
	Experimental groups			Cyclo-phosphamide	Challenge TNP-BSA
i.p.	1. Control	: BSA primed	i.p.	Cy	i.p.
	2. Tol	: d-BSA	i.p.	Saline	i.p.
	3. Tol	: d-BSA	i.p	Cy	i.p.
	4. Tol	: d-BSA	i.p.	Saline	i.sas.
	5. Tol	: d-BSA	i.p.	Cy	i.sas.
i.sas.	6. Control	: BSA primed	i.sas.	Cy	i.sas.
	7. Tol	: d-BSA	i.sas.	Saline	i.sas.
	8. Tol	: d-BSA	i.sas.	Cy	i.sas.

Figure 6.3.1a demonstrates that an almost completely unresponsive state was induced in mice receiving d-BSA i.p. As expected this suppression was abrogated by pre-treatment with Cy i.p. (Group 3). Antigenic challenge i.sas. also abrogated the tolerant state, and these animals (Group 4) showed higher responses than the Cy treated i.p. challenged mice (Group 3). Cy treatment i.p. further increased the numbers of anti-TNP PFC in the i.sas. challenged tolerant mice (Group 5) and it should be noted that in this group, the response was significantly higher ($p < 0.05$) than in mice which received both Cy and antigen i.p.

Injection of d-BSA into the SAS (Group 7) caused some hyporesponsiveness compared with the i.sas. primed control group, but failed to induce tolerance (Fig. 6.3.1b). Treatment of the i.sas. "tolerised" mice with Cy i.p. (Group 8) did not increase their response significantly, and in

Fig. 6.3.1

Induction of low zone tolerance in adult mice.



The results represent the mean of at least two experiments.

agreement with the findings presented in Tables 6.3.2 and 6.3.3 these animals had fewer spleen PFC than the controls (Group 6).

6.4 Discussion

There is ample evidence for the immunoregulatory role of helper and suppressor T cells in a variety of immune responses but to my knowledge this is the first report of an attempt to study the activity of these cells in the context of antigenic stimulation within the CNS.

The aim of the study was to evaluate the regulatory balance of helper and suppressor T cells in relation to the enhanced systemic antibody responses observed after introduction of antigen into the SAS. This was conducted by using two well established models:

(1) selective elimination of T suppressor cells with Cyclophosphamide; and

(2) induction of low zone tolerance to a carrier protein (BSA), a model which has been shown to be suitable for studying T cell suppression of carrier specific T helper cells (Brüner and Kölsch, 1981).

The principal findings are interpreted as follows:

The failure of the Cy treatment to increase the PFC response in i.sas. immunised animals and the unsuccessful attempts to induce T suppressor cell mediated tolerance by the i.sas. route (Group 7, Fig. 6.3.1b) suggests that introduction of antigen into the SAS results in a relatively ineffective

activation of T suppressor cell mechanisms. However, this route of immunisation also appears to cause vigorous activation of T helper cells, because it abrogated systemic T suppressor cell mediated tolerance without the help of cyclophosphamide (Group 4, Fig. 6.3.1a), and it also gave rise to higher PFC response in the Cy treated tolerised mice than shown by the corresponding i.p. immunised animals (Group 5 versus Group 3, Fig. 6.3.1a). Furthermore, the inability of i.sas. administration of Cy to enhance the PFC response suggests that there is no significant generation of suppressor cells within the CNS.

Taken together, the data therefore indicate that the enhanced immunogenicity of T-dependent antigens in the SAS is due to a combination of less effective activation of T suppressor cells and the generation of more vigorous T helper cell activity than takes place after immunisation by other routes.

The mechanism of this phenomenon remains to be elucidated. It is possible that the CNS contains a relatively high proportion of macrophages which preferentially interact with and generate T helper cells which would then leave the CNS and provide help for systemic antibody production. It is unlikely, especially within the short period of time used in these experiments, that the APC themselves leave the CNS for presentation of the antigen in the spleen and lymph nodes.

Further experiments should be carried out with other models of tolerance, different tolerogens and using the

transfer of lymphocytes and macrophages between the i.sas. immunised and tolerised animals.

C H A P T E R 7

THE EFFECT OF BRAIN INJURY AND BRAIN EXTRACTS ON THE ANTIBODY RESPONSE

7.1 Introduction

It has been demonstrated that brain trauma enhances the immunogenicity of systemically injected viruses and bacteria (Adams and Hopewell, 1970; Ed. Lancet, 1973) and evidence has also been presented which suggests that the immune system is under a regulatory influence of the neuroendocrine system (Besedovsky and Sorkin, 1977; Besedovsky et al, 1979). The concept of such regulation was introduced following the demonstration that neural activity increased within the hypothalamus just prior the peak of antibody responses and also that the immune response could be modulated by CNS lesions (Jankovic and Isakovic, 1973; Besedovsky and Sorkin, 1977).

It was suggested (Chapter 4) that the high antibody responses observed after i.sas. injection of antigens might at least in part be due to the release from inflamed brain tissue, of substances with "adjuvant" activity and/or alteration in the neuroendocrine regulation of the immune system resulting from the brain injury. Although injections of sterile saline into the SAS of control animals did not enhance the antibody responses, it was felt that the inflammation induced by antigens might have helped to increase the immunogenicity of the i.sas. introduced antigens. Therefore, further experiments were carried out in which the effect of brain extracts and more severe brain injuries (transcranial stab and X-irradiation) was studied in relation to antibody responses and high and low zone tolerance to BSA.

7.2 Materials and Methods

7.2.1 Animals

(CBA x C57BL) F1 male mice, 3-4 months old. Each experimental group consisted of at least 4 animals.

7.2.2 Antigens

Sheep Red Blood Cells (SRBC - TCS, Slough, England) in Alsever's solution were washed three times with normal sterile saline and injected i.p., i.sas. at various doses.

TNP-BSA was prepared according to Lewis and Goodman (1977) (see 3.2.2) and used at 100µg or 1mg i.p. and i.sas.

TNP-SRBC was prepared according to Rittenberg and Pratt (1969) (see 3.2.3.2) and used only for in vitro experiments.

De-aggregated BSA (d-BSA) prepared as in 3.2.4, was used at 10µg and 1mg for induction of low zone tolerance (LZT) and high zone tolerance (HZT) respectively.

7.2.3 Preparation of cell suspension

Spleen and lymph node cell suspensions were prepared as described in General Methods (3.7.1) and used for in vitro experiments.

7.2.4 Collection of mouse cerebrospinal fluid (CSF)

This was performed according to Carp et al (1971) (2.9).

Samples macroscopically free of blood contamination were pooled, mixed with equal volume of the corresponding serum, heat-inactivated and kept at -20°C.

7.2.5 Preparation of brain and liver membranes

These were prepared according to Mallet *et al* (1979), as described in General Methods (3.4). The final concentration was adjusted to 1.5mg/ml. The frozen samples were thawed (37°C) and injected together with the antigen.

7.2.6 Preparation of extracts

Homogenate of brain and liver as described in 3.5.1.

Crude saline extract as described in 3.5.2.

Brain lipid-proteolipid and ganglioside extracts as described in 3.5.3.

All the frozen samples were thawed (37°C) and, unless otherwise stated, injected *i.p.* together with the antigen.

7.2.7 Induction of tolerance

Low zone tolerance (LZT): This was performed according to Kölsch and Brüner (1981), as described in 6.2.

