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REVIEW ARTICLE

Thymic output, ageing and zinc

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Abstract The role of the thymus is vital for orchestration of T-cell development and maturation. With increasing age the thymus undergoes a process of involution which results in a reduction in thymic size, function and output. Until relatively recent it was not feasible to accurately measure the magnitude of age-related loss of thymic function. With the discovery of T-cell receptor excision circles (TRECs), which are the stable by-products of the newly generated T-cells, it is now possible to quantitatively measure the extent of thymic output. This review examines the available data on immune function and zinc deficiency and places them in the context of the aims of the ZINCAGE project which include the evaluation of the role played by zinc in maintaining thymic output in healthy elderly individuals.

Keywords Zinc · Thymic output · Ageing · TREC · Zinc deficiency · T-cell development · Immune system · Recent thymic emigrants

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Introduction

In general, old age is associated with increased incidences of infections, disease and poor health that is thought to arise as a result of the decreased ability of the immune system to protect the host from 'foreign' antigen or 'self' recognition. A key component in the provision of lifelong immunity to the host derives from the function of the thymus and the age-related changes that it experiences. The aim of this brief review is to examine the contribution made by Zinc on the immune system with particular reference to thymic output in elderly individuals.

Age related changes in the immune system

The thymus is a primary lymphoid organ located in the anterior mediastinum and produces T-cells throughout life although the number of T-cells it produces declines with age. In a young healthy adult (less than 30 years old) there are approximately 2×10^{11} T-cells of which 1–2% can be found within the blood, and up to 50% of these cells are contained within the ''antigen naïve'' population.

Generation of T-cells

Production of $\alpha\beta$+ T-cells in the thymus is a progressive step-wise differential process, in

which a small population of multipotential stem cells give rise to progeny populations. Stem cells migrating to the thymus are contained within the CD4–CD8– double negative (DN) population, a population which has been further subdivided on the basis of expression of CD44 and CD25. Progress from the most immature stage, CD44+CD25– (DN-1) requires the transient acquisition of CD25 so the cell first becomes CD44+CD25+ (DN-2) before becoming CD44–CD25+ (DN-3) and then the loss of CD44 when the population is CD44–CD25– (DN-4) (Godfrey et al. 1994, 1993; Wu et al. 1991). Cells within the DN-1 population are multipotential, whilst those at DN-2 have lost the capacity to form B cells, but can still produce either T-cells or dendritic cells (Shortman and Wu 1996; Wu et al. 1996). By the time the cells are within the DN-3 population they are committed to becoming T-cells and have undergone extensive rearrangement of the TCRβ chain genes (Capone et al. 1998). Expression of the TCRβ chain at the thymocyte surface requires a TCRα chain equivalent (Fehling and von Boehmer 1997) (the pre-TCRα) and these cells then undergo expansion and differentiation so that they become CD4+8+ thymocytes. These immature thymocytes are the largest subpopulation in the thymus and are located in the densely packed cortical region of each thymic lobule. It is in the double positive stage when the TCRα chain undergoes rearrangement (Petrie et al. 1993) after which there is TCR $\alpha\beta$-dependant selection. Many of these double positive cells fail to mature further, but a small percentage develop into mature thymocytes expressing either CD4 or CD8 alone and are located in the medullary region of each thymic lobe. Only a fraction of these cells are exported to the periphery as naive or virgin T lymphocytes.

These T-cells have not interacted with their cognate antigen. Their activation usually occurs once the antigen presented by an antigen presenting cell is met and requires a number of steps including recognition of the specific peptide antigen presented in the appropriate MHC molecule in conjunction with the necessary co-stimulatory molecules. In a successful response, activation of these antigen naïve T-cells leads to their clonal expansion and the generation of effector cells and the subsequent reduction in the amount and source of the antigen. This is then followed by a period of cell death since the immune system no longer requires large numbers of T-cells bearing that specific receptor. However some cells with this antigenic specificity remain to become memory T-cells and subsequently enter the memory T-cell pool. Repeated exposure of the immune system to a potential pathogen will be met by these memory T-cells and will lead to a response that is more rapid and of greater magnitude than the response following the initial exposure. This immunological memory provides the rational basis for protection by vaccination.

