ALLOGRAFTING AND THE T CELL SYSTEM

A MULTIPARAMETER ANALYSIS OF REJECTION IN THE RAT

ALLOGENE TRANSPLANTATIE EN HET T CEL SYSTEEM

Een multiparameter analyse van de afstoting in de rat

PROEFSCHRIFT

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CONTENTS

LIST OF ABBREVIATIONS

ALS	Anti-lymphocyte serum
APC	Antigen presenting cell
ARS	Autologous rat serum
ATG	Anti T cell globulin
ATN	Acute tubulis necrosis
ATXBM	T cell depleted animal
BSA	Bovine serum albumin
CD	Cluster of differentiation
CML	Cell mediated lympholysis
Con A	Concanavalin A
CPM	Counts per minute(s)
CTL	Cytotoxic T lymphocyte(s)
CTL-p	Cytotoxic T lymphocyte precursor(s)
DTH	Delayed type hypersensitivity
CyA	Cyclosporin A
Elisa	Enzyme-linked-immuno-sorbent-assay
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescence isothiocyanate
FNAB	Fine needle aspiration biopsy
HAT	Hypoxanthine-aminopterin-thymidine
HSC	Haemopoietic stem cell
IDC	Interdigitating cell(s)
IL-2	Interleukin-2
LCA	Leukocyte common antigen
LDA	Limiting dilution assay
LDH	Lactate dehydrogenase
LGL	Large granular lymphocyte(s)
MAF	Macrophage arming factor
MARG	Mouse anti IgG
MARS	Mouse anti-rat serum
MCA	Monoclonal antibody(ies)
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node(s)
MLR	Mixed lymphocyte reaction
NK cells	Natural killer cells
PALS	Periarteriolar lymphocyte sheath
PNA	Peanut agglutinin
RFE	Renal failure episode(s)
RIA	Radio-immuno-assay
RT	Room temperature
TdT	Terminal deoxynucleotidyl transferase
TCGF	T cell growth factor
TRITC	Tetra methylrhodamine isothiocyanate

LIST OF ANTIBODIES USED IN THE COURSE OF THIS THESIS

Antibody			Typical leukocyte subpopulation
Mouse	Human	Rat	recognized by MCA
		W3/13 - ER-1	All T lymphocytes
		MRC OX-19	
L3T4	anti-CD4[a]	W3/25 - ER-2	Helper T cell subpopulation
Lyt-1			
Lyt-2	anti-CD8[a]	MRC OX-8	Cytotoxic/suppressor T cells
		ER-9	T cell subpopulation
		ER-7	
		ER-3	
		ER-10	
		MRC OX-7	Rat Thy-1
		ER-14	
		ER-4	Bone marrow cells and thymocytes
		ER-13	Ia-like antigen common part
		F17-23-2	Ia-like antigen polymorphic part
		MARK-1	B lymphocytes
		ED-1	Macrophages

a. MCA directed against the CD4 and CD8

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

In an organism a complex and harmonious interaction of organs, tissues, cells and subcellular organelles occurs. A high degree of cooperation is necessary to sustain life. This delicate integrity is endangered by various threats from the outside world. The organism developed a defense mechanisms for protection like fighting and hiding. Also within the organism itself an intraorganismic defense mechanism is present, which is referred to as the immune system. This system is based upon three principles: recognition, processing and response. Recognition is a noncovalent interaction between a signal molecule, carrying information and a receptor molecule, which is able to recognize the signal. By this process the organism is capable to discriminate between itself (self) and the outside world (non-self). This information is analyzed (processing) and the organism can response to eliminate the intruding organism. The smallest unit able to provoke such a reaction is referred to as an antigen.

Within the animal world many different types of defense reactions can be distinguished differing in speed of the response, the presence or absence of memory, processing of the stimulating signals and the elimination of the antigenic intruders. Two major categories of defense can be distinguished with regard to the effector mechanism. The first is called the humoral system and is mediated by soluble factors in the body fluids, whereas the other category is the cellular system, because it is elicited by cells.

The **humoral reactions** can be divided into an **inducible** and a **noninducible** group, which refers to the ability and disability respectively, to counteract the exposure of an organism to antigen by increasing the level of the humoral factor. **Noninducible** factors are secreted into the body fluids at a constant rate. Already in 1916 Cantuzène discovered that if the landsnail *Helix pomatia* was infected with bacteria these bacteria clumped together due to the presence of an agglutinin after which they were removed. This factor is produced and

secreted at a constant rate, which is not influenced by external factors. Another example of this nature is provided by the complement system found in vertebrates. A number of proteins (complement factors) present in the serum can assemble into a complex molecular structure on the cell membrane. Subsequently various effector mechanisms can be activated, like punching holes in the target cell by the complex resulting in direct cell death. Also cellular effector reactions can be activated by these complement factors. The **inducible** humoral factors are normally absent or only present in small amounts in the body fluids. After the introduction of antigenic material a rapid increase of the factor can be seen, followed by a gradual decrease after withdrawal of the antigen. The lobster *Panulirus* produces a bactericidin that shows the described fluctuation after bacterial infection (Evans et al, 1968). A certain degree of memory can be observed in this species characterized by an enhanced reaction after a second exposure to the bacteria. The specificity, however, is low since other types of bacteria can also induce this secondary reaction. Antibodies in the vertebrates do have the same inducibility, but in contrast to the previous example they have an exquisite specificity for the antigen.

The **cellular defense system** can be divided into an aggressive and a nonagressive type. The nonagressive type can be illustrated by findings of Wilson (1907) in the sponge. Single cell suspensions of sponges were made and these cells were grown in seawater. These cells aggregated and were able to differentiate. After a few days functional sponges developed again. Repeating this experiment, but now by mixing cells of two different types of sponges, only cells of the same type aggregated. These findings implicated that such cells are able to discriminate between cells of the same type (self) and cells of another type (nonself).

Three types of aggressive cellular reactions can be distinguished: the hyperplastic reaction, engulfment and cellular cytotoxic reactions. The **hyperplastic** reaction can be illustrated with the coral *Hydractinia echinata* (Ivker, 1972). Colonies of the same type fuse upon contact with each other. Contact between different types produces abnormal hyperplastic stolons in one of the two colonies. These stolons strangle the other colony, cutting off the food supply, which results in death. The **engulfment** reaction is divided according to the size of the processed particles: pinocytosis (fluid droplets), phagocytosis (small particles) and encapsulation (large multicellular material). Pinocytosis and phagocytosis were for the first time described by Ernst Haeckel (1862) in a small

mollusk (*Thetys fimbria*). He found that after injection of indigo dye into the blood vessels many of the blood cells contained small particles of the dye within a few hours. More detailed analysis of this phenomenon revealed that cell projections upon contact between cells and dye was followed by enveloping and swallowing of the material. In the cell the dye was confined to a vacuole in which eventually enzymes were released resulting in degradation of the dye. The cellular **cytotoxic** reactions are primarily directed against intracellular parasites like virusses. These reactions are executed by specialized cells (killer or cytotoxic cells) that bind directly to an infected cell. Subsequently cell lysis is caused by changes in the permeability of the membrane. Obviously these cytotoxic cells are able to recognise an infected cell due to membrane alterations induced by the virus. This type of reaction will be discussed in detail later.

In vertebrates a higher degree of specialization of the defense system can be observed. Recognition, transfer of signals and effector function are executed by specialized cells. This diversification requires a special regulatory type of cells, that either enhance (helper cells) or suppress (suppressor cells) the response. Together with these specialized cells also less sophisticated defense cells are still present in vertebrates, which are still able to exert the ancient process of engulfment. This thesis will deal mainly with the immune system found in humans and rodents. Therefore further consideration will be given to the organization of the specific immune system in vertebrates.

The immune system of vertebrates is basically a dual system existing of a humoral and a cellular immune branch. Although both systems are capable to respond to any antigenic material, in most cases one defense line is favoured. The cellular system is more effective against cancer cells, fungi, parasites, intracellular viral infections and foreign tissues, whereas the humoral immune response is highly effective against extracellular phases of bacterial and viral infections. The cells responsible for both types of immune responses are the lymphocytes. They can be divided into (bone marrow derived) B lymphocytes and (thymus dependent) T lymphocytes. The cellular immune response is mediated by T cells. These cells can be activated by contact with an intruding organism resulting in a reaction that includes binding and killing of the organism. Other types of T cells are involved in the regulation of both cellular and humoral responses. B lymphocytes account for the humoral immune reponse. After recognition of the antigen B lymphocytes will start to proliferate

and differentiate into plasma cells which secrete antibodies. These antibodies can specifically bind to the antigen and thereby activate several elimination processes. Besides lymphocytes other types of cells play an important role in the immune sytem i.e. the so-called accessory cells. An important example of such accessory cells are the macrophages. These cells can attack virtually all non-self material in a non-specific manner by a primitive engulfment reaction. Furthermore, macrophages are very important for the accumulation, processing and presentation of antigenic material to lymphocytes. The immunogenicity of the antigen is highly increased by this kind of macrophage pretreatment. Also other cells of the hematopoietic system, such as dendritic reticular cells, can be involved in various stages of the immune response. This variety of response mechanisms results in a highly specific defense system, which is quite capable to counteract most antigenic intruders. One of the most appealing examples of such a foreign invader is an allogeneic organ or tissue transplant. Although the intrusion of this particular type of foreign material is very much wanted, the immune system will react in its natural way in an attempt to eliminate it by mounting a vigorous response.

TRANSPLANTATION

Transplantation is the transfer of living cells, tissues or organs from one part of the body to another or from one individual to another. We can distinguish between an orthotopic transplantation, when the graft is placed in the normal anatomical position, and a heterotopic situation, when the graft is replaced at an other site. Furthermore transplantation can be autogeneic (self), syngeneic (genetically identical), allogeneic (between two different inbred strains or different not inbred individuals) and xenogeneic (between different species).

The fate of a graft will be determined by the genetic relationship of the donor and acceptor. When no difference exists the graft will be accepted, otherwise the graft will be rejected. The allograft reaction can be divided into three phases. First a **recognition** phase: each transplantation will involve some damage of the transplanted cells and tissues. These cells will be phagocytized by leukocyte (macrophages, polymorphonuclear cells and granulocytes) of the host. Macrophages will process this cell debris and expose certain antigenic determinants on their surface. Subsequently these will be recognized by lymphocytes. Also

direct contact of lymphocytes with the antigenic sites within the graft
itself can occur. After this recognition phase, in which rather low
numbers of immunocompetent cells are involved, an **amplification** phase
follows. The activated lymphocytes proliferate and secrete various
substances that mobilize other cells such as macrophages, granulocytes, B
lymphocytes, natural killer (NK) cells and T lymphocytes. The last phase
is the **effector** phase in which a massive attack is mounted towards the
graft. Part of this attack is specifically directed against donor cells
carrying a foreign antigen but also a non-specific (recruited) attack
occurs, which may even include host cells (Review: Porter, 1983).
Allograft reactions can be distinguished in first set and second set
reactions. The latter type of response occurs when the acceptor is already
pre-sensitized and this process is characterized by an accelerated and
enhanced reaction. A more defined classification of the graft rejection
will be discussed in a later stage of this thesis.

The primary rule of transplantation is: a graft is rejected
whenever it possesses histocompatibility (H) antigens, which are absent in
the recipient. These antigens are encoded for the histocompatibility (H)
genes (Klein, 1975). About the total number of H-loci difference of
opinion exist, but the lowest estimations in rodents are thirty and figures
up to a hundred are mentioned. Counce et al (1956) described a marked
difference in intensity of allograft reaction caused by 3 loci as compared
to other loci. They introduced the notion of major and minor H-loci.
Later this division of 'strong' and 'weak' loci was thought to be rather
arbitrary, but nowadays it is evident that a fundamental difference exists
between one group of loci and the other H-loci. This group of loci, the
major histocompatibility complex (MHC) exerts the strongest effect on
allograft rejection. MHC-antigens can activate lymphocytes without the
context of MHC-antigens (unrestricted) whereas antigen recognition,
including minor H-antigens, only occurs in the context of MHC-antigens.

The MHC is a group of closely linked loci, that can be divided into
two separate classes, according to the structure of the molecules they
encode for (Fig. 1.1). The first group encodes for molecules which are
referred to as class I molecules. They consist of two polypeptide chains.
One of them, the heavy chain, with covalently attached to it one or two
carbohydrate moieties, has a comparable molecular weight (MW) in several
species: MW 44,000 in mouse (Klein, 1975; Klein, 1979) and MW 45,000 in rat
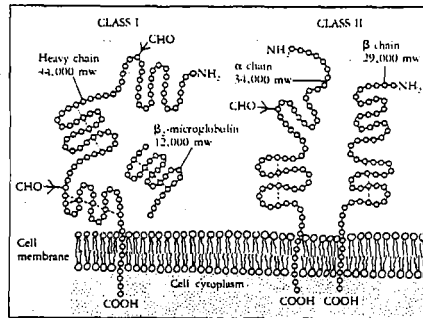(Sporer et al, 1978; Blankenhorn et al, 1978). This heavy chain consists

Figure 1.1 Structure of Class I and Class II molecules
CHO: sugar moiety

of an intracellular, a transmembrane and a extracellular segment. This last part consists of two disulfide loops and an amino terminal segment. A light chain, the so-called β2-microgobulin (β2M), is noncovalently attached to the heavy chain. β2M is a polypeptide with a molecular weight of 12,000, which·is folded in a single disulfide loop, with no direct attachment to the membrane. Class I antigens are found on all nucleated cells. The second group of MHC molecules are the Class II molecules. Class II molecules consist of two noncovalently bound polypeptides: an α chain with a molecular weight of 31,000 to 34,000 and a β chain with a molecular weight of 26,000 to 29,000 (Wettstein et al, 1981; Fukumoto et al, 1982). Class II antigens are normally found only on certain cells of the immune system. Although they are absent on resting T cells, activation of these cells results in class II expression. These antigens can also be induced on a wide variety of different cells (Mason, 1984).

The rat, a frequently used animal in transplantation research, has a MHC system comparable to that of other mammals. This gene complex, previously referred to as AgB system or H-1 complex is nowadays called the RT-1 system (Günther and Stark, 1977; Report of the first international workshop on alloantigenic systems in the rat, 1978; Workshop report, 1979). For a more detailed description of the MHC complex the reader is referred to Gill et al (1983). The structure of the RT1 complex is quite comparable to that of the MHC of the mouse: two class II loci (RT1.B and RT1.D) are enclosed by two class I loci (RT1.A and RT1.E) (Fig. 1.2). Another class I locus (RT1.C) is located outside the conventionally defined MHC. It is
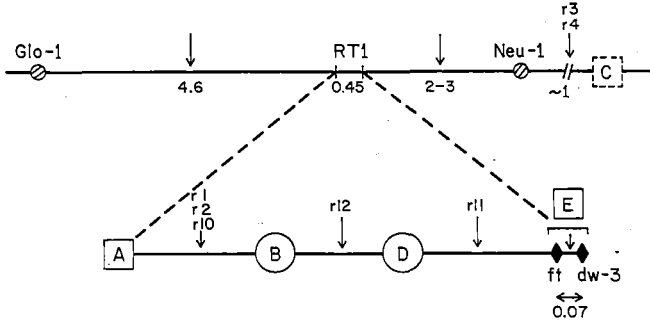
Figure 1.2 The structure of the major histocompatibility complex of the rat, the RT-1 system (Gill et al, 1983) Glo-1 and Neu-1 are loci controlling the activity of the glyoxalase and the neuramidase enzymes. The squares are the loci coding for the class I antigens RT-1.A and RT-1.E. The circles are the loci coding for the class II antigens RT-1.B and RT-1.D. The dw-3 (body weight) and ft (fertility) loci comprise the growth and reproduction complex. The numbers under the arrows are the map distance in cM. The r-numbers above the arrows refer to the recombinant strains.

suggested that this locus might be an equivalent to the Qa/Tla loci of the mouse (Stock and Günther, 1982). Blankenhorn et al (1983) demonstrated that the RT1.B and RT1.D loci code for I-A and I-E like class II molecules, respectively. They are associated with a strong mixed lymphocyte reaction (MLR) i.e. the proliferation of cells that is found, when lymphocytes of two genetically different individuals are cocultured. The capabilty to respond to small polypeptide antigens is also located in this region (Lobel and Cramer, 1981). The class I molecules have a high degree of homology with those of the mouse (Gill et al, 1983). Liebert et al (1982, 1983) found that they provide the major stimulus for cytotoxic T cell activity. Both class I and class II antigens can initiate the allograft reaction. This has been confirmed by many studies in the rat for transplantation of a variety of organs such as kidney, heart, pancreas and skin (Günther and Stark, 1977; Guttman et al, 1980; Pinto et al 1983; Gallico et al, 1979). The relative importance of the separate loci is still discussed. Gallico et al (1979) claim that a full MHC-difference is needed for a strong rejection. Guttmann et al (1980) reported that either a class I or a class II difference is sufficient. On the other hand, several studies with congenic rat strains showed a strong allograft reaction for a class II difference, whereas a mere class I difference resulted in a variety of reaction patterns ranging from weak to strong (Rozing et al, 1983; Lowry

and Gurley, 1983; Guttman et al, 1985). Therefore the influence of class I loci is not easy to interpret. Rozing et al (1983) suggested that the potential of the recipients to respond to MHC-antigens could be different for the various inbred strains. This would explain the different data reported by several groups. Another reason for the observed discrepancies is that the MHC-antigens in the graft are presented in various ways depending on the type of graft. In recent years the various lymphocyte populations and their relative importance in the allograft reaction have been extensively studied. B lymphocytes and the humoral immune response do participate in the allograft reaction. However, most authors agree, that the B cells are not able to iniate an allograft reaction by themselves. This has been confirmed by studies with athymic rodents i.e. nude mice and nude rats. Although recently for both nude mice as well as nude rats the presence of small numbers of T lymphocytes were reported (Raff, 1973; Brooks et al, 1980), these animals probably lack the right activation signal, since allogeneic as well as xenogeneic grafts are not rejected (Rygaard, 1969; Kindred, 1979; Festing et al, 1978). Similar results were obtained in another T cell deprived model in which animals are thymectomized and lethally irradiated followed by reconstitution of bone marrow (ATXBM). These animals also failed to reject allografts (Brunner and Cerottini, 1971). Although it is therefore well documented that T cells play a central role in the allograft reaction the actual cellular mechanism is still not completely understood.

T CELL SYSTEM

All cells of the haematopoietic system follow an analogous differentiation pathway. They are derived from a common cell, the pluripotent haematopoietic stem cell (HSC) (Till and McCullogh, 1961). A pluripotent HSC is an undifferentiated cell characterized by the potential for extensive proliferation, self-renewal capacity (formation of new HSC) and capability of differentiation (production of different hematopoietic cells). Also T lymphocytes derive from the HSC (Abrahamson et al, 1977). They differentiate into so-called precursor T cells or prothymocytes. These cells migrate to the thymus and after intrathymic proliferation and maturation they leave the thymus as T lymphocytes and subsequently migrate to the T cell domains of the peripheral lymphoid tissues. The fact that thymus lymphocytes originate from migrant cells rather than from

mesenchymal or epithelial elements of thymus itself was most convincingly demonstrated by Moore and Owen (1965) in fowl embryos. Haematopoietic cell chimerism was established between embryos of different sexes. This enabled analysis of the thymus lymphocytes using chromosomal sex markers. Dividing thymocytes were analyzed and about half of these cells originated from the other partner. Kadish and Bash (1976) defined these migrants (prothymocytes) as those cells that after transfer in lethally irradiated animals have the capacity to repopulate the thymus with donor specific lymphocytes. Prothymocytes in adult animals are mainly found in the bone marrow. The frequency of prothymocytes in bone marrow has been estimated in mice as <0.1% (Lepault et al, 1983).

Ontogenetic studies highly increased our knowledge of prothymocytes and their entry into the thymus. The earliest blood forming cells appear in the yolk sac, whereafter the site of blood formation is gradually taken over by the fetal liver and the fetal spleen (Moore and Owen, 1967; Owen and Ritter, 1969). In extensive studies in avian embryos using chicken-quail chimeras three successive waves of the influx of prothymocytes into the thymus were observed, suggesting discrete receptive periods in 5, 11 and 17 days old embryos (Jotereau et al, 1980; Jotereau and Le Douarin, 1982). The authors proposed the production of chemotactic factor(s) from the epithelial part of the thymus during these receptive periods.

The thymus gland consists of several different components. It originates from the primitive pharynx as an epitheliomesenchymal rudiment (Review, LeDouarin, 1977). It is generally accepted that the epithelium is endodermal in origin and derived from the third pharyngeal pouch, although some ectodermal participation has been proposed (Cordier and Haumont, 1980). The mesenchymal part that participates in the thymus structure is at least in part derived from mesenchyme of the pharyngeal arch. During ontogeny, at first an inner epithelial mass surrounded by a capsule of mesenchym (Jenkinson et al, 1981) is formed. Subsequently immigration of hematopoietic cells and antigen presenting cells (APC) from the fetal liver occurs (Bartlett and Pyke, 1982). In adults the thymus consists of two separate lobes, which are subdivided into small lobules (Hoshino, 1963; van Ewijk, 1984) consisting of distinct lymphoid as well as stromal cells. Each lobule can be divided into two regions: a peripheral cortical area and an inner medullary area, separated by the cortico-medullary region (CMR). The cortical area is densely populated with mainly small sized lymphocytes (85%-90% of all thymus lymphocytes), whereas the medulla contains

relatively few medium-sized lymphocytes (10%-15%) (Weissman, 1973).

Cortical lymphocytes can be distinguished from medullary cells by several criteria. For instance the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) is expressed by cortical cells, but not by medullary or mature T cells (Barton et al, 1976; Goldschneider et al, 1977; Gregoire et al, 1979). They also differ in their binding properties to the lectin peanut agglutinin (PNA). Cortical cells bind high levels of PNA in contrast to the low levels that are bound by medullary thymocytes. Also subpopulation markers proved to be useful tools to distinguish the separate cell populations in the thymus. Cantor and Boyse (1977) described an alloantigenic system on T lymphocytes of the mouse i.e Lyt 1 and Lyt 2. Peripheral T cells express only one of these molecules on their cell surface. The majority (70%) of the cortical cells, however, express both antigens Lyt1$^+$Lyt2$^+$ (Review Scollay and Shortman, 1984). The medullary cells (10-15%) are very similar to peripheral T cells since they express only one of these two antigens. Also an association between mature or immature phenotype and function can be found, since cortical cells are rather deficient in function and most medullary cells seem to be functional (Chen et al, 1982).

One of the first models of differentiation within the thymus was based upon studies of Weissmann (1973), who demonstrated that medullary cells can be derived from cortical lymphocytes. In this socalled 'straight through' model, proposed by Cantor and Boyse in 1977, prothymocytes enter the cortex, express an immature cortical phenotype (Lyt1$^+$2$^+$) and then mature as they migrate to the medulla, selectively loosing one of the two surface antigens. This model, however, is not in accordance with recent cell turnover data reported by several groups. Bryant (1972) determined a turnover time of approximately three days for the cortical lymphocyte population. The proliferation in thymus is predominantly located within the cortex since more than 95% of thymic mitoses are found in this area (Bryant, 1972). The number of cells that leave the thymus each day has been calculated by Scollay et al (1980). Intrathymic injection *in situ* of fluorescein isothiocyanate (FITC) was used for labeling the migrating cells. In adult mice only one percent of the thymocyte pool migrates to the periphery. The low number of migrants compared with the high rate of proliferation within the thymus indicates that many of the thymocytes never leave this organ. Biochemical data of McPhee et al (1979) confirmed such a high rate of intrathymic cell death. Although the proliferation rate in

the medulla is rather low, compared to that of the cortex, this area can produce enough cells to give rise to all emigrating cells (Scollay and Shortman, 1983). Several models have been proposed to comply with these controversial data. Shortman and Jackson (1974) suggest that prothymocytes are already committed into two lineages: one lineage migrates straight to the medulla and gives rise to functional T cells. The other lineage migrates to the cortex and differentiates to non-functional cells, the 'dead end cells'. Mathieson (1982) and Scollay (1983) have proposed the following model: they suggest that both functional subsets may develop independently. One subpopulation enters the subcapsular region of the cortex. Here these cells differentiate expressing the Lyt1 and the Lyt2 alloantigens. A selected minority of the cells of the cortex may develop to mature T cells and migrate to the medulla. Another subpopulation may develop in the medulla independently of the cortex.

Maturing T lymphocytes leave the thymus and migrate to the peripheral lymphoid tissues. The presence of specific homing receptors for spleen, lymph nodes and plaques of Peyer (Gallatin et al, 1983; Jalkanen et al, 1986) indicates a controlled lymphocytic traffic between blood and lymphoid organs. Some cells are heading for the spleen while others go to the lymph nodes. The number of cells emigrating each day from the thymus is four times greater than the total number of lymphocytes in the blood (Bryant, 1972, Scollay and Shortman, 1983). Furthermore most studies indicate that the lifespan of the circulating T cell population is extremely long (Gowans and Knight, 1964; Ford, 1969). The majority of the thymus migrants, however, apparently die shortly after reaching the lymphoid organs and only migrants encountering their specific antigen will be stimulated and transformed into a long lived cell. The expansion of the total peripheral T cell population seems to be largely antigen dependent. This is confirmed by the finding that the T cell mass in germfree animals is only about 10% of that found in normal animals Sprent and Miller (1979). Kendall Smith (1984) postulated therefore that the thymus is a developmental organ, that is active only during the growth period. In that period it generates the total T cell repertoire of the adult. Throughout life the expansion of this repertoire depends upon antigenic stimulation.

T lymphocytes have an important regulatory function in a great variety of immunological processes. Until recently T lymphocytes could only be identified in mice by anti Thy-1 alloantisera and in man by the property that they bind to sheep red blood cells (SRBC) or by functional

testing, such as mitogen response (Greaves and Janossy, 1972). A first indication for the existence of functionally different T cell subpopulations came from experimental studies, in which Gershon and coworkers (Gershon et al, 1974) showed that T cells provided help in the humoral immune response against T cell dependent antigens. Under certain circumstances they also displayed suppressive activity (Gershon and Kondo, 1972). Two functionally distinct subpopulations of T lymphocytes were initially defined by Cantor and Boyse (1975), which could be separated by different phenotype expression of surface antigens. T lymphocytes involved in helper phenomemona and delayed hypersensitivity expressed the Lyt1 alloantigen, whereas T lymphocytes active in cytotoxic as well as in suppressive reactions carried the Lyt2 alloantigen. Recently a more specific marker for the T helper cells in the mouse has been described i.e. the L3T4 antigen (Dialynas et al, 1983). Initially the Lyt system was defined with alloantisera. Raising this kind of specific alloantisera is complicated and difficult. Highly purified antigen is necessary for immunization and afterwards extensive absorption is required in order to remove non-specific antibodies. A major problem is also the isolation of antigens for which no specific antibody is available. With regard to the problems involved in raising specific antisera, the introduction of the hybridoma technique proved to be a major step forward. Already in the 1960's occasionally fusion of cells had been observed in cell cultures. The incidence could be increased by adding ultraviolet light treated Sendai virus or polyethyleneglycol (PEG). Köhler and Milstein (1975) demonstrated that also antibody secreting cells could be immortalized by fusion with myeloma cells. From the total mixture of fused cells, also referred to as hybrid cells, one specific cell can be isolated by subcloning. Subcloning consists of diluting the cells in such a fashion, that after distributionover wells of microculture plates, the majority of these wells contain growing cells which are derived from a single cell. Using this method, clones of identical hybrid cells can be isolated in large numbers. These cells produce monospecific antibodies, which can be obtained in large quantities. These so-called monoclonal antibodies (MCA) provide an invaluable and highly sensitive tool for the dissection of the immune system. In the recent years many MCA have been developed against surface antigens of different types of cells in various species. In humans for instance the equivalents of the Lyt 2 and L3T4 antigens were detected i.e. the CD8 and the CD4 alloantigens, respectively (Bernard et al, 1984). A

homology with the mouse system was also found for the function of these T cell subpopulations. Although a highly positive correlation exists between the surface alloantigens and the function of cells, it is not an absolute one. It became possible to maintain T cell lines in culture for long periods. Meuer et al (1984) isolated cytotoxic T lymphocyte (CTL) lines. Most of these CTL lines possessed the normal cytotoxic phenotype, but a few CTL lines expressed the helper phenotype. Several other exceptions have been described of the correlation between phenotype and function for cloned T cell lines in the recent years.

RATIONALE OF THE PRESENT STUDY

The immune system in vertebrates is a highly specialized type of defense system. Especially T lymphocytes play a dominant role in the management of the various parts of this defense. In transplantation the reaction against the foreign tissue is initiated by cells of this T cell system. In order to avoid an undesired allograft reaction it is necessary to obtain more information about the mechanism of the allograft reaction and especially about the role of the T cell system in this process. Subpopulations of T cells are involved in both effector and regulatory functions. These subpopulations take part in different functions, which highly correlate with the expression of distinct surface molecules. Therefore specific antibodies directed against these surface antigens are excellent tools for the monitoring and dissecting the immune system. After MCA became available for the human immune system, immunological monitoring of transplantation patients has been practised on a large scale. Particularly data on monitoring in kidney transplantation, which has been and still is the most successful and widely practised form of non-privileged organ transplantation, have been reported in the previous years.

In humans, however, the situation is very complicated. The complex structure of the human MHC (the human leukocyte antigen: HLA system) and the outbred situation of humans make it virtually impossible to find two individuals that are genetically identical. Therefore variable contributions of differences in MHC and non-MHC loci between donor and recipient will always be a complicating factor for the interpretation of the results. A major source of variation also is the mode of treatment of the allograft reaction and the different techniques of immunological monitoring. Many transplantation centres are using immunosuppressive

agents such as corticosteroids and antimetabolites; which are highly non-specific. Recently, treatment with the antibiotic Cyclosporin A, anti lymphocyte sera (ALS) and anti-T cell globulin (ATG) are also favoured. For the immunological monitoring of the graft some centres follow the clinical status of the patients, whereas other centres apply flow cytometric, histochemical or functional techniques for this purpose. Also the site of monitoring is different: monitoring within the graft itself or analysis of the peripheral blood lymphocytes is practised. It is clear, that the interpretation and the comparison of the clinical data is very difficult. Therefore experimental studies in animals are frequently used in order to obtain more information of the immunological basis of allograft rejection. Moreover these kind of studies are performed to achieve an improvement of immunosuppressive treatment. The rat is the animal of transplantation research since, in contrast to the mouse, kidney and heart transplantation is technically possible. At present congenic and recombinant inbred rat strains are available for immunogenetic research.

The analysis of the cellular composition of the immune system of the rat requires the production of specific antibodies. This thesis describes the optimalization of the hybridoma technique and the production of a series of mouse anti-rat MCA. Subsequently antibodies were selected for reactivity against surface molecules of T lymphocytes. From the available hybrid cell lines producing these type of antibodies, a representative number was selected for further study. New distribution patterns of lymphocytes in various stages along the T cell differentiation were detected. Characterization of these antibodies has been performed with regard to antibody properties, recognized surface molecules, but also the cells expressing these antigens in bone marrow, thymus and peripheral lymphoid organs. A possible involvement of these surface antigens in the function of the T cells was studied.

Subsequently, a series of transplantation experiments were performed, in which the alloreaction was studied using the selected panel of antibodies. Besides monitoring of alterations within the peripheral immune system and at the site of action (the graft), also changes in the functional repertoire were studied and will be described.

In the last part the immunosuppressive potential of the monoclonal antibodies has been studied in the initial phase after transplantation. The essential factors for immunosuppression will be discussed.

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Chapter 2

HYBRIDOMA TECHNIQUE

INTRODUCTION

A major contribution to the recognition and elimination of foreign invaders is provided by a highly specialized type of proteins: the antibodies or immunoglobulins (Ig). The molecule (Fig. 2.1) consists of four polypeptides, two heavy chains (H-chains) and two light chains (L-chains) held together by disulfide bridges. Each chain is divided in a variable (V) region and a constant (C) region. The V_L and V_H region form the V domain. The C-region of the H- and L-chains form the C_H domains. For a detailed description of the structure of the antibody molecule the reader is referred to Klein (1982).
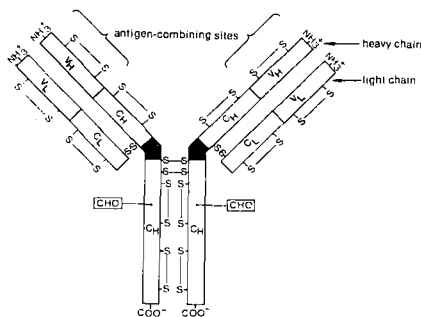
Figure 2.1 A schematic drawing of the human IgG antibody molecule.
(Hood et al, 1978)

Antibodies have two distinct functions which are reflected within the structure of the molecule: The two V-domains are involved in antigen recognition and binding. The C-domains are involved in effector functions such as complement activation. Two different forms of the Ig molecules are found: one is membrane-bound and located on the surface of B cells. This molecule is involved in antigen recognition and B cell activation. The second type is the secretory form of Ig, which is directly involved in antigen binding and elimination.

Antibodies are produced by a specialized type of lymphocytes: the B lymphocytes. B lymphocytes belong to the progeny of the pluripotent haematopoietic stem cell. In adults the precursor B cells differentiate within the bone marrow. From the moment a B lymphocyte is committed to antibody synthesis the antigen-binding specificity is irreversibly defined for this particular cell as well as for the complete progeny of this cell. Recognition and binding of the surface Ig molecules to antigen induces the proliferation and differentiation of B lymphocytes resulting in the production of plasma cells, all producing and secreting antibodies with the same antigen specifity as the original B cell.

Besides their essential function for the defense of the organism, antibodies are very important tools in immunological research through their ability to react specifically with foreign material. Immunization of animals with purified antigen will induce a strong humoral response, which results in an antiserum usually containing high amounts of specific antibodies. Such specific antisera have been applied in many detection techniques and are the basis of a wide variety of radio immuno assays (RIA) and enzyme linked immuno sorbent assay (ELISA) systems. However, there are also some disadvantages connected with the production of such antisera. The concentration of the antibodies will fluctuate in time, since the life span of the plasma cell is relatively short (2-3 weeks). Another complication is that of non-specificity. It is almost impossible to isolate pure antigen for antibody induction. Moreover, most antigenic sources contain a wide variety of determinants, each capable of activating its specific B cell. Even one particular determinant is capable to induce several clones of B cells. At first highly specific B cells will be switched on but especially after repeated immunization also B cells with a lower degree of specificity will be activated. The net result of an immunization is therefore the activation of many B cells, producing antibodies with a broad spectrum of specificities. Within this heterogeneous pool, various antibodies against the desired antigen are present, but also antibodies against other determinants. In order to obtain a specific antiserum extensive purification is required for the removal of the non-specific antibodies. For this purpose one needs the availabilty of these particular antigens. A variety of biochemical techniques is used to purify the required antigen.

The disadvantages of the development of conventional antisera motivated many investigators to search for new methods for the production

of specific antisera. The solution came from Köhler and Milstein (1975) by means of the hybridoma technique. They fused antibody-producing cells with mouse tumour cells. The hybrid cells continued to produce the antibody of the first fusion partner, but also inherited the property of continuous growth of the tumour cells. By cell cloning procedures they managed to select the progeny of a single cell, thereby, by definition, establishing a monospecific antibody producing line.

The hybridoma technique as such is based on the phenomenon of somatic cell fusion. The frequency of the fusion event can be increased by reagents like polyethyleneglycol (PEG), which induce alterations of the cell membrane. After fusion of the membranes also the cytoplasm and eventually the nuclei fuse. Fusion is a rare event even in the presence of PEG. Therefore the hybrid cells must be separated from the parental cells. A very elegant strategy has been devised by Littlefield (1964). The principle of this approach is that the main biosynthetic route of the pyrimidines and purines can be blocked by aminopterine (a folic acid antagonist). In normal cells DNA still can be synthesized via alternative pathways, provided that thymidine and hypoxanthine are available. Thymidine is processed by the enzym thymidine kinase (TK), whereas hypoxanthine requires the enzyme guanine phosphoribosyl transferase (HGPRT). Myeloma cells, deficient for one of these enzymes, can be obtained by growing cells in the presence of either 8-azaguanine or 5-bromodeoxyuridine. The cells, which are resistant to these drugs, fail to produce the enzym HGPRT or TK respectively . When these cells are grown in medium containing aminopterin, supplemented with thymidine and hypoxanthine (HAT-medium), they will die. In contrast, hybrids which received the ability to produce either TK or HGPRT from the the other fusion partner will survive in HAT-medium.

The hybridoma technique has distinct advantages over the conventional approach. One is obviously the immortalization of a single plasma cell. Like tumour cells, they can be kept in culture for an indefinite period of time. In this way a continuous source of antibody with a single specificity is available. Another advantage is the possibility to dissect complex biological systems without the necessity of having purified antigens for immunization or adsorption. Starting with a rather impure antigen for immunization, the fusion will produce hybrid cells against a wide variety of antigenic determinants. By subcloning, i.e. the production of clones of cells which are derived from a single

cell, the desired hybrid cells can be isolated from this pool of clones. These hybrid clones produce monospecific antibodies and, by screening the culture supernatants, it is possible to select one particular clone. This property of the hybridoma technique has made it an invaluable tool for the production of monoclonal antibodies (MCA) against polymorphic antigenic systems, such as antigens encoded for by the major histocompatibility complex (MHC) and surface antigens of B and T lymphocyte subpopulations. It is hardly possible to obtain such monospecific antibodies using the conventional approach. For the experiments described in this thesis recognition of various cell types constituing the immune system of the rat was required. The obvious choice for the development of these specific antibodies was the hybridoma technique. This chapter will describe and discuss the adaptation and optimization of this technique for the production of MCA against cellular antigens of rats. The hybridoma technique consists of two essential parts. The first one is the actual fusion in order to obtain the hybrid cells. The second part starts immediately after plating of the hybrid cells. This part depends on the availability of a fast and reliable technique for selecting the correct hybrid cells. In the first section the fusion technique will be discussed. In the second part several screening techniques will be compared and discussed.