High zone tolerance (HZT): Exactly the same protocol was used as for induction of LZT with the exception that each dose of d-BSA was 1mg.

7.2.8 Induction of brain injury

Stab wounds: The animals were anaesthetised and a horizontal transcranial stab made with a 27G needle through the right parietal region. The mortality rate was minimal and confined to the immediate post operative period. No alterations in behaviour, general health, body weight or food and water intake were observed in these animals.

Irradiation of the head and body is described in Chapter 2.10.

The animals were kept at 37°C in an incubator with microflow until complete recovery. The drinking water was sterile and contained antibiotics (neomycin sulphate 100mg/L and polymixin B 10mg/L).

7.2.9 Measurement of antibody responses

These were measured by a haemagglutination technique (as in 3.8.1) seven days after priming and five days after rechallenge. The results are expressed as mean $\log_2 \pm$ SE and $\log_{10} \pm$ SE respectively and represent the mean of at least 3 experiments.

The sera were pre-absorbed with the respective preparation (liver or brain) before titration of antibody levels.

Antibody producing PFC (as in 3.8.2.1) were determined either 4-5 days after priming or 5-7 days after rechallenge. For indirect PFC, a developing serum (rabbit anti-mouse IgG) diluted 1:100 was used. The results are expressed as mean

$\log_{10}$ ± SE of at least 3 experiments.

7.3 Results

7.3.1 The effect of s.c. injections of brain and liver membranes on the primary antibody response

In preliminary experiments groups of four mice were immunised i.p. or i.sas. either with SRBC (2×10^5) or TNP-BSA (100µg). Brain and liver membranes were resuspended in 3ml of sterile saline and 200µl injected s.c. immediately after the immunisation. The animals were killed 4 days later and antibody responses determined for the individual mouse. The results represent the mean of at least 2 experiments.

Injection of the brain membranes significantly ($p < 0.02$) increased the numbers of direct spleen PFC in mice immunised i.p. with SRBC or TNP-BSA (Table 7.3.1). In contrast, this treatment caused only a marginal increase in the numbers of PFC in the lymph nodes. Mice immunised i.sas. and treated s.c. with liver or brain membranes did not show significant increases in the antibody responses.

7.3.2 The effect of preparations from normal and brain-damaged animals on the antibody responses

Additional experiments were carried out to study further the effects of preparations from CNS tissue. Groups of four mice were primed and rechallenged i.p. with 2×10^5 SRBC mixed with equal volume (200µl) of crude brain extracts or preparations of brain membranes. Antibody titres were

Table 7.3.1

Antigen	Route of immunisation	PFC-spleen /10^7	PFC-LN /10^7
SRBC (2×10^5)	i.p.	3.77 ± 0.05 (5,899) $p < 0.02$	3.46 ± 0.20 (2,941)
	i.p-Bm	3.97 ± 0.07 (9,531)	3.54 ± 0.20 (3,530)
	i.sas.	3.91 ± 0.05 (8,282)	4.12 ± 0.06 (13,415)
	i.sas-Lm	4.03 ± 0.08 (10,904)	3.86 ± 0.13 (7,356)
TNP-BSA (100µg)	i.p.	2.29 ± 0.09 (196) $p < 0.02$	1.91 ± 0.12 (83)
	i.p-Bm	2.56 ± 0.06 (367)	2.22 ± 0.09 (167)
	i.sas.	2.70 ± 0.17 (507)	2.08 ± 0.03 (122)
	i.sas-Lm	2.54 ± 0.11 (353)	2.35 ± 0.11 (227)

Groups of 8 - 11 mice were primed either with SRBC or TNP-BSA and the numbers of PFC determined four days later. The results represent the mean of 2 and 3 experiments respectively. Brain membrane (Bm) and liver membrane (Lm) were injected s.c. just after immunisation.

determined 5 days after rechallenge.

Brains from 4 normal mice and from 4 mice which had received a brain stab 2 days previously were used to prepare the brain extracts and brain membranes. The protein content of these preparations was adjusted to 1.50mg/ml. The results shown in Table 7.3.2 represent the mean of three experiments.

When the brain preparations were injected <u>together</u> with antigen, the antibody responses were significantly enhanced both at the cellular (PFC) and the humoral level (Table 7.3.2). Extracts from brain-damaged mice had a greater effect ($p < 0.02$ to $p < 0.001$) than extracts from undamaged brains.

It should be pointed out that animals immunised <u>i.sas.</u> without brain extract showed higher antibody responses than any of the extract-treated groups.

Pooled CSF-serum from brain-damaged mice enhanced the antibody response, although not to the same degree as brain extracts ($p < 0.02$ to $p \leqslant 0.01$).

7.3.3 The effect of different brain fractions on the spleen PFC

Groups of 5 mice were primed and rechallenged <u>i.p.</u> with 2×10^6 SRBC mixed with an equal volume (200µl) of fractions prepared from normal and stabbed brains. The numbers of spleen PFC were determined 6 days after the rechallenge.

Table 7.3.2 The effect of preparations from normal and brain-damaged animals on the antibody responses

Groups / Brain Preparations		Direct-PFC				Indirect-PFC				Antibody Titres	
		/10^7 cells	p ≤	/spleen	p ≤	/10^7 cells	p ≤	/spleen	p ≤		p ≤
i.sas.	-	2.80±0.06 (642)		3.57±0.07 (3,786)		3.07±0.08 (1,187)		3.75±0.12 (5,678)		7.54±0.04 (189)	
i.p.	-	2.00±0.10 (101)		2.70±0.10 (509)		2.19±0.09 (158)		2.90±0.08 (794)		5.16±0.14 (65)	
i.p.	nBe	2.22±0.06 (169)	0.05	2.92±0.09 (848)	NS	2.48±0.07 (304)	0.02	3.18±0.08 (1,522)	0.02	6.45±0.14 (150)	0.001
i.p.	sBe	2.37±0.10 (236)	0.02	3.13±0.08 (1,379)	0.002	2.65±0.09 (453)	0.001	3.42±0.06 (2,649)	0.001	6.33±0.05 (88)	0.001
i.p.	sBm	2.05±0.08 (113)	NS	2.95±0.06 (909)	0.05	2.60±0.02 (399)	0.001	3.50±0.07 (3,225)	0.001	6.75±0.25 (112)	0.001
i.p.	CSF/serum*	2.30±0.06 (230)	0.02	3.03±0.05 (1,075)	0.01	2.48±0.07 (308)	0.02	3.21±0.06 (1,630)	0.01	6.62±0.26 (108)	0.001

nBe: extract from normal brain; sBe: extract from stabbed brain; sBm: membranes prepared from stabbed brains. * CSF-serum collected from brain-stabbed animals.

The p values refer to i.p. immunised animals which did not receive brain materials.

The following fractions were used (see 3.6.2, 3.6.3):

Fraction	Content
I (crude saline extract)	water soluble proteins
II (chloroform phase)	lipid and proteolipids
III (methanol phase)	gangliosides and non-proteolipid proteins

The concentration of the different fractions was adjusted to 1.50mg/ml in sterile saline.

The results (Table 7.3.3) show that when these brain preparations were injected together with the antigen there was a very marked and highly significant increase in the antibody responses ($p < 0.01$ to $p < 0.001$). All three fractions were active, but fraction I had the strongest activity and produced similar numbers of spleen PFC in i.p. immunised mice as that observed in i.sas. immunised animals which did not receive any "adjuvant". No significant difference was observed between the preparations made from normal and stabbed brains.

