Immune Reconstitution in Lymphoid Tissue Following Potent Antiretroviral Therapy

Jan Andersson, MD, PhD, and Thomas E. Fehniger, PhD
Department of Infectious Diseases, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden

Abstract
During untreated HIV-1 infection, a chronic state of immune activation and inflammation develops at the lymphoid tissue sites of viral replication. The early effect of potent combination drug therapy is a reduction in peripheral viral burden and a reduction in the production of inflammatory and type 1 cytokines. Further along in treatment there are trends toward normalization in the frequencies of CD8+ T-cells, CD4+ CD45RA+ cells, as well as CD4+ CD45R0+ cells. Finally, the CD1a+ dendritic cell network is re-established and germinal centers are reformed. Although this restoration of the lymphoid dynamic form is coupled to a reconstitution of peripheral blood T-cell function in vitro and by skin testing, sterilizing immunity to HIV-1 does not develop. Furthermore there is no heightened development of cytotoxic CD8+ T-cell function at the site of HIV-1 latency. This is evidenced by a massive recrudescence of HIV-1 viral replication within lymphoid tissue when therapy is stopped. The development of supplemental therapies, which reconstitute anti-HIV-1 immunity, will be required. Specific defects in anti-HIV-1 activity which occur in lymphoid tissue during infection include a downregulation of perforin expression by cytotoxic T-cells, the down regulation of the TCR signal transducing chain CD3ζ, and inadequate CD4+ T-cell help within the tissue compartment of immune regeneration.

Introduction
The treatment of HIV-1 infection with combinations of nucleoside analogs, protease inhibitors, and reverse transcriptase inhibitors has substantially reduced the incidence of clinical opportunistic infections and neoplastic disorders in patients

The Human Thymus: A New Perspective on Thymic Function, Aging, and HIV Infection

Carolyn M. Steffens,1 Giulia Marchetti,2 Alan Landay,1 and Lena Al-Harthi1
Department of Immunology and Microbiology, Rush Presbyterian St. Luke’s Medical Center, Chicago, IL1 and the Institute for Infectious and Tropical Diseases, University of Milan, Italy2

This work was supported by a grant number 02634-26-RGI from the American Foundation for AIDS Research (amfAR).

Abstract
Shortly after birth, the human thymus begins a life long process of involution, whereby the net size of the thymus is not altered but the organ is replaced by adipose tissue. As a result, it has long been believed that thymic involution is indicative of a nonfunctional organ. Recently, however, with the use of computed tomography analysis and innovative molecular approaches that measure T-cell receptor circles, indicative of recent thymic emigrants, doubt has been placed on that dogma. The thymus appears to be active in thymopoiesis throughout the adult life, albeit inversely correlated with age. Being faced with diseases that deplete T-cells such as the acquired immunodeficiency syndrome (AIDS), this recent finding has the potential to exploit novel approaches that enhance thymic output as a mecha-
Immune Reconstitution in Lymphoid Tissue Following Potent Antiretroviral Therapy

(Continued from pg. 65)

under therapy. This improvement in clinical course is partly related to a reconstitution of functional immune surveillance, which develops in parallel to reduction in peripheral HIV-1 viral burden. Immune reconstitution progresses at several levels in treated patients and is characterized by: i) an initial expansion of CD4+ CD45RO+ memory cells in peripheral blood mononuclear cell (PBMC), ii) a later occurring increase of “naive” CD4+ CD45RA+ T-lymphocytes along with declines of initially expanded CD8+ cell numbers in PBMC and lymphoid tissue (LT), and iii) a general reduction of T-cell activation both in PBMC and LT, in parallel with improved but not normalized response to recall antigens. However, the level of restored immune resistance capable of resolving malignancies and opportunistic infections does not achieve sterilizing immunity to HIV-1 itself. Thus, residual pools of HIV-1 which persist throughout therapy continue to be sustained. Consequently, when combination therapy is stopped, massive HIV-1 replication reoccurs within LT sites.

It is now clear that longterm combination drug therapy alone will not provide an adequate clinical “cure” in most patients. In addition, this therapy is not tolerated well by all patients, and many stop treatment on their own initiative. It is further acknowledged that some type of supplemental therapy is required, which could promote and maintain true HIV-1 specific immune reconstitution. For this to occur, we must increase our basic knowledge concerning the biological nature of the deficit in responsiveness promoted by HIV-1 infection. The design of effective immune reconstitution strategies will depend upon both the identification of correctable pathways and the validation of implemented strategies within the context of eradicating LT reservoirs of HIV-1 infection. We believe that any future therapy will need to address HIV-1 specific immune reconstitution at the site of active HIV-1 replication in LT. The diminishment of functional CD4+ T-helper and CD8+ cytotoxic effector activity at LT sites is a hallmark of this disease. Significant numbers of studies are addressing the many levels of lymphoid replenishment that are required for the establishment of cellular activities that limit HIV-1 replication and dissemination. In this review, we will present a variety of models which address several issues associated with the immunobiology of HIV-1 replication within LT in an effort to foster a strengthening in the conceptual development of future supplemental therapies.

Effects of Highly Potent Antiretroviral Therapy at Lymphoid Tissue

Cellular dynamics and the establishment of infection

Combination therapy with Highly Active Anti-retroviral Therapy (HAART) reduces the frequency of HIV-1 provirus containing cells within LT, but levels of HIV-1 DNA+ cells between one to five percent of all cells continue to be observed even after prolonged therapy. A key question is, how is this viral pool established? Does HIV-1 replication selectively damage specific immune competence during the initial phase of infection to favor viral dissemination and persistence? Already after the first weeks of primary HIV-1 infection, extensive HIV-1-replication occurs in CD4+CD45RO+ memory T-cells resident in LT. HIV-1 viral copy numbers are 10 to 1000-fold higher in LT as compared to peripheral blood. During this time, CD4+ T-cells are shown to be activated and proliferating at the site of infection. In parallel, CD8+ T-cell numbers increase four- to eight-fold over normal levels, as do cells expressing the chemokine receptors CCR5 and CXCR4. The state of prolonged hyperactivation coincides with the trapping of HIV-1 by follicular dendritic cells and the destruction of germinal centers. Over time, a decline of LT resident CD4+ T-cells as well as CD8+ T-cells occurs despite extensive recruitment and several fold increases in proliferative indices of these cells. In addition, follicular dendritic cell destruction occurs along with changes in the patterns of lymphoid tissue architecture as evidenced by proliferation of high endothelial venulae, lipid degeneration and, finally, fibrosis. Hypoplasia or diminishments in the density of resident LT cells is a common feature of HIV-1 disease progression. Using image analysis, an index of cellularity can be established by measuring the area within LT occupied by nucleated cells, in relationship to the total area of the tissue, which together encompasses cells, extracellular space, connective tissue, and vessels (Figure 1). LT is comprised of resident memory cells, recently recruited populations of naive, post thymic derived precursors, and peripheral memory cells which have migrated from tissue sites in response to antigens triggering HIV-1 infection diminishes CD4+ cellular pools through several possible pathways including direct cytotoxicity by mechanisms of viral replication, the elimination of CD4+ HIV-1 infected cells by cytotoxic T-cell activities, or by apoptosis through incom-
plete signaling which activate caspase pathways, or by specifically delivered Fas ligand binding substances.\textsuperscript{21}

The quantification of metabolically labeled cells at lymphoid sites has indicated that a dynamic of T-cell depletion occurs at the site of HIV-1 replication which is related to both a reduced life span of cells resident within LT and a diminished replenishment of newly synthesized CD4\textsuperscript{+}CD45RA\textsuperscript{+} cells.\textsuperscript{2,11,17,18}

T-lymphocyte activation and proliferation has also been investigated by quantification of Ki-67 antigen expression in LT. Initial increases in both Ki-67\textsuperscript{+} CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells occur in LT following HIV-1 infection. Ki-67\textsuperscript{+} CD4\textsuperscript{+} T-cells tend to decrease in untreated asymptomatic patients but levels of Ki-67\textsuperscript{+} CD8\textsuperscript{+} T-cells are maintained.\textsuperscript{2,22} Following combination drug therapy the numbers of CD4\textsuperscript{+} T-cells which are Ki-67\textsuperscript{+} may increase within LT. A further consequence of this therapy, is a reduction toward normalization in the levels of CD8\textsuperscript{+} T-cells present in LT as well as CCR5 and CXCR4 expression.\textsuperscript{2,15,23,24}

Local cytokine production

The production of certain cytokines within LT most likely plays an important role in creating an inflammatory environment that drives HIV-1 replication.\textsuperscript{5,15,25} Extraordinarily high levels of pro-inflammatory cytokine (IL-1\textalpha, IL-1ra, IL-12, TNF-\alpha), Type 1 T-cell inflammatory cytokines (IL-2, IFN-\gamma), and inflammatory proteins (CD68, calprotectin) are simultaneously produced at LT sites of established HIV-1 infection.\textsuperscript{5,15,26} In the LT of patients with established infection, Type 1 (IL-2, IL-12, IFN\gamma) cytokine production occurs at ten times the level of Type 2 (IL-4, IL-10) cytokine production.\textsuperscript{15} Is this extensive array of polarized cytokine production caused by the activation of cells in response to viral antigens or by some mechanism intrinsic to the process of HIV-1 replication within cells? The net effect of a persistent immune activation within the lymph node during untreated HIV-1 infection also involves upregulated chemokine synthesis, Fas and Fas-L expression, the shedding of the CD8 \alpha/\beta chains, and the downregulation of the TCR signaling ligand CD3z.\textsuperscript{13,21,23}

Together, this evidence is consistent with

**Figure 1.** The tonsil of a healthy subject stained with DAPI (left) shows a cellularity index of approximately 90\%. This index of the area occupied by cells versus total tissue area, is related to the dynamics of cellular recruitment, proliferation, migration, and cell death. A tonsil from an untreated, asymptomatic, HIV infected patient, shown at high magnification (right), exhibits reduced cellularity (~70\%) in comparison to uninfected control subjects.

**Figure 2.** Tonsillar biopsies from a patient sampled before HAART (A and C) and from the same patient after four weeks of HAART (B and D). Panels A and B illustrate (in brown) interferon \gamma producing cells; panels C and D illustrate cells expressing CD68.
an argument of HIV-1 pathogenesis in which the state of immune over-activation within tissue promotes a burn out of the CD4+ directed immune activities and incomplete HIV-1 specific cytotoxic lymphocyte (CTL) responses, while ineffective specific antibody driven immune mechanisms further fail to eliminate HIV-1 infected cells and free virions. An early event within LT following combination therapy is a substantial decrease in the production of both pro-inflammatory and Type 1 inflammatory cytokines at the sites of HIV-1 replication. After only four weeks of therapy, a general diminishment in the state of activation of LT cells is observed in conjunction with the reduction of local LT HIV-1 burden (Figure 2). Thus, by as yet unidentified mechanisms, local cytokine production can be affected significantly and altered by therapies directed at limiting the levels of replicating HIV-1. Overall, distinct changes in the patterns of cellularity within secondary lymphoid sites occur during combination therapy. Our studies have shown that the total cellularity in tonsils and cervical lymph nodes initially declines four weeks after introduction of highly potent retrovirus therapy. This could be related to migration of memory CD4+ cells (CD45RO+) out of LT and into the blood. However, at six months after therapy, a total increase in cellularity was observed due to expansion of both CD4+/CD45RA+ as well as CD45RO+ cells. In addition, CD1a+ DC cells also had increased, while the CD8+ T-cells declined from elevated levels by two- to three-fold (Figure 3). The initial effects of highly potent antiretroviral therapy on cell surface expression, cytokine, and chemokine levels in the tissue are summarized in Table 1.

The table is a summary of findings in several groups and is only representative for HIV-1 infected with a clinical response to highly potent antiretroviral treatment (>12 months).