FUSION

Since we wanted to produce monoclonal antibodies against rat cells, mice were immunized and mouse myeloma lines were used for hybridization. In all experiments, spleen cells of immunized BALB/c mice were used for fusion. In the fusion experiments rat cells, such as thymocytes or lymphoid tumour cells were used for immunization. Mice were primed intraperitoneally with 10^7 cells in phosphate buffered saline (pH 7.2). Four weeks later these animals were intravenously boostered with 5×10^7 cells. Four days later the spleen of the mice were isolated and single cell suspensions were prepared for the actual fusion. No adjuvants were required in the immunization procedure of these experiments. When raising MCA against antigens with low immunogenic properties or against soluble antigens, the application of adjuvants might be considered.

The hybridoma technique was performed according to the method of Fazekas de St.Groth and Scheidegger (1980). This technique was modified

for application in the rat system: single cell suspensions of spleens of immunized mice were prepared as described in detail previously (Rozing et al, 1977). Spleen cells (2×10^7) were fused with 5×10^7 SP2/0 myeloma cells (SP2/0-Ag14; Shulman et al,1978). The myeloma cells have to be grown half confluently and are brought in suspension at the time of fusion. Spleen cells and myeloma cells were mixed and washed in medium without serum. The cells were carefully resuspended in 1 ml PEG solution, which consists of 0.5 g PEG (PEG 4000 für die Gas-chromatographie, Merck, BRD) supplemented with 0.05 ml dimethylsulfoxid (DMSO) dissolved in 0.5 ml aquadest. This mixture is kept at 37°C for approximately 2 min followed by gradual dilution in buffered medium (without serum). The fused cells were plated in 24 well culture trays in a density of 10^4 spleen cells/well. Because the spleen cells are the limiting factor for the number of hybridoma cells, that can be formed the cellular input is expressed as the number of spleen cells of the fusion mixture. These wells contained feeder cells, i.e. Balb/c macrophages (2.5×10^4 cells/well), which were harvested from the peritoneal cavity and plated 24 hours before the fusion. The cells were cultured at 37°C in humidified air (5% CO_2) in RPMI-1640 medium (Seromed, BRD) containing 15% fetal calf serum (FCS; Batch nr. 100.378; Hyclone, USA). The FCS batch was selected for growth sustaining properties for hybrid cells in test fusion experiments. This culture medium contained HAT (Hypoxanthine, 10^{-4}M; Thymidine, 1.6×10^{-5}M; Aminopterin, 4×10^{-7}) for selecting the hybrids.

Myeloma cells lack the enzyme for the alternative pathway for DNA synthesis, whereas the spleen cells have only a limited lifespan. The hybrid cells with the enzyme producing capacity of the spleen cell and the immortality inherited from the myeloma cell will survive in this selective medium. After two weeks the HAT-medium can be removed gradually from the culture medium. At the same time the first growing hybrid clones can be detected with the microscope. Approximately one week later culture supernatants can be harvested for the primary screening of antibody production. Selection of hybrid clones should be done at an early stage in order to avoid maintenance of uncounted non-specific clones.

Plating large numbers of cells per well has been the approach in the original protocols (Goding, 1980). However, a high number of wells will contain more than one hybrid and overgrowth by non-producers will occur in many cases. For this reason we plated the fused cell in limiting numbers. A disadvantage of this approach, however, is that the rather

unstable hybrids are very intolerant to dilution. They seem to need the presence of other cells for growth. The application of feeder cells prevents the negative dilution effects. Reduction of the toxicity of the plastic culture trays has been suggested by Goding (1980), but the exact function of the feeder cells is still unknown. Using selected batches of FCS (data not shown) and feeder cells in the medium during the first weeks of culture, highly improves the yield of hybridomas, when the cells are plated in low numbers (Fazekas de St.Groth and Scheidegger, 1980). Plating of low numbers of cells (10^4 cells/well), as used in our protocol, normally results in approximately 70 percent positive wells. De Blas et al (1981) investigated the correlation between the percentage of wells with growth and the degree of monoclonality. The estimation of the number of clones in a single well was based on the Poisson probability model. According to this correlation, approximately 60 percent of the wells with growing hybrid cells (70% of all wells) contain clones derived from a single hybrid cell. This is in agreement with the high degree of monoclonality of the hybrid clones we obtained in a series of experiments.

The selected hybrid clones were grown to larger quantities and stored in liquid nitrogen. Hybridomas in this early period of culture can be lost for two major reasons. First of all they will lose a number of chromosomes with the possibility of losing either the capacity of antibody production or the growth property of the myeloma. The second problem is that part of the wells will contain more than one hybrid clone. In the case of mixed clones, due to the growth of more than one hybrid cell in one well, subcloning will be necessary in an early phase. Subcloning is done by plating hybrid cells in a low concentration (0.3 cells/well) in microtiter plates. These cells must be grown in the presence of feeder cells, as was described for growth of fused cells. Monoclonality of clones can be tested with isoelectricfocussing (IEF) as described by Nicolotti et al (1980). Culture supernatant of the parent clone, containing the produced antibodies is harvested and compared with supernatants of several subclones. These antibodies give a characteristic IEF pattern, which is fully identical if the parental hybrid cells were derived from a single cell and only partly identical if the parent cells were derived from more than one cells. In figure 2.2 culture supernatants of several parent clones (lanes A-G) are shown together with one subclone (lanes A-G).

For the further characterization of the antibodies and other experiments large quantities of MCA were required. For this purpose Balb/c
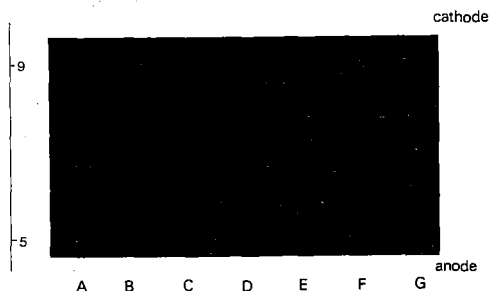
Figure 2.2 Screening of culture supernatants of seven hydridoma's
using agarose-IEF. Supernatants of parent clones (left
sides of lanes A-G) were tested together with
supernatants of a subclone (right side of lanes A-G).

female mice (15-30 weeks of age) were intraperitoneally injected with 3×10^6
hybrid cells. Tumour growth should be evident after 2-4 weeks and up to 10
ml of ascites fluid can be harvested from one mouse. The levels of
antibody in these ascites may range from 5 to 25 mg/ml. Occasionally some
hybrids fail to produce ascites and ascitis production may be favoured by
injecting 0.5 ml pristane (2,6;10,14-tetramethyl pentadecane) one week
prior to the injection of hybrid cells.

SELECTION OF THE HYBRIDS

One of the limitations of the hybridoma technique is the problem to
detect the hybrid clone which produces antibodies with the desired
specificity. The source of antigen used for immunization, but also for the
selection of the antibody produced by the hybrid cells is rather impure.
For this reason such a screening technique has to be highly sensitive for
the detection of small amounts of antibodies. It was the intention to
produce monoclonal antibodies against T cell subpopulations, therefore the
screening technique must also be able to detect antibodies, that recognize
only a small cell population of the cells used for the primary selection.
A fast automated system is required for screening large numbers of
supernatants for reactivity. Such a technique enables a primary selection
of hybrid clones in an early stage. This is important because growing,

expansion and cloning of hybrids are time and material consuming. For this purpose three techniques are compared for the specific use in the hybridoma technique.

Screening of large numbers of culture supernatants using either a radio-immuno-assay (RIA) or an enzyme-linked-immuno-sorbent-assay (ELISA) was made possible by coating 96-wells microtitration plates with target cells to (Brown et al, 1979). An elegant alternative is the analysis of the reactivity using a fluorescence technique. In the latter case a fluorescence activated cell sorter (FACS) is used. All three techniques were tested for the ability to detect a low concentration of antibody, but also to detect a small number of cells recognized by specific antibody.

For the RIA and ELISA techniques target cells were coated to wells of vinyl microtitration plates, as described by Stocker and Heusser (1978). Single cell suspensions were prepared of thymus of Lewis rats and Balb/c mice. All procedures i.e. washing and incubation were done in phosphate buffered saline (PBS,pH 7.2), supplemented with 1% bovine serum albumin (BSA) and 0.1% natriumazide unless stated otherwise. Target cells were adjusted to a concentration of 5.10^7 cells/ml in BSA free medium and subsequently injected into the wells (50 μl). The cells were spinned to the bottom and coated to the wells by adding 0.25% glutaraldehyde during 5 min. at 0 °C. This fixation reagent was removed by washing and the wells were subsequently filled with 150 μl PBS and stored at 4 °C until further use.

For all screening techniques affinity column purified rabbit-anti-mouse Ig (RAM-Ig) was used and subsequently (Fab)2 fragments were made by pepsin digestion. Affinity chromatography was performed with Sepharose-CL-4B coated with mouse Ig for positive selection of RAM-Ig and with rat Ig for removal of antibodies directed against the rat. This RAM-Ig(Fab)2 preparation was used for conjugation with enzyme, radiolabel and fluorochrome, respectively as described in the next sections. Purification of RAM-Ig is necessary because there is a high degree of conformity between mouse Ig and rat Ig, which will result in many cross reactive antibodies in the sera.

For RIA, RAM-Ig(Fab)2 was labelled with ^{125}I. For this conjugation the jodogen method was used (Markwell and Fox, 1978). The microtiter plates with the coated target cells were washed three times and subsequently filled with the unlabelled mouse antibody. After incubation for 1 hour at room temperature the wells were thoroughly washed and

incubated with the ^{125}I-RAM-Ig in an appropriate dilution. After this second incubation, which was performed under the same conditions, the unbound tracer was removed by washing. The single wells were isolated by cutting them from the plates with a hot wire cutter and the activity of the bound antibody was counted in the τ-counter.

For ELISA, RAM-Ig(Fab)2 was conjugated with the enzyme β-galactosidase in the presence of the coupling reagent m-maleimido-benzoyl-N-hydroxy-succimide ester according to the method of O'Sullivan et al (1978). Isolation of this conjugate was established by gel filtration on Sepharose-4B. The procedure of the first step is identical to the one used in the RIA. In the second incubation the β-galactosidase conjugate is added in optimal concentration and incubated for one hour at room temperature. The unbound conjugate is removed by washing and the quantity of conjugate bound to the coated target cells is determined by the reactivity towards the substrate 0-nitrophenyl-D-galacto pyranoside. This reaction is stopped with Na_2CO_3 (1M) and the concentration of the yellow reaction product was measured at 405 nm in a Titertek Multiscan.

For fluorescence staining, RAM-Ig (Fab)$_2$ was conjugated with fluoresceine isothiocyanate (FITC) antibody, dialysed against 0.1M carbonate buffer (pH 9.2).This was done by mixing RAM-Ig(Fab)$_2$ with FITC (1mg/ml DMSO) and incubating it for 2 hours at room temperature. The reaction was stopped by dialysis against PBS (pH 7.2), whereafter a fluorochrome/ protein ratio of 1.8 was found. Single cell suspensions of the thymus were prepared as described by Rozing et al (1977). The cells were washed and suspended in RPMI 1640 medium, supplemented with 1% BSA and 0.1% natrium azide (RPMI-BSA). After centrifugation of the cells, samples of approximately 10^6 cells were incubated for 30 min at 0°C, first with test antibody and subsequently with FITC-RAM-Ig(Fab)$_2$ at optimal concentration. The cells were washed with RPMI-BSA and resuspended in 1 ml. After staining the samples were analysed on a FACS II (Becton & Dickinson), equipped with an argon laser (Spectra Physics) at 488 nm. Forward light scatter windows were set to exclude erythrocytes and dead cells. Analysis of scatter and fluorescence signals was done with a Data ND-100 pulse height analyser. Cells were stained with a range of antibody dilutions and the mean fluorescence intensity of each dilution was determined.

The sensitivity to detect a low amount of antibody has been determined with a defined monoclonal antibody against Thy1.1. This mouse
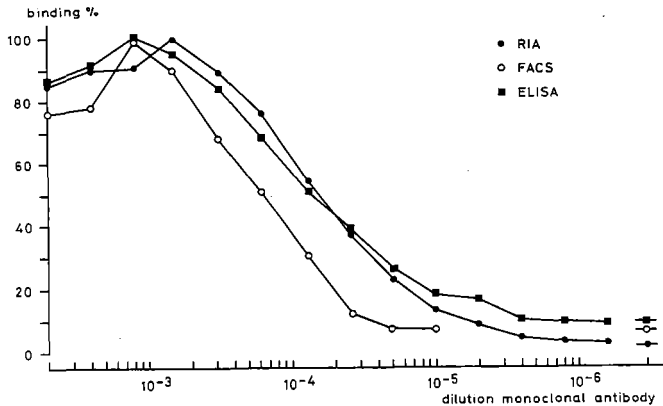
Figure 2.3 Dilution analysis of monoclonal antibody. Binding of various concentrations of MRC OX-7 (10 mg/ml) to rat thymocytes was tested in FACS, RIA and ELISA. The results are expressed as percentage of the highest response. The mean value of three indepent assays are shown, the standard error of the mean (SEM) did not exceed a value of 10%.

monoclonal anti-Thy-1..1 antibody (MRC OX-7; Mason and Williams, 1980) was purchased from Seralab, U.K. and is directed against rat Thy-1 and mouse Thy-1.1. It recognizes about 95% of the lymphocytes in the rat thymus. To test the sensitivity level of the various systems a dilution analysis of the anti-Thy-1.1 antibody (10 mg/ml) binding with rat thymocytes as target cells was done. For FACS analysis the mean fluorescence intensity per cell has been determined for the various dilutions, whereas for RIA and ELISA analysis, counts per minute (cpm) and extinction were measured,respectively. The results have been expressed as percentages, relative to the highest response, which was considered to be 100 percent (Fig. 2.3). Both RIA and ELISA systems proved to be superior to FACS analysis, since there was a 10-20 fold difference in sensitivity between the first two techniques compared to the FACS analysis. The detection limit is considered as the minimum concentration of antibody that can be distinguished from the background i.e. the background value plus twice the SEM of the back ground value. The detections limits as were determined in these experiments were 5 μg/ml for the FACS, 0.3 μg/ml for the RIA and 0.6 μg/ml for the ELISA, respectively. This showed that the RIA is slightly more sensitive than an ELISA. All three screening systems had sufficient,
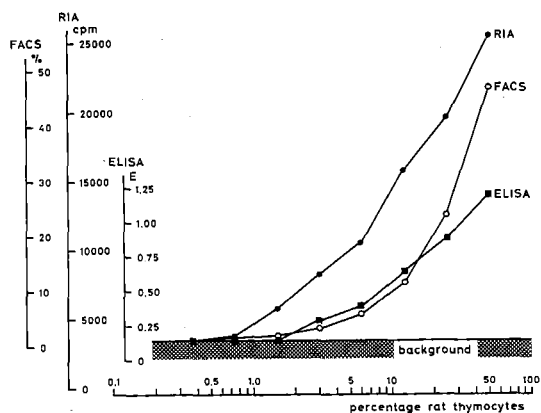
Figure 2.4 Comparison of the cellular sensitivity of RIA, ELISA and FACS analysis.
Binding of MRC OX-7 antibody to thymocytes of rat (LEW) and mouse (Balb/c)
in various combinations was tested. The background values of the
respective techniques were adjusted to the same level for a proper
comparison of the sensitivity.

though sometimes marginal (FACS), sensitivity to cover the normal observed
range of monoclonal antibody concentrations in culture supernatants (1-
10μg; personal observation).

For the sensitivity to detect a low percentage of positive cells
within a cell population thymocytes of Lewis rats (Olac, Bicester,UK) were
mixed with thymocytes of Balb/c mice. This technique can be used because
the anti-Thy-1 antibody is directed against the Thy1.1 allele, which is
present on rat thymocytes, whereas the Balb/c cells express the Thy1.2
allele. The cell mixtures contained the following percentages of rat
cells: 100, 50, 25, 12.5, 6.2, 3.1, 1.6, 0.8 and 0. Thus obtained mixtures
were coated on wells of microtitration plates as described before and
screened with monoclonal anti-Thy1.1 antibody in optimal concentration.
Subsequently these mixtures were incubated with the respective labelled
antibodies and analysed. For comparison the scales of the various
techniques were adjusted in figure 2.4, in order to fix the respective
background values at the same level. Data are expressed as cpm for RIA,
extinction for ELISA and percentage of cells positive for a given
fluorescence intensity in FACS analysis. From these data it appears that
both FACS and RIA have sufficient sensitivity to measure small percentages
(1.5%) of positive cells. ELISA is somewhat less sensitive, resulting in a

lower limit of 3% in these experiments. Furthermore, since the FACS also provides the percentage of positive cells, this way of analysis, in contrast to RIA and ELISA, discriminates between binding of the monoclonal antibodies to the entire population and binding to a (minor) subpopulation.

For detection of low concentrations of antibodies the RIA displays the highest sensitivity (see also Fig. 2.5), whereas the ELISA almost approaches the same degree of sensitivity. For the detection of minor populations of positive cells both the RIA and the FACS are highly sensitive. The advantage of analysis with the FACS is the possibility to discriminate between recognition of determinants on all cells or determinants only present on a minority of the population. For the RIA an ELISA such a discrimination is impossible. When similar experiments are performed using a marker, which is present at a low density (W3/13, approximately 3.8×10^4 molecules/cell) on the cell membrane (Williams et al, 1978), FACS proved to have a higher discriminating ability than RIA. On the other hand the sensitivity to detect low amounts of such an antibody is significantly lower than for the Thy-1.1 antibody (data not shown). Staining with low amounts of antibody will correlate with a decrease of fluorescence intensity. The intensity of positive cells with a low antigen density may approach the intensity of the negative population. Screening of culture supernatant with the FACS will therefore include the risk of missing several hybrids that produce specific antibody. Also the handwork
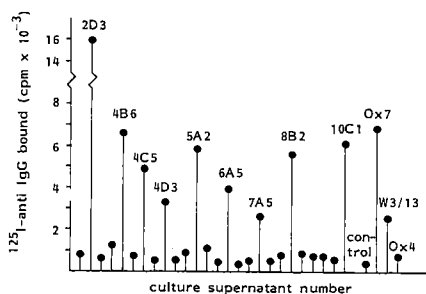

Figure 2.5 Primary screening (RIA) of culture supernatants of 30 hybrids for specific binding on rat thymus cells. The hybrids were selected for specific Ig production. MCA MRC OX-7 (Thy-1), W3/13 (T cells) and MRC OX-4 (B cells) were used as reference antibodies (Reference MCA are shown in Table 3.3).

involved in this technique is not suited for the screening of large numbers of samples. Both RIA an ELISA are highly sensitive techniques quite capable to detect specific antibodies within the normal concentration range found in culture supernatants. Moreover both techniques are highly suited for screening on a large scale. Therefore ELISA and RIA are the techniques of preference for primary screening in the hybridoma technique. The choice between these techniques will depend on the practical circumstances. RIA is the most sensitive and should be the technique of choice, when surface antigens on small percentages of target cells are be expected. ELISA is even faster than RIA and especially when large numbers of samples have to be screened, this is an important advantage. Although radioactivity is not involved, the substrate of β-galactoside is highly toxic. After the primary selection, analysis with FACS, using culture supernatants of further grown hybrid clones, will provide valuable information about the cellular distribution.

Fusion experiments

The hybridoma technique, with the modifications described in this chapter, is schematically illustrated in figure 2.6. A series of fusion

Balb/c mice
10^7 cells i.v.

SP2/0 fusion line
in vitro

6 weeks

5×10^7 cells
i.v.

4 days

Day 0: 2×10^7 spleen cells   X 5×10^7 SP2/0 cells

Plating in HAT-medium 10^4 cells per well
(15% FCS selected batch)

Day 17:   Primary screening using RIA, ELISA
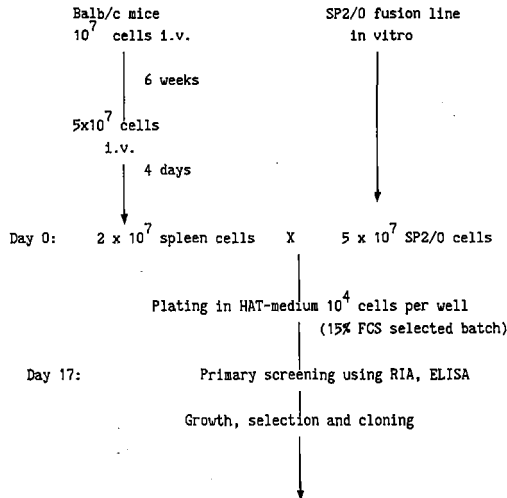
Growth, selection and cloning

Figure 2.6 Hybridoma technique used for the production of mouse anti-rat monoclonal antibodies

experiments were done for the production of monoclonal mouse anti-rat antibodies, which are directed against surface antigens of cells of the T cell lineage. Balb/c mice were immunized with various types of cells of the T cell lineage. After primary screening with RIA or ELISA a total of 230 hybrid clones was selected, that produced antibodies reacting with the target cells (summarized in Table 2.1). In figure 2.5 an example of such a primary screening using a RIA is shown. Positive hybrids were selected, subcloned and ascites was prepared for further experiments. Further selection and characterization of these monoclonal antibodies will be described in more detail in the next chapter.

In order to obtain monoclonal antibodies, directed against surface molecules of distinct T cell subpopulations, a series of hybridization experiments have been carried out. Most of the subpopulation markers described in mice and man are expressed on the majority of the thymocytes. Therefore thymocytes have been used extensively as a source of antigen for immunization. But also lymphoid tumour cells or spleen cells have served as a source of antigen (Table 2.1). Primary screening of growing hybrids in either a RIA or an ELISA was always done on the same cell source as used for immunization (see previous chapter).

Table 2.1 Hybrid clones selected for production of specific antibodies.

Experiment Nr	Clones	Immunizing[a] cells	Rat strain
1	26	Tumour cells	(AO x BN)F1
2	69	Thymocytes	Lewis
3	22	Thymocytes	Lewis
4	4	Thymocytes	Lewis
5	21	Cortison resistent thymocytes	Lewis
6	34	Lymphoid tumour cells	PVG
7	54	Lymphoid tumour cells	PVG

a. Cells used for immunization and primary selection.

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Chapter 3

CHARACTERIZATION OF MOUSE ANTI-RAT ANTIBODIES

PRIMARY CHARACTERIZATION

The 230 hybrids mentioned at the end of the previous chapter
(chapter 2.1), which were selected on the criterium of producing antibodies
with a positive reaction with the target cells used for immunization, were
further defined and characterized.

Immune histology on spleen and thymus was performed to check the anti-T
cell versus the anti-non T cell reactivity of the antibodies produced by
hybrids. Selected anti-T cell hybrids were then tested on cell
suspensions of spleen, thymus and lymph nodes in a flow cytometric
analysis assay (FACS) for the quantitation of their T cell reactivity.
Further insight was finally obtained on the specific reactivity of these
hybrids with defined lymphoid subsets in two colour fluorescence analysis
on lymph node cells.

Based on the data obtained in these experiments a final selection
of MCA was made with a differential recognition pattern within the T cell
population.

Immune histology

A first characterization of the reactivity pattern of the
monoclonal antibodies obtained with mature T lymphocytes as antigen was
established using immunohistochemistry on lymphoid organs as spleen and
thymus. Therefore tissue specimens were snap frozen and stored at -70°C.
Cryostate sections (6-8 μm) were picked up, air-dried, fixed in acetone for
10 minutes at room temperature (RT) and rinsed three times for 10 min. with
phosphate buffered saline pH 7.4 (PBS; 0.01M). The sections were covered
with mouse anti-rat monoclonal antibody and incubated for 60 min. at
roomtemperature (RT). Ascitis fluid containing the relevant MCA was used
at an appropriate dilution in PBS supplemented with 1% (v/v) normal rat
serum (NRS) and 2% (w/v) bovine serum albumin (BSA, Povite, Oss, The
Netherlands). After washing in PBS the incubation was repeated with rabbit

anti-mouse immmunoglobulin conjugated with horseradish peroxidase (Miles, Slough, England). The sections were stained for peroxidase activity using 3-3´ diaminobenzidine-tetrachloride (DAB; Sigma USA) in a concentration of 5 mg/ml in 0.05 M Tris/HCl buffer (pH 7.6), containing 0.01% hydrogen peroxide. A final incubation in 5% (w/v) Cu_2SO_4 solution was added to the procedure for improving the staining intensity.

The spleen is a highly vascularised organ, which can be considered as an encapsulated vascular filter. It filters the blood and can remove damaged blood cells and foreign substances. The spleen can be divided into two different compartments. The red pulp provides the microenvironment for differentiation of erythrocytes, granulocytes and megakaryocytes (reviewed by Tavassoli, 1975). The second compartment, the white pulp is primarily
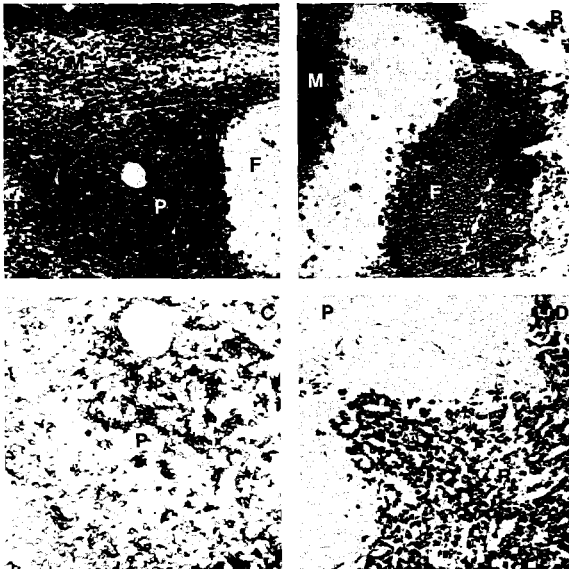

Figure 3.1 Screening of culture supernatants using immune peroxydase histology. Cryostate sections of the spleen were stained with culture supernatants of clone 1-7A6 (A), clone 3-11B2 (B), clone 3-11C6 (C) and clone 5-4B5 (D). Legends: follicles (F), marginal zone (M), red pulp (R) and PALS (P).
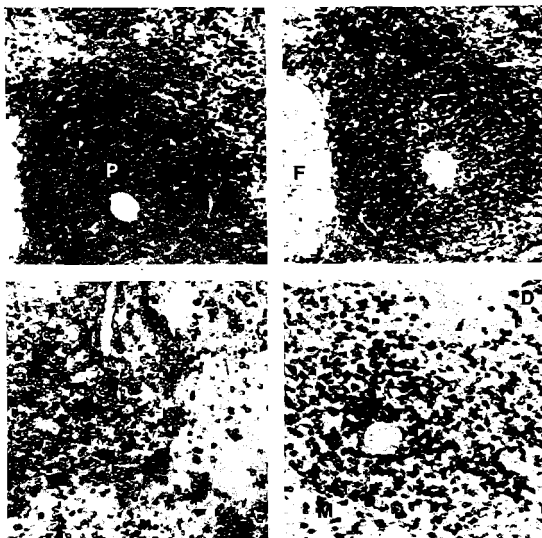
Figure 3.2 Screening of culture supernatants using immune peroxydase histology. Cryostate sections of
spleen were stained with supernatant of clone 1-7A6 (A), clone 5-7A3 (B), clone 5-4A3 (C)
and clone 4-7A6 (D). Legends: follicles (F), marginal zone (M), red pulp (R) and PALS (P).

lymphoid of nature. Three different areas can be distinguished in the
white pulp. In the center an arteriole is surrounded by a layer of
concentric sheats of reticular cells containing large numbers of lymphoid
cells. This is the peripheral arteriolar lymphatic sheath (PALS) where
mainly lymphocytes of the T cell lineage are found. Inserted at several
sites in the periphery of the PALS spherical and ovoid accumulations of
lymphocytes are located, the socalled follicles. The PALS is surrounded by
a loose meshwork of connective tissue, which is refered to as the marginal
zone. The follicles and the marginal zone are predominantly populated by B
cells (Gutman and Weissman, 1972; Weissman et al, 1976; Van Ewijk et al,
1977; Rozing et al, 1978). This distinct localization pattern of B and T
cells in the spleen enabled us to classify the recognition pattern of the
antibodies produced by the selected hybrids as specific for T cells, but
also T and B cells or non-lymphoid cells. Also a first indication on the

quantitative aspects of the reactivity among the cell population recognized was obtained using such an immunohistological procedure. This is illustrated by some examples of reaction patterns in figure 3.1 and figure 3.2.

In figure 3.1 several of the distribution patterns observed in this type of screening are shown. Many of the culture supernatants contained antibodies that were directed against surface antigens predominantly expressed by cells of the PALS area (Fig. 3.1A). Some of the antibodies reacted with cells located within follicles as well as in the marginal zone (Fig. 3.1B), whereas other antibodies appeared to react with cells throughout the white pulp (Fig.3.1C). A remarkably different distribution pattern as well as morphology of recognized cells can be observed in figure 3.1D. This antibody reacted with a non-lymphoid type of cells, mainly located in the red pulp. Most of the tested antibodies predominantly reacted with cells of the PALS and only with small numbers of cells in the follicles and marginal zone. With this type of antibody a variation in labelled cell numbers can be observed. In figure 3.2 four antibodies are shown which react with a diminishing number of cells in this area. From such antibodies that are apparently directed against T lymphocytes several representative antibodies were selected for further characterization. These MCA are listed in Table 3.1, together with the cells used for immunization and the number of the hybrid clone producing this antibody.

Table 3.1 Mouse monoclonal antibodies directed against
rat leukocytes

Antibody[a]	Cells used for immunization	Clone number
ER-1	Lymphoid tumour (AO xBN)F1	1-7A6
ER-2	Lymphoid tumour (PVG)	5-7A3
ER-3	Lymphoid tumour (PVG)	4-7A6
ER-4	Thymocytes (LEW)	2-2D3
ER-7	Lymphoid tumour (PVG)	5-4A3
ER-8	Thymocytes (LEW)	2-10C1
ER-9	Lymphoid tumour (PVG)	4-10B1
ER-10	Thymocytes (LEW)	2-10C1
ER-12	Spleen cells (LEW)	3-11B2
ER-13	Spleen cells (LEW)	3-11C6
ER-14	Lymphoid tumour (AO xBN)F1	1-4D4
ER-17	Thymocytes (LEW)	2-7A5
ER-18	Thymocytes (LEW)	2-4D3
ER-19	Lymphoid tumour (PVG)	4-8C1

a. ER stands for Erasmus University Rotterdam

In immune histochemistry a rough estimation of the number of positive cells for a given MCA can be obtained. Therefore a precise quantitation is still needed. For this purpose the relevant MCA have been analysed on the fluorescence activated cell sorter (FACS).

FACS analysis

The FACS is a highly sensitive tool for the quantification of positive versus negative cells. To do so, all antibodies, both those which were developed by us and those which were applied for reference purposes, were directly conjugated with fluorescein isothiocyanate (FITC) or with tetramethylrhodamine isothiocyanate (TRITC). Therefore antibodies were purified from ascites by ammoniumsulphate (50%) precipitation and DEAE chromatography. Subsequently the antibodies were conjugated under standard conditions as described by Goding (1976) with the appropriate fluorochrome. Single cell suspensions of spleen, lymph nodes and thymus were prepared and stained with FITC conjugated antibodies in a single step incubation as described in the second chapter. In these experiments PVG female rats, 12 weeks of age, were used. The cell suspensions were analysed on a FACS II (Becton Dickinson, USA) equipped with an argon laser (Spectra Physics) at 488 nm in the following setting: laser 200 mW, photomultiplier 560 V, fluorescence gain ranging from 4-16. Forward light scatter windows were
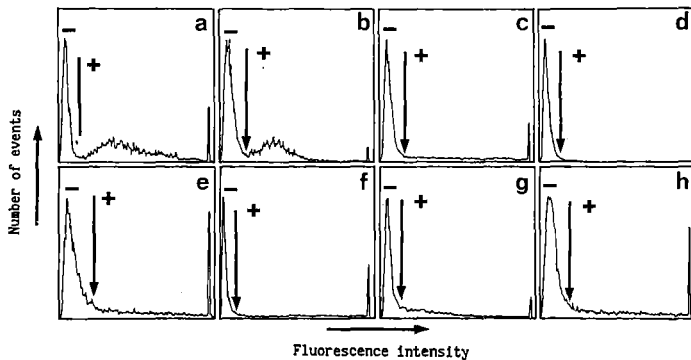

Figure 3.3 Fluorescence profiles of monoclonal antibodies on lymph node cells. On the horizontal axis the fluorescence intensity is shown, whereas the vertical axis represents the number of cells displaying a certain intensity a. ER-1, b.ER-2, c. ER-3, d. control (Leu-4) e. ER-7 f. ER-8 g. ER-9 and ER-10. The arrows indicate the separation between the positive and negative cells

used to exclude erythrocytes and dead cells. Scatter and fluorescence signals were analysed with a Nuclear Data ND-100 pulse height analyser interphased with a PDP-11 computer system (Digital, USA) for data storage and analysis. The fluorescence intensity of the cell is a reflection of the density of the labelled surface molecule. The data of all cells is combined in a fluorescence profile, in which by means of an electronic window the positive cells can be distinguished from the negative cells. The fluorescence profiles of several antibodies on lymph node cells are shown in figure 3.3. A clear distinction between positive and negative cells for the antibodies ER-1 and ER-2 can be detected (Fig. 3.3a and b). This clear distinction between cells which are negative and positive for these antibodies, is caused by the fact that the amount of surface antigens recognized by ER-1 or ER-2 is about equal on all positive cells and is quite different from negative cells. The other antibodies, on the other hand, display a remarkable heterogeneity in their staining profiles. No clear separation of positive and negative cells was possible, because cells were found displaying all grades of fluorescence intensity. Nevertheless, by comparing such profiles with relevant controls (Fig. 3.3d), it is possible to calculate the number of positive cells also for these MCA . By this method the percentage of positive cells has been determined in thymus, spleen and lymph nodes of young adult rats using a panel of MCA , which had

Table 3.2 Flow cytometric analysis of MCA
in various lymphoid organs

Antibody	Percentage[a]		
	Thymus	Spleen	Lymph Node
ER-1	98	49	58
ER-2	90	28	36
ER-3	92	20	21
ER-4	96	1	2
ER-7	94	25	31
ER-9	96	23	26
ER-10	98	25	28

a. Analysis was done with a FACS. For thymus, spleen and lymph nodes the scatter gates were set on the lymphocyte peak and percentage of positive cells is expressed as part of these cells. Mean percentage of positive cells in three separate experiments are shown. In these analyses 12 weeks old PVG male rats were used.

been selected for their T cell reactivity in immune peroxidase histology (Table 3.2).

As shown in Table 3.2 most antibodies recognized a considerable number of cells in the spleen and lymph nodes. The percentages, however, varied greatly. ER-1 for instance labelled 50% of the spleen cells and even more (58%) of lymph node cells. ER-4 on the other hand recognized only thymocytes, but no spleen or lymph node cells. The reactivity of other MCA was in between these two. However all MCA labelled the vast majority of thymocytes.

Two colour fluorescence analysis

The T cell reactivity of the selected MCA as suggested by immune peroxidase histology on spleen sections could be confirmed by double fluorescence analysis using specific B and T cell markers. For this comparison, but also in other experiments of this chapter, the selected antibodies were compared with a panel of reference antibodies which are summarized in Table 3.3. Part of these experiments were done on the FACS.

Table 3.3. Monoclonal antibodies used for reference purposes

Antibody	Target description	Reference
MARK-1	B-lymphocytes	Bazin et al, 1984
W3/13	T-lymphocytes	Williams et al, 1977
W3/25	T helper cells	Williams et al, 1977
MRC OX-4	B lymphocytes (Ia)	McMaster and Williams, 1979
MRC OX-8	T suppr./cytotoxic	Brideau et al, 1980
MRC OX-7	rat Thy-1	Mason and Williams, 1980
MRC OX-19	T-lymphocytes	Dallman et al, 1984

Therefore all antibodies, including reference MCA were labelled with FITC. At first all MCA were compared with a specific B cell marker. For this purpose a MCA that is directed against the kappa chain of the rat Ig (MARK-1), kindly provided by Dr.H.Bazin, Bruxelles, Belgium (Bazin et al, 1983), was used. Lymph node cells of Lewis rats (Lewis/Olac, Bicester, England) were stained with the MCA alone or in combination with MARK-1. When the MCA do not recognize B cells but only T cells, staining of cells with the MCA and MARK-1 at the same time must give a percentage of positive cells that is the complete summation of the figures of the single staining with

the ·MCA and of MARK-1. As shown in Table 3.4 all tested antibodies
demonstrated a complete summation in the double staining situation compared
with the individual figures. This kind of summation therefore confirms
that the tested antibodies do not react with B lymphocytes.

