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Differences in the regulation of CD4 and CD8 T-cell clones during immune responses

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The functional units of immune response are lymphocyte clones. Analysis of lymphocyte life span *in vivo* shows that the overall turnover of CD4 and CD8 lymphocytes does not differ greatly. Recently, molecular methods have been developed which allow a global analysis of T-cell clones responding to an antigen *in vivo*. We have used a sensitive, modified heteroduplex analysis to follow T-cell clones responding to Epstein–Barr virus in acute infectious mononucleosis (AIM). Strikingly, all the many large clones detected in freshly isolated AIM blood were found within the CD8 fraction. CD4 clonal populations responding to the soluble recall antigen tetanus toxoid could only be detected after *in vitro* re-stimulation. These data imply that CD4 responses may be more polyclonal than those of CD8 cells and that the size of CD4 clones is more tightly regulated. Several molecular mechanisms may contribute to this. Up-regulation of telomerase allows very large expansions of CD8 cells to occur without exhaustion of proliferative capacity.

Keywords: CD4; CD8; clone; life span; T cell; telomere

1. INTRODUCTION

An understanding of the population dynamics of lymphocytes is important for the more effective design of new therapies for diseases such as HIV, in which increased turnover of lymphocytes precedes the collapse of the function of the immune system (Hellerstein *et al*. 1999). Better understanding of how proliferation, survival and death of different subsets are controlled will also lead to more rational design of vaccines and immunomodulatory therapies targeted at different arms of the immune response. Since lymphocytes are clonal and in the case of T lymphocytes there is little evidence for somatic mutation, the important unit of function is not single T cells but T-cell clones; thus it is important to understand their life history.

In passing, it is worth noting that the importance of clones complicates the definition of 'life span' of T cells. Should it be time to extinction of the whole clone of cells bearing identical T-cell receptors or time to death or cell division of an individual cell? In this paper, we try to make clear exactly what is being referred to in any discussion of life span.

2. ESTIMATES OF LYMPHOCYTE LIFE SPAN IN HUMANS

Evidence from studies of the persistence of cells carrying chromosomal lesions induced by radiation indicates that some human T cells may survive without dividing for more than 20 years (Buckton *et al*. 1967). Subsequent experiments using similar methodology (Michie *et al*. 1992; Ramalho *et al*. 1995) demonstrated that there are distinct populations with very different rates of cell division and death. It was found that T cells expressing high-molecular weight isoforms of the leucocyte common antigen (CD45RA or naive T cells) divide (or die) infrequently, while those expressing low-molecular weight isoforms (CD45RO or memory T cells) divide (or die) more often (Michie *et al*. 1992).

The data from studies enumerating the persistence of stable and unstable chromosomal lesions have been re-examined more recently to compute several parameters of T-lymphocyte population dynamics (McLean & Michie 1995). Stable lesions allow cell division and the lesion is passed to one daughter cell. Loss of cells carrying stable lesions therefore allows an estimate of time to death of lymphocytes, irrespective of cell division. The average time to death of a T cell was estimated to be 20 years but of course the cell might have undergone many cell divisions during that time and the clone, of which that cell was a member, might well continue to exist. The presence of an unstable chromosomal lesion in a cell prevents completion of cell division and the cell dies. The rate of loss of cells carrying unstable lesions therefore provides a measure of the sum of the rate of cell division and intermitotic cell death. It was calculated that CD45RO cells divide (or die) on average every 154 days and CD45RA cells every 3.5 years. A simple calculation based on an estimate of around 5×10^{11} T cells in the human suggests that approximately 0.8×10^{9} CD45RO T cells divide every day, of which approximately two-thirds will be CD4 T cells, assuming that CD4 and CD8 cells behave similarly (table 1).

