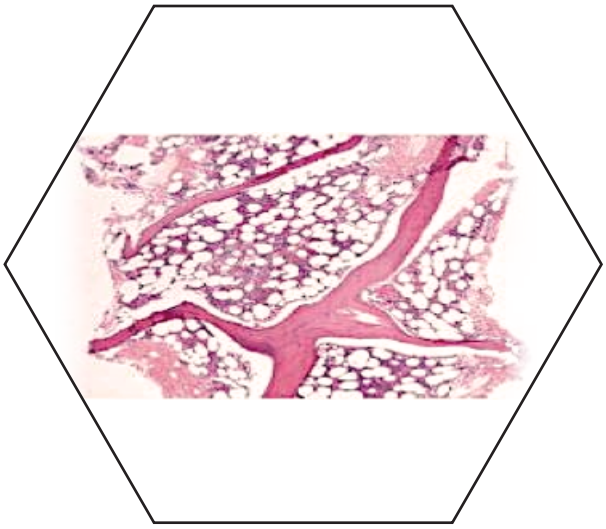

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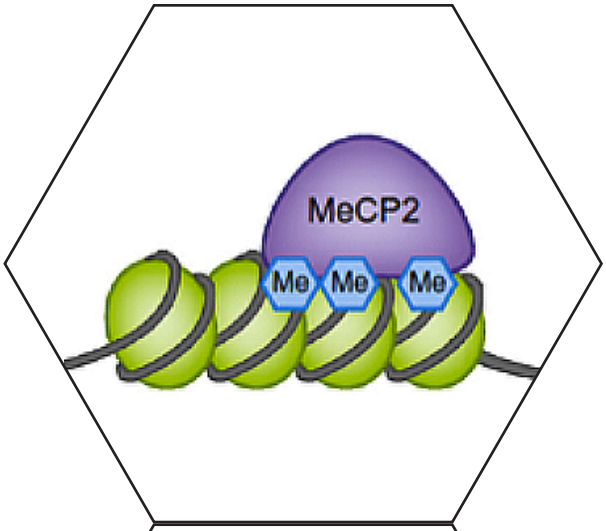

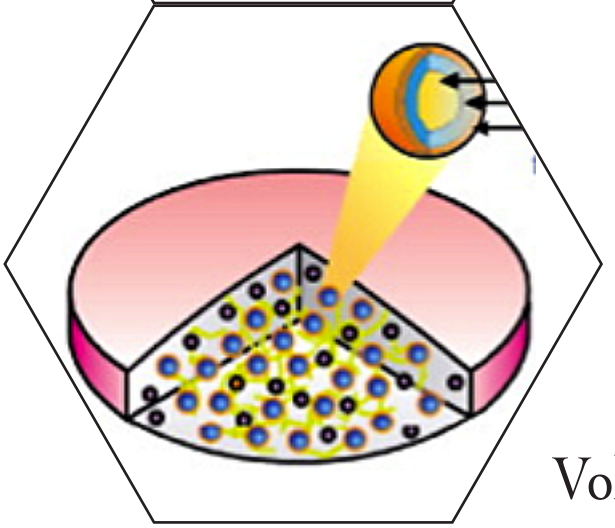

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The Lander College of Arts and Sciences at Touro in Flatbush

Throughout its 36-year history, Touro's Lander College of Arts and Sciences in Flatbush (with separate men's and women's schools) has provided cohorts of aspiring high school graduates from well-regarded yeshivas and seminaries with a foundation of academic excellence for professional career growth, in an environment that is supportive of the religious values of its students. Graduates have assumed leadership roles and continue to strengthen Jewish communities throughout the world.

Lander College of Arts and Sciences–Flatbush offers more than 25 majors and preprofessional options, and three joint undergraduate/graduate degree programs in occupational therapy, physical therapy and physician assistant studies with the School of Health Sciences. Honors tracks in biology, the health sciences, political science and psychology are currently offered.

Students are also required to complete a carefully designed core curriculum that emphasizes the development of communications skills, critical thinking and analytical competencies, computer literacy and quantitative reasoning. Enrollment in science courses, notably biology and chemistry, continues to increase, reflecting the career interests of premedical and health science students.

Faculty members continue to earn recognition for outstanding achievements, including Joshua November, Assistant Professor of Languages and Literature, who was selected as a finalist for the Los Angeles Times Poetry Book of the Year Prize in 2011; Karen Sutton, Assistant Professor of History, whose significant Holocaust analysis, The Massacre of the Jews of Lithuania, 1941–44, was published in 2008; and Atara Grenadir, Assistant Professor of Art, whose works were displayed at the Art Expo 2011 show in New York City.

Notable alumni distinctions of Touro's Lander College of Arts and Sciences in Flatbush include: David Greenfield (JD, Georgetown), elected to the New York City Council (44th Council District) in 2010; Dr. Israel Deutsch (MD, Einstein), appointed as Director of Brachytherapy at New York–Presbyterian Hospital/Columbia University; Yossi N. Heber (MBA, Wharton), President, Oxford Hill Partners; Dr. Haim Mozes (PhD, NYU), Associate Professor, Graduate School of Business, Fordham University; Vivian Schneck-Last, Managing Director, Goldman Sachs; and Sara Grossman Wiederblank, who published her fourth novel, Pass or Fail, in 2010. Alumni have published articles in the New York Law Journal, Bloomberg Law Reports, Institutional Investors Journal and other peer-reviewed journals.

The SCIENCE JOURNAL of the Lander College of Arts and Sciences-Flatbush

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Cover Pictures

Top: Efrat Bruck

Middle: Aliza Grossman Rubenstein

Bottom: Chedva Farkas

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GENETICALLY MODIFIED T-CELLS EXPRESSING CHIMERIC ANTIGEN RECEPTORS IN THE TREATMENT OF CANCER

Efrat Bruck

ABSTRACT

Dr. Carl June and his colleagues at the University of Pennsylvania have succeeded in treating patients with Chronic Lymphocytic Leukemia using gene therapy. Two of the three patients treated sustained a complete remission and one a partial remission. The procedure involved transducing the patients' T cells to express chimeric antigen receptors which target a particular protein found on both healthy and cancerous B cells. Following infusion of the newly transduced T cells, each patient developed clinical symptoms associated with an intense immune response. Shortly thereafter, tumors were completely eliminated in two of the patients and partially eliminated in the third. All three patients were pre-treated with conventional therapies and responded poorly. This study coalesces volumes of research in genetics, immunology, and molecular biology in what might just be the future of cancer treatment.

INTRODUCTION

Harnessing the body's own immune system to battle cancer has always been understood by medical scientists as the ideal avenue for treatment. An astounding Phase-1 Clinical Trial recently conducted at the University of Pennsylvania (UP) may prove to be a breakthrough in this area. The study represents a culmination of twenty years of intense research in immunology and gene therapy. Dr. Carl June and his colleagues at UP treated three patients having advanced chemotherapy-resistant CLL (chronic lymphocytic leukemia), a cancer of B-Cells. The trial involved transducing the patients' own T-cells to express chimeric antigen receptors, a fairly recent biomolecular invention, generally referred to as CARs (June et al. 2011). CARs, genetically engineered receptors, are generally comprised of a constant region that is similar to that of any T-cell receptor and a variable region that targets a specific antigen (Eshhar et al. 1989). With the correct genetic engineering and biomolecular construction, T-cells can be induced to express CARs that target a gamut of antigens. In the trial led by Dr. June, the patients' T-cells were transduced via an HIV-1 based lentiviral vector to express a CAR with specificity for CD19 (cluster of differentiation 19), a protein found on both healthy and cancerous B-cells. The modified cells are referred to as CART19 (chimeric antigen receptor T 19) cells.

Following exogenous transduction, the patients' T-cells were infused intravenously. Within one to three weeks, all three patients developed symptoms that were indicative of a serious immune response. The CAR T-cells expanded *in vivo* at least a thousand fold and successfully eliminated both healthy and cancerous B-cells. The effector-to-target (E/T) ratios observed in the patients were 1: 93,000; 1:2,200; and 1:1,000. Hence, in the patient with an E/T ratio of 1:93,000, an infusion of 1.4 x 10^{11} CART19 cells resulted in the remarkable elimination of 1.3 x 10^{12} CLL cells. Two of the three patients sustained complete remission, and one patient experienced a partial remission. Furthermore, in the two patients achieving complete remission, a population of memory CART19 cells was observed months after treatment, indicating the possibility of prolonged immuno-surveillance (June et al. 2011).

Efrat Bruck, B.S. '12, majored in Honors Biology.

CHIMERIC ANTIGEN RECEPTORS

The original construct of T-cells expressing chimeric antigen receptors was designed two decades ago by Dr. Zelig Eshhar and his colleagues at the Weizmann Institute of Science (Bridgeman et al. 2010). Their CAR T-cell was transduced with rearranged gene segments coding for the variable domain (V_H and V_L chains) of an anti-trinitrophenyl antibody attached to constant region (either alpha or beta) of a T-cell receptor. The transmembrane and cytoplasmic domains of a typical T-cell receptor were maintained. The resulting T-cells then produced an efficient immune response when exposed to trinitrophenyl. In response to the hapten, the CAR T-cells proliferated, produced interleukin 2, and targeted cell lysis (Eshhar et al. 1989).

The fact that T-cells can be endowed with antibody-type specificity is highly significant. Typically, T-cells only respond to an antigen that is bound to MHC (major histocompatibility) protein, either I or II. When antigen-presenting cells, such as macrophages; B cells; and dendritic cells, encounter an antigen, they process it. The procedure involves ingesting the foreign molecule, synthesizing an MHC molecule, fusing the two, and attaching this antigen-MHC complex to the plasma membrane of the cell. T-cells only respond to an antigen presented in this way. More specifically, CD4 cells react to antigens bound to MHC-II molecules, and CD8 cells respond to antigens that are bound to MHC-I molecules. The necessity of having an antigen bound to an MHC molecule in order to elicit an immune response in T-cells is known as MHC restriction (Tortora and Derrickson 2009).

MHC restriction can be circumvented with CARs. Since the CAR has the variable region of an antibody, it acts as one. Antibodies react with antigens in their native state to elicit an immune response. Thus, CARs can be used to target antigens that would normally not be presented with an MHC molecule (Eshhar et al. 1989), such as in the UP trial where the CARs targeted the CD19 protein found on B cells. In addition, in situations where MHC-I may be somewhat down-regulated by tumors as part of a strategy to inhibit immuno-surveillance, the use of CARs in the treatment of cancer may be more favorable (Bridgeman et al. 2010).

T-Cell activation usually requires two signals. One is delivered by the TCR-CD3 complex which interacts with the MHC-antigen complex. The other is delivered by co-stimulatory domain CD28 when it interacts with the co-stimulatory molecules (such as CD80 or CD86) found on the antigen presenting cells. Other co-stimulatory domains, such as CD137 or CD134 may also be necessary.

The optimal construction of CARs has been explored during the past two decades (Eshhar et al. 2001). Naturally, the precise design would depend on the target antigen and efficacy of the CAR. As illustrated in Figure 1, first generation CARs consist of the single-chain variable fragment (scFv) of an immunoglobulin specific for antigen (usually a tumor antigen), bound to a hinge region that crosses the cell membrane. The hinge region is attached only to the CD3- ζ chain of the TCR-CD3 complex which plunges into the cytoplasm and acts as the signaling domain.

To enhance effectiveness, second generation CARs have a co-stimulatory signaling domain, such as CD28, CD137 (4-1 BB), or OX40, inserted between the hinge region and the CD3- ζ chain (Urba and Longo 2011). For example, inclusion of a CD137 co-stimulatory signaling domain significantly enhanced *in vivo* persistence of CARs and antitumor activity in preclinical trials (Kalos et al. 2011, as described in

June et al. 2009). Third generation CARs incorporate various combinations of co-stimulatory domains (Urba and Longo 2011).

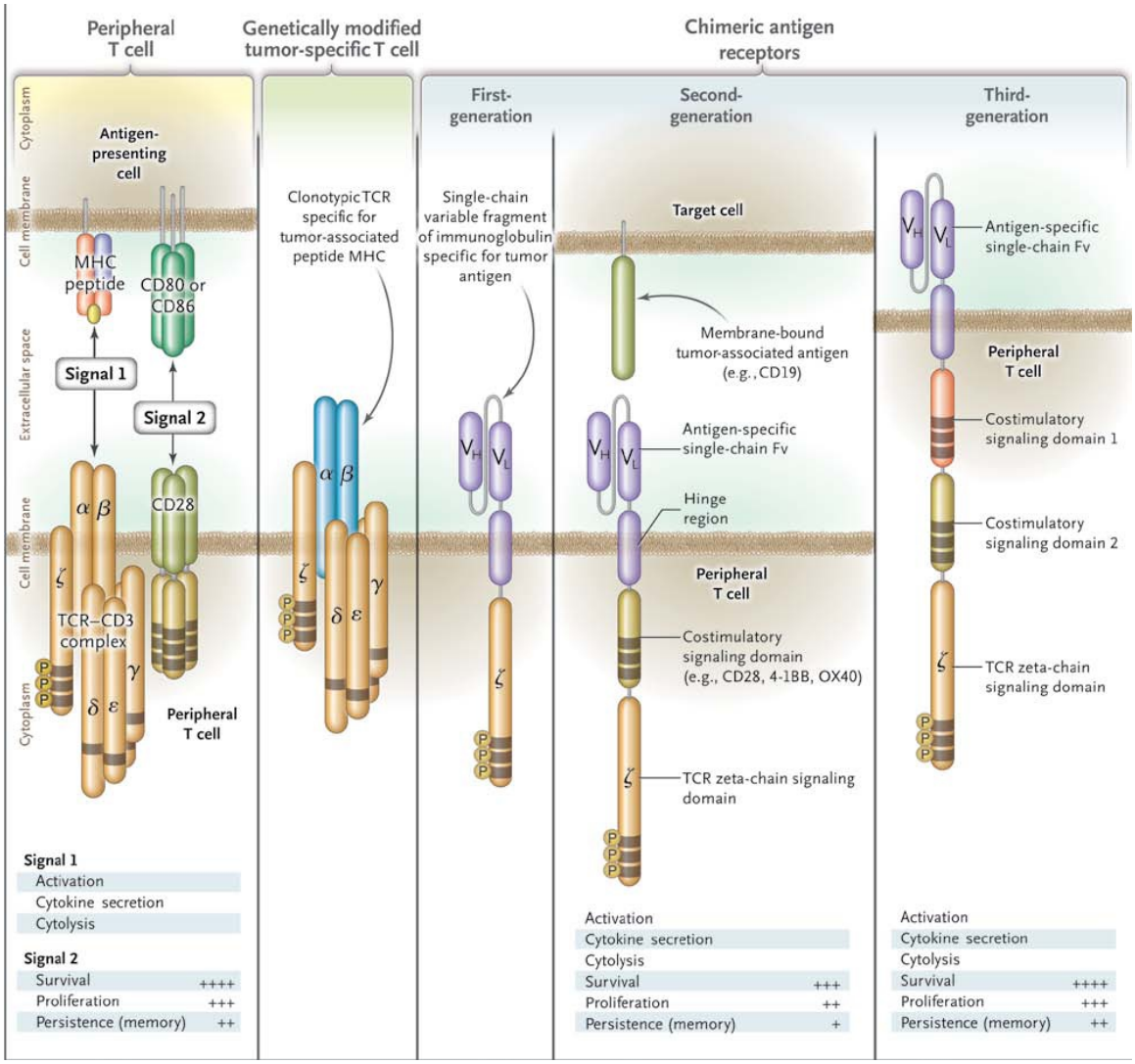

Figure 1: T-Cell Activation.
"Optimal T-cell activation requires a minimum of two signals. Signal 1 is delivered by the TCR–CD3 complex through interaction of the T-cell receptor (TCR) alpha and beta chains as they recognize peptide presented by a class I (CD8 T cells) or class II (CD4 T cells) major histocompatibility complex (MHC) molecule. Signal 2 is most commonly provided by the engagement of CD28 on the T cell with the costimulatory molecule CD80 or CD86 on the antigen-presenting cell. CD137 (4-1BB) and CD134 (OX40) also provide costimulation to T cells. The optimal combination of effector function, proliferation, and survival requires both signals. Delivery of signal 1 without costimulation, which often occurs for tumor-infiltrating lymphocytes and transgenic T cells encountering antigen on a solid tumor devoid of costimulatory molecules, leads to anergy and apoptosis, thereby limiting the antitumor response. The first-generation chimeric antigen receptors usually comprise of a single-chain variable fragment of an antibody specific for tumor antigen, linked to the transmembrane and intracellular signaling domain of CD3-zeta. Second-generation chimeric antigen receptors were developed to incorporate the signaling domain of a costimulatory molecule to improve T-cell activation and expansion. Third-generation chimeric antigen receptors include combinations of costimulatory domains." (Data are from Keith Bahjat, Ph.D.) Source: Urba and Longo 2011

Currently, expression of CARs on non T-cells is limited due to difficulties in transfection, although developments are underway. Furthermore, CARs only target molecules expressed on cell surfaces. Thus, intracellular tumor specific antigens would require a different approach (Bridgeman et al. 2010).

HIV-1 Based Lentiviral Vectors

A key factor in effectively transducing T-cells, as in any other aspect of gene therapy, is choice of the vector. Safety; long-term stability; versatility; and, sometimes, the ability to transduce non-dividing cells are all taken into account (Lu et al. 2004). In the UP trial, an HIV-1 based lentiviral vector was used. Lentiviruses (lenti is the Latin word for "slow") are thus named because of the long incubation period between infection and the onset of disease. What makes lentiviruses unique among retroviruses is their ability to infect non-dividing cells (Durand and Cimarelli 2011). Typically, a retrovirus must wait until the S phase of the cell cycle when it is afforded the opportunity of penetrating the nuclear membrane. Thus, it cannot infect the cell unless the cell is dividing. However, lentiviruses have the ability to integrate into the host's genome by penetrating the nuclear membrane on their own (Lu et al. 2004). This makes them highly useful in gene therapy whose main targets include the brain, lungs, liver, muscles, and hematopoietic system (Zufferey et al. 1998). In addition, transcriptional silencing has been observed in the use of onco-retroviral vectors and not in the use of lentiviral vectors. In fact, lentiviral vectors have successfully integrated into a variety of tissues (Vigna and Naldini 2000). Furthermore, these vectors are capable of carrying large transgenes of up to 18,000 bases (Coleman et al. 2003). One drawback of lentiviral vectors is that they integrate into the host's genome at random locations. Sometimes, this may activate nearby oncogenes. A number of patients in a clinical trial undergoing gene therapy for SCID – γC (severe combined immunodeficiency) syndrome developed leukemia as a result of the use of lentiviruses (Durand and Cimarelli 2011). Only one virus is known to incorporate itself at a specific site in the human genome—the adeno-associated virus that partially integrates into the human chromosome 19q13.42. Scientists are attempting to find a way to transpose the site-specificity of the adeno-associated virus to lentiviruses (Durand and Cimarelli 2011).

HIV Structure

The HIV virus (Figure 2) contains two copies of a single-stranded RNA measuring 9,749 nucleotides long enclosed in a capsid. Bound to the RNA are nucleocapsid proteins p6 and p7 (which prevent the RNA from digestion by nucleases) and enzymes such as reverse transcriptase and integrase. These enzymes allow the virus to transcribe DNA off its RNA and then integrate into

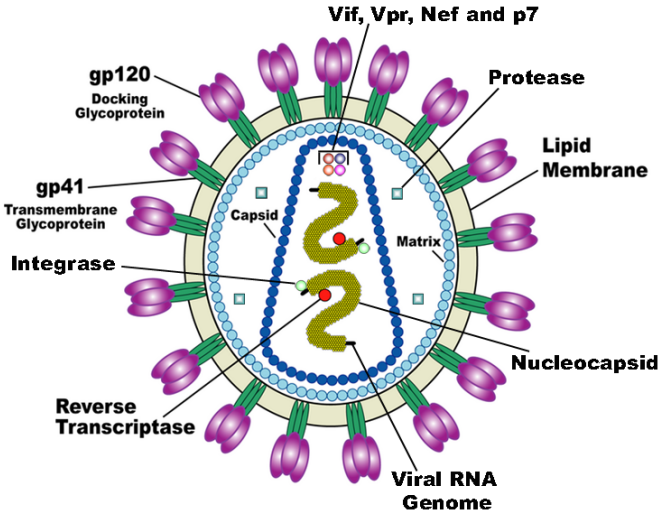

Figure 2: HIV Structure. Source: Henderson C.

the host's genetic material, respectively (Kuiken et al. 2008). Along the viral envelope are protein units that aid in attachment to the host cell. Each unit consists of three transmembrane subunits of glycoprotein 41 attached to three external subunits of glycoprotein 120 (Chan et al. 1997). Surrounding the capsid and anchoring these glycoproteins into the envelope is a matrix composed of the protein p17 (Kuiken et al. 2008).

HIV Genome

The typical HIV genome contains nine genes flanked by LTRs (long terminal repeats). The *gag*, *pol*, and *env* genes code for viral structural proteins. *Gag* codes for a p17 presursor, capsid protein p24, necleocapsid proteins p6 and p7, and spacer peptides. *Pol* codes for HIV protease, integrase, and reverse transcriptase. The *env* (envelope) gene codes for glycoprotein 160, a precursor to gp41 and gp 120 which are necessary for viral attachment to the host cell (Watts et al. 2009).

Tat and *rev* are two regulatory genes. *Tat* activates expression of the viral RNA, and *rev* promotes cytoplasmic export of *gag*, *pol*, and *env* transcripts. *Vif*, *nef*, *vpu*, and *vpr* are accessory genes critical for pathogenesis but not for replication (Vigna and Naldini 2000).

Construction of Safe HIV-1 Based Vectors

Constructing safe vectors presents many challenges. The virus must retain those genes necessary for efficient transduction of target cells. At the same time, genes that would enable the virus to reproduce and infect other cells following transduction must be eliminated.

Effective transduction using the two-plasmid production approach involves the introduction of two genomic constructs into the target cell: a transfer vector construct (Figure 3) and a packaging construct, also known as the helper plasmid (Figure 4). The transfer vector construct contains *cis*- acting sequences that are vital for the transfer and integration of the

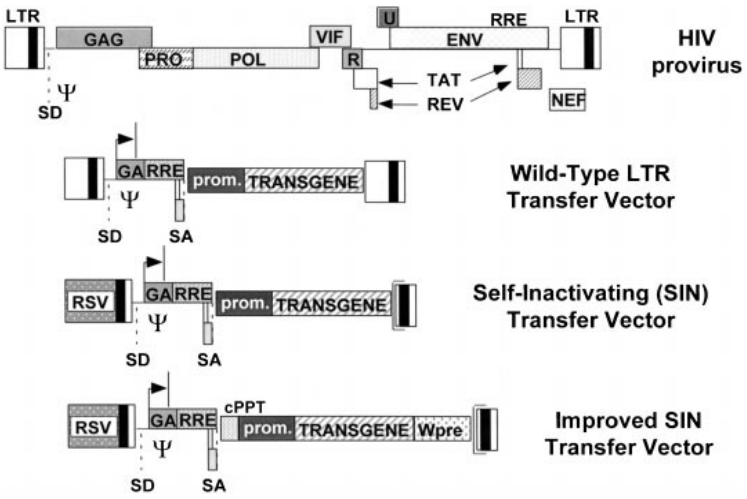

Figure 3: Various HIV-1 derived transfer vector constructs. Source: Vigna and Naldini 2000

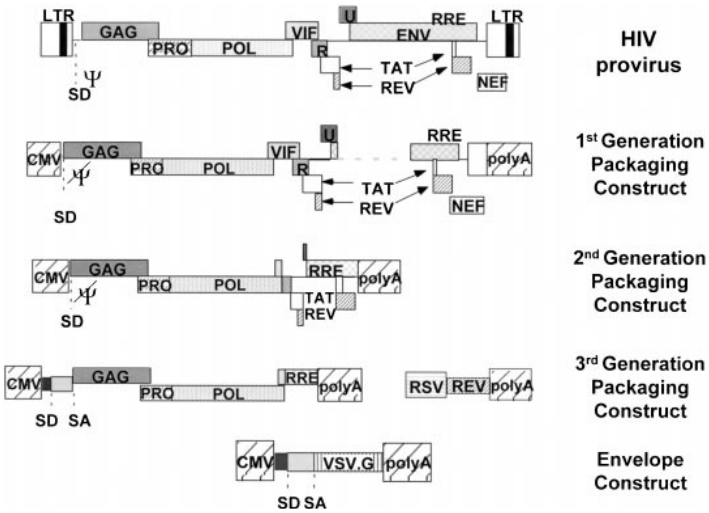

Figure 4: Various HIV-1 derived packaging constructs/helper plasmids. Source: Vigna and Naldini 2000

viral genome into a host's genome. The transgene is hooked onto the transfer vector construct. The packaging construct contains *trans*-acting genes that code for the essential viral proteins. When both constructs are introduced into the same cell, they express proteins necessary for encapsulation and integration of the transfer vector. As these vectors are carefully engineered to adhere to the highest standards of biosafety, this infectious process happens only once. This is due to efficient removal of genetic material that would enable the virus to proliferate and infect other cells.

In first generation HIV-1 derived packaging constructs, all genes necessary for the production of structural and accessory proteins are maintained; only *env* is eliminated. In second-generation constructs, the accessory genes, *vif*, *nef*, *vpu*, and *vpr* are also absent. In the third generation, *tat* and *rev* are eliminated as well, although *rev* is expressed by a separate construct that is flanked by a rous sarcoma virus promoter and a polyadenylation signal. Thus, only three out of the original nine genes are expressed.

