12 APR 2005

DIAGNOSTIC INDICATOR OF THYMIC FUNCTION

FIELD OF THE INVENTION

[0001] The present disclosure is in the field of immunology. In particular, it relates to diagnosing the ability of a thymus to be reactivated by inhibition of the effects of sex steroids on the thymus.

BACKGROUND OF THE INVENTION

[0002] The thymus is arguably the major organ in the immune system because it is the primary site of production of T lymphocytes. Its role is to attract appropriate bone marrow-derived precursor cells from the blood, and induce their commitment to the T cell lineage, including the gene rearrangements necessary for the production of the T cell receptor for antigen (TCR). Associated with this is a remarkable degree of cell division to expand the number of T cells and hence increase the likelihood that every foreign antigen will be recognized and eliminated. This enormous potential diversity means that for any single antigen the body might encounter, multiple lymphocytes will be able to recognize it with varying degrees of binding strength (affinity) and respond to varying degrees.

[0003] A strange feature of T cell recognition of antigen, however, is that, unlike B cells, the TCR only recognizes peptide fragments physically associated with MHC molecules. Normally this is self MHC (i.e., non-foreign MHC) and this ability is selected for in the thymus. This process is called positive selection and is an exclusive feature of cortical epithelial cells. If the TCR fails to bind to the self MHC/peptide complexes, the T cell dies by "neglect" – it needs some degree of signaling through the TCR for its continued maturation.

[0004] Following selection in the cortex, the developing thymocytes acquire functional maturation and migratory capacity and exit into the blood stream as naïve (not yet having contacted antigen) T cells. They circulate between the lymph and blood in search of antigen. If after 3-4 weeks they haven't been stimulated, they become susceptible to deletion from the peripheral T cell pool by other recent thymic emigrants. This system of thymic export and peripheral T cell replacement provides a continual replenishment of the quality of T cells, with homeostasis maintaining the appropriate levels.

[0005] While the thymus is fundamental for a functional immune system, releasing ~1% of its T cell content into the bloodstream per day, one of the apparent anomalies of

mammals is that this organ undergoes severe atrophy as a result of sex steroid production. This can begin even in young children but is profound from the time of puberty. For normal healthy individuals this loss of production and release of new T cells does not always have immediate clinical consequences. In fact although the aged thymus is atrophic and consists of less than 1% of its young counterpart, it still continues to release a very low level of new T cells into the blood stream.

[0006] These are insufficient, however, to maintain the optimal levels of peripheral T cell subsets. But this does mean that the thymus is not completely dormant, raising the possibility that it could be the target of therapy. With progressive aging, the decline in thymic export means that the status of peripheral T cells undergoes progressive change both quantitatively and qualitatively. On the one hand there is a gradual decrease in absolute T cell numbers in the blood with age as they die off through lack of stimulation. On the other hand, with each antigen contact, the relevant antigen-specific naïve T cells (those which have not yet encountered antigen) are stimulated and proliferate. A subset will progress to be effector cells and rid the body of the pathogen, but these eventually die through antigen-induced cell death. Another subset will convert to memory cells and provide long term protection against future contacts with that pathogen. Hence, there is a decrease in the levels of naïve T cells and thus a reduced ability to respond to antigen.

[0007] Aging also results in a selective decline in Th cells (characterized by expression of CD4) relative to Tc cells (expressing CD8) and imbalances in the ratios of Th1 to Th2 cells. This does not occur in the normal young because, as mentioned above, there is a continual supply of new T cells being exported from the thymus, which in turn provides a continual replenishment of the naïve T cell pool in the periphery.

[0008] Aging is not the only condition which results in T cell loss – this also occurs very severely for example in HIV/AIDS and following chemotherapy or radiotherapy. Again, in the young with an active thymus, the recovery of the immune system (through recovery of T cell mediated immunity) occurs relatively quickly (2-3 months), compared to post-puberty, when it can take 1-2 years because of the atrophic thymus.

[0009] There are thus several parameters which can influence the nature and extent of immune responses: the level and type of antigen, the site of vaccination, the availability of appropriate APC (antigen presenting cells), the general health of the individual and the status

of the T and B cell pools. Of these, T cells are the most vulnerable because of the marked sex steroid induced shut-down in thymic export which becomes profound from the onset of puberty.

[0010] Any vaccination program should therefore only be logically undertaken when the level of potential responder T cells is optimal in terms of both the level of naïve T cells representing a broad repertoire of specificity and the correct ratios of Th1 to Th2 cells and Th to Tc cells. The level and type of cytokines should also be manipulated to be appropriate for the desired response.

[0011] The ability to reactivate the atrophic thymus through inhibition of LHRH signaling to the pituitary provides a potent means of generating a new cohort of naïve T cells with a diverse repertoire of TCR types. This process effectively reverts the thymus to its prepubertal state, and does so by using the normal regulatory molecules and pathways which lead to optimal thymopoiesis.

[0012] The thymus is influenced to a great extent by its bi-directional communication with the neuroendocrine system (Kendall, 1988). Of particular importance is the interplay between the pituitary, adrenals and gonads on thymic function including both trophic (TSH and GH) and atrophic effects (LH, FSH and ACTH) (Kendall, 1988; Homo-Delarche, 1991). Indeed one of the characteristic features of thymic physiology is the progressive decline in structure and function which is commensurate with the increase in circulating sex steroid production around puberty (Hirokawa and Makinodan, 1975; Tosi *et al.*, 1982 and Hirokawa, *et al.*, 1994). The precise target of the hormones and the mechanism by which they induce thymus atrophy is yet to be determined.

[0013] Since the thymus is the primary site for the production and maintenance of the peripheral T cell pool, this atrophy has been widely postulated as the primary cause of an increased incidence of immune-based disorders in the elderly. In particular, deficiencies of the immune system illustrated by a decrease in T-cell dependent immune functions such as cytolytic T-cell activity and mitogenic responses, are reflected by an increased incidence of immunodeficiency, autoimmunity and tumor load in later life (Hirokawa, 1998).

[0014] The impact of thymus atrophy is reflected in the periphery, with reduced thymic input to the T cell pool resulting in a less diverse T cell receptor (TCR) repertoire. Altered cytokine profile (Hobbs *et al.*, 1993; Kurashima *et al.*, 1995), changes in $CD4^+$ and

$CD8^+$ subsets, and a bias towards memory as opposed to naïve T cells (Mackall *et al.,* 1995) are also observed. Furthermore, the efficiency of thymopoiesis is impaired with age such that the ability of the immune system to regenerate normal T-cell numbers after T-cell depletion is eventually lost (Mackall *et al.,* 1995).

[0015] However, recent work by Douek *et al.* (1998) has shown presumably thymic output to occur even in old age in humans. Excisional DNA products of TCR gene-rearrangement were used to demonstrate circulating, *de novo* produced naïve T cells after HIV infection in older patients. The rate of this output and subsequent peripheral T cell pool regeneration needs to be further addressed since patients who have undergone chemotherapy show a greatly reduced rate of regeneration of the T cell pool, particularly $CD4^+$ T cells, in post-pubertal patients compared to those who were pre-pubertal (Mackall et al, 1995). This is further exemplified in recent work by Timm and Thoman (1999), who have shown that although $CD4^+$ T cells are regenerated in old mice post BMT (bone marrow transplant), they appear to show a bias towards memory cells due to the aged peripheral microenvironment, coupled to poor thymic production of naïve T cells.

[0016] The thymus essentially consists of developing thymocytes interspersed within the diverse stromal cells (predominantly epithelial cell subsets) which constitute the microenvironment and provide the growth factors and cellular interactions necessary for the optimal development of the T cells. The symbiotic developmental relationship between thymocytes and the epithelial subsets that controls their differentiation and maturation (Boyd *et al.,* 1993) means sex-steroid inhibition could occur at the level of either cell type which would then influence the status of the other. It is less likely that there is an inherent defect within the thymocytes themselves since previous studies, utilizing radiation chimeras, have shown that bone marrow (BM) stem cells are not affected by age (Hirokawa, 1998; Mackall and Gress, 1997) and have a similar degree of thymus repopulation potential as young BM cells. Furthermore, thymocytes in aged animals retain their ability to differentiate to at least some degree (Mackall and Gress, 1997; George and Ritter, 1996; Hirokawa *et al.,* 1994). However, recent work by Aspinall (1997), has shown a defect within the precursor $CD3^-CD4^-$ $CD8^-$ triple negative (TN) population occurring at the stage of TCRγ chain gene-rearrangement.

12 APR 2005

Skewing of developing TCR repertoire towards, or away from, specific antigens.

[0017] The ability to enhance the uptake into the thymus of haematopoietic precursor cells means that the nature and type of dendritic cells can be manipulated. For example the precursors can be transfected with specific gene(s) which eventually become expressed in the dendritic cells in the thymus (and elsewhere in the body). Such genes can include those which encode specific antigens for which an immune response would be detrimental, e.g., autoimmune diseases, allergies and graft antigens

[0018] The genes can also encode antigens (also as peptides) for which an immune response is desired, e.g., tumor cells and invading microorganisms. In the latter case the level and affinity of the peptide would be manipulated to be low enough so as not to induce negative selection, but high enough to promote positive selection. We have shown that positive selection can involve multiple cell types: the cortical epithelium provides the specific differentiation molecules, and third party cells the MHC/peptide ligands.

[0019] The precursors can also be genetically modified by adding or deleting genes, such as those coding for soluble regulatory molecules, such as chemokines, cytokines and other molecules affecting any aspect of thymopoiesis and T cell development, activation, positive or negative selection, migration, and general status. This approach can be used to promote or retard thymic development or T cell responsiveness. It can be used to skew the T cell repertoire to specific antigens to create, for example, anti-viral and anti-tumor defenses. This approach can also be used to modulate the nature, organization and function of the thymic microenvironment.

Induction of tolerance

[0020] The most effective means of generating tolerance to self is through intra-thymic deletion (or anergy or induction of negative regulatory cells) of the potentially self reactive cells through negative selection, mediated most efficiently by intrathymic dendritic cells. As a corollary, the establishment of tolerance to exogenous or nominal antigens could be best achieved if dendritic cells expressing this antigen could be incorporated into the thymus. This form of tolerance may also be made more effective through the advent of inhibitory immunoregulatory cells. The mechanisms underlying the development of the latter,

however, are poorly understood, but again occur in the thymus and could involve dendritic cells.

[0021] In the case of hyperreactive T cells for which the target antigen is known, the haematopoietic stem cells can be transfected with the gene encoding the specific antigen. When these cells develop into dendritic cells in the thymus they will delete any new T cells arising which are potentially reactive to the nominal antigen.

[0022] The enormous clinical benefits to be gained through restoration of thymic function, represent an important strategy for the treatment of immunodeficiencies, particularly in the elderly, HIV patients and patients following chemotherapy. Furthermore patients who have functionally abnormal T cells can now be treated to remove all T cells, thereby stopping the disease, and then have their normal immunity restored by reactivation of thymic function by inhibition of sex steroid production. In the case of vaccination programs, the reactivation of the thymus will have profound improvements on the status of T cells and hence the nature, extent and quality of immune responses. Additionally, through presentation of donor cells during reactivation of the thymus, T cell populations can be modified to allow for tolerance of allogeneic and xenogeneic grafts. Moreover, regenerating populations of T cells can be genetically modified through gene therapy during thymic reactivation.

SUMMARY OF THE INVENTION

[0023] The present disclosure provides a diagnostic method for determining the susceptibility of a thymus to regeneration by inhibition of sex steroid production. In a preferred embodiment, the method provides an early determination of this susceptibility, preferably within a week, more preferably within 4 to 5 days, even more preferably within 2-3 days, and most preferably with 24 hours of initiation of inhibition.

[0024] In a particular embodiment sex steroid mediated signaling to the thymus is blocked by the administration of agonists or antagonists of LHRH, anti-estrogen antibodies, anti-androgen antibodies, passive (antibody) or active (antigen) anti-LHRH vaccinations, or combinations thereof ("blockers").

[0025] In a preferred embodiment, inhibition is caused by administering an LHRH agonist. Preferably a quick-acting antagonist such as Abarelix or Cetrorelix is administered. In an alternative embodiment, inhibition is caused by administering an LHRH agonist such as Zoladex or Leupron.