Functional data on the separated subsets suggested that CD45RA were naive and CD45RO memory T cells

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Table 1. *The turnover of human T cells*

(The figures given in the table for normal T-cell subsets assume that the overall turnover of CD4 and CD8 T cells is similar in healthy humans and are based on observations on total CD45RA and CD45RO T cells. It is assumed that there are around 5×10^{11} T cells in humans and approximately equal numbers of CD45RA and CD45RO cells. Half the CD45RA and CD45RO cells divide during 1277 or 154 days respectively, so that $2.5 \times 10^{11}/2 \times 1277$ or $2.5 \times 10^{11}/2 \times 154$ will divide every day.)

cell type	pool size	mean time days to division	number of divisions day^{-1}
CD45RA	*ca.* 2.5×10^{11}	1277	*ca.* 1×10^{8}
CD45RO	*ca.* 2.5×10^{11}	154	*ca.* 8×10^{8}
total T	*ca.* 5.0×10^{11}	275	*ca.* 9×10^{8}

(Merkenschlager *et al*. 1988; Merkenschlager & Beverley 1990) and that memory is carried not in long-lived resting cells, but by dividing cells (Michie *et al*. 1992). Evidence that reversion from the CD45RO to the CD45RA phenotype can occur suggests that the designation of the two populations as naive and memory cells may be an oversimplification. Better terms may be resting and antigen-activated cells, but whatever terms are used, the two subsets exhibit very different phenotypic and functional properties (Beverley 1996).

More recent experiments using ^{2}H-glucose to label DNA *in vivo* (Macallan *et al*. 1998; Hellerstein *et al*. 1999) have provided a direct estimate of 87 days for the mean survival times of CD4 and 77 days for CD8 T cells. The reason why these estimates are shorter than those obtained using chromosomal data is not clear but both methods may suffer from methodological difficulties. The stable isotope method may not be sufficiently sensitive to detect efficiently very slowly dividing populations, while all the chromosomal data has been obtained from irradiated patients who are initially lymphopenic and whose lymphoid system is therefore abnormal. The new data, however, provide evidence that the overall turnover of CD4 and CD8 T cells differs little.

3. TURNOVER OF T-CELL SUBSETS IN ANIMALS

Although there are few data measuring directly the life span *in vivo* of separated CD4 and CD8 T cells in normal humans, a number of studies have examined the dynamics of various T-cell subsets in animals. Most of these have been carried out in inbred mice but some data are available for a longer-lived species, the sheep. Direct measurement of the rate of cell division using re-infused dye-labelled lymphocytes shows that, on average, recirculating lymphocytes from subcutaneous efferent lymph divide once every 29.2 days. Reassuringly for human studies dependent on sampling peripheral blood, rates of division do not differ greatly among lymphocytes harvested from subcutaneous or mesenteric lymph, or blood (Young & Hay 1995). As is the case for humans, the overall average rate of division, which in this case includes B cells as well as large numbers of γδ T cells, conceals a large difference among subsets. The differences were revealed in earlier sheep experiments, which used the DNA precursor analogue bromodeoxyuridine (BrdU) administered *in vivo,* to label dividing cells. This has the advantage that the label incorporated into DNA can be detected with an antibody, allowing the phenotype of cells that have divided during the labelling period to be identified by dual- or triple-colour immunofluorescence. During an eight-day period of BrdU exposure *in vivo* almost 100% of cells with the phenotype and functional properties of antigen-activated ($CD45RA^{-}$) T cells labelled, while only very small numbers of resting ($CD45RA^{+}$) cells did so (Mackay *et al*. 1990). Furthermore, CD4 and CD8 T cells showed comparable levels of labelling.

In the mouse, numerous studies on lymphocyte population turnover have been carried out (Sprent *et al*. 1997). Many different strategies have been employed but among the least invasive are those that employ BrdU administered in the drinking water to label dividing cells *in vivo* (Rocha *et al*. 1990). The use of adult thymectomized mice allows a clearer picture of the turnover of peripheral T cells, avoiding confusion with recent thymic emigrants labelled in the thymus. Experiments using a short labelling period show that different phenotypic subpopulations label with very different kinetics. Broadly, the results confirm the human and sheep data, indicating that both CD4 and CD8 T cells with an antigen-activated phenotype divide rapidly. However, after longer BrdU administration periods, labelled cells with a 'resting' phenotype are found, indicating reversion from the antigen-activated to the resting state. 'Resting' cells may make up as much as 15–20% of BrdU labelled CD8 cells (Tough & Sprent 1994).