Age-related immune response to 'foreign' antigen

Since there are few completely sterile environments, each of us is confronted on a daily basis with different organisms, some of which could be pathogenic if we were not protected by our immune system. Our survival therefore, depends upon our immune system recognizing and responding successfully to a broad range of potential pathogens. Provided these pathogens do not result in our death, our immunological memory should increase, and analysis shows that this is indeed the case and that ageing is indeed associated with an increase in the number of memory T-cells. Theoretically then we should be able to cope with more infections as we get older; the immune system of a 90-year-old should be much better at coping with infection than the immune system, of a 20-year-old. Unfortunately this does not seem to be the case. Evidence from epidemiological, clinical and laboratory studies suggest an age related defect in the immune system. The epidemiological evidence reveals that older individuals are often the first to be affected by new or emerging pathogens. In the first outbreak of West Nile Virus in the USA in 1999 the median age of the 59 patients was 71 years, with 73% age 60 years or greater (Nash et al. 2001). In Israel in 2000 all of the victims of West Nile Virus were more than 78 years of age (Berner et al. 2002). Clinicians recognise that in addition to this susceptibility to new pathogens, older individuals often have difficulties in dealing with pathogens

which they have previously overcome. Common problems include reactivation of herpes zoster virus (Schmader 2001) or the increased immune response to cytomegalovirus (Pawelec et al. 2004), as well as the problems associated with the yearly return of influenza and respiratory syncytial virus (RSV). For example in the USA from 1990 to 1999, influenza and RSV accounted for 51,203 and 17,358 deaths annually, respectively (Thompson et al. 2003). Vaccination trials also reveal problems with inducing protection in the elderly. For example in a recent trial in which 45 healthy elderly (average age 74) and 37 healthy young controls (average age 28) were vaccinated with hepatitis B, all of the young individuals developed a protective titre compared to only 42% of the elderly cohort (Looney et al. 2001). A similar problem with vaccine cover occurs with influenza. Efficacy for influenza vaccine is between 70% and 90% in those under 65 but is reduced to 30–40% in those over 65 (Hannoun et al. 2004). Although several attempts have been made in the past to modify vaccines, either through alterations in their route of administration, or changes to their formulation by the inclusion of different adjuvants, the overall result has been a failure to improve the efficacy of vaccines in elderly individuals (Belshe et al. 2004; Looney et al. 2001). These trials would indicate that defects in the immune system rather than the deficiencies in the vaccine formulation are at the root of the problem. Attempts to link these epidemiological and clinical results with laboratory studies has shown that T-cells from elderly individuals produce poorer proliferative responses in vitro to stimuli which are normally mitogenic for T-cell from younger individuals (Pawelec et al. 1997). Moreover phenotypic analysis of T lymphocytes from older individuals reveals that they have a different profile of cytokine gene expression (Bui et al. 1994) compared with younger individuals and there may be increased numbers of senescent T-cells in older individuals (Effros et al. 2005). Like most somatic cells, T-cells have a limited replicative capacity and aging is often accompanied by the increase in the number of T-cells present in the blood which have reached this replicative limit (Effros and Pawelec 1997). As we noted above, a successful immune response requires clonal expansion of antigen specific cells and the accumulation of T-cells without the capacity to divide can only lead to a dysfunctional immune response and failure to protect the individual.

Factors influencing thymic function

As described previously the thymus plays a critical role in the orchestration and maturation of the T-cell arm of the immune systems. With increasing age the thymus is known to undergo a process of involution resulting in the accumulation of adipose tissue and a marked reduction in the generation of naïve T-cells that can be exported to the peripheral T-cell pool (Haynes et al. 2000b). As a result of regulatory homeostatic processes, the overall size of the T-cell pool remains constant due to compensatory expansion of the proportion of memory T-cells compared to naïve T-cells (Fry and Mackall 2002). Several physiological and pathological factors are known to interfere with the normal function of the thymus which in turn causes the thymus to experience atrophy, these include; infection, disease, ageing, pregnancy, puberty, physical and emotional stress, environmental conditions, alterations in hormonal and cytokine levels as well as deficiency of nutritional factors such as Zinc. A recent publications by Taub and Longo has reviewed in detailed the contribution made by these factors (Taub and Longo 2005). A summary of the proposed causes of thymic involution is seen in Table 1.