Transfer vectors house the transgene as well as GA and RRE, which are placed adjacently between an LTR and the internal promoter. GA is a derivative of the *gag* gene, while RRE (rev-responsive element) is a portion of the *env* gene (Vigna and Naldini 2000).

Elimination of most viral genes is necessary in order to avoid RCR (replication competent recombinants), which can happen if the *trans*-acting viral genes in the packaging constructs merge with the *cis*-acting sequences in the transfer vector. In fact, the biosafety of a vector depends on the extent of successful separation between the functions of the *cis*-acting and *trans*-acting viral genomes that comprise the transfer vector construct and packaging construct, respectively.

To avoid the emergence of RCR, self-inactivating (SIN) transfer vectors have been designed. In a wild-type LTR transfer vector (non-SIN), the LTRs are maintained. In the SIN transfer vector, there is a large deletion in the U3 region of the 3' LTR. Upon transduction, this deletion is duplicated upstream, resulting in inactivation of both LTRs (Vigna and Naldini 2000). Thus, during reverse transcription, the deletion is transferred to the proviral DNA, resulting in ineffective transcription of the LTRs. This avoids the hazardous production of complete vector RNA, since the LTRs contain promoter and enhancer sequences. In addition, sabotage of the LTRs prevents aberrant expression of sequences in the host's genome that are adjacent to the vector integration site. Transcription of the transgene is, instead, driven by an internal promoter that is nestled safely away from the cell's native genome (Zufferey et al. 1998).

The improved SIN transfer vector has some additional constituents. The central polypurine tract (cPPT) sequence (which provides increased transduction efficiency and transgene expression) of the *pol* gene is inserted just before the internal promoter in order to improve gene transfer performance. The post-transcriptional regulatory element of the woodchuck hepatitis virus (Wpre), which improves the performance of vectors, is inserted just after the transgene to enhance its expression (Vigna and Naldini 2000).

Lentiviral vectors, as observed by stable expression of marker genes *in vivo*, can successfully transduce a spectrum of cells. Neurons of adult rat brains were the first cells in which stable expression of lentiviral tranduction was observed (Vigna and

Naldini 2000, as described in Verma et al. 1996). Other cells that have been successfully transduced with lentiviral vectors include cells of the retina, liver cells of rodents (Miyoshi et al. 1997), human dendritic cells and macrophages (Schroers et al. 2000), and human CD34+ and CD38- hematopoietic cells (Case et al. 1999).

First Lentiviral Vector Evaluated in Human Cells,

VRX496 and its sister VRX496 were the first lentiviral vectors to be evaluated in human cells. The trial was conducted by Xiaobin Lu and his colleagues at the Sydney Kimmel Comprehensive Cancer Center of the John Hopkins School of Medicine. The HIV *env* gene is not eliminated from VRX496 and 496; instead, these vectors express a 937-base antisense to silence the gene. VRX496 contains a 186-base sequence that acts as a molecular marker and is derived from the Green Fluorescence Protein (GFP) gene. VRX496 has an enhanced GFP gene.

The cells transduced in this experiment were human CD4+ T lymphocytes. Human blood was obtained, and CD4+ cells were isolated with the magnetic activated cell-sorting system. Flow cytometry indicated a purity >95%. The cells were then cultured in X-vivo 15 media containing 10% human serum and the antibiotic gentamycin. In preparation for transduction, the cells were plated in a 24-well plate at 1 x 10^6 cells per well. VRX496 was added to the cells at 20 tranducing units per cell. Concurrently, to ensure activation and expansion of the cells, immobilized anti-CD3/CD28 (iCD3/28) antibodies were added at a ratio of three beads per cell as well as 100 U/ml of interleukin 2. The cells were then cultured for three days during which they were washed three times to remove the vector. The iCD3/28 beads were removed four days later. Finally, the lymphocytes were replated at half a million cells per ml. At this point, the culture was able to be maintained for a significant amount of time.

After seven days in culture, the tranduced cells were assessed for GFP expression which would indicate successful integration. Flow cytometry indicated that 99.4% of tranduced cells were positive for vector gene expression, while 99.9% of the control cells were negative.

To measure stability of transduction, another batch of CD4+ T lymphocytes were transduced at 20 TU/cell as well. The cells were allowed to expand 1.5-million-fold over 36 days during which GFP expression was monitored. GFP expression remained stable throughout the entire culture period, demonstrating consistent vector payload expression. To date, no RCR generation has been reported in the use of VRX496 and VRX496 (Lu et al. 2004).

Chimeric Antigen Receptors and Lentiviral Vectors in the Treatment of Cancer

The recent pilot study conducted by Dr. Carl June and his colleagues at UP involved three patients with chemotherapy-resistant CLL. Two of them had p53-deficient CLL, a 17p deletion that usually indicates poor response to conventional treatment. Before enrollment in the study, all the patients underwent standard therapies, such as rituximab, fludarabine and bendamustine. Nevertheless, all had significant tumor burdens right before the trial, including bone marrow infiltration and lymphoadenopathy. One patient also had peripheral lymphocytosis.

The lentiviral vector used, GeMCRIS 0607-793 (Figure 5), was produced by Lentigen Corp. using a three-plasmid approach. It contained the transgene CD-19-BB- ζ which coded for a second-generation chimeric antigen receptor (Kalos et al.

2011). The receptor was comprised of the single-chain variable fragment (scFv) from the human CD19-specific murine antibody (FMC63), a human CD8α hinge region, a human 4-1BB (CD137) co-stimulatory signaling domain, and a human CD3-ζ signaling domain (June et al. 2011). As previously mentioned, the inclusion of the 4-1BB signaling domain considerably enhanced anti-tumor activity and in vivo persistence of CARs in preclinical trials. This effectiveness was also observed in the clinical trial.

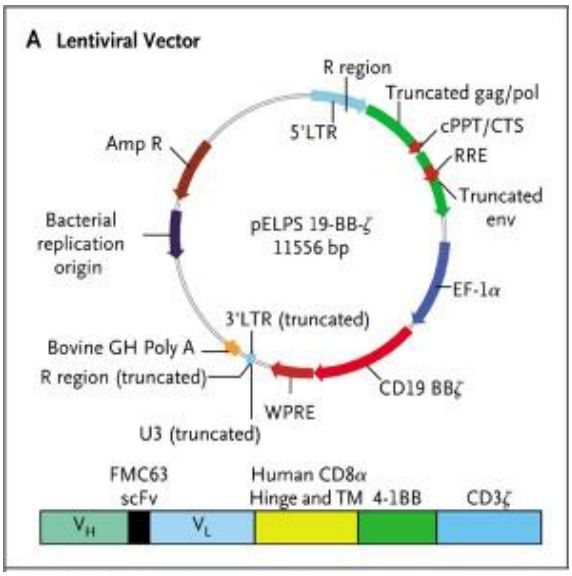

Figure 5: Lentiviral transfer vector construct used in the UP trial. The major functional elements of the transgene (represented by the sectional arrow labeled CD19BB ζ) are outlined in the bar at the bottom of Figure 5. Source: June et al. 2011

The patients' cells were obtained via leukapheresis. Anti-CD3/CD28 mAB-coated paramagnetic beads were used to positively select and activate T cells while remaining leukemic cells were depleted. GeMCRIS 0607-793, the lentiviral vector housing the transgene, was added to the culture and washed out three days later. The transduced cells were then allowed to expand for eight to ten days. Finally, the magnetic beads were removed by passing them through a magnetic field. The CART19 cells were collected, washed, concentrated, and cryopreserved in infusible medium (Kalos et al. 2011).

PATIENTS' RESPONSES

One to four days preceding the infusion of tranduced T-cells, all the patients underwent a round of lymphodepleting chemotherapy. Subsequently, each patient was infused intravenously with the transduced T cells over a three-day period as follows: 10%, 30%, and 60% of the dosage was infused on days one, two, and three, respectively. Patient UPN 03, the focus of this paper, began to have low-grade fevers associated with grade-2 fatigue two weeks after the infusion. Over the next few days, his temperature increased. Other symptoms, such as diarrhea, nausea, anorexia, and diaphoresis, were also observed. On day 22, he was diagnosed with tumor lysis syndrome, a metabolic complication that results when the kidneys are overburdened with a large load of destroyed tumor cells. The patient's uric acid and lactate dehydrogenase levels were above normal at 10.6 mg/dL and 1130 U/L, respectively. A creatinine level of 2.6 mg/dL indicated acute kidney injury. The patient was hospitalized and treated. His uric acid level returned to normal within 24 hours and the creatinine level within three days. He was discharged on the fourth day, and lactate dehydrogenase levels gradually decreased and returned to normal within a month.

On day 23 after infusion of the CART19 cells, CLL was absent from the bone marrow (BM) of UPN 03. By day 28, adenopathy was not palpable, and on day 31, CT scanning showed its resolution. In 198 out of 200 cells examined, FISH testing was negative for the p53 deletion. Flow cytometry indicated B-cell aplasia and no residual CLL. Three months later, CT scanning showed sustained remission. In addition, at

three and six months after infusion, studies of BM indicated no evidence of CLL as well as normal B cells (Figure 6). As of the publication of the study, remission has been sustained for ten months (June et al. 2011).

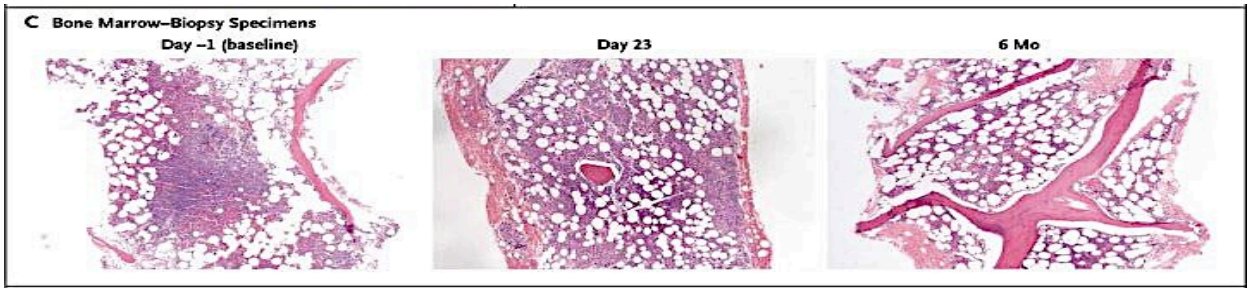

Figure 6: Bone marrow specimens of patient UPN 03. "The baseline specimen shows hypercellular bone marrow (60%) with trilineage hematopoiesis, infiltrated by predominantly interstitial aggregates of small, mature lymphocytes that account for 40% of total cellularity. The specimen obtained on day 23 shows residual lymphoid aggregates (10%) that were negative for chronic lymphoid leukemia (CLL), with a mixture of T cells and CD5-negative B cells. The specimen obtained 6 months after infusion shows trilineage hematopoiesis, without lymphoid aggregates and continued absence of CLL." Source: June et al. 2001

Patient UPN 02 also developed fevers and was hospitalized after CART19 infusion. Adenopathy was reduced somewhat, and p53 deficient CLL cells were eradicated from peripheral blood (PB). However, one month later, his BM was still infiltrated extensively with CLL cells. Thus, he had only sustained partial remission.

Patient UPN 01 also developed symptoms upon infusion. No CLL cells were detected in his blood one and sixth months later. BM studies were performed one, three, and six months after infusion and indicated total absence of CLL cells. Adenopathy was resolved as observed by CT scans one and three months after infusion. He remained in remission for over ten months as of the publication of the study (Kalos et al. 2011).

CART19 Effector-to-CLL Target Cell Ratio

In preclinical trials involving humanized mice, 2.2×10^7 CAR T cells were able to destroy tumors containing 1×10^9 cells (Kalos et al. 2011, as discussed in June et al. 2009). However, these calculations did not take into account *in vivo* expansion of the T cells. In the aforementioned UP trial, the three patients had their tumor loads estimated before infusion of the CAR T cells. This was done by calculating CLL cells in blood, bone marrow, and secondary lymphoid tissue. Patient UPN 03 had an estimated 8.8×10^{11} CLL cells in his bone marrow and 4.4×10^{11} CLL cells in secondary lymphoid tissue, totaling approximately 1.3×10^{12} tumor cells. His infusion contained only 1.4×10^7 CART19 cells. An astounding effector-to-target (E/T) ratio of 1:93,000 resulted in complete elimination of CLL cells. The overwhelming effectiveness of the CART19 cells is most likely due, in part, to their in vivo expansion. The E/T ratios observed in patients UPN 01 and UPN 02 were 1:2200 and 1:1000, respectively (Kalos et al. 2011).

Safety of CART19 Cells

No long-term toxicity, other than B cell aplasia, was observed as a result of CART19 infusion. The patients did, however, develop transient febrile reactions and other short-term symptoms indicative of a serious immune response that coincided with tumor destruction (June et al. 2011).

CYTOKINES

The patients' immune responses were accompanied by sharp increases in cytokines. Peripheral blood and bone marrow samples were analyzed and significant increases in interleukin-6, (IL-6), IL-8, IL-10, and interferon-γ (IFN- γ) were observed in patients UPN 01 and 02. Levels of chemokines, such as CXCL9 and CXCL10, also rose. Cytokine and CART19 cell levels both peaked at the same time, coinciding with the patients' clinical symptoms and eradication of tumor cells.

The chimeric antigen receptor used in this trial contained a 4-1BB signaling domain as opposed to CD28. CARs containing a CD28 signaling domain are associated with increased levels of IL-2 and tumor necrosis factor-α both of which are undesirable. Previous studies have shown that high levels of IL-2 suppress CAR T cells, and TNF-α is associated with cytokine-storm effects. Levels of IL-2 and TNF-α did not rise in any of the patients (Kalos et al. 2011).

IN VIVO EXPANSION, PERSISTANCE, AND BONE MARROW TRAFFICKING

On the first day after infusion, real-time PCR detected expression of the anti-CD19 CARs in patient UPN 03. The doubling time of CART19 cells was approximately 1.2 days. By day 21, a 3-log expansion of the cells was observed. CART19 cells comprised over 20% of circulating lymphocytes at peak levels, coinciding with the elevated levels of serum cytokines and the tumor lysis syndrome. Six months after infusion, CART19 levels were still significantly high, although they decreased by a factor of ten. The elimination half-life of the cells was 31 days. CART19 cells were also detected in the bone marrow beginning on day 23 and remained there for at least six months. Their half-life in the BM was 34 days, slightly longer than those in circulation. Three months after treatment, no CD19 or normal B cells were detected (June et al. 2011). Interestingly, none of the patients had an immune response targeting the CART19 cells even though they contained murine-derived segments. This may be due to the patients' severely compromised immune systems resulting from heavy pretreatment of CLL (Kalos et al. 2011).

LONG-TERM EXPRESSION AND ESTABLISHMENT OF MEMORY

In previous studies that have been conducted, CART cells have not been effective for prolonged periods of time. The long-term success of CART19 cells in the UP trial may be due to improved construction of the CAR. Several months after infusion, the values obtained by PCR for the prevalence of the CAR transgene closely matched those obtained by flow cytometry for the frequency of circulating CART19 cells. Blood and BM samples of UPN 03 that were analyzed by flow cytometry 169 days after infusion indicated the presence of CART19 cells and complete absence of B cells. In all three patients, PCR indicated that CART19 cells persisted for at least four months. At 71 days after infusion, 5.7% of the T cells in the blood of patient UPN 01 expressed CARs, and on day 286, 1.7% expressed CARs. Although small, these percentages indicate long-term expression of a CART19 population, possibly indicating long-term immunity too.

Polychromatic flow cytometry was also performed to study the function and phenotype of CART19 cells in patient UPN 03. On day 56, CART19 CD8+ cells expressed an effector memory phenotype which is normally stimulated by prolonged

exposure to an antigen. By day 169, some of the CART19 CD4+ cells expressed a central memory phenotype, as indicated by CCR7 and CD127 expression, both of which are associated with memory T cells. B cell progenitors in the BM could ensure that CART19 cells maintain a memory population, thereby providing long-term immunity to CLL with the use of CART19 cells (June et al. 2011).

CONCLUSION

Decades of research in genetics, immunology, and molecular biology have culminated in the ability of medical scientists to treat patients with CLL with autologous T cells. In the UP trial of Dr. Carl June and his colleagues, two out of three patients enrolled in the study have reached total remission, and one achieved partial remission. The engineering of chimeric antigen receptors, the ability to use lentiviral vectors to transduce T cells to express them, and the *in vivo* expansion and persistence of these CAR cells all represent monumental breakthroughs that will, hopefully, be transposed to other areas of medicine as well.

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TREATMENT OPTIONS FOR PARKINSON'S DISEASE

Sara Russ

INTRODUCTION

Parkinson's disease was first described and named Paralysis Agitans in 1817 by British physician James Parkinson (Lieberman 2004). Later on, it took on its current name after Dr. Parkinson. Parkinson's disease (PD) is a neurological disorder for which the cause is yet to be discovered. Like many other diseases, PD has numerous facets. Throughout all of its different stages, it presents with motor, as well as non-motor, symptoms (Simuni et al. 2009). Though estimates of people affected by PD are constantly being made, it is difficult to determine a precise and accurate number. This difficulty arises since symptoms are often mistaken for other diseases of the nervous system (jointly known as Parkinsonism) or are mistakenly attributed to the normal aging process. It is estimated that over 1 million people in North America are affected by this degenerative disorder. As life expectancy increases, incidence of the disease rises (Lang and Lozano 1998).

The mortality rate of PD patients is 2-5 times greater than age-matched controls; this alone indicates the significance of its impact. It is predicted that, by the year 2040, Parkinson's disease will surpass cancer as the second leading cause of death among the aged (Bennett et al. 1996).

NEUROPATHOLOGY

Parkinson's disease is characterized by slow movement, rigidity, and involuntary movement, which occur because of death to dopamine-producing neurons. Dopamine is the main neurotransmitter to be affected by the disease, although it is not the only one; serotonin, acetylcholine, and norepinephrine are other affected neurotransmitters. However, their contribution to clinical symptoms is unknown.

The striatonigral complex is the region of the brain that is the source for the majority of the brain's dopamine. It includes the putamen, caudate, and substantia nigra; the substantia nigra is the main source of dopamine in the region. There are 5 dopamine receptors (D1-D5) found in various brain regions that include the cortex, striatum, and limbic system (Macphee and Stewart 2007). Three of the receptors (D1, D2, and D3) are present in the basal ganglia. D1 and D2 receptors are known to promote voluntary movement; D3's function is unknown. The limbic system, which controls emotion, contains D3, D4, and D5 receptors; thus, the dopamine deficiency of PD leads to the cognitive and emotional impairments associated with PD (Rezak 2007).

The basal ganglia have a circuit that enables voluntary and involuntary movement (Macphee and Stewart 2007). Nigral cells have axons that extend into the putamen and caudate nucleus. The globus pallidus located near this complex regularly inhibits movement by releasing the neurotransmitter gamma amino butyric acid (GABA), an inhibitory neurotransmitter, to the thalamus, which prevents the motor cortex from being excited. The substantia nigra excites the caudate and putamen via the transmission of dopamine, which then informs the globus pallidus as to which forms of movement it should stop inhibiting. Thus, dopamine is necessary for motor output and decreased stimulation by dopamine causes decreased motor output (Kalat 2009).

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The distinguishing characteristic of Parkinson's disease was once thought to be the presence of Lewy bodies. However, not all forms of the disease have this pathological feature. For example, those that are classified as autosomal recessive cases do not have Lewy bodies. In autosomal dominant cases, however, Lewy bodies are seen (Macphee and Stewart 2007). Lewy bodies are spherically shaped protein masses and contain transparent cytoplasmic centers with halos surrounding them. The center consists of neurofilaments and other proteins accountable for proteolysis (protein breakdown) including parkin, ubiquitin, and alpha synuclein. In those patients who experience Lewy bodies, Lewy bodies appear in all affected areas of the brain (Dauer and Przedborski 2003). The mechanism of formation of Lewy bodies and their function in the pathology of the disease is not yet known (Lang and Lozano 1998).

Many areas of the brain are affected by the disease. The most significant damage occurs in the pars compacta, the posterior part of the substantia nigra (Macphee and Stewart 2007). This region is located in the midbrain, midway between the cerebral cortex and the spinal cord (Lieberman 2004). Most of the motor symptoms associated with the disease occur because of damage to this area. Motor symptoms begin to appear after the majority of the cells in this area are lost. Damage to the nucleus basalis, an area of the brain that produces an abundance of the neurotransmitter acetylcholine, is correlated with impaired memory and cognitive function in PD patients (Macphee and Stewart 2007).

The pars compacta has both anterior and posterior parts to it. The posterior part is pigmented with neuromelanin, whereas the anterior is considerably lighter, as it lacks this pigment. Normal aging is consistent with cell loss in the posterior region. PD, on the other hand, impairs the anterior region. This shows that Parkinson's disease is not an effect of advanced aging (Macphee and Stewart 2007).

Studies show that neurological symptoms of Parkinson's disease develop in an upward manner. The disease starts with damage to the brainstem and advances upward to the cerebral cortex. The disease can be divided into six stages, each associated with the onset of specific neurological symptoms (Macphee and Stewart 2007). In the first two stages, symptoms are not apparent (Davie 2008).

CAUSES

GENETIC CAUSES

Most incidences of PD occur sporadically, rather than from genetic causes (Wood-Kaczmar et al. 2006). Onset generally occurs at younger ages in genetic forms of the disease and pathological symptoms are different (Vila and Przedborski 2004). Genetic inheritance of PD occurs in both autosomal dominant and autosomal recessive forms (Wood-Kaczmar et al. 2006). Dominantly inherited Parkinson's was first discovered in the 1990s upon studying a familial case. Alpha-synuclein was the gene identified as causing PD (Cordato and Chan 2004). Several other genes involved in the onset of the disease have been located since that initial discovery (Vila and Przedborski 2004).

ALPHA SYNUCLEIN

A mutated *SNCA* gene, which encodes the alpha synuclein protein, has an altered sequence of nucleotides (Vila and Przedborski 2004) and therefore codes for a different protein product (Klug et al. 2006). Three types of mutations were found that link this gene to Parkinson's disease. These genetic mutations are only associated with

dominant inheritance (Wood-Kaczmar et al. 2006). Mutations in *SNCA* are thought to account for a small percentage of Parkinson's disease since it was found in a small number of studied hereditary cases (Lang, and Lozano 1998).

Alpha synuclein is made up of 140 amino acids and is present in several locations of the brain. It is found in the highest concentrations in the cortex, hippocampus (Wood-Kaczmar et al. 2006), hypothalamus, olfactory neurons, and substantia nigra. Neural and glial cells produce this protein, which is mainly found at pre-synapses. Alpha synuclein has the shape of an alpha helix and is found in vesicles that transport lipids.

Studies were done on mice by transferring a gene from another organism to replace the gene that encodes for alpha synuclein, in order to test its function in living organisms. Absence of alpha synuclein showed fewer proteins at synapses and fewer vesicles available for transport (Cordato and Chan 2004). Its function is not well understood, although it is thought to play a role in transmission of chemical messages, learning, and neuroplasticity (the ability for neurons to adapt to environmental stimuli) (Wood-Kaczmar et al. 2006).

Alpha synuclein can also be harmful. In high concentrations, these proteins cluster together to form polypeptides that are associated with various diseases. These unwanted protein accumulations are normally broken down by enzymes that specialize in getting rid of harmful material to the cell. Toxicity to neurons can happen if mutated *SNCA* produces alpha synuclein proteins that are more likely to clump together or are folded in such a way that makes it difficult for enzymes to break them down. These proteins are part of Lewy body formation and are thought to cause cell death by breaking down the proteins of neurons (Cordato and Chan 2004).

Leucine-Rich Repeat Kinase 2

A number of mutations in the gene that encodes for leucine-rich repeat kinase 2 (LLRK2) have been located in late-onset dominantly inherited PD. The most common one is substitution of the G2019S gene. This mutation generally accounts for a small percentage of genetically inherited PD. It contributes to a significantly greater percentage of PD in North African Arabs and Ashkenazic Jews. The role of this protein and its contribution to the disease is not yet understood (Wood-Kaczmar et al. 2006).

Parkin

Parkin is made up of 465 amino acids. It is one of many proteins that have a ring finger domain. Proteins that have this characteristic are able to act as ubiquitin ligases by breaking down harmful protein buildup, such as buildup of alpha synuclein. Mutations in the gene that encodes for parkin, *PARK2*, are due to recessive inheritance and are consistent with early-onset PD (Cordato and Chan 2004). Mutations of *PARK2* and alterations in parkin's post-translational state cause parkin's inability to target certain forms of alpha synuclein for breakdown, which can lead to build-up of alpha synuclein and toxicity to neurons (Vila and Przedborski 2004). In addition, mutations in other proteins do not permit parkin to carry out its job properly, resulting in toxic accumulation (Wood-Kaczmar et al. 2006). *PARK2* mutations cause death to cells of the substantia nigra and locus ceruleus (Cordato and Chan 2004).

Parkin functions as an ubiquitin enzyme marking its target cell for destruction. Lewy bodies form when a sequence of ubiquitin enzymes binds to a target protein and

is unable to break it down. In its mutated forms, parkin cannot bind to its target proteins; thus, Lewy bodies are not seen in this instance (Cordato and Chan 2004).