<table>
<thead>
<tr>
<th>Cytokine/Marker</th>
<th>HIV+ untreated</th>
<th>HIV+ HAART</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1, CD1a</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>CCR5, CXCR4</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>MIP-1α, MIP-1β, RANTES</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>CD32, CD28, CD4</td>
<td>↓</td>
<td>?</td>
</tr>
<tr>
<td>CD40-L</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>CD4+, CD45RA+</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>CD4+, CD45RO+</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>CD8+, granzyme A,B</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>CD8+, perforin</td>
<td>↑↓</td>
<td>→</td>
</tr>
<tr>
<td>CD8+, Fas-L</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

The table is a summary of finding in several groups and is only representative for HIV-1 infected with a clinical response to highly potent antiretroviral treatment (>12 months).

as well as CD45RO+ cells. In addition, CD1a+ DC cells also had increased, while the CD8+ T-cells declined from elevated levels by two- to three-fold (Figure 3). The initial effects of highly potent antiretroviral therapy on cell surface expression, cytokine, and chemokine levels in the tissue are summarized in Table 1.

One of the earliest events occurring after induction of combination therapy is the reduction of unregulated adhesion molecule expression on CD4+ helper T-cells. This is linked to reduced β-chemokine production and cell surface expression of the β-chemokine receptors CCR5 and CXCR4 (unpublished observations). These changes are followed by a downregulation of HLA-DR on CD38+ CD8+ T-cells as well as increases in CD44 expression. An increase in CD4+CD45RA+ cells is paralleled by an increase in CD1A on antigen presenting cells. Finally, as the follicular dendritic network is re-established, following two to three years

![Figure 3](image-url)
of therapy, a further increase in the frequency of CD1A expression is observed. Overall, the tendency for continuing reductions in inflammatory cytokine production occurs throughout the course of treatment.

**CD8⁺ T-cell Effector Activity in Lymphoid Tissue**

Cytotoxic cells are believed to play a major role in clearing HIV-infected cells from LT. However, despite extensive reports of cytotoxic T-cell activity within blood throughout the course of HIV infection, it is not yet established which principle cytotoxic mechanism is used by CD8⁺ T-cells to kill HIV-infected target cells. We know that CD8⁺ killing alone is not sufficient to prevent HIV-1 disease progression to AIDS. For optimal expression of cytotoxic function, both calcium-dependent exocytosis of perforin (and granzyme members) and FAS-mediated programmed cell death are dependent upon CD4⁺ cell help. The limited CD4⁺ help, which is delivered to effector cells within LT, is not adequate for establishing and maintaining CD8⁺ effector function. Recent findings indicate that one, and perhaps the major pathogenic mechanism which promotes HIV-1 dissemination is a lack of a synchronized upregulated granzyme and perforin expression in effector CTLs within LT.

This lack of coordinated expression of perforin and granzyme A likely contributes to an ineffective killing of viral infected cells. While granzyme A expression is significantly increased at LT sites, perforin is expressed in only one out of every ten of these granzyme-expressing cells. Since these two cytolytic effector molecules must be co-expressed in order to induce cytotoxicity of CD4⁺ HIV-1 infected cells, this finding can very well turn out to be of central importance for the life-long persistence of HIV-1 in infected individuals. In order for us to fully understand immune reconstitution of effector activity, we will need to better define the pathways of activation. Several potential targets have been identified for evaluation and validation in model effector/target systems. In addition to the induction of perforin expression in activated CD8 T-cells, the upregulation of the CD3ζ chain, the CD28 co-stimulatory molecule and CD40-L are also interesting platforms of study. All these molecules have been shown to be downregulated in untreated HIV-1 infected individuals. We have recently observed that even after one year of combination therapy, perforin expression is not restored within LT. Nor does combination therapy lead to downregulation of Fas-L, however, Fas expression is reduced in comparison to pre-treatment levels. Importantly there is no absolute positive correlation between the drop in HIV-1 load in lymphoid tissue and an improvement of anti-HIV immunocapacity by peripheral CD8⁺ effector cells. This central question regarding the nature of effector CD8⁺ cell activity at the site of HIV replication within LT will need to be resolved if cytotoxic T-cell activity is demanded in future therapies. It is most likely that attempts to redirect the local environment of cytokine production by CD4⁺ helper T-cells to promote these activities will be critical factors for re-establishing this immune function.

**Changes in the Thymic Function Induced by Highly Potent Antiretroviral Therapy**

The dynamic basis for T-cell depletion following progression of HIV-1 diseases remains controversial. It has recently been shown that in untreated HIV-1 infected subjects, the half-life for both CD4⁺ and CD8⁺ T-cells was less than one-third of that seen in healthy HIV-1 seronegative control subjects. This was compensated by an absolute increase in production of CD8⁺ but not CD4⁺ T-cell populations. However, following combination therapy which effectively suppressed HIV-1 replication, the production rates of circulating CD4⁺ and CD8⁺ T-cells were elevated considerably. The kinetic basis of increased CD4⁺ levels was due to a greater production rate and not a longer half-life. The dynamics of T-cell depletion during HIV-1 are influenced not only by the events which occur in LT at sites of HIV-1 replication but also within the sites of maturation such as the thymus. The thymus is the organ responsible for maturation and selection of T-lymphocytes. It has previously been reported that the thymus of adults infected with HIV-1 is generally inactive both because of HIV-1 related involution and viral destruction. However, abundant thymic tissue has been detected in as many as 50% of HIV-1 seropositive individuals at the age between 20 and 59 years based on computer tomography of the chest. These technologies have allowed us to gain insight into the process of immune reconstitution at thymic sites. These studies have shown that the immune reconstitution occurring in antiretroviral treated patients is now following the pathway previously observed in bone marrow transplanted individuals. The first subpopulation of expanding T-cells were memory CD8⁺ cells followed by naïve CD8⁺ T-cells while the CD4⁺ population, both memory and naïve, were considerably slower in their regeneration. It has also been shown that HIV-1 infected individuals which have been thymectomised because of myastenia gravis indeed have significant increases in CD4⁺ memory and later naïve T-cells following long-term treatment with highly potent antiretroviral therapy. Thus, these data imply that CD4⁺ T-cell maturation may be directed from extrathympic sites while CD8⁺ recovery seems more dependent upon thymic tissue interaction. In all, the kinetics by which CD4⁺ T-cell repopulation occurs in lymphoid tissues after treatment of HIV-1 infection may indeed be dependent on two levels of functional maturation, intra-thymically or extrathympically. In addition, growth factors such as IL-7, and perhaps IL-13, and co-stimulatory molecules like CD40-L may further influence maturation. Substantial and sustained increase in thymic output was generated in most patients tested under therapy, even in patients over 50 years of age. All together, this data suggests that HIV-1 replication or the products of the activation occurring within the thymus are capable of shutting down thymic function and its capacity to compensate for the increased loss of CD4⁺ cells. This finding may generate new abilities to increase the output of naïve CD4⁺ cells through growth factor supplementation along with the antiretroviral therapy.

**Immune Reconstitution at the Mucosal Site (MALT)**

Similar to what has been observed in lymph node, both duodenal and colon biopsies, indicate extensive HIV-1 repli-
cation occurring in parallel with an induction of hyper-immune activation, i.e., increased CD8⁺ T-cells, HLA-DR up-regulation, induced IFN-γ, IL-4, and β-chemokines, upregulation of chemokine receptors and increased turnover of T-cells, in particular CD8⁺ T-cells. No correlation was found between levels of peripheral blood CD4⁺ T-cells and duodenal or colon mucosal MALT CD4⁺ T-cells levels. However, much in line with lymph nodes, the CD8⁺ T-cells increased in frequency four- to eight-fold. The CD4 depletion is preceding the event noticed in blood and later on in lymph nodes. However, this is occurring at a time-point when the tissue shows persistent production of IFN-γ as well as IL-4, particularly noticed in the intraepithelial lymphoid cells. Very little attention has, until now, been directed to study effects of highly potent antiretroviral therapy on MALT tissue. Preliminary results indicate that the effect is much in line with what is found in lymphoid tissue in that the antiretroviral therapy treated patients show significant reduction in the number of CD8 cells, immune activation, local cytokine production, as well as granzyme A expression in CD8⁺ CTL (Figure 4).

Conclusions
The evaluation of the effects of combination retroviral therapy within tissue biopsies provides a means for better understanding the pathogenesis of HIV-1 infection at the site of viral replication and immune effector activity. We know little concerning the mechanisms that allow HIV-1 to remain sheltered from potentially neutralizing immune function. We know that treatment for more than two years with highly antiretroviral therapy allows measurable immune function to be re-established in both blood and tissue. We also know that these activities are not sufficient to eliminate reservoirs of latent or persistent virus. Combination therapy induces a reconstitution and repopulation of naive CD4⁺ and CD8⁺ T-cells within lymphoid tissue, and downregulation of the “superactivation state.” However, without any evidence for induction of HIV-1 specific neutralizing antibodies nor T-cell mediated cytotoxicity, the reconstitution established is mainly built by increased production of potentially reactive cells into the peripheral pool, and to a minor extent in reducing cell turnover. On the plus side, cell trafficking to and from LT seems to be much more normalized following potent antiretroviral therapy. There are still big holes in our knowledge at this time regarding the direction of future therapies. Of particular interest as targets of investigation are the nature of CD8⁺ cytoxic killing, the expression of HIV-1 specific peptides in the infected tissue on class I and class II molecules, the regulation of the CD4 and CD8 molecules on activated HIV-1 specific T-cells, the skewing of the expression of co-stimulatory molecules such as CD28, CTLA-4 as well as B7₁ and B7₂, and the abnormally high degree of down-regulation of CD3ζ signaling chain of the T-cell receptor within TL sites. All these data indicate the complexity of the disturbance in the immune response which appear during HIV-1 infection and which could be directly related to the hyperactivation state that uncontrolled HIV-1 replication generates and maintains. The current success of combinations of antiretroviral therapy treatment approaches are forming a platform from which we can improve strategies to mount an HIV-1 specific immune response. However, this approach probably requires more extensive studies based on specific interactions between HIV-1 and the host within lymphoid tissue.

Acknowledgements
The authors express gratitude for the continual support of reagent development for immunohistochemistry to Drs. Ed Morgan and Efthalia Chronopoulou of Pharmingen International, and to Dr. Monica Tsang of R&D Systems Inc.; and to Mr. Morris Bowles of Leica Microsystems Imaging Solutions for microscopic support and development.

Authors’ e-mail Addresses:
thomas.fehniger@draco.se.astra.com
jan.andersson@infect.hs.sll.se

References
2. Fleury S, De Boer RJ, Rizzardi GP et al.: Limited CD4⁺ T cell renewal in early HIV-1...


nism to reconstitute the immune system. In this review, we will revisit the role of T-cells in immunity, the relationship between thymic function and age, and closely examine the impact of HIV-mediated thymic dysregulation on thymopoiesis.

The Role of the Thymus in Immunity

The human thymus, a small organ located above the heart, provides a specialized microenvironment for the maturation, education, and selection of developing T-lymphocytes. However, the presence of mature T-cells in athymic individuals suggests that extrathymic sites of T-cell maturation may exist such as in the spleen, liver, bone marrow, and gut.

Nonetheless, the contribution of these sites is secondary to that of the thymus. The thymus is divided into two bilaterally symmetrical lobes that are further subdivided into lobules by invagination of a thin, fibrous connective tissue capsule (Figure 1A and 1D). The capsule surrounds the thymus and carries blood supply through vessels that extend to the center of the thymus. The area surrounding the vessels is the perivascular space, which is composed of lymphocytes, granulocytes, macrophages, mast cells, and adipose cells. The perivascular space functions as a delivery pathway and site of exchange between the stroma and the periphery.

Each of the numerous lobules of the thymus differentiates into two bilaterally symmetrical lobes that are further subdivided into lobules by invagination of a thin, fibrous connective tissue capsule (Figure 1A and 1D). Bone marrow-derived progenitor cells enter the thymus and migrate deeper into the cortex as they mature (Figure 1C).