Ageing

Histologically the thymus is composed of two key components; 1) Thymic epithelial space in which thymopoiesis occurs and 2) Nonepithelial perivascular space (Haynes et al. 2000a). The organ reaches a maximum size of approximately 25 cm^3 within the first 12 months of life (George and Ritter 1996). From this point thymopoietic thymic space has been observed to begin to atrophy shrinking in volume by 3% per year until middle age and by less than 1% per year for the remaining years of life reducing the capacity to develop thymocytes (Steinmann

Table 1 Proposed causes of Thymic involution

Proposed causes of thymic involution	References
Absence or inhibition of TCR receptor	Aspinall (1997)
Loss of self-peptides on thymic epithelial MHC	Hartwig and Steinmann et al. (1994)
Ageing of thymic stroma with loss of trophic cytokines	Hirokawa et al. (1982) Leiner et al. (1984) and Utsuyama et al. (1991)
Ageing of stem cell population	Tyan (1977) and Kadish and Basch et al. (1976)
Increased expression of certain cytokines as age increases	
• Leukaemia inhibitor factor (LIF)	Haynes et al. (1999) and Sempowski et al. (2000)
• Oncostatin M (OSM)	
• IL-6	
• Stem cell factor	
• Macrophage colony stimulating factor	
Corticosteroid suppression of thymus	Wyllie (1980)
Action of pituitary ACTH production which drives adrenal corticosteroid production	Akita et al. (1996)
Involution protects against autoimmune disease and cancer	Aronson (1991)
Wear and tear model	
Atrophy is due to a loss of the thymic microenviroment function	George et al. (1996) and Hirokawa et al. (1982)
The thymus as an energy expensive organ is allowed to involute after a full repertoire has been established in order that energy can be saved and invested for reproduction in germ line cells	Leiner et al. (1984) and Utsuyama et al. (1991)
Production of sex hormones	Sfikakis et al. (1998) Olsen and Kovacs (2001) Olsen et al. (1998) and Leposavic et al. (2001)

1986; Steinmann et al. 1985). At this rate it is estimated that total loss will occur by 105 years of age.

Zinc and the immune system

The importance of Zinc in animal systems has been known since 1934 although because of its ubiquity it was thought unlikely that alterations in zinc metabolism could lead to significant human disorders (Prasad 1998). More recently is has been demonstrated that zinc is an essential trace element for all forms of life and has been shown to be vital for numerous cellular metabolisms including, growth and development, the immune response, neurological function, and reproduction (Prasad 1985). The various interactions of zinc can be divided based on its involvement in catalytic, structural and regulatory functions (Chesters et al. 1993; O'Halloran 1993). It has been recognized that zinc deficiency can lead to a wide range of disorders and impairments of cellular function (Prasad 1985).

Zinc deficiency in humans

In the early 1960s the first case of zinc deficiency was described in a 21-year-old Iranian male. The striking effect of the zinc deficiency was observed as growth retardation, hypogonadism, severe hypochromic microcytic anaemia, enlarged liver and spleen, rough and dry skin, mental lethargy and a habit of eating clay (Prasad et al. 1961). The original description was considered to have arisen due to Iron deficiency as the symptoms were reversed when ferrous sulphate and animal protein was added to the diet. However, experimental animal studies examining the effects of iron deficiency on development noted that theses animals did not display the hypogonadism and the growth retardation, as observed in Iranian patient, whereas similar features were present in zinc deficient animals. Subsequent observation in a group of Egyptian patients infected with schistosomiasis and hookworm with similar symptoms to the original Iranian subject, found reduced zinc levels in plasma, red cells and hair. In addition, the zinc turnover rate was greater, the 24-h

exchange pool was smaller and the excretion of ^{65}Zn in the stool and urine was less than the control (Prasad et al. 1963).

Zinc deficiency has been identified in a number of disorders the most notable including sickle cell anaemia and acrodermatitis enteropathica. Individuals suffering from Acrodermatitis enteropathica, an autosomal recessive disease caused by a defect in zinc metabolism, experience thymic atrophy and impaired cell-mediated immunity resulting in increased susceptibility to infection and disease (Oleske et al. 1979). These symptoms are effectively corrected by supplementation with zinc.