Table 3.4 Double fluorescence analysis of
monoclonal antibodies

Antibody	Percentage[a]	
	MCA	MCA + MARK-1[b]
--	--	41.3
ER-1	55.9	97.5
ER-2	39.8	81.4
ER-3	17.4	57.9
ER-7	29.4	71.3
ER-9	27.0	70.1
ER-10	24.8	64.9

a. Analysis was done with a FACS II. Mesenteric
   lymph node cells (PVG rats; 12 weeks of age)
   were analysed with scatter gates set on the
   lymphocyte peak. Mean percentages of two
   separate experiments are shown.
b. MARK-1 is directed against the kappa chain
   on rat B cells.

Further experiments were done to relate the reactivity pattern
with three recently described mouse anti rat monoclonal anti T cell
antibodies. These MCA (W3/13, W3/25 and MRC OX-8) were kindly provided by
Dr.A.F.Williams, Oxford, England; Table 3.3). In contrast to the preceding
technique in which only one fluorochrome was used, the lymph node cells
were now double stained with FITC and TRITC conjugated antibodies. Two
fluorescence analysis enables a separation of cells according to the
phenotype. Cells may express either one of the surface antigens, defined
by the MCA or they may express both surface antigens. Using this technique
it can be defined whether the T cell subpopulations, defined by the newly
developed MCA , belong to the helper T cell subpopulation or to the
cytotoxic T cell subpopulations as defined by the MCA W3/25 and MRC OX-8.
The preparations were analysed on a Zeiss fluorescence microscope equipped
with an IV/F vertical illuminator and the appropriate filter combination
for red (TRITC-labelled cells) and green (FITC-labelled cells) distinction.
The two colour analysis, listed in Table 3.5, revealed that ER-1 and W3/13
recognize the same cell population. Since the ER-1 antigen was exclusively
expressed by W3/13^{+} cells (Exp. 1), this confirms ·that ER-1 recognizes all

T lymphocytes. W3/25 and MRC OX-8 recognize different cell populations (Exp. 2), although a small number of the the cells expressed both surface antigens. In the following experiments (Expts. 3-7) the antibodies were compared with W3/25 and MRC OX-8 expression. Comparison of ER-2 and W3/25 indicated that these antibodies too stain the same cell population (Exp.3). Not a single cell could be detected that was stained by only one of these two antibodies. Similarly to the W3/25 distribution (Exp.2) also a small number of ER-2$^+$MRC OX-8$^+$ could be detected (Exp.3). ER-3 and ER-10, on the other hand did stain all MRC OX-8$^+$ cells (Exp. 4 and 7), because only ER-3

Table 3.5 Two colour fluorescence analysis of lymph node cells

Exp.			Percentage[a]		
Nr	FITC	TRITC	Green	Red	Red+Green
1	ER-1	--	53.6	--	--
	--	W3/13	--	51.7	--
	ER-1	W3/13	0	0	52.5
2	W3/25	--	35.8	--	--
	MRC OX-8	--	18.9	--	--
	W3/25	MRC OX-8	34.6	19.2	2.1
3	--	ER-2	--	34.2	--
	W3/25	ER-2	0	0	35.9
	MRC OX-8	ER-2	17.1	33.6	1.2
4	--	ER-3	--	20.8	--
	MRC OX-8	ER-3	0	2.6	18.6
	W3/25	ER-3	33.4	18.6	2.5
5	--	ER-7	--	30.8	--
	MRC OX-8	ER-7	11.4	24.2	6.2
	W3/25	ER-7	11.2	5.2	26.4
6	--	ER-9	--	29.4	--
	MRC OX-8	ER-9	9.0	19.4	11.2
	W3/25	ER-9	12.8	9.6	22.5
7	--	ER-10	--	24.4	--
	MRC OX-8	ER-10	0	5.3	19.1
	W3/25	ER-10	28.6	18.6	6.1

a. Antibodies were directly conjugated with FITC and/or TRITC
b. Mesenteric lymph node cells of PVG rats (10 -12 weeks of age) were used. Analyses were done with a Zeiss fluorescence microscope and fluorescent cells are expressed as a percentage of all nucleated cells. Positive cells were green (FITC), red (TRITC) or red/green (TRITC + FITC)

(ER-10) positive cells or ER-3$^+$ (ER-10$^+$) MRC OX-8$^+$ double positive cells were found. Comparing these antibodies with W3/25 antibody showed that a minor subpopulation of the W3/25$^+$ cells were stained by these two antibodies. Finally, ER-7 and ER-9 recognize to a varying degree part of both W3/25$^+$ and the MRC OX-8$^+$ subpopulations (Exp. 5 and 6). Two colour fluorescence analysis of these antibodies with MRC OX-8 revealed that part of the cells expressed only one of the recognized surface antigens. However, also cells with both surface antigens were detected. These results were in agreement with the results with W3/25. Therefore, both the W3/25 and the MRC OX-8 defined subpopulations, are further divided into at least two different populations. The distribution of the described antibodies in the lymph node cells as defined by these experiments is schematically represented in figure 3.4.
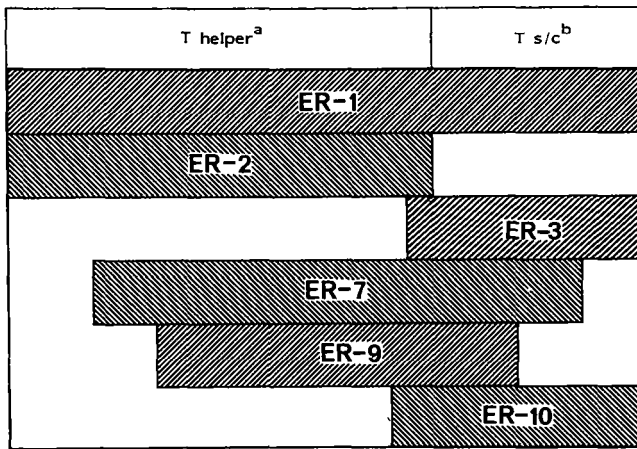

Figure 3.4 Subpopulations of peripheral T lymphocytes defined by the selected monoclonal mouse anti-rat antibodies.
a. T helper cell population defined by W3/25
b. T cytotoxic/suppressor population defined by MRC OX-8

In conclusion a panel of mouse anti rat monoclonal antibodies was obtained, that is directed against surface molecules of cells of the T cell lineage. ER-1 gives a pattern highly similar to that of W3/13. This is true for both the cellular distribution in thymus and peripheral lymphoid organs as well as for the FACS fluorescence profiles (data not shown). The other antibodies react obviously with only a part of the T lymphocytes in

spleen and lymph nodes. ER-2 recognizes (like W3/25) the helper T cell population, whereas ER-3 and ER-10 demonstrated a highly comparable reactivity with MRC-OX-8 in staining the suppressor/cytotoxic subset, though with a small, but significant percentage of the helper subset too. ER-7 and ER-9 are, in combination with existing antibodies most likely candidates for a further distinction of functional T cell subpopulations into smaller subsets.

Subpopulation of T cells in lymphoid follicles

From immunohistological studies it became obvious, that the small population of $ER-2^+ER-3^+$ cells observed in the two colour fluorescence analysis (Table 3.5) has a specific localization pattern. Some MCA reacted not only to cells in the specific T cell areas, but also reacted to a few cells in B cell areas (follicles). Since B cell reactivity of the MCA could be excluded, the presence of cells expressing specific T cell markers in such B cell regions was investigated in more detail. Secondary follicles, which develop upon antigenic stimulation can be distinguished from primary follicles. Secondary follicles consist of a central area (germinal centre) surrounded by a mantle zone (lymphocyte corona). They are important for the generation of (immunological) memory cells (Nieuwenhuis and Opstelten, 1984). From experiments using athymic animals it was clear that T cells are essential for the development of such secondary follicles (Jacobson et al, 1974). The way in which T lymphocytes are involved in the induction and persistence of these germinal centres is obscure. The presence of T cells in germinal centres was also reported in mice by Guttman et al (1972) and in man by Poppema et al (1981). These cells belonged mainly to the T helper population (Poppema et al, 1981; Rouse et al, 1982; Bailey et al, 1982). In the rat the reported data of T cells in the follicles were conflicting (Goldschneider and McGregor, 1973; Barclay, 1981 and Mayrhofer et al, 1983). The availability of several new antibodies directed against subpopulations of T lymphocytes offered an opportunity to define the phenotype of these cells more specifically.

For the primary follicles germfree AO and Spraque Dawley (SD) rats were used. For the secondary follicles AO rats were intraveneously immunized with 10^9 SRBC. Serial sections of spleen tissue were stained with a panel of antibodies and subsequently the bound antibody was determined with the peroxidase technique. The serial sections of germfree
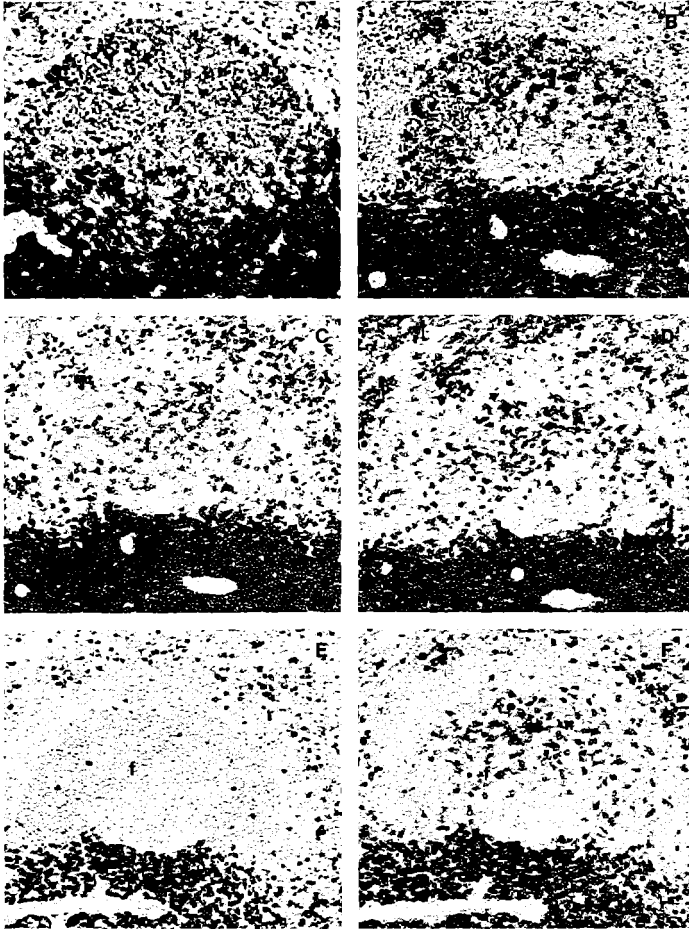
Figure 3.5. Immunoperoxidase histology of serial spleen sections of germfree and immunized rats. The sections of germfree animals were stained with MRC OX-19 (A), whereas the sections of immunized animals were stained with MRC OX-19 (B), ER-1 (C), ER-2 (D), MRC OX-8 (E) and ER-3 (F). f= follicle

rats revealed a low number of putative T lymphocytes within the primary follicles, as defined by antibodies that label all T lymphocytes like MRC OX-19 (Fig. 3.5A). The cells were randomly dispersed throughout the follicles and a part of the T cells belonged to the T helper population (ER-2 positive), whereas a somewhat smaller population was labelled with ER-3 and MRC OX-8 (data not shown). In the secondary follicles an increased number of cells were stained with the "pan T cell" antibodies, as illustrated for MRC OX-19 in figure 3.5A and B. These T cells were mainly found in the corona of the secondary follicles (Fig. 3.5B and C). For ER-2 (Fig.3.5D) the number of positive cells seemed to approach those observed for the pan T cell antibodies (Fig. 3.5B-C). The distribution of the various subpopulations revealed only a low number of MRC OX-8$^+$ cells in the corona of the germinal centres (Fig. 3.5E). Quite surprisingly ER-3 did not follow the expected MRC OX-8 pattern. This antibody (Fig. 3.5F) did recognize cell numbers to the same extent as ER-2 (Fig. 3.5D). Subsequently experiments using double immunohistochemical staining of tissue sections with ER-2/peroxidase and ER-3/alkaline phosphatase conjugates were done (F.Kroese et al, in preparation). Staining of spleen sections showed that the majority of ER-3 positive cells in secondary follicles expressed also the ER-2 antigen. The majority of the follicular T cells thus seem to be a subset of the T-helper population characterized by the presence of the ER-3 defined surface antigen. Whether this subpopulation of ER-2$^+$ ER-3$^+$ cells is unique for this area or is also present in the PALS still has to be studied. Also in the human the follicular T lymphocytes are a special subset of the T-helper subpopulation (Poppema et al, 1983; Porwit-Ksiazek et al, 1983). The Leu-7 determinant, normally present on natural killer (NK) cells is also expressed on the majority of T cells with a helper phenotype in the follicles. In this context, it should be emphasized that also the ER-3 defined antigen is expressed on NK cells of the rat (Reynolds, unpublished results). In addition to T cell regulation, recently NK cell regulation of B-lymphocyte responses were described by Brieve et al (1984). This unique subset of the helper T cells will be isolated for experiments to determine their role in the germinal centre activity.

From these studies it is obvious that several MCA of the selected panel, alone or in combination, enable a further specification of the T cell system, depending on site, differentiation stage or state of activation of the cells expressing such markers. This should be kept in

mind when evaluating the data obtained with such MCA in a highly aroused immune system due to a major antigenic challenge such as an allograft. In this respect also further insight in the actual structures recognized by the various MCA on the cell surface, and the possible role of these molecules in T cell functions is needed. Experiments performed to address these questions are described in the next section.

FURTHER CHARACTERIZATION

The primary characterization of the selected MCA described so far has been carried out on tissues from normal rats. In these tissues the immune system is in a homeostatic balance. After organ- or tissue transplantation the situation may change dramatically due to the severely increased antigenic pressure. Changes in cellular composition and phenotypical expression will probably occur, as compared to the normal resting situation. In order to interprete such changes properly one should have insight in the overall reactivity pattern of the relevant MCA . Therefore the selected panel of MCA was further analysed and characterized.

First the reactivity of the panel was established with T cells at early stages of their **differentiation** and **maturation process**. Furthermore information is needed on the target antigen not only on the cellular level, but also at the receptor level. Therefore the **cell surface antigens** recognized by several of these MCA were determined and their role in a variety of **T cell functions** established. Finally, also the **physical properties** of the MCA were investigated and described in the last part of this chapter.

Differentiation and maturation

Most if not all of the characteristics of the selected panel of MCA described so far were obtained using mature T cells in peripheral lymphoid organs and thymocytes. Since the surface markers recognized by these MCA may be considered as differentiation antigens, i.e. they discriminate between T cells and other cells, as well as within the T cell population, it was of interest to study the expression of these molecules also during the process of early prothymic T cell development. Therefore we looked at the reactivity of these antibodies in the bone marrow.

Bone marrow of PVG rats was collected by flushing the femora and tibia with RPMI-1640 supplemented by 0.1% BSA. Single cell suspensions were stained with FITC-conjugated antibody and analysed on a FACS II. Surprisingly most of the subpopulation markers, defined by these MCA reacted with a considerable number of cells in the bone marrow (Table 3.6). This was not expected since most T-cell markers in mice (Reinherz et al, 1980) and man (Ledbetter and Herzenberg, 1979), but also in the rat (Mason et al, 1983), are first expressed on cells in the thymus. Only ER-2 is almost negative in this compartment, which is a further similarity between this antibody and the reaction pattern described for W3/25 (Williams et al, 1977). For the other antibodies the reactivity ranged between about 65 percent (ER-1 and ER-10) and 22 percent for ER-4. Most antibodies reacted predominantly with the large cell fraction. ER-9 and ER-14 on the other hand, were found to be an exception, since they labelled an equal percentage of large and small cells. ER-4, labelled only cells of the small fraction. These cells have a lymphoid nature and possibly also the pro-thymocytes are included within this population. ER-3 and ER-10 did not react with a the same number of cells in the bone marrow, which is a further confirmation that these antibodies are directed against different surface molecules, as suggested before. The cell numbers recognized by

Table 3.6. Percentage of bone marrow cells
stained by mouse anti-rat antibodies

Antibody	Percentage[a]		
	small cells	large cells	all cells
ER-1	15	47	63
ER-2	--	--	3
ER-3	12	35	46
ER-4	21	2	22
ER-7	11	32	45
ER-9	25	31	58
ER-10	19	46	65
ER-14	15	15	31

a. Bone marrow was analysed with FACS using different scatter windows for total, small and large fractions. Percentages of the separate populations were expressed as part of all bone marrow cells. Mean percentage of three separate experiments is shown, in which 12-14 week old PVG male rats were used.

these antibodies in the bone marrow indicate that these surface antigens are also expressed by cells of other hematopoietic lineages. A further analysis of the reaction of these antibodies with non-lymphoid cells is in progress.

Pro-thymocyte differentiation

The precursor of the thymocytes, the prothymocyte, is a specialized cell, committed to the T-cell differentiation line and discrete from both the precursors of B-cells and the pluripotent stem cell (HSC) (Abrahamson et al, 1977; Jones-Villeneuve et al, 1980). However, based upon morphological and physical criteria such as buoyant density, sedimentation velocity and surface charge, prothymocytes cannot be distinguished from HSC and pre-B cells. For the rat it has been reported that the 25 kD Thy-1 molecule (Greiner et al, 1982) and the 95 kD W3/13 molecule (Dyer and Hunt, 1981) are present on prothymocytes. Both molecules, however, can also be detected on the HSC (Dyer and Hunt, 1981; Goldschneider et al 1978), pre-B cells and plasma cells (Ritter et al, 1978; Williams et al, 1977; Vaessen et al, 1985), some myeloid cell types and their precursors (Dyer and Hunt, 1981; Ritter et al, 1978). The W3/13 molecule is also present on all mature T cells (Goldschneider et al, 1978).

Although studies with these markers gave a great deal of information about the early phase of lymphoid differentiation, in rats more specific markers are still needed. The reactivity pattern of ER-4 in the bone marrow and the peripheral lymphoid organs suggested that this antibody could be such a marker. This reactivity pattern is summarized in Table

Table 3.7 Percentage of positive cells in several lymphoid tissues

Antibody	Bone marrow		Spleen		Lymph node	
	juv.[a]	adult[b]	juv.	adult	juv.	adult
ER-4	35	20	28	1	18	1
MRC OX-7	35	31	25	3	16	4
MRC OX-19	2	3	23	36	44	53

a. Juvenile: 4 weeks old rats
b. Adult: 12-20 week old rats

3.7. Cells of bone marrow, spleen and lymph nodes of adult (12-20 weeks of age) and young (4 weeks of age) Lewis rats (LEW/Ola, Bicester, England) were stained and analyzed with a FACS. MRC OX-7, as a marker for early cells defined by Thy-1 expression, and MRC OX-19, as a marker for mature T cells (Dallman et al, 1984) were used as reference antibodies.

All three antibodies recognized virtually all thymocytes in juvenile (data not shown) and adult rats (Table 3.2). The reactivity of ER-4 and MRC OX-7 was highly similar in the peripheral lymphoid organs at both ages; hardly any cells were labelled in adult animals whereas about 25% of the cells in young animals were recognized. While both antibodies recognized equal numbers of bone marrow cells (35%) in juvenile animals, ER-4 labelled a consistently lower percentage in the adult situation. Since in adult animals ER-4 did not react with mature T lymphocytes (Table 2.3), we analysed in the juvenile animals the relation between MRC OX-19 and ER-4 positive cells.

Table 3.8 Double analysis with monoclonal antibodies

Antibody	Percentage[a]	
	Bone marrow[b]	Spleen[c]
ER-4	21	28
MRC OX-7	31	25
MRC OX-19	2	23
ER-4 + MRC OX-7	39	nd
ER-4 + MRC OX-19	22	52

a. Percentage of all viable cells analyzed on a FACS II
b. Bone marrow from adult rats
c. Spleen cells from 4 week old rats
nd. not done

In spleen cells of young rats ER-4 and MRC OX-19 were expressed on different cell populations (Table 3.8), since the double staining percentage is a precise summation of the individual figures. Furthermore it is obvious from these data, that in bone marrow of adult rats there is a considerable overlap between the ER-4 and MRC OX-7 positive cells. Since staining with ER-4 and MRC OX-7 together did not give 52 percent positive cells (21% + 31%), but 39 percent, about 13 percent of these cells expressed the ER-4$^+$MRC OX-7$^+$ phenotype.

The reactivity of MRC OX-7 and ER-4 in young animals urged us to study the fluctuation of the distribution pattern of these antibodies

during ontogeny. From day 14 of gestation up to twenty weeks after birth
at several time points spleen and bone marrow suspensions were prepared and
analyzed. Only a low percentage (Fig. 3.6) of ER-4 or MRC OX-7 positive
cells were present in both organs at the time of birth. A fast increase of
the positive cells resulted in about 35 percent of ER-4$^+$ and MRC OX-7$^+$
cells in the spleen at three weeks after birth and in bone marrow (BM) one
week later. From this time point onwards the cells, binding to these
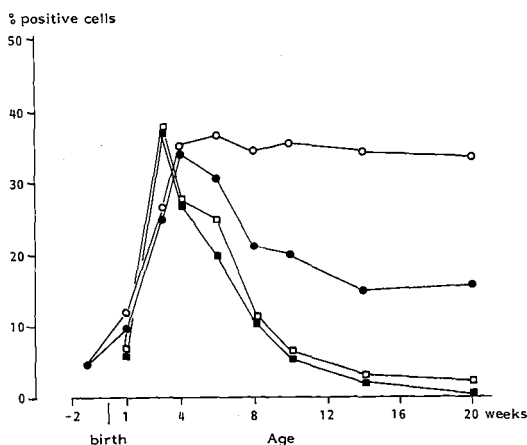markers, gradually disappeared from the spleen. In the bone marrow the

Figure 3.6 Reactivity of MCA ER-4 and MRC OX-7 in bone marrow
and pleen during ontogeny. The percentage ER-4 (●) and
MRC OX-7 (○) positive cells in bone marrow are shown.
Furthermore the ER-4 (■) and MRC OX-7 (□) positive
cells in the spleen are given. All values represent
the percentage of all viable cells

reactivity of MRC OX-7 remained at the same level, whereas ER-4 positive
cells diminished between 6 to 16 weeks after birth and remained present at
a level of about 15 percent for at least one year. BM cells of 7 weeks old
rats were stained with FITC-conjugated ER-4 antibody and sorted on FACS II
into an ER-4 positive and an ER-4 negative cell population. After sorting
the ER-4 positive bone marrow fraction had a purity of 95% and a viability
of 80%. The ER-4 negative fraction had a similar purity and a viability of
75 percent. From both populations cytocentrifuge preparations were made
for nuclear terminal deoxynucleotidyl tranferase (TdT) and cIg staining, as
well as for May Grünwald-Giemsa staining for morphological examination.
The ER-4 positive cell population consisted predominantly of small to

medium sized cells, with a lymphoid morphology. In this population only a few eosinophylic cells were observed. In the ER-4 negative cell population all cells from the erythroid and myeloid series were present. TdT staining was carried out with a test kit from Bethesda Research Lab. Inc. under standard conditions. In the second step TRITC-conjugated goat anti-rabbit Ig was used. In the ER-4 positive fraction approximately 5-10% of the cells were TdT positive and also part of the cells were cIg positive.

Barton et al (1980) described a lactate dehydrogenase (LDH) isoenzyme profile which is specific for rat thymocytes. Therefore the ER-4 sorted cell fractions were subsequently tested for the LDH pattern. The determination of the profiles was kindly done by Dr. J.Plum, State University, Gent, Belgium, according to the method described by Plum and De Smet (1980). In figure 3.7 the various profiles are shown. The most striking result was the absence of the LDH-5´ band in the ER-4 positive cells, representing a comparable profile as described by Barton et al (1980) for the prothymocytes. Both thymocytes and these ER-4^{+} bone marrow

Figure 3.7 LDH-isoenzyme profiles of bone marrow cells from 12 week old RP rats. In lane 1: FACS sorted ER-4 positive cells, lane 2: FACS sorted ER-4 negative cells, lane 3: unsorted stained cells and in lane 4: unsorted, unstained cells. From left to right hand the isoenzyme bands LDH-5, LDH-5´, LDH-4, LDH-3 and LDH-2 can be seen

cells lacked this isoenzyme. The ER-4 negative fraction contained this isoenzyme, but lacked the LDH-2 and the LDH-3 bands, while only a weak LDH-4 band compared with the ER-4^{+} cells was found. This population was very heterogeneous in morphology and consisted of all cell types from the myeloid and erythroid series.

In conclusion, in bone marrow the ER-4 antigen is only expressed by cells in an early stage of the T cell differentiation. Double labelling experiments with MRC OX-19 and ER-4 indicated furthermore that also in spleens of young animals the differentiation antigen recognized by ER-4 is not present on mature T-cells. Comparison of the cells recognized by ER-4 with those labelled by MRC OX-7 in the bone marrow showed also striking differences. In adult rats ER-4 reacted with a consistently lower percentage of bone marrow cells. Where ER-4 recognized predominantly small to medium sized cells with a lymphoid morphology, MRC OX-7 was also expressed by blastoid cells and cells of the erythroid and myeloid lineages. Also the LDH-profile of the ER-4 positive cells and the presence of TdT positive cells within this population support the hypothesis that ER-4 positive bone marrow cells belong to the early phase of T cell differentiation. Another confirmation for this hypothesis came from the ontogenic study. In this experiment a low percentage of ER-4 positive cells could be demonstrated in both BM and spleen just before and after birth. Shortly after birth the number of ER-4 positive cells started to increase, reaching maximal levels between 3 and 4 weeks after birth. Thereafter the numbers of positive cells started to decrease in these organs. At 16 weeks of age hardly any ER-4 positive cells could be detected in the spleen, whereas the percentage of positive cells in the bone marrow was only half of that found at 4 weeks of age. These observations strongly resemble the data given by Gregoire et al (1979) for the presence of prothymocytes, TdT^{+} cells, in the bone marrow and spleen. The absolute percentage of ER-4 positive cells in bone marrow exceeds the figures of TdT^{+} cells. The data therefore indicates that the TdT positive cells are present within the ER-4^{+} population. This difference between percentages might be due to the fact that not all prothymocytes are TdT[+] (Greiner et al, 1982; Gregoire et al, 1979). Possibly ER-4 antigen is expressed on all prothymocytes in contrast to TdT. The number of ER-4 positive cells (20%) in the bone marrow is considerably higher than reported by Lepault et al (1983) in earlier studies about the number of prothymocytes. They estimated approximately 0.1%, but in this respect

Wagner (1983) suggested recently that the number of prothymocytes must be rather high, in order to enable a selective and restrictive procedure in T-cell differentiation, which probably already takes place in the bone marrow, even before the cells go to the thymus. Therefore ER-4 in combination with other markers of the prothymocyte might be a valuable marker for the further definition of the prothymocyte and T cell differentiation.

Cell surface antigens

Molecular size

Since all antibodies reacted with virtually all thymocytes, the molecular weights of the surface antigens recognized by the panel of MCA have been determined on thymocytes. These cells were radiolabelled and immunoprecipitated according to Agthoven et al (1981).

Thymocytes were suspended in PBS at a concentration of $2\text{-}4\times10^7$ cells/ml and iodinated with 1 mCi sodium ^{125} Iodide (3.7 GBq/ml; Radiochemical Centre, Amersham, England) using the Iodogen method (Markwell and Fox, 1978). Iodinated cells, separated from unbound iodine by three washes in PBS with 2 mM potassium iodide were extracted in immunoprecipitation buffer (IPB) consisting of 0.14M NaCl in 20 mM Triethanolamine-HCl (pH8.6) supplemented with 0.2% (w/v) deoxycholate (DOC), 0.5%(w/v) Nonidet P40 (NP40), 1mM phenylsulfonylfluoride (PMSF) and 0.1 mg/ml trypsin inhibitor at a concentration of $2\text{-}4\times10^7$ cells/ml. The extracts were cleared of nuclei and cell debris by spinning at 10.000 g for 15 min at 4°C.

The supernatant was precleared with a heat-inactivated formalin fixed Staphylococcus aureus preparation (Pansorbin - Hoechst, BRD) and subsequently precleared twice with a complex of Pansorbin and normal rabbit serum and Pansorbin and normal mouse serum or twice with preformed complex of mouse Ig and rabbit anti-mouse Ig each for 1 hour at 4°C. Preclearing was performed to remove proteins that bind with mouse Ig or with Pansorbin. Precleared lysates (500 μl) were incubated overnight at 4-8°C with 50 μl of a preformed complex of the monoclonal antibody and the rabbit anti-mouse Ig.

The resulting precipitate was resuspended in IPB and washed on a discontinuous gradient consisting of one layer of 10% (w/v) sucrose, 0.5%
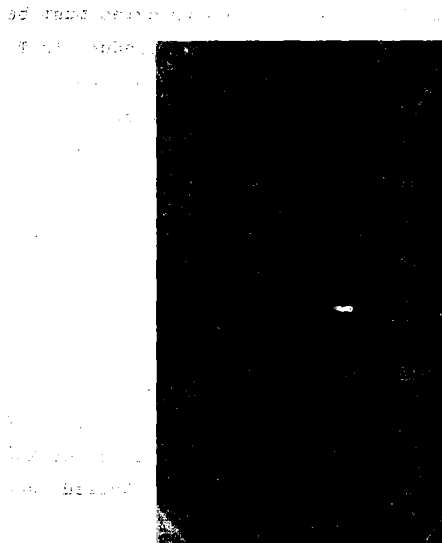
Figure 3.8 Immunoprecipitation of lysate of ^{125}I-labelled thymocytes on SDS-PAGE. Lane a. Control ascitis of SP2/0 cell line lane b. W3/13, lane c. ER-1 lane d. MRC OX-7, lane e. ER-4

NP40 in triethanolamine/NaCl (TBS) buffer and one layer of 30% (w/v) sucrose in TBS buffer. Immunoprecipitates were dissociated with 30 µl of sample buffer as described by Laemmli (1970). Sodium dodecylsulphate-polyacrylamid gelelectrophoresis (SDS-PAGE) was carried out on a gradient (7-18%) or continuous 12% acrylamide vertical slab gel according to a modification of the Laemmli technique. For autoradiography the gels were exposed to a X-ray film (Kodak XAR-5,USA). The following molecular weight markers (Bio-Rad, USA) were used in the PAGE for reference: lysozyme (14.4kD), soybean trypsin inhibitor (21.5 kD), carbonic anhydrase (31 kD), ovalbumin (45 kD), bovine serum albumin (66.2 kD), phosphorylase B (92.5 kD), β-galactosidase (116kD) and myosin (200kD).

For some of the antibodies the immunoprecipitations of ^{125}I-labelled thymocytes on SDS-PAGE are shown in figure 3.8 Both W3/13 and ER-1 were directed against a surface antigen of 95 kD, whereas MRC OX-7 and ER-4 recognized a 25 kD antigen. The results of the remaining antibodies are summarized in Table 3.9. ER-2 recognizes a 52 kD molecule, which is similar

to the molecular size that has been reported for W3/25 by Thomas and Greene (1983). ER-3 and ER-7, on the other hand, still have to be defined, since both antigens appeared to be highly sensitive for oxidation during the procedure of precipitation. These results suggest nevertheless that ER-3 is directed against a different antigen as MRC OX-8 and ER-10, since the latter antigens are not sensitive to oxidation. Furthermore, MRC OX-8 and ER-10 also recognize different structures on the cell membrane, since MRC OX-8 has been reported to label a complex of glycoproteins of 34, 39 and 67 kD (Thomas and Green, 1983), whereas ER-10 precipitated a band in the

Table 3.9 Molecular weight of the surface antigens recognized by mouse anti-rat monoclonal antibodies.

Antibody	M.W.[a]	Reference Antibodies	M.W.[a]	References
ER-1	95 kD	W3/13	95 kD	Standring et al, 1978
ER-2	52 kD	W3/25	52 kD	Thomas & Green, 1983
ER-3	n.d.	MRC OX-19	69 kD	Dallman et al, 1984
ER-4	25 kD	MRC OX-8	34+39+67	Thomas & Green, 1983
ER-7	n.d.	MRC OX-7	25 kD	Mason & Williams, 1980
ER-9	>100 kD			
ER-10	59-62 kD			
ER-14	25 kD			

a. The molecular weight is expressed in kilodalton (kD).

region of 59 - 62 kD. This means that, although these three MCA (MRC OX-8 - ER-3 - ER-10) all react with the suppressor/ cytotoxic subset of T cells, they recognize these cells through different receptors. This is well in agreement with the findings of small numbers of MRC OX-8^{−}ER-3^{+} and MRC OX-8^{−}ER-10^{+} cells in spleen and lymph nodes. Finally the ER-1 antigen is of the same size as the molecular weight reported for W3/13, i.e. a glycoprotein of 95 kD (see also Fig. 3.8). Two of the antibodies i.e. ER-4 and ER-14 are directed against a cell surface determinant of 25kD.

The apparent glycoprotein of this size (25 kD) on rat thymocytes is the Thy-1 antigen. It is found in a high density (10^6 mol/cell) on these cells (Williams et al, 1980). A first indication of an anti-Thy-1 specificity of ER-4 and ER-14 is obtained from experiments in which the activity of these (and other) antibodies was measured on thymocytes of AKR mice using a fluorescence staining technique. These mice carry the same allele as rats (Douglas, 1973). As shown in Table 3.10, ER-4, ER-14 and MRC OX-7, but not ER-2, reacted with AKR thymocytes. This is quite

Table 3.10 Binding of monoclonal antibodies to Thy-1
of the rat thymocytes

Antibody	Binding		Blocking
	AKR-thymus[a]	Thy-1[b]	Thy-1[c]
MRC OX-7	+	+	+
ER-4	+	+	+
ER-14	+	+	+
ER-2	−	−	−

a. All antibodies were conjugated with FITC and analysis
   done on a FACS II
b. Binding of antibody to purified Thy-1 was measured in
   a micro-ELISA
c. Blocking with purified Thy-1 of the antibody
   interaction with rat thymocytes

suggestive for a Thy-1 reactivity of the positive MCA. To get formal proof
the actual interaction of these antibodies to Thy-1 was tested. Purified
rat Thy-1 was kindly provided by Dr. A.F.Williams, Oxford, England. The
binding of the antibodies was measured in a micro-ELISA system, in which
purified rat Thy-1 was coated to the bottom of microtiter wells. Bound
antibody was determined with β-galactosidase conjugated rabbit anti-mouse
antibodies as described in the previous chapter. Purified Thy-1 has also
been used in a blocking assay. Therefore antibodies were preincubated with
Thy-1. Subsequently the remaining activity of these antibodies was
measured in a fluorescence assay on rat thymocytes. As shown in Table
3.10, both antibodies i.e. ER-4 and ER-14, showed the same reaction
pattern as the monoclonal antibody MRC OX-7. The latter antibody
recognizes the "classical Thy-1" molecule (Mason and Williams, 1980). The
ER-2 antibody (52 kd surface antigen), on the other hand was negative in
all three tests. It must be concluded therefore that all 3 MCA (ER-4 - ER-
14 - MRC OX-7) react with the Thy-1 molecule on the surface of rat
thymocytes.

        Nevertheless it is obvious, that ER-4 and ER-14 have major
differences, such as a differential recognition pattern in the thymus. ER-
14 gave a classical distribution pattern known for Thy-1 (Fig.3.9A), with
most cells equally stained and a slightly more intensive staining of the
subcapsular cells. ER-4, however, showed a quite different pattern.
Although all thymocytes (96%) were stained (Table 3.2), the intensity of
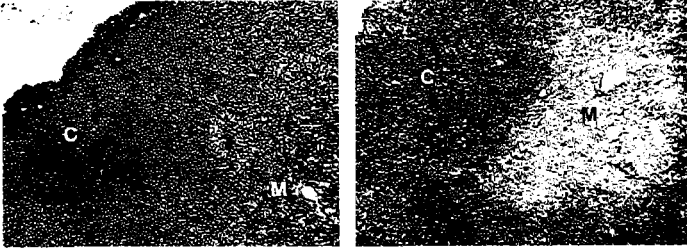the cortical cells exceeded that of the medullary cells by manyfold

Figure 3.9 Immune peroxydase histology of monoclonal antibodies ER-14 (A) and ER-4 (B) on sections
of the thymus. Legends: M, medulla; C, cortex.

(Fig.3.9B). In the FACS analysis these two populations could actually be
seen as two separate populations (data not shown). Also in the bone marrow
a different recognition pattern for these antibodies exists. ER-4, for
instance, recognized only small cells, whereas ER-14 reacted also with
large cells (Table 3.6). The most likely explanation for these findings is
that ER-4 and ER-14 react with different epitopes on the Thy-1 molecule,
which are expressed differently at various cell types.

Polymorphism

To exclude strain differences, a possible polymorphic distribution
of the recognized antigens was studied. Since all antibodies recognize the
majority of the thymocytes, these cells were used for these experiments.
Cells of eight inbred rat strains, displaying the same number of different
RT-1 haplotypes, were coated on microtiter plates and binding of all the
antibodies was measured using a RIA. Although a certain degree of
variation in binding properties could be observed between these strains all
antibodies showed a high degree of binding with all haplotypes (illustrated
for ER-1 and ER-2 in figure 3.10). Therefore it can be concluded that none
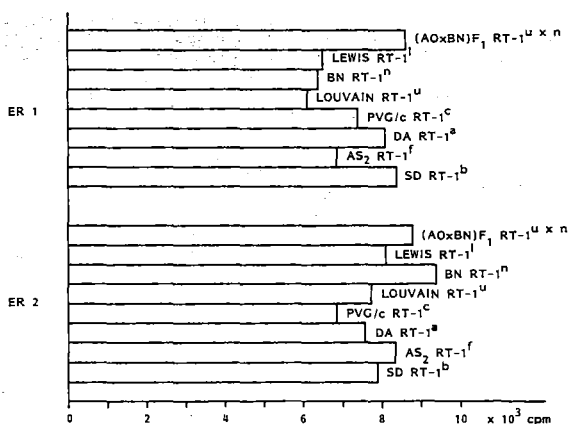of these antibodies detects a surface antigen displaying a polymorphic
distribution.