7.3.4 The effect of a brain stab injury on the antibody response in i.p. immunised mice

Groups of 4 mice were immunised i.p. with 2×10^7 SRBC and the numbers of PFC determined four days later. Brain injury was inflicted once with a 27G needle, at the time of the immunisation.

The mice with brain injury had significantly higher ($p < 0.05$ to $p < 0.01$) numbers of spleen PFC than unstabbed control animals (Table 7.3.4).

Table 7.3.3

Groups	Brain Fraction	PFC/spleen			
		Direct	p <	Indirect	p <
i.sas.	-	4.44 ± 0.45 (27,943)		4.49 ± 0.19 (31,504)	
i.p.	-	2.68 ± 0.15 (482)		2.91 ± 0.12 (829)	
i.p.	*n I	4.35 ± 0.04 (22,813)	0.001	4.61 ± 0.06 (41,147)	0.001
i.p.	n II	3.91 ± 0.14 (8,139)	0.002	4.06 ± 0.11 (11,706)	0.001
i.p.	n III	3.81 ± 0.17 (6,548)	0.01	4.16 ± 0.14 (14,733)	0.001
i.p.	*d I	4.34 ± 0.16 (21,982)	0.001	4.52 ± 0.12 (33,875)	0.001
i.p.	d II	4.21 ± 0.07 (16,244)	0.001	4.37 ± 0.06 (23,821)	0.001
i.p.	d III	3.86 ± 0.17 (7,383)	0.01	3.84 ± 0.18 (6,969)	0.01

* Preparations from normal (n) and damaged (d) brain.

Table 7.3.4

Groups	PFC-Direct		PFC-Indirect	
	/10^7	/spleen	/10^7	/spleen
i.p.	1.69 ± 0.27 (49)	2.70 ± 0.15 (504)	2.14 ± 0.26 (138)	3.14 ± 0.15 (1,399)
i.p.*	2.43 ± 0.12 (270)	3.50 ± 0.14 (3,305)	2.66 ± 0.08 (464)	3.75 ± 0.10 (5,674)

* Brain injury induced an increase in the numbers of spleen PFC ($p < 0.05$ to $p < 0.01$).

7.3.5 The effect of a brain stab injury on the secondary antibody response in i.p. immunised mice

The following protocol was used to test the effect of a brain stab injury on the secondary antibody response.

Groups of 4 mice were primed i.p. with SRBC (2×10^8) and rechallenged six days later with 2×10^4 SRBC by the same route. Brain injury was inflicted once with a 27G needle at the time of rechallenge, and the numbers of spleen PFC determined six days later.

The results (Table 7.3.5) confirmed the previous observation, that animals submitted to brain damage showed a significant enhancement of antibody responses ($p < 0.05$ to $p < 0.002$).

7.3.6 The effect of irradiation of the head on spleen PFC

These experiments were performed in order to assess the effect of generalised and diffuse brain injury on the antibody response.

Table 7.3.5

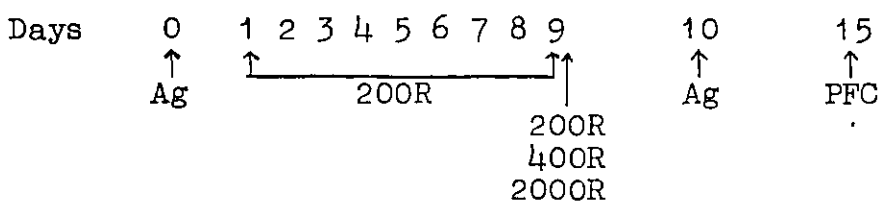
The effect of a brain stab injury on the secondary antibody response in i.p. immunised mice.

Groups	PFC-Direct		PFC-Indirect	
	/10^7	/spleen	/10^7	/spleen
i.p.	2.64 ± 0.09 (439)	3.61 ± 0.04 (4,087)	3.01 ± 0.04 (1,025)	3.97 ± 0.03 (9,550)
	$p < 0.02$	$p < 0.05$	$p < 0.01$	$p < 0.002$
i.p.*	3.09 ± 0.10 (1,258)	3.83 ± 0.07 (6,859)	3.52 ± 0.13 (4,107)	4.35 ± 0.07 (22,399)

* Transcranial brain injury.

Groups of 4-6 mice were primed and rechallenged 10 days later with 2 x 10^7 SRBC i.p. or i.sas. and the numbers of spleen PFC determined five days after the rechallenge. X-irradiation of the head, with the body shielded, or the body with the head shielded (Chapter 2.11), was performed 24 hours before the rechallenge, with doses ranging from 200R to 2000R. Further groups of six mice received a total of 1800R given in fractions of 200R for nine consecutive days.

Protocol



The results (Table 7.3.6) show that, regardless of the dose, irradiation of the head alone (body shielded) caused

Table 7.3.6

Groups	X Ray rads	PFC/spleen			
		Direct	p <	Indirect	p <
i.p.	-	3.32 ± 0.04 (2,120)		3.44 ± 0.16 (2,799)	
	200 (B)	3.54 ± 0.30 (3,480)	NS	4.01 ± 0.20 (10,383)	NS
	400 (B)	3.28 ± 0.11 (1,940)	NS	4.17 ± 0.06 (14,842)	0.01
	200 (H)	4.39 ± 0.04 (24,749)	0.001	4.69 ± 0.05 (49,320)	0.001
	2000 (H)	4.26 ± 0.17 (18,326)	0.001	4.97 ± 0.11 (93,478)	0.001
	1800* (H)	4.12 ± 0.07 (13,394)	0.001	4.39 ± 0.09 (24,789)	0.001
i.sas.	-	4.40 ± 0.06 (25,464)		4.92 ± 0 (84,191)	
	200 (B)	3.86 ± 0.17 (7,354)	0.02	4.51 ± 0.12 (33,085)	0.01
	400 (B)	3.78 ± 0.12 (6,045)	0.002	4.43 ± 0.11 (27,228)	
	200R (H)	3.91 ± 0.17 (8,253)	0.05	4.22 ± 0.21 (16,936)	0.02
	2000 (H)	4.10 ± 0.10 (12,591)	0.05	4.28 ± 0.07 (19,249)	0.001
	1800* (H)	4.08 ± 0.02 (12,265)	0.001	4.35 ± 0.07 (22,702).	0.001

* Mice received a total dose of 1800 rads given in fractions of 200 rads for nine consecutive days.

Results are expressed as mean $\log_{10}$ ± SE and geometric mean in parenthesis.

(B) X irradiation of the body with the head shielded.

(H) X irradiation of the head with the body shielded.

very marked increase ($p < 0.001$) in the numbers of PFC in the spleens of i.p. immunised mice and this effect was more pronounced in the indirect PFC.

The i.p. immunised group, which received 200R on the head for nine consecutive days showed less increase in PFC than the animals receiving single dose of 200R or 2000R. The mice in the first group lost weight and two out of six died during the experiment. In the i.p. immunised mice, irradiation of the body with the head shielded caused a slight increase ($p < 0.10$ to $p < 0.01$) in the numbers of indirect spleen PFC, probably due to inactivation of radio-sensitive suppressor cells.