12 APR 2005

[0026] In a preferred embodiment, the blocker(s) is administered by a sustained peptide-release formulation. Examples of sustained peptide-release formulations are provided in WO 98/08533, the entire contents of which are incorporated herein by reference.

[0027] In one embodiment, the diagnosis is accomplished by measuring the amount of thymic induced factors in a blood sample of the patient before and after initiation of inhibition.

[0028] In yet another embodiment, the invention is used to identify previously unidentified thymic factors.

[0029] In another embodiment, the diagnosis is accomplished by measuring thymic activity. In addition to the above, this will be achieved by determining levels of newly produced T cells identified by the presence in these cells of small circles of DNA termed T cell receptor excision circles (TREC's). These TREC's are produced as a normal part of T cell development in the thymus, in particular as a result of gene rearrangements in the formation of the T cell receptor for antigen. Basic increases in total T cell number (as measured by flow cytometry staining for CD3, CD4 and CD8) and shifts in their *in vitro* responsiveness to stimulation with anti-CD3 cross-linking can also be used to monitor thymic function but they are expected to take several days to weeks before any changes may be detectable.

DESCRIPTION OF THE FIGURES

[0030] Figure 1 A and B: Changes in thymocyte number pre- and post-castration. Thymus atrophy results in a significant decrease in thymocyte numbers with age. Aged (2-year old) mice were surgically castrated and analysed for (A) thymus weight in relation to body weight and (B) total cells per thymus, at 2-4 weeks post-castration. A significant decrease in thymus weight and cellularity was seen with age compared to young adult (2-month) mice. This was restored by castration. At 3-weeks post-castration thymic hypertrophy was observed and was returned to young adult levels by 4-weeks post-castration. Results are expressed as mean $\pm$1SD of 4-8 mice per group. ** = p $\leq$01; *** = p $\leq$0.001 compared to young adult and post-castration mice.

[0031] Figure 2 A-C: Aged (2-year old) mice were surgically castrated and analysed at 2 and 4 weeks post-castration for peripheral lymphocyte populations. (A) Total lymphocyte numbers in the spleen. Spleen numbers remain constant with age and post-castration. (B) The ratio of B cells to T cells did not change with age or post-castration, however (C) a significant decrease in the $CD4^+$:$CD8^+$ T cell ratio was seen with age. This was restored by 4-weeks post-

castration. Data is expressed as mean±1SD of 4-8 mice per group. *** = p ≤0.001 compared to young adult (2-month) and 4-week post-castrate mice.

[0032] Figure 3: Fluorescence Activated Cell Sorter (FACS) profiles of CD4 vs. CD8 thymocyte populations with age and post-castration. Aged (2-year old) mice were castrated and the thymocyte subsets analysed based on the markers CD4 and CD8. Representative FACS profiles of CD4/CD8 dot plots are shown for $CD4^-CD8^-$DN, $CD4^+CD8^+$DP, $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes. No difference was seen in the proportions of any CD4/CD8 defined subset with age or post-castration.

[0033] Figure 4: Aged (2-year old) mice were castrated and injected with a pulse of bromodeoxyuridine (BrdU) to determine levels of proliferation. Representative histogram profiles of the proportion of $BrdU^+$ cells within the thymus with age and post-castration are shown. No difference in the proportion of proliferating cells within the total thymus was observed with age or post-castration.

[0034] Figure 5 A-D: Effects of age and castration on proliferation of thymocyte subsets. (A) Proportion of each subset that constitutes the total proliferating population—The proportion of CD8+ T cells within the proliferating population is significantly increased. (B) However, a significant decrease in the proportion of DN (CD4-CD8-) thymocytes proliferating was seen with age. Post-castration, this was restored and a significant increase in proliferation within the $CD4^-CD8^+$ SP thymocytes was observed. (C) No change in the total proportion of $BrdU^+$ cells within the TN subset was seen with age or post-castration. However (D) the significant decrease in proliferation of the TN1 ($CD44^+CD25^-$) subpopulation with age is not returned to normal levels by 4 weeks post-castration. Results are expressed as mean±1SD of 4-8 mice per group. * = p ≤0.05; *** = p ≤0.001 compared to young adult (2-month) mice.

[0035] Figure 6 A-C: Aged (2-year old) mice were castrated and were injected intrathymically with FITC to determine thymic export rates. The number of $FITC^+$ cells in the periphery were calculated 24 hours later. (A) A significant decrease in recent thymic emigrant (RTE) cell numbers was observed with age. Following castration, these values had significantly increased by 2 weeks post-cx. (B) The rate of emigration (export/total thymus cellularity) remained constant with age but was significantly reduced at 2 weeks post-cx. (C) With age, a significant increase in the ratio of $CD4^+$ to $CD8^+$ RTE was seen and this was normalised by 1-week post-cx. Results are expressed as mean±1SD of 4-8 mice per group. ** = p ≤0.01; *** = p ≤0.001 compared to young adult mice. ^ = p ≤0.001 compared to castrated mice.

[0036] Figure 7 A-C: Changes in thymus (A), spleen (B) and lymph node (C) cell numbers following treatment with cyclophosphamide, a chemotherapy agent. Young (3-

12 APR 2005

month old) mice were depleted of lymphocytes using cyclophosphamide. Mice were either sham-castrated or castrated on the same day as cyclophosphamide treatment. (A) A significant increase in thymus cell number was observed in castrated mice compared to sham-castrated mice. (B) Castrated mice also showed a significant increase in spleen cell number at 1-week post-cyclophosphamide treatment. (C) A significant increase in lymph node cellularity was also observed with castrated mice at 1-week post-treatment. Results are expressed as mean±1SD of 4-8 mice per group. *** = $p \leq 0.001$ compared to castrated mice.

[0037] Figure 8 A-C: Changes in thymus (A), spleen (B) and lymph node (C) cell numbers following irradiation and castration on the same day. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate group at 2 weeks post-treatment. No difference in spleen (B) or lymph node (C) cell numbers was seen with castrated mice. Lymph node cell numbers were still chronically low at 2-weeks post-treatment compared to control mice. Results are expressed as mean±1SD of 4-8 mice per group. * = $p \leq 0.05$ compared to control mice; *** = $p \leq 0.001$ compared to control and castrated mice.

[0038] Figure 9 A-C: Changes in thymus (A), spleen (B) and lymph node (C) cell numbers following irradiation (625 Rads) one week after surgical castration. A significant increase in thymus regeneration was observed with castration (A). No difference in spleen (B) or lymph node (C) cell numbers was seen with castrated mice. Lymph node cell numbers were still chronically low at 2-weeks post-treatment compared to control mice. Results are expressed as mean $\pm$ 1SD of 4-8 mice per group. + = $p \leq 0.05$; ** = $p \leq 0.01$ compared to control mice; *** = $p \leq 0.001$ compared to control and castrated mice.

[0039] Figure 10: Changes in thymus, spleen and lymph node cell numbers following treatment with cyclophosphamide, a chemotherapy agent, and surgical or chemical castration performed on the same day. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate (cyclophosphamide alone) group at 1 and 2 weeks post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. (n = 3-4 per treatment group and time point). Chemical castration is comparable to surgical castration in regeneration of the immune system post-cyclophosphamide treatment.

[0040] Figure 11 A and B: Lymph node cellularity following foot-pad immunization with Herpes Simplex Virus-1 (HSV-1). Note the increased cellularity in the aged post-castration as compared to the aged non-castrated group (A). Bottom graph illustrates the overall activated cell number as gated on CD25 vs. CD8 cells by FACS (B).

[0041] Figure 12 A-C: Vβ10 expression on CTL (cytotoxic T lymphocytes) in activated LN (lymph nodes) following HSV-1 inoculation. Despite the normal Vβ10

responsiveness in aged mice overall, in some mice a complete loss of Vβ10 expression was observed. Representative histogram profiles are shown. Note the diminution of a clonal response in aged mice and the reinstatement of the expected response post-castration.

[0042] Figure 13 A-C: Castration restores responsiveness to HSV-1 immunization. (A) Aged mice showed a significant reduction in total lymph node cellularity post-infection when compared to both the young and post-castrate mice. (B) Representative FACS profiles of activated ($CD8^+CD25^+$) cells in the LN of HSV-1 infected mice. No difference was seen in proportions of activated CTL with age or post-castration. (C) The decreased cellularity within the lymph nodes of aged mice was reflected by a significant decrease in activated CTL numbers. Castration of the aged mice restored the immune response to HSV-1 with CTL numbers equivalent to young mice. Results are expressed as mean ±1 SD of 8-12 mice. ** = $p \leq 0.01$ compared to both young (2-month) and non-castrated mice.

[0043] Figure 14: Popliteal lymph nodes were removed from mice immunized with HSV-1 and cultured for 3 days. CTL assays were performed with non-immunized mice as control for background levels of lysis (as determined by ^{51}Cr-release). Results are expressed as mean of 8 mice, in triplicate ±1SD. Aged mice showed a significant ($p \leq 0.01$, *) reduction in CTL activity at an E:T ratio of both 10:1 and 3:1 indicating a reduction in the percentage of specific CTL present within the lymph nodes. Castration of aged mice restored the CTL response to young adult levels. * = $p \leq 0.01$ compared to young adult and post-castrate aged mice.

[0044] Figure 15 A and B: Analysis of $CD4^+$ T cell help and Vβ TCR response to HSV-1 infection. Popliteal lymph nodes were removed on D5 post-HSV-1 infection and analysed ex-vivo for the expression of (a) CD25, CD8 and specific TCRVβ markers and (b) CD4/CD8 T cells. (A) The percentage of activated ($CD25^+$) $CD8^+$ T cells expressing either Vβ10 or Vβ8.1 is shown as mean ±1SD for 8 mice per group. No difference was observed with age or post-castration. (B) A decrease in CD4/CD8 ratio in the resting LN population was seen with age. This was restored post-castration. Results are expressed as mean±1SD of 8 mice per group. *** = $p \leq 0.001$ compared to young and castrate mice.

[0045] Figure 16 A-D: Changes in thymus (A), spleen (B), lymph node (C) and bone marrow (D) cell numbers following bone marrow transplantation of Ly5 congenic mice. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate group at all time points post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. (n = 3-4

per treatment group and time point). Castrated mice had significantly increased congenic (Ly5.2) cells compared to non-castrated animals (data not shown).

[0046] Figure 17 A and B: Changes in thymus cell number in castrated and noncastrated mice after fetal liver reconstitution. (n = 3-4 for each test group.) (A) At two weeks, thymus cell number of castrated mice was at normal levels and significantly higher than that of noncastrated mice (*p $\leq$0.05). Hypertrophy was observed in thymuses of castrated mice after four weeks. Noncastrated cell numbers remain below control levels. (B) $CD45.2^+$ cells - CD45.2+ is a marker showing donor derivation. Two weeks after reconstitution donor-derived cells were present in both castrated and noncastrated mice. Four weeks after treatment approximately 85% of cells in the castrated thymus were donor-derived. There were no donor-derived cells in the noncastrated thymus.

[0047] Figure 18: FACS profiles of CD4 versus CD8 donor derived thymocyte populations after lethal irradiation and fetal liver reconstitution, followed by surgical castration. Percentages for each quadrant are given to the right of each plot. The age matched control profile is of an eight month old Ly5.1 congenic mouse thymus. Those of castrated and noncastrated mice are gated on $CD45.2^+$ cells, showing only donor derived cells. Two weeks after reconstitution subpopulations of thymocytes do not differ between castrated and noncastrated mice.

[0048] Figure 19 A and B: Myeloid and lymphoid dendritic cell (DC) number after lethal irradiation, fetal liver reconstitution and castration. (n= 3-4 mice for each test group.) Control (white) bars on the following graphs are based on the normal number of dendritic cells found in untreated age matched mice. (A) Donor-derived myeloid dendritic cells—Two weeks after reconstitution DC were present at normal levels in noncastrated mice. There were significantly more DC in castrated mice at the same time point. (*p $\leq$0.05). At four weeks DC number remained above control levels in castrated mice. (B) Donor-derived lymphoid dendritic cells—Two weeks after reconstitution DC numbers in castrated mice were double those of noncastrated mice. Four weeks after treatment DC numbers remained above control levels.

