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Subsets of CD4$^+$ T cells and their roles in autoimmunity

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SUMMARY

The CD4 molecule has a very restricted tissue distribution being found at high levels only on subpopulations of thymocytes and peripheral T cells. This finding implicated the molecule in the specialized actions of these cells and provided the impetus for studies directed at determining the function of the CD4 molecule itself and of those T cells that expressed it.

The first part of this paper reviews briefly some of the earlier work in this field in which Alan Williams played such a major role. The paper concludes with an account of more recent findings which reveal that CD4$^+$ T cells are themselves phenotypically heterogeneous and that the different subsets that can be identified mediate markedly different immunological functions. In particular studies with laboratory rats have shown that one subset plays an essential role in the prevention of autoimmunity.

This finding indicates that self tolerance cannot be accounted for entirely in terms of the deletion or irreversible inactivation of autoreactive T cells and raises a number of questions about how the immune response to self antigens is actively regulated and how possible deficiencies in this regulation may give rise to autoimmune disease.

1. INTRODUCTION

The CD4 antigen has a very limited tissue distribution being found on the cell membrane of a large subpopulation of thymocytes and peripheral T cells and at low levels on some macrophages (Williams *et al.* 1977; Jefferies *et al.* 1985). Membrane antigens, like CD4, that are expressed on only a limited range of cell types have proved invaluable in the analysis of the immune system in terms of its constituent cells. The monoclonal antibodies that react with such molecules have revealed a cellular heterogeneity among lymphocytes that was only partly recognized before these reagents became available. The monoclonal antibody (mAb) W3/25, produced by Alan Williams in collaboration with Galfre and Milstein in Cambridge (Williams *et al.* 1977), has a unique place in the history of the development of this field: it was the first to define the CD4 antigen and to be used to isolate CD4$^+$ T cells for functional studies (White *et al.* 1978). To appreciate the significance of this development, I shall review briefly the situation in cellular immunology before it took place.

2. LYMPHOCYTE HETEROGENEITY AND THE USE OF SEROLOGY TO STUDY IT

As Gowans showed (Gowans *et al.* 1962), small lymphocytes play a central role in the functioning of the adaptive immune response. These cells are the precursors for effectors that are involved in both the humoral and cell-mediated arms of the immune response and this diversity of function is reflected in the activity of these cells in immunity to all potential pathogens from viruses to parasites. Given this diversity an early question that faced immunologists was how was it that one cell type could mediate such a wide range of effector functions? Although small lymphocytes are morphologically very similar it became evident, from the work of Miller (Miller & Mitchell 1969) and others that these cells were functionally heterogeneous: lymphocytes obtained from the thymuses of mice were shown not to be precursors for antibody-secreting cells. These precursors were to be found among lymphocytes in bone marrow but these latter cells required some helper activity from the thymus-derived ones in order to differentiate into antibody-secreting plasma cells. These results, although invaluable in establishing that there was functional specialization among lymphocytes, did not permit any phenotypic distinction to be made between them. However, such a distinction was soon possible when it was demonstrated by the use of specific antibodies that the precursors of antibody-secreting cells expressed immunoglobulin on their surface (Jones *et al.* 1970) while murine lymphocytes of thymic origin expressed the cell surface molecule Thy 1 (Raff 1971). These applications of serology to the study of the functioning of the immune system illustrated the value of using antibodies to cell surface molecules to distinguish between morphologically similar lymphocytes but the technique was limited by