Figure 3.10 Binding of MCA'(A) ER-1 and (B) ER-2 to thymocytes of a panel of inbred rat strains with various RT-1 haplotypes. Thymocytes were coated on microtiter plates and binding was determined using a RIA. Mean background on the various thymocytes was 500 cpm.

The role of surface molecules in T cell function

Not only antigen-specific receptors are involved in T cell functions. Nakayama et al (1979) and Shinohara and Sachs (1979), however, demonstrated that anti-Lyt-2/3 antibodies inhibit the specific cytolytic T lymphocytes. This indicates that besides the antigen-specific receptors also non-specific differentiation antigens on T lymphocytes may play a role in the functional aspects of the T cells. Since these observations many surface molecules were reported to be involved in T cell functions (Ledbetter and Seaman, 1982; Swain, 1983; Reinherz et al, 1983 and Gunter et al, 1984).

In order to get an impression of the functional relevance of the recognized cell surface receptors on T cells, we studied the effect of various MCA on a panel of in vitro T cell responses, such as mitogenic activation, mixed lymphocyte reactivity and cytotoxic T cell response. We focussed in these experiments on the pan-T cell antibodies ER-1, W3/13 and MRC OX-19, the helper-T cell antibodies ER-2 and W3/25, the suppressor/cytotoxic T cell antibodies ER-3 and MRC OX-8 and MCA ER-9. The latter was selected for its highly interesting distribution pattern. First of all a possible mitogenic effect of the antibodies for resting T cells was tested, since such an activity has been described for the OKT3 antibody
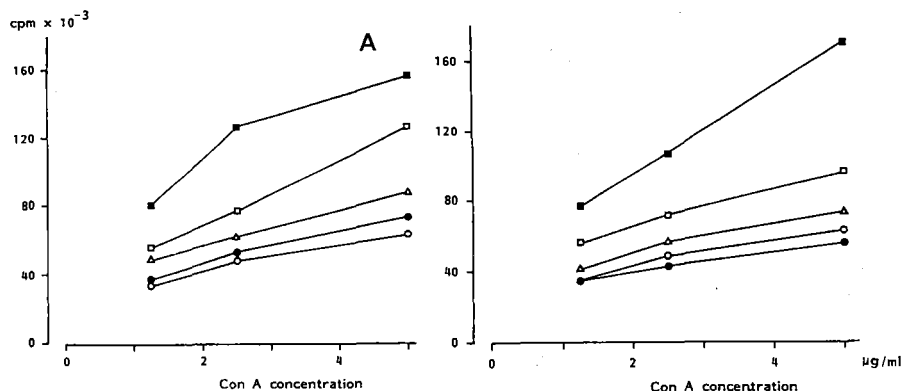
Figure 3.11 Influence of MCA ER-1 (A) and W3/13 (B) on the proliferative response of spleen cells cultured with Con A. The MCA were tested in concentrations of 0 (O), 0.1 (●), 1.0 (△), 10 (□) and 100 µg/ml (■).

(Reinherz et al, 1980). The following culture system was used (Rozing and Vaessen, 1979): Spleen cells were isolated and the cells were suspended in Hepes-buffered (30mM) RPMI-1640 medium supplemented with 2 g/L sodium bicarbonate, 20mM L-glutamin, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2×10^{-5} M 2-mercaptoethanol (MLR-medium) with 10% autologous heat-inactivated rat serum (ARS). The wells contained 200 µl of medium with 3×10^5 spleen cells, supplemented with antibody (0 – 0.1 – 1 – 10 – 100µg/ml). The cells were cultured in flat bottom microtiter plates (Costar, Cambridge, MA, USA) for 2, 3 and 4 days at 37°C in humidified air containing 5% CO_2. During the last four hours 1 µCi of (methyl ^{3}H)-thymidine (5 Ci/mmol; Radiochemical Centre, Amersham, England) was present in the cultures. The cells were harvested and the incorporation of ^{3}H-thymidine was determined by liquid scintillation counting. All tests were done in triplicate and the incorporation is shown as mean counts per minute (cpm). None of the antibodies tested had a mitogenic effect by itself (data not shown). However, several antibodies reacting with T cells of the rat are capable to suppress or enhance the proliferative response of mitogen activated T cells (Webb et al, 1979; Dallmann et al, 1982). Therefore spleen cells were cocultured with the mitogen concanavalin A (Con A) in the concentrations of 1.2 – 2.5 – 5.0 µg/ml.

        From the panel of tested antibodies the MCA W3/13 and ER-1 induced
an   increased ^{3}H-thymidine   incorporation   in   a   dose   dependent way from
approximately 50.000 cpm for the control experiment, without antibody, to
approximately 160.000 cpm after addition of 100 μg/ml of antibody (Fig.
3.11A and B). Also MRC OX-19 (data not presented) did show a comparable
effect on the mitogenic response, which is in agreement with data reported
by Dallman et al (1984) for this antibody. These three antibodies appeared
to act in a synergistic way with mitogen, since unstimulated cells were not
affected. No proliferation was observed when only MCA was added to the
culture. Only after the inductive signal of the mitogen they were able to
increase the degree of proliferation. The other antibodies (ER-2, ER-3,
ER-9,   W3/25   and MRC OX-8) did neither increase nor suppress the
proliferative response induced by Con A, as illustrated for MRC OX-8 and
ER-9 in figure 3.12. The experiments were repeated several times using
various rat strains (DA/Olac, AGUS/Olac, WAG/Rij, AO/Olac and PVG/Olac)
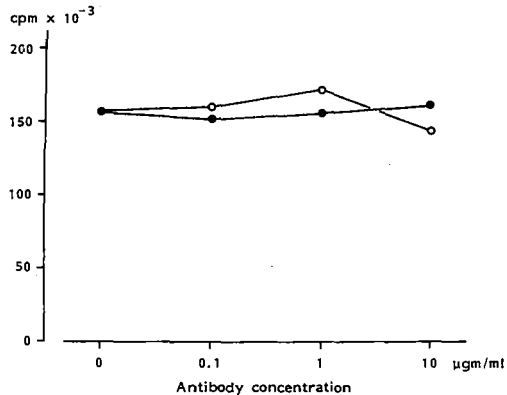with similar results.

Figure 3.12 The effect of MCA ER-9 (O) and MRC OX-8 (●) on the mitogen
response of Con A (5μg/ml) stimulated spleen cells.

        Subsequently the effect on the specific T cell response induced by
allogeneic surface antigens i.e. the mixed lymphocyte reaction (MLR) was
determined. MLR experiments were performed under the same conditions as
described for the mitogen response: responder cells of DA/Olac rats (3 x
10^5 cell/well) were cultured with the same number of irradiated (3000 rad)

stimulator spleen cells of RP/Rij rats. The cells were cultured during 5, 6 and 7 days and ^{3}H-thymidine was added 8 hours before the termination of the cultures.

The various antibodies showed a clear difference in their effect on the proliferative response of the mixed lymphocyte reaction. MCA ER-3 had no influence on the MLR (data not shown). ER-2 and W3/25, however, did almost completely suppress the MLR. For both antibodies the inhibition of the MLR displayed a similar dose dependent fashion (Fig.3.13A). MRC OX-19,
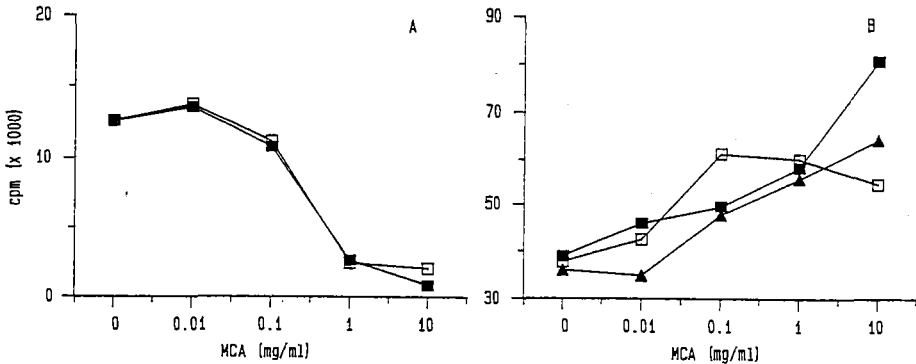

Figure 3.13 The influence of MCA on the proliferation induced by the MLR. In A the effect of ER-2 (□) and W3/25 (■) and in B the effect MRC OX-19 (■), ER-1 (□) and W3/13 (▲) are shown. Spleen cells of the DA rats were used as responders and irradiated RP spleen cells were added as stimulators cells.

W3/13 and ER-1, on the other hand, all seemed to increase the proliferation (Fig.3.13B) in the MLR, which is comparable to data reported by Dallman et al (1984) for MRC OX-19 and by Webb et al (1979) for W3/13. ER-9 suppressed the proliferative allogeneic response in a dose dependent way as is illustrated in figure 3.14. MRC OX-8, on the other hand, failed to show any influence on the MLR, even in the highest doses, which is in agreement with results reported by Green (1984). Repeating the experiments with other combinations of rat strains, such as DA/Olac and AO/Olac or AO/Olac and Agus/Olac provided similar results. ER-9, in the range of 10 – 100μg/ml, did almost reduce the proliferation to the background level. The surface antigen defined by ER-9 seems therefore to be involved in the specific alloreaction, since no effect of this MCA was seen on the mitogenic response induced by Con A.
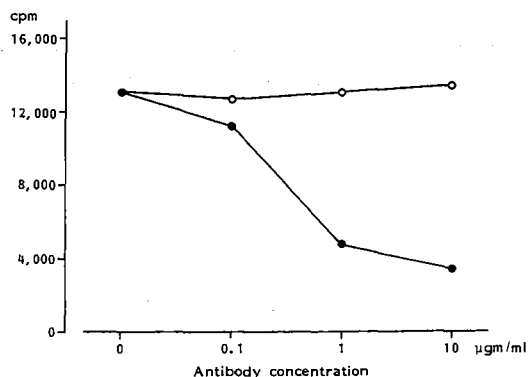
Figure 3.14 The influence of MCA ER-9 (●) and MRC OX-8 (○) on the MLR. Responder cells of DA/Olac rats were cultured during 6 days together with irradiated stimulator spleencells of RP/Olac rats. All tests were done in triplicate and the incorporation at various antibody concentrations are expressed as mean cpm.

In the last series of experiments the antibodies were studied for their effect on the cytotoxic T cell activity. Because cytotoxic effector cells are generated in the MLR, the antibodies can be tested on the generation of the cytotoxic T cell response by adding antibody in the MLR. By supplementing antibody in the cell mediated lympholysis assay the effect can be tested on the effector CTL . MLR cultures were set up between DA and RP spleen cells. Various concentrations of MCA were added at the initiation of the cultures to analyse their effect on the generation of cytotoxic T cells. Responders cells ($2x10^7$ spleen cells) were cultured with an equal number of stimulator cells (irradiated spleen cells) in 10 ml of MLR-medium supplemented with 10% autologous rat serum (ARS). These cells were grown for 6 days in 75 ml TC-flasks (Costar, England) at 37°C in humidified air containing 5% CO_2. The cells were collected and the viable cells (80-90%) were isolated using a Ficoll-Hypaque gradient (δ 1.077). These cells were washed and used as effector cells in the cell mediated lympholysis (CML) assay.

As target cells mitogen activated spleen cells of the stimulator strain were used. In 275 ml TC-flasks (Costar, England) $5x10^7$ spleen cells were cultured in 10 ml MLR-medium (5% FCS), containing 5μg of Con A, during 72 hours. Thereafter the viable cells were isolated by using Ficoll-Hypaque gradient and used for labelling. Ten million cells were labelled

with 300 μCi Na$_2$^{51}CrO$_4$ (7 -12Ci/mmol; Radiochemical Centre, Amersham, England) in 1 ml MLR-medium (pH 6.0-6.5; 5% FCS). After removing of the free radiolabel by extensively washing the cells were used as target cells in the CML assay.

The CML-assay was performed in round bottom tubes (2 ml). Target cells (10^4 cells) were combined with 10^6, 3x10^5 and 10^5 of the effector cells, respectively, in a total volume of 200 μl of MLR-medium (5% FCS). Prior to the incubation, the cells were centrifuged for 5 min at 50g, followed by an incubation of 3 hours at 37°C in humidified air containing 5% CO$_2$. The reaction was stopped by adding 1 ml of cold (4°C) medium and immediately centrifuged for 5 min at 800g (4°C) in order to separate the cellular ^{51}Cr from the free radiolabel. The supernatant was collected by decantation into counting tubes, whereafter the radioactivity was measured using a τ-counter. The release of ^{51}Cr in absence of effector cells cultured under identical conditions normally ranged between 200 - 800 cpm (spontaneous release). For the maximum release the labelled target cells were lysed with 0.5% (w/v) NP40 buffer, which resulted in the release of about 90% of the ^{51}chromium. For the expression of the cytotoxic activity the following formula was used:

$$\text{Specific release} = \frac{\text{exp.release} - \text{spont. release}}{\text{max.release} - \text{spont. release}} \times 100$$

To test the effect of the various MCA in the effector phase of the cytotoxic T cell response, the antibodies were added at the beginning of the incubation period of the CML in concentrations ranging from 0.1 to 300 μg/ml. None of the antibodies however, had any influence on the effector phase of the cytotoxic T cell reaction (data not shown). With regard to the effect of some of the antibodies in the induction phase, however, striking differences could be observed. No influences were noticed when ER-1, ER-3, W3/13 or MRC OX-19 were added to the cultures. As could be expected from their effect on the proliferative response in the MLR, the addition of ER-2 and W3/25 to the cultures inhibited the generation of cytotoxic T cells completely, probably through blocking of the inducer T cell, which is necessary for the differentiation of precursor cytotoxic T cells into mature cytotoxic T cells (data not shown). In contrast to their different reactivity pattern in the MLR, however, both ER-9 and MRC OX-8 inhibited the generation of the cytotoxic T cells in a dose dependent way. As is illustrated in figure 3.15 a dose of 10 μg of ER-9 reduced the
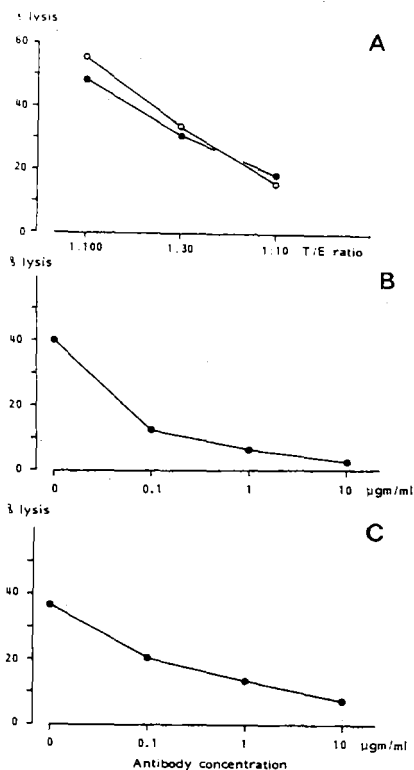
Figure 3.15 Effect of MCA   ER-3 (A),   MRC OX-8 (B) and ER-9 (C) on the generation of the cytotoxic T cells. Antibodies were added to MLR cultures of DA/Olac and irradiated RP/Rij spleen cells. The cytotoxic activity was determined with ^{51}Cr-labelled Con A blast cells of the RP rats. The effect of ER-3 (●) is shown for a concentration of 10µg/ml at various ratio's of the target and effector cells (T/E) together with a control experiment (O). The suppressive effect of ER-9 (B) and MRC OX-8 (C) is illustrated at a T/E ratio of 1:30.

cytotoxic activity more then 90% (Fig. 3.15B), while for MRC OX-8 a comparable suppression is seen at the same antibody concentration (Fig. 3.15C). The same type of inhibition was reproducible using other rat strain combinations.

When, as mentioned above, both antibodies were added in the effector stage of the cytotoxic T cell response (at the start of the CML-culture) the lysis of the targets remained unaffected (data not shown). Therefore it can be concluded that both MRC OX-8 and ER-9 are involved in the activation or differentiation of the CTL-p and not in the effector function. The effect of MRC OX-8 is most likely directly on the precursor cell, since the surface antigen of this antibody has been determined on both the precursor and the effector cytotoxic T cells (Dallman et al, 1982). It is possible that ER-9 also acts directly on the precursor cell,

but this antibody reacts also with part of the helper T cells as defined by the MCA ER-2. Helper T cells are the source of IL-2 (Cantrell, 1982) and probably also of differentiation factors required for CTL. (Wagner et al, 1982). Therefore the inhibitory effect of ER-9 may be caused by interaction of this antibody with the CTL-p or with the T helper cells or both. For this reason we studied a possible correlation between the
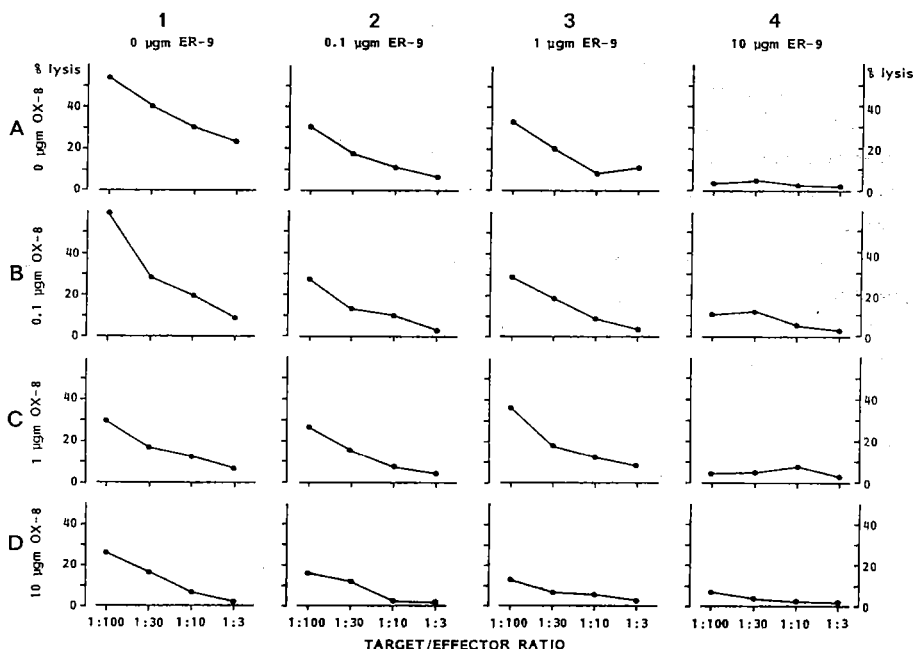

Figure 3.16 The correlation of the effect of MCA ER-9 and MRC OX-8 on the generation of the cytotoxic T cell activity. CTL were derived from DA rats and the stimulator and target cells of RP rats were used.

blocking effect of ER-9 and the effect of MRC OX-8 by adding various combinations of both antibodies at the same time. The results are shown in figure 3.16 and in row A the suppression of ER-9 is shown at various T/E ratios, whereas in the left column (1) the MRC OX-8 influence is demonstrated. These experiments were done with MLR generated spleen cells of DA rats tested for activity on target cells of RP rats. A maximum release of 10.972±373 cpm and a spontaneous release of 1.565±20 cpm was

found. Both MCA were capable to increase the degree of inhibition exerted by the other antibody as can be seen in the columns 2 and 3 for an additional effect of MRC OX-8 on the ER-9 influence and for the other combination in rows B and C. The degree of suppression between these two antibodies seems to be different: a complete blocking of the cytotoxic activity was only detected at a concentration of $10\mu g$ of MRC OX-8 in the 1:3 T/E ratio (D1). ER-9, on the other hand, showed a comparable blocking in all the tested T/E ratio's (column 4). The suppression of $1\mu g$ MRC OX-8 tested in the ratios of 1:100 and 1:30 (C1) was hardly increased after a tenfold increase of this antibody (D1). MLR cultures containing 1 and $10\mu g$ of MRC OX-8 in which no further blocking could be induced by this antibody, however, addition of ER-9 was able to further reduce the cytotoxicity (Fig. 3.16, rows C and D). Also MRC OX-8 was able to an additional inhibition of the reduction induced by ER-9 at 0.1 and $1.0\mu g/ml$ (Fig. 3.16, columns 1 and 2). Although these results indicate that ER-9 acts through another mechanism than MRC OX-8, it remains unclear which type of cell is the prime target for ER-9 to block the differentiation of the CTL-p cells. Both MCA may react with the same cells. On the other hand, ER-9 may also exclusively act through the helper T cells.

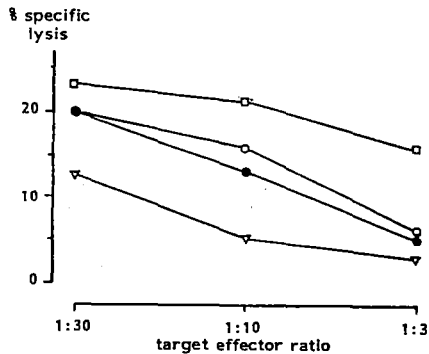Figure 3.17 Lymph node cells were sorted into ER-9 positive (□) and ER-9 negative (▽) cell fraction. These cell populations were tested for enrichment of precursor CTL specific for the class I antigens. Control experiments consisted of unseparated cells with (●) or without (○) ER-9 labeling.

In order to verify whether the precursors of the cytotoxic T cells express ER-9, the ER-9$^+$ cells were isolated and subsequently tested for

cytotoxic activity. Spleen cells were stained with FITC-conjugated ER-9 and the bright ER-9 positive cells were separated from the negative ones by sorting using flow-cytometry. MLR cultures were set up with these isolated fractions as responder cells. The isolated cells were supplemented with 10% of T cell growth factor containing supernatant to exclude shortage of IL-2. This T cell growth factor containing medium consisted of supernatant of spleen cell cultures stimulated with Con A (5μg/ml) for 24 hours. In figure 3.17 the results of a representative experiment are shown. An increased ability of the ER-9 positive cells to lyse target cells was found and a reduced capacity of the negative cell fraction. A possible interference of antibody bound to the cell surface with the generation of effector cells could be excluded, because lysis of targets by all cells labelled with ER-9 did not significantly differ from unlabelled, unseparated cells. These results suggest that the precursor of the cytotoxic T cell resides within the ER-9 positive cell population. On the other hand, preliminary data of FACS sorted cells, which were stimulated with Con A and tested for IL-2 production as described by Gillis et al (1978), indicate that the production of IL-2 is restricted to the ER-9 positive cells. This means that both the helper T cell and the precursor T

Table 3.11. Influence of antibodies on in vitro T cell function

MCA	Surface antigen (kD)	proliferation[a]		cytotoxic T cell[b]	
		Mitogenic	MLR	MLR	CML
ER-1	95	+[c]	+	0	nt
W3/13	95	+	+	0	nt
ER-2	52	0	-	-	0
W3/25	52	0	-	-	0
MRC OX-19	69	+	+	-	nt
MRC OX-8	34+39+67	0	0	-	0
ER-9	>100	0	-	-	0

a. The influence of MCA on the proliferative response by
   mitogenic stimulation or MLR.
b. The influence of MCA on the CTL activity when added in
   the MLR cultures or in the CML cultures.
c. Data of the experiments described in this chapter.
   + : increased; 0 : no effect; - : suppressed;
   nt : not tested.

cell do express the ER-9 surface antigen. The suppressive effect of ER-9
on the MLR (Fig. 3.14) seems to be caused by interference of ER-9 with the
inducer cell, because binding of ER-9 to these cells prevents proliferation
and the production of IL-2 and other factors. A mode of action through the
precursor T cell, however, cannot be excluded.
These data emphasize that ER-9 in combination with antibodies such
as ER-2 and MRC OX-8 enables a further dissection of the T cell compartment
into functional T cell subpopulations.

The results of the experiments studying the influences of the various
MCA on the functional in vitro assay show that also in the rat, besides the
T-cell receptor, other surface molecules are involved in the T cell
functions. In Table 3.11 the data in the rat system are summarized. At
least five different molecules seem to be involved in T cell functions.
Moreover we can distinguish also four or five different reaction patterns,
which are probably all effective in an early stage of T cell activation.

PHYSICAL PROPERTIES OF THE ANTIBODIES

Besides the cellular reactivity pattern also some physical
properties of the selected panel of MCA have been determined, such as
isotype, binding affinity, and complement-binding properties. This
information is essential for the further use of these antibodies in other
test-systems.

Table 3.12 Isotype and complement binding of
the MCA .

Antibody	Isotype[a]	Complement[b] binding
ER-1	IgG2a	++
ER-2	IgG1	-
ER-3	IgG2a	+
ER-4	IgG2a	++
ER-7	IgG2b	+
ER-9	IgG2b	++
ER-10	IgG2a	+
ER-14	IgG1	-

a. The isotype has been determined with a
   radial immunodiffusion assay.
b. Lysis of thymocytes using rabbit
   complement. Score: - (0-20%), + (20-
   60%) and ++ (60-100%)

Isotype

The isotype of these antibodies has been determined using a single radial immunodiffusion technique (Mancini et al, 1965). Gels consisting of a mixture of agarose (3%) and antibody, diluted in tris-barbital buffer (pH 8.6) were prepared. Wells, punched in the gel were filled with rabbit anti-mouse isotype antisera (Bionetics, England) in the appropriate dilution and a positive reaction resulted in a precipitation ring around the well. The isotypes, summarized in Table 3.12, all appeared to be of the IgG class. This is probably caused by the repeated immunisation procedure favouring a secondary response type with an increased presence of IgG producing plasma blasts at the time of fusion.

Complement binding

Thymocytes were isolated and brought upon a concentration of 5×10^7 cells/ml in RPMI-1640 supplemented with 5% FCS. From this suspension 50 μl were preincubated for 30 min at 0°C with antibody in various concentrations. Cells were washed and subsequently incubated for 30 min at 37°C with 100 μl rabbit complement (1/7 dilution v/v). Rabbit complement was tested for lytic activity and extensively absorbed with rat lymphocytes. Cells were washed and the viability was determined by staining the cells with 0.4% (w/v) trypan blue in 0.15 mM NaCl solution. The percentage of cell lysis was assessed by microscopic examination. Cell lysis (Table 3.12) varied from 80 - 90% for ER-9, ER-4 and ER-1, whereas several of the antibodies such as ER-3, ER-7 and ER-10 lysed approximately 50% of the cells. ER-2 and ER-14 did not show any binding of complement, which is in agreement with earlier findings for the IgG1 isotype from studies with alloantisera (Lems et al, 1983).

Affinity

For some of the in vivo experiments it was necessary to determine the degree of affinity of several monoclonal antibodies. These experiments will be described in the chapter 5. The affinity has been determined by binding experiments using radiolabelled antibodies. The interaction of antibodies and antigens has been described a.o. by Fazekas de St Groth (1979). In the situation of equilibrium the affinity of antibody binding

sites (paratopes; P) for an antigen binding site (epitope;E) can be measured by the ratio of complexed (x) and free reactants. The equilibrium constant, K, in this situation is defined by the equation:

$$K = \frac{[\text{free epitopes}]\,[\text{free paratopes}]}{[\text{complexes}]} \text{ or } \frac{[E-x][P-x]}{[x]}$$

The equation for large multivalent antigens, such as cells or bacteria, with a cell concentration [C], in which each cell carries n epitopes (n) is:

$$K = \frac{[nC-x][P-x]}{[x]} \qquad \text{(Fazekas de St Groth, 1979)}$$

The valency of the antibody can usually be ignored in this situation according to the author. In the example of a cell with n epitopes on it, an input concentration of [nC] epitopes which reacts with [P] paratopes, the separated fraction in this system will be the free antibody. When the fraction of complex bound antibody is α, ranging between 0 and 1, the complex is defined as $x \equiv \alpha P$. Rearrangement gives the following equation:

$$K = (nC - \alpha P)\,\frac{1-\alpha}{\alpha} \qquad \text{(Fazekas de St Groth, 1979)}$$

Under defined conditions the equilibrium constant (K) can be determined by the ratio between bound antibody and the total antibody concentration. This ratio was defined with [125]-I labelled antibodies in binding studies on rat thymocytes. The majority of these cells (>90%) are recognized by all the tested MCA . The antibodies were purified and the concentration was determined using the extinction at 280 nm. Purification was done with gelfiltration (Sephacryl S300) and DEAE chromatography and subsequently the MCA was conjugated with [125]-Iodine as described before. The unbound radiolabel was subsequently removed by gelfiltration. Binding studies with various cell concentrations and dilutions of the antibody were done to determine the biological activity of the antibody and the specific cell concentration and antibody dilution for the binding studies. The biological activity expressed as the percentage of total radiolabel that is able to bind to cells was >80 percent for all MCA tested.

The binding experiments were done under the following conditions: From a cell suspension with a concentration of 3-4 x 10^7 cells/ml, 100 μl was incubated with 150 μl of radiolabelled antibody for 3-5 hours at 0°C

(under rotation) in order to acquire a state of equilibrium. The reaction was halted by adding culture medium (2 ml RPMI1640; 0°C), immediately followed by centrifugation (5 min at 200g and 0°C) and separation of the cell pellet and the supernatant. The radioactivity in both fractions was measured using a τ-counter. These types of experiments require a highly purified source of antibody. The paratope concentration can be calculated from the radiolabel cpm/g purified antibody and this results for each MCA in the number of cpm/Mol of MCA (in this calculation for the molecular

Table 3.13 Affinity of mouse anti-rat monoclonal antibodies

Antibody	Radiolabel[a] (cpm x 10^3)		Bound/ total	Epitope conc. (nC) (Mol/1)[b]	Paratope conc.(P) (Mol/1)[c]	Mean value of affinity (K) (Mol/1)[d]
	Total[e]	bound				
ER-1	293,0	98,0	0.322	8.50×10^{-10}	1.09×10^{-9}	1.04×10^{-10}
	75,5	32,6	0.419		2.80×10^{-10}	
	21,2	9,4	0.433		7.85×10^{-11}	
	5,8	2,8	0.466		2.14×10^{-11}	
W3/13	437,0	38,1	0.0744	8.50×10^{-10}	1.94×10^{-9}	6.97×10^{-9}
	115,0	11,7	0.0890		5.11×10^{-10}	
	31,4	3,4	0.0965		1.39×10^{-10}	
	9,4	1,2	0.117		4.18×10^{-11}	
ER-2	292,0	38,4	0.119	3.99×10^{-10}	7.95×10^{-10}	2.30×10^{-9}
	76,9	11,0	0.131		2.09×10^{-10}	
	22,4	3,4	0.138		6.10×10^{-11}	
	6,5	1,1	0.160		1.76×10^{-11}	
W3/25	317,0	39,5	0.112	3.99×10^{-10}	8.32×10^{-10}	2.08×10^{-9}
	80,1	11,1	0.126		2.10×10^{-10}	
	23,0	3,3	0.131		6.03×10^{-11}	
	7,8	1,3	0.148		2.04×10^{-11}	

a. Thymocytes (4 x 10^6 in 250 µl) were incubated with ^{125}I-antibody. The total and the bound fractions were determined.

b. Epitope concentration: number of determinants per cell x 4.10^6 x 4000 : 2.602×10^{23} (Mol/1)

c. Paratope concentration (Mol/1): $\dfrac{\text{A cpm of MCA} \times 4000}{\text{cpm/g of MCA} \times 150,000}$

A: the total of MCA (cpm) shown in column e.

d. Mean value of equilibrium constant using the equation $K = (nC-\alpha P)(1-\alpha/\alpha)$ Fazekas de St Groth, 1979.

weight of the IgG a figure of 150,000 is used). The amount of radiolabelled MCA in the assay system (B cpm) expressed as Mol/1: A x 4000/ A (factor 4000 to calculate the activity from the 250 μl to liters). The cell concentration combined with the number of epitopes per cell gives the total concentration of epitopes. The latter information was not available

until recent sequential immunoprecipitation experiments, which will be described in the next chapter. The latter experiments showed that ER-1 is directed against the same surface molecule as the monoclonal antibody W3/13 and the same result could be established for ER-2 and W3/25. For these two antibodies the density of surface molecules on thymocytes has been determined (Williams and Mason, 1980). For W3/13 3.2×10^4 and for W3/25 1.5×10^4 determinants/thymocyte were reported. The number of epitopes/cell x total number of cells tested x 4000 gives the number of epitopes per liter. This figure has to be divided by Avogadro's constant to obtain the epitope concentration in Mol/l. Several binding experiments were done to determine the affinity. The results summarized in Table 3.13 showed that both ER-1 and ER-2 have a comparable degree of affinity as W3/13 and W3/25. The antibody ER-1 has even an equilibrium constant as high as 10^{-10} Mol/l, whereas the other antibodies range between 2.0 and 7.0 $\times 10^{-9}$ Mol/l.

Using the panel of herewith characterized MCA the second part of the experimental work was started: the analysis of the T cell system in relation to the allograft rejection. To do so two major types of approaches were used, in which these MCA played an essential role. **Firstly** they were used to monitor phenotypical changes in the T cell compartment after allogeneic kidney transplantation both in the central immune system and in the graft. **Secondly,** they were used to specifically interfere with the onset of the rejection process by using them as immunosuppressive agents which specifically eliminate or block certain T cells in this process.

The experiments describing these two experimental set-ups will be the main topic of the following two chapters.

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Chapter 4

RENAL ALLOGRAFTING IN THE RAT

INTRODUCTION

Transplantation research in the rat has a number of advantages as compared to other species. Technically it is possible, for instance, to perform vascularized organ grafts in rats, in contrast to mice. Also inbred rat strains, including congenic strains for immunogenetic research are readily available. In this way it is possible to study independently the parameters involved in the alloreaction, such as total or partial MHC disparities or non-MHC differences. Furtheron large numbers of animals, transplanted in a highly comparative donor recipient combination, enable a more detailed analysis of the immune status, at various times after transplantation. This can be done in the graft as well as in the peripheral lymphoid organs. Comparison of such data will provide further insight in the complex process of the alloreactivity after organ transplantation. With the availibility of an extensive panel of MCA against a variety of (functional) T cell markers, it became now possible to analyse the rejection process after allografting in detail.

Therefore a series of transplantation experiments was performed in allogeneic and isogeneic donor recipient combinations. At various days after transplantation, animals were sacrificed for a detailed analysis of the several lymphoid organs and of the renal graft itself. Besides characterization of the phenotype expressed by the cells involved in the rejection process also functional studies of the lymphoid cell population will be described.

In all transplantation experiments inbred male rats were used at an age of 10 - 20 weeks. PVG/Olac rats served as recipient and also as donor in the isogeneic test group, while the same rat strain received a kidney from the PVG.RT-1^a(DA)/Olac strain in the allogeneic combination. The PVG strain has a RT-1^c haplotype, whereas the RT-1 system of the PVG.RT-1^a(DA) strain has been derived from the DA strain, both strains are identical for the non-MHC background. The kidney transplantation technique was originally described by Fischer and Lee (1965) and later slightly modified by Tinbergen (1971). The donor rat, anesthetized with ether, was

heparinized (Thromboliquine, Organon, Oss, The Netherlands; 500 units i.v.) in order to avoid clotting. The kidney was removed and cuffs from donor aorta and vena cava are left on the kidney vessels to facilitate the anastomosis. The graft was transplanted in the abdominal cavity of the recipient, which was anesthetized in the same way as the donor. Transplantation consisted of end-to-side anastomosis of the renal artery and vein with the aorta and the vena cava of the recipient, respectively. The ureter-bladder anastomosis was made by insertion of the ureter into the bladder, where it was fixed onto the mucosa. The ischemia times normally ranged from 25 to 35 minutes. Within two days after the operation bilateral nephrectomy of the recipient kidneys was performed. The renal function of the graft was monitored by determination of the plasma urea level (P_{ur}) each day. The P_{ur} was measured in $10\mu l$ of plasma with automated kinetic methods on a computer-directed analyzer (Provoost and Molenaar, 1980).
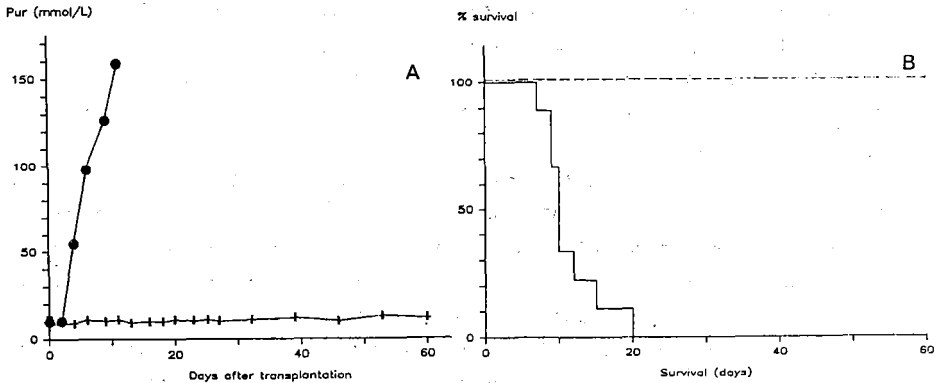

Figure 4.1 Survival of kidneys and function in PVG rats after kidney transplantation A. The renal function was illustrated by plasma urea levels in allografted (●) and an isografted (✚) recipient. B. Survival of allogeneic (—) and syngeneic (--) transplanted recipients kidneys. Recipients (PVG/Olac) were transplanted with PVG/Olac (syngeneic) or PVG.RT1[a](DA) (allogeneic) kidneys.