[0049] Figure 20 A and B: Changes in total and $CD45.2^+$ bone marrow cell numbers in castrated and noncastrated mice after fetal liver reconstitution. n=3-4 mice for each test group. (A) Total cell number—Two weeks after reconstitution bone marrow cell numbers had normalized and there was no significant difference in cell number between castrated and noncastrated mice. Four weeks after reconstitution there was a significant difference in cell number between castrated and noncastrated mice (*p $\leq$0.05). (B) $CD45.2^+$ cell number. There was no significant difference between castrated and noncastrated mice with respect to CD45.2+ cell number in the bone marrow two weeks after reconstitution. $CD45.2^+$ cell

number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same time point.

[0050] Figure 21 A-C: Changes in T cells and myeloid and lymphoid derived dendritic cells (DC) in bone marrow of castrated and noncastrated mice after fetal liver reconstitution. (n=3-4 mice for each test group.) Control (white) bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age matched mice. (A) T cell number—Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells—Two weeks after reconstitution DC cell numbers were normal in both castrated and noncastrated mice. At this time point there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells—Numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

[0051] Figure 22 A and B: Change in total and donor ($CD45.2^+$) lymph node cell numbers in castrated and noncastrated mice after fetal liver reconstitution. (n=3-4 mice for each test group.) (A) Total cell number—Two weeks after reconstitution cell numbers were decreased and there was no significant difference in cell number between castrated and noncastrated mice. Four weeks after reconstitution cell numbers were approaching normal levels in castrated mice. (B) $CD45.2^+$ cell number—There was no significant difference between castrated and noncastrated mice with respect to $CD45.2^+$ cell number in the spleen, two weeks after reconstitution. $CD45.2^+$ cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same time point.

[0052] Figure 23 A-C: Splenic T cells and myeloid and lymphoid derived dendritic cells (DC) after fetal liver reconstitution. (n=3-4 mice for each test group.) Control (white) bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age matched mice. (A) T cell number—Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived ($CD45.2^+$) myeloid dendritic cells—two and four weeks after reconstitution DC numbers were normal in both castrated and noncastrated mice. At two weeks there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived ($CD45.2^+$) lymphoid dendritic cells—numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

[0053] Figure 24 A and B: Changes in total and donor ($CD45.2^+$) lymph node cell numbers in castrated and noncastrated mice after fetal liver reconstitution. (n=3-4 for each

test group.) (A) Total cell numbers—Two weeks after reconstitution cell numbers were at normal levels and there was no significant difference between castrated and noncastrated mice. Four weeks after reconstitution cell numbers in castrated mice were at normal levels. (B) $CD45.2^+$ cell number—There was no significant difference between castrated and noncastrated mice with respect to donor $CD45.2^+$ cell number in the lymph node two weeks after reconstitution. CD45.2 cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same point.

[0054] Figure 25 A-C: Changes in T cells and myeloid and lymphoid derived dendritic cells (DC) in the mesenteric lymph nodes of castrated and non-castrated mice after fetal liver reconstitution. (n=3-4 mice for each test group.) Control (striped) bars are the number of T cells and dendritic cells found in untreated age matched mice. (A) T cell numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells were normal in both castrated and noncastrated mice. At four weeks they were decreased. At two weeks there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells—Numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

[0055] Figure 26: The phenotypic composition of peripheral blood lymphocytes was analyzed in human patients (all >60 years) undergoing LHRH agonist treatment for prostate cancer. Patient samples were analyzed before treatment and 4 months after beginning LHRH agonist treatment. Total lymphocyte cell numbers per ml of blood were at the lower end of control values before treatment in all patients. Following treatment, 6/9 patients showed substantial increases in total lymphocyte counts (in some cases a doubling of total cells was observed). Correlating with this was an increase in total T cell numbers in 6/9 patients. Within the $CD4^+$ subset, this increase was even more pronounced with 8/9 patients demonstrating increased levels of CD4 T cells. A less distinctive trend was seen within the $CD8^+$ subset with 4/9 patients showing increased levels, albeit generally to a smaller extent than $CD4^+$ T cells.

[0056] Figure 27: Analysis of human patient blood before and after LHRH-agonist treatment demonstrated no substantial changes in the overall proportion of T cells, CD4 or

CD8 T cells, and a variable change in the CD4:CD8 ratio following treatment. This indicates the minimal effect of treatment on the homeostatic maintenance of T cell subsets despite the substantial increase in overall T cell numbers following treatment. All values were comparative to control values.

[0057] Figure 28: Analysis of the proportions of B cells and myeloid cells (NK, NKT and macrophages) within the peripheral blood of human patients undergoing LHRH agonist treatment demonstrated a varying degree of change within subsets. While NK, NKT and macrophage proportions remained relatively constant following treatment, the proportion of B cells was decreased in 4/9 patients.

[0058] Figure 29: Analysis of the total cell numbers of B and myeloid cells within the peripheral blood of human patients post-treatment showed clearly increased levels of NK (5/9 patients), NKT (4/9 patients) and macrophage (3/9 patients) cell numbers post-treatment. B cell numbers showed no distinct trend with 2/9 patients showing increased levels; 4/9 patients showing no change and 3/9 patients showing decreased levels.

[0059] Figure 30 A and B: The major change seen post-LHRH agonist treatment was within the T cell population of the peripheral blood. In particular there was a selective increase in the proportion of naïve ($CD45RA^+$) CD4+ cells, with the ratio of naïve ($CD45RA^+$) to memory ($CD45RO^+$) in the $CD4^+$ T cell subset increasing in 6/9 of the human patients.

[0060] Figure 31: Decrease in the impedance of skin using various laser pulse energies. There is a decrease in skin impedance in skin irradiated at energies as low as 10 mJ, using the fitted curve to interpolate data.

[0061] Figure 32: Permeation of a pharmaceutical through skin. Permeability of the skin, using insulin as a sample pharmaceutical, was greatly increased through laser irradiation.

[0062] Figure 33: Change in fluorescence of skin over time after the addition of 5-aminolevulenic acid (ALA) and a single impulse transient to the skin. The peak of intensity occurs at about 640 nm and is highest after 210 minutes (dashed line) post-treatment.

[0063] Figure 34: Change in fluorescence of skin over time after the addition of 5-aminolevulenic acid (ALA) without an impulse transient. There is little change in the intensity at different time points.

[0064] Figure 35: Comparison of change in fluorescence of skin after the addition of 5-aminolevulenic acid (ALA) and a single impulse transient under various peak stresses. The degree of permeabilization of the stratum corneum depends on the peak stress.

DETAILED DESCRIPTION OF THE INVENTION

[0065] A characteristic feature of thymic function is that while it is of fundamental importance to the establishment and maintenance of the immune system and hence to the defense against infection and disease, it characteristically undergoes a profound age-dependent decrease in function to less than 5% of it maximal capacity. This becomes most pronounced following puberty, implicating a role for sex steroids.

[0066] Inhibition of sex steroids results, either directly or indirectly, in a major reactivation of thymic function, effectively reversing the atrophy. Given the broad range of patient age, diseases and treatments, however, it is anticipated that many patients will respond differently to this treatment, including some very poorly. Hence a new diagnostic early indicator of this responsiveness of the thymus to activation in the absence of sex steroids is provided to formulate rational clinical management of T cell based disorders.

[0067] Since the thymus is an endocrine organ, reactivation of thymic function involves release of not only new T cells into the blood stream after 2-4 weeks, but prior to this the thymus will also release increased levels of cytokines, even within hours of reactivation. These will be detectable in the blood or plasma. The present disclosure utilizes these released cells and molecules to detect the degree of response of a patient's thymus to inhibition of sex steroids. Provided here is a set of diagnostic techniques for making this determination.

Disruption Of Sex Steroid Signaling To The Thymus

[0068] As will be readily understood, sex steroid signaling to the thymus can be disrupted in a range of ways well known to those of skill in the art, some of which are described herein. For example, inhibition of sex steroid production or blocking of one or more sex steroid receptors within the thymus will accomplish the desired disruption, as will administration of sex steroid agonists or antagonists, or active (antigen) or passive (antibody) anti-sex steroid vaccinations. Inhibition of sex steroid production can also be achieved by administration of one or more sex steroid analogs. In some clinical cases, permanent removal of the gonads via physical castration may be appropriate.

[0069] In a preferred embodiment the sex steroid signaling to the thymus is disrupted by administration of a sex steroid analog, or preferably an analog of luteinizing hormone-releasing hormone (LHRH). Sex steroid analogs and their use in therapies and chemical castration are well known. Such analogs include, but are not limited to, Abarelix (US Pat. No. 6,197,337), Cetrorelix, Deslorelin (described in U.S. Patent No. 4,218,439), Eulexin (described in FR7923545, WO86/01105 and PT100899), Goserelin (described in US Pat. No. 4,100,274, US Pat. No. 4,128,638, GB9112859 and GB9112825), Leuprolide (described in US Pat. No. 4,490,291, US Pat. No. 3,972,859, US Pat. No. 4,008,209, US Pat. No. 4,005,063, DE2509783 and US Pat. No. 4,992,421), Dioxalan derivatives such as are described in EP 413209, Triptorelin (described in US Pat. No. 4,010,125, US Pat. No. 4,018,726, US Pat. No. 4,024,121, EP 364819 and US Pat. No. 5,258,492), Meterelin (described in EP 23904), Buserelin (described in US Pat. No. 4,003,884, US Pat. No. 4,118,483 and US Pat. No. 4,275,001), Histrelin (described in EP217659), Nafarelin (described in US Pat. No. 4,234,571, WO93/15722 and EP52510), Lutrelin (described in US Pat. No. 4,089,946), Leuprorelin (described in Plosker *et al.*) and LHRH analogs such as are described in EP181236, US Pat. No. 4,608,251, US Pat. No. 4,656,247, US Pat. No. 4,642,332, US Pat. No. 4,010,149, US Pat. No. 3,992,365 and US Pat. No. 4,010,149. The disclosures of each the references referred to above are incorporated herein by reference.

[0070] While the stimulus for thymic regeneration is fundamentally based on the inhibition of the effects of sex steroids and/or the direct effects of the LHRH analogues, it may be necessary to include additional substances which can act in concert to enhance the thymic effect. Such compounds could include but not be limited to Interleukin 7, members of the epithelium and fibroblast growth factor familes and keratinocyte growth factor. It is envisaged that these additional compounds would only be given once at the initial LHRH analogue application. In addition steroid receptor based modulators, which may be targeted to be thymic specific could be developed and used.

Delivery Of Agents For Chemical Castration

[0071] Delivery of the compounds of this invention can be accomplished via a number of methods known to persons skilled in the art. One standard procedure for administering chemical inhibitors to inhibit sex steroid signaling to the thymus utilizes a single dose of an

LHRH agonist that is effective for three months. For this a simple one-time i.v. or i.m. injection would not be sufficient as the agonist would be cleared from the patient's body well before the three months are over. Instead, a depot injection or an implant may be used, or any other means of delivery of the inhibitor that will allow slow release of the inhibitor. Likewise, a method for increasing the half life of the inhibitor within the body, such as by modification of the chemical, while retaining the function required herein, may be used.

[0072] Examples of more useful delivery mechanisms include, but are not limited to, laser irradiation of the skin, and creation of high pressure impulse transients (also called stress waves or impulse transients) on the skin, each method accompanied or followed by placement of the compound(s) with or without carrier at the same locus. A preferred method of this placement is in a patch placed and maintained on the skin for the duration of the treatment.

[0073] One means of delivery utilizes a laser beam, specifically focused, and lasing at an appropriate wavelength, to create small perforations or alterations in the skin of a patient. See U.S. Pat. No. 4,775,361, U.S. Pat. No. 5,643,252, U.S. Pat. No. 5,839,446, and U.S. Pat. No. 6,056,738, all of which are incorporated herein by reference. In a preferred embodiment, the laser beam has a wavelength between 0.2 and 10 microns. More preferably, the wavelength is between about 1.5 and 3.0 microns. Most preferably the wavelength is about 2.94 microns. In one embodiment, the laser beam is focused with a lens to produce an irradiation spot on the skin through the epidermis of the skin. In an additional embodiment, the laser beam is focused to create an irradiation spot only through the stratum corneum of the skin.