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the availability of antisera with single specificities. In most cases the cell surface molecules were not known (surface immunoglobulin was an exception) and could not, in general, be obtained by biochemical purification so these molecules were not available to use as immunogens to raise specific antibodies. Conventional serology did however, make one further contribution to the study of lymphocyte heterogeneity in that Cantor & Boyse (1976) raised alloantisera in mice to what later became known as the α and β chains of mouse CD8. These antisera were used to demonstrate that thymus-derived lymphocytes (so-called T cells) were composed of two subsets, one of which expressed the CD8 antigen and on activation killed target cells expressing the major histocompatibility complex (MHC) antigens to which the killer cells were sensitized. The other subset of T cells, while not effector cells in the cytotoxicity assays, were required to induce this killer activity in those that were CD8⁺. In fact Cantor & Boyse raised a second series of alloantisera, to the so-called Ly 1 antigen on T cells. It appeared from the functional studies with this antibody that it reacted with the complimentary, CD8⁻ subset of T lymphocytes. This interpretation turned out to be incorrect as the Ly 1 antigen was subsequently shown to be identical to murine CD5 which is expressed on all T cells (Ledbetter *et al.* 1981). These studies by Cantor & Boyse, while again demonstrating the value of the use of antibodies as tools to dissect the immune system, also showed the difficulties of preparing satisfactory antisera by conventional serological techniques.

3. MONOCLONAL ANTIBODIES AND THE CD4 ANTIGEN

As is widely recognized Köhler & Milstein (1975), by developing the hybridoma technique, solved the problem of producing monospecific antibodies when purified antigens are not available to use as immunogens. Alan Williams played the central role in the first application of this technique to the study of lymphocyte surface molecules (Williams *et al.* 1977). This work produced a remarkable range of monoclonal antibodies which illustrated the unique value of this approach to the study of immunology at both the molecular and cellular level. Among these monoclonal antibodies was one designated W3/25 directed against a surface antigen on a subset of rat T cells. This antigen was rat CD4. The human homologue of rat CD4 was soon identified (Reinherz & Schlossman 1980) and a little later mouse CD4 was also recognized (Swain *et al.* 1984). Its importance in the development and function of T lymphocytes has been firmly established (see this and other papers in this symposium) and it continues to be studied extensively.

4. THE FUNCTIONS OF CD4⁺ T CELLS

As the W3/25 monoclonal antibody (mAb) labelled only approximately 70–80% of rat T cells, depending on the strain of rat, it was possible to study functional heterogeneity of these T cells by isolating the CD4⁺ and CD4⁻ subsets using fluorescein-conjugated antibody and a fluorescence-activated cell sorter (FACS II Becton Dickinson, California). These experiments demonstrated that, whereas rat CD4⁺ T cells provided helper activity for B cells in the induction of antibody responses, the CD4⁻ cells, when activated in a semi-allogeneic host, inhibited this induction (White *et al.* 1978). Much of the work in cellular immunology in this laboratory since that time has been concerned with the use of other monoclonal antibodies to further fractionate rat CD4⁺ T cells and to define the functions of these subsets. This work is briefly described later in this article.

5. THE EFFECT OF ANTI-CD4 MONOCLONAL ANTIBODY *IN VITRO* AND *IN VIVO*

Studies on the relative activities of CD4⁺ and CD4⁻ rat T cells in the mixed leukocyte culture (MLC) showed that only the former cells proliferated vigorously to allogeneic dendritic cells (Mason *et al.* 1981). However, this proliferation was almost completely inhibited when the W3/25 mAb was included in the culture medium and inhibition required a concentration of mAb of no more than 100 ng ml^{-1} (Webb *et al.* 1979). This result implicated the CD4 molecule itself in the activation process but also showed that mAbs could be used as probes to obtain clues about the functions of molecules at the lymphocyte cell surface. These results were subsequently explained by evidence that the CD4 molecule interacts with Class II MHC molecules on the stimulating dendritic cells (Doyle & Strominger 1987).