First of all the graft survival of the isogeneic and the allogeneic transplanted groups were determined, in which the survival of the recipients has been taken as the endpoint of the graft survival. All isogeneic grafted animals survived for more than 60 days, with an excellent graft function as is illustrated in figure 4.1A. The animals that received an allograft, however, all died between 7 and 20 days after the

transplantation with a mean survival time (MST) of 11.3 ± 3.9 days. Already four days after the operation increased plasma ureum levels (20 – 50 mmol/L) demonstrated retarded renal function. This was followed by a further increase until a complete renal disfunction and as a consequence of this, the death of the animals resulted (figure 4.1A).

In the next series of experiments the recipients were sacrificed on various days after transplantation in order to evaluate in detail the process of the alloreaction. Kidney, spleen, mesenteric lymph nodes (MLN) and blood (heparinized) were isolated for further analysis. Part of kidney was snap frozen and stored at -70°C for immune histology. Another part of the kidney was fixed in cold phosphate buffered 4% formaldehyde solution (pH 7.4), subsequently dehydrated in ethanol and embedded in paraplast. Sections of 5 μ were stained with hematoxylin-eosin for immunopathology. Blood samples were drawn and the peripheral blood lymphocytes were isolated: the samples were centrifugated for 5 min. at 200 g and the leukocytes on top of the packed cells were isolated. This fraction, diluted in RPMI-1640 medium (v/v) was brought upon 2 ml of a Ficoll/Isopaque solution (δ: 1.077 g/ml). After centrifugation (20 min at 730g) the lymphocytes were isolated from the surface of the Ficoll/Isopaque layer and washed.

In the following sections the results of histological examination of the transplanted kidneys and the composition of the T cell population determined in cell suspensions of spleen, mesenteric lymph nodes and blood using flow cytometry will be described. Furthermore the graft infiltrating cells will be monitored using specific immune histology. In the last section of this chapter, data of experiments in which cells from the spleen, blood and the graft were tested for donor specific response will be presented.

PATHOLOGY

Animals were sacrificed at 4, 6 and 8 days after isogeneic and allogeneic kidney transplantation for immunopathological examination of the grafts. In the isogeneic combinations no signs of rejection could be detected. Only one animal (day 4) showed several small foci of infiltrating polynuclear cells, probably caused by a bacterial infection. This animal also differed in its reactivity as determined in fluorescence analysis. Therefore this animal has been removed from the study. In the

other isogeneic combinations at first (day 4) some signs of acute tubulis necrosis (ATN) could be detected (figure 4.2A), probably caused by surgery damage. Two days later (day 6) no signs of ATN were detected anymore in any of the grafts (n=5). Both at six as well as at eight days after transplantion (n=5) all kidneys showed a normal structure with no signs of rejection.

In the allogeneic combination a clear and acute cellular rejection was found as described by Porter et al (1967). At four days (n=6) infiltrating cells, widely scattered and mainly mononuclear in nature were found at perivascular, periglomerular and peritubular sites. Increased
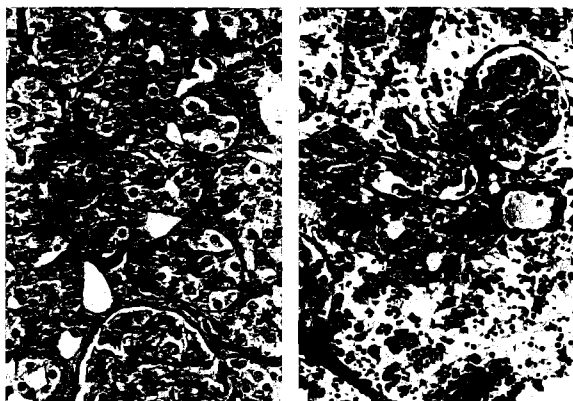

Figure 4.2 Pathology of the grafts after allogeneic kidney transplantation. A. ATN at three days after isogeneic kidney transplantation B. Allograft at 7 days after transplantation. Foci of RBC's and partial tissue damage can be seen

numbers were detected in grafts (n=7) at six days after transplantation, whereas at eight days (n=6) the numbers of infiltrating cells had significantly declined. Ischaemic damage was most likely the reason of ATN detected at four days. However, at six days necrosis is more severe and widespread, at this stage it is due to the rejection mechanism. Two days later ATN (day 8) is found throughout all grafts. One graft was completely rejected. Normal structures could not be recognized any more.

Interstitial oedema and foci of RBC's were detected in increasing intensity in some grafts at four days and in all kidneys at six and eight days (figure 4.2B). Arthritis with thrombus formation could be detected in 2 grafts at day 6 and in most of the organs at eight days.

The phenotype of the infiltrating cells as characterized by specific immuno-histochemistry on cryostat sections of the kidney will be described.

FLUORESCENCE ANALYSIS OF THE T-CELL POPULATION

In order to get insight into the complex rejection mechanism after organ transplantation and especially in the involvement of the immune system of the recipient, flow cytometric analysis was performed in spleen, mesenteric lymph nodes and peripheral blood at various times after transplantation using a panel of monoclonal antibodies. Single cell suspensions of spleen and mesenteric lymph nodes were prepared and labelled with FITC-conjugated MCA as described before. At the same time blood samples were drawn (Heparinized) and the peripheral blood lymphocytes (PBL) were isolated. Analysis of the labelled cell suspensions was done on FACS

Table 4.1 Panel of mouse anti-rat antibodies used for monitoring after renal transplantation

MCA	Reactivity		References
	Lymphoid	Non-lymphoid	
ER-1	T cells, Thymocytes	Non-lymphoid	Vaessen et al, 1985
	Early cells in BM	Bone marrow	This thesis
	B cells	cells	
MRC OX-19	T cells, Thymocytes		Dallman et al, 1984
ER-2	Helper T cells	Macrophages	This thesis
			Barclay, 1981
MRC OX-8	Suppr./Cytotoxic T-cells	NK cells	Brideau et al, 1980
			Cantrell et al, 1982
ER-9	T-cell subset	?	This thesis
	Thymocytes, Early cells in BM		
ER-4	Thymocytes, Early cells in BM	?	This thesis
ER-13[a]	B-cells, Activated T cells	Dendritic cells	Stet et al, 1984
F17-23-2[b]	B-cells, Activated T cells	Dendritic cells	Hart & Fabre, 1981
MARK-1	B-cells, Plasma cells		Bazin et al, 1984
ED-1	--	Macrophages	Dijkstra et al, 1985

a. Ia (common)
b. Ia (polymorphic: $RT-1^{u \text{ and } c}$ negative and $RT-1^{n \text{ and } a}$ positive.

II (Becton and Dickinson, USA) as described in the second chapter. A panel
of MCA, summarized in Table 4.1, was used for the monitoring of the immune
status. The composition of this panel has been defined by the reactivity
of these antibodies against various cells involved in the alloreaction.
The MCA ER-1 and MRC OX-19 recognize all T cells, whereas the T cell
subpopulations are recognized by ER-2 (T helper) and MRC OX-8 (T
suppressor/cytoxic). Besides a reactivity with the T-cells also a non-
lymphoid reactivity has been described for several antibodies (Table 4.1).
Also the B-cells, macrophages and the early T cells (ER-4 positive) can be
distinguished by MCA MARK-1, ED-1 and ED-1, respectively. For the
recognition of the class II MHC antigen two MCA were available. One (ER-
13) is directed against the monomorphic determinant, ER-13, the other (F17-
23-2) against a polymorphic determinant. Part of these MCA were used in
the fluorescence analysis, whereas all MCA were used in the analysis of the
graft infiltrationg cells described in the next section.

Besides the data as presented and discussed later in this section
several items of general importance came out of this analysis.

The **first** important phenomenon was the degree of variation between
the results of individual animals at the same day after transplantation.
This is illustrated in figure 4.3 with the percentages MRC OX-19 positive
lymph node cells of allografted and isografted animals. The individual
figures at 0, 4, 6, 7 and 9 days after transplantation are shown for both
the isogeneic and the allogeneic combinations. Compared with untreated
animals (day 0) only a minor variation can be seen in the isografted
animals, which is in agreement with a stable and non-activated situation.
The degree of variation the allografted animals, especially at six and
seven days after transplantation is higher than the isografted animals.
Because the rat strains were highly inbred, genetic variation within these
strains is minimal and subsequently it should be expected that also the
alloreactivity after transplantation might show a minor degree of
variation. In allografted animals activation probably occurs with some
time difference in the initiation of the immune response in the individual
animals. As a result of this also the effector phase may not be
synchronous, even in these highly identical inbred rats. Sufficient numbers
of experimental animals will reveal changes and shifts and will help to
resolve the mechanisms involved. But variability in data as found in this
study makes one sceptical about the possibility to get insight into the
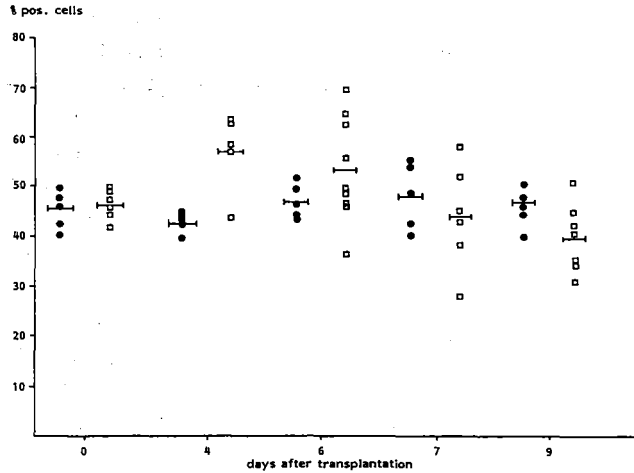ongoing process on an individual basis for diagnostic or prognostic

Figure 4.3 MRC OX-19 staining figures in mesenteric lymph node cells at various days after kidney transplantation in allogeneic (□) and the isogeneic (●) combination.

purposes, as is the case in clinical transplantation. All data shown furtheron are mean data of at least 5 animals.

The **second** item of importance is the way in which the infiltrating cell population is defined i.e. which marker is used to define such cells. This is illustrated for the T cell population by comparing three different methods to stain and monitor the T cell population. The T cell population is analyzed by the reactivity of MCA ER-1, MRC OX-19 and a combination of ER-2 and MRC OX-8. MRC OX-19 reacts with all T-lymphocytes in the peripheral lymphoid organs (Dallman et al, 1983). However, also activated T cells and cells with an immature phenotype have been reported after transplantation (Nanni Costa et al, 1983; Chatenoud et al, 1983). The reactivity of MRC OX-19 with early T cells and activated T cells still remains to be determined. For this reason also an estimation of the T cells by the expression of ER-1 antigen, as well by the summation of ER-2 and MRC OX-8 positive cells was done.The latter two approaches to enumerate the T lymphocyte population have a disadvantage: also non T cells express these surface antigens (Table 4.1), such as plasma cells (ER-1^{+}), macrophages (ER-2^{+}) and natural killer cells (MRC OX-8^{+}). In Table 4.2 the results of these experiments are summarized for the total T cell population. A comparison of the reactivity of MRC OX-19 with ER-1 revealed that only in the mesenteric lymph nodes of isogeneic grafted animals

comparable cell numbers were labelled, which is well in agreement with the
reactivity detected in the lymph nodes of untreated animals (chapter 3).
This illustrates that the choice of the MCA to define a population is quite
important for the interpretation of the results. All the markers indicate
an increase of the percentage of labelled cells shortly after allogeneic
transplantation, in lymph nodes and blood. Different patterns were found
during the later course of the rejection. In all organs a steady decrease
of MRC OX-19 and the ER-2 and MRC OX-8 reactivity in the last stage of the
rejection was detected (Table 4.2). This pattern was however, not followed
by the ER-1 reactivity. The reactivity of this marker remained unchanged
or, as in spleen, even increased. This suggests a significant involvement
of cells carrying the T cell determinant recognized by ER-1, but not the
other T cell markers, as will be discussed later. This illustrates the
necessity to bear continuously in mind that one should be reluctant to
translate directly phenotype changes into actual cellular (functional)
shifts.

Table 4.2 Flow cytometric analysis of the T cell population after
transplantation

Days after transpl.	Spleen			MLN			PBL		
	A[a]	B	C	A	B	C	A	B	C
Isogeneic									
0	34.5[b]	45.8	47.8	45.7	48.5	52.0	46.8	56.7	59.2
4	32.3	45.8	45.7	43.1	43.8	52.3	42.3	57.2	56.9
6	34.2	46.4	48.3	47.5	45.1	54.3	47.2	62.5	58.7
7	35.7	--	48.0	48.2	--	56.3	47.6	--	61.5
9	34.2	44.0	47.9	46.6	46.8	58.8	48.5	57.7	58.2
Allogeneic									
0	33.6	45.8	47.8	46.1	48.5	52.0	46.8	56.7	59.2
4	35.5	38.5	46.8	57.1	54.5	79.3	57.2	61.3	70.3
6	33.1	42.3	44.8	53.8	57.1	68.3	48.0	61.0	67.0
7	21.5	--	42.1	44.2	--	60.3	39.3	--	48.6
9	23.0	52.6	29.1	39.7	50.7	51.2	36.7	53.5	53.6

a. The total of T-lymphocytes has been expressed in three different ways:
   A. MRC OX-19 positive cells, B. ER-1 positive cells and C. cells positive
   for MRC OX-8 or ER-2.
b. PVG recipient rats received a kidney of PVG.RT1[a](DA/Olac) donors. For
   each time point after 5 animals were used for monitoring. Cells were
   and stained with directly FITC-conjugated MCA as described before. The
   standard error of the mean (SEM) did not exceed the 15 percent level
   (unless stated).

Thirdly, when comparing data at various times after transplantation one should not limit oneself to the relative reactivity as expressed in the percentages, but also absolute numbers should be determined. Since it has been reported that after allogeneic transplantation the weight of the spleen highly increases (Nemlander et al, 1982), also the absolute number of viable nucleated cells in spleen and blood has been determined. In the cell suspensions, prepared from the spleen and the peripheral blood, the total of viable nucleated cells was determined. Within the isografted animals the spleen and PBL cell numbers remained rather constant, suggesting a quiescent lympho-hematopoietic system. In the allogeneic grafted animals a decrease in the total number of nucleated spleen cells was detected four days after transplantation (Table 4.3). Subsequently a recovery of the cell numbers could be observed and even a increased number of nucleated cells was found at day 7 and 9 after transplantation both in the spleen and in blood.

Table 4.3 Total number of viable nucleated cells in spleen and blood after transplantation

Day	Number of rats	Spleen[a] $(x\ 10^8)$	Blood[b] $(x10^6)$
0	17	5.1 ± 1.8^c	8.4 ± 1.8
4	6	4.5 ± 1.6	6.6 ± 2.4
6	9	4.5 ± 1.8	9.5 ± 3.2
7	8	7.7 ± 1.7	14.8 ± 3.1
9	7	7.2 ± 1.5	13.5 ± 3.5

a. Single cell suspensions of the spleen were prepared and the leukocytes were counted on a cell counter.
b. Blood cells were counted after lysis of the red blood cells
c. Mean values ± SEM are shown

Isogeneic combination

When the percentage of cells expressing the various markers is determined at various times after transplantation in the spleen, lymph nodes and blood of isogeneic transplanted rats, no significant changes were found (Table 4.2). Although a mild, transient ATN was observed in these animals (previous section) at day 4, this had obviously no impact on the immunological status of the animals as determined by phenotyping of the lymphoid cell population, since also no changes were observed in overall cellularity in spleen and blood (data not shown).

Shifts found in allogeneic transplanted rats can therefore be attributed to the allograft directed rejection process, instead of surgical procedures.

Allogeneic combination

When looking in **spleen** at the percentages of cells positively stained in the allogeneic transplanted animals for all MCA including for ER-2, MRC OX-8 and ER-9 (data not shown), with the exception for ER-1, a decrease with time was found (Table 4.2). However, when corrected for the total number of cells, the absolute numbers revealed quite a different pattern. As shown in Table 4.4 no significant changes can be detected throughout the observation period for MCA MRC OX-19 and ER2 plus MRC OX-8. Shifts in the lymphoid cell compartment are therefore most likely caused by non-relevant changes in the non-lymphoid populations in the spleen.

Table 4.4 Change in cell numbers of various T-lymphocyte (sub)populations in blood after allogeneic transplantation

	MCA				
Days	ER-1	MRC OX-19	ER-2	MRC OX-8	ER-9
Spleen					
0	100[a]	100	100	100	100
4	74	94	86	90	93
6	81	88	93	72	86
7	--	94	126	123	107
9	162	102	93	98	93
Blood					
0	100[a]	100	100	100	100
4	85	103	152	92	105
6	122	122	187	162	168
7	--	157	183	223	247
9	152	135	204	192	184

a. Analysis of cells after staining with FITC-MCA has been performed on a FACS II. The absolute number of positive cells are expressed as percentage of the cell numbers detected in untreated animals. Figures within the squares are significantly increased, compared to the numbers of day 0.

However, only the ER-1 positive population increases significantly in the spleen as the rejection process goes on. This is true both for relative and absolute numbers (Table 4.2 and 4.4). These ER-1 positive cells in the later period of the rejection may be an indication for the presence of elevated numbers of cells with an immature phenotype. Baldwin et al (1982) reported that in untreated animals after transplantation a 40 to 50% decrease in cortical and medullary thymocytes was found. The possibility of immature cells was also confirmed by another MCA i.e. ER-4, which is mainly reactive with immature cells in the thymus (Previous chapter). In normal animals ER-4 reacted with 7.6 ± 2.1 percent of the spleen cells, but six days after allogeneic transplantation this reactivity was increased to 36.0 ± 3.1 percent. Therefore an influx of immature T-cells derived from the thymus may be partly spleen bound. The most likely candidates for such cells, displaying an immature phenotype is the cortical population of the thymus i.e. cells that express the surface antigen of ER-1, ER-4, ER-2, ER-9 and MRC OX-8 and are negative for MRC OX-19, this represents the dominant cell population of the cortex. Any medullary cell participitation of thymus migration would be difficult to detect since these cells have a mature phenotype. The presence of W3/25$^+$, MRC OX-8$^+$ in an activated immune system was reported by Dallman et al (1985) and also preliminary results of double fluoresence staining indicated the presence of this type of cells
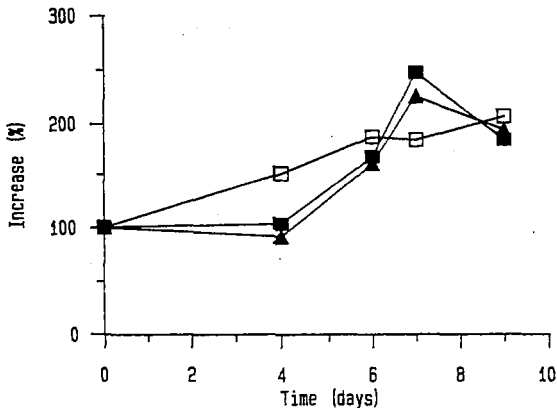

Figure 4.4 Fluctuation in the absolute number of T-lymphocyte subpopulations in blood after allogeneic kidney transplantation. The cell numbers stained with MCA ER-2 (□), MRC OX-8 (▲) and ER-9 (■) were analyzed on a FACS II. Cell numbers are expressed as percentage of the normal values detected in untreated animals. The figures are shown in Table 4.4.

upto 10 percent of spleen cells after stimulation (data not shown).

Although the T-cell population of the spleen after allogeneic transplantation seems to be in an equilibrium this is in fact a highly dynamic kind of equilibrium. Cell accumulation has been described after i.v. antigenic challenge, (Emeson, 1977). Also allografts induced an accumulation in the spleen (Ford, 1975; Satake et al, 1982; Kupiec-Weglinski et al, 1982) and in the graft (Chang and Sugarbaker, 1981) in the initial stage of the rejection, but declined later on. Intragraft labelling of infiltrating cells did confirm at least for these cells a considerable cell traffic between the graft and the spleen (Nemlander et al, 1982).

In **blood** variations in percentages for all of the markers analyzed obscured any upwards or downwards tendency, if present at all Table 4.2). However, if the absolute numbers were calculated a general clear-cut pattern was revealed (Table 4.4). All populations studied increased during time after transplantation, although sometimes with a different starting kinetics (Figure 4.4). ER-2, for instance, rises immediately at day 4, whereas the other markers remain at normal levels at first and get higher only at day 6 or later on (Table 4.4). Also the abundance of cells carrying the ER-2 and MRC OX-8 markers at later stages of the rejection period as compared to the overall T cell markers ER-1 and MRC OX-19 is striking. This is also suggestive for the presence of double positive cells for ER-2 and MRC OX-8 (Table 4.4).

Hardly any differences in percentage of positive cells between isogeneic and allogeneic rats could be found for the MCA MRC OX-19, ER-1, ER-2, MRC OX-8 and ER-9. However, this "quiet" picture is disturbed when looking at other MCA. Large differences were found for MCA ER-13, ER-4 and MARK-1. These latter antibodies recognize respectively class II MHC (Ia) positive cells (ER-13), thymocytes and prothymocytes (ER-4) and B cells (MARK- 1). This is illustrated in Table 4.5 for day 6 after transplantation. This change indicates that the blood is very actively involved in the rejection process from the start on. Since at day 6 no major quantitative differences in PBL's exist between the two groups (Table 4.3) this pattern is rather representative for what is going on in the blood. Normally the expression of class II MHC products is restricted to cells of the B cell lineage (Fukumoto et al, 1982). Since the number of B cells (MARK-1) did not change dramatically (Table 4.5), the higher expression of class II MHC antigens must be attributed to other cells than

B cells. Since also T cells can acquire class II MHC expression upon stimulation (Gillman et al, 1982) the increased ER-13 positivity is most likely caused by the presence of activated T cells. As in spleen also in the blood high numbers of cells carrying an immature T cell phenotype (ER-4) are found after allogeneic transplantation. These results demonstrate that in case of the allogeneic transplanted rats a considerable number of activated T-lymphocytes as well as a considerable amount of cells displaying an immature phenotype appear in the blood circulation. These observations have been confirmed in human renal allografts by the incidental finding of $CD8^+/CD4^+$ cells in the blood (Chatenoud et al, 1984).

Table 4.5 Phenotypic composition of PBL's after
6 days of transplantation

Antibody	Isogeneic	Allogeneic
MRC OX-19	48.1 ± 2.1	47.2 ± 4.1
ER-1	62.5 ± 2.2	61.0 ± 2.7
ER-2	43.6 ± 4.4	44.9 ± 1.9
MRC OX-8	15.1 ± 0.9	22.1 ± 3.0
ER-9	25.6 ± 3.0	33.3 ± 3.8
ER-4	10.0 ± 2.1	29.3 ± 6.9
ER-13	17.3 ± 2.2	39.0 ± 6.8
MARK-1	13.8 ± 1.5	17.6 ± 2.0

a. PBL's were isolated 6 days after transplantation,
   stained with FITC-conjugated MCA and analyzed
   using a FACS as described before. The mean
   percentage of positive cells ± SEM of 5 animals
   are shown for both combinations.

Furthermore, these data show that monitoring of the total T cell population and the two major subpopulations provide insufficient information about a rejection in progress. When the ratio between the helper and the suppressor/cytotoxic T-cells, which is a parameter used in many transplantation centres (Burton and Cosimi, 1983), are calculated for the control animals (isogeneic) a ratio of 2.87 ± 0.57 (n=6) is found and for the other group (allogeneic) a ratio of 2.48 ± 0.82 (n=8) is observed. It will be evident that in this model these parameters are unable to indicate a rejection for the individual animals. Although the untreated rejection model as used in this study, does not entirely reflects the clinical situation, where a low dose of immunosuppressive agents are applied in non-rejecting episodes, Guttman and Poulsen (1983) reported comparable $T_h/T_{s,c}$ ratio's both in well functioning and rejecting human

graft. The ratio may be a useful tool for the prediction of infections such as cytomegalovirus (Metselaar et al, 1986), but as indicator of a starting renal failure episode (RFE) it is inadequate. T cell activation markers recognizing receptors of IL-2 (Schubert et al, 1986) and transferrin (Hoshinaga et al, 1986; Brando et al, 1986) or the class II expression of T-lymphocytes and also immature markers such as described in this thesis seem to be more useful markers for indication of an activated T-cell system. For an absolute prognostic parameter for RFE in the recipient, however, only the measurement of a specific anti-donor response will give an adequate answer.

However, besides looking at the immune system of the recipient a more direct approach is monitoring of the graft itself. In the next section analysis of the infiltrating cells will be described using immunohistochemistry.

GRAFT INFILTRATING CELLS

To analyse the in situ immune reactivity, transplanted kidneys were removed at various times after grafting and used for immune histology. Part of the kidney was therefore snap-frozen in liquid nitrogen. Serial cryostat sections of the graft were stained with a panel of MCA, which are summarized in Table 4.1. The MCA used in this experiment are directed against T-cells (ER-1 and MRC OX-19), T cell subsets (ER-2, MRC OX-8 and ER-9), B-cells (MARK-1), macrophages (ED-1) and against class II MHC antigen (ER-13 and F17-23-2). The reactivity of the antibodies on the sections was visualized using a peroxydase technique as described in the third chapter. Six grafts were analyzed for each time point after transplantation.

As a control isogeneic grafts were stained and analyzed. In these animals small infiltrates both perivascular and peritubular, were detected starting at six days after transplantation, slightly increasing in number from day 12 on. However, the size of these infiltrates remained much smaller than the ones observed in the allogeneic group. Some reactivity was observed for ER-1, ER-2 and ED-1. Hardly any cells reacted with MRC OX-19 or with ER-9 in these animals, whereas only a minor part of the cells were MRC OX-8 positive.

In the allogeneic grafted animals two different types of infiltration can be seen after transplantation. First of all part of the

infiltrate is located in close vicinity of the arterioles i.e. the socalled perivascular infiltrates. These infiltrating cells are found in close connection with each other, such as is illustrated in figure 4.5. The remaining infiltrating cells, the socalled peritubular cells are scattered throughout the entire kidney and these cells are not found in clusters.

The perivascular infitrates increase in frequency and in size during the course of rejection. Several small (10-30 cells) infiltrates and incidentally a larger one (100-300 cells) were detected at four days after transplantation (figure 4.5). Whereas almost all cells were class II positive (ER-13), approximately 60 percent reacted with ER-1 (figure 4.5A). No B-cells (MARK-1) were found. Nearly equal numbers (±30%) of these cells reacted with ER-2 (Fig.4.5B), MRC OX-8 (C), MRC OX-19 (D) and ER-9 (E). Also macrophages (ED-1), with a branched shaped, non-lymphoid morphology were detected in these infiltrates with an estimated number of 30-40 percent (Fig. 4.5F), while ER-4 reacted with a low number of predominantly irregularly shaped cells. After day 3 the incidence of these perivascular infiltrates, but also the number of cells per infiltrate increased (day 4: Figure 4.6A), while at six days these infiltrates started to confluence (Figure 4.6B) resulting in large clusters of infiltrating cells. These clusters consisted of round cells and were found throughout the kidney from day 7 on (Figure 4.6C). In most of these infiltrates the MRC OX-8 positive cells were somewhat higher in number than the ER-2 reacting cells (3:2), whereas the ER-1 recognized approximately 70-80 percent of these cells, which seemed to be a summation of the cells stained by ER-2 and MRC OX-8. A decline of ER-9 positive cells was found, resulting in a low number of strong positive cells at day 6 and almost negative reactivity later on. Also the cells defined by MRC OX-19 decreased in number (20% at day 6) and the intensity of the staining was very low. An even further reduction to <10% was detected in last phase of the rejection.

At the peritubular sites, both in the cortex and the medulla, round shaped cells were detected at day 3 reacting with ER-1 and ER-13. Increasing numbers were found at day 4 and day 6, while in a later phase these numbers tended to decline, however, damage of tissues by the rejection mechanism interfered in several of these sections with a clear interpretation of these data. Only part of this type of cells was positively stained with MRC OX-8 (Figure 4.7B), while only a low number of round cells reacted with ER-2. The MRC OX-8 reactivity showed a comparable increase in these area's as described for ER-1. Besides these cells a
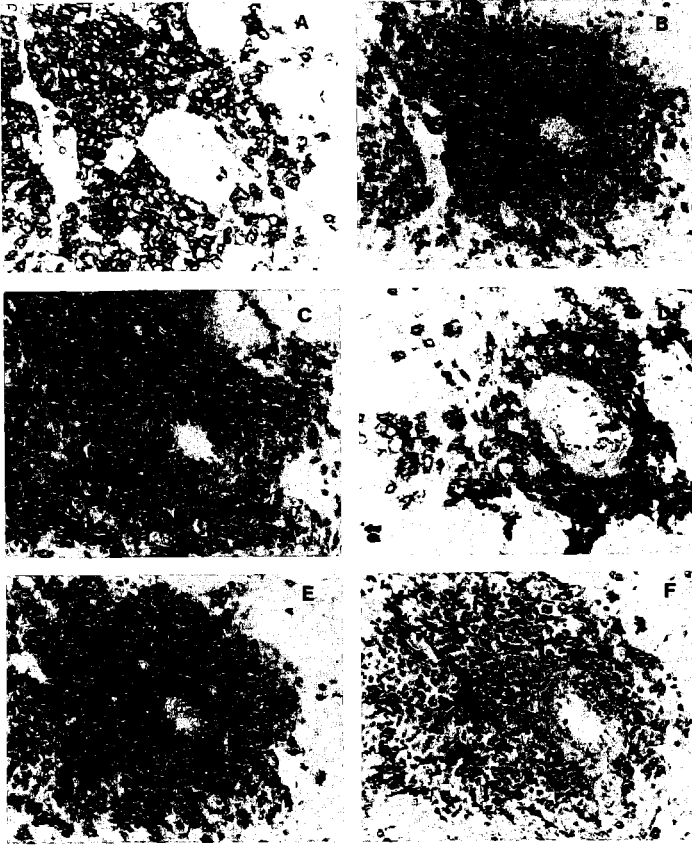
Figure 4.5 Perivascular infiltrate in renal allograft at 4 days after allogeneic kidney transplantation. Serial sections of the graft were stained with MCA ER-1 (A), MRC OX-19 (B), ER-2 (C), MRC OX-8 (D), ER-9 (E), and ED-1 (F) respectively.

Figure 4.6 Increased numbers of infiltrating cells after allogeneic transplantation. The ER-1 positive cell population is shown at day 4 (A), day 6 (B) and day 7 (C) respectively.
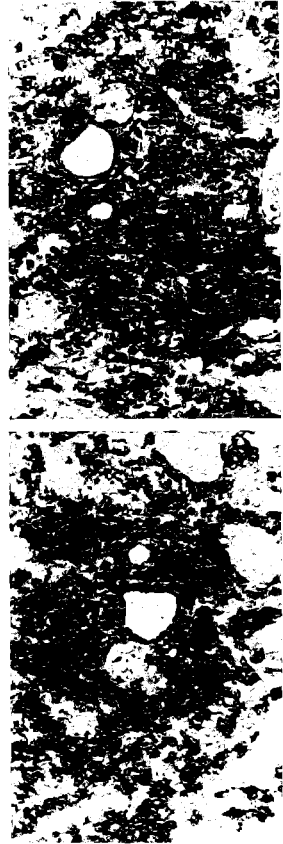
Figure 4.7 T-cell subpopulations of perivascular area's at 6 days after allogeneic kidney transplantation. Serial sections of the graft were stained with MCA ER-2 (A) and MRC OX-8 (B) respectively.

comparable number of monocytes (round shaped) and macrophages were detected in peritubular area's at day three after transplantation. These cells were defined by MCA ED-1. This was confirmed by the finding that a comparable amount of irregular shaped cells (macrophage-like) cells reacted with MCA ER-2 (Figure 4.7A). The number of macrophages increased at day 4 and at day 6 they were found in large numbers scattered throughout cortex and medulla. Some of the grafts at day 6 and in more cases at a later stage showed a declining number of these macrophages approaching the numbers detected at day 4. Only low numbers of ER-9 or MRC OX-19 positive cells were detected outside the vascular infiltrates and this reactivity even decreased after day 4.

The class II reactivity in these kidneys visualized by ER-13 was difficult to interprete, since the tubuli themselves were also positively stained. Additionally the Bowman's capsules and in the last days of the rejection episode also the endothelial cells of the arterioles became positive. From a comparison with the pattern obtained by staining with MCA F17-23-2, which recognizes only donor-type class II MHC antigens, it was nevertheless clear, that also the majority of infiltrating cells, both macrophage-like and lymphocyte-like, did express high levels of class II MHC antigens.

The described presence of predominantly CD8-like positive cells (MRC OX-8) in the graft and only a minor population of CD4-like (ER-2) cells was in accordance with observations by several authors (Renkonen et al, 1983; Claesson et al, 1985; Bradley et al, 1985). This was demonstrated by both cell isolation techniques and immunohistochemistry. The ratio between MRC OX-8 and ER-2 observed in allogeneic grafts might be the result of a more pronounced role of the CD8 defined population during the process of alloreaction against the graft. The lack of specificity of ER-2 and MRC OX-8 was a disadvantage for a clear interpretation of the results, since high numbers of large granular lymphocytes (LGL), which are MRC OX-8 positive (Reynolds et al, 1981) were reported by Nemlander et al (1983) to infiltrate the graft directly after transplantation. Also macrophages have been reported to infiltrate in large numbers in the graft (Christmas and MacPershon, 1982b; von Willebrand et al, 1979). These cells express the antigen defined by the MCA ER-2. In this respect a new MCA, specifically reacting with macrophages, (Dijkstra et al, 1985) is of importance. This MCA, ED-1 enabled a segregation of the macrophage and the helper T-lymphocyte recognition of MCA ER-2. ED-1 stained both round cells

(supposedly monocytes) and irregular shaped cells in the parenchyma. The results indicated that the T-helper cells were mainly located within the perivascular infiltrates together with suppressor/cytotoxic T-cells and some macrophages. The ED-1 positive macrophages were non-lymphoid in morphology, which was was in contrast to the ER-2 positive cells. The majority of ER-2 positive cells outside the perivascular areas showed a macrophage type of morphology. This seems to be confirmed by histological studies in man, where it has been shown that the lymphoid cells were almost completely located within the perivascular sites. Mononuclear phagocytes on the other hand were situated in other places (Christmas and MacPershon, 1982a).

The high expression of class II antigen on the donor type cells of the kidney is most likely induced by γ-interferon produced by activated T-lymphocytes (Pober et al, 1983; Morhen et al, 1984). The MCA ER-13 and F17-23-2 enable a separation of the class II antigens in recipient and donor type. These data revealed that the infiltrating cells at the perivascular sites do express high amounts of class II antigens. These sites contain T-lymphocytes of the CD4-like and CD8-like population together with macrophages, which may function as antigen presenting cells. Therefore these perivascular cuffs seem to represent lymphoid compartments of the recipient with a proper microenvironment for the host reaction against the graft. The presence of host derived macrophages suggests that graft antigen might be processed by these cells. Consequently the donor class I antigens, but also the non-MHC antigens may be presented in a MHC restricted fashion to the immunocompetent cells of the recipient, as has been suggested by Burakoff (1985). Another source of antigen may be the endothelial cells of the arteriole. A positive correlation of the class II expression of these cells with rejection has been reported (Häyry et al, 1981a and 1981b; de Waal et al, 1983; Milton et al, 1986) and the close relation of the perivascular infiltrates with the arterioles might indicate an antigenic role of the endothelial cells for the recipient infiltrating cells. In contrast to the first process this kind of response would be a non-MHC restricted anti donor class II response.

For a proper distinction between T-lymphocytes and other cell types the MRC OX-19 MCA might be a useful marker. This is certainly true for the initial posttransplantation period, when the antigenic expression seems to be rather normal. However, later on the reactivity pattern becomes more complicated. In the last stage of the rejection a remarkable decrease in

reactivity with both MRC OX-19 and ER-9 could be notified in all grafts. Normally it is assumed that the non T cell reactivity of MRC OX-8 and ER-2 (W3/25) is caused by the presence of many NK-cells (MRC OX-8) and macrophages (ER-2) and this causes the discrepancy between these MCA and MRC OX-19 (Bradley et al, 1985). As a consequence one has to assume that in our experimental system only a very low number of T-lymphocytes remain in the graft at the effector stage. This would be highly in contradiction with most data in rejection of grafts (Forbes et al, 1983; Christmas and MacPershon, 1982b; Tilney et al, 1975). With the assistance of the MCA ED-1 this partial non-lymphoid reactivity of ER-2 could be verified. In the perivascular sites the number of ER-2$^+$ cells and their morphology are different from the ED-1 population. This suggests that at least part of the ER-2$^+$ MRC OX-19$^-$ cells do not belong to the macrophage lineage. Although it was not possible to separate the MRC OX-8 positive cells into T-cells and NK-cells, Nemlander et al (1983) reported that most LGL's leave the graft at approximately four days. The large number of MRC OX-8 positive cells in the graft at day 7 and later on may be in part NK-cells, but at least one has to assume that also a considerable number of these cells belong to the T-cell lineage. In contrast to the graft the cell numbers recognized by both MRC OX-19 and ER-9 in the recipient at the same time after transplantation was comparable with the other subset markers. Therefore this depletion seems to be specific for the graft during the effector phase. An explanation for this phenomenon can be found in the unique degree of activation, since in the graft a high degree of specific activation is combined with activation induced by lymphokines. In the other lymphoid organs including blood only donor specific activation occurs and consequently the activation will not be to same extent as in the graft. Functional studies described in the chapter 3 showed that the surface molecules defined by these two MCA (MRC OX-19 and ER-9) are involved in the initial stage of T-cell activation. It may well be that these surface antigens are lost after the earliest process of activation or that modification of the antigen in this stage interferes with binding of these antibodies. In the recipient a depleted reactivity might not be observed in the total pool of the lymphocytes. The depletion of the MRC OX-19 population may also be partly explained by immature T-lymphocytes. An influx of cortical thymocytes has been reported by Baldwin et al (1982) in the end stage of the rejection. The low numbers of ER-4 positive cells in the grafts might be an indication for this observation and also the low

intensity of MRC OX-19 staining in the grafts after day 6. Cortical thymocytes express this antigen in a lower density than medullary and peripheral T-lymphocytes (Dallman et al, 1982). These data, but also the results of the fluorescence analysis in spleen and blood after transplantation favour an influx of immature T-lymphocytes, which are probably partly MRC OX-19 negative.