[0074] Several factors may be considered in defining the laser beam, including wavelength, energy fluence, pulse temporal width and irradiation spot-size. In a preferred embodiment, the energy fluence is in the range of 0.03-100,000 J/cm^2. More preferably, the energy fluence is in the range of 0.03 - 9.6 J/cm^2. The beam wavelength is dependent in part on the laser material, such as Er:YAG. The pulse temporal width is a consequence of the pulse width produced by, for example, a bank of capacitors, the flashlamp, and the laser rod material. The pulse width is optimally between 1 fs (femtosecond) and 1,000 μs.

[0075] According to this method the perforation or alteration produced by the laser need not be produced with a single pulse from the laser. In a preferred embodiment a

perforation or alteration through the stratum corneum is produced by using multiple laser pulses, each of which perforates or alters only a fraction of the target tissue thickness.

[0076] To this end, one can roughly estimate the energy required to perforate or alter the stratum corneum with multiple pulses by taking the energy in a single pulse and dividing by the number of pulses desirable. For example, if a spot of a particular size requires 1 J of energy to produce a perforation or alteration through the entire stratum corneum, then one can produce qualitatively similar perforation or alteration using ten pulses, each having 1/10th the energy. Because it is desirable that the patient not move the target tissue during the irradiation (human reaction times are on the order of 100 ms or so), and that the heat produced during each pulse not significantly diffuse, in a preferred embodiment the pulse repetition rate from the laser should be such that complete perforation is produced in a time of less than 100 ms. Alternatively, the orientation of the target tissue and the laser can be mechanically fixed so that changes in the target location do not occur during the longer irradiation time.

[0077] To penetrate the skin in a manner that induces little or no blood flow, skin is perforated or altered through the outer surface, such as the stratum corneum layer, but not as deep as the capillary layer. The laser beam is focussed precisely on the skin, creating a beam diameter at the skin in the range of approximately 0.5 microns - 5.0 cm. Optionally, the spot can be slit-shaped, with a width of about 0.05-0.5 mm and a length of up to 2.5 mm. The width can be of any size, being controlled by the anatomy of the area irradiated and the desired permeation rate of the fluid to be removed or the pharmaceutical to be applied. The focal length of the focusing lens can be of any length, but in one embodiment it is 30 mm.

[0078] By modifying wavelength, pulse length, energy fluence (which is a function of the laser energy output (in Joules) and size of the beam at the focal point (cm^2)), and irradiation spot size, it is possible to vary the effect on the stratum corneum between ablation (perforation) and non-ablative modification (alteration). Both ablation and non-ablative alteration of the stratum corneum result in enhanced permeation of subsequently applied pharmaceuticals.

[0079] For example, by reducing the pulse energy while holding other variables constant, it is possible to change between ablative and non-ablative tissue-effect. Using an Er:YAG laser having a pulse length of about 300 μs, with a single pulse or radiant energy and irradiating a 2 mm spot on the skin, a pulse energy above approximately 100 mJ causes partial

or complete ablation, while any pulse energy below approximately 100 mJ causes partial ablation or non-ablative alteration to the stratum corneum. Optionally, by using multiple pulses, the threshold pulse energy required to enhance permeation of body fluids or for pharmaceutical delivery is reduced by a factor approximately equal to the number of pulses.

[0080] Alternatively, by reducing the spot size while holding other variables constant, it is also possible to change between ablative and non-ablative tissue-effect. For example, halving the spot area will result in halving the energy required to produce the same effect. Irradiation down to 0.5 microns can be obtained, for example, by coupling the radiant output of the laser into the objective lens of a microscope objective. (e.g., as available from Nikon, Inc., Melville, NY). In such a case, it is possible to focus the beam down to spots on the order of the limit of resolution of the microscope, which is perhaps on the order of about 0.5 microns. In fact, if the beam profile is Gaussian, the size of the affected irradiated area can be less than the measured beam size and can exceed the imaging resolution of the microscope. To non-ablatively alter tissue in this case, it would be suitable to use a 3.2 J/cm^2 energy fluence, which for a half-micron spot size would require a pulse energy of about 5 nJ. This low a pulse energy is readily available from diode lasers, and can also be obtained from, for example, the Er:YAG laser by attenuating the beam by an absorbing filter, such as glass.

[0081] Optionally, by changing the wavelength of radiant energy while holding the other variables constant, it is possible to change between an ablative and non-ablative tissue-effect. For example, using Ho:YAG (holmium: YAG; 2.127 microns) in place of the Er:YAG (erbium: YAG; 2.94 microns) laser, would result in less absorption of energy by the tissue, creating less of a perforation or alteration.

[0082] Picosecond and femtosecond pulses produced by lasers can also be used to produce alteration or ablation in skin. This can be accomplished with modulated diode or related microchip lasers, which deliver single pulses with temporal widths in the 1 femtosecond to 1 ms range. (See D. Stern et al., "Corneal Ablation by Nanosecond, Picosecond, and Femtosecond Lasers at 532 and 625 nm," Corneal Laser Ablation, Vol. 107, pp. 587-592 (1989), incorporated herein by reference, which discloses the use of pulse lengths down to 1 femtosecond).

[0083] Another delivery method uses high pressure impulse transients on skin to create permeability. See U.S. Pat. No. 5,614,502, and U.S. Pat. No. 5,658,892, both of which

12 APR 2005

are incorporated herein by reference. High pressure impulse transients, e.g., stress waves (e.g., laser stress waves (LSW) when generated by a laser), with specific rise times and peak stresses (or pressures), can safely and efficiently effect the transport of compounds, such as those of the present disclosure, through layers of epithelial tissues, such as the stratum corneum and mucosal membranes. These methods can be used to deliver compounds of a wide range of sizes regardless of their net charge. In addition, impulse transients used in the present methods avoid tissue injury.

[0084] Prior to exposure to an impulse transient, an epithelial tissue layer, e.g., the stratum corneum, is likely impermeable to a foreign compound; this prevents diffusion of the compound into cells underlying the epithelial layer. Exposure of the epithelial layer to the impulse transients enables the compound to diffuse through the epithelial layer. The rate of diffusion, in general, is dictated by the nature of the impulse transients and the size of the compound to be delivered.

[0085] The rate of penetration through specific epithelial tissue layers, such as the stratum corneum of the skin, also depends on several other factors including pH, the metabolism of the cutaneous substrate tissue, pressure differences between the region external to the stratum corneum, and the region internal to the stratum corneum, as well as the anatomical site and physical condition of the skin. In turn, the physical condition of the skin depends on health, age, sex, race, skin care, and history. For example, prior contacts with organic solvents or surfactants affect the physical condition of the skin.

[0086] The amount of compound delivered through the epithelial tissue layer will also depend on the length of time the epithelial layer remains permeable, and the size of the surface area of the epithelial layer which is made permeable. The properties and characteristics of impulse transients are controlled by the energy source used to create them. See WO 98/23325, which is incorporated herein by reference. However, their characteristics are modified by the linear and non-linear properties of the coupling medium through which they propagate. The linear attenuation caused by the coupling medium attenuates predominantly the high frequency components of the impulse transients. This causes the bandwidth to decrease with a corresponding increase in the rise time of the impulse transient. The non-linear properties of the coupling medium, on the other hand, cause the rise time to decrease. The decrease of the rise time is the result of the dependence of the sound and

particle velocity on stress (pressure). As the stress increases, the sound and the particle velocity increase as well. This causes the leading edge of the impulse transient to become steeper. The relative strengths of the linear attenuation, non-linear coefficient, and the peak stress determine how long the wave has to travel for the increase in steepness of rise time to become substantial.

[0087] The rise time, magnitude, and duration of the impulse transient are chosen to create a non-destructive (i.e., non-shock wave) impulse transient that temporarily increases the permeability of the epithelial tissue layer. Generally the rise time is at least 1 ns, and is more preferably about 10 ns.

[0088] The peak stress or pressure of the impulse transients varies for different epithelial tissue or cell layers. For example, to transport compounds through the stratum corneum, the peak stress or pressure of the impulse transient should be set to at least 400 bar; more preferably at least 1,000 bar, but no more than about 2,000 bar.

[0089] For epithelial mucosal layers, the peak pressure should be set to between 300 bar and 800 bar, and is preferably between 300 bar and 600 bar.

[0090] The impulse transients preferably have durations on the order of a few tens of ns, and thus interact with the epithelial tissue for only a short period of time.

[0091] Following interaction with the impulse transient, the epithelial tissue is not permanently damaged, but remains permeable for up to about three minutes.

[0092] In addition, the new methods involve the application of only a few discrete high amplitude pulses to the patient. The number of impulse transients administered to the patient is typically less than 100, more preferably less than 50, and most preferably less than 10. When multiple optical pulses are used to generate the impulse transient, the time duration between sequential pulses is 10 to 120 seconds, which is long enough to prevent permanent damage to the epithelial tissue.

[0093] Properties of impulse transients can be measured using methods standard in the art. For example, peak stress or pressure, and rise time can be measured using a polyvinylidene fluoride (PVDF) transducer method as described in Doukas et al., Ultrasound Med. Biol., 21:961 (1995).

12 APR 2005

[0094] Impulse transients can be generated by various energy sources. The physical phenomenon responsible for launching the impulse transient is, in general, chosen from three different mechanisms: (1) thermoelastic generation; (2) optical breakdown; or (3) ablation.

[0095] For example, the impulse transients can be initiated by applying a high energy laser source to ablate a target material, and the impulse transient is then coupled to an epithelial tissue or cell layer by a coupling medium. The coupling medium can be, for example, a liquid or a gel, as long as it is non-linear. Thus, water, oil such as castor oil, an isotonic medium such as phosphate buffered saline (PBS), or a gel such as a collagenous gel, can be used as the coupling medium.

[0096] In addition, the coupling medium can include a surfactant that enhances transport, e.g., by prolonging the period of time in which the stratum corneum remains permeable to the compound following the generation of an impulse transient. The surfactant can be, e.g., ionic detergents or nonionic detergents and thus can include, e.g., sodium lauryl sulfate, cetyl trimethyl ammonium bromide, and lauryl dimethyl amine oxide.

[0097] The absorbing target material acts as an optically triggered transducer. Following absorption of light, the target material undergoes rapid thermal expansion, or is ablated, to launch an impulse transient. Typically, metal and polymer films have high absorption coefficients in the visible and ultraviolet spectral regions.

[0098] Many types of materials can be used as the target material in conjunction with a laser beam, provided they fully absorb light at the wavelength of the laser used. The target material can be composed of a metal such as aluminum or copper; a plastic, such as polystyrene, e.g., black polystyrene; a ceramic; or a highly concentrated dye solution. The target material must have dimensions larger than the cross-sectional area of the applied laser energy. In addition, the target material must be thicker than the optical penetration depth so that no light strikes the surface of the skin. The target material must also be sufficiently thick to provide mechanical support. When the target material is made of a metal, the typical thickness will be 1/32 to 1/16 inch. For plastic target materials, the thickness will be 1/16 to 1/8 inch.

[0099] Impulse transients can be also enhanced using confined ablation. In confined ablation, a laser beam transparent material, such as a quartz optical window, is placed in close contact with the target material. Confinement of the plasma, created by ablating the target

material by using the transparent material, increases the coupling coefficient by an order of magnitude (Fabro et al., J. Appl. Phys., 68:775, 1990). The transparent material can be quartz, glass, or transparent plastic.

[0100] Since voids between the target material and the confining transparent material allow the plasma to expand, and thus decrease the momentum imparted to the target, the transparent material is preferably bonded to the target material using an initially liquid adhesive, such as carbon-containing epoxies, to prevent such voids.

[0101] The laser beam can be generated by standard optical modulation techniques known in the art, such as by employing Q-switched or mode-locked lasers using, for example, electro- or acousto-optic devices. Standard commercially available lasers that can operate in a pulsed mode in the infrared, visible, and/or infrared spectrum include Nd:YAG, Nd:YLF, $C0_2$, excimer, dye, Ti:sapphire, diode, holmium (and other rare-earth materials), and metal-vapor lasers. The pulse widths of these light sources are adjustable, and can vary from several tens of picoseconds (ps) to several hundred microseconds. For use in the new methods, the optical pulse width can vary from 100 ps to about 200 ns and is preferably between about 500 ps and 40 ns.