The suppression of T cell activation *in vitro* by W3/25 mAb prompted studies on possible *in vivo* effects. It was shown that the paralysis induced in Lewis rats by the injection of guinea-pig myelin basic protein (MBP) was completely inhibited by prior injection of W3/25 mAb and that the disease, termed experimental allergic encephalomyelitis, could be reversed if injection of antibody was delayed until paralysis had developed (Brostoff & Mason 1984). This demonstration of the potential therapeutic uses of mAbs has been amply confirmed (Qin *et al.* 1993) but further work is required to determine the mechanism of the effect. However, it has been established that the prevention of paralysis in Lewis rats by the injection of W3/25 mAb does not destroy the CD4⁺ T cells that respond to MBP and give rise to the paralysis (Sedgwick & Mason 1986) and it seems probable that the antibody has a more subtle effect than simply blocking T cell activation. It is possible that the antibody induces an anti-MBP response that inhibits the activation of the CD4⁺ T cells that cause the paralysis. This explanation requires that CD4⁺ T cells are themselves heterogeneous, for which there is much evidence (Powrie *et al.* 1990; Mosmann 1991) and that one subset can inhibit the activation of another (Locksley *et al.* 1991). Support for this explanation is provided by the demonstration that presentation of the encephalolitogenic peptide of MBP by B cells prevents the induction of experimental allergic encephalomyelitis although CD4⁺ T cells with the poten-

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tial to cause paralysis are still generated (Day *et al.* 1992).

6. SUBSETS OF CD4⁺ T CELLS

Since the first identification of the $CD4^+$ subset of T cells numerous studies in man, mouse and rat have shown that these cells are involved in a wide range of immune reactions. In addition to their helper activity in B cell responses already referred to, they mediate allograft rejection, graft-versus-host disease, delayed type hypersensitivity and, for some anti-viral responses at least they play an essential role in the generation of $CD8^+$ cytotoxic T cells from inactive precursors. Finally they have been implicated in virtually all autoimmune diseases both in man and in experimental animals. As may be anticipated, rats and mice subject to procedures that delete $CD4^+$ T cells show major deficiencies in immune responses and the complications in AIDS can be attributed to the loss of $CD4^+$ T cells that is associated with this disease.The diversity of immunological activities displayed by $CD4^+$ T cells has raised the question of whether there is functional specialization within this population. Two approaches have been made to answer this question: first to look for phenotypic differences between $CD4^+$ T cells and attempt to correlate these with differences in immunological function, and second to look for functional differences between various $CD4^+$ T cell clones. Both approaches have been fruitful (Powrie *et al.* 1990; Mosmann 1991) but it is with the former that the work to be described has been concerned.

7. THE CD45 ANTIGEN AND SUBSETS OF CD4⁺ T CELLS

The leukocyte-common antigen, CD45, was discovered by conventional serological techniques in mice (Trowbridge 1978) and rats (Standring *et al.* 1978) but it was only with the advent of monoclonal antibodies and gene sequencing that the complexity of this molecule became fully appreciated (Thomas 1989). The molecule exists at the leukocyte cell surface in a number of different isoforms generated by differential splicing of mRNA transcribed from three different exons A, B and C that encode for amino acid sequences near to the extracellular NH_2 terminus of the molecule. One isoform, designated CD45RO, has none of the A, B or C exons expressed so that, in principle eight different isoforms of CD45 can be generated. It is not known how many of these are actually expressed at the surface of any one cell but it is evident that there is heterogeneity of expression among lymphocytes. Approximately 50% of human $CD4^+$ T cells express CD45RA but not CD45RO while the remainder are $CD45RA^-O^+$ (Streuli *et el.* 1987; Terry *et al.* 1988). However, further heterogeneity can be demonstrated using antibodies to CD45RB so that at least three subsets of human $CD4^+$

Table 1. *Functions of rat CD4⁺ T cell subsets in vivo*

($CD4^+$ T cells change their CD45RC phenotype as they mature and also when activated (see figure 1). The phenotypes in tables 1 and 2 are those expressed when the cells under study are isolated before their function is determined *in vivo* or *in vitro*.