A participation of B-lymphocytes and plasma cells in the rejection mechanism has been described by several authors (Duarte et al, 1981; Renkonen et al, 1983; Garovoy et al, 1979). Besides an indication of increased numbers of plasma cells in the spleen after transplantation we could not confirm this, since hardly any of these MARK-1 positive cells could be detected in the grafts. Since the acute type of rejection is predominantly a T-cell defined mechanism it was suggested that the presence of B-cells in the graft was caused by a polyclonal activation as an spin-off of the strong T-cell activation (Duarte et al, 1981), which was supported by *in vitro* findings of Garovoy et al, 1979 and Pobor et al, 1983. Another explanation is that the B-cell response is directed against non-MHC antigens (Häyry et al, 1984). In the congenic strain combination used in our experiments no difference in non-MHC systems exists and this correlates with the absence of a humoral component in the rejection process.

Whereas the monitoring of the immune system of the recipient only provided indirect indication of an ongoing rejection episode, phenotypical analysis within the graft itself gives more direct information. Although also in isografts, in a non-rejecting situation, graft infiltrating cells are found the high degree of class II induction is not found in these animals. Therefore monitoring within the graft itself seems a better parameter for the prognosis of a RFE than screening in the blood. The recently described technique of fine needle aspiration biopsy (FNAB) can be applied with a high frequency without causing disrupture of the organ (Häyry and von Willebrand, 1981).

Besides the phenotype of the various populations of immunocompetent cells a more direct approach is to measure the donor specific immune response of the recipient. In the next section this donor specific response of the host is described.

SPECIFIC CYTOTOXIC T-CELL RESPONSE

In addition to studying changes in composition of defined lymphocyte subsets through cell surface marker analysis after allogeneic organ transplantation as a reflection of an ongoing rejection, one can also focus on the specific allogeneic response itself and try to correlate such measurements with the data obtained in the other types of analyses. Although such a response comprises a variety of immunological effector functions we limited ourselves to the cytotoxic T cell reaction, since this is considered to be a major contribution to the actual damaging process during rejection (Cerottini and Brunner, 1974; Moreau et al, 1985; Bradley et al, 1985). This section describes experiments, which were performed to analyze on a comparative and quantitative basis the specific cytotoxic T cell (CTL) response both in the central lymphoid system as well as in the graft at various times after transplantation. In order to do so the number of precursor CTL was determined using the method of limiting dilution assay (LDA), which was extensively described by Lefkovits and Waldman (1979). The CTL activity was determined with a micro assay system of the MLR for the generation of CTL. The method, basically comparable to the macro assay described in the previous chapter, needed adaptation to cell growth under limiting dilution conditions.

Assay system

The precursors of the CTL (CTL-p) were determined in the spleen, the blood and in the graft at various times after transplantation. Both single cell suspensions of spleen and the isolation of PBL were done as described before. The renal graft was isolated and minced into small pieces and subsequently carefully squeezed through a vitallium wire mesh (0.003 inch). This suspension was filtered through nylon-gauze to obtain a single cell suspension, whereafter the lymphocyte fraction was isolated by Ficoll/Isopaque gradient (δ 1.077) centrifugation as described before. The viability of such cells always exceeded the 90 percent value. Variable cell numbers of responder cells were injected into wells of microtitration plates (round bottom shaped) together with 10^5 of irradiated stimulator cells (3000 rad). The cell mixture was grown in RPMI-1640 culture medium supplemented with growth sustaining factors, which will be described in detail. Cells were grown for 6 days in a total volume of 200 μl of medium.

After six days the CTL activity was determined by the capacity to lyse target cells in a CML-assay. The target cells used in the CML were Con A stimulated (72 hrs) spleen cells, that were labeled with ^{51}Cr and lysis of the target cells results in release of the ^{51}Cr. For the CML-assay 10^4 target cells (20 μl of medium) were injected in each well and the suspension was carefully mixed by pipetting. After four hours of incubation the supernatants were collected with supernatant harvesting cartridges (Skatron, Oslo, Norway) and the radioactivity was determined in γ-counter.

For a proper LDA it is necessary to test the sensitivity of the culture conditions in order to verify that only the tested cells are present in limiting amounts. The optimalization of the culture system requires much attention, for example IL-2 containing supernatant was described as an essential component for this test system (Wagner et al, 1980; Miller, 1982). The medium was supplemented therefore with FCS and T-cell growth factor (TCGF) as a source of exogenous IL-2 and other differentiation factors. The source of FCS was selected by testing several batches of FCS for growth sustaining under limiting dilution conditions as described in the second chapter. For these experiments FCS of Boehringer (Mannheim, Germany), batch nr. 688.829 has been used in a final concentration of 10 percent. As a source of TCGF supernatant Con A stimulated spleen cells harvested after 48 or 72 hours of culture was used. The supernatants were tested for IL-2 content using a IL-2 dependent T cell line (CTLL) as described before (Gillis et al, 1978). TCGF supernatant was added to the culture medium in a concentration of 5% (v/v). Besides these two factors also 2 x 10^5 irradiated thymocytes of the responder strain as feeder cells were added to each well. Furthermore the availibility of adequate numbers of accessory cells for the growth, differentiation and function of the CTL-p cells need attention, while on the other hand also suppressor cells might introduce a complicating factor to the system (Corley et al, 1978).

The sensitivity of the LDA to detect CTL-p in the rat, was tested with spleen cells of DA/Olac (RT-1^a) rats at four days after transplantation of a kidney of RP/Rij (RT-1^{u,l}) rats. Spleen cells were isolated and MLR cultures of these cells with irradiated spleen cells of the donor strain were started. In the CML-assay the presence of effector cells was tested on target cells of the WAG/Rij strain, which express the same class I MHC antigen as the donor strain. The spleen cells were tested

100-

80-

% lysis

60-

40-

20-

0.06 0.125 0.5   1.0   2.0   3.0   4.0   5.0   6.0   8.0   10.0 12.0
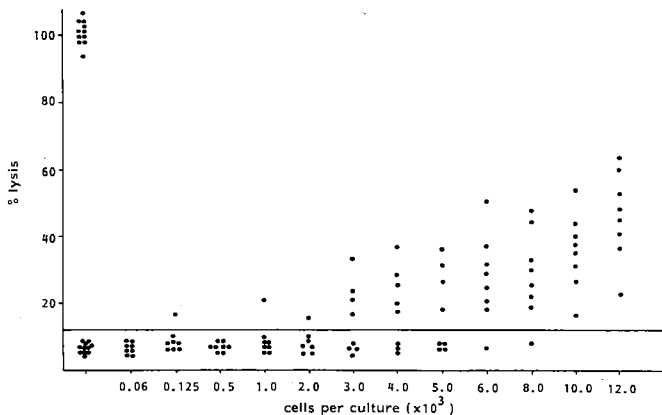
cells per culture ($\times 10^3$)

Figure 4.8 Donor specific cytotoxicity of spleen cells under limiting dilution conditions. Cells of DA rats were cultured with irradiated cells of RP rats and the effector cells were tested for specific cytotoxicity with target cells of WAG rats. In the first collumn the spontaneous [51]Chromium release and the maximal release are shown. The specific release of the cultures has been expressed as percentage of the maximal release.

in numbers of 60 to 1.2×10^4 cells per well and each dilution was tested in 8-fold replicate cultures. The percentage of lysis was expressed as: $cpm_{exp.}/cpm_{total} \times 100$, in which $cpm_{exp.}$ is the radioactivity in the culture supernatant of test cultures and cpm_{total} is the maximal release, defined by lysis of the same number of target cells, as described before. Cultures were regarded to be positive, when the radioactivity counted in the culture supernatant exceeded the value of the spontaneous release plus 3 times the standard deviation. At a cell dose of 5×10^2 per well incidental cultures became positive, whereas at a cell number of 1.2×10^4 all cultures showed a positive target cell lysis (Figure 4.8). In the macro system described in the previous chapter the same number of target cells were used, but in that system 10^5 effector cells were required to obtain a reproducible target cell lysis. This indicates that the culture system used in this experiment is more sensitive than the one used in the macro system.

For the estimation of the frequency of the CTL-p within a cell population it should be defined whether the data of the dose response experiments correspond with a single hit model. This model assumes that one single CTL-p cell is needed for a positive cytotoxic reponse. With the

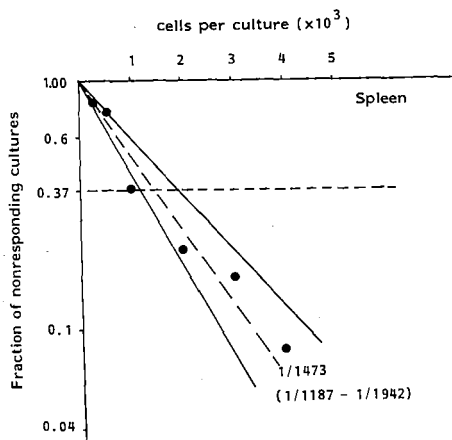cells per culture ($\times 10^3$)

Figure 4.9 The estimated frequency of the donor class I specific CTL-p in unseparated spleen cells at 8 days after transplantation. The frequency has been calculated by the method of weighed mean estimation and the 95% confidence interval is shown (Taswell, 1981). Each dose of spleen cells was tested in 24 fold replicate cultures and the fraction of negative responding cultures was determined as described in the text.

assumption that the test cell suspension is homogeneous the distribution of these cells is described by the Poisson probability distribution (Haight, 1967). In the single-hit model only a discrimination between positive and negative cultures is made and distinction between cultures with one or more responder cells is not possible. Therefore the incidence of the negative cultures is the important factor in LDA analysis. The fraction of negative cultures (no cytotoxic activity) is defined by the zero term of the Poisson distribution: $Fo = e^{-u}$. This can be transformed in its logarithimic form: $u = -\ln Fo$, in which Fo is the ratio of negative cultures and the total of tested cultures and u is the mean number of CTL-p per well. When the fraction of negative cultures reaches the value of 0.37 the number of CTL-p per well (u) has the value 1. When the fraction of the non-responding cultures, on a log scale, are plotted against the cell input per well, on a linear scale, a straight line corresponds with single hit kinetics (Lefkovits and Waldman, 1979). More complex statistical methods to calculate the frequency and test the single hit model were described by Taswell (1981).

To test the applied culture conditions for detection of a single CTL-p, spleen cells of DA recipients at 8 days after allogeneic transplantation were isolated and MLR-cultures with limiting amounts of these responder cells were started and each dose was tested in 24 replicate cultures. In the CML-assay the specific target cell lysis was determined with WAG derived target cells and subsequently the fraction of responding versus the nonresponding cultures was determined and plotted (figure 4.9) as described above. The CTL-p frequency was calculated using the method of the weighed mean estimation with the 95% confidence interval (CI) as described by Taswell (1981). The frequency of donor specific (class I antigen) CTL-p in the spleen had an estimated value of 0.000679 ± 0.000084 with a 95% CI between 0.000842 and 0.000515 i.e 1 CTL-p cell on 1473 spleen cells.

A primary criterion for the validity of this estimation is the agreement of the data with the expected Poisson distribution. A plot of the fraction of nonresponding cultures against the cell dose should give a straight line for single-hit kinetics. Fitting of these data to a straight line can be tested by the Chi square test of goodness of fit, which gives a corresponding probability value for the tested hypothesis. The results of the experiment was tested and a Chi square value of 2.5582, with 5 degrees of freedom was calculated, which corresponds with a probability value of 0.42 (Tables of chi-square distribution). This value allows a satisfactory acceptance of the single-hit model for the the tested cell population, since the hypothesis is not rejected on a 5% level of significance (Lefkovits and Waldman, 1979; Taswell, 1982). The culture conditions applied in the micro assay system are therefore adequate to sustain cell growth in limiting numbers. Furthermore these data indicate that no suppressor cells are actively involved in this system. The latter would result in a highly different kind of kinetics (Lefkovits and Waldman, 1979). Although the Poisson distribution decribed above can be taken as evidence that a single CTL-p is detected, it must be emphasized that the efficiency of detection of CTL-p cannot be assessed with certainty. Therefore the frequency values described in the remaining sections should be regarded as minimal estimates.

Transplantation analysis

In a series of transplantation experiments the fluctuation of the estimated frequency of the CTL-p was determined in spleen, blood and in the
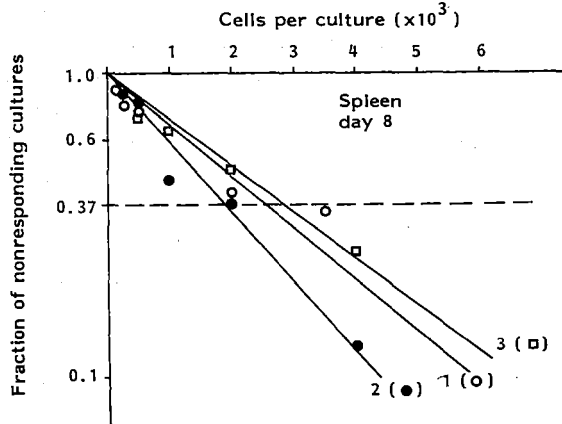

Figure 4.10 Variation of the estimated CTL-p frequency after allogeneic (day 8) transplantation. Spleen cells of 3 recipients were isolated and tested in in a single experiment. Each cell dose has been tested in 8 fold replicate cultures.

graft. For several reasons a somewhat different setting of the experimental approach was chosen. First of all no data exist on either the values of allospecific CTL-p frequencies in the rat system or on the degree of variation after transplantation. Therefore a wide range of different cell doses had to be tested in order to make sure that the relevant cell doses were included in the test range. Furthermore we focussed on the MHC class I directed CTL activity by using specific cell targets. For a correct comparison the time schedule for transplantation was designed in such a way, that on the same day always two animals of each time point (day 4,6 and 8) and one control animal (day 0) were available for testing in one experiment. Data below represent a minimum of the separate experiments in which at least five individual rats were tested for each time point. DA rats were sacrificed at 4, 6 and 8 days after transplantation (RP donors). The cell doses were tested in 8 or 16 fold replicate cultures. After the MLR phase with RP derived stimulator cells, the generated CTL were tested for target cell lysis of two different types of target cells. The class I

directed CTL activity was determined with cels derived from WAG rats, which share the same haplotype for the class I antigens with RP rats. The lysis with PVG (RT-1^c) targets was determined for the estimation of the nonspecific activity.

Table 4.6 Estimated values of the frequency
of CTL-p in DA rats.

Day	Spleen	Blood	Kidney
0	1/13,402	1/25,900	nt
4	1/3,283	1/2,690	1/10,491
6	1/486	1/731	1/2,871
8	1/2,594	1/486	1/669

a. The results are shown of a representative
experiment. All animals were sacrificed at
the same day and tested as described.

Although a low number of cultures was tested for a specific reponder cell dose the variation of the estimated CTL-p frequency remained acceptable as is illustrated in figure 4.10. Three independent LDA of spleen cells, 8 days after transplantation gave estimated CTL-p frequencies of 1/2,541, 1/1,923 and 1/2,871 respectively.

The data of a representative experiment in which the CTL-p were determined at various days after transplantation are shown in figure 4.11 and the calculated CTL-p frequencies are summarized in Table 4.6. In the unprimed animals (day 0) the donor specific CTL-p frequency of 1/13,400 in the spleen and 1/24,900 in blood. Four days after transplantation a 4 fold increase was observed in the spleen (1/3,283) and even a 10 fold increase in blood (Figure 4.11A and B). A further increase of the CTL-p cells was notified two days later: a 34 fold increase in spleen (1/731) compared with day 0 and a 26 fold elevation in blood. In the last phase of the rejection (day 8) a reduction of the CTL-p in the spleen (1/2,594) was found, whereas the high incidence in the blood remained at the same level (1/480).
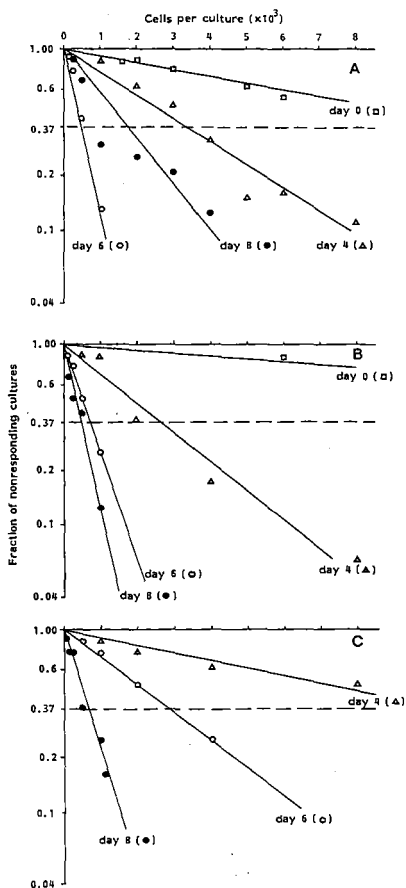
Figure 4.11 The influence of allogeneic kidney transplantation on the number of the CTL population directed against the class I MHC antigen of the donor. The results of a representative experiment are shown in which the CTL activity has been determined in spleen (A), blood (B) and the graft (C) at various days after transplantation. The experimental conditions are described in the text.

As shown in figure 4.11 the CTL-p frequency in the graft itself started to increase later than in spleen and blood. At four days after transplantation the incidence of CTL-p in the graft (1/10,491) was 3 to 4 times lower than in spleen and blood. The estimated frequency within the graft reached a value at day 6 of 1/2,871, which is comparable to the values detected in blood and spleen at day 4. In contrast to the spleen

the level kept increasing after that. This resulted in a frequency of 1/669 at 8 eight days after transplantation, which is approximately the same value as detected in blood.

In order to test the presence of non-specific target cell lysis the ^{51}Cr-release of PVG target cells was determined under identical conditions as described for the specific CTL response. With an exception of blood and allograft at day 8 all experiments showed a frequency corresponding with a value of <1/100,000. In blood the frequency increased at day 8 towards a frequency of with 1/26,000 and 1/35,000, which is only 1.4 - 1.8 percent of the specific CTL-p frequency. In the graft a non-specific killing was detected at the same time with a frequency of 1/18,779 - 1/27,010 correlating with 3.6 and 2.5% of the donor specific target cell lysis. These data prove that the obtained values of the class I directed CTL-p frequencies are not influenced by elevated numbers of nonspecific CTL.

Table 4.7 Frequency of precursor CTL in spleen,
blood and kidney after allogeneic
transplantation.

Day	Spleen	Blood	Kidney
0	9 - 16[b]	>25	n.t.
4	2.8 - 4.1	1.6 - 3.2	10.1 - 14.1
6	.0.3 - 1.0	0.5 - 1.1	1.9 - 2.9
8	1.8 - 2.6	0.4 - 1.3	0.5 - 1.0

a. Frequency is expressed as the number of cells
   (x 10^3) containing one precursor of a donor
   specific cytotoxic T-cell. The results of
   representative experiment are shown.
b. The range of the data determined in four
   independent experiments are shown.
   n.t.: not tested

The estimated frequency of the CTL-p in the spleen of untreated rats ranged from 1/9,000 - 1/16,000, which seems to be lower than values reported in other species. MacDonald et al (1980) reported in mice frequencies ranging between 1/170 - 1/1000 and Buurman et al (1983) described in dogs a frequency estimate of 1/1,900 - 1/3,000 in blood. Lefkovits and Waldman (1979) on the other hand reported estimated values in unseparated spleen and lymph node populations in mice between 1/1,000 and 1/10,000. These figures are however, determined in fully mismatched

combinations i.e. both MHC and non-MHC differences were involved. This is also the case in outbred species, such as man and dog. In mice the anti class I CTL-p frequency ranged from 1/500 - 1/3,000 (MacDonald et al, 1980). The low frequency of CTL-p in the rat might be caused by species differences. Definite speculations however should await more data from a variety of combinations of rat strains to exclude differences in allogeneity or responder status between the various rat strains.
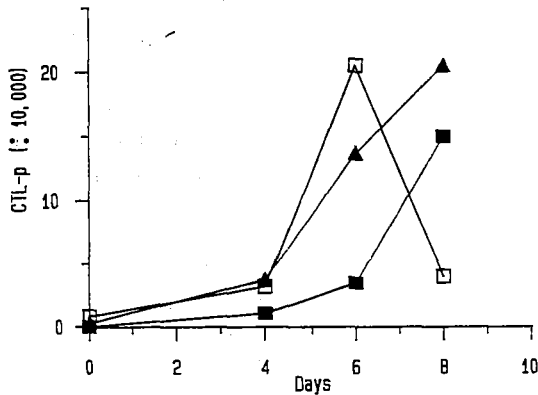

Figure 4.12. The frequency of the estimated donor specific CTL population after transplantation in the recipient, as determined in spleen (□), blood (▲), and graft (■).

DISCUSSION

Donor specific cytotoxic T cell activity

The results of the described experiments showed that allografting has a clear impact on the number of donor specific CTL in blood and spleen (Table 4.7 and Fig. 4.12). In both organs a maximal level is reached at 6 days after transplantation, whereafter a reduction in the spleen was found. The elevation detected in blood is even more pronounced when it is expressed in absolute numbers than in relative figures, since during the later stage of the rejection an increased number of nucleated cells is found in the blood (Fig.4.12). The reduction of the CTL in the spleen may be correlated an increase. Nemlander et al (1982) demonstrated that during the first days after transplantation large numbers of cells migrate to the

graft, but also a comparable number could be found to leave the graft. After 5 - 6 days the cell traffic declines, whereafter the immune response in the graft seems to evolve independently.

The maximal levels of CTL are reached at a rather late stage of the rejection process, especially within the graft itself (day 8). Assuming that a high density of antigen·on the target cells correlates with an increased vulnerability, the kinetics of the class I expression on the graft cells seems in agreement with a late role of the specific CTL. The expression of this MHC-antigen on the graft tissue is highly increased, starting at day 1, but a maximal expression of this antigen was found at day 5 approaching a 30 fold increase as compared to control animals (Milton et al, 1986). Also the *in vitro* analogue of the CTL reactivity requires a MLR-culture of six days for optimal CTL response. Although the *in vivo* activation process may be different, an important role of the specific CTL in the initial phase of the rejection is highly doubtful. Specific CTL response is a rather late event. Nevertheless both histologically and functionally already during the first days clear signs of rejection are found. Significant numbers of MRC OX-8 positive cells are already present in the graft from day 4 on. The presence of such cells with the supposed phenotype of cytotoxic T cells seems to be in contradiction with the functional data, which suggest only a later involvement of CTL in the rejection process. However, the presence of MRC OX-8 positive cells may at least in part be due to an influx of LGL in the graft early after transplantation (Nemlander et al, 1983). LGL, which are also MRC OX-8 positive, are supposed to be the rat analogue for NK cells (Reynolds et al, 1981). In this respect it is striking that the immunospecificity of the infiltrating cells early after transplantation is indeed rather low (Tilney et al, 1978; Tilney and Ford, 1974).

T cell subpopulations in allograft rejection

Major changes early after transplantation, however, seem to be in the ER-2 positive helper/inducer subset. The role of the various T-cell subpopulations in allograft rejection has been a highly controversial matter. Already for a long time it was generally accepted that the donor-specific cytotoxic T-lymphocyte was the dominant cell type in mediating graft rejection (Cerottini and Brunner, 1974). Transfer experiments in thymectomized, irradiated and bone marrow reconstituted mice and rats also,

showed that not the cytotoxic T-cell subset, but the helper T-cell subpopulation is required to restore the skin graft immunity (Loveland et al, 1981; Dallman et al, 1982; Loveland and MacKenzie, 1982a). Moreover Loveland and MacKenzie (1982b) demonstrated a correlation of the ability to reject skin allografts and to mount a delayed-type hypersensitivity (DTH) reaction to alloantigens in B-mice reconstituted with helper T-cells. They suggested that the alloreaction was mediated by cells with a helper T-cell phenotype, which mediate a DTH reaction. Heidecke et al (1984) furthermore performed experiments in which they investigated the relative role of T cell subsets and IL-2 for the rate of the rejection. Transfer of all T-cells, supplemented with IL-2 conditioned medium into allografted B-rats, gave acute rejection. The same type of rejection was seen after transfer of helper cells and Ts/c cells in a normal ratio in the presence of IL-2. Transfer of only helper cells on the other hand resulted also in rejection, but the rate of rejection was never as fast as for the the other two combinations. Moreover IL-2 conditioned medium alone did not change the rejection time. These studies reemphasized the role of the DTH in allograft rejection originally suggested by Brent et al (1962). However, the interpretation of these data became obscured by the observation of Hall et al, who described many MRC OX-8 positive cells within the graft after pure T-helper reconstitution of B-rats. These cells might belong to the NK-cell lineage, but Dallman (Ph.Phil. thesis) was able to show that precursors of cytotoxic T-cells of recipient origin were still present in such animals. Moreover LeFrancois and Bevan (1984) provided evidence in T-cell deprived mice, which were reconstituted with syngeneic T-helper cells (only differing in Thy-1 haplotype) that during allograft rejection the majority of the newly generated cytotoxic T-cells were host derived.

Thus at present there remains a large deal of controversy over the dominating cell type mediating the allograft rejection. Lowry and coworkers (Lowry et al, 1983; Gurley et al, 1983; Lowry and Gurley, 1983) on one hand emphasized the role of the DTH activity in the process of graft rejection. They noticed clear graft rejection without any detectable MRC OX-8 positive cells in the graft. The observed dominance of W3/25 (ER-2) positive cells in their experiments, by them explained as DTH effector-inducer cells, could be due, however, to other factors as well. For instance also the anti-class II cytotoxic T cell and its precursor seems to be W3/25 (ER-2) positive. This latter category of cells was suggested by similar results in mice (Dialynas et al, 1983), man (Krensky et al, 1982;

Spits et al, 1982) and in rats (Mason et al, 1983).

On the other hand the role of specific cytotoxicity in the rejection of allografts has been emphasized by a variety of data. In Cyclosporine A (CyA) treated, non rejecting allograft recipients the nonspecific cytotoxicity remained unchanged, whereas the specific activity was markedly decreased (Mason and Morris, 1984). Also in enhanced grafts an absence of allo-antigen specific CTL was demonstrated, while NK-activity was comparable to untreated recipients (Bradley et al, 1985). Moreover a high frequency of the T-cell clones established from rejecting human kidneys were cytotoxic to donor lymphocytes (Moreau et al, 1985). Illustrative is also the fact that treatment of transplanted monkeys with MCA directed against CD8^{+} cells resulted in prolonged skin graft survival (Jonker et al, 1985; Jonker et al, 1986).

Allograft rejection

The most likely explanation for the observed duality in data seems to be that allografts can and will be rejected by both delayed type hypersensitivity reactions and specific cytotoxic T cells. In untreated and fully mismatched donor recipient combinations both mechanisms may be involved. Only the time of action and the actual site will probably differ.

Our data suggest that effective levels of specific cytotoxicity appear rather late during acute rejection. Nevertheless, as mentioned before, rejection starts almost immediately after transplantation. At first, aspecific infiltration occurs, since also in isografts and autografts some infiltrating cells are seen (our data, Häyry et al, 1979; Christmas and MacPershon, 1982b). In the case of an allograft, however, at that moment the first activation process starts. Donor specific T-lymphocytes within the infiltrate may be directly activated by graft derived cells such as class II positive dendritic cells (Lechler and Batchelor, 1982) and passenger leukocytes (Guttman et al, 1969). Indirect activation may take place by cell debris, caused by ischaemic damage, phagocytized by macrophages and subsequently presented to immunocompetent cells. Both T-helper cells for the CTL differentiation and for DTH reactivity seem to be involved in this initial stage. An important role for MHC class II antigens seems to be indicated in this process, since treatment of allografted mice (skin) with monoclonal anti-Ia antibody not

only prolonged the graft survival, but also suppressed the delayed
hypersensitivity response. (Williams and Perry, 1985). The activation of
these cells results in the production of IL-2, T-cell differentiation
factors and τ-interferon, which subsequently induce differentiation and
maturation of donor specific CTL. On the other hand lymphokines produced
by a T_{DTH} cells might be the explanation of some confusing observations.
The role of infiltrating macrophages remains poorly understood, since these
cells were also detected in isografts, CyA treated grafts and in enhanced
situations (Mason and Morris, 1984; Bradley et al, 1985). Assuming that
such cells require an activation factor for active participation of the
graft rejection, the macrophage arming factor produced by the the T_{DTH} cell
would be a likely candidate. Analogous to the macrophages, τ-interferon
produced in this stage has been reported to activate and potentiate the
function of NK-cell populations (Kawase et al, 1981). Milton et al (1986)
reported a difference in the time that the class I expression starts to
increase on graft cells as compared to the induction of class II
expression. Lymphokines that activate macrophages and other leukocytes,
which produce α-interferon (Clemens and McNurlan, 1985) might explain the
difference between class I and class II expression, because this factor
induces only the class I expression (Fellous et al, 1982). In a later
stage elevated levels of τ-interferon produced by activated T-cells will
result in the induction of class II expression and a further increase of
class I expression.

There might also be a difference in the site were the various
immune responses against the graft take place. Infiltrating cells will be
activated at the graft site, but macrophages, after phagocytosis and
remigration to the spleen, are likely candidates for the immune response
outside the graft. The high degree of cell traffic from the graft to the
spleen indicates that a large number of sensitized cells migrate to the
spleen. Cells do probably not selectively enter the spleen, since no
specific homing receptors are known sofar, but it is assumed that antigen-
induced cellular trapping occurs (Frost and Lance, 1974; Frost, 1974; Zatz
and Gershon, 1974). Such trapped cells find a proper microenvironment in
the spleen for activation resulting in differentiation and proliferation.
Shortly after transplantation such a spleen located immune reaction seems
more suited for the CTL differentiation, since the conditions in the graft
are probably not optimal for the complex cellular interactions required for
this CTL-differentiation. Therefore the spleen is most likely the prime

source of specific CTL in the initial phase, which is in agreement with the earlier appearance of increased numbers of specific CTL in spleen, compared to the graft (our data). Specific CTL leave the spleen and they accumulate preferentially within the graft, not by a specific migration, but probably by specific trapping in the graft (Tilney et al, 1978; Tilney and Ford, 1974).

Finally, also another component of the allograft response should be pointed out. Although the alloresponse in general is thought to be primarily non-MHC restricted, one can envisage the existence of certain MHC-restricted responses directed against anti-non-MHC antigen in the process of allograft rejection as suggested by Bukaroff (1985). In this respect the high concentration of infiltrating cells in the close vicinity of the arterioles must be remembered. Since these arterioles are donor derived, this environment could well be the obvious site for such types of reaction in the graft. Also outside the graft such a response might have an important impact on the overall reactivity pattern, both functionally and phenotypically.

In conclusion

Research on the mechanism of allograft rejection is inhibited by a number of complicating factors. Function and phenotype of a cell are not absolutely correlated. Also differences in place and time between various immune reponses are found. Furthermore the role of minor subpopulations in the mechanism of rejection is obscure.

Although the phenotypical expression of a cell is a valuable tool in immune research it is also a highly confusing parameter. Rat cells which express the CD4 (ER-2) antigen may belong to the following functional populations: (1) T cells with a DTH reactivity, (2) anti class II CTL, (3) cells with a helper function for anti-class I CTL differentiation, (4) class II restricted anti-non MHC CTL and (5) macrophages. On the other hand cells carrying the CD8 (MRC OX-8) antigen are involved in (1) NK activity, (2) non restricted anti MHC CTL activity, (3) class I restricted anti non MHC or (4) anti class II responses. Even more complicating is the factor that cells may not be restricted to the expression of either the CD4 or the CD8 antigen. The presence of elevated numbers of $CD4^+CD8^+$ cells in highly activated situations may reflect a possible phenotypical switch of the $CD4^+$ or $CD8^+$ T cell populations. T-cell clones confirm a flexibility

of the phenotypical make-up versus cell function. CD8^{+} clones, secreting a whole variety of factors, including IL-2, γ-interferon and MAF were described (Widmer and Bach, 1981), while CD4^{+} T-cell clones exerted cytotoxic activity in combination with production of various T cell factors. When in vitro T-cell clones are a reflection of the functional potential of T-lymphocytes *in vivo*, it must be also considered that the normal immune system found in healthy, non-stimulated individuals may be a poor reflection of what actually goes on in the immune reponse during a rejection period. It may well be that normally infrequent cells, become important in highly activated situations such as an allograft rejection. This might especially be true in situations where a normal immune reaction is blocked by immunosuppressive treatment.

Although as described above two dominant types of rejection are favoured by most investigators several alternative pathways of the immune response are suggested. These different immune responses will most likely collaborate in the rejection mechanism, acting at different times and different sites in the graft. Depending on the experimental system the results will be in favour of one of these pathways. The importance of such alternative pathways of alloreaction remains to be determined. For instance the class II directed CTL seems rather low in number for an effective participitation of the rejection mechanism. At specific sites, for instance in the perivascular sites in close proximity with class II positive endothelial cells of the arterioles, they could play an important role in rejection.

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Chapter 5

IMMUNOSUPPRESSIVE PROPERTIES OF MCA

INTRODUCTION

In man immunosuppressive treatment after transplantation will always be necessary to suppress the alloreaction of the immune system of the recipient towards the graft. The current immunosuppressive agents are not specific and also the normal immune response is inhibited. A more specific immunosuppression is therefore wanted. One of the approaches has focussed on the cells that are responsible for the rejection, i.e. the lymphocytes. The immunosuppressive potential of antisera that are directed against lymphocytes, the socalled anti-lymphocyte sera (ALS), was discovered about three decades ago. The first demonstration of this immunosuppressive activity of ALS is ascribed to Inderbitzin (1956), who reported the inhibition of skin reaction of delayed type hypersensitivity after treatment with ALS. Later on Waksman et al (1961) also found an inhibition of autoimmune disease and a prolongation, although weak, of skin graft survival. The main interest in ALS, however, started after a striking prolongation of skin graft survival in ALS treated rats as described by Woodruff and Anderson in 1963 and 1964. After these publications many studies have been performed on the antigen specificity as well as the antibody properties, which are essential for the immunosuppressive properties of ALS (reviewed by Lance et al in 1973).

ALS is a complex reagent, directed against a multitude of antigen specificities and it consists of a mixture of different types of antibodies. This is a major problem for the elucidation of its mode of action. Several cell types, mainly lymphoid in nature, can be used as immunogens. As far as specificity is concerned, lymphocytes are an essential component of the cell mixtures used in the immunization procedure. With regard to the antibody itself, it is emphasized that the activity is almost completely restricted to the IgG fraction. The IgM fraction can not prolong graft survival in a normal test system. Only a minimal immunosuppressive activity could be demonstrated for this fraction, when administered in a high dose in closely spaced intervals after transplantation. Moreover Capel et al (1983) showed, that after fractionation of a polyclonal mouse anti-rat serum into an IgG1 fraction

and an IgG2 fraction, the immunosuppressive activity was exclusively detected within the IgG2 subclass. The IgG1 subclass failed to do so in all experiments. However, this finding seems to be rather species specific, since the same authors (Lems et al, 1983) found that in polyclonal rat anti-mouse sera the IgG1 and the IgG2 fraction showed a comparable immunosuppressive activity. The necessity of an intact IgG molecule was shown by Lance et al (1973), since neither $F(ab)_2$ nor Fab' fragments had any effect on the graft survival. Possibly the Fc portion of the molecule, which mediates many diverse functions, such as complement fixation and opsonization, is an important factor for the immunosuppressive activity of the antibody. Another relative simple explanation of the differences in the activity of the IgG fraction on one side, and the IgM fraction or the IgG fragments on the other side may be found in a variation of the biological half life time of such inocula. IgG has a rather low rate of elimination in blood (6-7 days), whereas IgM (2-2.5 days), $F(ab)_2$ and Fab' fragments (about 6 hours) are much faster removed from the circulation (Lance et al, 1973 review; Spiegelberg and Weigle, 1965).

Thus, a lot of uncertainties and controversies still exist about the relative importance of the antibody composition of immunosuppressive ALS. Furthermore the importance of the antigen specificity of ALS remains to be solved. MCA offer a simple solution to address the problems in both area's. Moreover if MCA can substitute ALS, a constant and unlimited source of antibody can be achieved.

Immunosuppression

With the availibilty of a panel of well defined MCA against rat cell surface markers, as described in the previous chapters, it became thus possible to address the main questions on the immune properties of ALS. The questions are: Which are the target antigens or cells, that are important for immunosuppression and which are the restrictions on the side of the antibody for immunosuppression?

For this purpose ascites fluids containing the various MCA were tested for immunosuppressive activity in a skin graft model. Transplantation was done as described by Capel et al (1983): tail skins of PVG.RT-1[a](DA) rats were grafted onto the dorsal flank of PVG rats. The grafts were fixed with Nobecutane[R] spray and the recipients were treated i.p. with 0.5 ml of ascites fluids at days 0, 2 and 4 after grafting. All

ascites were tested for active binding before use.