[0102] Impulse transients can also be generated by extracorporeal lithotripters (one example is described in Coleman et al., Ultrasound Med. Biol., 15:213-227, 1989). These impulse transients have rise times of 30 to 450 ns, which is longer than laser-generated impulse transients. To form an impulse transient of the appropriate rise time for the new methods using an extracorporeal lithotripter, the impulse transient is propagated in a non-linear coupling medium (e.g., water) for a distance determined by equation (1), above. For example, when using a lithotripter creating an impulse transient having a rise time of 100 ns and a peak pressure of 500 barr, the distance that the impulse transient should travel through the coupling medium before contacting an epithelial cell layer is approximately 5 mm.

[0103] An additional advantage of this approach for shaping impulse transients generated by lithotripters is that the tensile component of the wave will be broadened and attenuated as a result of propagating through the non-linear coupling medium. This propagation distance should be adjusted to produce an impulse transient having a tensile component that has a pressure of only about 5 to 10% of the peak pressure of the compressive component of the wave. Thus, the shaped impulse transient will not damage tissue.

12 APR 2005

[0104] The type of lithotripter used is not critical. Either an electrohydraulic, electromagnetic, or piezoelectric lithotripter can be used.

[0105] The impulse transients can also be generated using transducers, such as piezoelectric transducers. Preferably, the transducer is in direct contact with the coupling medium, and undergoes rapid displacement following application of an optical, thermal, or electric field to generate the impulse transient. For example, dielectric breakdown can be used, and is typically induced by a high-voltage spark or piezoelectric transducer (similar to those used in certain extracorporeal lithotripters, Coleman et al., Ultrasound Med. Biol., 15:213-227, 1989). In the case of a piezoelectric transducer, the transducer undergoes rapid expansion following application of an electrical field to cause a rapid displacement in the coupling medium.

[0106] In addition, impulse transients can be generated with the aid of fiber optics. Fiber optic delivery systems are particularly maneuverable and can be used to irradiate target materials located adjacent to epithelial tissue layers to generate impulse transients in hard-to reach places. These types of delivery systems, when optically coupled to lasers, are preferred as they can be integrated into catheters and related flexible devices, and used to irradiate most organs in the human body. In addition, to launch an impulse transient having the desired rise times and peak stress, the wavelength of the optical source can be easily tailored to generate the appropriate absorption in a particular target material.

[0107] Alternatively, an energetic material can produce an impulse transient in response to a detonating impulse. The detonator can detonate the energetic material by causing an electrical discharge or spark.

[0108] Hydrostatic pressure can be used in conjunction with impulse transients to enhance the transport of a compound through the epithelial tissue layer. Since the effects induced by the impulse transients last for several minutes, the transport rate of a drug diffusing passively through the epithelial cell layer along its concentration gradient can be increased by applying hydrostatic pressure on the surface of the epithelial tissue layer, e.g., the stratum corneum of the skin, following application of the impulse transient.

Diagnostic Indicators Of Thymic Function

A. Known Markers

[0109] Certain markers are associated with the activation of the thymus. By following the concentration of any one, or any combination, of these markers, one can monitor the level of activation of the thymus. Changes in the levels of these marker molecules pre-and post-activation of thymic function can be examined using bioinformatics. For example, two-dimensional gel electrophoresis of plasma (i.e., blood depleted of all cells by centrifugation) is performed on patients' samples pre- and post-inhibition of sex steroids. The differentially expressed "dots" on the gels are recorded and analyzed by computer.

1. Interleukin-7 (Il-7)

[0110] The major lymphopoietic and thymopoietic cytokine produced by thymic cortical epithelial cells, IL-7 is essential for the proliferation and differentiation of immature thymocytes (von Freeden-Jeffry *et al.*, 1995; Komschlies *et al.*, 1995; Peschon *et al.*, 1994). Triple negative cell development requires interaction with IL-7 (Oosterwegel *et al.*, 1997; Moore *et al.*, 1993), which acts primarily by inducing *bcl-2* expression and inhibiting programmed cell death of immature thymocytes (Akashi *et al.*, 1997; Maraskovsky *et al.*, 1997). Treatment with IL-7 alone has been demonstrated to reverse both the increase in apoptosis and decline in thymopoiesis within the $CD44^{+}CD25^{+}$ (TN2) and $CD44^{-}CD25^{+}$ (TN3) subsets, corresponding to the location of TCR β-chain rearrangement, in aged mice (Andrew & Aspinall, 2001).

[0111] Immune recovery in mice after T cell-depleted bone marrow transplantation has been documented to be enhanced following administration of IL-7, suggesting the production of IL-7 may be one of the mechanisms regulating *de novo* production of T cells after bone marrow transplantation (Bolotin *et al.*, 1996). Analysis of IL-7 serum levels in patients before and after bone marrow transplantation by ELISA revealed an inverse relationship to absolute lymphocyte count (Bolotin *et al.*, 1999). Studies measuring IL-7 levels in HIV-infected pediatric and adult patients also indicate a strong inverse correlation between IL-7 and absolute CD4 counts and lesser but significant correlations with CD3 and CD8 counts (Fry *et al.*, 2001).

[0112] The mechanism underlying the increase in circulating IL-7 are unclear but it has been suggested that decreased T cell numbers result in diminished IL-7 receptor availability leading to increased levels of free IL-7 with no change in IL-7 production. That is, binding to lymphocytes that express IL-7 receptors (Bolotin *et al.*, 1999) homeostatically

regulates circulating IL-7 levels. An alternative mechanism is the direct upregulation of IL-7 in response to lymphopoenia through the interaction of T cells and IL-7-producing cells via a soluble mediator or through direct contact within the lymphoid microenvironment (Fry *et al.*, 2001).

Normal IL-7 levels

[0113] In children aged 6-months to 5.5 years, the normal mean concentration of IL-7 is 10.7 ± 3.9 pg/ml. In adults aged 22.2 to 53.5 years the mean is appreciably lower, at 3.1 ± 2.5 pg/ml. It has thus been suggested that IL-7 levels may be determined by age since IL-7 levels are highest in infants less than one year of age and lower in children and adults (Bolotin *et al.*, 1999). This would support previous studies which demonstrated an age-dependent decline in thymopoietic capacity in chemotherapy and bone marrow transplant patients beginning in adolescence (Mackall *et al.*, 1995; Weinberg *et al.*, 1995). Moreover studies of bone marrow stroma from aged mice have shown decreased secretion of IL-7 with age (Stephan *et al.*, 1998).

[0114] According to an embodiment of the present disclosure, concentration of IL-7 in a patient's blood or serum is monitored before and after administration of the agent(s) that block sex steroid mediated signaling to the thymus. Rise in the concentration of IL-7 within 2-3 days, preferably within 24 hours, more preferably within 2-3 hours, of administration of the agent(s) signifies that the thymus is responding to blockage of the sex steroid activity. Concentration of IL-7 is periodically monitored to determine the level of activation of the thymus over time.

2. Facteur Thymique Serique (FTS)

[0115] It is now largely established that the immune and neuroendocrine systems cross-talk by using similar ligands and receptors. The thymic-hypothalamus/pituitary axis constitutes a bi-directional circuit where the ascending feedback loop is effected by thymic factors of epithelial origin. Aside from modulating the release of peptidic hormones and neuropeptides, thymic hormones act mainly to promote the phenotypic maturation of progenitor cells from the bone marrow and to modulate mature T cell function (Ritter and

12 APR 2005

Crispe, 1992). Hence thymic hormones may be important in a large spectrum of pathological conditions ranging from immunodeficiencies to neuroendocrine diseases.

[0116] FTS or thymulin is a nonapeptide hormone secreted exclusively by the thymic subcapsular and medullary cells (Ritter and Crispe, 1992). Essential for both early and late stages of T cell differentiation as well as T cell function, FTS also induces expression of several T cell markers, and promotes T cell functions such as allogeneic cytotoxicity, suppressor functions and IL-2 production (Ritter and Crispe, 1992).

[0117] FTS titers in children gradually increase with increasing age from 2.69 ± 1.10 at a few days of age to 4.77 ± 0.44 at a few years of age, then gradually decrease to 0.66 ± 0.26 at 36 years of age to old age (Consolini *et al.*, 2000). As the thymus is physiologically under neuroendocrine control, peptide hormones and neuropeptides influence age-related fluctuations in FTS levels. As noted above, impaired hormonal activity has been shown to be associated with age-related thymic atrophy (Consoloni *et al.*, 2000). In particular, thymic atrophy is most evident following the rise in serum sex steroid levels following puberty (Fabris *et al.*, 1997). Moreover FTS secretion by thymic epithelial cells is enhanced by growth hormone (Mocchegiani *et al.*, 1990).

[0118] Zinc has been shown to be important in cellular immunity (Prasad *et al.*, 1988), which is not surprising since FTS is biologically activated upon binding one molecule of zinc (Zn-FTS) (Bach, 1983). As zinc turnover is usually reduced with age (Panerai and Sacerdote, 1997), it has been postulated that the low FTS levels in old age can be related to a zinc deficiency (Mocchegiani and Fabris, 1995). Indeed it was found zinc treatment in elderly patients restores thymic secretory activity (Morcchegiani *et al.*, 1990). However, *in vitro* studies on addition of zinc ions to plasma from adolescent patients did not restore the biological activity of FTS, indicating that the decreased FTS levels in adolescence is more likely related to the decline of thymic activity than zinc deficiency (Consolini *et al.*, 2000).

[0119] In an embodiment of the present disclosure, the concentration of FTS in a patient's blood or serum is monitored before and after administration of the agent(s) that block sex steroid mediated signaling to the thymus. Rise in the concentration of FTS within 2-3 days, preferably within 24 hours, more preferably within 2-3 hours, of administration of the agent(s) signifies that the thymus is responding to blockage of the sex steroid activity.

Concentration of FTS is periodically monitored to determine the level of activation of the thymus over time.

3. Thymosin And Thymopoietin

[0120] In contrast to FTS which begins to decline after 20 years of age in humans, thymosin-alpha 1 and thymopietin serum levels seem to decline as early as 10 years of age (reviewed in Bodey *et al.*, 1997). Castration appears to increase thymosin-alpha 1 and thymosin-beta 4 serum levels as found in male rats (Windmill and Lee, 1999).

[0121] In an embodiment of the invention, the concentration of thymopoietin, thymosin-alpha 1, thymosin-beta 4, or combinations thereof are measured before and after administration of the agent(s) that block sex steroid mediated signaling to the thymus. Rise in the concentration of any of these compounds or combinations within 2-3 days, preferably within 24 hours, more preferably within 2-3 hours of administration of the agent(s) signifies that the thymus is responding to blockage of the sex steroid activity. Concentration of any of these compounds or combinations is periodically monitored to determine the level of activation of the thymus over time.

B. Newly Identified Markers

[0122] In addition to the known markers for thymic activation, several additional markers have been identified and used, based on the methods of the present disclosure.

[0123] Procedures for obtaining these markers can mimic those for following the already identified markers. For example, 2D gel electrophoresis can be used and the intensity of the various spots monitored over time. The spots will usually correspond to individual proteins, although occasionally there may be overlap or concurrence of spots from two or more different proteins. The identity of the molecules is revealed by solid phase amino acid sequencing. A new molecule(s) so identified as being altered in expression (increase or decrease) as a result of thymic activation will form the basis of a new diagnostic test for thymic responsiveness to loss of sex steroids.

T Cell Analysis

[0124] Monitoring of T cell production is another method that may be used to determine activation of the thymus. Techniques such as flow cytometric analysis of whole peripheral blood, detection of proliferating cells by monitoring the marker Ki67, and TREC analysis are among the methods known to those of skill in the field for such monitoring. In an

12 APR 2005

embodiment of the invention, numbers of T cells, as well as proliferating T cells, are determined before and after administration of the agent(s) that block sex steroid mediated signaling to the thymus. Rise in the number of any of these T cells or combinations within 2-3 days, preferably within 24 hours, more preferably within 2-3 hours of administration of the agent(s) signifies that the thymus is responding to blockage of the sex steroid activity. Concentration of any of these T cells or combinations is periodically monitored to determine the level of activation of the thymus over time.SMALL ANIMAL STUDIES

Materials and Methods

Animals

[0125] CBA/CAH and C57Bl6/J male mice were obtained from Central Animal Services, Monash University and were housed under conventional conditions. Ages ranged from 4-6 weeks to 26 months of age and are indicated where relevant.

Castration

[0126] Animals were anesthetized by intraperitoneal injection of 0.3 ml of 0.3 mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5 mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline. Surgical castration was performed by a scrotal incision, revealing the testes, which were tied with suture and then removed along with surrounding fatty tissue.