The two types of mutations (Wood-Kaczmar et al. 2006) in the *PARK2* gene (missense and nonsense mutations) cause very similar symptoms, such as early onset (usually occurring before age 30) (Cordato and Chan 2004; Vila and Przedborski 2004), degeneration at a slower pace, receptiveness to levodopa (a medication used to treat motor symptoms of PD) (Macphee and Stewart 2007), and susceptibility to side effects of levodopa early on (Cordato and Chan 2004). Mutations in the *PARK2* gene account for 50 percent of early-onset familial PD and for 77 percent of early-onset sporadic cases (Wood-Kaczmar et al. 2006).

PARK7

The gene *PARK7* codes for DJ-1, a protein with many functions. It is found scattered in many areas of the brain. Several recessive mutations link this protein to PD. Compared to mutations in *PARK2*, mutations in DJ-1 account for a small amount of early onset PD. Mutations occur in various areas of the gene, yet they cause the same symptoms (Vila and Przedborski 2004). The normal function of DJ-1 is to stabilize proteins, while protecting them from damage by free radicals. Studies on mice that lack DJ-1 have shown dysfunction of dopamine pathways, causing motor disability (Wood-Kaczmar et al. 2006).

Environmental Causes

The development of PD in a group of drug users was linked to the intake of a drug containing methyl-phenyl-tetrahydropyridine (MPTP). MPTP has been shown to cause degeneration specifically in the nigral cells, by decreasing complex 1 (the first enzyme of the respiratory chain leading to the production of ATP) activity of the mitochondria. The discovery that environmental toxins can lead to the onset of PD has led researchers to extend their studies to pesticides and herbicides to test their contribution to the disease (de Lau and Breteler 2006). Rotenone is an agent commonly used by gardeners to prevent unwanted plant growth. Repeated exposure to this chemical in low doses has also been shown to cause degeneration specific to nigral cells. Studies show that exposure to this pesticide is linked to the formation of Lewy bodies, a major component of the PD, whereas exposure to MPTP has not yielded such results (Jenner 2001). It has been hypothesized that exposure and accumulation of heavy metals, such as aluminum, amalgam, copper, iron, manganese, zinc, etc., in the substantia nigra may cause an increased risk of PD by causing oxidative damage (de Lau and Breteler 2006).

Symptoms

Motor Symptoms

A number of motor features, including bradykinesia (slow movement), a resting tremor, muscle rigidity, and postural instability, distinguish PD from Parkinsonism. There are several scales to assess the rate of decline in PD patients. Two widely used scales are the Hoehn and Yahr, and the Unified Parkinson's disease rating scale (UPDRS). The Hoehn and Yahr, a scale that ranges from 0 (no symptoms) to 5 (bedridden) evaluates how far the disease has progressed. The UPDRS, on the other hand, is used to determine speed of progression of the disease. This scale is currently being modified to include non-motor symptoms of PD (Jankovic 2007).

The most apparent symptom of the disease is bradykinesia, or decreased movement. Bradykinesia adds difficulty to daily activity management, leading to an inability to initiate, plan, multitask, and/or carry out tasks in a consecutive order. Slow movement affects fine motor function such as buttoning a garment and handling utensils. Spontaneous movement, such as blinking, facial expressions, and arm swaying while walking, is also impaired. These hindrances are due to reduced dopamine activity, which causes a decrease in motor output (Jankovic 2007). Slowness of movement is related to the patient's emotional state, not their motor abilities. Thus, when an external stimulus, such as a yell of "Fire" or a signal telling them to beware of an obstacle, informs them of the need for quick movement, they regain the ability to move quickly (Jankovic 2007).

According to studies (Gelb et al. 1999), the most common symptom of PD (Jankovic 2007), which occurs in 79-90 percent of patients, is a resting tremor. Hand tremors begin on one side of the body and spread to the other. It is noticeable on the lateral parts of the hand or leg. Tremors may also affect the chin, jaw, lips, and legs. Tremors are not apparent while sleeping or in the course of action (Jankovic 2007).

Another feature seen in PD is stiffness, which can occur in several areas, such as the ankles, hips, neck, shoulders, and wrist. Stiffness can be accompanied by pain. A study found that rigidity, along with tremor and imbalance, was associated with an increased risk of PD in individuals who initially showed no signs of Parkinsonism (Jankovic 2007).

Rigidity can also cause bending of the elbows, knees, neck, and/or trunk. It can also lead to striatal hand or foot, which occurs when the thumb or big toe is extended while the joint by the knuckles and other toes are bent. These abnormalities usually occur later on in the disease and are generally associated with early onset of the disease (Jankovic 2007).

Postural instability usually arises in later stages of the disease. This symptom is the source for many falls that often cause hip fractures. One study indicated a wait of nine years for patients to experience their first fall. Interestingly, patients who fear falling show an increased incidence of falling. Unlike other symptoms, postural imbalance is generally untreatable by therapy (Jankovic 2007).

NON-MOTOR SYMPTOMS

Non-motor aspects of PD are currently receiving increased attention, since they affect the quality of life of patients significantly (Macphee and Stewart 2007). Non-motor features consist of autonomic dysfunction, sleep disorders, and impaired cognitive function (Jankovic 2007).

Autonomic dysfunction includes sweating, constipation, erectile dysfunction, orthostatic hypotension (a sudden decrease in blood pressure when the patient stands up) (Jankovic 2007), and reduced olfaction (Chaudhuri et al. 2006). Constipation is a common symptom that can serve as a precursor to PD. A study done over a period of 24 years showed that men who originally had constipation were three times more likely to develop PD, following a 10 year interval. Both elevated and decreased sex drive were reported by patients. Ninety percent of PD patients develop problems with their sense of smell. A number of studies concluded that decreased sense of smell is an early sign of motor symptoms in PD. Many relatives of patients who reported reduced

olfaction but did not report any other symptoms of PD were later diagnosed with PD (Chaudhuri et al. 2006).

Sleep disruption was once thought to be a side effect of treatment for Parkinson's. Several doctors now believe that it is a component of the disease. About one third of PD patients have rapid eye movement sleep behavior disorder where a dream accompanies dramatic motor movement. Insomnia occurs in over 50 percent of patients (Jankovic 2007). Sleepiness during daytime hours is common and may result from sleep disruption at night or as an outcome of treatment (Macphee and Stewart 2007). Degeneration of neurons in the brain's sleep regulation centers in the thalamocortical pathway and brainstem contribute to sleep disorders (Chaudhuri et al. 2006).

Studies have found decreased cognitive ability in nearly 85 percent of PD patients, while close to half of them developed dementia. Dementia in PD patients usually accompanies neuropsychiatric disorders. A study comprising 537 PD patients showed that close to 50 percent experienced anxiety, apathy, depression, and hallucinations. Other studies reveal that many patients display various forms of impulsive behavior, such as cravings, hypersexuality, obsession with shopping, pathological gambling, etc. These behaviors develop as a result of taking levodopa (Jankovic 2007), a medication used to treat PD (Macphee and Stewart 2007).

PREVENTION

Several case-control and population-based studies from numerous countries showed a significantly decreased risk for PD among cigarette smokers. The mechanism of cigarette smoking decreasing the risk of PD is not well understood. It is possible that nicotine in cigarettes stimulates dopamine release.

Several studies also found consumption of coffee to be consistent with a reduced risk for PD. The effective component in coffee is perhaps caffeine, because other studies showed a decreased risk for PD from taking in other sources of caffeine. According to studies, caffeine has a greater protective effect on men than woman (de Lau and Breteler 2006).

A third factor that may reduce risk of PD is alcohol consumption. Some studies found a decreased risk with alcohol consumption. Others, though, found no association (de Lau and Breteler 2006).

DIAGNOSIS

The main symptoms of PD at the time of diagnosis include rigidity, bradykinesia, and a resting tremor (Davie 2008). Postural instability is usually not present during diagnosis since it typically manifests itself later on in the disease (Jankovic 2007). Symptomatic features are generally not present on both sides of the body. Handwriting change and decreased facial expressions can be detected. Decreased olfaction can be reported since it is usually an early symptom of PD. Post-mortem studies showed that diagnosis of PD by a neurologist reflected a 25 percent misdiagnosis rate. Patients who were diagnosed in a clinic that specialized in movement disorders indicated less diagnostic inaccuracy. It is thus important to see an expert in the field to ensure proper diagnosis (Davie 2008).

There are several Parkinsonism disorders that can easily be mixed up with PD. Diagnosis of PD normally takes place in a clinical setting. Sometimes brain images using magnetic resonance imaging (MRI) and computed tomography (CT) are

necessary to rule out other Parkinsonism diseases. Using single emission computerized tomography (SPECT), other conditions can be ruled out (Davie 2008).

TREATMENT OF MOTOR SYMPTOMS

LEVODOPA

Each patient requires treatment dedicated to his or her specific symptoms and needs (Rezak 2007). Since the 1960s, levodopa has been the main drug used in treatment of PD (Schapira et al. 2006). Levodopa greatly reduces symptoms of rigidity and bradykinesia, but has a lesser effect on tremors (Macphee and Stewart 2007). Levodopa is currently the most effective drug on the market. However, since levodopa's effectiveness lasts for about ten years (Rezak 2007), it is held back from patients or given at low doses until it is absolutely necessary.

In order to reduce its side effects, levodopa may be combined with other drugs. Levodopa, a dopamine precursor, is usually combined with a decarboxylase inhibitor (Macphee and Stewart 2007), such as carbidopa or benserazide. These inhibitors occupy receptors on the enzyme dopa decarboxylase, thus preventing levodopa's conversion to dopamine before it reaches the brain. Decarboxylase inhibitors help alleviate side effects that accompany levodopa such as excessive sweating, low blood pressure, and nausea. Another drug that may be combined with levodopa and carbidopa is entacapone. This drug inhibits catechol-o-methyl-transferase, an enzyme that breaks down neurotransmitters, causing increased uptake of levodopa in the intestines.

Even when combined with other drugs, there are possible side effects that accompany levodopa (Rezak 2007). After five years of taking levodopa, 50 percent of patients may develop (Lieberman 2004) dyskinesia, motor fluctuations, hallucinations, sleepiness, nausea, and/or low blood pressure (Rao et al. 2006). Levodopa induced motor fluctuations can range between wearing off of dosage and unsystematic severe on and off motor functioning. Levodopa's half-life of 60-90 minutes causes a spurt of dopamine receptor activation. The occurrence of continuous abrupt spurts of dopamine receptor activation at the same time as the constant death of dopamine-producing cells causes abnormal receptor activation known as the "on-off" phenomenon. Initially, levodopa is prescribed very sparingly in order to protect patients from its adverse side effects (Rezak 2007).

DOPAMINE AGONISTS

Dopamine agonists activate dopamine receptors by mimicking the actions of dopamine (Lieberman 2004). They include bromocriptine, cabergoline, lisuride, pergolide, pramipexole, ropinerole, and rotigotine (Macphee and Stewart 2007). Dopamine agonists, which have been previously prescribed together with levodopa, are currently being given alone as an early PD treatment, thereby delaying the administration of levodopa. Pramipexole, ropinerole (Rezak 2007), and rotigotine are currently the most prescribed dopamine agonists (Davie 2008). Studies comparing the effects of pramipexole and ropinerole against levodopa have shown a decreased rate of progression of PD with levodopa use. The dopamine agonists, however, showed less motor complications (Rao et al. 2006). With advancement of the disease, dopamine receptor agonists are commonly taken with levodopa/carbidopa to reduce levodopa's accompanied motor complications (Lieberman 2004). Possible side effects of

pramipexole and ropinerole can include low blood pressure, dyskinesia, abnormal sleep patterns, impulsive behavior, and cognitive/psychiatric impairments. Unlike the other dopamine agonists, rotigotine is administered through a transdermal patch and is absorbed over a 24-hour period, thereby continuously stimulating dopamine receptors. Pergolide, bromocriptine (Rezak 2007), cabergoline, and lisuride are not prescribed that often since they cause cardiac valve degeneration (Davie 2008).

MONOAMINE OXIDASE-B INHIBITORS (MAO-B)

The enzyme monoamine oxidase-B (MAO-B) is responsible for degrading most of the dopamine in the basal ganglia. Selegiline and rasagiline are two drugs that inhibit this enzyme causing increased levels of available dopamine (Rezak 2007). Like dopamine agonists, selegiline can either be used alone to treat symptoms while delaying levodopa therapy or be joined with levodopa to reduce motor fluctuations (Macphee and Stewart 2007). Insomnia and nausea are potential side effects of this drug (Rao et al. 2006). Rasagiline is a newer and more powerful MAO-B inhibitor than selegiline. Rasagiline is an effective monotherapy in early PD as well as a preventer of motor fluctuations when taken with levodopa later on in the disease's progression (Rezak 2007). Compared to a placebo, when taken with levodopa, rasagiline has shown to decrease motor fluctuations by one more hour per day (Macphee and Stewart 2007).

CATECHOL-O-METHYLTRANSFERASE INHIBITORS (COMT-I)

Catechol-O-methyltransferase is an enzyme present in several locations, including the intestines, liver, kidneys, neural cells and glial cells. The goal of tolcapone and entacapone, the two catechol-O-methyltransferase inhibitors (COMT-I), is to prevent motor fluctuations resulting from the wearing off of levodopa's effects between doses (Macphee and Stewart 2007). These drugs can improve symptoms and effectively reduce the dose of levodopa (Rao et al. 2006). COMT-I drugs inhibit metabolism of levodopa by COMT in the gastrointestinal tract, thus increasing the amount of levodopa reaching the substantia nigra and thereby enabling levodopa's conversion to dopamine. In addition to increasing the availability of levodopa to the brain, tolcapone and entacapone also prolong the duration of levodopa's metabolism. COMT inhibitors can be good treatments for early Parkinson's disease. They delay the onset of levodopa's motor complications by stabilizing the blood concentration of levodopa and effectively decreasing peak dosage, via the increase of levodopa's half-life (Rezak 2007).

COMT-I drugs can have negative effects as well. Diarrhea can occur as a side effect (Rao et al. 2006), necessitating discontinuation of the drug. The addition of COMT inhibitors to levodopa therapy causes increased dopaminergic stimulation, possibly resulting in dyskinesia. Such cases may call for decreased dosage. Since tolcapone has been shown to cause hepatotoxicity, the FDA requires patients' liver enzymes to be monitored (Rezak 2007); it is thus given only when entacapone proves to be ineffective (Macphee and Stewart 2007). Although it has a shorter half-life and is less effective, entacapone is still more frequently used (Rezak 2007).

AMANTADINE

Amantadine, initially an antiviral drug, was discovered to be useful in PD treatment (Lieberman 2004) by improving dopamine release from presynaptic

terminals (Rezak 2007) and acting as an anticholinergic agent, which prevents glutamate activation of N-methyl-D-aspartate (NMDA) receptors (Lieberman 2004). Overstimulation of NMDA receptors are linked to dopaminergic death in PD. Amantadine's impact on treating symptoms is greatest for tremor and it has its greatest effects in the early PD stages. It has also shown to reduce dyskinesia associated with levodopa (Rezak 2007). Side effects of this drug may include hallucinations, confusion, hypotension, nausea, and edema (Rao et al. 2006).

Table 1: FDA Approved Medications for Parkinson's Disease

Medication	*Adverse effects*	*Indications and comments*
Anticholinergics		
Benztropine (Cogentin), trihexyphenidyl (Artane)	Dry mouth, dry eyes, constipation, hypotension, cognitive impairment, urinary retention	Useful for symptomatic control of Parkinson's disease (benefits are mild to moderate); associated with more adverse effects than other drugs
Carbidopa/levodopa		
Immediate- and sustained-release carbidopa/levodopa (Sinemet)	Nausea, somnolence, dyskinesia, hypotension, hallucinations	Levodopa is the most effective medication and remains the primary treatment for symptomatic Parkinson's disease; no added benefit for motor complications with sustained-release versus immediate-release preparations
COMT inhibitors		
Entacapone (Comtan)	Diarrhea; exacerbates levodopa adverse effects; bright orange urine	Useful for managing motor fluctuations ("wearing-off" effect) in patients taking levodopa; levodopa dose may need to be reduced if dyskinesia appears
Tolcapone (Tasmar)	Diarrhea; exacerbates levodopa adverse effects; rare liver failure (liver function monitoring needed)	
Dopamine agonists		
Bromocriptine (Parlodel)	Nausea, headache, dizziness	Useful for early and advanced disease
Pergolide (Permax)	Somnolence; hallucinations; nausea; edema; fibrosis of cardiac valves, lung, and retroperitoneum; retroperitoneal and pulmonary fibrosis	Useful for the initial treatment of parkinsonism and as adjunct therapy in patients taking levodopa
Pramipexole (Mirapex), Ropinirole (Requip)	Nausea, sleep attacks, edema, hallucinations, hypotension	Useful for early disease and in patients with Parkinson's disease and motor fluctuations
MAO-B inhibitors		
Selegiline (Eldepryl)	Nausea, insomnia, drug interactions with other MAO inhibitors/tyramine	Useful for symptomatic control of Parkinson's disease (benefits are mild to moderate) and as adjuvant therapy for patients with Parkinson's disease and motor fluctuations
Rasagaline (Azilect)	Weight loss, hypotension, dry mouth, drug interactions with other MAO inhibitors/tyramine	
NMDA receptor inhibitor		
Amantadine (Symmetrel)	Nausea, hypotension, hallucinations, confusion, edema	Useful for treating akinesia, rigidity, tremor, dyskinesia

FDA = U.S. Food and Drug Administration; COMT = catechol O-methyltransferase, MAO-B = monoamine oxidase-B; NMDA = N-methyl-D-aspartate.

Source: Cordato and Chan 2004

ANTICHOLINERGICS

Anticholinergic drugs also treat tremor in PD. Trihexyphenidyl, benztropine, and procyclidine, are the most commonly used anticholinergic drugs. Due to their adverse effects, they should not be given to the elderly. Side effects of anticholinergic drugs can include blurred vision (Rezak 2007), dry mouth, hypotension, constipation and cognitive impairment (Rao et al. 2006).

SURGERY

Brain surgery used to be a common method for treating tremor and rigidity in Parkinson patients, although success rates varied and detrimental risk factors such as death were involved. However, with the advent of levodopa in the 1960s, which proved to be a safer and more effective option for treatment, the idea of surgery diminished. With advances in neuroimaging, surgery has regained popularity as a treatment for PD among those who do not respond to drug therapy anymore (Lieberman 2004).

With greater understanding of the neuropathology involved in PD, two ablative procedures are renewably performed (Arle and Alterman 1999) to help control symptoms of PD (Lieberman 2004). A thalamotomy involves destroying part of the ventrolateral thalamus, a brain region involved in transmitting signals that control movement. This procedure significantly reduces tremor but shows little improvement to symptoms of rigidity and bradykinesia. Death rates are less than 1 percent due to better targeting and lesion technique. Since thalamotomy does not significantly improve many symptoms of PD, it is less favored than pallidotomy (Arle and Alterman 1999).

In a pallidotomy, the globus pallidus, a brain area responsible for involuntary intermittent movements in PD patients, is targeted (Lieberman 2004). Pallidotomy has been shown to greatly reduce rigidity, bradykinesia, and tremor. Patients taking levodopa can also benefit from this procedure because it decreases the "off" period in their motor fluctuations. Better UPDRS scores were reported for up to 2 years following this procedure (Arle and Alterman 1999).

Deep brain stimulation has become more common now because of its effectiveness. The head of the patient is placed in a stereotaxic frame, a halo-like device. Surgeons can spot the thalamus, globus pallidus, or the subthalamic nucleus through MRI on the brain. The skull is pierced, using a drill, and a probe is placed deep inside the brain to reach the target tissue. The probe transmits a burst of electricity, which causes the brain region's electrical activity to normalize reversing PD symptoms. This is a safe surgery and results can be seen immediately (Lieberman 2004).

STEM CELL THERAPY

Currently there is no cure for PD. Embryonic stem cells and stem cells that come from fetal brains and adult bone marrow have been successfully transformed into functioning dopamine producing cells. However, there are several setbacks. Use of embryonic stem cells is controversial (Arias-Carrion and Yuan 2009) as many people believe that fetal tissue should not be used for research as a fetus is unable to consent (McLaren 2001). Many believe that adult stem cells have much to offer for future treatment of degenerative diseases such as PD. Previously, adult stem cells have been scarce, but recent progress enables their use in large quantities. Adult stem cells can be

implanted into the brain where it can differentiate into neural cells. With a better understanding of the immune system and successful transplantation, the use of stem cells is under serious consideration for treating the various dimensions of PD. It is hypothesized that stem cells should come from the very same patient who will derive benefit from it; however, not much is known about this type of implantation. The current goal under study is to test the use of autologous stem cell transplant in animals, and further the findings to clinical studies (Arias-Carrion and Yuan 2009).

CONCLUSION

Parkinson's disease is a multi-dimensional disease for which there is currently no cure. Much advancement has been made in the treatment of PD symptoms as opposed to curing it. Current research is giving increased attention to treatment of non-motor symptoms since it greatly affects the quality of life of patients. Additionally, increasing focus is being given to stem cell research as a potential cure for Parkinson's disease by using the patient's own stem cells to repair their damaged dopaminergic tissue.

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HOW ARE DRUG THERAPEUTIC LEVELS MAINTAINED WHILE AVOIDING DANGEROUS SIDE EFFECTS ASSOCIATED WITH CONVENTIONAL IMMEDIATE-RELEASE DOSAGES?

Chedva Farkas

INTRODUCTION

Ever since the discovery of bacteria and their role in the disease process in the mid-1800s, scientists have been heavily involved in the discovery and development of drug therapies and their mechanism of action in the human body. Shortly after the initial discovery came the era of drug discovery of the late 1800s-early 1900s, also known as the "drug revolution" (Dash and Cudworth 1998). Although the drug revolution led to the rapid discovery of many new drugs, pharmacological factors such as dosage forms, drug delivery systems, plasma drug levels, and how all these factors contribute to the efficacy of a drug were poorly understood. It wasn't until the mid-1900s that these factors were identified (Dash and Cudworth 1998). One particular factor which, once discovered, turned into a main focus in pharmacological research and lead to improvements in drug efficacy is centered on the importance of maintaining a steady therapeutic drug concentration level in the plasma.

The most conventional method for drug administration has always been through the use of oral products, such as tablets and capsules. These conventional oral drug products are formulated to release the active drug immediately after oral administration to obtain complete and rapid drug absorption in the body and immediate therapeutic effects (Shargel et al. 2004). However, once the body fully absorbs the drug, plasma drug concentration levels decline precipitously, possibly falling below the minimum effective plasma concentration (MEC), resulting in a loss of therapeutic activity. Before falling below MEC, the patient must be administered another dosage to maintain therapeutic effects (Shargel et al. 2004). Clearly, the conventional dosage form leads to a peak-and-valley curve of drug plasma levels versus time. This peak-and-valley pattern can have adverse effects since peaks, high plasma concentration of drugs (a result of frequent dosing), can cause toxicity, and valleys, low drug concentration in the plasma, may lead to sub-therapeutic levels and a possible buildup of drug resistance by the body's immune system (Dash and Cudworth 1998). How are drug therapeutic levels maintained while avoiding dangerous peak-and-valley side effects that often occur after the administration of conventional immediate-release dosage forms?

In order to maximize the therapeutic effectiveness of a drug while avoiding potential side effects that result from large fluctuations in drug blood levels, optimal concentration of drug in blood plasma must be sustained continuously (Breimer et al. 1984). In the past, the only known way to maintain a steady concentration of a drug's level in the plasma was through intravenous (IV) administration of the drug at a constant rate. Although steady IV administration is effective, it generally requires a health care professional to monitor the plasma drug concentration and cannot be performed at home (Dash and Cudworth 1998). In recent years, continued advances in pharmaceutical sciences have given rise to modern technological processes which

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provide alternate drug delivery systems that can maintain a steady therapeutic drug-plasma level while avoiding the inconveniences of IV administration and the possible dangers of frequent oral dosing (Chen et al. 2010). "Modified-release drug products" is the general term used by the US Pharmacopeia (USP) to describe products that "alter the timing and/or rate of release of the drug product to accomplish constant therapeutic levels not offered by conventional immediate release products" (Chen et al. 2010).

A major subdivision of modified-release products includes drug products with extended-release (also referred to as controlled-release) characteristics. Examples of extended-release drug products primarily include prolonged-action drug products and sustained-release drug products. Prolonged-action drug products are designed to slowly release the active drug substance in a way that provides a continuous supply over a period of time, thereby avoiding rapid and peak drug absorption in the plasma. Sustained-release drug products are designed to deliver an initial therapeutic dose followed by a slower steady release of drug that equals the rate of drug elimination from the body, resulting in minimal plasma drug concentration fluctuations (Shargel et al. 2004). Figure 1 portrays a plasma drug concentration versus time profile for both oral and sustained release delivery systems. This graph proves the value of an extended-release drug delivery system over the conventional frequent oral dosage since the sustained-release delivery system clearly stays in the desirable therapeutic and relatively homeostatic level while the oral immediate-release pill fluctuates between toxic and ineffective levels (Dash and Cudworth 1998). Currently, extended-release drug products encompass a wide range of products ranging from extended-release oral products, transdermal patches, and even implantable drug systems.