    The results of these experiments are shown in Table 5.1. Untreated animals rejected the graft in 9-10 days (Mean survival time: MST 9.5). Out

Table 5.1 The effect of MCA on the graft survival in the rat[a]

Target antigen	Antibody	Subclass	MST(days)
Control		—	9.5[b]
Leukocyte common antigen	ER-17	IgG1	10.7
	ER-18	IgG2a	10.1
	ER-19	IgG2a	9.9
All T cells	ER-1	IgG2a	_15.9_
	ER-1 F(ab)$_2$	F(ab)$_2$	10.6
	HIS-17[c]	IgG1	_14.9_
	W3/13	IgG1	9.2
T helper cells	ER-2	IgG1	_15.0_
	W3/25	IgG1	_13.7_
T cytotoxic/suppressor	MRC OX-8	IgG1	8.5
	ER-3	IgG2a	8.1
	ER-10	IgG2a	9.2
T cell subset	ER-7	IgG2a	9.7
	ER-8	IgG1	8.9
	ER-9	IgG2a	7.5
	ER-11	IgG2a	8.7
	MRC OX-22[d]	IgG1	8.7

a. Tail skin of PVG.RT-1[a](DA) rats was grafted on PVG recipient
   rats. The recipients were treated with 0.5 ml ascites fluid on
   days 0,2 and 4 after grafting.

b. MST is the mean survival time of 5 animals. Underlined figures
   represent significant prolonged graft survivals. SEM ranged
   between 1 and 1.5 days.

c. Kroese et al,1985

d. Spickett et al, 1983

of the 16 MCA tested in this model only four proved to be immunosuppressive. The negative results of the other MCA were not caused by low antibody concentration in the ascites fluids, since increasing the amount of antibody per administration did not influence the results (data not shown). It appears that also for MCA an intact molecule is required for immunosuppression, because the prolongation of the graft survival of ER-1 (Table 5.1) is completely abrogated when F(ab)$_2$ fragments of this antibody are tested in the same concentration.

Much to our surprise three out of the four immunosuppressive antibodies (HIS-17, ER-2 and W3/25) were of the IgG1 subclass (Table 5.1), which seems to be in contradiction with the results obtained with the polyclonal IgG1 fraction as described by Capel et al (1983).

An explanation for this finding could be that the antigenic specificities of these MCA are normally not found in the IgG1 fraction of polyclonal antisera. Therefore it was tested whether the fine specificities of these antibodies are present within the normal repertoire of a polyclonal ALS. The MCA were radiolabelled with [125]-Iodine and binding to spleen cells of PVG rats as well as the ability of mouse anti-

Table 5.2 Inhibition of binding of MCA
to PVG spleen cells

MCA	Percentage of inhibition[a]	
	MARS	NMS[b]
ER-1	87[c]	2
W3/13	54	0
HIS-17	81	1
ER-2	75	0
W3/25	83	3

a. [125]I MCA and serum (1/2 dilution)
   were incubated with spleen cells (PVG).
b. NMS: normal mouse serum
c. Inhibition:Bound MCA (cpm) - Bound MCA
   + MARS/NMS (cpm) x 100/ Bound MCA

rat serum (MARS) to inhibit this binding was determined using a similar technique as described in the second chapter. As can be seen in Table 5.2, MARS is capable to inhibit the binding of all tested antibodies, varying from 54% of W3/13 binding up to a 87% suppression of the ER-1 binding. Thus the specificities defined by these MCA are present in the normal repertoire of MARS. A restriction of these specificities to the IgG2 subclass in polyclonal sera was neither the case, as illustrated for ER-2. As shown in figure 5.1 both the IgG1 and the IgG2 fractions of polyclonal ALS were able to block the binding of MCA ER-2, demonstrating that both fractions contain antibodies against this specificity. The data of the blocking experiment (Fig. 5.1) and the immunosuppressive properties of MCA of the IgG1 isotype indicate, that also the polyclonal IgG1 fraction contains suppressive antibodies. Probably the frequency and the efficiency of immunosuppressive antibodies within the IgG2 subclass is the

highest, but potentially also antibodies of the other Ig (sub)classes can be immunosuppressive. A lower frequency combined with a lower efficiency of the IgG1 antibodies in polyclonal sera may be the cause of the negative results observed for the IgG1 fraction. The observation that, in contrast to IgG1 of polyclonal MARS, monoclonal IgG1 antibodies can be immunosuppressive is therefore probably due to a very powerful selection by the hybridoma technique of normally, existing specificities in the IgG1 fraction of MARS. This data is once more an indication that MCA do not always behave in a similar way as their polyclonal members of the family.

As far as the antigen specificities of the immunosuppressive MCA in this study are concerned, it is obvious that all of them are reactive with T cells. ER-1 and HIS-17 (Kroese et al, 1985) react with all T cells, whereas ER-2 and W3/25 recognize the T helper population. This indicates that the cell population recognized by the MCA is an important factor for immunosuppression: In this model only MCA against all T cells or the helper
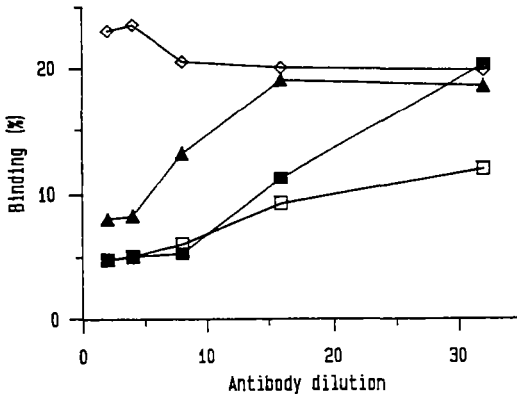
Figure 5.1. Inhibition of ER-2 binding on rat spleen cells by polyclonal anti-lymphocyte serum (MARS: □), the IgG1 fraction (MARG1: ▲) or the IgG2 fraction (MARG2: ■). Control is normal mouse serum (NMS: ◇).

T cell population were suppressive. All MCA against surface antigens on the suppressor/cytotoxic T cells failed to prolong graft survival in our model. However, in other systems MCA specific for such cells were effective, since immunosuppressive activity of anti-CD8 antibodies in humans have been reported (Jonker et al, 1986; Cosimi, 1983). Very

recently Claesson et al (1985) also reported prolongation of survival of heart and kidney in rats with MRC OX-8. The model used and especially the precise time course of the MCA treatment in relation to the onset and progress of the rejection of the transplant are probably essential for the desired immunosuppressive effect of the MCA, as will be discussed in detail later in this chapter.

The leukocyte common antigen (LCA) is expressed on all leukocytes, including all T lymphocytes. Nevertheless all MCA against this antigen did fail to influence the graft survival in this study. This is in agreement with earlier findings of Fabre et al (1981) with polyclonal as well as monoclonal anti-LCA antibodies. These results indicate that reactivity with the proper cell population is not conclusive, but also the surface molecule on those cells is an important factor for immunosuppression.

The MCA against all T cells (ER-1, W3/13 and HIS-17) and against all helper T cells (ER-2 and W3/25) were selected for further study, on the mechanism of immunosuppression. Strikingly only 2 out of 3 pan-T cell antibodies were suppressive (ER-1 and HIS-17 versus W3/13). These differences in immunosuppressive behaviour were not caused by variations in physical properties between the various MCA. As described in chapter 3 also affinity differences between the antibodies cannot account for the variation in their immunosuppressive potential (ER-1/W3/13: chapter 3; HIS-17 was determined at a comparable K: 2.6×10^{-10} mol/l) Also complement binding properties (chapter 3) and the isotype of these antibodies do not give any correlation with the immunosuppressive activity.

To exclude furthermore the possibility of significant differences in specific antibody content in the various ascites fluids, a dosis-response study was done with the selected MCA. For a proper comparison of the various MCA the specific biological activity of each was determined. Therefore the MCA were labeled with ^{125}-Iodine and the ratio of bound antibody (cpm) versus the total radiolabel (bound/total) was determined on spleen cells. The ratio of bound/total in the situation of an excess of antigen (5×10^8 cells) reflects the degree of 'biological activity' i.e. antibody, which is able to bind with the surface antigen on the target cells. The dose of the ascites is corrected according to this ratio and it is expressed as dose specific activity. If for example ascites is radiolabelled and binding experiments with high cell numbers show a maximum of 55 percent of the radiolabel to bind to the cells, the specific activity

is 55% of 1 ml, i.e. o.55 ml. The dosis effect of the panel of antibodies expressed in this way is shown in figure 5.2. As can be seen from this figure W3/13 remains non-suppressive even when high doses of MCA were used for treatment. The differences between ER-1/HIS-17 and W3/13 can not be attributed to simple dose differences in antibody concentration. The prolongation induced by ER-2 and W3/25 treatment (Fig. 5.2A) is much more profound than the effect of ER-1 and HIS-17 (Fig. 5.2B). These results indicate again, that probably the surface antigen is one of the important factors defining the extent of suppressive activity of the antibody.
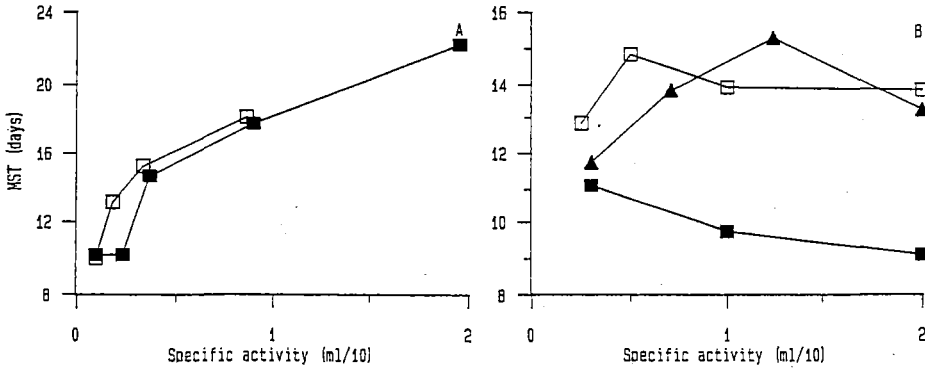
Figure 5.2 The survival of skin grafts in relation to antibody dose (ml). A. Treatment of the recipient with ER-2 (□) and W3/25 (■). B. Treatment of recipients with MCA W3/13 (■), ER-1 (▲) and HIS-17 (□).

We focussed our attention more in detail on the surface antigens recognized by these MCA. First, we determined whether the MCA with a comparable reactivity pattern might recognize the same determinant on the target cells. Therefore ER-1, W3/13 and HIS-17 on one hand and W3/25 and ER-2 on the other hand were studied for their blocking capacities in a binding assay. Purified MCA were labelled with [125]-Iodine as described before and their binding on thymocytes was tested using a RIA system. Various concentrations of unlabelled antibodies were incubated together with the radiolabelled antibody and the binding of the latter antibody was measured.

The results of the binding studies of ER-2 and W3/25 were somewhat confusing. Even in the highest excess of ER-2 the binding of labelled

W3/25 was not affected (Fig. 5.3D), but surprisingly in the reciprocal
situation low amounts of W3/25 inhibit the binding of labelled ER-2 up to
50 percent (fig. 5.3C). Increasing amounts of W3/25, however, did not
further influence the binding of labeled ER-2. The determinants recognized
by W3/25 and ER-2 therefore have to be located within a short distance from
each other, either on the same molecule or on closely associated molecules.
To investigate the target molecule of these antibodies immunoprecipitation
studies were performed. Thymocytes were surface labelled and the lysates
were used for immunoprecipitation of the various MCA as described in
chapter 3. For the target antigens of ER-2 and W3/25 the same molecular
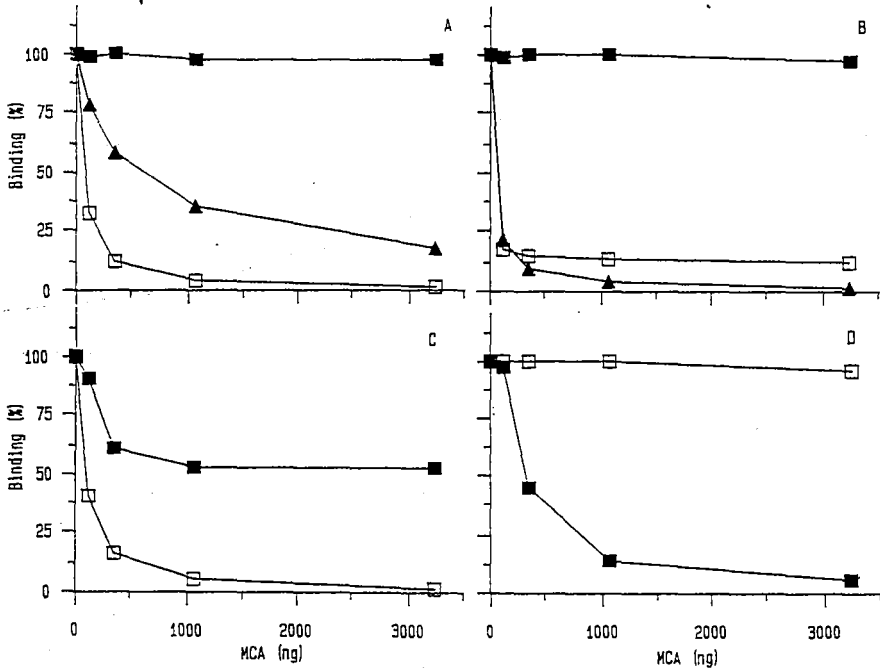
Figure 5.3 The influence of MCA on the binding of several antibodies to rat thymocytes. Fixed
amounts of ^{125}-Iodine conjugated antibody were mixed with a increasing amount of unlabelled
antibody. Binding of the labelled antibody was subsequently measured using a RIA (as described
before). Binding of labelled MCA A: ER-1, B: HIS-17, C: ER-2 and D: W3/25 are shown.
Unlabelled MCA HIS-17 (▲), ER-1 (□) and W3/13 (■) (A and B); ER-2 (□) and W3/25 (■)
(C and D).

weight (52 kD) was determined (Table 5.3), which is in agreement with the
data reported for the W3/25 antigen by Thomas and Green (1983). To clarify
whether these molecular weights represent different molecules of the same
size or that W3/25 recognizes a different epitope on the same surface

molecule as ER-2, sequential immunoprecipitation of these antibodies was necessary. Cells were surface labelled (^{125}-Iodine) and separate portions of the same cell lysate were repeatedly precipitated with control ascitis (SP2/0) (Fig.5.4,lane A), ER-2 (lane B) and W3/25 (lane E) respectively. The last two lysates were precipitated with the same antibodies until no more antigen could be detected by autoradiography. Since no ER-2 antigen and W3/25 antigen, respectively is present in these lysates (lanes C and F), they were tested for the presence of the other antigen by precipitation with W3/25 and ER-2 respectively. W3/25 did not precipitate any antigen (lane D), whereas for ER-2 only a small band could still be detected (lane

Figure 5.4 Sequential immunoprecipitation of ER-2 and W3/25 with surface labelled rat thymocytes. Autoradiography of immunoprecipitates on gels after SDS-PAGE are shown of Sp2/0 (control): lane A. MCA ER-2: lane B and W3/25: lane E. Lysates were cleared by repeated precipitation of the antigens of ER-2: lane C and W3/25: lane F. Both antigen free lysates were subsequently precipitated with the other antibody. Lane D: precipititation with W3/25 of ER-2 antigen cleared lysate and lane G: precipitate with ER-2 of W3/25 antigen cleared lysate.

G). If two independent antigens had been recognized this band should have the same intensity as the one of the initial lysate which is shown in lane B. Therefore this small band in lane G must be ascribed to a technical cause. Therefore these data support the notion that ER-2 and W3/25 are directed against different, closely linked, epitopes on the same surface molecule.

In figure 5.3A the results of the binding assay with radiolabelled ER-1 are shown. First of all a complete inhibition of binding of labelled ER-1 is demonstrated by the unlabelled form of this antibody. Also HIS-17 is capable to inhibit the binding of ER-1 upto about 80 percent (Figure 5.3A). A comparable inhibitory effect of ER-1 on the binding of labelled HIS-17 has been found (Fig. 5.3B). This pattern of mutual blocking capacities indicates that ER-1 and HIS-17 recognize most likely the same molecule. The minor variation in the degree of blocking between unlabelled ER-1 and HIS-17 on labelled ER-1 and labelled HIS-17, respectively might point out that these two antibodies react with different, but closely linked epitopes on the same molecule. Even in the highest concentrations W3/13 fails to influence the binding of either labelled ER-1 (Fig. 5.3A) or the binding of labelled HIS-17 (Fig.5.3B). This means that the determinant recognized by W3/13 is either located on another surface molecule or, when the determinant is located on the same molecule as ER-1 and HIS-17, it is not in a close range.

ER-1, HIS-17 and W3/13 give a similar reaction pattern with all T cells in terms of histology, FACS analysis and all other criteria tested sofar. Of these three MCA ER-1 and HIS-17 were shown in the above described blocking experiments to recognize similar or closely related epitopes on the same cell surface molecule. W3/13 might react with another molecule or with a non-associated epitope on the same molecule. Therefore the molecular size of these antibodies was determined using immunoprecipitation. As could be expected ER-1 and HIS-17 (Table 5.3) are directed against surface antigens of the same size. The figure of 95 kD is the same as the one reported by Standring et al (1978) for the W3/13 marker (Table 5.3). In the same type of sequential immunoprecipitation experiment, as described for ER-2 and W3/25, also ER-1 and W3/13 were

Table 5.3 Molecular weight of surface antigens

Antibody	Mol.weight[a] (kD)	Antibody	Mol.weight[a] (kD)
ER-1	95	ER-2	52
HIS-17	95	W3/25	52
W3/13	95		

a. Molecular weights of the surface antigens were determined using immunoprecipititation experiments as described in the chapter 3.

tested. From the results of this experiment (data not shown), it could be established that ER-1 and W3/13 are directed against the same surface molecule. This proves, that W3/13 is directed against another epitope on the same 95 kD molecule as the one of ER-1 and HIS-17.

The data sofar show that testing of a panel of MCA representing 11 different surface molecules on the same cells, only antibodies directed against two molecules possess immunosuppressive activity in the model used. Even a differential reactivity pattern of antibodies directed against the same molecule can be observed, as illustrated by the cluster of antibodies reacting with the 95 kD molecule. Since the only apparent difference seems to be on the level of the epitope, this suggests that recognition of a certain surface antigen alone is not sufficient, but also the epitope is important for immunosuppression. The role of the epitope is also confirmed by human data of MCA directed against the CD8 antigen. Only part of these antibodies are immunosuppressive (Jonker et al, 1986), which indicates the existence of an active site on the CD8 molecule, which has to be recognized for immunosuppression. In mice, Lems et al (1985) suggested the presence of socalled dominant epitopes, that are decisive for immunosuppression.

In vivo behaviour

Differences in effectiveness of monoclonal antibodies might be caused by trivial reasons as antibody degradation rate and variations in distribution. Besides the fine antigen specificity therefore also the *in vivo* behaviour of the five antibodies was studied. First of all biological life time in the blood has been determined. PVG rats were i.v. injected with 1 mg of purified antibody and at various times blood samples were drawn, whereafter the plasma fraction was isolated and stored at -20 °C. These samples were tested for the presence of specific antibodies by binding to thymocytes using a highly sensitive Elisa technique. All antibodies showed the same type of clearance in the circulation, which is illustrated for W3/13 in figure 5.5. Within four hours the concentration reached a maximum, which was maintained until 48 hours after injection. Thereafter a gradual decrease was observed until after approximately 90 hours the concentration reached the background level.

Subsequently the localization of injected antibody was monitored in various organs. Antibodies were radiolabelled with [125]Iodine and 8-12μg of
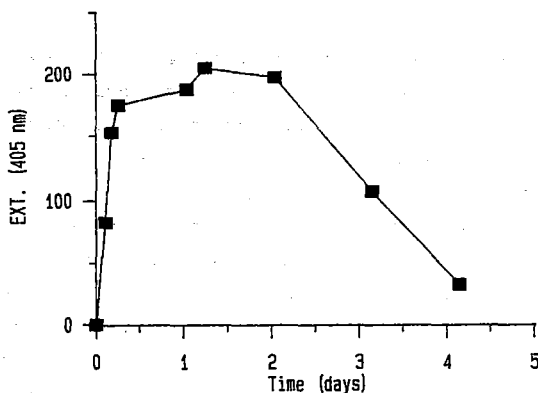
Figure 5.5 Serum levels of MCA W3/13 after a single dose (i.v.).
Specific antibody binding on thymus cells has been
determined using an Elisa technique (described in chapter
2). The extinction of the reaction product was measured
at 405 nm.

antibody was i.v. injected in PVG male rats (10-12 weeks of age). The rats
were sacrificed after 24, 48 and 72 hours respectively: for each antibody
five animals per time point were used. After isolation of the organs the
radioactivity was counted using a τ-counter and the localization was
expressed as cpm/g organ.

After 24 hours, all antibodies, with the exception of W3/13, showed
a high preference for the spleen, while in the mesenteric lymph nodes the
localization ranged from high for ER-2 to hardly any for W3/25,
intermediate levels were found for the other antibodies. A fast decrease
of antibody in time is especially found in organs with a high specific
localization such as the spleen (Fig. 5.6). Almost a complete
disappearance of the antibodies from the blood could be seen within 24
hours after injection, with exception of W3/25 and W3/13. This is in
contradiction to the results of the biological half life time (Figure 5.5).
The low dose of antibody used in these studies (8-12μg), compared to the
experiment of the biological life time (1 mg) is most likely the reason for
the a faster removal of antibody from the circulation for some of the
tested MCA compared with the results of the biological life time.

The low amount of localization of W3/13 in all tested organs (except
in the blood) might be explained by inabilility of ^{125}I-W3/13 to bind to
cells or by aggregation of this antibody. However, when this labelled
antibody was tested for specific binding to spleen cells it appeared to
have a good binding capacity. The possibility of aggregation was tested by

i.v. injection of comparable amounts of antibody in Balb/c mice. Almost all of the antibody remained in the blood and hardly any antibody was found within the liver and other organs. Aggregated antibody should correlate in these mice with elevated activity in the liver, which was not detected. In experiments with the other MCA the same type of localization in Balb/c mice was detected. Therefore it can be concluded that these results of W3/13 cannot be explained by the inability of this antibody to bind to the target cells or by aggregation.

A low activity was detected for all antibodies in the thymus despite of the high cell numbers that express the relevant surface antigens in that organ. It appears therefore that direct entry into the thymus is blocked for these antibodies. Since neither an increased activity was found after 48 and 72 hours, it seems that also indirect entry of the
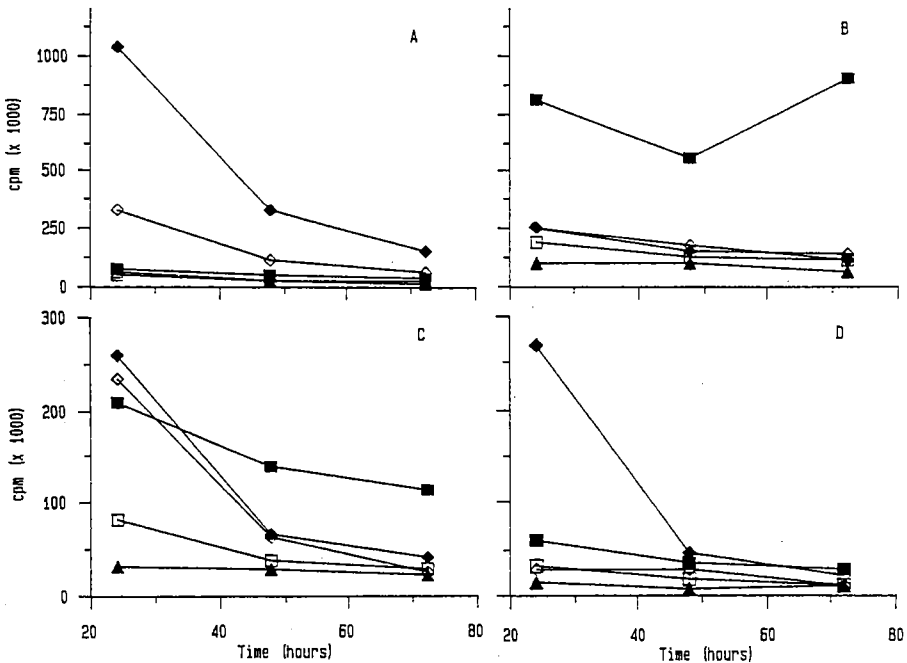
Figure 5.6 Localization of MCA in spleen (◆), lymph node (◇), blood (■), liver (□) and thymus (▲) at 24, 48 and 72 hours after a single i.v. dose. The results are shown for A. ER-1, B. W3/13, C. W3/25 and for D. ER-2.

antibodies via the lymph system does not occur on a large scale. This limited entrance into the thymus of the rat, confirmed by Holmdahl et al (1985) for W3/13, W3/25 and MRC OX-8, is in contrast to the results of Kruisbeek et al (1983 and 1985) in the mouse. The latter authors showed that MCA directed against Class II (Ia) molecules freely enter the thymus. The 'classical' thymus-blood barrier (Raviola and Karnovski, 1972) seems not to function for all antibodies, since apparently some are able to penetrate through the blood-thymus barrier.

A likely explanation for the absence of accumulation of W3/13 in the spleen was provided by data described by Holmdahl et al (1985). They showed that after W3/13 treatment no W3/13 positive T-lymphocytes could be demonstrated anymore in the spleen and lymph nodes already after two hours. After 14 days a recovery could be noticed. It took however as long as 5 weeks before a normal cellular distribution in the spleen was observed. Staining with W3/25 and MRC OX-8 in the same spleen of the W3/13 treated animals revealed nevertheless only a partial depletion of T-lymphocytes. Therefore it can be concluded that after W3/13 treatment, only part of the cells leave the spleen and furtheron that the remaining cells within the spleen have lost the ability to bind the W3/13 antibody. This explains our findings, that we were unable to detect any W3/13 binding in the lymphoid organs at 24 hours after injection. After treatment with ER-1, HIS-17 (data not shown), ER-2 and W3/25 a high activity was detected in the spleen (figure 5.6), especially at 24 hours after injection. These observations suggest that for these antibodies, in contrast to W3/13, both cells and surface antigen are still present. This is in accordance with the findings for W3/25 by Holmdahl et al (1985). They did not detect any discernable change in the cellular distribution pattern in the spleen after W3/25 treatment: the cells as well as the surface antigen are still present.

The same experimental approach as was described for determination of the biological life time in blood was used for analysis of the effect of injected MCA on the peripheral blood lymphocytes (PBL) after a single dose of antibody (1 mg antibody; i.v.). At various times after injection blood samples were drawn and the peripheral blood lymphocytes were isolated: the samples were centrifuged for 5 min. at 200 g and the leukocytes on top of the packed cells were isolated. This fraction, diluted in RPMI-1640 medium (v/v) was brought upon 2 ml of Ficoll/Isopaque solution (d: 1.077 g/ml). After centrifugation (20 min at 730 g) the lymphocytes were isolated from the surface of the Ficoll/Isopaque layer. Alterations within the

lymphocyte population were monitored by staining with FITC-conjugated antibodies and analyzed using a FACS. The availability of several MCA against the same lymphocyte population enabled a more detailed analysis. First of all the cell population can be monitored, but also fine analysis of the surface antigens can be done. The cells can still be present, while the surface antigens are lost by shedding or modulation. It is evident that cells cannot be detected by staining with antibodies directed against an antigen, which is lost. For this reason also staining with MCA directed against other surface antigens on the same cells was done. MRC OX-19, reacting with all T-cells (Dallman et al, 1984), is such a MCA, since this antibody is directed against a surface molecule of 69 kD. Therefore the presence of the T-lymphocytes was analyzed for the MRC OX-19 reactivity. For analysis of the surface antigens on these cells, MCA directed against different epitopes on the same surface molecule may be used. In the blocking studies (Fig. 5.5) it was shown that W3/13 does not interfere with binding of ER-1 or HIS-17.

　　　　Treatment with ER-1 resulted in a fast elimination of T lymphocytes from the blood. This is illustrated in figure 5.7A by the MRC OX-19 staining 4 hours after ER-1 treatment, together with the fluorescence profile of untreated PBL´s. At the same time after W3/13 treatment the MRC OX-19$^+$cells are also decreased (figure 5.7B), but the depletion of T-cells by this antibody was less dramatic than observed after injection of ER-1. Also after a single dose of ER-2 and W3/25 part of the cells disappeared from the circulation. In figure 5.7C the PBL´s were stained with MRC OX-19, 2.5 hours after a single dose of ER-2. The fluorescence profile shows a decreased number of MRC OX-19 positive cells. In figure 5.7D it is illustrated that cells carrying the surface antigen of W3/25 are completely disappeared at 2.5 hrs after treatment with MCA W3/25, since no ER-2 positive cells could be detected. The cells are are disappeared and not only the surface antigen is lost from the cells. This can be concluded, because the reduction of ER-2 positive cells is of the same magnitude as the one that could be observed for MRC OX-19 (illustrated in Fig. 5.7C and D). In all experiments antibody treatment resulted in elimination of the cells that express the specific surface antigens. None of these experiments using these antibodies indicated the existence of modulation of the surface antigens or only a coating by the antibodies. The data of T-lymphocyte fluctuations after MCA treatment are shown in figure 5.8. The number of MRC OX-19 positive cells at a given time was compared with the
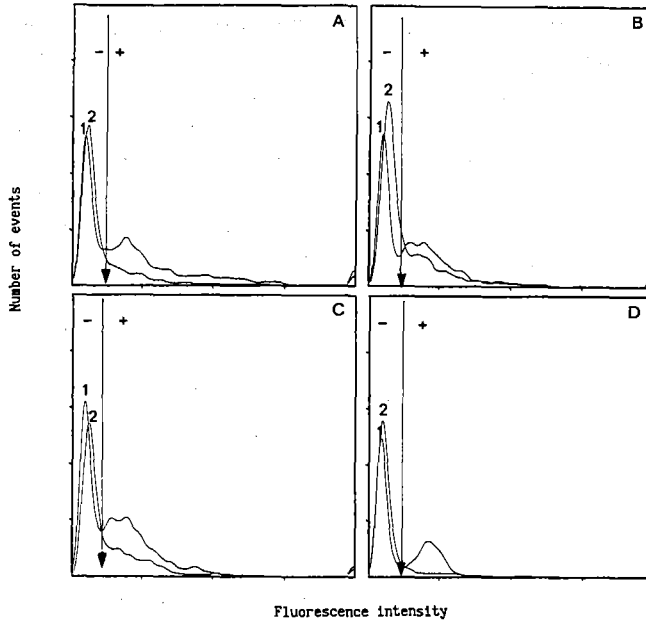
Figure 5.7 Fluorescence staining profiles of PBL's of PVG rats after a single dose of
MCA (i.v.). Horizontal axis: Fluorescence intensity, vertical axis:
number of events. Fluorescence staining of treated (2) and control
(1) animals are shown. A. PBL's stained with MRC OX-19 at 4 hrs after a
ER-1 dose. B.PBL's stained with MRC OX-19 at 4 hrs after a W3/13 dose.
C.PBL's stained with MRC OX-19 at 2.5 hrs after a ER-2 dose. D. PBL's
stained with ER-2 at 2.5 hrs after a W3/25 dose. The arrows indicate
the separation between positive cells and negative cells.

cell number of untreated animals. A dramatic decrease of T lymphocytes in
the blood is detected after one dose of ER-1 and even after three days the
recovery of T-cells is only 20 percent of the original number. The
fluctuations after W3/13 and HIS-17 treatment are less severe, but also for
these antibodies only a partial recovery is detected. The influence of ER-
2 and W3/25 is somewhat different since the drop of T-cell numbers is less
dramatic and within three days the recovery phase has already started
(Fig.5.8B).

These experiments showed no difference between the non-
immunosuppressive MCA W3/13 and the other immunosuppressive MCA (ER-1 and
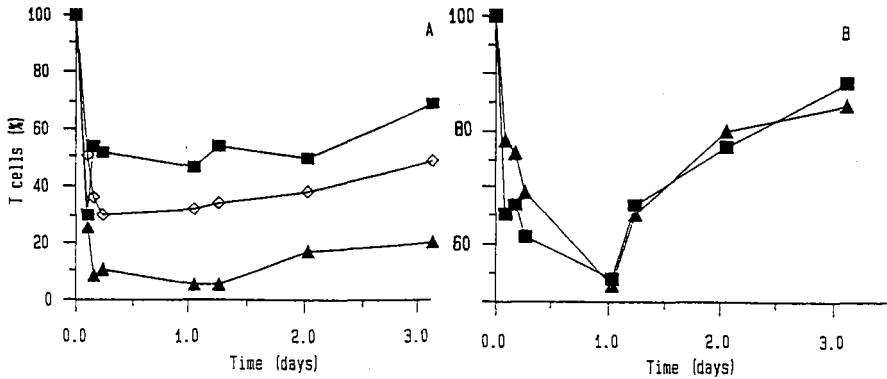HIS-17) on elimination of T cells in the blood. The observation, that

Figure 5.8 Effect of i.v. treatment of MCA on peripheral blood lymphocytes. A. treatment
with MCA W3/13 (◇), ER-1 (▲) and HIS-17 (■). B. ER-2 (■) and W3/25 (▲).
PBL's were stained with MRC OX-19 and analyzed with a FACS II. The positive
labelled were expressed as percentage of the control figures.

effective elimination of the target cell population is not totally
correlated with immunosuppression, was confirmed too by the data obtained
with ER-9. This non-suppressive antibody is highly effective in
elimination of its target cell population from the blood (data not shown).
Comparable observations have been reported for human anti-CD4 and anti-CD8
antibodies: immunosuppression did not fully correlate with properties such
as elimination, coating or modulation (M.Jonker, personal communications).

These *in vivo* experiments intended to compare the behaviour of
immunosuppressive and non-suppressive MCA in the organism. The biological
life time may be important for a suppressive status of antibodies, but this
was not the case for the tested antibodies, since a comparable fluctuation
was detected in the blood. Immunosuppression was not correlated with the
effect on the T-lymphocytes either. Treatment resulted in elimination of
the T cells from the blood for all these antibodies. Non-suppressive
antibodies eliminated as effective as the suppressive ones. Strikingly, a
different kind of localization in spleen was observed for W3/13, as
compared to the other antibodies. Only part of the cells were eliminated
by W3/13 treatment, but the remaining cells did not express the W3/13
determinant anymore. Most likely W3/13 cannot block the T cell function,
due to the modulation of the determinant and as a consequence a normal T
cell activation after transplantation and rejection is still found.

The above described *in vivo* experiments were done in untreated animals, in which the immune system was not activated. The introduction of a graft will result in an activation of the immune system. The immunosuppressive effect of the antibodies will most certainly be affected by the alloreaction of the recipient towards the graft.

The *in vitro* experiments described in the chapter 3 showed that both the 95 kD antigen and the 52 kD antigen are involved in the function of the T-lymphocytes. The data on the 95 kD antigen indicate that this antigen acts early in the activation process in a co-stimulatory way. W3/25 and ER-2 acting on the 52 kD molecule, on the other hand, caused a clear inhibition of the MLR response (see for W3/25 data also: Webb et al, 1979; our data). This effect acts directly on T cells and not through antigen presenting cells (Mason et al, 1980). Jefferies et al (1985) showed that the 52 kD antigen in the rat is the analogue of the CD4 antigen in man. CD4 is important in the cellular interaction between T cells and antigen presenting cells. It has been suggested that this molecule binds to a nonpolymorphic part of the class II antigen, whereas for the CD8 antigen a comparable binding to the class I antigen has been suggested (Swain, 1983). The evidence, however, remains conflicting (Dialynas et al, 1983; Watts et al, 1984; Greenstein et al, 1984 and Strassman et al, 1984). Table 5.4 summarizes the influence of several monoclonal antibodies on the in vivo T cell function. The data of the *in vivo* (Table 5.1 and 5.4) and

Table 5.4 Influence of MCA on in vivo
T cell function[a]

MCA	Surface molecule (kD)	In vivo[a] T cell function
ER-1	95kD	-
W3/13	95kD	0
HIS-17	95kD	-
ER-2	52kD	-
W3/25	52kD	-
MRC OX-19[b]	69kD	-

a. Effect of MCA on the skin graft survival as described in this chapter 0 : no effect;
   - : suppressed
b. Data reported by Claesson et al, (1985)

of the *in vitro* (summarized in Table 3.11) experiments indicate a positive correlation between immunosuppression and surface antigens, that are directly involved in the T cell function. This is supported by data of other species. Prophylactic treatment with anti-CD4 and anti-CD8 antibodies in man (Cosimi et al, 1981) and primates (Jonker et al, 1984) and by antibodies directed against 'activation' markers such as treatment with anti-Ia antibodies (Lems et al, 1986), MCA directed against the Interleukin-2 receptor (Shapiro et al, 1987; Kupiec-Weglinski et al, 1986) and the MCA CLB1 (Billing et al, 1984), are in support of such a correlation. We therefore propose the following model: the immune regulation is controlled by surface molecules on T lymphocytes, that are directly involved in their function. Only antibodies that are directed against certain epitopes on such surface antigens are immunosuppressive.

Data of the CD4 molecule are in agreement with this model. This molecule is involved in the interaction of T cells and antigen presenting cells (APC). For T cell activation two signals or a combination of these two signals are required: first binding of the T cell receptor and secondly binding to the 52 kD antigen. Binding of antibody (W3/25 or ER-2) to the CD4 molecule interferes with the interaction between the T cell receptor and its antigen. Thus T cell activation either in vitro (MLR) or in vivo (alloresponse) is suppressed by these antibodies.