Bromodeoxyuridine (BrdU) incorporation

[0127] Mice received two intraperitoneal injections of BrdU (Sigma Chemical Co., St. Louis, MO) (100 mg/kg body weight in 100µl of PBS) at a 4 hour interval. Control mice received vehicle alone injections. One hour after the second injection, thymuses were dissected and either a cell suspension made for FACS analysis, or immediately embedded in Tissue Tek (O.C.T. compound, Miles INC, Indiana), snap frozen in liquid nitrogen, and stored at –70°C until use.

Flow Cytometric analysis

[0128] Mice were killed by CO_2 asphyxiation and thymus, spleen and mesenteric lymph nodes were removed. Organs were pushed gently through a 200µm sieve in cold PBS/1% FCS/0.02% Azide, centrifuged (650g, 5 min, 4°C), and resuspended in either PBS/FCS/Az. Spleen cells were incubated in red cell lysis buffer (8.9g/liter ammonium chloride) for 10 min at 4°C, washed and resuspended in PBS/FCS/Az. Cell concentration and

12 APR 2005

viability were determined in duplicate using a hemocytometer and ethidium bromide/acridine orange and viewed under a fluorescence microscope (Axioskop; Carl Zeiss, Oberkochen, Germany).

[0129] For 3-color immunofluorescence thymocytes were routinely labeled with anti-αβTCR-FITC or anti-γδ TCR-FITC, anti-CD4-PE and anti-CD8-APC (all obtained from Pharmingen, San Diego, CA) followed by flow cytometry analysis. Spleen and lymph node suspensions were labeled with either αβTCR-FITC/CD4-PE/CD8-APC or B220-B (Sigma) with CD4-PE and CD8-APC. B220-B was revealed with streptavidin-Tri-color conjugate purchased from Caltag Laboratories, Inc., Burlingame, CA.

[0130] For BrdU detection, cells were surface labeled with CD4-PE and CD8-APC, followed by fixation and permeabilization as previously described (Carayon and Bord, 1989). Briefly, stained cells were fixed O/N at 4°C in 1% PFA/0.01% Tween-20. Washed cells were incubated in 500µl DNase (100 Kunitz units, Boehringer Mannheim, W. Germany) for 30 mins at 37°C in order to denature the DNA. Finally, cells were incubated with anti-BrdU-FITC (Becton-Dickinson).

[0131] For 4-color Immunofluorescence thymocytes were labeled for CD3, CD4, CD8, B220 and Mac-1, collectively detected by anti-rat Ig-Cy5 (Amersham, U.K.), and the negative cells (TN) gated for analysis. They were further stained for CD25-PE (Pharmingen) and CD44-B (Pharmingen) followed by Streptavidin-Tri-colour (Caltag, CA) as previously described (Godfrey and Zlotnik, 1993). BrdU detection was then performed as described above.

[0132] Samples were analyzed on a FacsCalibur (Becton-Dickinson). Viable lymphocytes were gated according to 0° and 90° light scatter profiles and data was analyzed using Cell quest software (Becton-Dickinson).

Immunohistology

[0133] Frozen thymus sections (4µm) were cut using a cryostat (Leica) and immediately fixed in 100% acetone.

[0134] For two-color immunofluorescence, sections were double-labeled with a panel of monoclonal antibodies: MTS6, 10, 12, 15, 16, 20, 24, 32, 33, 35 and 44 (Godfrey *et al.*, 1990; Table 1) produced in this laboratory and the co-expression of epithelial cell determinants was assessed with a polyvalent rabbit anti-cytokeratin Ab (Dako, Carpinteria,

12 APR 2005

CA). Bound mAb was revealed with FITC-conjugated sheep anti-rat Ig (Silenus Laboratories) and anti-cytokeratin was revealed with TRITC-conjugated goat anti-rabbit Ig (Silenus Laboratories).

[0135] For BrdU detection, sections were stained with either anti-cytokeratin followed by anti-rabbit-TRITC or a specific mAb, which was then revealed with anti-rat Ig-Cγ3 (Amersham). BrdU detection was then performed as previously described (Penit et al., 1996). Briefly, sections were fixed in 70% Ethanol for 30 mins. Semi-dried sections were incubated in 4M HCl, neutralized by washing in Borate Buffer (Sigma), followed by two washes in PBS. BrdU was detected using anti-BrdU-FITC (Becton-Dickinson).

[0136] For three-color immunofluorescence, sections were labeled for a specific MTS mAb together with anti-cytokeratin. BrdU detection was then performed as described above.

[0137] Sections were analyzed using a Leica fluorescent and Nikon confocal microscopes.

Migration studies

[0138] Animals were anesthetized by intraperitoneal injection of 0.3ml of 0.3mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline.

[0139] Details of the FITC labeling of thymocytes technique are similar to those described elsewhere (Scollay *et al.*, 1980; Berzins *et al.*, 1998). Briefly, thymic lobes were exposed and each lobe was injected with approximately 10μm of 350 μg/ml FITC (in PBS). The wound was closed with a surgical staple, and the mouse was warmed until fully recovered from anaesthesia. Mice were killed by CO_2 asphyxiation approximately 24h after injection and lymphoid organs were removed for analysis.

[0140] After cell counts, samples were stained with anti-CD4-PE and anti-CD8-APC, then analyzed by flow cytometry. Migrant cells were identified as live-gated $FITC^+$ cells expressing either CD4 or CD8 (to omit autofluorescing cells and doublets). The percentages of $FITC^+$ CD4 and CD8 cells were added to provide the total migrant percentage for lymph nodes and spleen, respectively. Calculation of daily export rates was performed as described by Berzins *et al.* (1998).

[0141] Data analyzed using the unpaired student 't' test or nonparametrical Mann-Whitney test was used to determine the statistical significance between control and test results

12 APR 2005

for experiments performed at least in triplicate. Experimental values significantly differing from control values are indicated as follows: *p≤ 0.05, **p≤ 0.01 and ***p≤ 0.001.

Results

The effect of age on thymocyte populations.

(i) Thymic weight and thymocyte number

[0142] With increasing age there is a highly significant (p≤0.0001) decrease in both thymic weight (Figure 1A) and total thymocyte number (Figure 1B). Relative thymic weight (mg thymus/g body) in the young adult has a mean value of 3.34 which decreases to 0.66 at 18-24 months of age (adipose deposition limits accurate calculation). The decrease in thymic weight can be attributed to a decrease in total thymocyte numbers: the 1-2 month thymus contains ~6.7 x 10^7 thymocytes, decreasing to ~4.5 x 10^6 cells by 24 months. By removing the effects of sex steroids on the thymus by castration, regeneration occurs and by 4 weeks post-castration, the thymus is equivalent to that of the young adult in both weight and cellularity (Figure 1A and 1B). Interestingly, there is a significant (p≤ 0.001) increase in thymocyte numbers at 2 weeks post-castration (~1.2 x 10^8), which is restored to normal young levels by 4 weeks post-castration (Figure 1B).

[0143] The decrease in T cell numbers produced by the thymus is not reflected in the periphery, with spleen cell numbers remaining constant with age (Figure 2A). Homeostatic mechanisms in the periphery were evident since the B cell to T cell ratio in spleen and lymph nodes was not affected with age and the subsequent decrease in T cell numbers reaching the periphery (Figure 2B). However, the ratio of $CD4^+$ to $CD8^+$ T cell significantly decreased (p≤ 0.001) with age from 2:1 at 2 months of age, to a ratio of 1:1 at 2 years of age (Figure 2C). Following castration and the subsequent rise in T cell numbers reaching the periphery, no change in peripheral T cell numbers was observed: splenic T cell numbers and the ratio of B:T cells in both spleen and lymph nodes was not altered following castration (Figure 2A and B). The decreased CD4:CD8 ratio in the periphery with age was still evident at 2 weeks post-castration but was completely reversed by 4 weeks post-castration (Figure 2C).

(ii) αβTCR, γδTCR, CD4 and CD8 expression

[0144] To determine if the decrease in thymocyte numbers seen with age was the result of the depletion of specific cell populations, thymocytes were labeled with defining markers in order to analyze the separate subpopulations. In addition, this allowed analysis of

the kinetics of thymus repopulation post-castration. The proportion of the main thymocyte subpopulations was compared with those of the normal young thymus (Figure 3) and found to remain uniform with age. In addition, further subdivision of thymocytes by the expression of αβTCR and γδTCR revealed no change in the proportions of these populations with age (data not shown). At 2 and 4 weeks post-castration, thymocyte subpopulations remained in the same proportions and, since thymocyte numbers increase by up to 100-fold post-castration, this indicates a synchronous expansion of all thymocyte subsets rather than a developmental progression of expansion.

[0145] The decrease in cell numbers seen in the thymus of aged animals thus appears to be the result of a balanced reduction in all cell phenotypes, with no significant changes in T cell populations being detected. Thymus regeneration occurs in a synchronous fashion, replenishing all T cell subpopulations simultaneously rather than sequentially.

Proliferation of thymocytes

[0146] As shown in Figure 4, 15-20% of thymocytes are proliferating at 4-6 weeks of age. The majority (~80%) of these are DP with the TN subset making up the second largest population at ~6% (Figure 5A). Accordingly, most division is seen in the subcapsule and cortex by immunohistology (data not shown). Some division is seen in the medullary regions with FACS analysis revealing a proportion of SP cells (9% of CD4 T cells and 25% of CD8 T cells) dividing (Figure 5B).

[0147] Although cell numbers are significantly decreased in the aged thymus, proliferation of thymocytes remains constant, decreasing to 12-15% at 2 years (Figure 4), with the phenotype of the proliferating population resembling the 2 month thymus (Figure 5A). Immunohistology revealed the division at 1 year of age to reflect that seen in the young adult; however, at 2 years, proliferation is mainly seen in the outer cortex and surrounding the vasculature (data not shown). At 2 weeks post-castration, although thymocyte numbers significantly increase, there is no change in the proportion of thymocytes that are proliferating, again indicating a synchronous expansion of cells (Figure 4). Immunohistology revealed the localization of thymocyte proliferation and the extent of dividing cells to resemble the situation in the 2-month-old thymus by 2 weeks post-castration (data not shown). When analyzing the proportion of each subpopulation which represent the proliferating population, there was a significant ($p<0.001$) increase in the percentage of CD8

T cells which are within the proliferating population (1% at 2 months and 2 years of age, increasing to ~6% at 2 weeks post-castration) (Figure 5A).

[0148] Figure 5B illustrates the extent of proliferation within each subset in young, old and castrated mice. There is a significant ($p \leq 0.001$) decay in proliferation within the DN subset (35% at 2 months to 4% by 2 years). Proliferation of CD8+ T cells was also significantly ($p \leq 0.001$) decreased, reflecting the findings by immunohistology (data not shown) where no division is evident in the medulla of the aged thymus. The decrease in DN proliferation is not returned to normal young levels by 4 weeks post-castration. However, proliferation within the CD8+ T cell subset is significantly ($p \leq 0.001$) increased at 2 weeks post-castration and is returning to normal young levels at 4 weeks post-castration.

[0149] The decrease in proliferation within the DN subset was analyzed further using the markers CD44 and CD25. The DN subpopulation, in addition to the thymocyte precursors, contains αβTCR+CD4-CD8- thymocytes, which are thought to have downregulated both co-receptors at the transition to SP cells (Godfrey & Zlotnik, 1993). By gating on these mature cells, it was possible to analyze the true TN compartment ($CD3^-CD4^-CD8^-$) and these showed no difference in their proliferation rates with age or following castration (Figure 5C). However, analysis of the subpopulations expressing CD44 and CD25, showed a significant ($p<0.001$) decrease in proliferation of the TN1 subset ($CD44^+CD25^-$), from 20% in the normal young to around 6% at 18 months of age (Figure 5D) which was restored by 4 weeks post-castration. The decrease in the proliferation of the TN1 subset, was compensated for by a significant ($p \leq 0.001$) increase in proliferation of the TN2 subpopulation ($CD44^+CD25^+$) which returned to normal young levels by 2 weeks post-castration (Figure 5D).

The effect of age on the thymic microenvironment.