Pulse chase experiments also indicate that overall there is a switch with time from the BrdU-high to BrdU-low phenotype, indicating dilution of label by cell division. Once again, BrdU-high and BrdU-low cells are found with both resting and antigen-activated phenotypes, indicating that there is an imperfect correlation between the surface-antigen expression of a cell and its prior life history. This finding is in line with earlier studies in rats (Bell & Sparshott 1990; Sparshott & Bell 1994) and humans (Rothstein *et al*. 1991; Ramalho *et al*. 1995), suggesting that reversion from an activated state to the phenotype of a long-lived non-dividing, small lymphocyte may occur.

4. THE PROLIFERATIVE CAPACITY OF CD4 AND CD8 T CELLS

The picture of the population dynamics of peripheral lymphocytes obtained from studies in intact humans, sheep and adult thymectomized mice, is supported by a variety of cell transfer experiments. These provide additional information on the behaviour of T-cell subsets. Early results obtained in athymic nude mice or rats showed that peripheral T cells had a considerable proliferative capacity, expanding to 'fill the space available' (Rocha *et al*. 1989). More recent cell transfers of resting lymph-node lymphocytes into severe combined immunodeficient (SCID) mice maintained under clean conditions demonstrated that the transferred cells proliferated only relatively slowly, many cells retaining a resting phenotype

over several months (Sprent *et al*. 1991). This is in line with evidence that transferred polyclonal or T-cell receptor (TCR)-transgenic CD4 or CD8 T cells retained stable naive or memory phenotypes and cell numbers over prolonged periods of time unless exposed to antigen (Bruno *et al*. 1995; Boursalian & Bottomly 1999).

In the presence of their specific antigen, transferred cells have the capacity for extensive expansion. Thus in the studies described above of CD4 and CD8 TCR-transgenic cells, at least a two log increase in cell number was seen when the transferred cells were confronted with antigen. In similar studies of transferred TCR-transgenic lymphocytic choriomeningitis virus (LCMV)-specific T cells, a 1000-fold increase was seen in virus-infected mice so that at the peak of the response the transgenic cells made up 70% of CD8 T cells in the (normal) recipient (Zimmermann *et al*. 1996*a*). Recently, similarly high percentages of antigen-specific CD8 T cells have been revealed in normal mice following virus infection, using major histocompatibility complex (MHC)-peptide tetramers for direct visualization of peptide-specific cells (Murali-Krishna *et al*. 1998). There is much less data available for CD4 T cells, though overall frequencies of CD4 and CD8 cells responding during an immune response, as measured by limiting dilution, are thought to be similar (Doherty *et al*. 1996). However, the limiting dilution method does not detect effector T cells.

What conclusions can be drawn from these data? In all species so far examined, there is a remarkable similarity in the overall picture of the population dynamics of T cells. When cell division is measured, it is found that two more or less distinct populations can be detected with very different kinetics (table 1). In humans, CD45RO cells divide approximately eight times more rapidly than CD45RA cells. Data from other species appear broadly in line with this. Only minor differences have been noted in the behaviour of CD4 and CD8 T cells at the population level.

The data from cell transfer experiments show that the most profound stimulus for proliferation is antigen (Mackall *et al*. 1996; Zimmermann *et al*. 1996*a*) and that peripheral T cells have extensive capacity for cell division. Studies of human T-cell clones *in vitro* strongly suggest that this is not unlimited and that clones do eventually undergo senescence, with a mean of 35 population doublings (Pawelec *et al*. 1997).

5. DETECTION OF CLONAL T-CELL RESPONSES *IN VIVO*

Until recently it has been difficult to track the responses of clonal populations of lymphocytes *in vivo*. TCR transgenic mice provide populations, which can be followed using monoclonal antibodies raised against the receptor. Transfer of the monoclonal population into normal mice provides a model where a single clone can be followed against a polyclonal background. However, this is an artificial situation since in a normal immune response, many clones respond at the same time. Experiments with responding cells that make use of a very restricted TCR beta chain variable gene (TCR Vβ) repertoire, detectable by an anti-TCR Vβ antibody have demonstrated rapid early clonal expansion of T cells following antigen administration, but the restricted use of V-genes makes this also a non-physiological situation (McHeyzer-Williams & Davis 1995). Use of MHC-peptide tetramers allows direct visualization of T cells responding to a particular peptide but requires prior identification of target peptides and does not give a picture of the whole response (Altman *et al*. 1996; Murali-Krishna *et al*. 1998).