There are several interesting factors associated with Zinc which warrant further investigation to elucidate its contribution to cellular immunity. First, a hallmark of zinc deficiency in animal models is the development of age-independent thymic atrophy (Prasad 1985). Second, individuals with zinc deficiency are known to suffer from increase susceptibility to infection and disease indicative of poor immune function. Third, with increasing age there is a decreased ability to absorb Zinc in the gut therefore increasing the likely of individuals become deficient of Zinc (Fraker and King 2004). Fourth, studies in aged mice have shown that drinking water supplementation with zinc sulphate can increase thymic mass and possibly thymopoiesis (Fraker and King 2004). Fifth, Zn deficiency has been noted as a secondary disorder in disease such as diabetes, AIDS, Down's Syndrome and select cancers (Keen and Gershwin 1990). Sixth, Zinc supplementation has been shown to increase thymulin secretions in aged mice (Mocchegiani and Fabris 1995) and human (Prasad et al. 1988) suggesting a beneficial role for thymic function. Taken together these factors provide compelling reasons for investigating the potential impact to be made by Zinc on the immune system of free living old people.

Unlike age-related thymic atrophy many of the factors mentioned are associated with transient or reversible atrophy. This may indicate the extent to which factors within the thymic microenvironment influence the regulation of cellular immunity. Where physiological resources become limited, for example in the case of Zinc deficiency, the immune system may prioritize first line defence function above more luxurious functions i.e. increasing the T-cell repertoire (Fraker and King 2004; Fraker et al. 2000). The may lead to increased likelihood of thymic atrophy unless additional signals are received which prevent this process. Potential factors have been reported to prevent or reverse the thymic atrophy, these include; interleukin-7 (IL-7) (Andrew et al. 2001, 2002; Henson et al. 2005; Imami et al. 2000; Phillips et al. 2004; Virts et al. 2006); Ginkgo biloba leaf extract EGb 761(Tian et al. 2003) and Melatonin (Tian et al. 2001).

For the remainder of this review we will examine the approaches employed for assessing thymic output and how this can be used to explore the effects of Zinc within the elderly population.

Methods of quantifying thymic function

Assessment of the ability of the thymus to produce T-cells is dependent on providing a suitable marker of thymic function. This has proved to be problematic. Until recently, thymic output could only be indirectly quantified by either taking measuring the number of phenotypically naïve T-cells in the circulation or by correlating the CD4+ naïve T-cell count with computer tomography chest scan measurements of thymic volume (Poulin et al. 1999). Neither of these however has proven to be a reliable measure of thymic function (Zhang et al. 1999).

The profound effects of stress on thymopoiesis have ruled out intrathymic assays on T-cell production (Poulin et al. 1999). Alternative strategies have concentrated on T-cells in the periphery. T-cells that have entered the periphery undergo only a few cellular divisions after leaving the thymus are referred to as recent thymic emigrants (RTEs) (Zhang et al. 1999). Several studies in experimental animals have characterized the phenotype of RTEs. It has been demonstrated by Kong et al. that RTEs in chickens express the chT1 thymocyte antigen. In rats and mice the identified RTEs, include $RT6^{hi+}$ $CD45RC^{hi+}$ T-cells in rats (Hosseinzadeh and Goldschneider 1993), and intermediate expression of heat stable antigen (HSA) in mice (Penit and Vasseur 1997) Table 2.

Table 2 RTE markers used in previous studies

Species	Marker	Comment	References
Chicken	chT1	chT1 antigen decreases 12 days after thymectomy	Kong et al. (1998) and Kong et al. (1999)
Rats	$Thy1^{+}CD45RC^{+}RT6^{+}$	Marker of RTEs	Hosseinzadeh et al. (1993)
Mouse	HSA^{int+}, $Qa\text{-}2^{hi+}$	Marks approx 50% RTEs in lymph node & spleen	Penit et al. (1997)

Similar attempts in humans to characterize RTEs has proved to be more difficult. Firstly, the expressions of CD62L and CD45 isoforms have been used to identify RTEs in humans, with the unconvincing results. After emigration $CD45RA^{+}$ naive T-cells can have a long quiescent lifespan, proliferate in an antigen independent manner or convert to $CD45RO^{+}$ memory or effector T-cell phenotype (Bell and Sparshott 1990). Alternatively the naive marker $CD45RA^{+}$ can be acquired by memory T-cells (Douek et al. 2000b). As a result, in the human adult, $CD45RA^{+}$ T-cells may be naïve, but may not represent RTEs. Secondly, Aspinall and co-workers have shown that $CD4^{+}$, $CD45RA^{+}$, $CD95(fas)^{+}$ T-cells as well as $CD8^{+}$, $CD45RA^{+}$, $CD95(fas)^{+}$ T-cells decline with age. However, the use of this combination of T-cell markers for RTEs has yet to be validated (Aspinall et al. 1998). Thirdly, (Hassan and Reen 1998) suggest that human cord blood express thymocyte-like characteristics such as rapid rates of apoptosis and enhanced proliferation in the presence of IL-7 and so could represent RTEs.