[0150] The changes in the thymic microenvironment with age were examined by immunofluorescence using an extensive panel of MAbs from the MTS series, double-labeled with a polyclonal anti-cytokeratin Ab.

[0151] The antigens recognized by these MAbs can be subdivided into three groups: thymic epithelial subsets, vascular-associated antigens and those present on both stromal cells and thymocytes.

(i) Epithelial cell antigens.

[0152] Anti-keratin staining (pan-epithelium) of 2 year old mouse thymus, revealed a loss of general thymus architecture with a severe epithelial cell disorganization and absence of a distinct cortico-medullary junction. Further analysis using the MAbs, MTS 10 (medulla) and MTS44 (cortex), showed a distinct reduction in cortex size with age, with a less substantial decrease in medullary epithelium (data not shown). Epithelial cell free regions, or keratin negative areas (KNA's, van Ewijk *et al.*, 1980; Godfrey *et al.*, 1990; Bruijntjes *et al.*, 1993).) were more apparent and increased in size in the aged thymus, as evident with anti-cytokeratin labeling. There is also the appearance of thymic epithelial "cyst-like" structures in the aged thymus particularly noticeable in medullary regions (data not shown). Adipose deposition, severe decrease in thymic size and the decline in integrity of the cortico-medullary junction are shown conclusively with the anti-cytokeratin staining (data not shown). The thymus is beginning to regenerate by 2 weeks post-castration. This is evident in the size of the thymic lobes, the increase in cortical epithelium as revealed by MTS 44, and the localization of medullary epithelium. The medullary epithelium is detected by MTS 10 and at 2 weeks, there are still subpockets of epithelium stained by MTS 10 scattered throughout the cortex. By 4 weeks post-castration, there is a distinct medulla and cortex and discernible cortico-medullary junction (data not shown).

[0153] The markers MTS 20 and 24 are presumed to detect primordial epithelial cells (Godfrey, *et al.*, 1990) and further illustrate the degeneration of the aged thymus. These are present in abundance at E14, detect isolated medullary epithelial cell clusters at 4-6 weeks but are again increased in intensity in the aged thymus (data not shown). Following castration, all these antigens are expressed at a level equivalent to that of the young adult thymus (data not shown) with MTS 20 and MTS 24 reverting to discrete subpockets of epithelium located at the cortico-medullary junction.

(ii) Vascular-associated antigens.

[0154] The blood-thymus barrier is thought to be responsible for the immigration of T cell precursors to the thymus and the emigration of mature T cells from the thymus to the periphery.

[0155] The MAb MTS 15 is specific for the endothelium of thymic blood vessels, demonstrating a granular, diffuse staining pattern (Godfrey, *et al*, 1990). In the aged thymus,

MTS 15 expression is greatly increased, and reflects the increased frequency and size of blood vessels and perivascular spaces (data not shown).

[0156] The thymic extracellular matrix, containing important structural and cellular adhesion molecules such as collagen, laminin and fibrinogen, is detected by the mAb MTS 16. Scattered throughout the normal young thymus, the nature of MTS 16 expression becomes more widespread and interconnected in the aged thymus. Expression of MTS 16 is increased further at 2 weeks post-castration while 4 weeks post-castration, this expression is representative of the situation in the 2 month thymus (data not shown).

(iii) Shared antigens

[0157] MHC II expression in the normal young thymus, detected by the MAb MTS 6, is strongly positive (granular) on the cortical epithelium (Godfrey *et al.*, 1990) with weaker staining of the medullary epithelium. The aged thymus shows a decrease in MHC II expression with expression substantially increased at 2 weeks post-castration. By 4 weeks post-castration, expression is again reduced and appears similar to the 2 month old thymus (data not shown).

Thymocyte emigration

[0158] Approximately 1% of T cells migrate from the thymus daily in the young mouse (Scollay *et al.*, 1980). We found migration was occurring at a proportional rate equivalent to the normal young mouse at 14 months and even 2 years of age (Figure 5) although significantly ($p \leq 0.0001$) reduced in number. There was an increase in the CD4:CD8 ratio of the recent thymic emigrants from ~3:1 at 2 months to ~7:1 at 26 months. By 1 week post-castration, cell number migrating to the periphery has substantially increased with the overall rate of migration remaining constant at 1-1.5%.

EXAMPLES

EXAMPLE 1: T Cell Depletion

[0159] In order to remove the abnormal T cells, the patient underwent T cell depletion. One standard procedure for this step is as follows: The human patient received anti-T cell antibodies in the form of a daily injection of 15mg/kg of Atgam (xeno anti-T cell globulin, Pharmacia Upjohn) for a period of 10 days in combination with an inhibitor of T cell activation, cyclosporin A, 3mg/kg, as a continuous infusion for 3-4 weeks followed by daily tablets at 9mg/kg as needed. This treatment did not affect early T cell development in the

patient's thymus, as the amount of antibody necessary to have such an affect cannot be delivered due to the size and configuration of the human thymus. The treatment was maintained for approximately 4-6 weeks to allow the loss of sex steroids followed by the reconstitution of the thymus. The prevention of T cell reactivity may also be combined with inhibitors of second level signals such as interleukins or cell adhesion molecules to enhance the T cell ablation.

[0160] Because in many cases it is not possible to reduce only the antigen-specific T cells which cause the disease, the whole population of T cells, including the pathological ones, is depleted. This depletion of peripheral T cells markedly retards the disease. Simultaneously, however, because of the lack of T cells, it induces a state of generalized immunodeficiency which means the patients are highly susceptible to infection, particularly viral. Even B cell responses will not function normally in the absence of appropriate T cell help.

EXAMPLE 2: Sex Steroid Ablation Therapy

[0161] The patient was given sex steroid ablation therapy in the form of delivery of an LHRH agonist. This was given in the form of either Leucrin (depot injection; 22.5mg) or Zoladex (implant; 10.8 mg), either one as a single dose effective for 3 months. This was effective in reducing sex steroid levels sufficiently to reactivate the thymus. In some cases it is also necessary to deliver a suppresser of adrenal gland production of sex steroids, such as Cosudex (5mg/day) as one tablet per day for the duration of the sex steroid ablation therapy. Adrenal gland production of sex steroids makes up around 10-15% of a human's steroids.

[0162] Reduction of sex steroids in the blood to minimal values took about 1-3 weeks; concordant with this was the reactivation of the thymus. In some cases it is necessary to extend the treatment to a second 3 month injection/implant.

EXAMPLE 3: Alternative Delivery Method

[0163] In place of the 3 month (or 3 times one month) depot or implant administration of the LHRH agonist, alternative methods can be used. In one example the patient's skin may be irradiated by a laser such as an Er:YAG laser, to ablate or alter the skin so as to reduce the impeding effect of the stratum corneum.

[0164] A. Laser Ablation or Alteration: An infrared laser radiation pulse was formed using a solid state, pulsed, Er:YAG laser consisting of two flat resonator mirrors, an

Er:YAG crystal as an active medium, a power supply, and a means of focusing the laser beam. The wavelength of the laser beam was 2.94 microns. Single pulses were used.

[0165] The operating parameters were as follows: The energy per pulse was 40, 80 or 120 mJ, with the size of the beam at the focal point being 2 mm, creating an energy fluence of 1.27, 2.55 or 3.82 J/cm^2. The pulse temporal width was 300 μs, creating an energy fluence rate of 0.42, 0.85 or 1.27 x 10^4 W/cm^2.

[0166] Subsequently, an amount of LHRH agonist is applied to the skin and spread over the irradiation site. The LHRH agonist may be in the form of an ointment so that it remains on the site of irradiation. Optionally, an occlusive patch is placed over the agonist in order to keep it in place over the irradiation site.

[0167] Optionally a beam splitter is employed to split the laser beam and create multiple sites of ablation or alteration. This provides a faster flow of LHRH agonist through the skin into the blood stream. The number of sites can be predetermined to allow for maintenance of the agonist within the patient's system for the requisite period of time.

[0168] B. Pressure Wave: A dose of LHRH agonist is placed on the skin in a suitable container, such as a plastic flexible washer (about 1 inch in diameter and about 1/16 inch thick), at the site where the pressure wave is to be created. The site is then covered with target material such as a black polystyrene sheet about 1 mm thick. A Q-switched solid state ruby laser (20 ns pulse duration, capable of generating up to 2 joules per pulse) is used to generate the laser beam, which hits the target material and generates a single impulse transient. The black polystyrene target completely absorbs the laser radiation so that the skin is exposed only to the impulse transient, and not to laser radiation. No pain is produced from this procedure. The procedure can be repeated daily, or as often as required, to maintain the circulating blood levels of the agonist.

EXAMPLE 4: Sample Collection

[0169] Selected patients were bled immediately prior to receiving the LHRH analogue to inhibit sex steroid production, and at short time intervals (typically during the first 24-72 hours) after the application of the LHRH analogue. Blood was centrifuged (750gav) to sediment cells and the plasma collected. The plasma samples were compared by subjecting them to analysis of concentration of particular thymic marker molecules.

EXAMPLE 5: Flow Cytometry Analysis Of Whole Peripheral Blood

[0170] 20µl of the appropriate antibody cocktail was added to 200µl whole blood and incubated in the dark, RT for 30min. For removal of RBC, 2ml of FACS lysis buffer (Becton-Dickinson, USA) was then added to each tube, vortexed and incubated 10min, RT in the dark. Samples were centrifuged at 600_{gmax}, supernatant removed and cells washed twice in FACS buffer. Finally, cells were resuspended in 1%PFA for FACS analysis.

EXAMPLE 6: Ki67 Analysis

[0171] For detection of proliferating cells, lysed samples were incubated for 20min, RT, in the dark in 500µl of 1x FACS permeabilising solution (Becton-Dickinson, USA). Washed samples were incubated with either anti-Ki67-PE or anti-Ki67-FITC (or the appropriate isotype controls) for 30min at RT, in the dark. Samples were then washed and resuspended in 1%PFA for analysis.

[0172]

Antibody Cocktails:

1. CD27/CD45RA/CD45RO/CD4 or CD8
2. CD62L/CD45RA/CD45RO/CD4 or CD8
3. γδTCR/αβTCR/CD28/CD4 or CD8
4. CD69/CD25/CD152/CD3
5. CD11b/CD11c/CD56/CD3
6. CD19/CD117/CD34/CD3
7. CD3/CD4/CD8/HLA-DR
8. For Ki67:
    a) CD4 or CD8/CD45RO/CD27 followed by Ki67-PE or IgG1-PE
    b) αβTCR/CD8a/CD8b followed by Ki67-FITC or IgG1-PE

EXAMPLE 7: Detection Of Intracellular Cytokines

[0173] 200µl of whole blood was stimulated with soluble purified anti-CD3 (5µg/ml) and anti-CD28 (10µg/ml) for 6 hours at 37°C, 5%CO_2. Brefeldin A (final concentration10µg/ml) was added during the final 4 hours to limit cytokine secretion from the activated cells. Following stimulation, samples were incubated for 15min, RT with 20µl of 20mM EDTA in PBS. Samples were then surface stained with anti-CD4-FITC and anti-CD8-

CyChrome. Following lysis and permeabilisation, cells were stained with anti-IL-4-PE and anti-IFNγ-APC or the appropriate isotype controls. Unstimulated cells were used as a control for activation.

EXAMPLE 8: Preparation Of PBMC

[0174] Purified lymphocytes were used for T-cell stimulation assays and TREC analysis. 10-50ml of peripheral blood was diluted 1:1 with RPMI-Heparin. Diluted blood was carefully layered over ficoll-hypaque at a ratio of 2:1 blood:ficoll. Tubes were centrifuged at (800_{gmax}) for 25 min at RT. Following centrifugation, the plasma layer was removed and stored at -20°C for analysis of sex steroid levels. The buffy coat layer was removed and diluted with RPMI-Heparin. Tubes were centrifuged at 25°C for 15min at (600_{gmax}), followed by a second wash at 400_{gmax} for 10min. Supernatant was removed and cell counts performed in duplicate using a haemocytometer. Cells not used for stimulation assays were resuspended in freezing media and stored at –70°C overnight, before transferring to Liquid Nitrogen prior to TREC analysis. Plasma collected following ficoll purification was stored at –20°C prior to analysis of sex steroid levels.