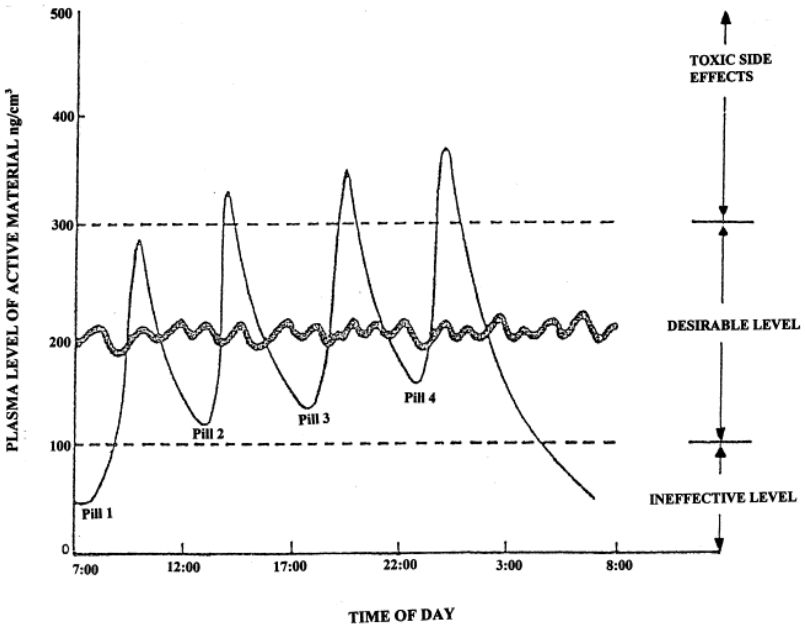

Figure 1: plasma drug concentration versus time profile for both oral and sustained release delivery systems. Source: Dash and Cudworth 1998

Before investigating the actual pharmaceutical applications used to create extended-release drug products, it is important to understand the possible biopharmaceutical factors that determine how a drug works in the gastrointestinal tract and the various pharmacokinetic properties that determine a drug's rate of absorption and release in the human body.

DRUG- ABSORPTION

As mentioned above, modified-release pharmaceutical preparations are generally designed to produce drugs with slow and uniform in-vivo absorption. In view of the fact that many of these oral modified release drugs are designed to remain intact in the gastrointestinal (GI) tract longer than the original immediate-release drugs, the anatomy and physiology of the GI tract plays a key role in the dosage and absorption of these drug products (Shargel et al. 2004). There are a number of physiological factors along the GI tract that affect drug release rate and uptake from the modified-release product. They include variations in pH, GI motility phases, presence of food, gastric emptying, presence of bacteria, enzymatic activities, varying permeability along the GI tract, volume of intestinal juice, and other factors as well (Lobenberg et al. 2000). Clearly, in-vivo behavior of modified-release oral drugs can be extremely complex considering all of the interacting mechanisms and changes occurring at every second in the gut (Varum et al. 2010). Researchers have, therefore, studied gut physiology and the mechanisms of digestion extensively with the hope that they can get a grasp on the in-vivo mechanisms and thereby produce maximally effective modified-release drugs.

The stomach is the first organ along the gastrointestinal track that a drug reaches. The general role of the stomach in the digestive system is to mix entering foodstuffs with digestive juices and then empty this mixture periodically into the small intestine (Shargel et al. 2004). Although this may seem to be a simple and predictable cycle, the movement of food and drugs through the stomach is greatly affected by the physiological state of the stomach, thereby affecting the time and location of the release of a drug from its dosage form (Shargel et al. 2004 and Varum et al. 2010). One particularly significant physiological factor which affects the ultimate time a drug is released from its dosage form depends upon the presence or absence of food in the stomach at the time of drug intake.

FACTORS THAT INFLUENCE ABSORPTION

The presence or absence of food has a major role in gastric emptying and gastric retention (Varum et al. 2010). In the presence of food, the stomach is considered to be in a digestive phase, whereas when lacking food, the stomach is considered to be in an interdigestive phase (Shargel et al. 2004). In the interdigestive phase, also known as the fasted state, gastric motility is under the control of a series of cyclical fluctuations in contractile activity commonly referred to as the migrating motility complex (MMC) (Higaki et al. 2008). The MMC is composed of four phases and can take up to about 30-40 minutes to be completed. Phase I is characterized by a total lack of activity. Phase II follows with an increase in the number and intensity of contractions. Phase III is characterized by large-amplitude peristaltic contractions that end with a strong "housekeeper contraction" which causes a massive gastric emptying of everything left in the stomach. Phase IV is an intermediate phase and acts as a transition period between the strong contractions of Phase III and Phase I of the next MMC cycle (Higaki et al. 2008 and Shargel et al. 2004). In the presence of food, on the other hand, MMC is basically abolished and low-amplitude peristaltic contractions take over, enabling gastric emptying of only small molecules into the duodenum (Higaki et al. 2008). Food particles larger than 2mm are retained in the stomach during the digestive phase (Shargel et al. 2004). Clearly, gastric emptying of

drugs (particularly tablets larger than 2mm) occurs mainly during Phases II and III of MMC in the fasted state (Higaki et al. 2008). Therefore, drugs larger than 2mm that are administered during a fasted state will be emptied out of the stomach fairly quickly, leading to a faster release of the drug into the patient's plasma. However, if the same large drug is administered in the digestive stage, it may remain in the stomach for a few hours until Phase II/III of the next MMC occurs.

Another factor that is known to have an important effect on gastric emptying and gastric retention is the caloric content of meals eaten around the time of drug administration. High caloric content meals generally show a delay in gastric emptying of both food and drug. In one particular study, a multiple-unit dose failed to empty for up to ten hours post-dosing in volunteers who consumed a high caloric meal (Varum et al. 2010). If, for example, a certain drug is modified to be released a few hours after administration with the intent that, at that time, the drug will be in the small intestine, a highly caloric meal might retain the drug in the stomach for too long and lead to a release of the drug in the stomach instead of the small intestine. Clearly, researchers can greatly benefit from knowledge of factors affecting gastric retention and emptying when formulating modified-release dosages.

Many studies were done to determine the effects of the fed/fasted state along with the caloric content of a meal on gastric emptying of a modified- release drug. One experiment in particular hypothesized that gastric emptying of different-sized enteric-coated pellets (enteric coating of pellets is a modified-release formulation characteristic that prevents dissolution in the stomach but allows rapid dissolution in the small intestine) would occur at different rates with the smaller pellets emptying in the fed state and the larger pellets emptying in the fasted state (Rhie et al. 1998). In this experiment, 12 healthy individuals were each given 0.7mm caffeine (CAFF) and 3.6mm acetaminophen (APAP) along with a viscous caloric meal at levels of 4000, 6000, and 8000 cP. The CAFF and APAP pellets were both enteric-coated spherical pellets formulated with sucrose nonpareils as the core which was coated with several suspension layers of the active ingredient, thereby achieving a target diameter, drug potency, and enteric coat level to aid in the release of the drug at a specific location. Gastric motility patterns were recorded using the monometric catheter, a technological device where the peaks on the machine represent the start and end of the different stages, such as Phases II and III of the MMC cycle. Blood samples were also obtained throughout the experiment in order to assess the plasma profiles of the drug, focusing specifically on the time when the drug was first detected in the plasma. Plasma results demonstrated that CAFF from the 0.7mm enteric-coated pellets were consistently (with all the different caloric level meals) measured in the plasma before the APAP from the 3.6mm enteric-coated pellets. Additionally, upon observing the timing of the release of the pellet dose, the results indicated that plasma profiles were strikingly superimposable upon the gastric motility patterns noted by the monometric catheter (especially with the 4000 cP meal); the time that CAFF from the smaller pellet was detected in the plasma correlated with the spikes on the monometric catheter which represented a fed state phase, backing up the assumption that small pellets are released from the stomach during the digestive phase. In contrast, APAP pellets were first observed in the plasma at the same time as the onset of the fasted contractile activity (Phases II and III). Overall, the meal viscosity levels in this experiment did not significantly affect the rate of drug absorption (Rhie et al. 1998). Knowledge of how

factors of gastric emptying affect the release of a drug assists in the development of modified release drugs that can take advantage of these factors.

It is important to note that, although most studies prove that a larger dosage form results in longer gastric retention, there have been studies that proved that different size dosages had no effect on the timing of gastric emptying. Clearly, more research should be done on this complex issue. Perhaps one of the many other physiological factors in the stomach, such as a pH level of 1-2 in the presence of food and 3-5 in the absence of food, can alter gastric emptying times if the experiment is not properly controlled.

The stomach empties its contents into the small intestine which provides a large surface area for drug absorption and where transit time of solids takes approximately 3-5 hours (Varum et al. 2010). Data obtained from various research projects is too varying to make conclusions on the effects fed and fasted states have on the transition of dosage forms along the small intestine. However, it was observed in numerous studies that the administration of modified-release multiple-unit enteric-coated dosage forms before eating resulted in a faster small intestinal transit time compared with the transit time of the drug in a fasted state (Varum et al. 2010). This information is extremely valuable. For example, if a drug is meant to be absorbed in the proximal small intestine, such a drug should be administered in pre-fed patients, resulting in modified-release of the drug in the upper GI tract. Administration of the drug in a fasted state might give the drug enough time to travel as far as to the colon where colonic conditions may make it impossible to be absorbed. Additionally, the presence of bacteria in the terminal part of the small intestine and its pH level of about 6 may also affect drug release in the small intestine.

The large intestine is the next and last step for a drug to be absorbed in the GI tract. A lack of fluids in the colon, besides for in the rectum, makes it difficult to absorb drugs passing through. The presence of bacteria in the colon can perhaps affect the absorption of modified-release drugs in the colon as well (Shargel et al. 2004).

The high variability in GI transit presents significant implications for the in-vivo performance of drugs in modified-release systems that are intended to delay or sustain release of drugs (Varum et al. 2010). The following studies represent how modified-release drug products can take advantage of the physiological conditions of the GI tract. Scientists do numerous studies on the gastrointestinal effects on a drug before creating a modified-release version of the drug. The majority of these experiments use the novel technique of pharmaco-scintography to assess regional drug absorption in humans. This technique works by co-administering a radiolabeled placebo pellet along with the coated modified-release drug of choice. The radiolabeled pellet's gastrointestinal transit is then monitored through a gamma camera. Blood samples are also generally collected periodically to assess plasma concentration of the drug in question and then compared to the scintigraphic results to help determine the exact time and gastrointestinal location of drug release (Basit et al. 2004).

One specific study was performed to evaluate the GI transit, release, and absorption of budesonide, a drug used for the treatment of inflammatory bowel disease (IBD), from its mutimatrix MMX® formulation. Budesonide's MMX formulation is designed to release the drug throughout the entire colon at a controlled rate (Brunner et al. 2006). Previously, budesonide had only been formulated to treat IBD in the right-sided colonic region in Chron's disease, and in left-sided ulcerative colitis. Therefore, in order to orally treat distally located IBD, gastric- resistant, extended-release budesonide tablets characterized by a multimatrix structure have been developed to allow a prolonged and steady release of budesonide along the entire colon at a controlled rate. This experiment tested the efficacy of the drug's prolonged-release characteristics on twelve healthy males. The volunteers were administered the budenoside multimatrix tablets, along with a ^{152}Sm-oxide tablet that was transformed into a γ-ray-emitting compound to be used for scintigraphy. Scintigraphic scans and blood samples were taken and compared periodically. MMX®-budesonide tablets were detected by scintigraphic imaging in the ascending colon between 4 and more than 24 hours after dosing, as is depicted in 2, and the drug left the descending colon at 12 to more than 24 hours post-dosing.

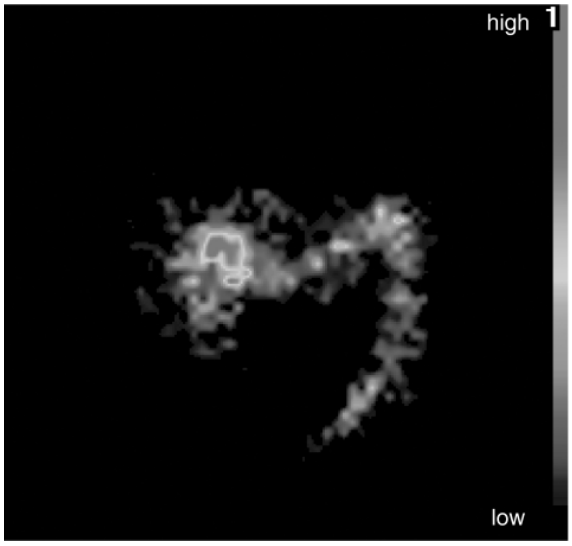

Figure 2: Scintigraphic imaging of the colon. Source: Brunner et al. 2006

An estimated 96% of the budesonide was absorbed in the target region (between the ascending and descending colon) as was calculated by the area under the curve (AUC), represented by the $AUC_{target/}AUC_{24}$ ratio where the AUC_{target} represented the plasma AUC where radioactivity was detectible in the target region, and the AUC_{24} was attained from the plasma AUC values of budesonide observed over the entire 24-hour period (Brunner et al. 2006). Clearly, this modified budenoside formulation was successful in delivering its active drug throughout the entire colon. Additionally, although budesonide plasma concentrations were first observed after 6.8 ± 3.2 h, maximum plasma concentrations were reached about seven hours later. The time difference between the initial detection of budesonide in the plasma and budesonide's time of max concentration (t_{max}) verifies the sustained-drug release characteristics of the budesonide MMX® tablets.

Phase two of this experiment tested the effect of food on budesonide pharmacokinetics. Plasma samples taken after the administration of a highly caloric and fatty meal reflected the fact that budesonide absorption decreased by 30% in relation to those who took the pill under fasted conditions (Brunner et al. 2006). This decrease of drug absorption may justify the administration of this drug with a meal to limit its potency and thereby improve the safety profile as a drug. Conclusively, the formulation of MMX®-budesonide tablets have clearly utilized knowledge of gastrointestinal transit as an aid in producing a sustained-release drug suitable for

targeted drug-delivery to the colon. However, further research should be done on this subject, since colonic infected individuals, for whom this drug is meant, may react differently to the drug than healthy individuals on whom the experiment was done.

Another study evaluated the absorption of the drug Ipsapirone along the human GI tract. In man, Ipsiparone, an anti-depressant, has an absorption half-life of less than 0.25 hours and an elimination half-life of about 1-3 h. This noticeably rapid absorption leads to a rapid peak in plasma concentration of the drug which often causes vertigo, dizziness, and dysphoria (Fuhr et al. 1994). Researchers have, therefore, studied the bioavailability (a term used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation) of Ipsapirone after its administration at different regions of the GI tract to help develop an effective and safe modified-release drug. Researchers hoped that a modified-release version of Ipsapirone would reduce unwanted side effects since the modified-release dosage form does not release its entire dose in one region, thereby avoiding a rapid plasma peak of the drug. Ipsapirone-HCl was administered to four healthy males orally, rectally, and locally in different locations along the GI tract by a remote control drug delivery device. Plasma results indicated that there was a 2-3-fold increase in the bioavailability of the drug administered directly into the colon than when the drug was administered into the upper GI tract. This 2-3-fold increase indicates an equivalence of a 5 mg dose of ipsapirone-HCl released in the colon with a 10-15 mg dose given orally. Oral doses of this size (10-15 mg) were tolerated, and administration of 5 mg ipsapirone into the colon was deemed safe. These results fulfill the criteria for the development of a prolonged-release preparation with 5 mg ipsapirone-HCl. Through modifications of the drug's coating, the drug could be modified to be released only in the colon; such a modification makes it unnecessary to administer a full 10-15 mg immediate-release dose which loses most of the drug by the time it reaches the colon. Considering the fact that this was a small sample of only four males, further research should be done to test if rectal administration of this drug to the colon really equals oral prolonged-release administration (Fuhr et al. 1994).

An example of a new oral modified-release drug that was formulated based on knowledge and applications of the varying characteristics of the GI tract is Rifamycin SV MMX (Di Stefano et al. 2011). Rifamycin SV is an oral, non-absorbable antibiotic which can be used in the treatment of colonic bacterial infections. These tablets are formulated using a multimatrix structure which, like budesonide, delivers the active drug ingredient (200mg of sodium rifamicyn SV) directly to the colon. In immediate-release drugs, the active ingredient would be released immediately upon administration, and maximum bioavailability in the colon would not be achieved (Di Stefano et al. 2011). With the modified-release tablets, however, the maximum bioavailability of the active ingredient is achieved, and the biological effect is optimized at the target region, the colon.

The Rifamicyn SV MMX tablet contains a double-matrix system. Microparticles of the active ingredient are dispersed in a lipophilic matrix which is in turn dispersed in a hydrophilic matrix. The hydrophilic coating inhibits the penetrations of fluids into the tablet, thereby lowering the rate of drug dissolution in the upper GI tract. The tablet is coated with a pH dependent, gastro-resistant polymer film which also inhibits dissolution of the tablet in the upper GI tract. This MMX tablet begins to disintegrate when the pH is ≥ 7. With the pH-sensitive and hydrophilic

coating, the tablet reaches the cecum intact and it is there that the release of the drug begins. The pH-sensitive coating disintegrates, and the intestinal fluids interact with the hydrophilic coating which forms an outer gel mass that slows diffusion of the antibiotic into the colonic lumen (Di Stefano et al. 2011). As the tablet progresses towards the rectum, debris of the gel mass disaggregates and releases the antibiotic directly near the mucosa of the rectum. Studies done through pharmaco-scintigraphic investigations clearly demonstrate an effective colonic delivery of this drug, supporting the production of rifamycin in a modified-release formulation.

These studies all give us valuable insight into the natural absorption of different drugs. Using this knowledge allows for the creation of drugs specially suited for different releases.

PHARMACOKINETICS

Pharmacokinetics is the study of the time-course of drug concentrations in the body, based on various characteristics of drug absorption, distribution, metabolism, and excretion (Mager 2006). As stated above, conventional drug delivery systems very often rely on frequent dosing in order to attain a therapeutic level, which leads to large fluctuations in drug blood levels. The frequency at which a conventional dosage must be given is dependent on two pharmacokinetic properties: elimination half-life ($t_{1/2}$) and therapeutic index (TI) (Sood and Panchagnula 2003). Half-life is the time interval in which half of the active drug in a system is lost, and therapeutic index is the concentration range in which a drug works (Sahin and Benet 2008). Knowing the half-life of a drug assists pharmaceutical companies in setting dosage frequency intervals so that the right amount of active drug will be in circulation at all times, since the dosing interval directly influences the ratio of maximum (C_{max}) to minimum (C_{min}) blood drug concentrations. Although this seems quite simple, it should be noted that there are many other factors involved in half-life and other pharmacokinetic properties and equations which are beyond the scope of this paper. Obviously, drugs with shorter half-lives require a more frequent administration in order to keep blood-drug concentration levels within the therapeutic index. The goal of controlled-release drugs is to release the active drug ingredient at a sufficient rate, frequency, and dose so that the ratio C_{max}/C_{min} is maintained at an effective steady state throughout the therapy without having to be so dependent on half-life and therapeutic indexes which often leads to the necessity of frequent dosing (Sood and Panchagnula 2003). Drugs with zero-order kinetics are generally the easiest to deal with when producing controlled-release dosage forms because they are predictable. They release the active ingredient at a constant rate that is independent of the concentration of the reactants; it is much easier to create a modified-release formulation when the drug's exact rate of release is already known.

RELEASE CONTROL MECHANISM

The knowledge of the gastrointestinal and pharmacokinetic implications on a drug's efficacy aided researchers in manufacturing extended-release drugs which can help in lowering the fluctuations in blood-drug levels. Most extended-release drugs are manufactured through the use of a matrix structure. The drug is suspended or dissolved within the matrix and/or within a rate-controlling membrane through which the drug diffuses (Shargel et al. 2004). In this context, the matrix refers to an inert solid in which the drug is suspended throughout and diffuses out of quite slowly.

There are basically three different types of modified release matrix mechanisms. In the first type, the drug is dissolved in a matrix material that is coated with a soluble coating. In such a case, the rate of drug release depends entirely on the matrix material; a porous matrix will lead to a fast absorption of water, resulting in rapid drug release from the matrix material, whereas a less porous matrix will not absorb much water right away and will, therefore, cause a slower and lengthier drug release. The second system is that of a matrix with an insoluble membrane. When a drug is prepared in such a manner, its release is not only dependent on the permeability of the matrix; it is dependent on the membrane's permeability as well. The third system is a matrix tablet with a combined membrane where the membrane becomes porous after dissolving the soluble part of the membrane in water. Aside from matrix and membrane factors in creating extended-release products, most extended-release drugs release their drug product as a result of a combination of processes including dissolution, permeation, and diffusion. Water permeation is probably the most important factor that drug manufacturers consider since, as the influx of water into the product is controlled, the rate at which the drug dissolves is essentially controlled. Once the drug dissolves, drug diffusion out of the tablet/capsule is further controlled by the permeability of the membrane (Shargel et al. 2004). All of these factors are considered and used to aid in the development of modified-release drugs.

Oral Extended-Release

Of all the different kinds of extended-release products, oral extended-release products prove to be the simplest and easiest to manufacture, lowest cost, high level of reproducibility, and stability (Siddique et al. 2010). The following examples touch the surface of the numerous kinds of applications that can modify the release of oral drugs to maintain homeostatic conditions in the human body.

Gum-Type Matrix Tablets

Gum-type matrix tablets are manufactured with a matrix that swells in the presence of water to form a gel-like consistency. This gel is generally very thick and, therefore, provides a barrier for drug diffusion out of the tablet. Such a matrix is commonly formed with excipients (carrier of active drugs) such as methylcellulose, gum tragacanth, Veegum, and alginic acid. The thickness of the gel caused by these excipients provides controlled-release results in that they prevent drug dissolution until the gel-like matrix breaks up and the drug completely dissolves and gradually diffuses into the intestinal fluid (Shargel et al. 2004).

Diazepam, a benzodiazepine drug used for acute management of severe seizures, is an example of a drug that has been manufactured in a gum-type matrix tablet, thereby providing sustained-release of this drug for hours (Shargel et al. 2004). A study was done on Diazepam which investigated the feasibility of incorporating Diazepam, a poorly water-soluble drug, into solid-lipid nanoparticles (such as a gum-type matrix) that can offer rapid onset (for early termination of seizures) and prolonged-release of the drug for long-acting protection against further seizures. The particular characteristics of the solid-lipid nanoparticles (SLNs) used (such as the SLN's biocompatible lipid core but ampiphillic outer shell in this case) allowed for immediate and prolonged release which, in essence, is the main characteristic of a sustained release drug system. Different SLN formulations were then tested to see which combination of lipid matrices and surfactant would result in the best prolonged-

release results. (SLN formulations composed of Tween 80 as a surfactant and lipid matrix of 5% Compritol ATO 888 and 5% Imwitor 900k provided the best in vitro prolonged-release effects for diazepam (Abdelbary and Fahmy 2009).)

Clearly, a gum-type/lipid matrix is one effective manner of prolonging the release of a drug, thereby preventing the need of frequent dosing and dangerous fluctuations of blood drug levels. However, it should be noted that since this experiment tested results in vitro, more research should be done to obtain in vivo results which would be more beneficial in determining the actual effects of such drugs in the human body.

Polymeric Matrix Tablets

Of recent, drug manufacturers have been very interested in using polymeric material in the matrix to prolong the rate of drug release. The significance of polymeric matrix tablets over other kind of matrix tablets is that polymeric matrix tablets can prolong the release of a drug to last for days or even weeks (Shargel et al. 2004). The polymers that are available for drug formulation include hydrophilic polymers, such as polylactic acid and polyglycolic acid, and hydrophobic polymers, such as ethylene-vinyl acetate copolymer (EVA). The hydrophilic polymers release the drug gradually while the hydrophobic polymers release the drug over a much longer period of time because of its hydrophobic characteristic which blocks water from entering and causing drug dissolution. Additionally, the rate of drug release can be further controlled by combining different polymers, such as adding a hydrophobic polymer to a hydrophilic one to decrease the rate of the drug's release. Light, heat, and other factors may also be administered to change the properties of the polymers being used (Shargel et al. 2004).

One particular study investigated the influence of polymer level and type of some hydrophobic polymers including hydrogenated castor oil; Eudragit RS100; Eudragit L100; and some fillers, mainly mannitol; dibasic calcium phosphate dihydrate; and anhydrous dibasic calcium phosphate on the release rate and mechanism of the drug baclofen. Results showed that a high polymeric content (40%) in the matrix lowered the release rate of baclofen, whereas a low polymeric content (20%) elevated the rate of drug release from the matrix. Additionally, hydrogenated castor oil was proven to be the polymer that caused the strongest retardation of drug release (Abdelkader et al. 2008). Thus, in addition to knowing the kinetics and half-life of a particular drug, the correct combination of polymers can be formulated to bring about the perfect amount and rate of drug release.

Multiple Unit Pellets

Modified-release dosage forms which have been proven to control the release of the active drug, thereby reducing the side effects associated with peak and trough drug plasma levels, have been improved upon in the development of modified-release multiple unit pellets. Whereas single-unit formulations contain the active ingredient within one single tablet or capsule, multiple-unit dosages are manufactured so that a number of discrete particles are combined into one dosage form. These particles could come in the form of pellets, granules, sugar seed (non-pareils), mini-tablets, or powders which contain within them the active ingredient. One advantage of multiple unit pellets over single unit pellets is that when taken orally, multiple unit pellets spread over a large surface in the GI tract, and the particles behave like liquids that

can leave the stomach shortly after entering without waiting for the series of waves to push them along the GI tract (Abdul et al. 2010). In addition, the fact that the pellets disperse throughout the GI tract improves the drug's bioavailability and can reduce drug concentration in a specific location which might otherwise have lead to toxicity. Additionally, sometimes if a drug is released too early along the GI tract, it can irritate the gastric mucosa. Multiple unit pellets definitely reduce the risks associated with premature drug release because of the rapid transition of smaller enteric-coated pellets along the GI tract. Failure of some of these units from reaching their target will also not be as consequential as the failure of a single-unit dose. Theophylline (Gyrocap) is an early example of a beaded form extended-release pellet (Shargel et al. 2004). The frequency of adverse reactions such as nausea, headache, and vertigo, were greatly reduced after the administration of Theophylline in pellet form as opposed to a liquid form. The reduction of side effects in multiple pellet dosage forms comes from the fact that, unlike liquid, the multiple pellet dosage allows drugs to be absorbed gradually instead of rapidly (Shargel et al. 2004).