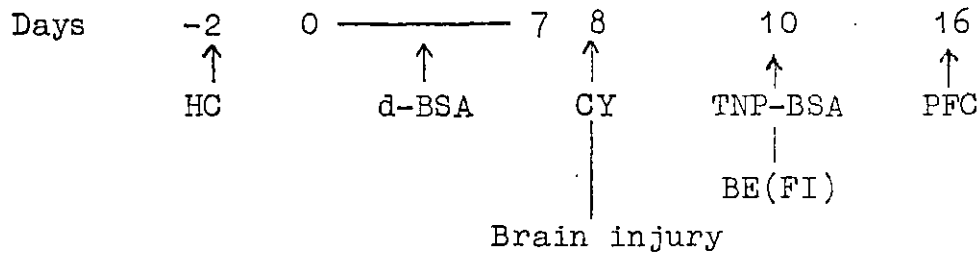
In the i.sas. immunised mice, the same protocol of irradiation had the opposite effect, namely a marked reduction in PFC, regardless of the site and dose of the irradiation.

7.3.7 Termination of low zone tolerance by CNS injury

A series of experiments was performed to study the effect of a brain injury and brain extract (Fraction I) upon LZT induced with d-BSA (Brüner and Kölsch, 1981).

Groups of five mice were pre-treated with 2.5mg of HC-acetate i.p. and then injected i.p. for seven consecutive days with d-BSA (10µg). The protocol used in these experiments is described in detail elsewhere (see 6.2).

Protocol



The effect of the following procedures on LZT was studied:

(1) Cyclophosphamide treatment (2.5mg injected i.p. 48 hours before antigen rechallenge)(TNP-BSA).

(2) X-irradiation of the head (2000 and 400R).

(3) Brain stab injury (27G needle).

(4) Injection of brain extract (Fraction I), together with the antigen (TNP-BSA).

The animals were killed six days after the rechallenge for detection of IgG anti-TNP PFC in the spleens and the results in Table 7.3.7 represent the mean of two experiments.

In agreement with previous findings, the protocol used for induction of LZT resulted in an almost complete TNP unresponsiveness which was readily abrogated by Cy treatment. This tolerance was also consistently broken by irradiation of the head 48 hours prior to the antigen rechallenge, and administration of the antigen mixed with brain extract was even more effective in overcoming the tolerance. Brain stab injury inflicted 48 hours before the antigen rechallenge also broke the tolerance.

Table 7.3.7

Groups	Treatment				PFC-Indirect /10^7	p <
	Tol	Cy	Brain Injury: Stab	Brain Injury: X Ray		
BSA primed	-	+	-	-	3.31 ± 0.10 (2,078)	
BSA-TOL	+	-	-	-	2.07 ± 0.13 (120)	0.001
BSA-TOL + Cy	+	+	-	-	3.12 ± 0.15 (1,329)	0.001
BSA-TOL	+	-	+	-	2.68 ± 0.16 (489)	0.02
BSA-TOL	+	-	-	2000R	2.83 ± 0.05 (691)	0.001
BSA-TOL	+	-	-	400R	2.79 ± 0.09 (624)	0.001
BSA-TOL* + BE	+	-	-	-	3.25 ± 0.14 (1,813)	0.001

* i.p. tolerant mice received normal brain extract (Fraction I) together with TNP-BSA.

7.3.8 Time course of the effect of brain injury in the recovery of LZT

Additional time course studies were conducted for the irradiation, and as shown in Table 7.3.8, irradiation four days prior to the antigen rechallenge was optimal for breaking the LZT, and this timing of the irradiation was in fact as effective as the Cy treatment.

It is unlikely that the high responses in the animals irradiated 4 days prior to antigen rechallenge was due to spontaneous recovery, because irradiation given 6 days before the antigen rechallenge was only marginally effective in breaking LZT.

7.3.9 The effect of irradiation of the head on HZT

Experiments were performed in order to assess whether irradiation of the head could also influence a state of HZT induced with i.p. injection of d-BSA.

Cy treatment was not very effective in breaking this tolerance, possibly because it is not mediated via suppressor cells to the same extent as LZT. It was nevertheless broken by irradiating the head with 400R 48 hours before the antigen rechallenge (Table 7.3.9).

It is possible that the effect of irradiation on HZT would have been stronger, if the time schedule which was optimal for breaking LZT had been used (Table 7.3.8).

Table 7.3.8

Time course of the effect of brain injury in the recovery of LZT.

Groups	Treatment			PFC-Indirect	p <
	Tol	Cy	CNS-X Ray	$/10^7$	
BSA-primed	-	+	-	3.31 ± 0.10 (2,078)	
BSA-TOL	+	-	-	2.07 ± 0.13 (120)	0.001
BSA-TOL + Cy	+	+	-	3.12 ± 0.15 (1,329)	0.001
BSA-TOL*	+	-	day 2	2.79 ± 0.09 (624)	0.001
BSA-TOL*	+	-	day 4	3.10 ± 0.10 (1,275)	0.001
BSA-TOL*	+	-	day 6	2.58 ± 0.18 (388)	0.05

* Irradiation of the head (400R) with the body shielded before antigen rechallenge.

Table 7.3.9

The effect of irradiation of the head on HZT.

Groups	Treatment			PFC-Indirect /10^7	p <
	Tol	CY	CNS-X Ray		
BSA-primed	-	+	-	3.31 ± 0.10 (2,078)	
BSA-TOL	+	-	-	2.08 ± 0.05 (122)	0.001
BSA-TOL + Cy	+	+	-	2.56 ± 0.04 (365)	0.001
BSA-TOL*	+	-	+	2.68 ± 0.15 (484)	0.01

* Irradiation of the head with the body shielded (400R) 48 hours before antigen rechallenge.

7.4 Discussion

The results presented in this Chapter show that stab and X-ray injuries of the brain, as well as materials extracted from brain tissue, enhance systemic antibody responses and can even break down tolerant states. Furthermore, serum and CSF collected from brain-damaged animals also increased the numbers of spleen PFC. Moreover, injection of brain extract (Fraction I) or irradiation of the head proved as effective as Cy in terminating low zone tolerance.

It has been demonstrated that high quantities of synaptic membranes are released into the CSF and that galactocerebrosides, MBP and cerebrosides can be detected in

the systemic circulation after CNS injury (Lim et al, 1972; Paterson et al, 1981). X-irradiation of the head with 2000R doses is known to temporarily damage the neuroglial cells with a subsequent proliferation of astrocytes and microglial cells (Gilmore, 1979).

LZT is known to be mediated by Cy sensitive T suppressor cells acting on carrier specific T helper cells (Brüner and Kölsch, 1981) and abrogation of this state can be achieved by using treatments which selectively eliminate suppressor T cells or enhance helper cell function (Weigle, 1973; Benacerraf and Unanue, 1981a; Scibienski, 1981).

It is unlikely that the total T suppressor cell activity of the body was significantly affected by the irradiation of the head, because the body was shielded and the only lymphoid structures irradiated were those present in the head and the upper neck regions (Uede et al, 1981; Cogburn and Glick, 1981). It is also unlikely that elimination of suppressor cells from this limited amount of lymphoid tissue was responsible for the abrogation of LZT. Moreover, irradiation of the head suppressed the antibody response to i.sas. injected SRBC.

Taken together these observations suggest that following brain injury, there might be a release from the brain of substances with adjuvant activity capable of enhancing the activity of T helper cells.