**Figure 1.** Infant thymus and age-related involution. An infant thymus and an involuted adult thymus are shown in A and B, respectively. Structures of interest from each thymus are schematically highlighted in D and E. Stages of T-cell development are illustrated in C, where bone marrow (BM)-derived progenitors enter the thymic cortex and undergo a process of maturation and selection. Intrathymic T-cell precursor (ITTP) migrate through the cortex and give rise to CD4⁺CD8⁺ double positive (DP) T-cells which, in the medulla, become either CD4⁺ or CD8⁺ single positive (SP) T-cells that exit the thymus through blood vessels (V) and into the periphery as recent thymic emigrants. Histology photos of the infant and adult thymus were kindly provided by Dr. Jerome Loew at the Department of Pathology at Rush Presbyterian St. Luke’s Medical Center, Chicago, IL.
The cortex consists of densely populated immature thymocytes, epithelial cells, and scattered macrophages, while the inner medulla contains the mature thymocytes, epithelial cells, dendritic cells, and Hassall’s corpuscles. As the developing thymocytes move through the cortex and into the medulla, they receive signals for proliferation, receptor gene rearrangement, selection, and maturation.

Thymic events involved in T-cell development are schematically represented in Figure 2. Population of the thymus begins during fetal gestation when bone marrow-derived progenitor cells expressing the CD34 molecule migrate to the subcapsular epithelium and enter the thymus. Once in the subcapsular zone of the thymus, the progenitor cells interact with thymic stromal cells and are exposed to growth factors such as interleukin-7 (IL-7) and stem cell growth factor. These events induce the progenitors to proliferate and express the CD2 and CD7 cell surface markers. Expression of these two molecules identifies the developing progenitors as cells committed to the T-cell lineage. Since these early thymocytes still lack the CD4 and CD8 molecules characteristic of mature T-cells, they are referred to as “double negative (DN)” thymocytes.

Expression of the adhesion molecule CD44 on the thymocytes and movement of the cells into the thymic cortex characterize the next stage of development. At this stage, all genes encoding the T-cell receptor are still in their germline configuration. As T-cell development proceeds, expression of the CD25 molecule (α chain of the IL-2 receptor) is upregulated and CD44 is downregulated. At this stage (CD44+CD25−), the rearrangement of the T-cell receptor (TCR) β-chain genes begins. Once the TCR β-chain genes are successfully rearranged, expression of CD25 is downregulated. If the β-chain gene rearrangement is unsuccessful, development of the thymocyte will be blocked at the CD44+CD25− stage.

The successfully rearranged β-chain then pairs with a surrogate α-chain (pTα), forming a complex which will be delivered to the cell surface and expressed along with the CD3 molecules. Expression of this β-chain/pTα complex induces a signal that arrests further rearrangement of the β-chain genes, resulting in allelic exclusion at the β-chain locus. These thymocytes are also triggered to proliferate, express the CD4 and CD8 cell surface molecules, and begin the rearrangement of the α-chain genes. The developing CD4+CD8+ thymocytes are now referred to as “double positive (DP)” cells.

During thymocyte proliferation, the enzymes responsible for gene rearrangement are suppressed. Once proliferation is complete, these enzymes become active again and initiate TCR α-chain gene rearrangement. Because the genes encoding the α-chain are not restricted by the allelic exclusion observed for the β-chain genes, cells expressing an α-chain can continue to rearrange the α-chain genes until positive selection occurs.6 The result

---

Figure 2. Pathway of T-cell development in the thymus. A detailed process of T-cell synthesis in the thymus is illustrated. Cell surface expression of CD44, CD25, CD3, CD4, and CD8 are designated where appropriate; pTα;β refers to the formation of a chimeric precursor chain for the αβ TCR.
of this process is low level expression of the αβ T-cell receptor with the CD3 molecules on the surface of DP thymocytes.

Developing thymocytes continue to move through the thymic cortex where they undergo a further selection process that defines the TCR repertoire. While in the cortex, DP cells come into close contact with thymic cortical epithelial cells expressing major histocompatibility complex (MHC) molecules. Developing T-cells that sufficiently recognize self-peptide/MHC complexes are positively selected and migrate to the medulla. In the medulla, the thymocytes mature and increase the surface expression of TCR and CD3 molecules. Expression of either CD4 or CD8 will be downregulated in the medulla, depending on whether the TCR engaged MHC class I or class II proteins.

Almost 95% of developing thymocytes do not migrate out of the thymus. Thymocytes that express the TCR but do not sufficiently recognize self-MHC complexes fail to be positively selected and die in the thymus via apoptosis. Those thymocytes that recognize the self-MHC or self-peptide complexes with high affinity are eliminated, as they could be autoreactive if released into the periphery. This process is called “negative selection” and results either in anergy or death of the cell, also via apoptosis.

Cells that survive the process of development and selection in the thymic stroma emerge from the thymus as either CD4+ or CD8+ single positive cells with high level expression of αβ TCR and CD3 molecules. After completing these stages of differentiation, the thymocytes migrate to the periphery and are referred to as recent thymic emigrants (RTE) representing a vast TCR repertoire.

Thymopoiesis and Age

The thymus is fully developed at birth, with absolute growth continuing only through the first year of life. After birth, the process of thymic involution begins, which may be regulated by a number of gonadal and thymic hormones. The human thymus remains relatively the same size for up to 80 years, but over this period of time, the organ undergoes an almost complete lipomatous atrophy. In fact, in individuals older than 50 years, the fibrous capsule of the thymus predominantly surrounds adipose tissue.

As seen in Figure 1A, a newborn’s thymus is fully developed and functional, consisting mainly of active sites of thymopoiesis (the thymic epithelium). A clear distinction between the outer cortex and the inner medulla are also clearly observed. However, examining the thymus of an older individual (>65) illustrates predomi-
nately perivascular space (adipose tissue) that surrounds areas of true thymic tissue (Figure 1B). Additionally, a clear distinction between the cortex and the medulla cannot be easily made.

The process of involution, as marked by thymic atrophy and architectural changes, occurs in stages that begin with an enlargement of the perivascular space. The connective tissue septae dividing the lobules begin to disappear and are gradually replaced by single fat cells and adipose tissue. Further development of adipose tissue underneath the capsule separates the capsule from any remaining true thymic tissue. The medulla is eventually lost but islands of thymic epithelium remain.

Four phases have been established to characterize the level of thymic involution. Phase I, seen in newborns to 10 years of age, is characterized by a progressive decline in total lymphatic tissue, equivalent to a decline of five percent of lymphatic tissue per year. Phase II, seen in 10- to 25-year-old individuals, is characterized by a decrease in epithelial space that is correlated with a maximal increase in perivascular space. Phase III, seen in 25- to 40-year-old individuals, is demonstrated by a decline in both perivascular space and thymic epithelium accompanied by an increase of fatty atrophy at an estimated rate of five percent per year. Finally, phase IV, seen in individuals 40 years and older, is characterized by a slow involution of the remaining lymphatic tissue at a rate of 0.1% per year. End stage involution is characterized by lipomatous atrophy.

It has long been believed that age-related thymic involution is accompanied by a non-functional thymus. However, recently a number of investigators have challenged that hypothesis by demonstrating that thymopoiesis still continues through life albeit inversely correlated with age. Thymic samples from human donors up to 49 years old undergoing car-
diac surgery still contained intrathymic T-cell precursors and mature thymocytes, suggesting that the adult thymus is still able to sustain T-cell differentiation. Additionally, computed tomography (CT) studies have indicated that abundant thymic tissue is still present despite age-related thymic involution. Innovative molecular approaches, including the measurement of T-cell receptor excision circles, have also shed some light on thymic function and age as well as on the impact of HIV drug therapy on thymopoiesis.
Figure 3. Mechanism of TCRβ (A) and TCRα (B) TREC generation. A. In TCRβ TREC generation, DJ recombination must occur prior to V recombination. A recombination signal sequence (RSS) flanks each gene to be recombined. RSS consists of a heptamer, a spacer sequence that is 12 or 23 bp in length, and a nonamer sequence. Recombination activating enzymes recognize RSS and cleave the DNA only between gene segments possessing 12 or 23 bp spacer sequence (the 12/23 rule). Cleaved sites form an extrachromosomal DNA circle, referred to as T-cell receptor excision circle (TREC). Removal of TREC allows for the formation of a recombined chromosomal gene encoding a specific VDJ TCR. B. Common to all TCRα germline DNA is an intervening sequence for the δ gene. In order to generate a VJ recombination event, the intervening δ gene is excised, in the same mechanism described for the β chain TREC, to initially generate a signal TREC (Dδ, Jδ, Cδ). A second TREC is then formed corresponding to the coding joint (Vα, Vδ, and Jα).
TABLE 1. CHEMOKINE CO-RECEPTOR EXPRESSION DURING T-CELL DEVELOPMENT

<table>
<thead>
<tr>
<th>Cell phenotype</th>
<th>CXCR4</th>
<th>CCR5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thymus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Periphery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; or CD8&lt;sup&gt;+&lt;/sup&gt; naive</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; or CD8&lt;sup&gt;+&lt;/sup&gt; memory</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; naive</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; memory</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* ++++, ++, and – denote the level of chemokine co-receptor expression, corresponding to high, medium, low, or no expression, respectively.

Values are based on data from references 19 and 20.
TN, ITTP, DP, SP, early bone marrow progenitors, stromal cells, and endothelial cells. In vitro studies have demonstrated, mostly through detection of integrated HIV DNA, that intrathymic precursors of the T-cell lineage (CD4^+CD8^-, CD4^+CD8^+, and CD7^+CD3^+CD4^hi, CD8^hi) are targets for HIV-1 infection.  

Primary immature thymic lymphocytes (CD4^+CD8^+, CD4^+CD8^-, or CD3^+CD4^-CD8^-) have also been shown to be susceptible to HIV (HTLV-IIB and LAI strains) infection.  

Productive infection of HIV is specifically induced by the interaction of thymocytes with autologous thymic epithelial cells (TEC).  

To determine the effect of HIV on human thymic tissue, various animal model systems have been used. The SCID-Hu model is a small animal model designed to support thymic and hematopoietic differentiation in vivo. Human fetal liver and human fetal thymus fragments are co-implanted into mice homozygous for the severe combined immune deficiency (SCID) defect, resulting in the development of a conjoint functional human organ that supports normal development of competent human thymocytes. HIV infection of thymus/liver (Thy/Liv) implants results in severe and preferential depletion of immature (CD4^+CD8^-) human thymocytes. Defects in the thymic microenvironment, and defective thymopoiesis. The depletion of human thymocytes, predominately in the cortex, is via apoptosis, however, and only ten percent of these cells undergoing apoptosis are infected, indicating that an HIV-independent cytopathic pathway exists.  

Infection of macaques with simian immunodeficiency virus (SIV) provides another model for the study of HIV-induced disease in humans. Thymic infection of juvenile macaques with pathogenic clones of SIV resulted in increased apoptosis in thymic cells and depletion of thymic progenitors. Immunohistochemical analysis has provided direct evidence of viral DNA and RNA in SIV-infected cells. Similar depletion of CD4^+ cells has been observed following infection of pig-tailed macaques with virulent strains of the chimeric simian/human immunodeficiency virus (SHIV).  

Analysis of human thymic tissue from HIV-infected infants, children, and adults demonstrated HIV RNA and proteins in the thymus, providing direct in vivo evidence for thymic infection. HIV infection of the thymus not only leads to severe depletion of thymocyte sub-populations (CD3^+CD4^-CD8^+ and CD3^-CD4^-CD8^-), contributing to decreased thymic output, but also causes a number of physiological abnormalities including thymus involution even among infants with AIDS. HIV infiltration of the thymus with mature plasma cells, reduction in the number of thymic epithelial cells, degenerative changes in intrathymic blood vessels, and depletion of Hassall's corpuscles in most cases but if present these structures are calcified.  