The effector T cell clones isolated so far produce IFN-γ, IL-2 and TNF-β.)

function	$CD45RC^{low}CD4^+$ subset	$CD45RC^{high}CD4^+$ subset
alloreactivity (local)	weak	strong
alloreactivity (systemic-graft versus host disease)	none	strong
precursor for B cell help in primary immune responses	no	yes
mediator of B cell help in secondary immune responses	strong	weak
effector T cell clones	yes	no

Table 2. *Functions of rat CD4⁺ T cell subsets in vitro*

(Rat and mouse serum contains an inhibitor of IL-2 activity which has not been fully characterized but which is absent from foetal calf serum. $CD45RC^{low}CD4^+$ cells produce less IL-2 on activation than do $CD45RC^{high}CD4^+$ cells and are more susceptible to inhibition by IL-2 antagonists.)

function	$CD45RC^{low}CD4^+$ subset	$CD45RC^{high}CD4^+$ subset
proliferation in the MLC	yes, in foetal calf serum but not in rat serum	yes, in foetal calf serum *and* in rat serum
proliferation to Con A	yes, in foetal calf serum but not in rat serum	yes, in foetal calf serum *and* in rat serum
IL-2 production in MLC and in response to Con A	low levels	high levels
mRNA for IL-2 on activation	low levels	high levels
IFN-γ production	low levels	high levels
mRNA for IFN-γ on activation	early but transient	sustained
meditor of B cell help in secondary immune responses	positive	negative
mRNA for IL-4 on activation	high levels	very low levels

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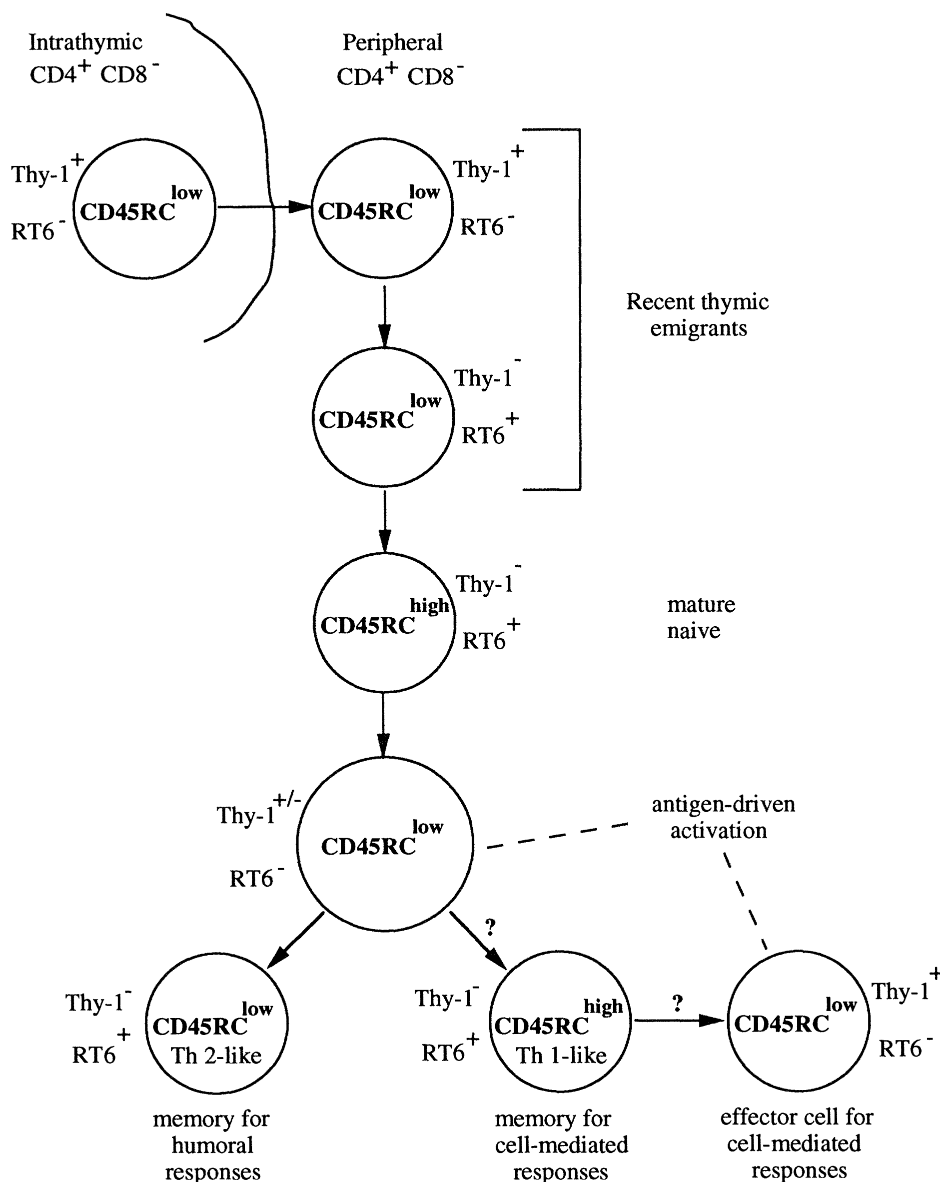