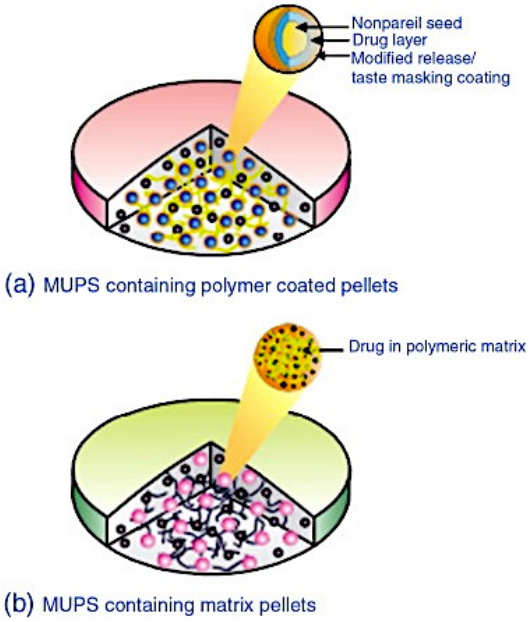

Figure 3: MUPS. Source: Abdul et al. 2010

Because it is difficult to compact the multiple pellets into one system, there are currently only a few multiple-unit tablet products available. They include Beloc® ZOK, Antra® MUPS, and Prevacid® SoluTb™. The difficulties in compaction arise when trying to fuse the pellets together without fusing them mistakenly into non-disintegrating matrixes. If the pellets are fused into a non-disintegrating matrix, they can no longer provide the benefits of a multiple-unit pellet system that disintegrates into individual pellets in the GI fluids leading to a more uniform concentration of active drug in the body (Abdul et al. 2010). Scientists should, therefore, develop safe ways to compact pellets so that the maximum benefits of multiple-unit pellets can be available to the world of modified-release drugs.

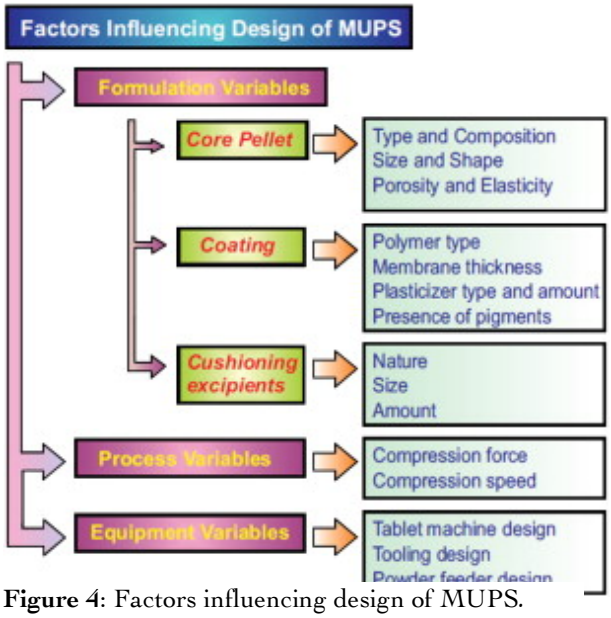

Figure 4: Factors influencing design of MUPS. Source: Abdul et al. 2010

TRANSDERMAL DRUGS

Transdermal drug delivery systems have recently turned into a promising field of study for scientists involved in the production of extended-release drug products. The transdermal drug delivery system, generally in the form of a patch, is designed to deliver the active medication across the skin in a controlled rate over an extended period of time (Shargel et al. 2004). Transdermal drug delivery systems obviously provide benefits over immediate-release oral dosages since the controlled rate of drug delivery eliminates frequent dosing which leads to dangerous plasma level peaks and valleys. Transdermal drugs also seem to have added benefits over the oral modified-release drugs. One major benefit is that drugs administered transdermally avoid hepatic first-pass metabolism. Such a feature can greatly benefit individuals who are hepato-compromised, since the drug avoids the liver altogether. Transdermal delivery routes also completely avoid passage through the GI tract. In this case, poorly bioavailable drugs can clearly take advantage of avoiding the GI tract through a transdermal route (Paudel et al. 2010).

Although transdermal patches may vary, most designs store the active drug in a reservoir that is enclosed on one side with an impermeable backing and with an adhesive on the other side that contacts the skin (Prausnitz and Langer 2008). There are generally four layers in a patch: an impermeable backing membrane, a drug reservoir, a semi-permeable membrane that may serve as a rate-limiting barrier, and an adhesive layer. Nitroglycerin is a drug that is commonly administered transdermally and formulated in the described manner (with the four layers). Nitroglycerin delivered transdermally may provide protection against angina (chest pains that results from a lack of oxygen-rich blood reaching heart muscles) for hours whereas as sublingual (oral) tablets only provide relief for a few minutes (Shargel et al. 2004).

Although transdermal drugs do seem to be a fantastic way to administer drugs at a controlled rate while avoiding possible complications that arise along the GI tract, they have not yet taken over the controlled-release drug market because of several limitations. One such limitation is that the skin, the most important natural barrier against the efficacy of transdermal drugs, only allows moderately lipophilic and low molecular weight drugs to cross over transdermally (Paudel et al. 2010). The solubility of the drug across the skin rather than the concentration of the drug in the patch is the most important of the rate-controlling factors of a transdermal drug (Shargel et al. 2004). Therefore, overcoming low skin permeability through different chemical and physical means has become an active field of research in order to allow many more pharmaceutical products entrance into the world of transdermal drugs (Paudel et al. 2010). Other factors such as humidity and temperature can also affect the rate of absorption across the skin (Shargel et al. 2004).

Interestingly enough, transdermal drug delivery has been proven to provide an even more stable blood level of drug than provided by oral dosages (Shargel et al. 2004). Thus, if the drug is found to be successful against transdermal barriers, scientists will probably be interested in manufacturing the drug almost exclusively as a patch because of the stable blood levels, avoidance of GI tract, and other benefits of transdermal drugs over oral modified-release dosages. Many experiments have, therefore, been done to compare the effects of transdermal versus oral dosage forms. For example, a series of experiments were done to determine the dose-response effects

of oral versus transdermal selegiline on anti-depressant-like activity in rats. Rats received selegiline orally by gavage (0-100 mg/kg) or via transdermal patches (0-4.8 cm^2; 0-8.7 mg/kg) daily for seven days. Antidepressant-like activity was then determined in two ways. The first was through the forced-swim test in which immobility and latency times during a five-minute forced swim test were measured. The latency time, which is the measurement of the swimming time from the beginning of the trial until the onset of immobility, increases under therapeutically effective anti-depressants. The second way through which anti-depressant activity was assessed was through assaying the cerebral cortices of the rats after day seven for MAO-A and MAO-B activities, since inhibition of MAO-A is an indication and requirement for clinical improvements in depressed patients. Results demonstrated that selegiline is an effective anti-depressant, as represented in the forced-swim test after both oral and transdermal delivery, that the anti-depressant-like effect of selegiline requires greater than 70% inhibition of MAO-A activity, and that the transdermal delivery of slelgiline is 10-20 times more potent than the oral selegiline in producing both its anti-depressant-like effects and in inhibiting cortical MAO-A (Gordon et al. 1999). Clearly, transdermal administration of selegiline, which bypasses first-pass metabolism, allows for the usage of lower doses than in oral administration.

Scientists have also put massive focus on the development of a transdermal treatment for menopausal syndromes. One particular experiment was done to determine the efficacy of Busipirone hydrochloride (BH) administration in animal models in the treatment of the main menopausal syndromes of hot flushes and anxiety (Shumilov and Touitou 2010). With oral administration, BH is rapidly absorbed in the GI tract and undergoes extensive first-pass metabolism, so it has a very short elimination half-life. Because of the short half-life elimination, efficient oral treatment requires frequent dosing. Scientists hypothesized that administering this drug transdermally would avoid the drawbacks of oral treatment. This study was therefore done to test the efficacy of the BH transdermal system using ethosomes (vesicular carriers that enhance permeation through the skin). Figure 5 represents the plasma drug concentration profiles following transdermal administration of 15 mg/kg BH from a formulation containing 30 mg/g drug and a single dose of 3 mg/kg oral administration of aqueous drug solution.

Clearly, when administered transdermally, the drug was present in rat plasma for a much longer period compared to the oral administration, 12 hours versus 4 hours, respectively. The continuous delivery of BH into the bloodstream under transdermal administration can offer sustained efficacy with reduced side effects, a huge benefit in the world of modified-release drugs. Additionally, the application of BH ethosomal system on the skin of rats caused a decrease in the temperature at three hours after administration and continued for a total period of 6 hours, proving that transdermal BH could be effective against hot flushes. This should, therefore, be further researched in humans (Shumilov and Touitou 2010).

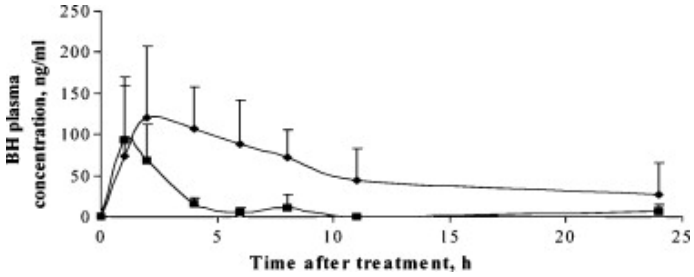

Figure 5: Plasma drug concentration profile following transdermal versus oral administration of BH.
Source: Shumilov and Touitou 2010

Another study was done to compare the efficacy of the Lidocaine Patch 5%, a transdermal, noninvasive treatment, versus Naproxen 500 mg two times daily for the treatment of Carpal Tunnel Syndrome. Results of this experiment proved that Lidocaine Patch 5% provided a comparable level of pain relief to the oral alternative while avoiding risk factors associated with systemic and invasive treatments (Nalamachu et al. 2006).

Transdermal delivery definitely presents an attractive alternative to the oral delivery of drugs. Transdermal delivery has provides an advantage for poorly bioavailable drugs in that it avoids hepatic first-pass metabolism (which can prematurely metabolize drugs) and the GI tract (Prausnitz and Langer 2008 and Gordon et al. 1999). Transdermal delivery of drugs also eliminates frequent dosing and allows for steady, controlled delivery which lowers plasma level peaks and valleys (Paudel et al. 2010). Transdermal drugs are also non-invasive and can generally be self-administered—a big advantage for patients requiring long-term treatment (Paudel et al. 2010). The advantages of the transdermal route over the conventional oral route have led to much interest in producing modified-release drugs through a transdermal route. Eventually, when transdermal challenges, such as permeation of the hydrophobic skin barrier and possible skin irritation from patches, are eliminated, transdermal drugs may become a popular choice on the modified-release drug market.

Implantable Drug Delivery Systems

In the late 1930s, Deasby and Parkes began researching sustained release implantable drug delivery systems as a possible solution to the problem of high plasma concentrations of drugs that may lead to toxicity or low drug levels that may cause sub-therapeutic levels found with immediate-release products (Dash and Cudworth 1998). This relatively novel idea involves implanting a drug delivery system that has been previously modified to release the active drug in a controlled manner into the human body at a specific location. Implantable systems are geared specifically to deliver drugs to a specific site, thereby reducing the amount of drug necessary and limiting its side effects, since the drug does not have to travel throughout the body before reaching its target. Pharmaceutical literature has shown that when the drug used is selective to its site of action, fewer drugs need to be administered. In this manner, drugs which were previously too unstable to administer in-vivo because of bodily temperature and pH conditions that may diminish the drug's efficacy can now be administered directly to the site requiring treatment. Another advantage of implantable drug delivery systems over conventional oral dosages is that while an oral dose may need to be administered one, two, or even multiple times daily, some of the implantable systems have been developed to last as long as five years with minimal monitoring as opposed to administering the oral dose one, two, or even multiple times daily (Dash and Cudworth 1998). These advantages notwithstanding, since the system is to be implanted, care must be taken that it be biocompatible with the human environment. All materials used must be chemically inert, non-carcinogenic, hypoallergenic, and mechanically stable so that the human body does not reject the implantable system.

There are two main classes of implantable drug delivery: drug implants and implantable pumps containing the drug (Dash and Cudworth 1998). The class of drug implants can be further divided into non-degradable and biodegradable implant

systems. One common form of the non-degradable implant system is the matrix system. In this system, the drug is dispersed inside the matrix material, and slow diffusion of the drug out of the matrix provides sustained release of the drug. However, the kinetic release of the drug is not at a constant (zero-order) rate since it depends on the volume fraction of the drug ingredient in the matrix. A non-zero order release rate can be hard to handle in sustained-release drugs since it is difficult to predict its rate of release. A second common form is the reservoir-type system which contains a compact drug core within a permeable non-degradable membrane. The permeability and thickness of this membrane controls the rate of diffusion of the drug into the body. This type of system generally releases its drug at a constant zero-order kinetic release rate because the drug is released based on the properties of diffusion; as soon as some drug is swept away by the surrounding material in the body, more drug diffuses out of the reservoir, leading to the constant rate of diffusion (Dash and Cudworth 1998). Levonorgesterol (LNG), a sustained-release birth control, is the most commonly used reservoir system. In this system, the hormone LNG is encapsulated in a silicone membrane and is implanted on the underside of the upper arm. This LNG system has been proven to effectively provide sustained-release of LNG for up to five years.

However, there are still some problems with non-degradable implants that prevent such a system from being an extremely popular sustained-release route. One issue is that minor surgery is required to insert and remove the implant system, especially with non-degradable systems because they do not disintegrate in the body. Additionally, there is also the fear that the membrane will rupture and lead to "drug dumping," causing drug-plasma concentration to exceed maximum safety levels and have toxic side effects. This fear is especially relevant with reservoir systems because the membrane is the only barrier blocking the drug from diffusing throughout the body, unlike the matrix systems in which the drug must first dissolve out of the matrix and then out of the membrane. Therefore, although implantable systems do seem like a fantastic approach to modified-release drugs, there are clearly some factors that make scientists wary of its use. Biodegradable drug implant systems improve a bit upon non-degradable drugs since the polymers used in biodegradable systems are eventually absorbed or excreted by the body. Surgery to remove the system is avoided, so patients are more accepting to the idea of an implantable drug. However, since the polymers in such a system are biodegradable, many added factors have to be considered. For example, in order to maintain the sustained-release characteristics of the drug in-vivo, the degradation rate of the polymer must be maintained at a constant rate as well as the drug, since the release kinetics, solubility, and diffusion of the drug depend upon the degradation of the polymer (Dash and Cudworth 1998). Factors such as pH, temperature, and increased surface area can lead to early erosion of the degradable system. All of these factors, therefore, become a major challenge when developing a biodegradable system with extended-release goals. The reservoir and matrix systems of biodegradable implants are similar to the non-degradable systems except for the fact that the material used in biodegradable implants is degraded in-vivo at a controlled rate as the drug is released.

The second major class of implantable drug delivery systems is the implantable pump containing the drug. Implantable pumps provide precise control of delivery rate that biodegradable and non-degradable systems cannot provide. Pump systems have

been made possible through advances in micro technology which developed small enough pumps that release drugs at a controlled rate as result of an electronically generated pressure difference gradient (Dash and Cudworth 1998). Implantable drug delivery systems have definitely created a breakthrough in sustained-release drug therapy. However, the limitations mentioned above, such as possible dose dumping and the need for surgery, along with the costliness of these products limits the use of implantable systems. Hopefully, the future will bring possible methods to lower the cost of these products so that implantable drug delivery systems can be used as standard therapeutic practice.

CONCLUSION

Oral, transdermal, and implantable extended-release drug products definitely offer many important advantages over immediate-release dosage forms. The most important advantage is that extended-release characteristics allow for a sustained therapeutic drug-blood level, providing a clinical response in patients that lasts much longer and steadier than with immediate-release products. This sustained therapeutic level also reduces fluctuations between a drug-plasma minimum and maximum that comes from a multiple dose regimen of an immediate-release product. In this manner, the side effects that come as result of the highly fluctuating drug-blood concentrations, such as toxicity and sub-therapeutic levels, are basically eliminated. Another advantage of extended-release products is that such products generally lead to better patient compliance, because taking an extended-release oral dosage once a day, applying a patch once a week, or inserting a pump that lasts five years, is much more convenient than having to remember to take the dosage multiple times a day to maintain a therapeutic level. Clearly, drugs with a short half-life, which under immediate-release characteristics would need to be given frequently, will greatly benefit from an extended-release formulation that can lower dosage frequency and maintain efficacy over a longer duration of time.

One major concern that came with the introduction of extended-release drugs was that although extended-release products would lower fluctuations, they would not provide the same effective therapeutic levels as the immediate-release counterpart. However, all of the studies mentioned in this paper show that the extended-release products do provide the same effective therapeutic effects along with the benefit of lowering fluctuations of the blood-drug levels.

However, some concerns have yet to be resolved. One such concern is the possibility of dose dumping and the difficulty of removing the spilled drug from the body. Immediate-release products definitely have the benefit over extended-release products in this case since it is a much smaller dosage that is causing the adverse reaction and is, therefore, not as toxic, and it is also easier to remove. Another concern is the lack of in-vivo testing conducted on human models. In-vivo testing on human models is crucial since different gastro-intestinal factors and in-vivo characteristics can perhaps interact with the different formulations in possibly destructive ways. Ideally, the study should be done on patients with the disease the drug is meant to treat in order to see if and how the disease affects the release rate and/or efficacy of the drug. Sometimes, slight differences between different individuals, such as caloric content of a meal, body temperature, and weight, can also affect drug release and must, therefore, be taken into account. It may not either be worthwhile to formulate an oral extended-

release drug for products that are administered at high dosages for the practical reason that the size of the pill will be too large to swallow. Therefore, all these factors must been studied extensively to determine the cost/benefit ratio of the extended-release drug.

Clearly, there are a couple of considerations that must be acknowledged when dealing with extended-release products. More research should be done in-vivo to determine if the disadvantages of the extended-release products can be reduced, thereby providing the benefits of lowering fluctuations in blood-drug plasma and maintaining a homeostatic profile without the added disadvantages. For example, further research should determine whether products demonstrate sustained-release characteristics without dose dumping and if products are consistent with minimum patient-patient variations. Further research should also definitely be done on transdermal and implantable drugs since they have the benefit of completely avoiding the intricacies of the gastro-intestinal tract. Perhaps the transdermal and implantable methods can be applied to many more drug products, thereby benefitting patients in the long run.

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EPIGENETICS: A POSSIBLE MECHANISM OF MEMORY

Aliza Grossman Rubenstein

INTRODUCTION

The mind-body connection has fascinated philosophers and scientists for centuries. How is it possible that consciousness arises from a lump of matter known as the brain? How does neurons' firing affect choice and beliefs? How do the electrochemical properties of the brain allow for the memory of events long after they've occurred? One of the most studied of these areas is that of memory. Researchers seek to understand the biological basis behind memory and how that biology is affected in individuals suffering from memory disorders.

Why is memory so difficult to comprehend from a biological standpoint? There are several facets of memory that must be satisfied by its biological mechanism: acquisition, consolidation, and extinction. To acquire a memory, the brain must first be stimulated by the environment. The outside world must be able to influence the biology behind memory. Once acquired, the memory must have the ability to become permanent, or consolidated, to accommodate long-term memory. The biological mechanism must be switched on by the environment, and remain on. Last, once memories are present, there must be a mechanism that allows them to be forgotten (otherwise known as extinction). These three requirements must be found in any biological mechanism that seeks to explain memory.

Recently, researchers have begun exploring the field of epigenetics in relation to memory. Epigenetics (literally "over genetics") is a term coined by Conrad H. Waddington to describe regulation of genetic expression by modifications of the genome that are independent of DNA sequence (Reichenberg et al. 2009; Sananbenesi and Fischer 2009). Originally, epigenetics was only used to describe stable, heritable modifications. Today, though, this distinction has blurred with the realization that epigenetic factors can be both transient and dynamic, occurring fleetingly in response to environmental factors (Day and Sweatt 2011). This is especially true in the field of cognitive epigenetics, as it is this flexibility of epigenetics that enables it to act in cognition.

Epigenetics is well suited as a candidate mechanism to explain memory as it satisfies the three requirements of the biological mechanism of memory. The epigenome is dynamic and can change in response to environment, allowing the encoding of a memory in response to a stimulus. Once activated, it may be relatively stable, thereby storing the memory across time (Day and Sweatt 2011). Despite its relative stability, its dynamic nature does allow for change, thus ensuring the possibility of extinction of the memory. Epigenetics, therefore, may be able to encode, store, and allow for extinction of memory.

EPIGENETICS

Epigenetics encompasses several processes, which may be present separately or may interact to express complex phenotypes. The two factors that have garnered the most research in the field of memory are DNA methylation and histone modifications. Both factors modify the genome, influencing transcription.

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Chromatin

In order to adequately discuss DNA methylation and histone modifications, it is necessary to first examine the organization of the genetic code. The genome consists of three levels of organization: the actual DNA sequence, the histones around which DNA is wrapped, and the chromatin, the highest level. Changes at both the DNA level and the histone level may influence the transcription of the chromatin. Chromatin is divided into two categories, according to its degree of transcription: euchromatin and heterochromatin. Euchromatin is more open and transcriptionally active while heterochromatin is less accessible to transcriptional machinery and is, therefore, transcriptionally silenced (Nelson and Monteggia 2011; Sananbenesi and Fischer 2009). Epigenetic changes at either the DNA level or histone level can alter heterochromatin to become transcriptionally active, or euchromatin to become transcriptionally silenced.

DNA Methylation

The two major pre-transcriptional epigenetic modifications are those that occur at the DNA level, such as DNA methylation, and those that occur at the histone level, such as histone modification. DNA methylation consists of adding a methyl group to the 5' carbon of cytosine within CpG sequences (sequences of cytosine-guanine nucleotides). These CpG sequences often repeat within the genome, especially within the promoter regions of genes, and are then known as CpG islands (Lubin 2011).

Methylation and Demethylation: Mechanisms

DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosylmethionine to the 5' carbon of cytosine (Lubin 2011). There are three distinct DNMTs in humans: DNMT1, which is primarily involved in ensuring that the methylation pattern of DNA is conserved during DNA replication, and DNMT3a and DNMT3b, which are able to methylate DNA *de novo*, at a previously unmethylated position (see Figure 1) (Nelson and Monteggia 2011). DNMTs are selective in their methylation, only methylating specific cytosines. One possible selectivity mechanism is that DNMT1s are targeted towards hemimethylated CpG islands, or islands that have methyl groups attached on one side. A second targeting technique is that of histone modification marks, which

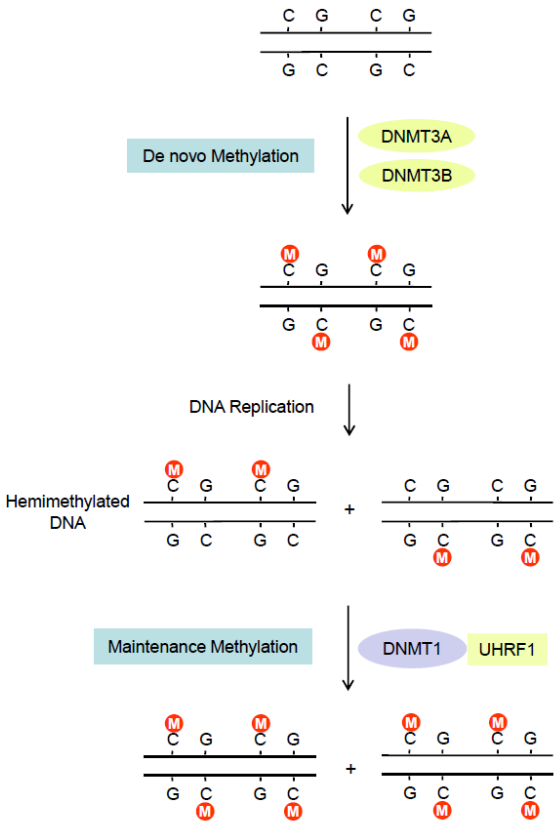

Figure 1: De novo methylation occurs via DNMT3a and DNMT3b, while maintenance replication occurs via DNMT1, which targets hemimethylated DNA. Source: Yu et al. 2011

will be discussed shortly. Yet a third possibility involves small non-coding RNAs, although this has not been described yet in mammals (Yu et al. 2011). This selectivity of DNMTs facilitates the creation of a methylation pattern that can be tissue-specific and event-dependent.

Methylation via DNMTs is a clear and well-known process; however, scientists are still investigating whether that process is reversible, allowing demethylation to occur (see Figure 2). While passive demethylation (i.e. demethylation due to a lack of DNMT activity) is widely accepted, active demethylation remains a puzzle. Methylation creates a covalent bond; as such, it is considered the most stable epigenetic mark (Yu et al. 2011). Over the past decade, though, there have been several reports of active demethylation.