**Immune Reconstitution and the Thymus in HIV-infected Patients**  
Lessons drawn from the AIDS Clinical Trial Group (ACTG), evaluating the efficacy of highly active antiretroviral therapy (HAART), have shown that while HAART is successful in decreasing viral load, this reduction is not accompanied by the complete normalization of the immune response. Nonetheless, sustained increases in CD4^+ cell numbers were observed which may be from redistribution of T-cells from lymphoid organs to the periphery, clonal expansion, or new T-cell synthesis. Recent studies have extended support to the latter. The adult thymus, despite involution, continues to contribute to the generation of de novo/naive T-cells, as measured by TREC and immunostaining of pockets of thymopoiesis. HIV infection, however, appears to reduce thymopoiesis but at least in one study this reduction was reversed by suppression of virus replication by HAART. To the contrary, Lewin and colleagues also demonstrated increases in thymopoiesis following HAART but only in individuals that had lower levels of thymopoiesis prior to HAART. Their data indicate that thymopoiesis may not be affected in all HIV patients but when it is it can still be normalized by viral suppression. In either case, these data suggest that in some patients, the level of thymopoiesis may be enhanced after potent suppression of HIV replication.  

**Concluding Remarks**  
The finding that the adult thymus can still contribute to new T-cells in the periphery, that HIV can effect thymopoiesis in some individuals, and that potent suppression of HIV replication can return thymic output to levels comparable to that seen in age-matched groups, all stress the need to design future therapeutic approaches that can exploit the capacity of the adult thymus to repopulate the periphery with functional naive T-cells. These strategies are of great benefit to AIDS patients, DiGeorge athymic syndrome patients, and cancer patients following intensive chemotherapy. Some of these strategies include thymic transplants, whereby a fetal thymus is transplanted into the arm (Richard Hong, University of Vermont, personal communication) or thigh of immunodeficient patients. Thymus transplant was successfully applied to a few DiGeorge athymic syndrome patients, as determined by restoration of T-cell function in the periphery and expansion of the TCR repertoire. Thymic transplants were also applied to 15 AIDS patients with 8 of 15 demonstrating transient increases in only the CD8 T-cell compartment. This study was conducted prior to potent antiretroviral drugs that can suppress HIV replication. These disappointing results may be due to reinfection and destruction of de novo T-cells, suggesting that, in the case of HIV, thymic transplants must be accompanied by either potent anti-HIV drugs or that the new T-cells should be genetically modified to resist HIV infection, a prerequisite that is far from being a successful common clinical practice. Alternatively, cytokine therapy may enhance the pool of naive T-cells. IL-7 has been shown to induce the proliferation of naive cells without inducing their switch into memory cells, as indicated by the expression of the CD45RO cell surface marker. Collectively, for the first time, it is theoretically possible to target strategies that can enhance thymic output in the adult patient in an effort to reconsti-
tute the immune system of not only AIDS patients but also in any pathology that depletes the T-cell pool.

Author's e-mail Address
laharth@rush.edu

References


41. Chen L, Nugeyre MT, Barre-Sinoussi F, Israel N: High-level replication of human immunodeficiency virus in thymocytes requires NF-kappaB activation through interaction with

Human Herpesvirus 6 (HHV-6): Could it Play A Role in the Etiopathogenesis of Multiple Sclerosis (MS)?

L.F. Kastrukoff, MD and E.E. Thomas, MD, PhD

Demyelinating Disease Study Group, Division of Neurology, University of British Columbia and Department of Pathology and Laboratory Medicine, BC Children’s Hospital, Vancouver, BC, Canada

The etiology of multiple sclerosis (MS), a demyelinating disease involving the human central nervous system (CNS), remains unknown. Available information suggests that it is multifactorial and implicates inherited susceptibility, an autoimmune component, and environmental factors which may be infectious in nature. Although specific viruses are known to cause a number of chronic neurological diseases, evidence from epidemiology, serology, and virology studies which would implicate a role for viruses in the etiopathogenesis of MS is less exacting. Additional support for a viral etiology does, however, come from experimental animal models of virus induced CNS demyelination. These studies demonstrate that different families of viruses are capable of inducing demyelination and raise the possibility that different viruses could play a role in MS rather then a specific “MS virus.” The models also suggest that causation is multifactorial. The induction of CNS demyelination in many of these models requires the interaction of a specific virus with a specific immune repertoire determined by the host’s genetic makeup. The actual mechanisms mediating the interaction can vary from one system to the next and may include “hit and run” as in the case of acute disseminated encephalomyelitis, “molecular mimicry” where homologous peptide sequences of various viruses and CNS antigens such as encephalitogenic regions of myelin basic protein may be shared, and “epitope spreading” where immune responses develop because of de novo priming of self-reactive T-cells to sequestered autoantigens released secondary to virus-specific T-cell-mediated demyelination.

While human herpesvirus 6 (HHV-6) has only recently been implicated in the etiopathogenesis of MS, the idea that members of the herpesvirusidae family of viruses may play a role in this disease is not new. Herpes simplex virus (HSV) was isolated from the brain of a MS patient by Gudnadottir et al. in 1964 and from the cerebrospinal fluid (CSF) of a second MS patient by Bergstrom et al. Furthermore, Fraser et al. using Southern blot hybridization, were able to identify HSV DNA in the CNS of a number of MS patients. The interpretation of these results was made difficult however by the fact that HSV DNA was also found in the CNS of controls without MS. Recently, Sanders et al. have extended these studies by using polymerase chain reaction (PCR) and Southern blot hybridization techniques. They also found HSV DNA to be present in the CNS of both MS patients and controls but found viral DNA to be more likely associated with active rather than inactive plaques. Although these associations are very intriguing, they are not proof that HSV is responsible for the induction of CNS demyelination in MS patients. Even if it were to be shown that HSV is capable of inducing CNS demyelination, the presence of viral DNA in the CNS of both MS patients and con-
controls would suggest that other factors besides virus are required. In an experimental animal model, HSV-1 has been shown to induce recurrent multifocal CNS demyelination but only in specific strains of mice and with specific strains of virus.\textsuperscript{20-22} In some murine strains, HSV-1 can be found throughout the CNS following oral inoculation with virus but without the development of demyelinating lesions.\textsuperscript{21} Other members of the Herpesviridae family of viruses have also been implicated in the etiopathogenesis of MS. Evidence implicating Epstein-Barr virus (EBV) in MS has recently been reviewed by Munch et al.\textsuperscript{23} While Ross et al.\textsuperscript{24} make a case for an association between varicella-zoster virus (VZV) and MS using an epidemiologic approach. Using PCR, Sanders et al. were able to find viral DNA from EBV and cytomegalovirus (CMV) in the CNS of both MS patients and controls but at a lower incidence than HSV, HHV-6 or VZV.\textsuperscript{19} It is not known if different members of the Herpesviridae family of viruses may contain conserved genetic sequences which are important to the induction of CNS demyelination in susceptible hosts.

HHV-6, a beta-herpesvirus, was first isolated in Robert Gallo's laboratory at the National Cancer Institute in 1986.\textsuperscript{25} It was initially identified in the peripheral blood of immunosuppressed patients affected by various lymphoproliferative diseases. The molecular and structural characteristics of HHV-6 have been extensively reviewed by Lusso\textsuperscript{26} and Braun et al.\textsuperscript{27} There are two major subgroups or variants of HHV-6 (A and B) which have been defined on the basis of DNA restriction analysis, in vitro tropism, and antigenic relationships defined by monoclonal antibodies.\textsuperscript{28} Furthermore, multiple strains of the variants exist. Examples of these include strains GS and U1102 of the A variant and strains Z29 and HST of the B variant.\textsuperscript{29} Strains of variant B can be further designated as belonging to one of two groups: group 1 or group 2. Although the variants differ genetically, immunologically, and biologically, they do share a tropism for T-lymphocytes (primarily CD4 T-cells), macrophages,\textsuperscript{30} and NK cells. Infection by HHV-6 is the most ubiquitous of all human herpesviruses\textsuperscript{31} and is present throughout different geographic areas.\textsuperscript{32}

The virus is likely acquired through transmission of oropharyngeal secretions\textsuperscript{26} and within the first one to three years of life.\textsuperscript{33} HHV-6 is detectable post-mortem in the CNS of many neurologically normal adults\textsuperscript{34} and it has been suggested that the CNS may be the sole site of persistence of this virus.\textsuperscript{35}

Clinically, HHV-6 causes exanthem subitum (roseola infantum) in children.\textsuperscript{36} Only HHV-6B strains definitively have been proven to cause disease. Although infection with this virus is often mild, it can cause a spectrum of illness including neurological disease.\textsuperscript{37} Seizures are a frequent complication of primary HHV-6 infection in infants while encephalitis and meningoencephalitis are more rare.\textsuperscript{38} In those cases of virus-associated CNS disease, HHV-6 DNA can frequently be detected in the CSF.\textsuperscript{39,40} HHV-6 CNS infections also have been reported in immunosuppressed patients such as bone marrow transplant patients\textsuperscript{41} and adults/children with AIDS.\textsuperscript{42,43} It is of interest that in several of these cases, the neuropathology involved either extensive demyelination of the subcortical white matter or focal areas of demyelination.\textsuperscript{40,41,44}

Recently, HHV-6 has been implicated in the etiopathogenesis of MS. Evidence to support this comes from several avenues of investigation including anecdotal reports, serological studies in serum and CSF, detection of viral nucleic acid in serum and CSF, and detection of HHV-6 in the CNS. Among the anecdotal reports, Merelli et al.\textsuperscript{45} reported the development of an acute encephalopathy in a MS patient. The patient was a known carrier of HHV-6 as specific viral genomic sequences were detected in peripheral blood mononuclear cells (PBMC) but not CSF prior to the event. During the acute encephalopathy, CSF was positive for HHV-6 genomic sequences at a time when new lesions were identified on the MRI. Furthermore, Carrigan et al.\textsuperscript{46} reported the case of a young woman who died of a subacute demyelinating disease which clinically and pathologically were diagnosed as MS. Using an immunohistochemical approach, the authors were able to identify a disseminated active HHV-6 infection which correlated with the areas of demyelination. A similar case was reported by Novoa et al.\textsuperscript{47} where a young woman, who developed a fulminating demyelinating encephalomyelitis, had HHV-6 associated with the demyelinating lesions as detected by immunohistochemistry and PCR both on brain biopsy and brain tissue at the time of autopsy. Although one could argue that these two cases were “atypical” for MS, it would likely have been the diagnosis were it not for the newer diagnostic techniques. Furthermore, it is reminiscent of those patients who originally were diagnosed as suffering from MS but where later the diagnosis was changed once it was realized that a separate condition, TSP/HAM is caused by HTLV-1.

Several serological studies have looked at differences in antibodies to HHV-6 in MS patients and controls. Sola et al.,\textsuperscript{48} using an immunofluorescence analysis of sera, found anti-HHV-6 antibody titers to be significantly higher in 126 MS patients compared with 500 normal controls. However, as PCR of the PBMC was only positive for HHV-6 in one MS and one control, they interpreted their results as being more consistent with immune impairment observed among MS patients rather than resulting from reactivation of a latent infection. In a similar type of study, Wilborn et al.\textsuperscript{49} studied 21 MS patients and compared the results with 19 patients with facial palsy, seven with Guillain-Barre syndrome, and 16 controls. Using an ELISA analysis of sera they also found anti-HHV-6 antibody titers to be significantly higher than in control groups. PCR of the PBMC was negative for HHV-6 in both the MS and controls. In contrast, Nielsen et al.\textsuperscript{50} studied 189 serum samples from MS patients at different stages of their disease and compared them with 190 serum samples from age and sex matched controls. Only two of the controls and none of the MS patients were sero-negative for antibodies to HHV-6. No significant difference was found in the anti-HHV-6 IgG titers between MS patients and controls using a competitive ELISA technique. In this study, PCR of the PBMC was not performed for HHV-6. When taken together the results support the ubiquitous nature of infection with HHV-6 as most individuals were sero-positive for this virus. It is less clear if higher titers of antibody to HHV-6 are a consistent finding among
MS patients. Recently, Soldan et al. (51) using an EIA, examined serum for antibodies to HHV-6 p41/38 early antigen from 36 (22 relapsing–remitting [R/R], 14 chronic progressive[CP]) MS patients, 31 other neurological diseases (OND), 21 other inflammatory diseases (OID), and 14 normal controls. Although there was no difference in the anti-HHV-6 IgG response to HHV-6 p41/38 early antigen, there was a significantly elevated IgM response in the R/R group. Similar differences in IgG or IgM antibodies to EBV or CMV were not identified. In one cohort of this study, IgM antibodies to p41/38 HHV-6 early antigen in the CSF were not found among 18 RRMS, 6 CPMS, or 18 HAM/TSP patients. HHV-6 DNA was not identified, using PCR, in the PBMC of 47 normal controls or patients with OND or OID. It was found however in 15 (14 RRMS, 1 CPMS) of 50 MS patients. The authors suggested that the results represented a reactivation of latent HHV-6 in MS patients but they were not prepared to conclude that HHV-6 is the causative agent of MS.