T-cell clones within a polyclonal population can be detected by sequencing a number of TCR Vβ clones isolated from the polymerase chain reaction (PCR) product of that Vβ family to derive frequency data but this is laborious (Posnett *et al*. 1994). More global methods depend on the detection of variation in TCR Vβ complementarity-determining region three (CDR3) length (Cochet *et al*. 1992), or specific CDR3 sequences detected by heteroduplex analysis (Wack *et al*. 1996) against a polyclonal background. The latter method is at least a log more sensitive than the former and can detect a monoclonal T-cell population at a frequency of $< 1{:}10\,000$ against a polyclonal background (Maini *et al*. 1998). We have used this method to study acute infectious mononucleosis (AIM). In AIM, clonal bands may be detected in all TCR Vβ families in fresh, unstimulated peripheral blood (Maini *et al*. 2000), indicating the presence of a large number of expanded clones. In AIM, there is a CD8 lymphocytosis and these cells have an activated phenotype, as do many CD4 cells. Nevertheless, fractionation of the blood T cells indicates that all the expanded clones are in the CD8 subset.

To reach the threshold of detection it is likely that a clone must contain at least 10^8 cells. Sequencing of the PCR product of Vβs with prominent clonal bands indicates that some clones may be considerably larger; up to 10% of CD8 T cells, or greater than 10^9 cells. These data led us to consider two questions. The first is how can memory persist for long periods of time following massive clonal expansion if clones have a limited capacity for division (Pawelec *et al*. 1997)? And the second is whether there is a difference in the regulation of clone size in CD4 and CD8 T cells.

6. HOW DO CD8 CLONES SURVIVE?

In animal models, following the acute stage of infection and CD8 lymphocytosis, the frequency of virus-specific cytotoxic T-lymphocyte precursors (CTLp) is maintained at an elevated level, though the CD8 cell count returns to normal (Murali-Krishna *et al*. 1998; Zimmermann *et al*. 1996*b*). This is also the case in Epstein–Barr virus (EBV) infection, with T-cell clones detected at the onset of infection, persisting over prolonged periods (Silins *et al*. 1996). Since there is continuing cell division among memory phenotype cells, it is presumably necessary that cells enter the memory pool with a proliferative reserve even after the massive clonal expansion seen in AIM (Callan *et al*. 1996, 1998). Since shortening of telomeres is known to be associated with cell division (Hastie *et al*. 1990; Vaziri *et al*. 1993), we investigated telomere length in AIM T cells and to our surprise found that telomeres are longer in the CD8 than the CD4 subset (Maini *et al*. 1999). Telomere shortening can be prevented by the enzyme telomerase (Lundblad & Wright 1996) and telomerase activity is greatly up-regulated in the acute stage

Table 2. *Differences in regulation of CD4 and CD8 clones*

	CD4	CD8
clone size	moderate	very large
CD45 phenotype of clonal population	CD45RO	varied
telomerase up-regulation during activation *in vivo*	yes	yes
susceptibility to bystander activation	low	high
downregulation of IL2-R	rapid	slower
susceptibility to fas-mediated apoptosis	high	low
signalling through CTLA-4	inhibits proliferation	no inhibition

of AIM in both the CD4 and CD8 subsets (Maini *et al*. 1999). A similar finding has been obtained in germinal centres, where rapid cell division of B cells occurs during affinity maturation and immunoglobulin class switching (Weng *et al*. 1997). Following resolution of AIM, telomere lengths in the CD4 and CD8 subsets return to an equal length as is the case for normal controls. It remains unclear whether the observed shortening of CD8 telomeres during resolution of AIM is due to death of cells with the longest telomeres or continuing cell division after downregulation of telomerase (Weng *et al*. 1996). Alternatively, the presence of a smaller number of EBV-specific CD8 cells in the memory pool after apoptotic death of the majority (Akbar *et al*. 1993), may no longer bias telomere length measurement. Nevertheless up-regulation of telomerase and preservation of telomere length seems to be an important mechanism allowing massive CD8 clonal expansion during an acute illness as well as entry of some of the responding cells into the memory pool with a preserved proliferative capacity.