Figure 1. Lineage of rat CD4+ T cells.

T cells, namely: CD45RA+B+, CD45RA−B+ and CD45RA−B− can be identified (Mason & Powrie 1990). In the rat a monoclonal antibody MRC OX-22, that reacts with the C exon product of the CD45 gene, divides rat CD4+ T cells into two subsets; CD45RC^{high} CD4+ and CD45RC^{low} CD4+ with a ratio of frequencies of about 2:1 (Spickett *et al.* 1983).

A series of studies have shown that these two phenotypically distinct subsets mediate different T cell functions (Fowell *et al.* 1991). Tables 1 and 2 summarize these findings. Examination of these shows that there is good concordance between the *in vivo* and *in vitro* results but also shows that there is a complex association between the level of CD45RC expressed on CD4+ T cells and the function of those cells. The reason for this complexity was made apparent when studies were carried out on the way that CD45RC expression varies with the state of maturation and activation of CD4+ T cells. As figure 1 illustrates, thymocytes and recent thymic emigrants are CD45RC^{low} but as they mature the cells become CD45RC^{high}. However, on encounter with specific antigen CD45RC expression is again downregulated and remains low on memory cells that provide helper activity for secondary B cell responses (Kampinga *et al.* 1992; Mason 1992). In contrast there is some evidence that memory cells for cell-mediated reactions re-express CD45RC at high levels: CD45RC^{high}CD4+ T cells, when activated *in vitro*, are vigorous producers of IFN-γ and this is not a characteristic of naive T cells which produce only IL-2 on primary activation (Dohlsten *et al.* 1988). However, rat T cell clones that are actively synthesizing IFN-γ are again CD45RC^{low} (Sedgwick *et al.* 1989). These changes in the level of expression of CD45RC with maturation, differentiation and activation have made it necessary to define subsets of CD4+ T cells using additional markers that distinguish recent thymic migrants from more mature cells and activated cells from resting ones (Fowell *et al.* 1991; Kampinga *et al.* 1992; Mason 1992). Some of

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these markers are shown in figure 1 and it will be noted that the expression of these too requires cautious interpretation.