Also the 95 kD molecule is involved in the T cell function. The in vitro data suggest that binding to this molecule gives a co-stimulatory signal during mitogenic or allogeneic activation. The in vivo results, however, seem to be in conflict with a co-stimulatory function, because suppression of the immune response was found for ER-1 and HIS-17. This indicates that the in vivo stimulation may occur through another mechanism than the in vitro activation. In this respect it is tempting to speculate on the nature of the antigen presentation in both systems. The antigenic stimulus in an in vitro MLR assay is supposedly a direct non-restricted allogeneic signal. In such a system antibodies against the 95kD molecule would give an additional signal and do not interfere with this process of non-restricted allo-stimulation.

Besides this non-restricted alloreactivity as can be observed in *in vitro* systems one can imagine the existence of a restricted anti-alloresponse *in vivo*. In the latter system the allo-MHC antigens should be recognized in the context of self-MHC. The most likely site for this type of antigen presentation, would be at the level of the APC. The APC might

express on its cell surface processed allo-antigens in association with self-MHC determinants to the T cell system. Perhaps this type of reaction is the most prominent mode of activity early after transplantation, which would fit in well with the observed primary reactivity of (class II MHC restricted) $CD4^+$ cells early after allografting. In this way the early steps in T cell activation in vivo after allografting would be more MHC-restricted anti-allo responses. In that case, one has to assume that also in the in vivo situation, antibodies against the 95 kD molecule give a stimulatory signal. However, by binding to the molecule these antibodies interfere with the MHC-restricted interaction process, thereby preventing the specific activation mechanism. In this proposed model ER-1 and HIS-17 would bind and interfere. As a result no activation of the T cell system and no rejection would occur. W3/13 on the other hand, has a strong modulating effect on the 95 kD molecule, resulting in the disappearance of the specific epitope on the receptor. As a consequence the cell receives its stimulatory signal, but since the epitope is no longer present, the antibody can no longer bind to the cell, and will not interfere with any cell function. As a result normal activation of the T cell system occurs and the allograft will be rejected. Obviously the MHC-restricted anti-alloresponse is only part of the rejection mechanism. The residual activity is not hampered by MCA binding. Therefore activation will take place, as can be concluded from the fact that also with ER-1 and HIS-17 only a prolongation of graft survival is found and never a complete abolishment of the rejection.

For the mechanism of immunosuppression defined by these MCA the time of application, i.e. presence during the initial phase of transplantation is probably essential. In experimental studies in which antibodies were used for treatment of a renal failure episode the mechanism is basically different from the treatment in the initial phase. In the former situation the immune system is in an activated state. During this rejection episode a massive depletion of T cells, after for instance anti-thymocyte globuline (ATG) treatment, appears to be effective for a reversal of the alloreaction (Shield et al, 1979; Simonian et al, 1983). The approach of our studies involved a non-activated T cell system and interference in the recognition phase of the immune response. As a consequence different surface molecules are most likely involved and immunosuppression will be controled at another level. Whether a comparable type of immunosuppression is possible in an activated status, as proposed for the recognition phase remains to be defined.

Conclusion

The data of these studies show that several restrictions exist for an antibody to be immunosuppressive. Although the affinity of the antibodies may be important, these experiments give no further information about this factor, since all tested antibodies had a high affinity for their respective determinant. Furthermore the IgG2 subclass appears to be the most suited isotype (at least in the rat), though another isotypes can also be immunosuppressive. The complement binding capacity of the IgG2 isotype may even be a negative factor, because in the clinical situation a massive cell death resulting from treatment with such an antibody will cause unwanted side effects. This would favour the production of MCA of other isotype such as the IgG1 subclass. The data strongly indicates that an immunosuppressive MCA has to be directed to surface antigens, that are directly involved in T cell function. MCA directed against such surface molecules showed that also the epitope that is recognized is decisive for immunosuppression. The results described in this chapter have to be evaluated in regard to the time of application of the antibodies. They were injected only in the initial phase of the allogeneic reaction and this will be most certainly an important factor in the observed interpretation of the results. A model for the mechanism of immunosuppression has been proposed, which can be tested with the panel of antibodies. Furtheron the proposed mechanism of immune regulation has to be evaluated in other approaches such as treatment in combination with cyclosporin A or treatment of an rejection episode.

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Chapter 6

CONCLUDING REMARKS

Today it has been almost thirty years ago, that the first clinical organ transplantation was performed. Since that time major progress has been achieved and the knowledge of the factors controlling the fate of the graft has been greatly improved. Especially the introduction of modern technology, such as monoclonal antibodies, T cell cloning and DNA recombinant techniques, has deepened our understanding.

Both patient and graft survival has been increased as compared to those pioneering days of clinical transplantation. Improvement in the fields of surgical techniques, organ preservation and matching of donor and recipient for the most important genetic factors as well as better protocols for immunosuppression are responsible for this progress. The knowledge about the major genetic complex involved in rejection, i.e. the MHC has expanded over the last years. The high doses of immunosuppressive agents used in the first protocols have been replaced by more specific and other methods of immunosuppression. In earlier protocols for instance azathioprine and prednisolone were used for immunosuppression, whereas nowadays especially cyclosporin A has is favoured as an immunosuppressive agent in transplantation. Also combined regimes of ATG with other immunosuppressive agents or pretransplant blood transfusions have been shown to prolong graft survival.

However for the immune status after transplantation and especially during a renal failure episode (RFE), many transplantation centres still rely predominantly on clinical parameters and direct measurement of the renal function. After the introduction of the first anti-lymphoid subset MCA´s in man many efforts were done to establish a prognostic or diagnostic value for these reagents in relation to an RFE. At first monitoring of the helper and cytotoxic/suppressor T cell population in transplant patients seemed hopeful. However in Cy A treated patients these values do not change significantly during the course of rejection.

Thus, in spite of the improved knowledge of factors involved in transplantation, the actual mechanism of induction and rejection remains poorly understood. This is mainly caused by the complex composition of the immune system, which is a network of interconnected cell interactions and feed back loops. The immune system has furthermore various alternative

pathways to accomplish its task, i.e. the elimination of foreign tissue.

Transplantation research in man is hampered by several complicating factors. For instance the genetic variation within the human species is extensive and it is almost impossible to find genetically identical individuals. Moreover the clinical background of patients, but also methods of immunosuppressive treatment differ. Additionally, extensive variations in methods applied for monitoring and matching between centres exist. Therefore comparison of clinical transplantation data should be done with utmost care.

For these reasons transplantation research in animals is essential for fundamental research of the mechanism of the rejection process. Experimental studies in animals as described in this thesis have several major advantages as compared to the clinical situation. In contrast to the human system experiments can be performed with inbred animals. This makes it possible to compare the individual results and to reproduce the experimental conditions for more detailed studies. The basics of the immune response against the graft can be investigated in untreated animals with a defined course of rejection. Both the effects induced by the graft in the recipient as well as within the graft itself can be analyzed. For such an analysis one needs the availibility of sufficient tools to be able to dissect the immune system to a certain extent. Such tools include among other things the possibility to recognize and test a variety of immune cell sub-populations in these experimental animals. The first part of this thesis deals therefore with the development and fine characterization of a panel of monoclonal antibodies, directed against specific cell surface determinants on T cells of the rat. Much attention was payed to analyse the overall recognition patterns of these MCA´s, since it is unknown what type of data would come out of the use of such antibodies in the analysis of a highly activated immune system as one can expect after allogeneic organ transplantation. Correct interpretation of the results obtained can only be achieved through such a "complete" insight into the fine specificity of the MCA´s. From this point of view it was also important to specify the precise cell surface molecule recognized by an MCA and its possible involvement in specific cell functions.

With this knowledge a multidirectional approach was started to analyse the factors involved in the rejection process. To minimize the variations and complicating contributions to the allogeneic response, alterations in the immune response were investigated in a donor-recipient

combination only differing in antigens coded by the MHC. In such recipients changes in immune status were measured at various times after kidney transplantation both in the graft as well as in the immune system of the recipient. These phenotypical data were correlated with a simultaneous analysis of functional changes within the T cell system.

These type of experiments and the results obtained indicated the vaste complexity of the rejection phenomenon. Even in a defined model as used in these studies a variety of processes occur. The experiments here should be considered as a first trial to look at the alloresponse after organ transplantation from a variety of angles. Therefore, conclusions drawn from these studies can only point out at which level further investigations are needed.

In immunosuppressed individuals the mechanism will be even more complicated. For instance Cy A interferes with T cell activation and prevents production of lymphokines. Still irreversible rejection episodes are found in such patients. This illlustrates that the immune system can escape from suppression under certain conditions. During immunosuppression normally infrequent and supposedly insignificant types of immune responses may become important. The class II restricted CTL's and other peculiar cells may dominate in immunosuppressed situations.

Finally, also more specific means of immunosuppression are required, since the immunosuppressive treatments used sofar in transplantation all have the major disadvantage that they affect the whole immune system. Only the undesired response against the graft should be the target of an ideal method of immunosuppression. One way to achieve this goal would be the use of very specific MCA's as immunosuppressive agents. In the final part of this thesis a start was made to get insight in this complex material. We could show, that not only by massive removal of certain T cells, but more importantly also by direct interference with essential T cell functions anti-T cell MCA's can be very efficient in preventing or at least delaying the onset of rejection. Several important features in terms of recognition of the relevant MCA's in relation to their immunosuppressive properties were discovered. A model of interaction of these MCA's with the immune system during rejection was proposed and discussed. Although up till now only prolongation of graft survival and not a complete abolishment of the rejection process could be achieved, these studies indicate the enormeous potential of this type of approach in the future.

SUMMARY

The integrity of an organism needs an intraorganismic defense system to protect itself against disease-causing microorganisms. This socalled immune system is in vertebrates provided by two systems, which are both capable to respond specifically to most foreign substances. Generally one of these two is favoured, depending on the place, the actual form and the way the antigenic substance is presented to cells of the immune system. An allogeneic transplant is for the immune system a foreign substance and the graft will induce a normal type of activation of the immunocompetent cells, whereupon a cascade of recruited cells will destroy the graft. From studies with T cell deprived animals we know that both the initiation of the immune response as well as the final effector phase against the graft are predominantly caused by cells of the T cell system. Therefore most of the immunological transplantation research has been focussed on the T cell system. The T cell system is, however, a complex network of various specialized cells, which have regulatory and/or effector functions. The T cell system exists of myriads of cellular interactions and the system possesses various alternative pathways to obtain the desired result. Cell functions takes place through specialized surface molecules. Specific antibodies against such surface determinants make it possible to recognize the various cell populations.

This thesis describes an experimental study of the T cell system in relation with allogeneic organ transplantation. The experiments were performed in rats. Inbred rat strains as well as congenic rat strains are readily available. These inbred strains enable transplantation in fixed donor-recipient combinations. Data obtained from one allografted animal can therefore directly be compared with data obtained from another animal. For a dissection of the T cell system of the rat in these type of studies specific antibodies were required directed against various subpopulations of the T cells. Since hardly any of such antibodies were available for the rat, the first part of this thesis describes the development of such antibodies, using the hybridoma technique. Since in the course of the alloreaction due to activation and differentiation the phenotypical expression of cells may change it was necessary to characterize the MCA´s in regard to the surface antigen, which they recognize, the cells that carry these antigens and their function during T cell activation.

The literature indicates conflicting data about the mechanism of

the rejection. Two different routes of activation of the lymphoid system and graft destruction have been described. In the first model the rejection process is mediated through cytotoxic T cells, which by direct cell-contact will lyse and destroy the foreign material. The second response mechanism is the delayed type hypersensitivity reaction, in which regulator T cells through mediators arm the mononuclear phagocytic system, which thereafter acts as the effector mechanism in graft rejection. The role of small subpopulations of immature T cells or mature T cells in the alloreactions is unknown. The aim of these studies was to perform a multiparameter analysis of the T cell system during rejection. First of all a panel of MCA's was selected to define quantitative changes in the major subpopulations, to detect signs of activation and a possible presence c.q. influx of other subpopulations. Both in the graft and in the recipient the transplantation induced alterations, which were screened at fixed time intervals. Besides mere monitoring of the major cell streams also the graft directed response of the recipient was studied. Finally, the interference with the rejection process was studied by a selected number of the MCA's. In this work the mode of action of the immunosuppressive effects of antibodies was studied in detail.

In **chapter 1** the fundamentals of the immune system are described. Illustrations of basic forms of defense both aspecific and specific are given. A high degree of specialization of the immune system is found in the vertebrates with specialized cells, both in regulatory and effector functions.

One of the most appealing examples of foreign invasion is the transplantation of allogeneic organs or tissues. The genetics controling the alloreaction of the immune system, especially in the rat, is reprted. Cells of the T cell system play an central role in the allograft reaction and the differentiation and maturation of cells of this lineage are described.

The study of the T cell system of the rat in the course of the alloreaction of the recipient towards the grafts requires specific antibodies, which are directed against various components of this system. For this purpose the hybridoma technique was the obvious choice for the production of such antibodies.

In **chapter** 2 several aspects of the hybridoma technique have been described. Large numbers of growing hybrid cells require a fast and sensitive method of selection. Therefore three techniques were compared for application in the hybridoma technique. Both the RIA and ELISA were shown to be excellent techniques for the primary selection of hybrid cells. Analysis with the fluorescence activated cell sorter seems not sensitive enough to detect a low concentration of antibody, but in a later stage with selected hybrid clones this technique provides additional information about the cellular reactivity of MCA´s. The hybridoma technique was subsequently used in a series of experiments in which a large number of hybrid cells were obtained producing specific monoclonal antibodies directed against a variety of rat leukocytes.

The further characterization of these MCA´s directed against rat leukocytes is described in **chapter 3**. Immune histology on spleen and thymus revealed information about the reactivity of these antibodies with the lymphocyte populations in these organs. Quantitative data of the cellular reactivity of selected antibodies were obtained using flow cytometric analysis. A majority of lymphocytes in the thymus and a variety of cell numbers in the lymphoid organs reacted with these MCA´s. Using double marker and two colour fluorescence analysis it could be defined that these antibodies recognize T cells and not B cells, moreover they react with various parts of the total T cell population in the periphery.

For a proper application in transplantation research it was necessary to define the precise recognition of these MCA´s also with cells in the early stages of the T cell differentiation. Almost all MCA´s reacted with a substantial number of bone marrow cells indicating a recognition of other cells in early stages of the myeloid differentiation lines. A monoclonal antibody could be selected that does react with thymocytes and precursor T cells, but not with mature T cells.

The size of the various surface antigens recognized by these newly developed MCA´s was defined. An example of two MCA´s, directed against different determinants of the same surface molecule and displaying divergent reactivity patterns is shown. Furthermore several of these antigens could be shown to be directly involved in the T cell function. By testing the influence of monoclonal antibodies on a variety of in vitro immunological assays several patterns of stimulatory or inhibitory nature were established. Most active MCA´s were directed against determinants

invloved in early stages of the activation process after allogeneic
stimulation. Some exerted their effect through the inducer subset, others
through the precursor cytotoxic subset. Finally the physical properties of
these MCA´s, such as isotype, complement binding abilities and affinity,
which are important for the biological effects of these antibodies are
described.

Based on these results a panel of MCA´s was selected. This panel
was used in the study of the mechanism of the allograft rejection.

Chapter 4 shows data of experimental studies on the mechanism of
the graft rejection. Inbred congenic rat strains were used for kidney
transplantations. The donor recipient combinations only differed for the
MHC complex. After defining the model by functional and pathological
parameters the immune system was analyzed in situ in the recipient as well
as in the graft at various times after transplantation.

Flow cytometric analysis of the immune system in the spleen and in
blood after transplantation has been done. The results emphasize once more
the importance of using inbred strains for such experiments. Reduction of
the variation in the results is necessary for a clear evaluation of the
results. Also the consequences of the method to define subpopulations must
be taken into consideration. Furthermore the absolute cell numbers of T
cell subpopulations are a better reflection of the ongoing rejection
process than the relative numbers. MCA´s directed against mature T cell
markers seem to indicate a rather unaffected immune system in the spleen.
Data with other markers, however, indicate a dynamic situation in this
organ as illustrated for instance by the influx of immature T cells,
probably thymus derived. In blood the helper T cell population reacts
shortly after transplansplantion by a significant increase in cell numbers.
The other subpopulations seem to be involved in a later stage of the
rejection. The situation is illustrated in blood at six days after
transplantation showing that monitoring of the normal lymphocyte
subpopulations does not reflect the activated status of the immune system
in contrast to analysis with antibodies directed against activation markers
and socalled immature T cell markers.

The graft infiltrating cells are analyzed using specific immune
histology. In allografted animals two major types of infiltrating cells
are found. Cell concentrations in close connection with the arterioles of
the graft (perivascular infiltrates) and scattered cells throughout the

graft (tubular infiltrates). Both infiltrates increase in cell numbers
during the course of rejection. In the perivascular infiltrates the
cytotoxic/suppressor T cells are detected in higher numbers than the helper
T cells. Macrophages enter the graft right after transplantation and they
are located in both infiltrates. The high class II expression on graft and
host cells indicate a high degree of activation of the cells of the immune
system. The helper T cells are predominantly located in the perivascular
clusters. These compartments, containing both T cell subpopulations and
macrophages may be the site of a specific immune reponse of host cells
against the graft.

One of the major participants of the immune reponse against a graft
i.e. the donor class I antigen directed CTL was functionally determined.
The determination of the precursors of the CTL's in the rat using the
technique of limiting dilution is described. Using this technique the
frequency of the CTL-p has been determined at various days after allogeneic
transplantation. Data of spleen and blood, but also in the graft is
described. During the first days after transplantation the frequency
highly increases in the recipient spleen and blood. The number of donor
specific CTL-p's in the graft on the other hand increases only at a later
stage during the rejection process. Our data indicate an early role for
the T-helper cell population, possibly both as cells displaying DTH
activity as well as cells involved in the induction of the CTL
differentiation. Lateron a more pronounced role both phenotypically and
functionally of the cytotoxic T cell subset is found.

In **chapter 5** the application of MCA's as immunosuppressive agents
in transplantation is described. A large number of MCA's were tested in a
skin graft model. Only a few antibodies, reacting either with all T cells
or with helper T cells, have the ability to prolong the graft survival.
Five MCA's were selected for studying the factors, that are decisive for
the immunosuppressive properties of an antibody. In contrast to polyclonal
antibodies the immunosuppressive MCA's are not restricted to specific
isotypes. Furthermore the immunosuppressive ability of these antibodies
seems to be correlated with particular epitopes on some specific surface
molecules. The *in vivo* behaviour of these MCA's such as the biological
half life time, the localization in various organs and the analysis of the
T cell population in blood was studied after a single dose of MCA.
Elimination of the T cell population in blood was found both for the

immunosuppressive antibodies as well as for the antibody lacking this ability. In the spleen a rather different type of localization was found for the non-suppressive antibody compared with the suppressive ones. The *in vitro* experiments in chapter 3 showed that both surface molecules recognized by these 5 MCA's are involved in the function of the T cells. The immunosuppressive properties of antibodies are probably connected with this function. Based on the data of these experiments a hypothesis on the mechanism of immunosuppression is suggested: immunosuppressive antibodies have to be directed against surface molecules, that are directly involved in the T cell function. The data of these experiments are discussed in view of this model. Besides the specific T cell receptor complex several surface molecules are involved in cell interaction processes. Binding to such antibodies results in blocking of these interactions and consequently suppression of the immune response.

SAMENVATTING

Voor de integriteit van een organisme is een intraorganistische afweer noodzakelijk om zich te kunnen beschermen tegen micro-organismen, die ziekten kunnen veroorzaken. Dit zogenaamde immuunsysteem bestaat in vertebraten uit twee afzonderlijke systemen, die beide in staat zijn om specifiek te reageren tegen de meeste vreemde substanties. In het algemeen heeft één van beide systemen de voorkeur, afhankelijk van de plaats, de precieze vorm en de manier waarop de antigene stof aan de cellen van het afweersysteem wordt aangeboden. Een allogeen transplantaat is voor het immuunsysteem een vreemde substantie en dit transplantaat zal een normale activatie van de immuuncompetente cellen veroorzaken, waarna een groot aantal gerekruteerde cellen het transplantaat zullen vernietigen. Studies met dieren, waaruit de T cellen zijn verwijderd, hebben ons geleerd, dat deze cellen een centrale rol vervullen bij de initiele en de effector fase van de immuunresponse tegen het transplantaat. Daarom heeft een aanzienlijk deel van het transplantatieonderzoek zich gericht op de cellen van het T cel systeem. Dit systeem is echter een zeer complex netwerk van verschillende cellen, die een regulerende en/of een effector funktie bezitten. Het T cel systeem bestaat uit een zeer groot aantal cellulaire interacties en het systeem heeft verschillende mogelijkheden om een bepaald doel te bereiken. Celfunkties worden via speciale oppervlaktemolekulen uitgevoerd en met behulp van specifieke antilichamen, gericht tegen dergelijke moleculen is het mogelijk om de afzonderlijke celpopulaties te herkennen.

Dit proefschrift beschrijft een experimentele studie van het T cel systeem in relatie tot de allogene orgaantransplantatie. De experimenten werden uitgevoerd in de rat. Momenteel zijn inteeltstammen en congene stammen voor de rat gemakkelijk te verkrijgen. Met behulp van deze inteelt stammen is het mogelijk om in gedefinieerde donor-ontvanger combinaties te transplanteren. Gegevens van allogeen getransplanteerde dieren kunnen daarom onderling vergeleken worden. Voor een juiste evaluatie van het T cel systeem van de rat in dit type onderzoek is het noodzakelijk om de verschillende T cel subpopulaties te onderscheiden met behulp van specifieke antilichamen. Omdat dit soort antilichamen nauwelijks beschikbaar zijn voor de rat beschrijft het eerste deel van dit proefschrift de ontwikkeling van dergelijke antilichamen met behulp van de hybridoma techniek. Tijdens het proces van de allogene reaktie zullen

cellen veranderen van fenotype als gevolg van aktivatie en differentiatie. Daarom was het noodzakelijk om de monoklonale antilichamen (MCA's) te karakteriseren ten aanzien van het oppervlakteantigeen, die ze herkennen, de cellen die het antigeen tot expressie brengen en de funktie van dit antigeen tijdens T cel aktivering.

De literatuur geeft tegenstrijdige gegevens voor het mechanisme van de afstoting. Twee verschillende modellen worden beschreven voor de aktivering en de destructie van het transplantaat. In het eerste model wordt het afstotingsproces gemedieerd door cytotoxische T cellen, die via direct celkontakt de vreemde materie zullen lyseren en vernietigen. Het tweede response mechanisme is de vertraagd type overgevoeligheids reaktie, waarbij regulerende T cellen d.m.v. mediatoren het mononucleaire fagocytaire systeem zullen aktiveren. Dit laatste systeem fungeert vervolgens als effector mechanisme voor de afstoting van het transplantaat. Naast deze twee response types blijft de rol van kleine subpopulaties onrijpe T cellen of uitgerijpte T cellen in de allogene reaktie een onbekende factor. Het doel van deze studies was een multiparameter analyse van het T cel systeem tijdens de afstoting. In de eerste plaats werd een panel MCA's geselekteerd om kwantitatieve veranderingen in de belangrijkste subpopulaties te kunnen bepalen en om aanwijzingen van aktivatie en een mogelijke influx van andere subpopulaties te kunnen ontdekken. Zowel in het transplantaat als in de ontvanger zullen veranderingen worden geinduceerd door het transplantaat. Behalve monitoring van de belangrijkste veranderingen in de samenstelling van de celpopulatie werd ook de repons, die gericht is tegen het transplantaat bestudeerd. Ten slotte werd een studie gemaakt van de beinvloeding van het afstotingsproces m.b.v. een selektie van MCA's. Hierbij werd het mechanisme van de immunosuppressieve werking van de antilichamen in detail bestudeerd.

In **hoofdstuk 1** wordt de fundamentele werking van het immuunsysteem beschreven. Dit wordt geïllustreerd met basale vormen van zowel aspecifieke als specifieke manieren van verdediging. In vertebraten treffen we een hoge mate van specialisatie van het immuunsysteem aan. Dit wordt gemedieerd door speciale cellen, die een regulerende hetzij een effectorfunktie bezitten.

Een van de meest aansprekende voorbeelden van vreemde substantie is de transplantatie van allogene organen of weefsels. De genetische controle van de allogene reaktie van het immuunsysteem, met name in de rat, wordt

besproken. Bij de transplantaatafstoting spelen de cellen van het T cel systeem een centrale rol en daarom wordt de differentiatie en de maturatie van de cellen van deze lijn beschreven.

Voor de studie van het T cel systeem in de loop van de allogene reaktie van de transplantaatontvanger tegen het transplantaat zijn specifieke antilichamen nodig, die verschillende componenten van dit systeem herkennen. Voor de produktie van dergelijke antilichamen was de hybridoma techniek een logische keuze.

In **hoofdstuk 2** worden verschillende aspecten van de hybridoma techniek beschreven. De aanwezigheid van grote aantallen groeiende hybride cellen maken een snelle en een gevoelige methode voor selektie noodzakelijk. Daarom werden drie verschillende technieken met elkaar vergeleken in het kader van de specifieke condities voor gebruik in de hybridoma techniek. Zowel de RIA als de Elisa blijken uitstekende technieken te zijn voor de primaire selektie van hybride cellen. Analyse met behulp van de fluorescence activated cell sorter (FACS) is niet in staat om lage concentraties antilichamen te detecteren. In een later stadium echter, na selektie van hybride clonen, geeft deze techniek extra informatie over de cellulaire reaktiviteit van de geproduceerde MCA´s. Vervolgens werd de hybridoma techniek toegepast in een aantal experimenten, waarin een groot aantal hybridoma´s werden verkregen, die specifieke monoklonale antilichamen produceren gericht tegen verschillende leukocyten van de rat.

In **hoofdstuk 3** wordt een meer gedetailleerde karakterisering beschreven van deze MCA´s, die gericht zijn tegen leukocyten van de rat. Met behulp van immuunhistologie op milt en thymus kan informatie verkregen worden over de reaktiviteit van deze antilichamen met de lymfocytenpopulaties in deze organen. Kwantitatieve gegevens van geselekteerde antilichamen ten aanzien van de cellulaire reaktiviteit werden verkregen met behulp van een flow-cytometrische analyse. Het grootste deel van de lymfocyten in de thymus en een variabel deel van de T cellen in de periphere lymfoide organen reageerden met deze MCA´s. Gebruik makend van dubbelmarker en twee kleuren fluorescentietechnieken kon worden vastgesteld, dat deze antilichamen T cellen en niet B cellen herkennen en tevens, dat zij met verschillende delen van de totale T cel populatie reageren.

Voor een optimale toepassing in het transplantatieonderzoek was het noodzakelijk om de exacte herkenning van deze antilichamen, ook die ten aanzien van cellen in de vroege fase van de T cel differentiatie te bepalen. Bijna alle antilichamen reageren met een aanzienlijk deel van de cellen in het beenmerg, hetgeen een indicatie is, dat ook cellen, in een vroeg stadium van de andere myeloide differentiatie reeksen, worden herkend. Voor één monoklonaal antilichaam kon worden vastgesteld, dat dit antilichaam reageert met thymocyten en met precursor T cellen, maar niet met rijpe T cellen.

De grootte van de verschillende oppervlakte antigenen, die door deze nieuw ontwikkelde MCA´s worden herkend, werd vastgesteld. Een voorbeeld wordt gegeven van twee MCA´s, beiden gericht tegen verschillende determinanten van hetzelfde oppervlakte molecuul, echter met een divers reaktiepatroon. Tevens kon voor een aantal van deze antigenen worden aangetoond, dat zij direct zijn betrokken bij de funktie van de T cel. Door de invloed van de monoklonale antilichamen te testen in een aantal immunologische *in vitro* bepalingen werden verscheidene patronen gevonden, die een stimulerend hetzij een remmend karakter, vertoonden. De meeste van deze actieve MCA´s waren gericht tegen determinanten, die in een vroege fase van het activeringsproces na allogene stimulatie zijn betrokken. Sommige antilichamen oefenen hun effect uit op de inducer subpopulatie, terwijl anderen via de precursors van de cytotoxische subpopulatie werken. Tenslotte worden fysische eigenschappen van deze MCA´s beschreven, zoals het isotype, het vermogen om complement te binden en de affiniteit. Dit is van belang voor de biologische activiteit van deze antilichamen.

Gebaseerd op deze gegevens werd een panel monoklonale antilichamen geselekteerd. Gebruik makend van dit panel werd het mechanisme van de allogene transplantaat afstoting bestudeerd.

Hoofdstuk 4 geeft de data, die betrekking hebben op experimentele studies van het mechanisme van de transplantaat afstoting. Hiervoor werden niertransplantaties uitgevoerd met behulp van congene inteeltrattestammen. In de combinatie van donor en ontvanger was alleen een verschil ten aanzien van het MHC complex. Na de definïering van het model door middel van funktionele en pathologische parameters werd een analyse van het immuunsysteem van de ontvanger in situ, maar ook in het transplantaat op verschillende dagen na transplantatie uitgevoerd.

Analyse van het immuunsysteem na transplantatie werd uitgevoerd in

de milt en het bloed met behulp van flow-cytometry. Het resultaat benadrukt eens te meer het belang van inteeltstammen voor dit type onderzoek, omdat voor een duidelijke evaluatie van de resultaten het noodzakelijk is om de variatie in de gegevens te minimaliseren. Men dient zich bewust te zijn van de konsekwenties, die het gevolg zijn van de methode, die gekozen is om een bepaalde subpopulatie te definiëren. Verder blijken de absolute celaantallen een betere reflektie te zijn van het afstotingsproces dan de relatieve cel aantallen. MCA's, die gericht zijn tegen rijpe T celmarkers, laten in de milt bijvoorbeeld nauwelijks veranderingen van het immuunsysteem zien, wanneer de relatieve celaantallen worden gehanteerd. Gegevens verkregen met behulp van andere markers echter laten zien, dat dit een dynamische situatie is. Dit wordt byvoorbeeld geïllustreerd door de influx van onrijpe T cellen, waarschijnlijk afkomstig uit de thymus. In het bloed reageert de helper T cel populatie kort na transplantaties met een duidelijke toename in aantal cellen. De andere subpopulaties schijnen pas in een later stadium van de afstotingsreaktie betrokken te zijn. Monitoring van de normale, rijpe lymfocytenpopulaties geeft in tegenstelling tot analyse met antilichamen gericht tegen activerings markers of zogenaamde onrijpe T cel markers, geen juist beeld van de geactiveerde status van het immuunsysteem. Dit laatste wordt geïllustreerd door de situatie in het bloed op 6 dagen na transplantatie.

De cellen, die infiltreren in het transplantaat werden geanalyseerd met behulp van specifieke immuunhistologie. In de allogeen getransplanteerde dieren werden twee belangrijke types infiltrerende cellen waargenomen. Celconcentraties in de nabijheid van de arteriolen van het transplantaat (perivasculaire infiltraten) en cellen, die door het hele transplantaat voorkomen (tubulaire infiltraten). Beide infiltraten nemen toe in celaantal tijdens het verloop van de afstoting. In perivasculaire infiltraten worden meer cytotoxische/suppressor cellen aangetroffen dan helper T cellen. Macrofagen komen direct na de transplantatie het transplantaat binnen en worden in beide infiltraattypes aangetroffen. De hoge expressie van het klasse II antigeen op de cellen van het transplantaat en die van de ontvanger suggeren een hoge mate van activatie van de cellen van het immuunsysteem. De helper T cellen zijn voornamelijk gelokaliseerd in de perivasculaire clusters. Mogelijk zijn deze compartimenten, die zowel T cellen als macrofagen bevatten, de lokatie van de specifieke immuunresponse van ontvangercellen gericht tegen het transplantaat.

Eén van de belangrijkste participanten van de immuun response werd funktioneel bepaald. Dit is de aktiviteit van de cytotoxische T lymfocyten (CTL's) tegen het klasse I antigeen van de donor. De bepaling van de precursor cellen van de CTL's (CTL-p) in de rat werd uitgevoerd met behulp van de limiting dilution assay (LDA). Met behulp van deze techniek is het mogelijk om een inzicht te krijgen in de frekwentie van de CTL-p op verschillende tijdstippen na transplantatie. Gegevens van de milt en het bloed, maar ook in het transplantaat zelf worden beschreven. Tijdens de eerste dagen na transplantatie neemt de frekwentie sterk toe in de milt en in het bloed van de ontvanger. In het transplantaat zelf neemt het aantal donor specifieke CTL-p's pas in een later stadium van de afstoting toe. Onze gegevens indiceren een vroege rol voor de helper T cel. Dit kunnen cellen zijn, die een vertraagd type overgevoeligheidsreactie tot expressie brengen of cellen die betrokken zijn bij de inductie van de CTL differentiatie. In een later stadium wordt een meer geprononceerd rol voor de cytotoxische T cel subpopulatie gevonden, zoals blijkt uit fenotypische en functionele gegevens.

In **hoofdstuk 5** wordt de toepassing van MCA's als immuunsuppressieve agentia beschreven. Een groot aantal MCA's werd getest in een huid transplantatiemodel en hierbij bleek, dat slechts enkele antilichamen in staat zijn een verlenging van de transplantaatoverleving te bewerkstelligen. Deze MCA's herkennen alle T cellen of alleen de helper T cellen. Vijf antilichamen werden vervolgens geselekteerd voor een studie van de factoren die bepalend zijn voor de immuunsuppressieve eigenschappen van een antilichaam. In tegenstelling tot polyklonale antilichamen is immuunsuppressie bij monoklonale antilichamen niet beperkt door het isotype. Verder lijkt er een correlatie te bestaan tussen de immuunsuppressieve eigenschappen van deze antilichamen en bepaalde epitopen op enkele specifieke oppervlaktemoleculen. Het *in vivo* gedrag van deze antilichamen werd bestudeerd na een enkelvoudige dosis van het MCA d.m.v. bepaling van de biologische half waarde tijd, de lokalisatie in verschillende organen en de analyse van de T cel populatie in het bloed. Eliminatie van de T cel populatie in het bloed werd aangetoond voor zowel de immuunsuppressieve MCA's als voor het antilichaam, dat deze eigenschap mist. In de milt werd voor dit laatste antilichaam een afwijkende lokalisatie gevonden in vergelijking met de andere MCA's. Uit de *in vitro* experimenten beschreven in hoofdstuk 3, blijken deze twee

oppervlaktemoleculen, waartegen de 5 MCA´s zijn gericht, ook betrokken te zijn bij de funktie van de T cel. De immuunsuppressieve eigenschappen van deze antilichamen zijn hoogst waarschijnlijk gerelateerd met deze funktie. Gebaseerd op de gegevens van deze experimenten werd een hypothese voorgesteld voor het mechanisme van immuunsuppressie: Immuunsuppressieve antilichamen moeten gericht zijn tegen antigenen, die direct betrokken zijn bij de funktie van de T cel. De resultaten van deze experimenten worden in het kader van deze hypothese bediscusseerd. Naast het T cel receptorcomplex zijn verscheidene andere oppervlaktemoleculen ook betrokken bij het proces van celinteractie. Binding van antilichamen, die gericht zijn tegen dergelijke antigenen zal resulteren in een blokkering van de celinteractie en als gevolg daarvan zal een suppressie van de immuunresponse plaats vinden.

CURRICULUM VITAE

Na het behalen van het diploma MULO-B aan de "Prs. Wilhelmina"
school te Utrecht in 1963 ben ik gedurende de periode van 1963 tot 1970
werkzaam geweest bij het Centraal Instituut voor Voedingsonderzoek TNO
(CIVO-TNO) te Zeist als aankomend analist. Door middel van studie tijdens
de avonduren behaalde ik in 1966 het diploma leerling analist (chemische
richting) bij de Stichting tot Opleiding van Assisterend
Laboratoriumpersoneel (SAL) te Utrecht. In de daarop volgende periode
volgde ik bij het Stichts Avondlyceum te Utrecht onderwijs en sloot deze
periode af met het behalen van het HBS-B diploma in 1970. In datzelfde
najaar begon ik met de studie Biologie aan de Rijksuniversiteit te Utrecht
en het kandidaatsexamen werd in 1974 afgelegd. Het doctoraal examen, met
de hoofdvakken Immunologie en Microbiologie werd in het voorjaar 1977 met
goed gevolg afgelegd.

Na tijdelijk teruggekeerd te zijn bij het CIVO-TNO te Zeist begon
ik in 1979 mijn werkzaamheden bij de Erasmus Universiteit te Rotterdam. Na
een korte periode bij de afdeling Heelkunde, waar betrokken was bij het
tranplantatie onderzoek bij de hond, kwam ik in januari 1980 op de afdeling
Inwendige Geneeskunde. In de periode, die daarop volgde werd op het
laboratorium Experimentele Nefrologie van deze afdeling het onderzoek
verricht, dat in dit proefschrift wordt beschreven.

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Nawoord

Het onderzoek, dat in dit proefschrift wordt beschreven is tot stand
gekomen door de inzet en de hulp van een groot aantal mensen. De goede sfeer
en het werken in teamverband, waarbij iedereen bij alle experimenten betrokken
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mate bijgedragen tot het bereikte resultaat. Ook in de laatste fase, tijdens
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inspanning, onze eerste relevante monoklonale antilichamen opleverde. Frans
Tielen heeft zich in de afgelopen jaren onmisbaar gemaakt o.a. door het
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