The third avenue of investigation involves the identification of HHV-6 nucleic acid in the blood or CSF of MS patients. Martin et al. (52) were not able to identify HHV-6 DNA in blood using PCR in over 116 samples obtained from MS, optic neuritis and OND. Similar results were obtained by Wilborn et al. (49). In contrast, Sola et al. (48) were able to identify viral DNA in the blood but only in 1 of 31 MS patients and 1 of 24 normal controls while Merelli et al. (53) reported the presence of HHV-6 nucleic acid in PBMC of 3 of 56 MS patients. Soldan et al. (51) reported a higher rate with 30% of 50 MS patients having viral DNA in their blood but none of 47 controls. Recently, Mayne et al. (54) used two separate probes to target different regions within the HHV-6 genome. Depending on which probe was employed, detection of HHV-6 genome ranged from 11.7 to 23.5% in controls, 3.1 to 23% in RRMS, and 14.2 to 28.5% in CPMS. Martin et al. (52) did not find HHV-6 DNA in any of 115 CSF samples obtained from patients with MS, optic neuritis, or OND. In contrast, Wilborn et al. (48) did find viral DNA in 3 of 21 MS patients but not in their controls. Similar results were reported by Liedtke et al. (55) in acellular CSF. HHV-6 was detected in 4 of 36 MS patients, 2 of 27 neuro-AIDS patients, but in none of their control group.

The fourth avenue of investigation involves the detection of viral DNA in the CNS and their relation to MS lesions. Challoner et al. (39) used representation difference analysis to search for pathogens in MS brains. Using PCR in 86 brain specimens, HHV-6 DNA was identified in >70% of MS cases and controls. These results along with studies by Luppi et al. (34) and Saunders et al. (19) indicate that HHV-6 is a commensal virus of the human brain. Not all investigators however have found HHV-6 DNA in the CNS of MS patients. (43) Using DNA sequencing, Challoner et al. found most of the HHV-6 were variant B group 2 in nature but one isolate was found to be variant B group 1. In most of the 49 MS patients, viral titers were similar to that found in controls. However, in two MS patients, markedly higher titers were found. Further study of the viral isolates from these two MS patients found Hind HI restriction site polymorphisms which suggested that there may be sub-types of HHV-6 variant B group 2 which differ in their biological behavior. Immunocytochemistry studies employing antibodies to HHV-6 virion protein 101K and DNA binding protein p41 were of particular interest. Nuclear staining of oligodendrocytes by these antibodies was found in 12 of 15 MS patients but not in controls suggesting a fundamental difference between the two groups. Further support for this observation comes from in vitro studies where HHV-6 was found to have a tropism for oligodendrocytes and microglia but not neurons. (27)

Although evidence is mounting for a possible role of HHV-6, along with other members of the Herpesviridae family of viruses, in the etiopathogenesis of multiple sclerosis, it is important to realize that association does not prove causation. Historically, the search for infectious agents which may play an etiologic role in MS has been a difficult one and of necessity must raise a healthy skepticism whenever new viral agents are claimed to play a role. Nevertheless, the potential of Herpesviruses, and HHV-6 in particular, to play a role represents an exciting development in MS research and one which hopefully will become clear in the near future.

Author’s e-mail Address
lornefk@exchange.ubc.ca

References
17. Baringer JR, Pisani P: Herpes simplex virus


HUMAN RETINAL PIGMENT EPITHELIAL CELLS INFECTED WITH TOXOPLASMA GONDII UPREGULATE mRNA EXPRESSION AND SECRETE IL-1, IL-6, GM-CSF AND INTERCELLULAR ADHESION MOLECULE-1

Chandrasekharam N. Nagineni, 1 Barbara Detrick, 2 and John J. Hooks 1

1 Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892; 2 Department of Pathology, Johns Hopkins University Medical Institutions, Baltimore, MD

Objective: Retinochoroiditis caused by the Toxoplasma gondii infection is one of the common causes of uveitis, an inflammatory disease of the eye. Recently, we have demonstrated T. gondii replication in human retinal pigment epithelial cells (HRPE), a critical regulatory cell type in the retina. In the present study, we have examined the cytokine secretion by HRPE cells in response to T. gondii (RH strain) infection.

Methods: Replication of the parasite in the cultures was monitored by immunofluorescence staining of the cells using T. gondii specific antibodies. Supernatant fluids were collected from the control and T. gondii infected HRPE cultures at various time points and the levels of cytokines determined by ELISA. Total RNA prepared from the cultures was used for Northern blot and RT-PCR analyses.

Results: Progressive increase in the secretion of IL-1, IL-6, and GM-CSF was observed as a function of post-inoculation time. After four days of inoculation, when most of the cells were infected with the parasite, the levels of cytokine in the media were IL-1β (con 0.5 vs T. gondii 11.5 pg/ml), IL-6 (con 259.6 vs T. gondii 2476.3 pg/ml) and GM-CSF (con 35.4 vs T. gondii 291.8 pg/ml). In contrast, T. gondii infection of HRPE cells did not increase the secretion of TNF-α, IL-4, IL-10, IL-12 and IL-15. However, significant secretion (con 7.8 vs T. gondii 35.3 ng/ml) of intercellular adhesion molecule-1 (ICAM-1) was observed in T. gondii infected HRPE cells. Northern and RT-PCR analyses indicated elevated levels of mRNA for IL-1β, IL-6, GM-CSF and ICAM-1 suggesting transcriptional regulation.

Conclusion: The secretion of these inflammatory molecules by HRPE cells in response to T. gondii infection may be an important factor that is involved in the regulation of pathophysiological processes during T. gondii induced retinochoroiditis.

A NEW FULLY AUTOMATED ELISA ON THE COBAS CORE™ FOR THE DETECTION OF B2-GLYCOPEPTIDE 1 ANTIBODIES RELEVANT FOR THE DIAGNOSIS OF THE ANTIPHOSPHOLIPID SYNDROME (APS)

D. Block, A. Buscemi, H.P. Lehmann, and J.W. Zolg
Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland

Objective: We have developed this qualitative two-step indirect ELISA with highly purified human B2-Glycopeptide 1 (B2 GP1) on the solid phase intended for use on the fully automated Cobas Core immunoanalyzer. This assay is part of the so-called “Rheuma Information Level” (Cobas Core Anti-B2-GP1 EIA, CRP EIA Quant, RF EIA Quant and HEP2 ANA EIA) which is intended to be used for the “screening” of patients with suspected systemic autoimmune disorders.

Methods: 57 sera from patients with APS were derived from three rheumatological centers in Israel, Italy and Switzerland. Control sera were obtained from 120 healthy blood donors. The samples were tested with the new Cobas Core Anti-B2-GP1 EIA and manual ELISAs from 3 commercial suppliers.
Results: Clinical sensitivities for APS were 91.2% for Cobas Core, 91.2% for Competitor 1, 89.5% for Competitor 2 and 87.7% for Competitor 3 Anti-ß2-GP1 EIA. All 120 blood donor sera tested were negative in the Cobas Core Anti-ß2-GP1 EIA, whereas 2/120, 7/120 and 9/120 were detected positive by Competitor 1, Competitor 2 and Competitor 3 Anti-ß2-GP1 EIA, respectively.

Conclusion: The Cobas Core Anti-ß2-GP1 EIA showed comparable performance to three commercial manual ELISAs. Its further advantage is reduction of workload by fully automated handling of samples.

PILOT STUDY OF CYTOKINES RECOVERED FROM WOUND FLUID OF NON-HEALING WOUNDS

G. Baron, 1 G. Marshall, 2 E. Henninger, 2 A. Herbert, 2 T. Hunt, 3 and C. Fife 1, 2

1 Hermann Hospital, 2 The University of Texas Houston Health Science Center Medical School, Houston, TX, 3 University of California, San Francisco, San Francisco, CA

Objective: Wounds which are refractory to standard care may result from systemic factors such as diabetes mellitus, chronic venous insufficiency, rheumatoid disease, sickle cell anemia, or external insults. The non-healing wound bed environment may be regulated by a local network of interacting cytokines produced as a result of external insults. The non-healing wound bed environment may represent immune response dysregulation at the local level. Longitudinal studies of cytokine populations recovered from the non-healing wound bed during the course of patient’s wound healing therapy may prove useful in understanding the local wound environment associated with the healing process, and contribute to strategies for wound care therapy design.

EVALUATION OF ENZYME IMMOUNOASSAY FOR ROUTINE ANTI-NUCLEAR ANTIBODY SCREENING IN A LARGE REFERENCE LABORATORY

J. Shieh-Ngai, 1 J. Griffiths, 2 P. Conboy, 2 V. Cappel, and N. Cappel 2

1 Kaiser Permanente Regional Laboratory, Berkeley, CA; 2 Helix Diagnostics, West Sacramento, CA

Objective: With the increasing demands on the clinical laboratory to reduce labor and material costs and the dilemma of an ever shrinking number of experienced clinical laboratory scientists, the prospect of utilizing an objective, automatable enzyme immunoassay for ANA testing is very appealing. The objective of this study was to determine if enzyme immunoassay could adequately function as a routine method for ANA screening compared with IFA-ANA, specific autoantibody detection and clinical findings.

Methods: A total of 705 routine, consecutive ANA sera were screened according to manufacturer’s instructions, by IFA (HEp-2 ANA, Zeus) and four commercially available EIA methods (Helix Diagnostics, West Sacramento, CA, DiaSorin, Stillwater, MN, Wampole-Zeus, Cranbury, NJ and Pharmacia & UpJohn, Glendale, AZ) for the presence of anti-nuclear antibodies. Discrepant samples were evaluated for the presence of detectable, specific autoantibodies by EIA and immunoblot techniques. Select sera underwent a clinical chart review for further clarification. Additional “challenge sera” were also tested by EIA as materials allowed.

Results: The Wampole-Zeus EIA ANA test was eliminated from the evaluation due to lack of specificity and sensitivity. Final sensitivity and specificity calculations were based on positive values defined as IFA ANA results of > 2+ at 1:80 and/or detection and verification of the presence of specific autoantibodies. The remaining tests demonstrated the following performance characteristics:

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix Diagnostics</td>
<td>97%</td>
<td>99%</td>
</tr>
<tr>
<td>DiaSorin</td>
<td>82%</td>
<td>100%</td>
</tr>
<tr>
<td>Pharmacia &amp; UpJohn</td>
<td>78%</td>
<td>88%</td>
</tr>
</tbody>
</table>

Conclusion: This study demonstrated the ability of a high quality EIA method to adequately serve as a routine screening method for the detection of anti-nuclear antibodies. Helix Diagnostics demonstrated superior performance and was selected for this purpose. Kaiser Permanente Regional Reference Laboratory plans to utilize the Helix EIA-ANA as a first line screen for ANA. Positive EIA results will then be tested in a 1:80 and 1:320 dilutions on HEp-2 IFA to determine patterns of fluorescence. With this approach we will be able to screen out the negative sera (approximately 85% of the total) with an objective, automated EIA technique. The remaining 15% will be tested on a limited basis with IFA in order to provide physicians with continuity of results. In addition to being clinically comparable, a cost analysis has demonstrated that the EIA ANA is less costly and time consuming compared with FANA.
SIGNIFICANCE OF SEROLOGICAL METHODS IN THE DIAGNOSIS OF CELIAC DISEASE (CD)
Manoj Rajadhyaksha, J. Schwartz, V. Ramsperger, M. Risbood, and V. Kumar
IMMCO Diagnostics. Inc., Amherst, NY 14228

Objective: To study the diagnostic efficacy of various serological methods towards the diagnosis of CD.