EXAMPLE 9: T Lymphocyte Stimulation Assay

[0175] For mitogen stimulation, PBMC were plated out in 96-well round-bottom plates at a concentration of 1 x 10^5/well in 100µl of RPMI-FCS. Cells were incubated at 37°C, 5% CO_2 with PHA in doses from 1-10µg/ml. For TCR-specific stimulation, cells were incubated for 48 hours on plates previously coated with purified anti-CD3 (1-10µg/ml) and anti-CD28 (10µg/ml). Following plaque formation (48-72 hours), 1µCi of ^{3}H-Thymidine was added to each well and plates incubated for a further 16-24 hours. Plates were harvested onto filter mats and incorporation of ^{3}H-Thymidine was determined using liquid scintillation on a β–counter (Packard-coulter, USA).

EXAMPLE 10: TREC Analysis

[0176] Detection of TRECs is perfomed by purifying new helper T cells (Th; e.g., CD4+, CD45RA+ CD27+) and cytotoxic T cells (Tc; e.g., CD8+, CD45RA+ CD27+) by flow cytometry and then TREC analysis using specific DNA probes and RT-PCR.

A. Cell Sorting

[0177] Frozen samples were rapidly thawed, washed in FACS buffer containing1mM EDTA and 1% Human Serum and centrifuged (600_{gmax}, 5min., 4°C). Cells were incubated

with anti-CD4-FITC, anti-CD3-APC and anti-CD45RA-PE for 30min., RT, washed and fixed by the drop-wise addition of 1ml of 3% Formalin in PBS. Samples were incubated for a further 30min., washed and resuspended in 500µl FACS buffer for sorting. Four populations were obtained: $CD3^+CD4^+CD45RA^+$; $CD3^+CD4^+CD45RA^-$; $CD3^+CD4^-CD45RA^+$ and $CD3^+CD4^-CD45RA^-$.

B. DNA Isolation

[0178] Cells were sorted directly into PCR grade 0.5ml eppendorfs, centrifuged (8min, 2500_{gmax}) and resuspended in Proteinase K (PK) digestion buffer ($2x\ 10^5$ cells/ 20µl of a 0.8mg/mL solution). Proteinase K (PK) was added to the PCR digestion buffer just prior to use. Samples were incubated for 1 hour at 56°C followed by 10min at 95°C to inactivate the proteinase. Lysed samples were stored at –70°C prior to RT-PCR.

C. Real Time-PCR using Molecular Beacons

[0179] This technique is described in Zhang et al., 1999. Primers for signal-joint TRECs were 5'-AAAGAGGGCAGCCCTCTCCAAGGCAAA-3' (SEQ ID NO:1) and 5'-AGGCTGATCTTGTCTGACATTTGCTCCG-3' (SEQ ID NO:2). Primers for coding-joint TRECs were 5'-CCTGTTTGTTAGGGCACATTAGAATCTCTCACTG-3' (SEQ ID NO:3) and 5'-CTAATAATAAGATCCTCAAGGGTCGAGACTGTC-3' (SEQ ID NO:4). DNA was extracted from the cells using Proteinase K digestion. PCR conditions were: 95°C for 5 min, followed by 90°C, 60°C and 72°C, each for 30s, for 30 or 35 cycles as indicated. Each PCR reaction contained 1U platinum *Taq* polymerase, 1.8mM $MgCl_2$, 0.2mM dNTPs, 12.5µM each primer and 1.7 nmol (5µCi) ^{32}P-labelled dCTP in 50µl platinum *Taq* buffer.

EXAMPLE 11: Radioimmunoassay

[0180] Detection of sex steroid levels in patient sera (frozen following Ficoll-Paque centrifugation) was performed using a ^{125}I-Testosterone radioimmunoassay (RIA). Prior to the assay, all reagents, samples and controls were brought to room temperature. Control tubes had either buffer alone - non-specific binding (NSB) tube or 0ng/ml testosterone standard (B_0). Buffer alone, standards (0-10ng/ml testosterone) or test samples were added to each tube, followed by sex binding globulin inhibitor (SBGI) to limit non-specific binding of the radio-labelled testosterone. The ^{125}I-testosterone was added to each tube followed by an anti-testosterone antibody (except for the NSB tubes). Tubes were then incubated at 37°C for 2 hours. Following this, a secondary antibody was added to all tubes which were incubated for

a further 60 mins following vortexing. Tubes were centrifuged (1000_{gmax}) for 15 mins, supernatant removed and the precipitate counted on a Packard Cobra auto-γ counter. Triplicate cpm results were averaged and a standard curve constructed using the formula for percent bound Testosterone (B/B_0):

$$\%B/B_0 = \frac{\text{Sample - NSB}}{B_0 - \text{NSB}}$$

Sample = average cpm of particular test sample

NSB = average cpm of non-specific binding tube

B_0 = average cpm of 0ng/ml standard (total binding tube)

The level of testosterone in each test sample was determined from the standard curve. The plasma was subjected to protein analysis based on 2D gel electrophoresis followed by computer based bioinformatics to determine the presence of indicators of thymic function.

12 APR 2005

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12 APR 2005

CLAIMS:

1. A method of identifying one or more thymic factors, or one or more indicators of thymic function, the method comprising detecting a difference in the concentration of a marker in a blood sample which has been taken from a patient after disruption of sex steroid mediated signalling as compared to the concentration of the marker in a sample which was taken prior to said disruption, wherein the detected difference(s) identifies a new thymic factor or a new indicator of thymic function.

2. The method of claim 1 wherein the blood samples are treated to separate out plasma and an analysis is performed on the plasma.

3. The method of claim 1 or claim 2 wherein the analysis of the samples includes two dimensional gel electrophoresis.

4. The method of claim 3 wherein identification of the new thymic factor or new indicator is determined using solid phase amino acid sequencing.

5. A method of identifying a change in thymic activation status, the method comprising detecting a difference in the concentration of a marker in a blood sample which has been taken from a patient after disruption of sex steroid mediated signalling as compared to the concentration of the marker in a sample which was taken prior to said disruption, wherein the detected difference identifies a change in thymic activation status.

6. The method according to any one of claims 1 to 5 wherein the marker is a protein.

7. A diagnostic method to determine a patient's susceptibility to thymic activation, the method comprising measuring the amount of an indicator present in a blood sample obtained from the patient after the patient had received a medicament to disrupt sex steroid mediated signalling, wherein the indicator is selected from the group consisting

12 APR 2005

of: interleukin 7, interleukin 4, Interferon gamma, FTS, thymosin, thymulin, thymopoietin, a thymopoietic cytokine, a neuropeptide, and the level of indicator is diagnostic of the ability of patient's thymus to undergo activation, and wherein the thymosin is not α1 thymosin or β4 thymosin.

8. The method of claim 7 further comprising measuring the amount of the indicator present in an additional blood sample which was obtained from the patient at a different time, wherein the level of indicator in the two samples is compared and a difference is diagnostic of the ability of the patient's thymus to undergo activation.

9. A diagnostic method to identify a poor responder to thymic activation comprising measuring the amount of an indicator present in a blood sample obtained from a patient after the patient had received a medicament to disrupt sex steroid mediated signalling, wherein the indicator is selected from the group consisting of: interleukin 7, interleukin 4, Interferon gamma, a cytokine, FTS, thymulin, thymopoietin, a thymopoietic cytokine, a neuropeptide, a thymosin, a thymic hormone and a thymopoietic hormone, and the level of indicator is diagnostic of the ability of patient's thymus to undergo activation.

10. The method of claim 9 further comprising measuring the amount of the indicator present in an additional blood sample which was obtained from the patient at a different time, wherein the level of indicator in the two samples is compared and a equivalence is diagnostic of the inability of the patient's thymus to undergo activation.

11. A method according to any one of claims 1 and 4 to 10 wherein the blood sample has been treated to produce serum or plasma.

12. The method according to any one of claims 1 to 11 wherein the blood sample was taken within about one week of disruption of sex steroid signalling.

12 APR 2005

13. The method according to any one of claims 1 to 11 wherein the blood sample was taken within about 4 to 5 days of disruption of sex steroid signalling.

14. The method according to any one of claims 1 to 11 wherein the blood sample was taken within about 2 to 3 days of disruption of sex steroid signalling.

15. The method according to any one of claims 1 to 11 wherein the blood sample was taken within about 24 hours of disruption of sex steroid signalling.

16. A diagnostic method to determine a patient's thymic activation state comprising determining the level of TREC DNA in a sample comprising T cells which have been obtained from the patient following administration of a medicament to disrupt sex steroid mediated signalling.

17. The method of claim 16 wherein PCR is used to determine the level of TREC DNA in the T cells.

18. The method of claim 17 wherein the PCR is performed using at least one primer comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4.

19. The method according to any one of claims 16 to 18 the method further comprising determining the level of TREC DNA in a sample comprising additional T cells which have been obtained from the patient at a different time, wherein the level of TRECs in the two T cell samples is compared.

20. The method according to any one of claims 1 to 19 wherein the patient is post-pubertal.

21. The method according to any one of claims 1 to 20 wherein disruption of sex steroid mediated signalling includes blocking of one or more sex steroid receptors.

22. The method according to any one of claims 1 to 21 wherein disruption of sex steroid mediated signalling includes the inhibition of sex steroid production in the patient.

23. The method according to any one of claims 1 to 22 wherein disruption of sex steroid mediated signalling lowers the concentration of a sex steroid in the patient.

24. The method according to any one of claims 1 to 23 wherein disruption of sex steroid mediated signalling results in chemical castration of the patient.

25. The method according to any one of claims 7 to 24 wherein the medicament comprises a compound selected from the group consisting of: sex steroid antagonists, sex steroid agonists, sex steroid analogs, anti-sex steroid vaccines, steroid receptor modulators, LHRH analogs, LHRH-R agonists, LHRH-R antagonists, anti-LHRH vaccines, anti-estrogen antibodies, anti-androgen antibodies, and combinations thereof.

26. The method according to claim 23 wherein the medicament comprises a compound selected from the group consisting of: Buserelin, Cysterelin, Decapeptyl, Deslorelin, Gonadorelin, Goserelin, Histrelin, Leuprolide, Leuprorelin, Lutrelin, Meterelin, Nafarelin, Triptorelin, Abarelix, Cetrorelix, Eulexin, Leucrin, Zoladex, and combinations thereof.

27. The method according to any one of claims 7 to 24 wherein the medicament comprises an LHRH-R agonist and an LHRH-R antagonist.

28. The method according to any one of claims 7 to 24 wherein the medicament comprises a slow release formulation.

29. The method according to any one of claims 7 to 28 wherein the medicament comprises a compound that disrupts sex steroid mediated signalling to the thymus.

30. A kit when used for determining a patient's susceptibility to thymic activation comprising a compound that disrupts sex steroid mediated signalling.

31. The kit of claim 30 wherein the compound is selected from the group consisting of: sex steroid antagonists, sex steroid agonists, sex steroid analogs, anti-sex steroid

12 APR 2005

vaccines, steroid receptor modulators, LHRH analogs, LHRH-R agonists, LHRH-R antagonists, anti-LHRH vaccines, anti-estrogen antibodies, anti-androgen antibodies, and combinations thereof.

32. The kit of claim 30 wherein the compound is selected from the group consisting of: Buserelin, Cysterelin, Decapeptyl, Deslorelin, Gonadorelin, Goserelin, Histrelin, Leuprolide, Leuprorelin, Lutrelin, Meterelin, Nafarelin, Triptorelin, Abarelix, Cetrorelix, Eulexin, Leucrin, Zoladex, and combinations thereof.

33. The kit according to any one of claims 30 to 32 further comprising one or more components selected from the group consisting of:

a reagent that can specifically bind to an indicator of thymic function;
a cytokine;
a growth factor;
an immunosuppressant;
an anti-T cell antibody;
a suppressor of adrenal gland function; and
an oligonucleotide primer to detect TRECs; and
combinations thereof.

34. The kit of claim 33 wherein the indicator is a thymic factor identified by the method of any one of claims 1 to 4.

35. The kit of claim 33 wherein the indicator is selected from the group consisting of: interleukin 7, interleukin 4, Interferon gamma, FTS, thymulin, thymopoietin, a neuropeptide, a thymosin, a thymic hormone, a thymopoietic cytokine and a thymopoietic hormone, wherein the thymosin is not $\alpha 1$ thymosin or $\beta 4$ thymosin.

36. The kit of claim 33 wherein at least one primer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4.