7. CD4 AND CD8 CLONAL REGULATION

Very few studies have analysed simultaneously CD4 and CD8 clonal behaviour during a specific immune response. Nevertheless, data from normal individuals indicate a profound difference between CD4 and CD8 T cells. Several studies have noted that large clones are frequently seen among fresh-blood CD8 cells, while they are seldom detected in the CD4 subset. The number of detectable CD8 clones increases with age. In young normal donors, a few clones are seen in separated CD8 but almost never CD4 cells, while with increasing age, expanded clones are readily detectable in the CD8 CD45RO and RA subset, but only later in CD4 cells (Schwab *et al*. 1997; Wack *et al*. 1998). In the mouse, similar data show that large clonal CD8 expansions accumulate in aged mice (Callahan *et al*. 1993; Ku *et al*. 1997).

In EBV, although we could easily detect many clones in fresh, separated CD8 cells and these could be followed for over a year, we did not detect clonal expansions in separated CD4 cells. This prompted us to examine under what circumstances antigen-specific CD4 cells could be detected. We studied the response to the recall antigen tetanus toxoid and could show that following stimulation *in vitro*, specific TCR Vβ clonal bands could be detected readily using heteroduplex analysis. The pattern of bands for a given donor was stable in repeated experiments over time, was not altered by *in vivo* immunization with tetanus toxoid, differed from that detected after stimulation with another antigen (PPD), and the clones were found exclusively in the CD4 subset (Maini *et al*. 1998). Others have detected CD4 clonal populations transiently in peripheral blood while CD8 expansions persisted for longer (Masuko *et al*. 1994), and an allergen-specific clone, shown to persist for several years, was estimated to be present at a very low frequency (Wedderburn *et al*. 1993). These data suggest either that large CD4 clonal expansions are more transient than CD8 clones, a hypothesis supported by some murine experiments (Doherty *et al*. 1996), or that responding CD4 clones do not recirculate in the blood for long after antigen activation. In one example at least, a hepatitis C virus-specific CD4 clone was found in the liver and not blood (Minutello *et al*. 1993), but the data in old mice and humans suggests that this is not a general explanation for the failure to detect CD4 clonal expansion.

These data raise the question of what regulates clone size among T cells (table 2). Antigen or cross-reacting antigen is clearly an important driving force for both CD4 and CD8 cells (Selin *et al*. 1994). In addition, CD8 memory cells have been shown to proliferate readily *in vivo* in response to inducers of type 1 interferon (Tough *et al*. 1996), suggesting that bystander cytokine production is a mechanism promoting persistence of CD8 clones. As well as mechanisms promoting clonal survival and expansion, those that terminate responses will be important in determining the size of clones. Activated CD4 cells become refractory to interleukin-2 earlier than do CD8 cells (Gullberg & Smith 1986) and are susceptible to apoptosis mediated through the CD95 (fas) pathway (Singer & Abbas 1994; Sytwu *et al*. 1996) while CD8 T cells are resistant *in vivo* (Ehl *et al*. 1996; Zimmermann *et al*. 1996*b*). In contrast, in the absence of signalling through CTLA-4, murine CD4 cells show uncontrolled proliferation while CD8 cells do not (Chambers *et al*. 1997).

8. CONCLUSIONS

These data suggest that the regulation of CD4 and CD8 T-cell clones may be very different. In acute systemic viral infections, many expanded CD8 clones are readily detected and the clonal cells persist for long periods of time at an elevated frequency. The clonal population can often be demonstrated in CD8 subpopulations with diverse phenotypes, suggesting that no single marker, including CD45 isoform expression, can be used to distinguish CD8 T cells, which have encountered antigen (Hamann *et al*. 1997). In contrast, large CD4

clones are rarely detected in blood and can usually only be detected after *in vitro* re-stimulation. These data strongly suggest that CD4 clones are generally maintained at a smaller size. Clonal populations responding to soluble recall antigens are generally detected only in the CD4 CD45RO subset, except in old age, suggesting that the rate of reversion to the CD45RA phenotype may be slow among CD4 T cells. A variety of molecular mechanisms contribute to these differences in the regulation of CD4 and CD8 clones.

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