Introduction: Celiac disease is a chronic digestive disorder caused by an autoimmune reaction against gluten, a protein found in wheat. It is characterized by damage to the absorptive villi and hyperplasia of the crypts in the small intestine. Clinical presentation of CD most commonly reflects disturbed small bowel function: diarrhea, weight loss and anemia from malabsorption of iron and folic acid. Active CD increases risks of gastrointestinal malignancy, infertility, osteoporosis and epilepsy and other neurological syndromes. CD is treated with a gluten free diet. Serological tests have been developed to aid in diagnosis of CD (anti-endomyosial antibody (EMA) and anti-reticulin antibody (ARA), immunofluorescence (IFA) tests and antigliadin antibody (AGA) ELISA test methods. The EMA test is very sensitive and specific. Yet it requires years of experience for accurate identification of immunofluorescence patterns that are specifically associated with CD. Recently, the antigen associated with CD was identified as tissue transglutaminase (tTG). The current opinion is that the EMA antibodies specifically bind to tTG to give the peculiar reticular pattern observed in the EMA reactions. The tTG enzymes are ubiquitous and highly conserved across species. With a well characterized diseased and control population we have evaluated the efficacy of all the serological diagnostic tests for the identification of CD cases using well-characterized sera from confirmed celiacs and non-celiacs.

Methods: 774 well characterized CD cases, based on their intestinal biopsy results, age, sex, diet and onset of disease were tested on all the four currently established serological methods of CD diagnosis. This population was tested for EMA, ARA, AGA, and anti-tTG antibodies, along side with age matched and sex matched adult and pediatric control population (65 adult normal and 50 normal children sera). Besides, the biopsy confirmed CD cases tested above, 284 suspected CD cases were also tested.

Results: Of the IFA test methods, the EMA showed greatest sensitivity (100%) and specificity (100%) for the detection of CD cases. The AGA IgG ELISA had the highest sensitivity (98%) but lower specificity for the detection of CD patients. In contrast the AGA IgA ELISA had the highest specificity (99%) but lower sensitivity (80%) for the identification of CD patients. It was thus quite desirable to have an ELISA system that could have sensitivity and specificity similar to EMA. We report here that our Anti-tTG ELISA has comparable sensitivity (95%) and specificity (100%) in comparison to EMA, for the diagnosis of CD. This is noteworthy as earlier reported commercial anti-tTG ELISA kits have lower sensitivities of detection.

Conclusion: Anti-tTG is a very sensitive and specific diagnostic test method for CD diagnosis.

USE OF AUTOANTIBODY PROFILES IN A PEDIATRIC POPULATION TO SOLVE DISCREPANT PATIENT TEST RESULTS
V. Demarest and T. Charbonneau
New York State Department of Health, Wadsworth Center, Albany, NY

Introduction: Current recommendations for the diagnosis of perinatal HIV transmission require analysis of multiple specimens; one submitted within the first week of life and follow-up at 4 to 8 weeks and again at 16 to 24 weeks of age. An infant is considered infected after two positive HIV DNA PCR test results and not infected after two negative results with one performed at 16 to 24 weeks of age. When the follow-up testing is inconsistent with the initial test results i.e. non repeatedly reactive positive, one must consider a possible specimen mix-up by either the laboratory or the submitting institution.

Objective: The Antibody Profile Assay (AbP, Miragen) has been widely used to confirm and match biological specimens in adults, but its use in a pediatric population has not been substantiated. The laboratory sought to determine whether the development of autoantibody profiles early in life preclude the use of AbP for resolving discrepant pediatric test results.

Methods: Specimens were plasma aliquots, stored at -20°C, from infants submitted for diagnosis of perinatal HIV-1 transmission. Two different cohorts were selected for analysis: i) 45 specimens, drawn at three different time intervals from 15 infants and ii) 9 maternal/child pairs. All specimens were analyzed by the Miragen Antibody Profile Assay as per manufacturers instructions. Since the immunoassay is qualitative, maternal/infant pairs were analyzed at the same time, as well as the triplicate infant specimens, to minimize assay to assay variations. All results were coded by two separate individual readings.

Results: For the 15 infants analyzed over three time periods, no single infant demonstrated an identical autoantibody profile for all specimens submitted during the first year of life (age range 0.14 to 56 weeks). Six infants (40%) had similar profiles between the first and second specimen submitted (average age difference 4 weeks) while 9 infants (60%) demonstrated slight mismatches in the banding pattern (7 weeks). There were no complete mismatches for this early time period. Conversely, when the time difference between specimens was greater than 20 weeks, 53% of the infants had slight mismatches while 40 % demonstrated complete mismatches (average age difference 25 weeks). A single infant’s specimen drawn at 8 weeks exhibited and exact match with a specimen drawn at 28 weeks. In the maternal/child cohort, 8 of the 9 pairs (89%) had an exact match between the maternal and infant specimens (average age of infant 1.2 weeks). The single mismatched mom/baby pair was from an infant whose initial specimen was submitted at 7 weeks of age. Furthermore, slight mismatches between mothers/infants were noted for infants’ specimens drawn between 6 to 8 weeks of age and complete mismatches when infants were greater than 12 weeks.

Conclusion: The data indicates that although the Miragen assay may be successful in resolving discrepant testing results on adult
patients, it would be unsuitable in a pediatric population. Presumably, the development of the infant immune system and the loss of maternal antibody during the first year of life contributes to the disappearance of some autoantibody bands and the development of others. Alternatively, the data suggests that the assay may be useful in a nursery setting when there is a need to match mothers to their newborns.

QUANTITATION OF FETAL RED BLOOD CELL BY FLOW CYTOMETRY — EXPERIENCE OF TWO YEARS OF CLINICAL TESTING

J.C. Chen and B.H. Davis
William Beaumont Hospital, Royal Oak, MI

Objective: Determination of fetal hemoglobin (HbF) containing red blood cell is useful in the management of fetomaternal hemorrhage and treatment of sickle cell diseases. The flow cytometric method (FCM) has been proposed to replace the manual Kleihauer-Betke test because of its objectivity and sensitivity.1, 2 We report the evaluation of results from two years of FCM clinical testing with emphasis to fluorescence intensity (FI) of HbF staining and cell size by light scatter (CS).

Methods: erythrocytes are fixed with 0.05% of glutaraldehyde for 10 min and permeabilized by exposure to 0.1% Triton X-100 for 5 min. The cells (approx. 5 X 10^8 RBCs) were incubated with fluorescein isothiocyanate-labeled monoclonal HbF antibody (Caltag, Burlingame, CA) for 20 min. The cells were analyzed by 2 FACScan cytometers. Data analysis was performed with Winlist software (Verity, Topsham, ME). The regions of analysis for HbF negative cell, F-cell and fetal RBC were determined by using controls spiked with 1% fetal cord RBCs.

Results: 3.4 percent of the 602 patients were found to be positive by FCM (>0.1% of fetal RBC or 7.5 ml of fetal blood). 99.2 percent of patients had less than 30 ml of fetal blood detected, the volume of Rh positive fetal blood in an Rh negative woman that is adequately treated by a standard 300 mg of RhIG for immune suppression. Five patients (0.83 percent), with average of 218 ml, had more than 30 ml of fetal blood in circulation. The FI of HbF negative, F-cell, fetal RBC cell populations are 4.06+0.34, 55.68+7.94 and 471.64+137.62 mean channels (MCh), respectively. There is a significant FI difference between HbF negative cell, F-cell and fetal RBC (t<0.01, n=11). The FI and CS of the controls spiked with 1% fetal cord RBCs.

Conclusions: i) The variations of fetal RBCs’ FI and CS are smaller than those of fetal RBC. Sequential sampling of a patient with 4.13% of fetal RBC bled, showed a qualitative change in two-parameter histograms.

References:

A NEW FULLY AUTOMATED ELISA ON THE COBAS CORE™ IMMUNOANALYZER FOR THE SENSITIVE DETECTION OF C-REACTIVE PROTEIN (CRP) IN RHEUMATOID SERA

J. Kaufmann, J. Stumpf, H.P. Lehmann, and J.W. Zolg
F. Hoffmann-La Roche Ltd, Roche Diagnostics, Basel, Switzerland

Objectives: The COBAS Core CRP EIA Quant is an in vitro diagnostic test for the qualitative measurement of CRP in human serum or plasma. It is intended for use on the Cobas Core immunodiassay analyzer for “screening” of patients with suspected systemic autoimmune disorders. A new application for the measurement of CRP is discussed within the field of rheuma entrance screening. This CRP EIA is part of the of the so-called “Rheuma Information Level” (Cobas Core Anti-ß2-GP1 EIA, CRP EIA Quant, RF EIA Quant and HEp2 ANA EIA).

Methods: We have compared the performance of the Cobas Core CRP EIA QUANT with a sensitive immunoturbidimetric method (Cobas Integra CRP Lx). 144 sera were selected from patients with different rheumatic diseases. 50 non-rheumatoid sera were analyzed as a control group.

Results: Both methods showed excellent correlation (r = 0.99). All non-rheumatoid sera contained CRP levels below 10 µg/mL. 88% of the samples have concentrations <3µg/mL, 50% <1µg/mL. Patients with various rheumatoid disorders showed CRP levels between 0.07 and 141.8 µg/mL. 31% of the samples are measured above 10 µg/mL. 22% of the remaining sera are below 1µg/mL.

Conclusion: The COBAS Core CRP EIA Quant fulfills the needs for CRP measurement in rheumatoid sera in the context of the Rheuma Information Level on the Cobas Core immunoanalyzer. In patients with rheumatic disorders the CRP levels are clearly increased compared to non-rheumatoid persons. Since a subtle increase of CRP levels is observed in two thirds of the specimen, it is obvious that a sensitive measurement of CRP concentrations is necessary to contribute the assessment of rheumatic disorders on the information level.

EVALUATION OF A NEW TISSUE TRANSGLUTAMINASE IGA AUTOANTIBODY ASSAY IN CELIAC DISEASE PATIENTS AND IN PATIENTS WITH HIGH AND LOW IGA LEVELS

E. Dolan, R.A. Ambacher, and L. Cook
Lahey Clinic, Burlington, MA

Objectives: Anti-endomysial antibody (EMA) measurements have in the last few years been shown to have significant clinical usefulness in the diagnosis and monitoring of Celiac Disease. Last year the specific endomysial antigen, tissue transglutaminase (tTG), was identified and recently an FDA approved EIA kit
Results were identified by performing IgA quantitations on 300 sequenced with decreased or absent IgA that were HCV antibody positive, 10 samples with IgA levels over 500 mg/dl, and 19 samples with IgA deficiency. Other samples tested included 10 samples EMA positive with a variety of titers, and the remaining 85 were antibody testing were run in the EIA tTG assay. Of these, 40 were A total of 125 samples submitted to the laboratory for endomysial antibody preparation adsorbed with primate tissues supplied by Inova Diagnostics and used according to the manufacturer’s instructions. The anti-human IgA conjugated antibody was an IgG conjugate taken from another Inova EIA assay kit. EMA slides containing monkey esophagus tissue were obtained from Scimedx and used according to the manufacturer’s instructions. The anti-human IgA fluorescent antibody was supplied in the kits. The anti-human IgG conjugated antibody was a specific antibody preparation adsorbed with primate tissues supplied by Inova Diagnostics. The IgG and IgA Gliadin 96 well microtiter plate kits were obtained from Inova Diagnostics and used according to the manufacturer’s instructions.

A total of 125 samples submitted to the laboratory for endomysial antibody testing were run in the EIA tTG assay. Of these, 40 were EMA positive with a variety of titers, and the remaining 85 were EMA negative. Other samples tested included 10 samples that were HCV antibody positive, 10 samples with IgA levels over 500 mg/dl, and 19 samples with decreased or absent IgA that were identified by performing IgA quantitations on 300 sequential samples submitted for endomysial antibody testing.