It should be noted that irradiation of the head alone or the body with the head shielded, although markedly increasing the numbers of spleen PFC in the i.p. immunised mice, had a

striking opposite effect in the i.sas. immunised animals (Table 7.3.6). In this respect, X-irradiation had a similar effect as Cy treatment (Tables 6.3.1, 6.3.3). This paradoxical effect indicates that the mechanism responsible for the humoral hyper-responsiveness after antigen injections into the SAS is X-ray and Cyclophosphamide sensitive, and that it is partially operating within the CNS itself. It should be possible to elucidate further the cellular basis of this phenomenon by carrying out time course and cell transfer experiments.

It could be argued that the increased antibody levels induced by the brain extract were due to brain antibodies which cross-reacted with SRBC or TNP. However, in contrast to the microsomal brain fraction, brain homogenates are weak immunogens, even after repeated injections with complete Freund's adjuvant (Shek and MacPherson, 1971).

Damage to brain structures may disturb the neuroendocrine regulation and sympathetic innervation of lymphoid organs with consequent increase in antibody production (Besedovsky and Sorkin, 1977; Besedovsky et al, 1979; Roszman et al, 1982; Miles et al, 1981), but the effect of X-irradiation of the head on the neuroendocrine regulation of the immune system has not been studied.

The findings presented in this chapter do not distinguish whether changes in neuroendocrine regulation of the immune system or "adjuvant" activity is the major mechanism responsible for the high antibody titres and breakdown of

tolerance resulting from the i.sas. injections. Further experiments using more selective brain injuries and analysis of serum, CSF and brain extracts from normal and brain-damaged animals in relation to the development of antibody responses might elucidate which is the main operative mechanism.

CHAPTER 8

GENERAL DISCUSSION

8 General Discussion

8.1 Biological significance

The brain has been considered a privileged site in relation to transplantation immunity (Medawar, 1948; Raju and Crogan, 1977). The relatively long survival of i.c. transplanted grafts is believed to be due to an impairment in both the afferent and efferent arcs of the immune response, due to a lack of lymphatic system within the CNS (afferent privilege) and the presence of the BBB (efferent privilege) (Barker and Billingham, 1977). However, the intercellular spaces within the brain parenchyma have free communications with the SAS and also with the lymphatic system of the head and neck regions (Cserr, 1974; Bradbury, 1981). The CSF-lymphatic outflow is very rapid and as has been demonstrated by several groups (Casley-Smith et al, 1976; Oehmichen, 1978; Bradbury and Cole, 1980) and confirmed in this thesis (Chapter 5), particles injected into the CNS can be recovered in cervical lymph nodes within a very short period of time. It is, therefore, difficult to see how lack of drainage into the lymphatic system can account for extended graft survival in the brain. Moreover, migration of macrophages from brain parenchyma into the perivascular space has also been described (McKeever and Balentine, 1978). Suppressor mechanisms both humoral (Barker and Billingham, 1977; Raju and Crogan, 1977) and cellular (Raju and Crogan, 1977) have also been implicated in graft tolerance in the brain, but experimental support for these suggestions has not yet been obtained.

There is ample evidence that sensitised lymphocytes can pass the blood-brain barrier and infiltrate the brain parenchyma (Scheinberg et al, 1966; Stensaas and Horsley, 1975; Wilkerson et al, 1978; Lublin et al, 1981), thus the most likely cause of the extended graft survival in the brain is a low level of sensitisation.

The data presented in this thesis strongly indicate that the brain is less privileged with respect to humoral immunity. The enhanced immunogenicity of SAS and i.bp. introduced antigens was only observed with T-dependent and not with type 2 T-independent antigen (TNP-Ficoll) (Mond et al, 1980)

Several mechanisms were proposed and studied in an attempt to explain this phenomenon:

1) CNS macrophages may be unusually effective as antigen presenting cells;

2) Antigen presentation within the CNS may lead to relatively ineffective activation of suppressor cells and/or vigorous induction of helper cell activity;

3) Injection of antigens into the CNS may produce an inflammatory reaction which could release substance(s) with
   (a) adjuvant activity, and/or
   (b) alter the neuroendocrine regulation of the immune system.

Macrophages are heterogeneous with respect to expression of Ia molecules, and the proportion of Ia positive and Ia

negative cells has been shown to vary in different tissues (Cowing et al, 1978; Beller and Unanue, 1980) and also to be influenced by environmental factors (Calamai et al, 1982). The relationship between these two macrophage populations is not well established, although there is evidence that Ia positive macrophages are involved in antigen presentation and subsequent interaction with T helper cells, whereas Ia negative macrophages play a major role in inflammation (Beller et al, 1980). Recent studies by Britz et al (1982) indicate that some specialised antigen presenting cells with high levels of Ia and H-2D expression (Langerhans cells and splenic dendritic cells) activate helper/effector T cells and also contra-suppressor cells, thereby generating an immune response which is resistant to host suppressive mechanisms.

The ability of CNS macrophages to present antigens, and their level of Ia expression has so far not been studied, however, Hart and Fabre (1981) described the presence of Ia positive dendritic cells in the choroid plexus and meninges of rats. The results presented in Chapter 5 of this thesis suggest that macrophages in the CNS may be highly efficient in antigen presentation.

On balance the results presented in Chapter 6 and Chapter 7 indicate that the enhanced immunogenicity of i.sas. injected antigens is due to a vigorous induction of helper activity rather than ineffective activation of suppressor cells. This is demonstrated by the fact that i.sas. injected TNP-BSA was capable of breaking down T suppressor cell mediated tolerance (Chapter 6).

The findings that in i.sas. immunised animals, i.sas. cyclophosphamide treatment (Chapter 6) or irradiation of the head (Chapter 7) decreases the numbers of spleen PFC indicate that the mechanisms amplifying the antibody response to i.sas. injected antigen are sensitive to these agents. It is difficult to reconcile this with the observation that antigen-activated suppressor T cell population is more radiosensitive than the antigen-activated helper T cell population (Basten et al, 1975; Doria et al, 1982).

The failure to induce tolerance by injecting d-BSA into the subarachnoid space (Chapter 6) could be due to ineffective induction of suppressor T cells. However, it is also possible that CNS macrophages are able to take up and present d-BSA to T helper cells. The findings, presented in Chapter 7, that brain injury is capable of enhancing antibody responses and breaking down tolerance in adult animals were unexpected. The most likely explanation for this phenomenon is that substances with adjuvant activity are released from injured or inflamed brain tissue. This is further supported by the observation that brain extract could enhance antibody production and abrogate tolerance (Chapter 7). In addition, CNS macrophages may be more responsive to adjuvants than macrophages located in other tissues.

Brain homogenate is known to contain mitogenic activity (Mirsky, 1980) and it can also enhance formation of active E rosettes (Offner et al, 1981). Recently Fontana et al (1981) described a lymphokine capable of increasing proliferation and differentiation of cultured astrocytes. This factor was named

glial cell stimulating factor (GSF). Furthermore, LPS and BGG can stimulate astrocytes to produce immunoregulatory factors such as prostaglandin E and an interleukin 1-like substance (Fontana et al, 1982). These observations suggest that astrocytes may participate in the regulation of immune responses within the CNS.

There is ample evidence that the neuroendocrine system can modulate various immunological functions (Besedovsky and Sorkin, 1977; Besedovsky et al, 1979; Williams and Felten, 1980; Roszman et al, 1982; Miles et al, 1981). It is possible that at least some of the observations described in this thesis could be due to a disturbance of these neuroendocrine regulatory mechanisms, but the presented data do not allow any definitive conclusion in this respect.