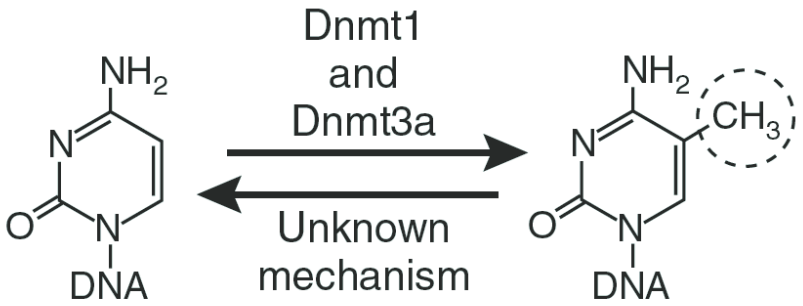

Figure 2: Cytosine group is methylated by DNMT1 and DNMT3a, as well as DNMT3b (not shown in figure) and demethylated by an unknown mechanism, although there are some possible explanations for this mechanism. Source: Korzus 2010

Two candidate mechanisms for active demethylation exist. One is an excision repair-based mechanism involving growth arrest and DNA damage-inducible (Gadd45α and Gadd45β) proteins. A recent study found that overexpression of Gadd45 proteins was correlated with global DNA demethylation while Gadd45 knockdown was correlated with hypermethylation. Presumably, Gadd45 proteins recruit DNA damage repair machinery, which replaces the methylated cytosine with an unmethylated cytosine (Barreto et al. 2007). A second possibility is that ten-eleven translocation 1 protein causes demethylation by converting 5-methylcytosine to 5-hydroxymethylcytosine, which leads to demethylation (Yu et al. 2011). Although it appears that active demethylation occurs, further research is required to elucidate its exact mechanism.

Regulation of Methylation

Methylation is regulated by DNMTs; however, how are DNMTs regulated? The exact mechanisms that regulate the expression of DNMTs are currently unclear (Nelson and Monteggia 2011). It is known, however, that DNMTs are actively regulated across different regions of the body and across a lifetime. Both DNMT1 and DNMT3a are expressed differentially in cortical neurons, especially in interneurons, in the brain of human adults. Fascinatingly, the expression of DNMT3a is greater during embryogenesis and then declines into adulthood while the expression of DNMT3b increases into adulthood (Lubin 2011). This active regulation of DNMTs indicates the dynamic nature of DNA methylation.

Impact of Methylation

DNA methylation generally causes transcriptional repression through two possible mechanisms (see Figure 3). First, it can act as a docking site for proteins that contain a methyl-binding domain, such as the methyl-CpG binding protein2 (MeCP2). These proteins, especially MeCP2, can recruit histone-modifying enzymes that aid in the formation of heterochromatin (Nelson and Monteggia 2011). Second, the methylated cytosine residues hinder transcription by repelling transcriptional activators (Yu et al. 2011). The usual result of DNA methylation is, therefore, transcriptional repression.

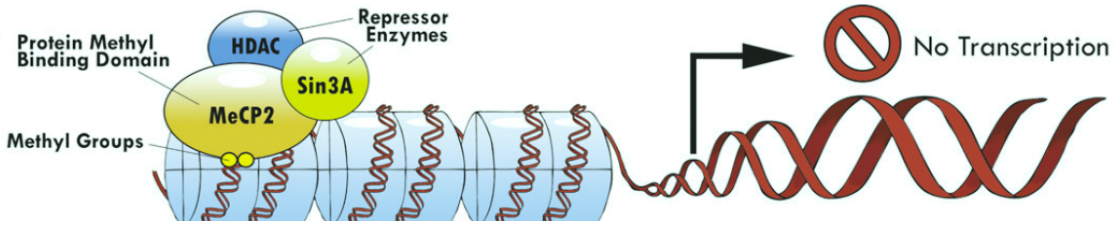

Figure 3: DNA methylation causes MeCP2 to bind to DNA, which recruits repressor enzymes, suppressing transcription. Source: Lubin 2011

Histone Modifications

The second major pre-transcriptional epigenetic regulation occurs at the histones, through histone modification. Before examining histone modifications, further explanation regarding the nature of a histone is necessary. A histone is a small, highly basic protein consisting of a globular domain and a flexible N-terminus. The histone binds DNA, which is wrapped around it. Eight histones, linked as an octamer, form a nucleosome, which consists of two molecules of each core histone: H2A, H2B, H3, and H4. The N-termini of the histones protrude from the nucleosome and are referred to as "histone-tails" (Sananbenesi and Fischer 2009). It is these histone tails that can be modified by the addition of one of several molecular groups: acetyl, methyl, phosphate, SUMO, or ubiquitin. Each of these molecular groups influences transcription differently (Mikaelsson and Miller 2011).

Modification: Mechanism

The modification of the histone tail by each group is catalyzed by a different group of enzymes. The only class of enzymes that is explored in much detail in terms of memory is that of the acetyl group. The acetyl group consists of histone acetyltransferases (HATs), which add the acetyl group, and histone deacetyltransferases (HDACs), which remove an acetyl group (see Figure 4) (Nelson and Monteggia 2011). HDACs form a significant part

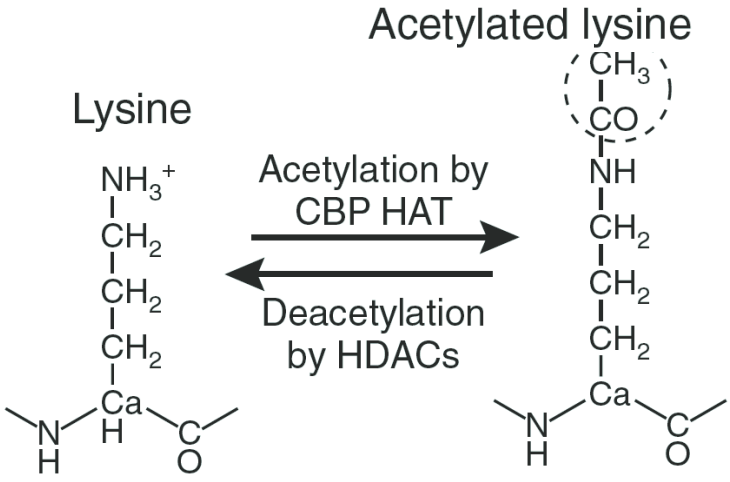

Figure 4: Acetylation of histone tails occurs via HATs, and deacetylation occurs through HDACs. Source: Korzus 2010

of cognitive epigenetics. Two classes of HDACs are Class I HDACs (HDACs 1, 2, 3, 8) and Class II HDACs, which can be further divided into Class IIa (4, 5, 7, 9) and Class IIb (6, 10). Class I HDACs are generally localized to the nucleus, although HDAC3 can be found in both the cytoplasm and the nucleus. These HDACs are highly expressed in the mature neuron, with HDAC3 having the highest expression levels in the hippocampus, cortex, and cerebellum (Haggarty and Tsai 2011; McQuown and Wood 2011). Class II HDACs shuttle between the nucleus and cytoplasm in response to phosphorylation signals and are more specifically expressed (Nelson and Monteggia 2011). Much interaction occurs between HDACs. HDAC3, for instance, can interact with HDACs 4, 5, 7, and 10 (Haggarty and Tsai 2011).

HDACs can be inhibited using small molecule probes called HDAC inhibitors, or HDACis. These probes inhibit histone deacetylase activity, thus increasing histone acetylation and generally increasing gene upregulation. Three classes of probes that have been explored in terms of memory are carboxylic acids (e.g. butyrate and valproate), hydroxamic acids (e.g. trichostatin A and suberoylanilide hydroxamic acid), and ortho-aminoanilines (e.g. MS-275) (Haggarty and Tsai 2011). Potentially, HDACis can be administered therapeutically to change the histone acetylation level.

Impact of Modification

Each group has a different effect on transcription. Acetyl and phosphate groups, which are negatively charged, reduce the affinity of the histone tail for the negatively charged DNA backbone, so the DNA has a more "open" conformation and is more easily transcribed (Lubin 2011). Additionally, acetyl groups facilitate recruitment of bromo-domain coactivators, such as chromatin-associated proteins and histone acetyltransferases, which bind the acetyl-lysine motifs and promote transcription (McQuown and Wood 2011). SUMOylation and ubiquitination have the opposite effect on transcription (Lubin 2011). Methylation, on the other hand, can activate or repress transcription depending on the methylation site and the number of methyl groups that are added. It influences transcription by serving as a docking site for activator/repressor proteins to restructure chromatin, not through its electrical charge (Lubin 2011). The varied effects of the groups on transcription allow for a large degree of precision in epigenetic regulation.

Cross-talk Between DNA Methylation and Histone Modification

DNA methylations and histone modifications often interact, producing interesting results. First, DNA methylation may change the pattern of histone modifications. Methylated CpG islands recruit proteins that interact with HDACs to mediate repression of target groups. Second, histone marks can target DNMTs to specific DNA sequences. This effect is demonstrated at the unmethylated histone H3K4, which becomes a docking site for DNMTs, resulting in *de novo* DNA methylation and transcriptional repression (Lubin 2011). Third, histone modifications are often interdependent. A hypoacetylated H3 tail is targeted in phosphorylation of H3S10 residues, and the phosphorylation of H3S28, methylation of H4K20, and dimethylation of H3K4 are interdependent on HDAC3 (McQuown and Wood 2011).

Memory and Synaptic Plasticity

Even with an understanding of epigenetics, an understanding of memory is still required. Memory on a cellular and molecular level is a much studied but little understood phenomenon. The accepted model of memory claims that memories are encoded as connections between neurons. In this model, neuronal connections are altered as new memories are encoded, either by growing new dendrites or altering synaptic strength (Squire 2011). This model is known as synaptic plasticity.

Long-term potentiation (LTP) and long-term depression (LTD) are two examples of synaptic plasticity. LTP refers to the enhanced synaptic transmission between two neurons, resulting from a short burst of high-frequency stimulation of the presynaptic fibers. LTD can be seen as the opposite of LTP; it involves decreased synaptic transmission due to low-frequency stimulation of the presynaptic fibers (Squire 2011). Both phenomena are persistent, lasting for hours or even weeks, yet they are triggered by a transient stimulus (Squire 2011). LTP, LTD, and long-term memory (LTM) share this feature of a short stimulus affecting long-term neuronal change, as well as input specificity and an inability to be maintained in the presence of protein synthesis inhibitors (Day and Sweatt 2011). Interestingly, enhancements or impairments in spatial memory are demonstrated in animals that have increases or decreases in hippocampal LTP, respectively (Nelson and Monteggia 2011). These shared features strongly suggest that LTP is involved in memory formation.

The next question that scientists ask involves the molecular changes that occur with LTP and LTM. The rapid turnover of proteins within a cell belies the long-term effects of LTP (Day and Sweatt 2011). There must be some kind of maintenance molecule to overcome the loss of acquired changes (Yu et al. 2011). Crick postulated in 1984 that this maintenance molecule would form multimers or at least dimers, with each monomer able to exist in a modified (+) or unmodified (-) mode. If a monomer is (+), then even if molecular turnover causes a newly synthesized linked monomer to be (-), the maintenance enzyme would alter it to become (+) (Yu et al. 2011). This feature matches the activity of DNMTs in methylation of hemimethylated DNA perfectly (see above). Additionally, since both LTP and LTM cannot be maintained in the presence of protein synthesis inhibitors, it seems that changes in gene expression are a necessary component of memory formation (Day and Sweatt 2011; Nelson and Monteggia 2011). Together, these features suggest that epigenetic regulation may act to mediate LTP, and through LTP, memory formation.

Epigenetics and Memory

Evidence of Epigenetic Changes with Learning

The Global Epigenome

In the past decade, much evidence has shown that the epigenome changes in response to learning or memory events. The DNA methylation status of the genome changes drastically. According to Sananbenesi, exposing rats to fear conditioning causes a brief rise in the hippocampal levels of DNMT3a and DNMT3b, presumably changing DNA methylation levels (Sananbenesi and Fischer 2009). This was found to be true in a study performed by Feng and his colleagues that found a global 20% demethylation in DNMT1 and DNMT3a conditional double knockout mice that exhibited memory impairments. That study also demonstrated that 84 genes were upregulated more than 1.5-fold and 7 genes were downregulated more than 1.5-fold in

these double knockout mice (Feng et al. 2010). It has been shown that contextual fear conditioning triggers *de novo* DNMT gene expression in the adult hippocampus (Lubin 2011). DNA methylation appears to be affected in learning and memory.

Besides causing changes in DNA methylation, learning and memory events appear to alter histone modifications. According to Mikaelsson, novel taste learning in rats resulted in heightened acetylation of histone H2A and H4 in the insular cortex (Mikaelsson and Miller 2011). Rats displayed a transient increase in histone acetylation 1 hour after exposure to fear conditioning (Mikaelsson and Miller 2011; Sananbenesi and Fischer 2009). Correspondingly, HAT activity, which serves to acetylate histones, increased in the amygdala following cued fear conditioning, an amygdala- and hippocampal-dependent form of associative learning (Nelson and Monteggia 2011). Learning and memory events appear to cause increased histone acetylation.

Other histone modifications altered by learning include phosphorylation and methylation. Increased histone H3 phosphorylation occurs at Ser10 residue in hippocampal area CA1 during the formation of contextual fear memory (Mikaelsson and Miller 2011). Contextual fear conditioning also increases histone H3 phosphoacetylation, H3K4 trimethylation, and H3K9 dimethylation in the hippocampus (Nelson and Monteggia 2011). Thus, increases in histone phosphorylation and methylation are involved in learning and memory formation.

BEHAVIORAL PHENOTYPE OF EPIGENETIC CHANGES

Behaviorally, learning and memory appear to be dependent on DNA methylation and histone modifications changes. In the aforementioned study by Feng and his colleagues (2010), it was shown that the DNMT double knockout mice, which had lost much of their ability to dynamically regulate DNA methylation levels, performed poorly in the Morris water-maze task, a hippocampus-dependent learning and memory task. Generally, DNMT double knockout mice had impaired spatial learning and memory ability. Other studies sought to determine the effect of the blockade of DNMT activity using DNA methylation inhibitors as opposed to mutated mice. It was found that inhibition of DNA methylation by intrahippocampal injections of 5-aza or zebularine (two DNMT inhibitors) severely impaired memory consolidation (Sananbenesi and Fischer 2009). These studies seem to prove DNMTs are necessary for long-term memory (LTM) formation.

A second vital epigenetic change in memory and learning is that of histone modifications. HATs, which act to acetylate histones, include the cAMP response element-binding (CREB)-binding protein (CBP) and P300. Both are necessary for hippocampal synaptic plasticity and long-term memory formation in both novel objects and contextual fear conditioning paradigms (Mikaelsson and Miller 2011; Sananbenesi and Fischer 2009). In fact, mouse models that lack CBP and its HAT function demonstrate attenuated histone acetylation as well as impaired LTM (McQuown and Wood 2011). LTM is seemingly dependent on HATs.

Since HATs appear to be necessary for LTM, one would expect HDACs to be negative regulators of LTM; this has been shown to be true in mutant mouse models. Mice deficient in HDAC5 show enhanced learning in cocaine conditioned place preference, while mice that overexpressed HDAC4 or HDAC5 demonstrated a weakened expression of cocaine conditioned place preference (McQuown and Wood

2011). Additionally, HDAC2 deficient mice experienced enhanced memory formation and synaptic plasticity while forebrain overexpression of HDAC2 (but not HDAC1) caused impaired memory formation and synapse formation (Nelson and Monteggia 2011). A study undertaken by McQuown and her colleagues demonstrated that HDAC3 deficiency enhanced LTM formation to such a degree that HDAC3 deficient mice that received subthreshold training (3 minutes) in a novel object recognition test did experience LTM formation, and those mice that had completely lost HDAC3 function retained this memory for seven days, which is longer than the normal retention of object memory (2011). It seems that HDACs negatively regulate LTM.

Several studies have sought to confirm that HDACs negatively regulate LTM by examining the effect of HDAC inhibitors (HDACis) in wild-type mice. The previously mentioned study by McQuown and her colleagues also found enhanced learning in wild-type mice that were injected with an HDACi, RGFP136 (2011). These mice experienced LTM even with subthreshold training conditions. A second study, performed by Vecsey and his colleagues (2007), proved that HDAC inhibition in the hippocampus enhances memory consolidation for hippocampus-dependent learning by microinjecting mice with trichostatin A (TSA), an HDACi, directly after conditioning, and measuring their level of freezing when exposed again to the conditioned context. Mice injected with TSA showed a notable enhancement in memory as compared to control groups. This study also ruled out HDACi inhibition enhancement of memory retrieval, as opposed to consolidation, by microinjecting the mice with TSA four hours before re-exposure to the conditioned context: results were comparable for experimental and cued conditioning (Vecsey et al. 2007). Additionally, the study repeated the protocol using non-hippocampal dependent conditioning in order to prove that the memory enhancement is due to the microinjections into the hippocampus; again, no differences were found between the TSA- and control-treated mice (Vecsey et al. 2007). It appears that HDACis do enhance LTM formation in wild-type mice.

HDACis have also been found to ameliorate loss of other epigenetic functions, such as those caused by inhibition of DNMTs or loss of HATs. DNMT inhibitors impair memory, but this effect appears to be reversed by administration of TSA, an HDACi, prior to the test (Day and Sweatt 2011). Loss of CBP, as mentioned above, impairs LTM, but not short-term memory, in a number of learning and memory tests. Administration of HDACis to *Cbp* mutant mice restores their memory function, probably because some CBP HAT activity remained active (Nelson and Monteggia 2011). Oddly enough, when the same HDACis were administered to CBP conditional knockout mice, which lacked any expression of CBP in excitatory neurons of the forebrain, no restoration of memory function was observed; this was most probably due to the complete deficiency of CBP (Nelson and Monteggia 2011). A similar effect was observed in the aforementioned study by Vecsey and his colleagues (2007). Memory enhancement was observed in $CREB^{+/+}$ mice that were injected with TSA but not in the CREBαΔ (CREB-deficient) mice that were injected with it. It appears that HDACis can act in a limited capacity to reverse memory impairment caused by deficiencies in other epigenetic functions.

Effect of Epigenetic Changes on LTP and LTD

After concluding that epigenetic changes heavily influence memory formation, the question is whether they influence synaptic plasticity (e.g. LTP and LTD). DNA methylation does appear to play a role in both LTP and LTD. A number of studies in mutant mouse models demonstrate that DNA methylation is necessary for LTM formation. In a study performed by Feng and his colleagues (2010), the mice that were deficient in DNMT1 and DNMT3a, showed attenuation of LTP and enhanced induction of LTD. Importantly, DNMT KO mice show no LTP after 1-2 hours, but the base response is still there, so the DNMTs must be acting in memory acquisition, not in initial synaptic transmission (Day and Sweatt 2011). One explanation of why DNA methylation is so significant in LTP focuses on MeCP2, one of the mechanisms by which DNA methylation acts to repress transcription. MeCP2 mutant mice show impairments in hippocampal LTP, hippocampal LTD, and cortical LTP, while MeCP2 overexpressing mice show enhanced hippocampal LTP. Apparently, DNA methylation may mediate LTP through MeCP2.

Other studies have sought to confirm that DNA methylation influences synaptic plasticity by examining the results of treatment with DNMT inhibitors. Brain slices treated with DNMT inhibitors show no LTP after 1-2 hours, but the base response is still there, so the DNMTs must not be influencing the synaptic transmission itself (Day and Sweatt 2011). Specifically, the LTP in hippocampal slices that were treated with the DNMT inhibitors zebularine and 5-aza was shown to be reduced in magnitude (Nelson and Monteggia 2011). DNA methylation is significant to synaptic plasticity.

Histone acetylation, as well, appears to play a role in synaptic plasticity, as studies using mutant mice models demonstrate. HDAC2 overexpressing mice showed impaired hippocampal LTP (Nelson and Monteggia 2011) as well as decreased dendritic spine density and synapse number (Haggarty and Tsai 2011), while HDAC2 forebrain-specific KO mice showed enhanced LTP (Nelson and Monteggia 2011) and increased synapse number (Haggarty and Tsai 2011). Additionally, heterozygous *Cbp* mutant mice showed impaired hippocampal late-phase LTP with a normal stimulation protocol; however, with a stronger stimulation protocol, no impairment was observed. Histone acetylation is crucial for synaptic plasticity.

Several studies have sought to confirm the involvement of epigenetic changes in synaptic plasticity, using the effects of HDACis on wild-type mice. The study performed by Vecsey and his colleagues (see above) demonstrated that HDACis enhanced LTP in hippocampal slices from wild-type mice (Vecsey et al. 2007). Their controls included mice that were injected with TSA and actinomysin D, a substance that prevents transcription, to prove that HDACis act through transcription-dependent mechanism. Most HDACis appear to have this effect on LTP. Treatment of hippocampal slices with TSA and sodium butyrate, an HDACi, resulted in enhanced LTP induction at Schaffer-collateral synapses, while treatment of amygdala-containing slices with TSA resulted in enhancement of forskolin-induced LTP (Nelson and Monteggia 2011). Last, treatment of hippocampal slices with suberoylanilide hydroxamic acid enhanced late-phase LTP in wild-type mice but had no effect on HDAC2 KO mice, showing that HDACis are effective due to their effect on HDACs

(Nelson and Monteggia 2011). HDACis do enhance LTP in wild-type mice, confirming a role for histone acetylation in LTP.

Other studies examined the ability of HDACis to compensate for the effects of other epigenetic deficiencies. HDACis compensate for the loss of DNMT activity through DNMT inhibitors; treatment with TSA prior to testing reverses the effect of DNMT inhibitors on LTP (Day and Sweatt 2011). Additionally, HDACis can somewhat attenuate the effects of a loss of CBP function. The treatment of hippocampal slices with suberoylanilide hydroxamic acid does ameliorate the LTP deficit generally observed in $Cbp^{+/-}$ mice (Nelson and Monteggia 2011). However, TSA treatment of CREBαΔ mice and mice with a genetic disruption between CREB and CBP did not enhance LTP. Apparently, HDACis can compensate somewhat for the loss of other epigenetic changes.

MECHANISM OF EPIGENETIC CHANGES AND MEMORY

Now that it is clear that epigenetic changes are involved in memory, the next step is to determine how they influence memory. Intriguingly, as explained above, memory appears to be dependent on both histone acetylation and DNA methylation (see Figure 5). This is rather incongruous as histone acetylation increases transcription while DNA methylation decreases transcription. The apparent inconsistency can be explained by viewing these epigenetic modifications as gene-specific, so that histone acetylation upregulates some genes whereas DNA methylation downregulates other genes. There are many genes, as well as some non-histone substrates of histone-modifying enzymes, that are regulated by these epigenetic changes.

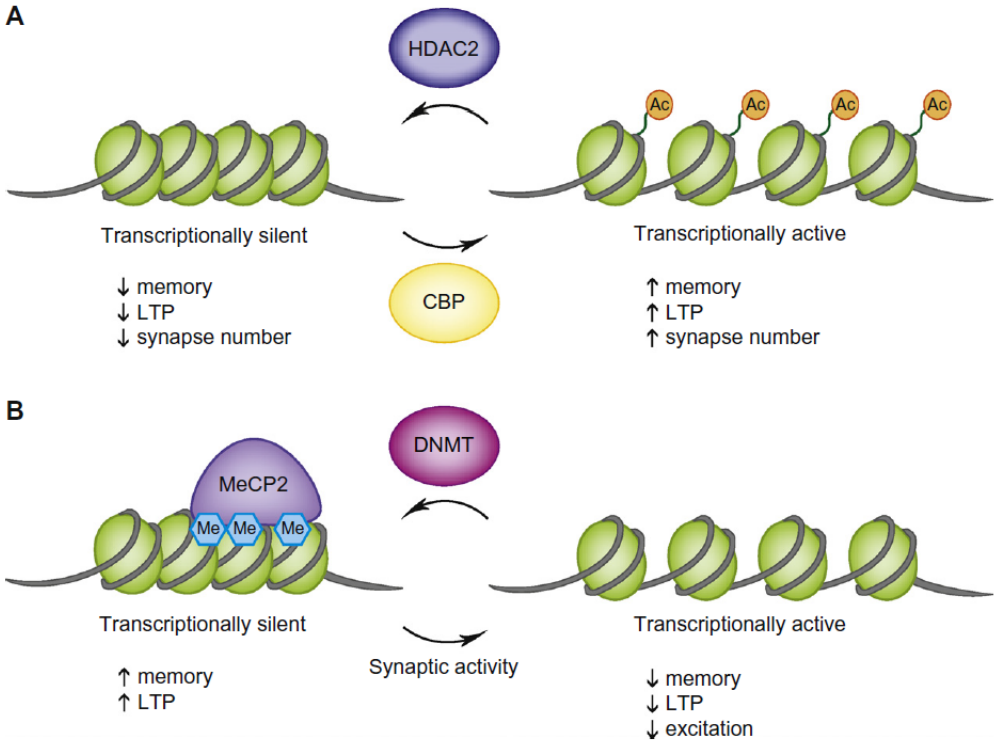

Figure 5: Histone acetylation and DNA methylation are necessary for memory formation and LTP. Histone deacetylation and DNA demethylation impair memory formation and LTP. Source: Nelson and Monteggia 2011

Epigenetic Proteins

Interestingly, several enzymes that are instrumental in epigenetics are themselves regulated by epigenetics. First, DNMT1, the enzyme that methylates DNA, is itself a target of histone methylation enzymes (Lubin 2011). Second, HDAC1, which acts to deacetylate histones, is a substrate of histone acetylation enzymes (Lubin 2011). Third, the HATs p300, CBP, and p300/CBP-associated protein, are their own targets; each can act to acetylate itself, a process known as auto-acetylation (Lubin 2011). They can also be deacetylated by HDAC3, in combination with a corepressor known as nuclear receptor co-repressor 1 (McQuown and Wood 2011). Histone-modifying enzymes modify both DNA methylation enzymes and other histone-modifying enzymes.