8. THE ROLE OF SUBSETS OF CD4⁺ T CELLS IN AUTOIMMUNITY

Given that T cell and B cell receptors for antigen are generated by random gene rearrangement of the elements that encode them it is inevitable that self tolerance is an acquired characteristic of the immune system. Although this fact has been recognized for almost half a century, long before the molecular basis of antigen recognition was established, the mechanisms underlying self tolerance are still imperfectly understood. In what follows, self tolerance among T cells will be discussed because although there is some evidence that autoreactive B cells are deleted from the B cell repertoire there are data to show that this is not universally so. Consequently self tolerance depends crucially on the maintenance of non-reactivity to self by T cells. Essentially two types of mechanism have been considered. In the first of these, autoreactive T cells are deleted intrathymically as they encounter antigen at an immature phase of their development (Kappler *et al.* 1988) or become anergic if, having left the thymus, they come into contact with extra thymic, tissue-specific self antigens presented on tissues and cells that lack an essential co-stimulating capacity (Mueller *et al.* 1989). Alternatively they may not be anergized but simply remain inactivated because of the absence of these co-stimulating signals (Ohashi *et al.* 1991). In contrast to these mechanisms, which depend in one way or another on an intrinsic failure of potentially autoreactive T cells to become activated, the second mechanism proposed for self tolerance suggests that this is, in part, a regulatory process that involves the direct participation of T cells that prevent autoimmunity. The distinguishing feature of these two types of mechanism is that procedures that render an experimental animal relatively lymphopoenic should not break self tolerance if this is maintained by mechanisms of the first type; i.e. those for which non-reactivity to self is an acquired but intrinsic property of potentially autoreactive cells: however, induced lymphopoenia might be expected to result in autoimmunity if tolerance requires the active participation of regulatory T cells.

It has been shown that appropriately selected strains of rats, thymectomized as adults and subject to a number of low doses of γ irradiation become relatively lymphopoenic and develop autoimmune thyroiditis (Penhale *et al.* 1975) and/or diabetes (Fowell *et al.* 1991). Detailed studies have shown that the autoimmune diabetes requires CD8⁺ T cells and these can be found in normal healthy syngeneic donors (in neither strain of rat studied does thyroiditis or diabetes develop spontaneously). Further, the diabetes can be prevented by the injection of a particular subset of CD4⁺ T cells into pre-diabetic recipients. The protective cells have the phenotype $CD45RC^{low}$, RT6⁺ Thy1⁻ $TCR\alpha\beta^+$, CD4⁺ (Fowell *et al.* 1991) and cells with this phenotype make mRNA for IL-4 on activation and provide helper activity for B cells in secondary immune responses (see table 2 and relevant references). In contrast the complementary subset of CD4⁺ T cells, that expresses CD45RC at high levels, when injected into syngeneic nude rats produces manifestations of autoimmunity in several organs (Powrie & Mason 1990) and again, the $CD45RC^{low}$ cells are protective. These results provide compelling evidence that autoreactive T cells are present in normal animals and that a T cell-dependent mechanism is involved in preventing the activation of those cells. Other data supporting this conclusion can be found in the literature (reviewed in Fowell *et al.* 1991).

At present it is not known how the protective subset of CD4⁺ T cells prevents autoimmunity. There is evidence that the intestinal flora influence the incidence of disease (Penhale & Young 1988) and it is possible that the lymphopoenia compromises the immunity of the gut mucosa. The mesenteric lymph nodes and the thoracic duct lymph contain a very high frequency of activated T cells in pre-diabetic rats and this observation supports this hypothesis. Whether dietary antigens, bacterial antigens or super-antigens are the principal inducers of this activation remains to be determined and whether some of these activated T cells are autoreactive is also unknown.

Many past and present members of the MRC Cellular Immunology Unit have contributed to the studies of the functional activities of CD4⁺ T cells and it is not practical to list them all. Their names will be found in the references cited in the text but I should like to record the pleasure that I have had working with them.

The key role that Alan Williams played, especially in the earlier phases of the work, is universally recognized and is the reason that this meeting has taken place. His contribution to the Cellular Immunology Unit can be gauged to some extent by the volume and quality of the work produced by the Unit while he was Director, and also by the standards that he set in terms of scientific integrity and commitment.

Finally I should like to acknowledge the secretarial assistance of Roz Sainty in the preparation of this manuscript.

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