8.2 Clinical implications

The observations reported in this thesis may have important implications for certain diseases affecting the CNS, including multiple sclerosis and SSPE. A common feature in these diseases is synthesis of oligoclonal immunoglobulin within the CNS (Paterson and Whitacre, 1981; Tourtellotte et al, 1981; Mehta et al, 1982). Such synthesis has even been demonstrated after cerebral infarcts, suggesting that brain injury may lead to polyclonal activation of B cells (Röstrom et al, 1981).

The vigorous activation of T helper cells which is suggested by the data presented in this thesis, could, at least in part, be responsible for these phenomena. Another

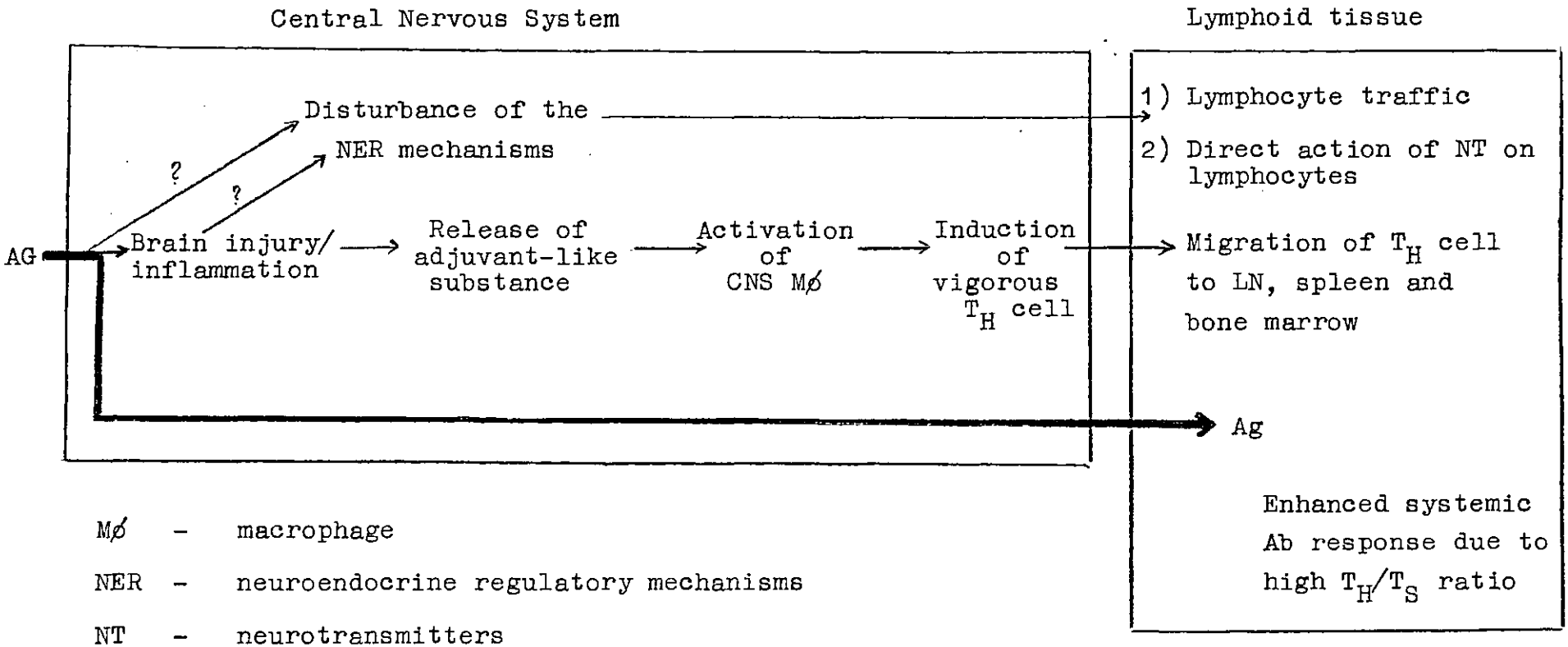
consideration which arises from the findings is the possibility that brain injury may contribute to autoimmune response against self antigens which are tolerated at low zone suppressor cell dependent level.

8.3 Proposition of a model

The precise mechanism(s) underlying the enhanced immunogenicity of antigens injected into the subarachnoid space, remains to be determined. It is tempting, however, to propose a provisional model as an attempt to explain the various observations reported herein.

This model proposes that antigens injected into the CNS induce a local inflammatory reaction with consequent release of adjuvant substances capable of stimulating CNS macrophages. These activated macrophages are particularly efficient in generating T helper cells which activate B cells either within the CNS itself or in the lymph nodes and spleen where a substantial proportion of i.sas. injected antigen is localised. In addition, the inflammatory reaction in the CNS may also affect the neuroendocrine regulation of the immune system.

Diagram of the proposed model



Mø - macrophage

NER - neuroendocrine regulatory mechanisms

NT - neurotransmitters

T_H - T helper

T_S - T suppressor cell

8.4 Concluding remarks

The studies reported in this thesis suggest that the mechanism which amplifies antibody responses when T-dependent antigens are injected into the subarachnoid space is:

1) operating both inside and outside the CNS;

2) sensitive to cyclophosphamide and X-irradiation;

3) a combination of effective antigen presentation, generation of vigorous T helper cell activity and relatively ineffective activation of suppressor cell mechanisms.

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T-Dependent Antigens are More Immunogenic in the Subarachnoid Space than in Other Sites

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Summary

Systemic antibody responses were compared in mice injected with antigens into the subarachnoid space (SAS) and into conventional sites. Introduction of T-dependent antigens into the SAS induced higher systemic antibody responses than injections into any other sites. This applied to both soluble and particulate antigens and was more apparent in secondary than in primary responses. The effect was not observed with a T-independent antigen and not consistently with high doses of sheep red blood cells (SRBC).

It was concluded that the subarachnoid space is a highly efficient site for the induction of systemic antibody production, in particular to low amounts of T-dependent antigens.

Key words: *Antigens – Mice – Sheep red blood cells – Subarachnoid Space – T-dependent antigens T-independent*

Introduction

Although the central nervous system (CNS) is devoid of a lymphoid system and lacks direct lymphatic drainage into lymph nodes, intense local and systemic immune responses are observed in animals with experimental allergic encephalitis and in patients with subacute sclerosing panencephalitis.

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Essick showed by injecting erythrocytes into the subarachnoid space, that some of the cells lining this space fulfil all criteria for macrophages, and he observed macrophages with erythrocyte inclusions in cervical and submaxillary lymph nodes several weeks after the injections (Essick 1920), suggesting that macrophages can migrate from the SAS into cervical lymph nodes. Such migration has also been suggested by other workers (Prineas and Wright 1978; Bradbury and Cole 1980). It therefore seems likely that these cells can carry antigens from the SAS and present them to antigen reactive cells in lymph nodes.

Survival of histoincompatible grafts in the CNS has been studied by several groups with conflicting results. The balance of available evidence suggests that graft rejection is relatively inefficient in the CNS, and the brain is therefore generally considered to be a partially privileged site with respect to graft survival (Lodin et al. 1977). This delay in graft rejection has in part been attributed to lack of direct lymphatic drainage into lymph nodes (afferent privilege), and partly to the interference of the blood–brain barrier with the extravasation of effector cells (efferent privilege) (Barker and Billingham 1977).