Memory Genes

Several genes that are known to be involved in memory are regulated via epigenetics. The promoter of *reelin*, which enhances LTP, shows a robust response when exposed to DNMT inhibitors while learning (Mikaelsson and Miller 2011). DNMT inhibitors also modify DNA methylation in the adult brain at the promoter of *bdnf*, a gene that is crucial for memory (Day and Sweatt 2011). Electroconvulsive treatment, which causes LTP, decreases the methylation level of specific regulatory regions of *bdnf* (Yu et al. 2011). Interestingly, *bdnf* promoters are differentially methylated in memory. *Bdnf* exon I promoter is demethylated by chronic network activity caused by picrotoxin treatment of cultured neurons, as well as contextual exposure to living animals; the methylation level of this promoter is correlated with object recognition memory task performance (Yu et al. 2011). *Bdnf* exon IV promoter, which is generally basally-repressed by MeCP2, becomes demethylated and, thereby, expressed following high potassium induced neuronal depolarization in rodent primary neuron culture (Yu et al. 2011). Interestingly, *bdnf* demethylation may be active, caused by Gadd45β, as discussed above, since Gadd45β-KO mice displayed no significant demethylation at regulatory region of *bdnf* exon IX in response to electroconvulsive treatment, thus downregulating *bdnf* expression (Lubin 2011). Both *reelin* and *bdnf* are regulated by DNA methylation.

Bdnf is also regulated by histone modifications. Fear conditioning causes the upregulation of *bdnf* exons I and IV, which is associated with increased histone acetylation and phosphorylation at those promoters. Additionally, extinction of fear conditioning in mice is associated with an increase in histone H4 acetylation around the promoter of *bdnf* exon IV. *Bdnf* is regulated epigenetically to influence LTM formation.

Two memory suppressor genes, *Re1n* and *Pp1* are also regulated by DNA methylation. Interestingly, although DNMT inhibitors upregulate *Re1N* and *Pp1*, DNMT1 and DNMT3a conditional double knockout mice have normal expression of both genes (Feng et al. 2010; Nelson and Monteggia 2011). PP1 may actually participate in regulating other epigenetic modifications at the promoters of *Creb* and *nuclear factor-kappa B (NF-κB)*. *Pp1* and *Re1N* may be epigenetically modified during memory formation.

Transcription Genes

Several genes that are involved in transcription experience epigenetic modification during memory formation. *Nr4a1* and *Nr4a2*, which are both immediate

early genes (IEGs), acting to transcribe other genes, are regulated by histone acetylation. After TSA-induced memory enhancement, CBP-dependent expression of *Nr4a1* and *Nr4a2* occurred (Vecsey et al. 2007). Additionally, increased *Nr4a2* expression was observed in the area of focal HDAC3 deletion in the dorsal hippocampus two hours after subthreshold training (McQuown and Wood 2011). Remarkably, the silencing of *Nr4a2* through small interfering RNA attenuates the memory enhancing effects of HDAC3 deficiency in novel object memory (Haggarty and Tsai 2011), indicating that *Nr4a2* may interact with CBP and/or HDAC3 in their epigenetic roles, besides for being regulated by them.

A second IEG is *Egr1*, which is also influenced by histone acetylation. *Egr1* is upregulated in the hippocampus by associative learning. This appears to be mediated by BDNF, which causes HDAC2 to leave chromatin. H3 and H4 in the *Egr1*promoter are thereby acetylated, causing transcription. The expression of *Egr1* is affected by histone acetylation.

A third transcription factor is NF-κB, previously discussed in the context of PP1, which may act to regulate it. The promoter of *NF-κB* experiences reduced phosphorylation during novel object recognition (Mikaelsson and Miller 2011). NF-κB itself has been implicated in the induction of synaptic plasticity and initial formation of LTM. One of its subunits, p65/Re1A, is actually activated as one of the non-histone substrates of histone-modifying enzymes. It is the target of both histone methylation and histone acetylation enzymes (Lubin 2011). HDAC2 acts to negatively regulate it, and treatment with TSA results in prolonged p65 acetylation with a resulting increase in NF-κB DNA binding activity as well as enhanced memory formation. This enhancement in memory is attenuated by inhibitors of NF-κB DNA binding activity, indicating that this may be a mechanism whereby HDACis cause enhanced memory formation (Lubin 2011). NF-κB is regulated epigenetically to influence memory formation.

Other IEGs and transcription factors include *c-Fos*, transcription factor p53, and MEF2. Increased *c-Fos* expression was noted in the area of focal HDAC3 deletion in the dorsal hippocampus two hours after subthreshold training (McQuown and Wood 2011). Transcription factor p53 is a non-histone substrate of histone-methylating and histone-acetylating enzymes (Lubin 2011). MEF2, a transcription factor important for regulation of structural plasticity genes, can be deacetylated by HDAC3, thus terminating the transcription of plasticity genes.

Immune Function Genes

Interestingly, several genes that are involved in immune function may be involved in memory as well, including *MHC 1*, *Stat1*, and *calcineurin*. *MHC 1* is highly upregulated in DNMT double knockout mice (Feng et al. 2010). *Stat1*, which is important for synaptic function in CNS and learning/memory, is also highly upregulated in neuronal cells of DNMT double knockout mice (Feng et al. 2010). *Calcineurin* is regulated by a methylation change that occurs in contextual fear conditioning (Yu et al. 2011). These genes may be involved in signaling pathways that influence memory formation.

Other Non-histone Targets of Epigenetic Enzymes

There are several other non-histone substrates of chromatin-modifying enzymes. The estrogen receptor alpha is targeted by both histone methylation and histone acetylation enzymes. Both tubulin and the glucocorticoid receptor are substrates of histone-acetylating enzymes. The function of these proteins in memory is unclear.

Regulation of Epigenetic Changes in Memory

Epigenetics clearly influences memory via regulation of genetic transcription. How, though, is epigenetics itself modulated by the physical cause of memory, neuronal stimulation? Clearly, increased synaptic activity triggers DNA methylation changes and histone modification changes. N-methyl-D-aspartate receptor activation is a crucial part of the signaling pathway. It activates MAPK signaling which is instrumental in hippocampal H3 acetylation (Sananbenesi and Fischer 2009). The ERK/MAPK pathway is crucial for heightened acetylation of H2A and H4 in insular cortex due to novel taste learning in rats, histone acetylation associated with hippocampus-dependent fear memory, and histone H3 phosphorylation during formation of contextual fear memory (Mikaelsson and Miller 2011). Additionally, N-methyl-D-aspartate receptor activation is actually linked to both *bdnf* DNA methylation and changes in the levels of histone H3K4me3 at the *bdnf* promoter IV in response to contextual fear conditioning (Lubin 2011). Other pathways that may be involved in DNA methylation mediation are the protein kinase C and NF-κB pathways (Lubin 2011). Synaptic activity may act through N-methyl-D-aspartate receptor activation to initiate signaling cascades that cause epigenetic modifications to occur.

Future Directions

Although it is clear that epigenetics is heavily involved in memory formation, there are still many questions that must be clarified. These questions can be classified into three categories: those regarding memory alone, those involving epigenetic changes and memory, and those investigating therapeutic potential of epigenetics. According to Haggarty (2011), it is crucial to understand the roles of the individual genes implicated in memory in order to comprehend the ways in which epigenetic modifications affect them. Before understanding memory, it is unfeasible to understand how epigenetics affects memory.

A second direction is to examine the ways in which epigenetic modifications affect memory. One problem involved is that of cross-talk between epigenetic modifications (e.g. histone acetylation affecting DNA methylation, etc.). Researchers should undertake to study the epigenome and neuron as a whole, investigating all aspects of memory formation in order to understand how they interact (Haggarty and Tsai 2011). Another part of epigenetic modification that must be clarified is that of how a cell-wide modification affects synapse selectivity. Although there are several theories that attempt to explain this, no studies have examined it in depth (Day and Sweatt 2011). A third difficulty is that of differentiating between epigenetic modifications that are transient and activity-induced and can therefore be implicated in acquisition, and those that are more stable and likely involved in consolidation (Yu et al. 2011). Answering these puzzles is a significant step in understanding epigenetics.

The last class is that of examining the potential of HDACi as a therapeutic drug. The memory enhancement ability of HDACi seems to indicate its utility as a therapeutic drug for cognitive diseases such as Alzheimer's disease and Huntington's disease. Several questions must be answered, though, before it can be used clinically. First, the selectivity of different HDACis must be determined. Since non-histone substrates and histone substrates are involved, it is necessary to determine how different HDACis will affect each of them. Currently, most HDACis affect all HDACs, which may be too general for therapeutic utility. More research should be undertaken to find other, more specific, HDACis (Haggarty and Tsai 2011). Additionally, the absorption, distribution, metabolism, excretion, and pharmacokinetics of HDACis must be studied (Haggarty and Tsai 2011). Although HDACis have great potential, much must be answered before they can be used.

CONCLUSION

Epigenetic modifications play a large role in memory formation. They modify genetic expression of many genes and proteins that are involved in transcription and memory formation. Although it is still unclear how exactly epigenetics fulfills the requirements of a molecular mechanism of memory formation, it definitely holds much potential for future research and investigation of its role. The mind-body connection may be elucidated after all.

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IMATINIB RESISTANCE IN PHILADELPHIA CHROMOSOME-POSITIVE CHRONIC MYELOID LEUKEMIA

Rivky Kops

Chronic myeloid leukemia (CML) is a disorder of blood stem cells in bone marrow, which leads to a rapid production of white blood cells. Of the patients diagnosed with CML, 95% have the Philadelphia (Ph) chromosome, which means that chromosome 22 is smaller than regular (22 q-). Historically, the median survival time for chronic phase CML patients was four to five years, while the accelerated and blast (profusion of immature red blood cells in circulation) phases had a much shorter survival time. Recently, due to the revolutionary new drug imatinib, CML patients diagnosed early have a higher survival rate. Nevertheless, some patients may show resistance to imatinib, and alternative treatments must be considered (Hochhaus and La Rosée 2004).

CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia originates in a single pluripotent bone marrow stem cell. It accounts for approximately 15% of all leukemia cases (Liesveld and Lichtman 2011). As shown in Figure 1 below, the long ("q") arms of chromosome 9 and 22 swap DNA, resulting in a longer chromosome 9 (9q+) and shorter chromosome 22 (22q-).

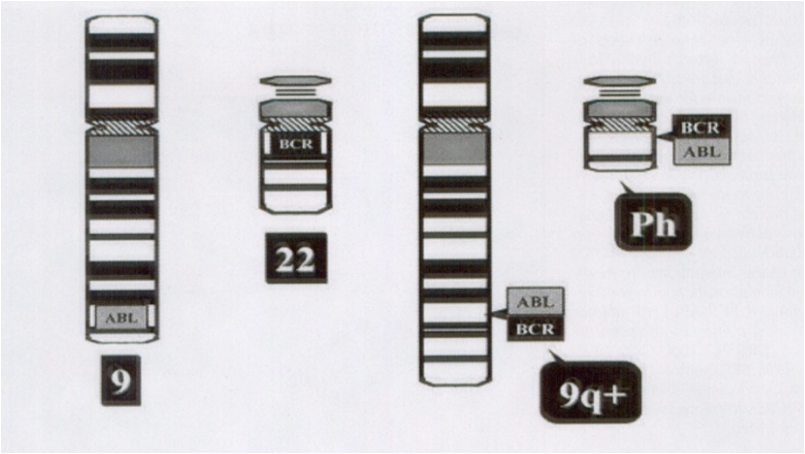

Figure 1: Normal chromosomes 9 and 22, and 9q+ and 22q- resulting from the reciprocal BCR-ABL translocation. Source: Litzow 2006

Chromosome 22q- is known as the Philadelphia chromosome and is the identifying characteristic of CML in over 90% of cases. It occurs when the Abelson oncogene (ABL) of chromosome 9 at 9q34 fuses with the breakpoint cluster region (BCR) of chromosome 22 at 22q11. BCR is a multiplier gene, and ABL codes for a tyrosine kinase which is heavily suppressed in healthy cells (Encyclopedia of the Human Genome). The resulting BCR-ABL gene codes for a constitutively active tyrosine kinase which induces rapid stem cell differentiation by inducing cell growth and bypassing signals that block cell mitosis. These new BCR-ABL+ stem cells have

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lower proliferation capacities compared to normal stem cells, but the tremendous increase in stem cells results in a net increase in leukocytes . Granulocytes, and often megakaryocytes, are the main cells arising from the malignant stem cells. However, studies have also found erythroblasts and macrophages with the Philadelphia chromosome, leading to the belief that CML arises in a pluripotent stem cell (Liesveld and Lichtman 2011).

Early stage chronic myeloid leukemia is asymptomatic; the abnormally high percentage of white blood cells compared to red blood cells is frequently only detected as part of a routine complete blood count. Common CML symptoms are anemia; extreme blood granulocytosis; splenomegaly; early satiety and unintentional weight loss; and, seldom, thrombocytosis. A chronic phase is usually followed by an accelerated phase, which is characterized by blasts making up 15% of the red blood cells circulating in the bloodstream. Historically, median survival rate in the accelerated phase has been one to two years, with many dying before reaching blast crisis. Blast phase, characterized by 30% immature cells, had a median survival of three to six months. Ninety percent of patients are diagnosed in the chronic stage. In the chronic stage, survival rates and remission rates are more optimistic compared to the accelerated and blast phases (Pemmaraju et al. 2011).

There are three markers of remission: hematological, cytogenetic, and molecular response. Complete hematological remission is marked by white blood cell counts returning to normal levels of less than 10 x 10^9/L and the disappearance of CML symptoms. However, while quality of life is much improved with the normalization of white blood cell counts, hematological remission is a poor indicator of long-term survival. Cytogenetic response means that cells bearing the Ph chromosome are not being produced. Partial cytogenetic response is defined as 1 to 35% of metaphases remaining Ph positive, while complete cytogenetic response means that no mature cells bear the Ph chromosome. Complete molecular response means that there are no detectable BCR-ABL transcripts (Pemmaraju et al. 2011). In an IRIS (International Randomized Study of Interferon and STI-571) trial, which compared the efficacy of drugs targeting CML, achieving a complete cytogenetic response was determined to be the most important factor in long-term survival. Of the patients achieving complete cytogenetic response but incomplete molecular response, the five-year survival rate was 98% (Wetzler et al. 2012). Therefore, achieving a complete cytogenetic response was established as the goal of treatment (Pemmaraju et al. 2011). Progression-free survival, which means that patients have not progressed from the chronic phase to a more advanced state, is also used as a benchmark of successful therapy, since patients in the chronic phase usually experience few side effects and have a higher quality of life.

Because the vast majority of CML patients display the Ph chromosome, the BCR-ABL fusion is the best target for therapy to treat CML. Imatinib is the first truly targeted drug to inhibit a tyrosine kinase (Marx 2001), and it has granted CML patients a hopeful prognosis.

IMATINIB

Imatinib, a highly targeted tyrosine kinase inhibitor, uses hydrogen bonding to bind to the contact site in the inactive configuration of the BCR-ABL kinase. This

hinders the ATP binding site so that the affected cell has no source of energy for proliferation and survival. This process is shown in Figure 2.

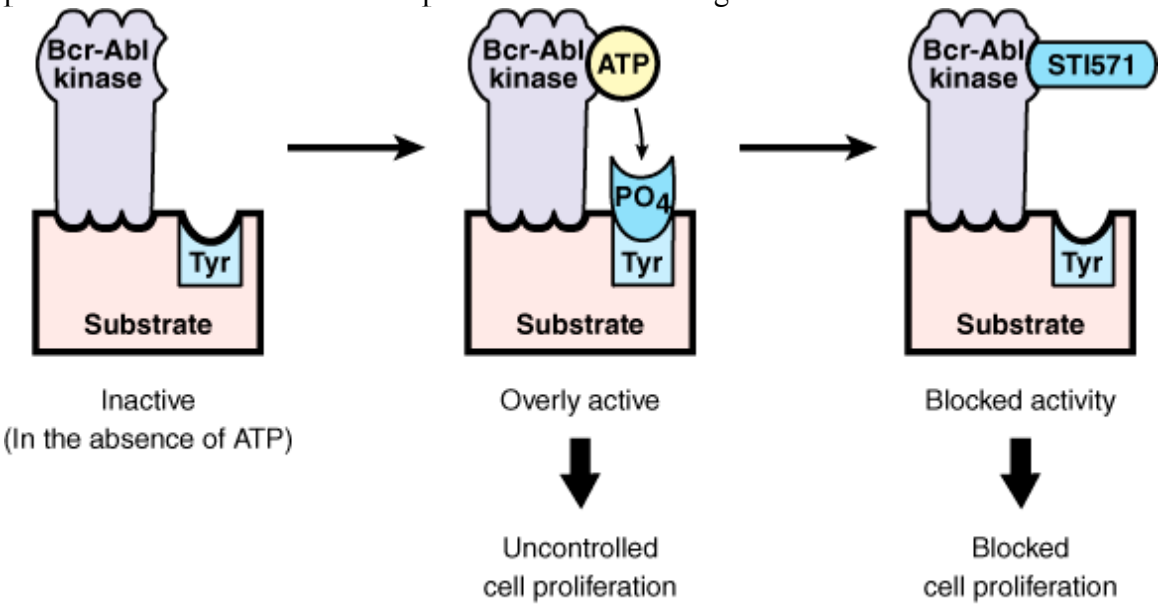

Figure 2: Imatinib (STI571) filling ATP binding site of BCR-ABL tyrosine kinase. Source: Feng et al. 2010

Imatinib also induces apoptosis in Philadelphia-chromosome-positive cells without affecting Ph negative cells (Moen et al. 2007). It has a very high success rate, and patients diagnosed in the early chronic phase now have an estimated survival of 20 to 30 years. The rate of complete cytogenetic response for early chronic phase is about 80%, and five-year progression-free survival is 96.7%. Imatinib is well tolerated, with less than 3% of patients showing resistance to it (Pemmaraju et al. 2011). The structure of imatinib is shown in Figure 3.

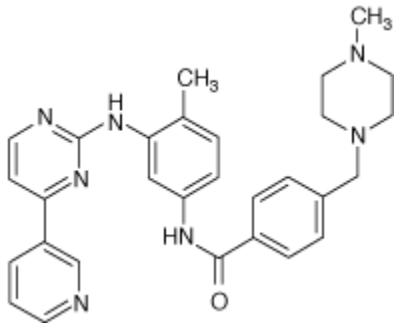

Figure 3: Structure of imatinib. Source: Chabner et al. 2011.

Prior to the introduction of imatinib, patients were treated with interferon (IFN-α), which treated CML more effectively than standard chemotherapy. IFN-α was successful in achieving a complete cytogenetic response in 5 to 25% of patients. Combining IFN-α with cytarabine yielded better results and a greater probability of survival. In an IRIS trial, patients were randomly chosen for either IFN-α with cytarabine or imatinib. Imatinib showed significantly higher rates of complete cytogenetic response and lower toxicity levels than IFN-α with cytarabine. At 19 months, the percent of imatinib-treated patients achieving complete cytogenetic remission was 79%, compared to 11% achieved in patients treated with IFN-α with cytarabine. At the five-year follow-up, the percent of complete cytogenetic remission from imatinib had increased to 82%. There are no five-year follow-up data for the group taking IFN-α because most of that group switched to imatinib treatment. Additionally, health-related quality of life was maintained among the patients on imatinib, while those receiving IFN-α with cytarabine experienced a deterioration in

quality of life. Imatinib was therefore established as the primary therapy for chronic myeloid leukemia (Moen et al. 2007).

The standard dose for imatinib is 400 mg/day, with doses under 300 mg/day yielding unsatisfactory results. The TOPS (Tyrosine Kinase Inhibitor Optimization and Selectivity) trial investigated the possible benefit of doubling imatinib dose to 800 mg/day. While patients taking 800 mg/day initially fared better, the percentages of complete cytogenetic responses over a longer period were nearly equal: 64% for 800 mg/day and 58% for 400 mg/day. Since long-term toxicity levels have not been assessed for the higher dose, the standard dose is currently 400 mg/day (Pemmaraju et al. 2011).

Imatinib, unlike previous treatments for CML, is unique in that it can effect a hematological remission for patients in the accelerated and blast phases (Marx 2001). However, 4-5% of patients on imatinib, particularly those who have progressed past the chronic stage, become resistant to the drug and relapse (Biotech Business Week 2006), including 80% of patients in blast crisis (Marx 2001). Others may never achieve remission with imatinib.

IMATINIB RESISTANCE

Imatinib resistance results from BCR-ABL gene amplification and point mutations. Some causes of resistance can be countered by dose escalation, while others render imatinib useless.

Some patients relapse due to gene amplification, which leads to increased kinase activity. The BCR-ABL gene produces more tyrosine kinase than standard dose imatinib (400 mg/day) can counter. The tyrosine kinase produced by BCR-ABL overexpression causes too many leukemia cells to be produced. While imatinib still functions properly, the leukemia cells proliferate at an even faster rate than usual. If the patient has not become imatinib resistant, an increased dosage of 600 or 800 mg/day may overcome the rapid proliferation of cells and bring about a remission. In particular, patients who achieved a complete cytogenetic remission and then lost the remission benefit from increased dosage (Pemmaraju et al. 2011).

Some patients are completely resistant to imatinib. Imatinib resistance is categorized as either primary or acquired. Primary resistance means that the patient never responds to the medication. Acquired resistance means a loss of imatinib-benefit after previously benefiting from it, which can be on a hematological, cytogenetic, or molecular level (Hochhaus and La Rosée 2004).

Point mutations, individual changes in gene sequencing, often confer drug resistance. They are thought to occur due to the inherent genetic instability of cancerous cells (Marx 2001). The dominant mechanism of imatinib resistance is genetic mutation in the kinase domain (ABL portion) of BCR-ABL. The mutated Ph chromosome contains an amino acid residue different than the regular BCR-ABL oncogene, coding for a slightly different tyrosine kinase. Since imatinib competitively inhibits the BCR-ABL tyrosine kinase by snugly fitting into the contact site, any change in the binding site can prevent it from binding effectively. Additionally, mutations often lock the protein in its active configuration, and imatinib binds only to the inactive configuration. Imatinib's pronounced specificity in binding makes resistance common (Liesveld and Lichtman 2011).

There are four main regions of the protein that are prone to resistance-conferring mutations: the ATP binding loop, or P-loop; the imatinib contact site; the catalytic domain; and the activation loop, which controls catalytic activity and changes conformation depending on protein activation (Litzow 2006). Although over 40 point mutations have been identified, 85% of mutations occur at seven amino acid residues: M244V, G250E, Y253F/H, and E255K/V in the P-loop; T315I at the contact site; M351T and F359V in the catalytic domain (Cang and Liu 2008). Usually only a single point mutation is detected in an imatinib-resistant patient, but occasionally patients have multiple mutations (Hochhaus and La Rosée 2004).

P-loop mutations account for 36-48% of all mutations (Cang and Liu 2008). The tyrosine kinase is composed of two flexible loops: the P-loop and the activation loop (An et al. 2010). When a mutation occurs in the P-loop, the configuration changes, causing the activation loop to fold outward into the active configuration and remain that way. Imatinib binds only to the inactive form of BCR-ABL and is, therefore, ineffective against most forms of P-loop mutations (Litzow 2006).

Contact site mutations confer high levels of resistance as well. The 315th amino acid of the tyrosine kinase serves as a contact point for imatinib. The threonine present at 315 forms a hydrogen bond with imatinib, a step that is crucial in order for imatinib to fill the ATP binding pocket (Hochhaus and La Rosée 2004). The switch of threonine to an isoleucine residue confers resistance in two ways. First, isoleucine does not form a hydrogen bond with imatinib like threonine does, so imatinib cannot form the bond critical for the drug's inhibitory effect on tyrosine kinase. Second, isoleucine is bulkier than threonine, so it acts as a "gatekeeper" by adding residue to the contact site, sterically hindering imatinib (Tanaka et al. 2010). In fact, the T315I mutation confers resistance to all second-generation tyrosine kinase inhibitors as well, as will be expounded upon later.

According to the MD Anderson algorithm for treating CML, if a patient has not achieved a complete hematological response within three months from the start of treatment, the patient is deemed imatinib resistant and switched to a different treatment method. Likewise, a patient who has not achieved a complete cytogenetic remission at 18 months is switched to a different therapy. If at any point in imatinib treatment, a patient relapses and loses a level of remission, it indicates a need for alternative treatment (Pemmaraju et al. 2011). The MD Anderson algorithm for CML treatment is shown in Figure 4.

ALTERNATIVES

Several alternative forms of treatment have been suggested for patients not responding satisfactorily to imatinib. They include allogeneic stem cell transplantation, novel tyrosine kinase inhibitors, aurora kinase inhibitors, and reactive oxygen species generators.