Results: Essentially all EMA+ Celiac disease patient samples had positive tTG results with the quantity of antibody present roughly correlated with the EMA titer. A few tTG positive, EMA negative samples were identified in the patient population and in a control group of samples with high IgA levels. The majority of these discrepant samples were probable false positive tTG assays and were from patients with evidence for other autoimmune diseases (ANA, SS-A, or Smooth Muscle antibody positive). Additional testing is ongoing in an effort to further characterize the tTG reactions for these samples.

All 19 low IgA serum samples submitted for EMA testing had negative IgA and IgG tTG, IgA and IgG EMA, and IgA gliadin antibodies, but about 50% had IgG gliadin antibodies. The IgG gliadin antibody positive results were not correlated with Celiac disease.

Conclusion: The tTG EIA assay results correlated well with the IFA EMA assay results and we now use the tTG assay for routine clinical testing because it is more rapid, less expensive, and less subjective than the EMA assay. Based on the 3% frequency of the “false positive” tTG results in our testing population and our in-house routine EMA positive rate of 6%, all samples to be tested in our laboratory are currently screened by the EIA tTG assay and then the specificity is confirmed with an IFA EMA assay.

Our studies of samples with decreased IgA levels indicated that IgG gliadin testing has too high a rate of false positive results to be clinically useful. Further studies with additional decreased IgA levels who have Celiac disease need to be performed in order to determine whether any of the IgG specific assays have a clinical role.

**REGENERATION AND TOLERANCE FACTOR (RTF): A CORRELATE OF HIV-ASSOCIATED T-CELL ACTIVATION**

T.S. Givens, B.K. DuChateau, J.S. Boomer, M.P. Westerman, A. Gilman-Sachs, K.D. Beaman

1Clinical Immunology Laboratory and Department of Microbiology/Immunology, Finch University of Health Sciences/The Chicago Medical School, North Chicago, IL 60064; 2Department of Medicine, Mount Sinai Hospital, Chicago, IL 60608

Objective: To determine the relationship between regeneration and tolerance factor (RTF) and expression of activation markers on CD4+ and CD8+ T-cells from human immunodeficiency virus (HIV)-infected individuals. HIV infection causes extensive phenotypic alterations in lymphocytes. Cellular markers that are normally absent or expressed at low levels on quiescent-T-cells are upregulated throughout the disease course. Recently, we reported that expression of the transmembrane form of regeneration and tolerance factor (RTF) was significantly increased on cells from HIV-seropositive (HIV+) compared to HIV-seronegative (HIV-) individuals. Because T-cells from HIV+ individuals express markers reflecting chronic activation, we hypothesized that these activated cells would coexpress RTF.

Methods: Sodium heparin anti-coagulated blood samples were collected from 26 HIV-infected and 18 uninfected individuals. Direct immunofluorescent staining of whole blood was performed using combinations of phycoerythrin (PE)-cyanin 5.1 (PC5)-conjugated anti-CD4 or anti-CD8, fluorescein isothiocyanate (FITC)-conjugated anti-RTF monoclonal antibody (mAb), and PE-conjugated anti-CD38 or anti-HLA-DR. Using three-color flow cytometric analysis, CD4+ and CD8+ T-cells were examined for dual expression of RTF and markers of activation (CD38 and HLA-DR). Plasma samples from HIV+ individuals were assessed for increased β2-microglobulin concentrations.

Results: The mean channel fluorescence (MCF) of RTF expression was higher on T-cells from HIV+ compared to HIV- (p <0.0001) individuals. HIV+ individuals had also higher percentages of RTF+CD38+ (p < 0.0001) and RTF+HLA-DR+ (p = 0.0001) CD4+ T-cells than HIV- individuals. In HIV+ individuals, increased percentages of CD4+ T-cells that were RTF+, RTF+CD38+, and RTF+HLA–DR+ correlated inversely with the absolute number and percentage of CD4+ T-cells and correlated positively with plasma β2-microglobulin concentrations. HIV+ individuals had higher percentages of CD8+ T that were RTF+CD38+ (p = 0.0001) and RTF+HLA–DR+ (p = 0.0010). In HIV+ individuals, the increased percentages of CD8+ T-cells that were RTF+HLA–DR+ correlated inversely with the percentage of CD4+ T-cells, and high percentages of CD8+ T-cells that were RTF+CD38+ correlated positively with plasma β2-microglobulin levels.
Conclusion: The depletion of CD4+ T-cells in HIV-infected individuals is strongly related to increased expression of RTF and activation markers. Hence, the presence of membrane-associated RTF may explain the vulnerability of CD4+ T-cells to activation-induced (and perhaps spontaneous) apoptosis during HIV infection. These findings strongly attest that increased RTF expression is a correlate of HIV-associated immune activation.

COMPARISON OF AN ENZYME IMMUNOASSAY (EIA) TO TWO INDIRECT FLUORESCENT ANTIBODY (IFA) TESTS FOR THE DETECTION OF IMMUNOGLOBULIN A ANTIBODY AGAINST ENDOMYSIUM

T.D. Jaskowski,1 C. Schroder,1 C.M. Litwin,1,2 T.B. Martins,1 and H.R. Hill1,2
1Associated Regional and University Pathologists (ARUP) Institute for Clinical and Experimental Pathology; 2Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT

Objective: Recently, the endomysial antigen has been identified as the protein cross-linking enzyme known as tissue transglutaminase (tTG). Our objective was to compare a novel enzyme immunoassay that detects IgA antibody against tTG to two standard IFA methods utilizing thin tissue sections of rat kidney/stomach (KS) and primate esophagus (PE) as substrates to detect IgA antibody against endomysium (EMA).

Methods: Sera from 100 patients suspected of having gluten-sensitive enteropathy (GSE) and 28 sera possessing various antibodies used for EIA cross-reactivity studies were included in the study. IFA tissue slides were purchased or donated by MarDx Diagnostics (Carlsbad, CA) and EIA for tTG were donated by INOVA Diagnostics (San Diego, CA). Additional tests performed routinely in our laboratory were utilized to further assess sera from patients suspected having GSE. These tests include IgA antigliadin antibody (AGA; an in-house developed EIA) and IgA antireticulin antibody (ARA; IFA tissue slides purchased from MarDx) and are part of the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) revised criteria for diagnosing GSE.

Results: When compared to IFA using KS, the tTG EIA had a sensitivity of 87.5%, was 97.1% specific and had an overall agreement of 94.0%. When compared to IFA using PE, the tTG EIA had a sensitivity of 91.7%, was 89.5% specific and an overall agreement of 90.0%. When the KS IFA was compared to the PE IFA for EMA, the KS IFA had a sensitivity of 95.8%, was 97.1% specific and had an overall agreement of 90.0%. When the KS IFA was compared to the PE IFA, the KS IFA had a sensitivity of 91.7%, was 89.5% specific and an overall agreement of 90.0%. The majority of sera that were positive for tTG, but negative by IFA (KS, n=2/PE, n=8) possessed IgA antibodies against gliadin and/or reticulin. Six of n these eight sera with negative results by PE IFA were positive by the KS IFA.

Conclusion: We conclude that the tTG EIA compares well to both KS and PE IFAs when detecting IgA antibody against endomysium. We do not recommend the use of the PE IFA to detect EMA since this IFA had many negative sera that were positive for tTG (n=8), AGA (n=7) and ARA (n=6).

EFFECTS OF HYDROXYCHLOROQUINE ON ACTIVATION OF PLATELETS BY ANTIPHOSPHOLIPID ANTIBODIES

Ricardo Espinola, Silvia Pierangeli, and E. Nigel Harris
Morehouse School of Medicine, Atlanta, GA

Objective: Antiphospholipid (aPL) antibodies have been associated with recurrent thrombosis (arterial and/or venous) and recurrent pregnancy loss. In previous studies, our group has demonstrated that aPL cause thrombosis in an animal model of thrombosis in vivo. Furthermore, prothrombotic properties of antiphospholipid (aPL) antibodies may be explained in part by their ability to enhance the activation of platelets pre-treated with low doses of ADP or thrombin. The antimalarial drug hydroxychloroquine (HQ) has been used successfully in prevention of post-operative thrombosis and in treatment of patients with SLE or APS. In one study, administration of (HQ) reversed the thrombogenic properties of aPL in mice. However, the mechanism of action of HQ in preventing thrombosis is not clearly understood. In order to explore this further, the effects of HQ on activation of platelets by aPL in the presence of a thrombin agonist was studied.

Method: Platelets from a healthy donor were treated with suboptimal doses of a thrombin agonist receptor peptide (TRAP) and affinity purified aPL antibodies (aPL), in the presence and in the absence of hydroxychloroquine (1 mM). The changes in the expression of GPIIb/IIIa (CD41a), GPIIb (CD42b), GPllla (CD61) and GMP140 (CD62p) on platelet membrane by flow cytometry (Cytoquant, Diagnostica Stago, France), were used as indicators of platelet activation.

Results:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression in MoAb Molecules/Platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>53412</td>
</tr>
<tr>
<td>GPIIIa</td>
<td>41921</td>
</tr>
<tr>
<td>GMP140</td>
<td>56480</td>
</tr>
<tr>
<td></td>
<td>749</td>
</tr>
</tbody>
</table>

Conclusion: The data shows that TRAP increased the expression of GPIIb/IIIa, GPIIIa and GMP140 and decreased the expression of GPIb, on platelet surface as expected. APL antibodies further enhanced the expression of GPIIb/IIIa and GPIIIa, but did not further affect expression of GPIb and GMP140. The effects of aPL and TRAP on expression of platelet surface markers of activation was completely abrogated by HQ, suggesting at least one possible mechanism by which HQ may prevent thrombosis. This may have important implications in treatment of thrombosis in APS patients.

COMPARISON OF FOUR ASSAYS FOR THE DETECTION OF ANTIPHOSPHOLIPID AND ANTI-B2GLYCOPROTEIN 1 ANTIBODIES

S.S. Pierangeli, L.K. Silva, I. Abreu, J. Byam, and E.N. Harris
Morehouse School of Medicine, Atlanta, GA; Louisville APL Diagnostics, Inc., Doraville, GA; and University of Lisbon, Lisbon, Portugal
Objective: The anticardiolipin (aCL) ELISA is the most frequently test used in the diagnosis of Antiphospholipid Syndrome (APS). Although a sensitive test, aCL ELISA may be positive in a variety of infectious (syphilis, HIV, Q fever) and autoimmune diseases (RA). It has been reported that the use of phosphatidylserine (PS), a mixture of phospholipids or β2glycoprotein 1 (β2GP1) alone as antigens (particularly when β2GP1 is coated onto oxidized plates), enables more specific detection of aPL antibodies associated with APS. This study proposes to compare the specificities and sensitivities of three commercially available kits: i) a Kit that utilizes β2GP1 coated on treated plates (INOVA Diagnostics, La Jolla, CA; ii) an antiphospholipid ELISA that utilizes a mixture of phospholipids, instead of CL alone (AphL® ELISA KIT, Louisville APL Diagnostics, Inc., Louisville, KY; and iii) a flow cytometry assay for detection of aCL and aPS antibodies (aCL/aPS® FACS Kit, Louisville APL Diagnostics, Inc), with the aCL ELISA (bench method).

Method: Eighty-nine samples with confirmed diagnosis of APS, 42 samples from “non-APS” patients (these including RA, syphilis, HIV, Q fever, other infectious diseases) were analyzed in this study. The samples were tested in the three commercial kits according to the manufacturer’s instructions and in the aCL ELISA bench as described elsewhere (Br J Haematol. 1990:74:1-9). Sensitivities (%) and specificities (%) were calculated for the four assays.