The capacity of the CNS to mount humoral immune responses has been much less investigated. However, two groups have reported that introduction of xenogeneic red cells into the SAS leads to higher systemic antibody response than injections into conventional sites while injection into the brain parenchyma itself resulted in antibody levels similar to those achieved by subcutaneous immunization (Panda et al. 1965; Sterzl and Lodin 1978).

The purpose of the present study was to compare systemic antibody responses induced by different doses of T-dependent and T-independent antigens injected into the CNS and sites commonly used for immunization.

Material and Methods

Mice

Male (CBA × C57) F_1 strain mice were used at the age of 3–4 months.

Antigens

Sheep red blood cells (SRBC) were used as a *T-dependent particulate antigen,* and trinitrophenyl-bovine serum albumin (TNP-BSA) as a *T-dependent soluble* antigen. The latter was prepared by reacting 300 mg of a protein dissolved in 15 ml of 0.28 M cacodylate buffer pH 11.0 with 180 mg of trinitrobenzene sulfonate previously dissolved in 15 ml of cacodylate buffer. The reaction was allowed to proceed for 6 h at room temperature with constant stirring followed by exhaustive dialysis against water and lyophilization. All procedures were done in a foil-wrapped container to prevent photodecomposition. The molar ratio of hapten:carrier was measured spectrophotometrically, and found to be 12:1.

TNP-$_{14}$ aminoethyl carboxymethyl:50 Ficoll (TNP-AECM:Ficoll) was prepared (Inman 1975) and used as a *T-independent antigen*. For the in vitro tests SRBC were coupled with TNP by the method of Rittenberg and Pratt (1969).

Immunization

All antigens were suspended in 0.85% NaCl. For subarachnoid (i.s.), liver (i.l.) and brain parenchyma (i.b.p.) injections 10 or 100 μl of antigen were used but 200 μl for subcutaneous (s.c.), intravenous (i.v.) and peritoneal (i.p.) injections. SAS immunization were performed, through the esfenoglenoid foramen with the animals under anesthesia (Sagatal 1 mg/kg injection intraperitoneally). The antigen preparations were injected slowly with microsyringe (Hamilton micro measure BV-710 LT) and a small needle (microlance B-G 10×4, 5 26G 3/8-Ind. Brasileira) penetrating no more than 2 mm. Mice with bleeding after SAS injection were discarded from the experiments. For injections into the brain parenchyma, the head and neck regions of the mice were shaved, thoroughly cleaned and a parasagittal incision made in the overlaying skin. A posterior parietal 2-mm diameter hole was drilled (1200–14000 rpm) and 10 μl of the antigens injected slowly with Hamilton syringe (701 N). Following injection the wounded surface was irrigated with sterile saline, the hole closed with Zinc Cement No. 1 (SS White-Ind. Brasileira) and the incision sutured with small clips. Four mice were used for each injection site in all experiments.

Measurement of antibody responses

Sera from the 4 mice in each experimental group were pooled and antibody levels titrated in a direct haemagglutination assay.

Primary responses were tested 7 days after the first antigenic challenge and secondary responses 5, 7, 10, 20 and 30 days after the second challenge.

Enumeration of antibody forming cells (AFC)

This was done either 4–5 days after priming or 4–7 days after rechallenge. Suspensions of spleen and lymph node cells were prepared by squeezing the tissues through stainless steel sieves (mesh 80/inch). The cells were washed twice with medium (Hank's BSS with 0.35 g/l sodium bicarbonate; Flow Lab., Great Britain) containing 10% of fetal calf serum. The number of AFC was determined for individual mice by the direct and indirect haemolytic plaque technique as modified by Cunningham and Szenberg (1968).

Results

Injection of SRBC into SAS induced significantly higher systemic antibody levels during the secondary response than immunization by all the other routes (Table 1). TNP-BSA had a similar but usually more marked effect which was also observed during the primary response (Fig. 1). In contrast, the T-independent antigen, TNP-Ficoll, did not show enhanced immunogenicity in the subarachnoid space, neither in the primary nor in the secondary response (Table 2).

The antibody responses for the 3 antigens had a similar time course when injected i.v. and i.s. (Fig. 1). With 2×10^5 SRBC the difference between i.v.- and i.s.-induced antibody levels was maximal 10 days after the second antigenic challenge, while a 4-fold difference in antibody titre was observed with TNP-BSA during both the primary and the secondary response.

TABLE 1

PRIMARY AND SECONDARY ANTIBODY RESPONSES TO 2×10^6 SRBC

Route of immunization	Number of experiments	Agglutination titres (GMT ± SEM)	
		Primary response [a]	Secondary response [b]
s.c.	3	14.6 ± 8.7	29.3 ± 17.4
i.v.	5	46.4 ± 11.4 [c]	204.8 ± 31.3 [d]
i.s.	5	59.2 ± 20.1 [c]	614.4 ± 102.4 [d]
i.l.	3	37.3 ± 14.1	130.0 ± 71.6

[a] Measured 7 days after priming.
[b] Measured 5, 7, 10, 20 and 30 days after rechallenge and the highest titre for each route recorded.
[c] Primary i.s. versus primary i.v. ($P > 0.1$).
[d] Secondary i.s. > secondary i.v. ($P < 0.01$).

The effect of varying antigen doses was studied by injecting 10 groups of mice with different numbers of SRBC ranging from 10^8 to 10^3 per animal. The results of this experiment are presented in Fig. 2 as a ratio of antibody titre in each group of i.s.-injected animals over the titre for the corresponding i.v.-injected group. Although there was a tendency towards higher primary and secondary antibody responses in the SAS-injected groups throughout the dose range this was only consistently observed in animals receiving less than 10^7 SRBC.

In the experiment presented in Fig. 2 the secondary responses were only measured 5 days after the second challenge. It was demonstrated in separate experiments that this was the time when the i.s./i.v. ratio tended to be highest in animals injected with large doses of SRBC ($\geq 2 \times 10^6$) while with lower doses ($\leq 1 \times 10^6$) this ratio did usually not reach maximum until 10 days after rechallenge. Higher i.s./i.v. ratios would therefore probably have been observed for the secondary responses in the low-dose range if the antibody levels had been measured later.

A series of experiments were carried out to compare antibody levels and numbers

TABLE 2

PRIMARY AND SECONDARY RESPONSES TO 10 μg OF TNP-Ficoll

Route of immunization	Number of experiments	Agglutination titres (GMT ± SEM) [a]	
		Primary response	Secondary response
s.c.	3	32.0 ± 16.0	18.7 ± 7.1
i.v.	7	64.0 ± 12.1 [b]	64.0 ± 12.1 [b]
i.s.	7	91.0 ± 15.6 [b]	66.2 ± 17.3 [b]
i.l.	3	53.3 ± 10.6	34.6 ± 16.2

[a] Recorded as described in Table 1.
[b] Primary and secondary i.s. versus i.v. ($P > 0.1$).