T-cell receptor rearrangement excision circles: TREC marker

In their study of thymic function in mice Kong and co-workers used T-cell receptor rearrangement excision circles (TREC) as a marker which signified the developmental proximity of cells to the thymus (Kong et al. 1998). Douek et al. applied this marker to humans in the study of thymic output in HIV patients following highly active antiretroviral therapy (HAART) (Douek et al. 1998). The concentration of TREC in peripheral blood was used to evaluate thymic output.

TRECs are formed during the generation and expression of the T-cell antigen receptor (Livak and Schatz 1996). The recombination of V, D and J gene segments in T-cells is responsible for the creation of functional TCR chains and the diversity of the TCR repertoire (Al-Harthi et al. 2000; Hochberg et al. 2001). Studies found that a common requirement for the productive rearrangement of the TCRα locus was the deletion of the TCRδ locus which it encompasses. Two rearrangement events occur during this process producing a signal joint TREC (sj TREC) and a coding joint TREC (cj TREC).

Studies on the cell specificity of TRECs have found that they were present in phenotypically naive T-cells while absent in memory T-cells, γ–δ T-cells and B-cells (Douek et al. 1998; Kong et al. 1998; Zhang et al. 1999). These results confirmed TRECs as being unique to naive α–β T-cells. TRECs were also observed not to replicate during mitosis and to be diluted during in vitro cell division. For every $\log^{10}$ increase in $CD4^{+}$ and $CD8^{+}$ T-cells after stimulation with anti-CD3 and anti-CD28 antibodies there was an equivalent decrease in TRECs per μg of cellular DNA. Consequently as cell number increases the total number of TRECs remained unchanged leading to a decrease in TRECs on a per cell basis. TREC levels in peripheral blood T-cells are therefore a measure of thymic output and of peripheral expansion.

In summary the advantages of TREC as a marker were found to be; 1) TRECs are associated with RTEs, 2) they are stable and 3) TRECs are not replicated during mitosis and are therefore diluted out with each cellular division. TRECs can therefore act as markers for the replication history of a cell being indicators of active thymopoiesis.

TREC measurements in the elderly

To date, very few studies have measured thymic output in the elderly population and many of

those that do rarely investigate beyond the age 80 years. Of these studies, it has been shown that the TREC marker is detectable in individuals' up to 80 years of age, and in few cases the detectable TREC levels have been relatively high (Aquino et al. 2003; Arellano et al. 2006; Douek and Koup 2000a). In a recent study by Nasi and colleague they examine TRECs and immunophenotypic marker (i.e. naïve, effector memory and central memory) to investigate thymic output in 44 centenarians. In 84% of centenarians, no TREC marker was detectable. In addition the composite of the T-cell pool highlighted an increase in the proportion of effector and central memory cells. Interestingly, the level of IL-7 and IL-7 receptor were shown to be higher in females compared to males (Nasi et al. 2006). A number of possible arguments to explain these results include; 1) residues of thymic lymphopoietic islets, or 2) represent long-living lymphocytes that have not yet encountered their antigen.

Currently work is ongoing as part of the collaborative European study, ZINCAGE, that is focused on investigating the impact of Zinc on the elderly population. By making use of the TREC assay, as described above as a marker for thymic output, early results indicate that low level thymic function is maintained in free living people into the tenth decade of life prior to zinc supplementation (unpublished data). Further studies are ongoing to assess the impact made by Zinc supplementation.

Conclusion

Age-related changes in the thymus leading to a reduction in thymic function and output are believed to influence the ability of an individual to combat infection and disease in old age. Zinc has been shown to be essential for a wide range of cellular function with deficiency leading to transient disorders that can be corrected if zinc levels are restored. It has been demonstrated that low level thymic output is maintained into the tenth decade of life, a question which still remains to be answered is whether zinc supplementation can enhance the functionality of the immune system. One aims of the ZINCAGE project, to investigate the role of zinc on the immune function of free living old people, will represents a powerful tool for understanding how to successfully combat the lifelong challenges.

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