Results:

<table>
<thead>
<tr>
<th>Assay</th>
<th>Clinical Sensitivity (%)</th>
<th>Clinical Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCL ELISA (Bench)</td>
<td>96.6</td>
<td>79</td>
</tr>
<tr>
<td>anti-β2GP1 Kit</td>
<td>75</td>
<td>82</td>
</tr>
<tr>
<td>AphL ELISA Kit</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>aCL/aPS FACS Kit</td>
<td>96.6</td>
<td>84</td>
</tr>
</tbody>
</table>

Conclusion: The AphL ELISA Kit, the anti-β2GP1 Kit and the aCL/aP FACS Kit appear to be more specific than the aCL ELISA for the detection of aPL antibodies present in APS. The aCL, the AphL ELISA Kit and the aCL/aPS FACS Kit were superior in sensitivity to the anti-β2GP1 Kit. ACL ELISA may be used for the initial screening of patients with APS and a more specific assay (such as the AphL ELISA Kit the aCL/aPS FACS Kit or the anti-β2GP1 ELISA may be used for the confirmation of APS.

NEW FULLY AUTOMATED ELISA ON THE COBAS CORE™ FOR THE DETECTION OF RHEUMATOID FACTORS (ALL CLASSES), IGA-RF AND IGM-RF. CORRELATION TO AN AGGLUTINATION ASSAY

H. Faust, C. Geier, H.P. Lehmann, G. Schneider, and J.W. Zolg
F. Hoffmann-La Roche Ltd, Roche Diagnostics, Basel, Switzerland

Objective: The performance the COBAS Core RF (IgG, IgM, IgA), RF (IgA), and RF (IgM) EIAs has been compared to an established agglutination assay (COBAS INTEGRA RF Latex). The COBAS Core RF EIA Quanti is part of the so-called “Rheuma Information Level” (COBAS Core Anti-β2-GP1 EIA, CRP EIA Quanti, RF EIA Quanti and HEP2 ANA EIA) intended for use on the fully automated Cobas Core immunoanalyzer for “screening” of patients with suspected systemic autoimmune disorders.

Methods: Sensitivities of the three EIAs and correlation coefficients compared to the COBAS Integra assay were determined by testing 70 selected samples from rheumatoid arthritis (RA) patients.

Results: The following sensitivities were obtained: 90.0%, 61.4%, 92.9% and 91.4% for the COBAS Core RF (IgG, IgM, IgA), RF (IgA), RF (IgM) EIAs and the COBAS Integra RF Latex, respectively. The obtained correlation coefficients are: 0.891, 0.485, 0.911 for the COBAS Core RF (IgG, IgM, IgA), RF (IgA), RF (IgM) EIAs, respectively compared to the COBAS Integra RF Latex.

Conclusion: Sensitivities of the four investigated assays are comparable. The data correlate well, indicating that all three assays predominantly detect RF-IgM. The COBAS Core system additionally allows the detection of IgA-RF. Its clinical relevance in RA patients has been shown in different clinical studies.

THE EXPRESSION OF REGENERATION AND TOLERANCE-FACTOR (RTF) CORRELATES WITH HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION AND NOT WITH HEPATITIS C VIRUS (HCV) INFECTION

C.C. Sung,1 T.S.Givens,1 B.K. Duchateau,1 J.S. Boomer,1 M.P. Westerman,2 A.Gillman-Sachs,4 and K. D. Beaman4
1Clinical Immunology laboratory, Finch University of Health Sciences/The Chicago Medical School; 2Mount Sinai Hospital, Chicago, IL

Objective: Regeneration and tolerance factor (RTF) is a protein with potent immunomodulating properties. It has been suggested that RTF may participate in driving or sustaining a Th 2 cytokine response. Progression to AIDS is thought to be related to a Th 2 type response. Interestingly, HIV-infected patients coinfected with hepatitis C virus (HCV) had increased percentages of CD3+T-cells expressing RTF as compared to patients with HIV and no secondary infection. Whether the increased expression of RTF in those patients is due to HCV coinfection is unclear. The purpose of this study is to determine if increased expression of RTF is due to HIV infection or if another viral infection would cause similar results.

Methods: Three-color flow cytometric analyses was performed on peripheral venous blood collected in sodium heparin anticoagulated tubes. The appropriate local institutional review boards approved this study. Samples from 25 HIV infected subjects and 4 HCV seropositive individuals as well as peripheral blood samples obtained from 18 normal individuals at FINCH University of Health Sciences/The Chicago Medical School were studied. Within 24 hours after collection, 100 µl aliquots of blood were incubated with 10 µl of antibodies (Coulter) to either: CD45-FITC/CD14-PE, IgG1-FITC/IgG1-PE as isotype controls, CD3-PC5, CD4-PC5, CD8-PC5. Next, 25 µl of FITC conjugated RTF monoclonal antibody was added. After labeling, samples were washed, lysed using the Coulter Clone Immuno-Lyse reagents and immediately analyzed using a Coulter Epics XL-MCL flow cytometry after fixation.
Results: The mean percentage of CD4+ T-cells expressing high levels of RTF was 26.01 ± 2.97 for HIV infected individuals, 6.05 ± 2.95 for HCV seropositive and 9.23 ± 1.04 for normal individuals. For the CD8+ T-cells, the mean percentage of cells expressing RTF is 33.76 ± 3.17 for HIV infected individuals, 7.88 ± 5.32 for HCV seropositive and 16.77 ± 2.67 for normal individuals. Two-fold increase in the percentage of CD3+ cells expressing RTF was seen in individuals with both HIV and HCV infection as compared to individuals with HIV but no HCV infection.

Conclusions: The expression of RTF is upregulated on CD4+ and CD8+ lymphocytes from HIV infected patients as compared to HIV infected or normal individuals. Importantly, increased expression of RTF correlated with the presence of the secondary infection (HCV infection) in these HIV infected individuals. These data suggest that the expression of RTF may be a clinically relevant marker for monitoring the health of patients with HIV. The expression of regeneration and tolerance factor (RTF) correlates with human immunodeficiency virus (HIV) infection and not with hepatitis C virus infection.

EVALUATION OF THE SERODIA® TP-PA GELATIN PARTICLE AGGLUTINATION TEST FOR THE DETECTION OF ANTIBODIES TO TREPONEMAL PALLIDUM

P.T. Tsui, R. Cadotte, and R. Emmadi

Divisions of Immunology and Molecular Diagnostics, Cook County Hospital, Chicago, IL

Objective: We have evaluated the Serodia Particle Agglutination Test (PA) for the detection of TP Antibodies and compared to Enzyme immunoassay (TP-EIA) and Microhemagglutination (TP-MHA) Methods.

Methods: The TP-PA test was performed in parallel with TP-EIA and TP-MHA on a total of 247 serum samples. The sera used included sera previously found to contain non-treponemal and/or treponemal antibodies, sera submitted to our laboratory for tests other than serological tests for syphilis, sera containing antibodies to HIV and/or hepatitis, anti-nuclear antibodies, rheumatoid factor and sera from IV drug users. One serum supplied by the manufacturer was used as a confirmatory standard.

Results: TP-EIA positive samples were assayed by TP-PA methodology and an overall concordance of 99.6% was determined. For non-reactive samples the specificity was 99%. One sample was weakly reactive by EIA and negative by RPR, MHA and PA methods. Among reactive TP-PA and TP-MHA samples using score of 1+ to 4+, a 94.3% agreement was found. All reactive MHA samples were tested reactive by TP-PA. One sample was TP-PA positive, MHA non-reactive and TP-EIA equivocal. Within assay duplicate testing of 10 weak reactive and 12 strong reactive sera, TP-PA and TP-MHA yielded endpoint titers within 1 to 2 doubling dilution on a single assay run.

Conclusion: Our observation to date suggests TP-PA test is in good correlation with TP-EIA and is more sensitive and specific than TP-MHA for the detection of Treponemal antibodies in serum. TP-MHA may be replaced by TP-PA as a confirmatory test.

THE EFFECT OF PROTEIN KINASE ACTIVATORS/INHIBITORS ON TH1 AND TH2 CYTOKINES INDUCTION

S. Yousefi, H. Enomoto, J. Ocariz, N. Vaziri, and T. Cesario

University of California, Irvine, Irvine, CA

Objectives: We have previously shown that the nature of the inducing agent significantly influences whether Th1 or Th2 cytokines will predominant in the culture of human peripheral blood mononuclear cells (PBMC). Lately we have been studying the effect of protein kinase C (PKC) activators/inhibitors on the Th1 and Th2 cytokines induction by hPBMC.

Methods: Freshly isolated hPBMC were induced using A23187 in the presence and absence of PKC activators/inhibitors. Samples of supernatants and mRNAs were harvested at different time intervals. Supernatants were tested for specific cytokines using ELISA assays. mRNA samples were transcribed and amplified by semi-quantitative PCR.

Results: We demonstrated that induction of gamma interferon (IFN) and IL-2 (Th1 cytokines We) were enhanced ten fold or more in the presence of PMA or Mezerein (PKC activators). Yields of IL-4, IL-5 and IL-10 were either not enhanced at all (IL-4) or increased less than five fold (IL-5/IL-10). The presence of PMA was also shown to enhance accumulation of mRNA for gamma IFN, IL-2, IL-5 and IL-10 but not IL-4. Whereas Staurosporin and Bis-indolylmalideimide (PKC inhibitors), both reduced yield of all five cytokines over a range of normal concentrations. Yields of IL-5, however, were statistically significantly less affected than the yields of other cytokines.

Conclusion: IL-2 and gamma IFN are more affected by PKC activators than are IL-4, IL-5 and IL-10. The changes in cytokine protein levels are reflected in the accumulation of cytokine specific mRNA. The effect of PKC activator on cytokine specific mRNA may differ somewhat in different individuals. This could be a reflection of the number of the specific cell types. Activation of PKC was associated with a slightly longer half life of the mRNA for all cytokines tested.
Information for Contributors

In general, the style adheres to the *ASM Style Manual for Journals and Books*. As a backup, follow *The Chicago Manual of Style*.

**Case Reports**

Reports documenting interesting case presentations are invited for possible publication in the *Clinical Immunology Newsletter*. Submitted case reports should be written concisely and should contain:

a) a brief clinical history summarizing the symptoms and course of the illness; b) a description of immunologic and other laboratory tests performed; and c) the results of these laboratory tests as related to the clinical observation of patients and the outcome of the disease process and therapy, if appropriate.

**References**

Authors are responsible for the accuracy of references, which should include complete publication information. References should be numbered consecutively in order of citation, and cited in the text by those numbers. Some typical examples of references are listed below. Consult this issue for additional examples of *Newsletter* reference format.


**Letters**

Letters expressing opinion(s) or offering technical ideas and procedures will be considered for publication (subject to editing) provided they are signed by all authors and do not exceed two typewritten (double-spaced) pages.

**Questions and Answers**

Questions about immunologic techniques, laboratory procedures for immunology, and general problems in clinical immunology are welcome. They will be answered by the editorial board or by selected individuals in the field.

**Information about Meetings**

Announcements or reports of meetings and conferences of interest to clinical laboratory immunologists will also be considered for publication.

**Preparation and Submission of Material**

All material submitted for publication in the *Newsletter* should be typed double-spaced (including references) on standard 8 1/2 x 11 paper. Please send the original typescript along with two copies and diskette to:

M.R.G. O’Gorman, M.Sc., Ph.D., Editor in Chief
Assistant Professor of Pediatrics
Northwestern University
Director, Diagnostic Immunology and Flow Cytometry Laboratories
Children's Memorial Hospital
2300 Children’s Plaza
Chicago IL 60614

**Reprints**

The corresponding author of each article will receive 3 offprints free of charge. Additional offprints may be ordered at the rates listed on the order form accompanying proofs.
Upcoming Articles

- HIV Therapeutic Vaccines
- Homocysteine and Immune Injury
- Immune Reconstitution After Combination Retroviral Therapy
- Fibrin(ogen) and Inflammation: Current Understanding and New Perspectives
- Phagocytic Recognition of Apoptotic Leukocytes
- Flow Cytometry-based Procedures for the Measurement of Apoptosis Applicable to the Investigation of HIV Infection