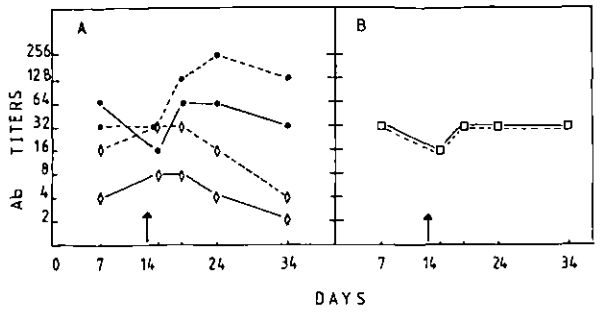

Fig. 1. Time course of systemic antibody responses in groups of 4 mice immunized intravenously or subarachnoidally. Priming on day 0, rechallenge on day 14 (arrows). The same antigen dose used for priming and rechallenge: 2×10^5 SRBC, 100 μg TNP-BSA and 10 μg TNP-Ficoll. *A*: ●———● SRBC i.v., ●- - - - -● SRBC i.s., ◇———◇ TNP-BSA i.v., ◇- - - - -◇ TNP-BSA i.s., *B*: □———□ TNP-Ficoll i.v., □- - - - -□ TNP-Ficoll i.s.

of AFC in the spleen and lymph nodes of mice immunized by different routes. Mice immunized with SRBC by the i.s. route consistently showed 4–20-fold higher numbers of AFC compared with mice receiving i.p. or i.v. injections. This applied to a wide dose range of SRBC and also to mice immunized with TNP-BSA. In contrast, no increase in spleen or lymph node AFC numbers was observed in mice receiving i.s. injections of TNP-Ficoll.

Table 3 gives the results of one representative experiment in which 2×10^6 SRBC and 10 μg TNP-Ficoll were used for both priming and rechallenge. Data for the lymph nodes are not presented since they always agreed very closely with those obtained from the spleens. With T-dependent antigens the difference between the numbers of AFC in i.s.- versus i.p.- or i.v.-immunized mice was always highly significant within each experiment ($P < 0.002$ to $P > 0.001$) indicating that the high antibody levels in i.s.-injected mice may largely have been due to antibody production outside the CNS. It should be noted that in the experiment reported in Table 3

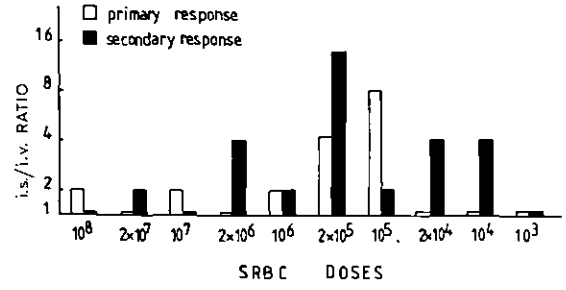

Fig. 2. The effects of varying doses of SRBC on systemic antibody responses of mice immunized intravenously or subarachnoidally with SRBC. Primary responses were measured 7 days after priming and secondary responses 5 days after rechallenge. Each column represents a ratio of the mean antibody titre of 4 i.s.-injected animals over the mean titre of the corresponding i.v.-injected groups.

TABLE 3

ANTIBODY RESPONSES IN MICE IMMUNIZED BY DIFFERENT ROUTES WITH TNP-Ficoll AND SRBC

Groups of 4 mice were primed with 10 μg TNP-Ficoll or 2×10^6 SRBC and rechallenged 7 days later with the same antigen dose injected into the same site. PFC were determined 4 days after rechallenge for TNP-Ficoll and 7 days after rechallenge for SRBC. PFC values are expressed as mean $\log_{10}\pm$SE and geometric mean in parenthesis.

Route of immunization	T-independent response (TNP-Ficoll)			T-dependent response (SRBC)		
	PFC/spleen		Reciprocal Ab. titre	PFC/spleen		Reciprocal Ab. titre
	Direct	Indirect		Direct	Indirect	
Not immunized	2.17±0.20 (151)	2.27±0.24 (186)	<1	1.75±0.08 (56.5)	1.6±0 (40)	<1
Subcutaneous	ND	ND	ND	1.86±0.13 (72.6)	1.9±0 (80)	8
Peritoneal cavity	4.34±0.06 (22327)	4.34±0.09 (22086)	128	2.18±0.12 [a] (152)	2.85±0.0 [a] (387)	32
Subarachnoid space	4.36±0.12 (23379)	4.31±0.10 (20633)	128	3.35±0.08 [b] (2246)	3.24±0.07 [b] (1753)	128
Brain parenchyma	ND	ND	ND	2.60±0.16 (466)	2.30±0.16 (220)	64

[a] versus [b]: $P<0.002$ by Student's t-test.
ND=not done.

immunization directly into the brain parenchyma gave rise to a similar antibody response as i.p. immunization.

Discussion

In this paper observations are reported which indicate that injection of T-dependent antigens into the subarachnoid space of mice gives rise to a more vigorous systemic antibody response than injections into other sites which are conventionally used for immunization. In contrast, T-independent antigen did not have this effect. Other workers using only dog red cells (Panda et al. 1965) or SRBC (Sterzl and Lodin 1978) have also reported that subarachnoid injections result in high systemic antibody responses compared with immunization by other routes, but to our knowledge this is the first demonstration that only T-dependent and not T-independent antigens show enhanced immunogenicity when injected into the subarachnoid space.

Immunization into the brain parenchyma has previously been shown to induce a systemic antibody response which is similar in magnitude to that observed after s.c. immunization (Panda et al. 1965). We found that intracerebral inoculation gave somewhat higher antibody levels than s.c. injections. Although it is difficult to exclude minor antigen leakage into the subarachnoid space in these experiments, it seems likely that the brain parenchyma is less 'privileged' with respect to antibody responses than cellular immunity.

A variety of mechanisms can be suggested to explain the vigorous responses after subarachnoid injection of antigens and we are currently investigating the following.

Since the elevated antibody responses were only observed with T-dependent antigens, regulatory T cells or macrophages are likely to be involved. Although available data indicate that suppressor T cells may to some extent act directly on B cells, suppressor activity on antibody production is probably largely mediated via helper T cells (Gershon 1980). Assuming that T-independent responses are therefore less affected by suppressor T cells than are T-dependent responses, the high antibody levels could be due to relatively ineffective induction of T suppressor activity when antigens are introduced directly into the subarachnoid space. Alternatively the subarachnoid macrophage population might contain relatively few cells capable of rapid antigen degradation and a correspondingly high proportion of antigen presenting cells. This possibility is consistent with the observation that the difference in antibody titre was more pronounced when small amount of antigen was used. It should also be pointed out in this context that T-independent antigens may generally be less easily degraded in the body than T-dependent substances (Coutinho and Möller 1974). Lastly, it is conceivable that trauma or inflammatory reaction affecting the CNS may cause the release of substances with adjuvant activity or disturb regulatory functions which may be exerted by the CNS on antibody production (Besedovsky 1977; Jankovic 1980).

It is tempting to speculate that the observation reported in this paper could be relevant to the high systemic antibody levels associated with certain chronic infec-

tions of the CNS, notably subacute sclerosing panencephalitis and visna. Interestingly, maedi, a persistent lung infection in sheep with a virus which is closely related to the visna virus (Thormar and Helgadottir 1965), may give rise to somewhat lower and less sustained virus specific antibody titre than visna (Gudnadottir and Kristinsdottir 1967). Furthermore, oligoclonal bands, some of which are virus specific, can be demonstrated within a few days of cerebral infarction (Roström et al. 1981), again suggesting a relative hyperactivity of B lymphocytes when activation is triggered within the CNS. Further studies of the mechanisms underlying this hyperactivity could increase our understanding of certain immunopathological conditions of the central nervous system.

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