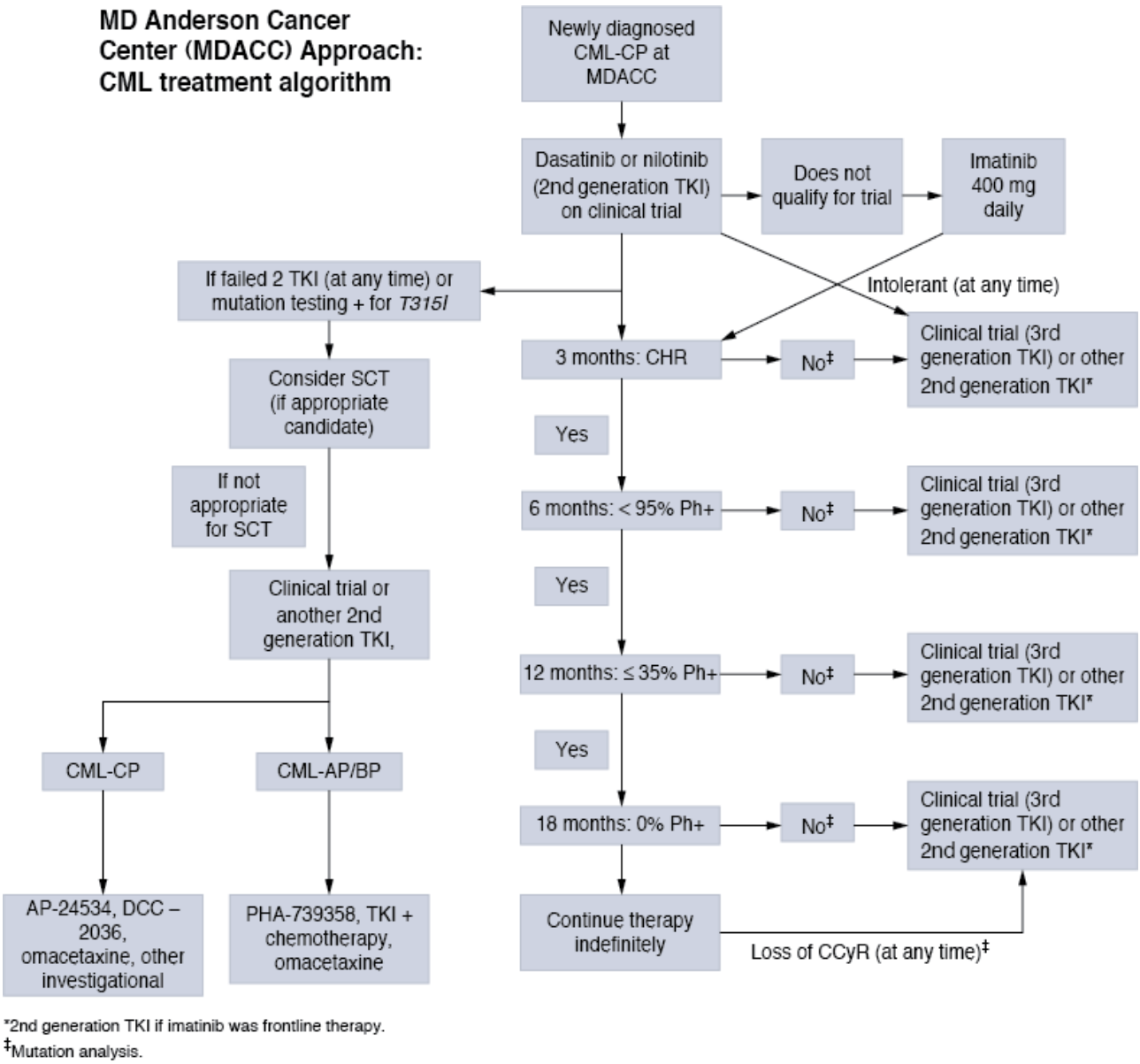

Figure 4: The MD Anderson algorithm for treatment of CML. Patients who fail to achieve the desired remission level within the specified timeframe or relapse are switched to a different therapy. Source: Pemmaraju et al. 2011

Allogeneic stem cell transplantation (SCT) is currently the only potentially curative treatment for CML (Linker and Damon 2011). All other therapies control the BCR-ABL oncogene expression; allogeneic SCT destroys the stem cells bearing the gene (Pemmaraju et al. 2011). SCT involves heavy chemotherapy and/or radiation regimens to destroy the diseased cells, ridding the body of the disease, and the transplant of healthy stem cells to the patient's bone marrow to replenish the cells destroyed by chemotherapy. Autologous SCT, in which healthy stem cells are extracted from the patient prior to chemotherapy and reintroduced afterwards, is sometimes used (Liesveld and Lichtman 2011). However, autologous SCT is associated with high morbidity levels due to the high-dose chemotherapy preparatory regimen. Allogeneic SCT uses stem cells from a well-matched donor which are infused

with mature donor leukocytes. This is more effective than autologous SCT because the grafted leukocytes recognize any remaining cancerous stem cells as foreign and attack them. This graft-versus-malignancy effect successfully eradicates malignant stem cells not destroyed by the preparatory regimen. Therefore, the pre-transplant regimen does not need to be as rigorous, making allogeneic SCT therapy an option for older patients (ages 60-75) who cannot tolerate standard high-dose chemotherapy (Linker and Damon 2011). Prior to the introduction of imatinib, allogeneic SCT was the treatment of choice for younger patients with a well-matched donor. However, allogeneic SCT has high morbidity levels due to incidence of graft-versus-host, in which the donor's cells attack the host. Therefore, with the introduction of imatinib, allogeneic SCT is no longer first-line therapy for chronic phase CML. However, it still remains useful for treating patients with poor response to imatinib and in patients with the T315I mutation (Liesveld and Lichtman 2011).

Following the success, and mindful of the flaws, of imatinib, several second-generation tyrosine kinase inhibitors (TKI) were created. These TKIs counter different imatinib resistance-conferring mutations. The three second-generation TKIs that are currently available are dasatinib, nilotinib, and bosutinib. Each has a distinct advantage over imatinib.

Dasatinib is a powerful TKI that inhibits many tyrosine kinases. It exhibits 300 times greater potency against the unmutated form of BCR-ABL than imatinib. Unlike imatinib, it binds to both the active and inactive forms of BCR-ABL and is, thus, unaffected by P-loop mutations (Cang and Liu 2008). Although effective against most mutations, it is ineffective against T315I, V299L, F317L, and a few others. Dasatinib has impressive results; over 50% of patients in chronic phase who failed imatinib therapy achieved a complete cytogenetic response with dasatinib. The standard dose for dasatinib is 100 mg daily, based on minimal toxicity levels and maximum performance. Dasatinib has few side effects and is overall well tolerated (Pemmaraju et al. 2011).

Based on dasatinib's effectiveness against imatinib-resistant CML, a study was conducted using dasatinib as front-line therapy in newly diagnosed chronic phase CML. Results were impressive and swift. Within six months, 90% of patients achieved complete cytogenetic remission (compared to historical records of ≈80% with imatinib); within 12 months, 45% of patients had further improved to major molecular remission; and within 24 months, 71% achieved major molecular remission (Pemmaraju et al. 2011). The structure of dasatinib is shown in Figure 5.

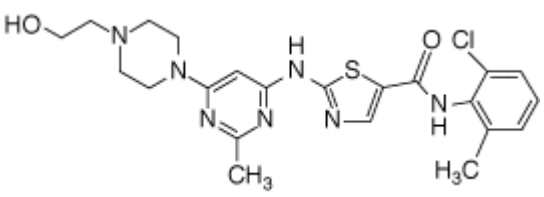

Figure 5: Molecular formula for dasatinib. Dasatinib binds both the active and inactive configurations of ABL-BCR tyrosine kinase. Source: Chabner et al. 2011

Nilotinib is structurally similar to imatinib but modified to increase drug potency and selectivity. Like imatinib, it binds to the inactive configuration of BCR-ABL, locking the activation loop in the closed form to block the ATP binding site. Unlike imatinib, however, nilotinib forms hydrogen bonds with the amino acids at 286 and 381, two residues not prone to mutation (Chabner et. al. 2011). In a study of 321 chronic phase CML patients who failed imatinib treatment, 46% achieved a complete cytogenetic response with nilotinib. Nilotinib has an advantage over imatinib because

it is effective against nearly all mutations, with the exception of T315I (Pemmaraju et al. 2011). Some P-loop mutations have shown in-vitro resistance to nilotinib, so patients with P-loop mutations might benefit more from dasatinib (Cang and Liu 2008). Nilotinib is approved at 400 mg twice daily and is well tolerated (Pemmaraju et al. 2011).

Due to nilotinib's high success rate in imatinib-resistant patients, studies were conducted using nilotinib as the initial therapy for early chronic phase CML. Over 90% of patients achieved a complete cytogenetic response within six months, and an astounding near 80% of patients achieved a major molecular remission by 12 months. More significantly, one study showed that by 12 months, less than 1% of patients had progressed to the accelerated or blast phases, compared to 4% in patients receiving imatinib (Pemmaraju et al. 2011). The structure of nilotinib is shown in Figure 6.

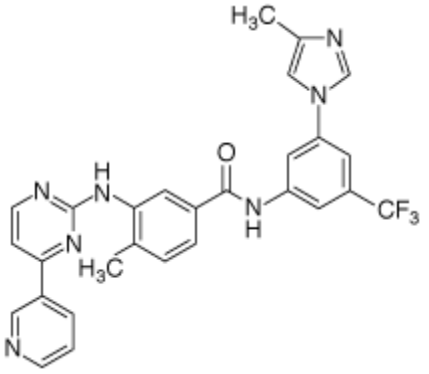

Figure 6: Molecular structure of nilotinib. Like imatinib, nilotinib binds only the inactive conformation of the BCR-ABL protein. Source: Chabner et al. 2011

Bosutinib is another potent TKI currently in development. It is 30-50 times stronger than imatinib against unmutated CML and is active against almost all BCR-ABL mutations. Bosutinib has success rates similar to the other second-generation TKIs; over 40% of chronic phase CML patients who switch to bosutinib because of imatinib resistance achieve a complete cytogenetic response. Bosutinib has an advantage over other TKIs due to its greater selectivity. Unlike the other TKIs, bosutinib has less off-target outcomes, which is theorized to reduce toxicity associated with other TKIs. Unfortunately, though, as with the other TKIs, bosutinib is ineffective against the T315I mutation (Cang and Liu 2008).

The efficacies of the three second-generation TKI's, dasatinib, nilotinib, and bosutinib, are summarized in Table 1.

Despite the efficacy of second-generation TKIs, none are successful in combating the T315I, and alternate therapies are necessary.

Aurora kinase inhibitors are a new class of CML therapy. Aurora kinases have been implicated in intensifying certain cancers, so combatting aurora kinases with aurora kinase inhibitors holds promise for controlling CML (Tanaka et al. 2010). MK-0457 was the first aurora kinase inhibitor to show activity against T315I. It binds to the amino acid at 381, and not at 315, thereby avoiding the steric clash with isoleucine (Quintas-Cardama and Cortes 2008). However, despite the promising results of MK-0457, trials were stopped due to concerns of cardiotoxicity (Cang and Liu 2008). Other aurora kinase inhibitors, like XL228, PHA-739358, KW-2449, and AT9238, are in various stages of clinical trials (Quintas-Cardama and Cortes 2008). Though the introduction of aurora kinase inhibitors in T315I+ CML is recent, results of early trials look promising since aurora kinase inhibitors do not bind to T315 and, therefore, are unhindered by isoleucine.

Another alternative for imatinib- and TKI-resistant CML is the use of reactive oxygen species (ROS). ROS are the main catalysts of redox dysregulation and oxidative stress within cells, especially cancerous cells (Wondrak 2009). The BCR-

Table 1: Response to Second-Generation TKI's

PERCENT RESPONSE											
	Dasatinib				***Nilotinib***				***Bosutinib***		
	P	P	yBP	yBP	P	P	yBP	yBP	P	P	P
	=387	=174	=109	=48	=321	=137	=105	=31	=146	=51	=38
Median follow-up (mo)	5	4	2	2	4						
% Resistant to imatinib	4	3	1	8	0	0	2	2	9	R	R
% Hematologic Response		9	0	0	4	6	2	9	5	4	6
CHR	1	5	7	9	6	1	1	3	1	4	6
% Cytogenetic Response	R	4	6	2	R	R	R	R	R	R	R
Complete	9	2	6	6	6	0	9	2	4	7	5
Partial	1				5	2	0	6	3	0	8
% Survival (at 12 months)	6 (15)	2 (12)	0 (12)	0 (5)	7 (24)	7 (24)	2 (12)	2 (12)	8 (12)	0 (12)	0 (10)

CP, chronic phase; AP, accelerated phase; BP, blast phase; MyBP, myeloid blast phase; LyBP, lymphoid blast phase; NR, not reported. Source: Pemmaraju et al. 2011

ABL oncogene promotes ROS-generated redox imbalances, and this dysregulation can be manipulated to induce cell death. The introduction of exogenous ROS-generating species increases oxidative stress in cancerous cells, leading to rapid protein degradation and cell death (Zhang et al. 2008). Redox imbalances operate under synthetic lethality, which means that only cancerous cells are killed, not normal cells (Wondrak 2009). PEITC (β-phenylethylisothiocyanate) is a naturally-occurring ROS-generating agent. In one experiment, when introduced in cells bearing normal BCR-ABL and cells with the T315I mutation, PEITC completely inhibited cell growth, raised oxidative levels twofold, and caused over 60% cell death (Zhang et al. 2008). The viability of ROS-generating agents like PEITC in vivo has not yet been established, but early research shows promise (Wondrak 2009).

SUMMARY

Chronic myeloid leukemia is no longer as frightening a diagnosis as cancer can be. The historic median survival of 4-5 years is a thing of the past. Novel targeted drugs can restrain BCR-ABL+ stem cells to the point that the disease is virtually undetectable. Imatinib, the first molecularly targeted anticancer therapy, is so effective that over 95% of patients maintain progression-free survival at five years. Even the few patients who are resistant to imatinib are not left without hope. Allogeneic stem cell transplants can cure the disease in healthy patients with a well-matched donor. Second-generation tyrosine kinase inhibitors can effect remission in most patients bearing imatinib-resistant BCR-ABL mutations. Dasatinib, nilotinib, and bosutinib are second-generation TKIs with differing potentials against the different mutations. Though none of these TKIs can counter the T315I contact site mutation, other therapies can. Aurora kinase inhibitors and ROS-generating agents are in various stages of clinical trials and show tremendous potential for treatment of T315I+ CML. Aurora kinase inhibitors do not bind to the threonine at 315, so they are not rendered ineffective in blocking the tyrosine kinase activity. ROS-generating agents prevent proliferation and induce cell death by critically raising intracellular oxidative levels in cancerous cells. With the wealth of scientific research and experimentation that has abounded regarding CML in the past 15 years, chronic myeloid leukemia has become a truly treatable disease.

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MOLECULAR MECHANISM OF XY GONADAL DYSGENESIS

Griendy Indig-Weingarten

One of the fundamentals of human sociology is the characterization of the people around us based on gender. We tend to think of gender as a strict binary system where the option is clear: boy or girl. Although society usually honors this dichotomy, biology allows more flexibility to the definition of male versus female. Estimates state that one in every 2000 births is one with a disorder of sex development (The Intersex Society of North America 2006). Some of the disorders are visually obvious while others are only discovered later on in life. Regardless of when the disease first becomes obvious, all of these disorders constitute a variation along the standard development of a male or female. Therefore, to understand disorders of sex development, one must first understand what constitutes normal sex development. As in all of embryology, development is controlled by many different genetic codes and the pathways they encode. Therefore, variation along any gene responsible for any part of the sex differentiation mechanism will result in a deviation from the expected.

NORMAL SEX DEVELOPMENT

The identity of the sperm that successfully penetrates the ovum is the official beginning of sex determination. When meiosis of the spermatocyte is completed, the mature haploid spermatozoa can either have an X or a Y as its twenty-third chromosome. When this particular sperm penetrates the ovum, the identity of its twenty-third chromosome determines the genetic sex. This is the first step of sex determination. Further sex development is divided into two parts: primary sex determination and secondary sex determination. Primary determination involves the development of the fetus's gonads. Once formed, the gonads start producing hormones which initiate secondary sex development. Secondary sex development includes the development of duct systems and external genitalia. Secondary sex development is only complete after puberty (Gilbert 2010).

PRIMARY SEX DIFFERENTIATION

Regardless of their karyotype, initially all embryos present a common set of genital structures. These include the gonads, the internal duct system, and the external genitalia. The development of the genital system begins at the fourth week of embryonic life. The entire genital system is derived from the intermediate mesoderm and urogenital sinus except for the gametes which emigrate from the mesoderm of the yolk sack. The intermediate mesoderm differentiates to form the adrenal cortex and the start of the urogenital ridge originating as the nephrogenic cord. The urogenital ridge is the site of the development of the urinary and reproductive system. The cephalic portion of the nephrogenic cord further matures to form the pronephros, which is mostly an embryonic structure. The more caudal portions of the nephrogenic cord form the mesonephros and the pronephric duct which later forms the mesonephric duct. The genital ridge arises along the middle portion of the mesonephros. This is the central location for the development of the gonadal and genital structures. The caudal (posterior) portion of mesonephric duct fuses with mesonephric tubules and continues to extend and connect to the urogenital sinus.

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Then the most caudal area of the nephrogenic ridge further develops into the metanephros which is the permanent kidney (Figure 1) (McMahon et al. 2008).

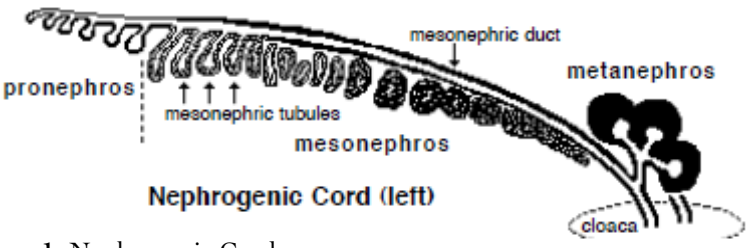


Figure 1: Nephrogenic Cord.
Source: http://vanat.cvm.umn.edu/TFFlectPDFs/LectEmbUrinarySys.pdf.

GONADS

The gonads are unique embryonic structures because they are the only bipotential organs. They can develop into ovaries or testes. Gonads usually appear at week four but remain undifferentiated until week seven. At about 3-4 weeks, the mesonephros is at its largest size, and it is at this point that the epithelial layer of the mesonephros, also known as the coelomic epithelium, starts to proliferate into the surrounding mesenchyme, or undifferentiated loose connective tissue, and form the primary sex cords. These cords will surround the entering germ cell to form the gonadal cord. At this point, the gonadal tissue will start to differentiate to testes or ovaries.

MALE GONADAL DEVELOPMENT

An XY fetus, at around the eighth week, will start developing testicular tissue. This begins with the differentiation of the gonadal cord into the Sertoli supporting cells. These cells form processes that surround the primordial germ cells and eventually mature into Sertoli cells. At the same time, cells migrating from the mesonephros, called peritubular myoid cells, form a single layer around the gonadal cords. These peritubuluar myoid cells contribute to the structural integrity of the testis cord and will support spermatogenesis later in the adult male's life (Buehr et al. 1993). It is at this point (around the eight week) that the gonadal cord is recognizable as the first form of the testis cord. The testis cord then comes in contact with the mesonephric tubules which are connected to the mesonephric duct, causing a capsule called the tunica albuginea to form around the testes. Meanwhile, the testis cords continue to create a dense network called the rete testis. The rete testis functions later on as the carrier of sperm from the seminiferous tubules out of the testicles through the efferent duct which was derived from the mesonephric tubules. At 20 weeks, the testis cord forms the straight and convoluted seminiferous tubules. The straight tubules are connected to the rete testis and do not have any germ cells within them. The convoluted tubules are a continuation of the seminiferous tubules and contain the primordial germ cells. Spermatogenesis takes place in the convoluted tubules (Celio et al. 1999). Once the testis is formed, some primordial germ cells differentiate into a pre-sperm state called T1-prospermatogonia and remain that way until puberty (Gilbert 2010).

Another important part of testicular development is the differentiation of the Leydig cells. These cells originate from mesenchyme, or the undifferentiated loose connective tissue in between the testis cords. These Leydig cells eventually produce testosterone which will be crucial to further male differentiation. Because of its steroid

producing nature, Leydig cells tend to cluster near the testicular vasculature (Wilhelm 2007).

Female Gonadal Development

In the case of an XX embryo, the gonads usually develop into ovaries. The ovaries start developing during the eight week. In the absence of testis determining genes, the genital ridge will differentiate into ovaries. However, unlike testis, in which the testis cord forms early on in embryogenesis, the functional unit of the female gonads—the ovarian follicles—completely mature only at puberty. During fetal development, primordial follicles are formed. These follicles are usually formed around 16-24 weeks after fertilization (Celio et al. 1999). The ovarian development begins with the migration of the primordial germ cells into the genital ridge and the gonadal cord partially atrophies. Later on, the primordial germ cells proliferate and form the primordial oocyte, or the oogonium. At around the fifth month, the oogonia are arrested at prophase of the first meiosis and are subsequently surrounded by pregranulosa cells which differentiated from the gonadal cord. These granulosa cells are connected with cellular bridges and high levels of vasculature. One of the key differences between the male and female primordial germ cells is that while all the male cells will continue to proliferate and mature to sperm from the age of puberty throughout a lengthy adult life, only the female cells that have matured and were arrested at prophase of the first meiotic division in utero will mature into ooctyes.

Molecular genetics of gonad development

Testis pathway

Genetic sex is defined as the presence of an XX or XY karyotype. In either case, one X will be inactivated, allowing the X or the Y to be the defining chromosome in sex determination. The Y chromosome carries a dominant testis-determining gene which actively promotes testicular development. Absence of the gene, regardless of the identity of the rest of the chromosome, will cause the embryo to go into "default" development and continue to develop as a female. It is for this reason that sex determination is sometimes equated with testis determination. This was proven by Alfred Jost when he castrated rabbits in utero and the rabbits' development continued according to the female pattern (Jost 1972). Later research showed the testis-determining gene to be the *SRY* gene on the short arm of the Y chromosome. In fact, the research that discovered the connection between the *SRY* gene and testis determination was carried out on XY females and Turner Syndrome patients (Berta et al. 1990).

SRY: Sex- determining region Y

The *SRY* gene is located on the upper short arm of the Y chromosome. In studies using XX reversed males and XY reversed females, results showed that a fetus with the Y short arm, even when missing the long Y arm, would develop testis. Accordingly, any individual missing the Y short arm would not develop testis. Additionally, if the *SRY* gene is inserted in the genome of a normal XX mouse, the XX mouse would develop testis (Koopman et al. 1991). The *SRY* gene is expressed in the bipotential gonads immediately before differentiation of the Sertoli cells begins, at around eight weeks, and is deactivated a few days later (Cotinot et al. 2002). Although *SRY* is deactivated after a short window, while active, it synthesizes the *SRY*

transcription factor whose primary role is to activate the *SOX9* gene, allowing differentiation to continue even after *SRY* is deactivated.

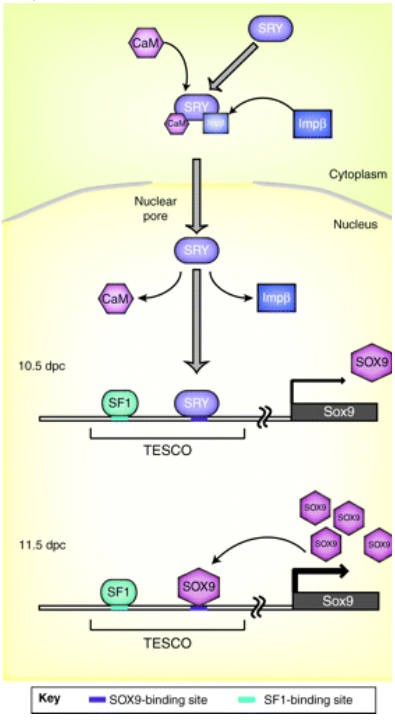
SOX9

The *SOX9* gene is located on the seventeenth chromosome. SOX genes are called transcription factors due to their nature of transcribing proteins that can bind to specific locations on DNA to control gene activity. *SOX9* is involved in many developmental processes, particularly skeletal formation. In fact, the role of *SOX9* in testis formation was discovered when a link between *SOX9* and a condition called campomelic dysplasia was discovered. Campomelic dysplasia patients exhibit multiple skeletal abnormalities. Interestingly enough, however, 75% of the 46 XY patients with campomelic dysplasia were sex-reversed females (Cooke et al. 1985). This led researchers to discover that when *SOX9* is activated in the genital ridge, it induces testis formation. Studies have shown that mice missing the *SRY* gene but possessing an extra copy of the *SOX9* will develop as male. Accordingly, mammals possessing the *SRY* gene but lacking the *SOX9* gene will not develop male (Huang et al. 1999).

SOX9 works in several different manners:

- It activates its own promoter, creating a positive feedback loop which allows it to be active for a longer period of time (Sekido and Lovell-Badge 2008).
- It blocks genes that are involved in ovary formation (de Santa Barbara et al. 2000).
- It can bind and activate different genes necessary for testicular development.
- It promotes the gene expression necessary to produce fibroblast growth factors that are critical for Sertoli Cells precursors.
- It binds to the gene that promotes the production of anti-mullerian factor (de Santa Barbara et al. 2000).

SOX9 is detected in the somatic cells in the human fetal gonads as early as the sixth week. When the Sertoli cells start differentiating, *SOX9* is expressed in the nuclei of the cells (de Santa Barbara et al. 2000). Studies done on *SOX9* have found that it very often works together with a nuclear receptor called steroidogenic factor-1 (SF-1) which is activated by the *NR5A1* gene. The relationship between the *SRY*, *SOX9*, and SF1 is explained in Figure 2.



In the cytoplasm, SRY is bound by calmodulin (CaM) and importin β (Impβ), which recognize the N- and C-terminal nuclear localization signals (NLSs) on SRY, respectively, and recruit it to enter the nucleus. At 10.5 dpc, SRY and steroidogenic factor 1 (SF1) bind directly to specific sites ('TESCO', testis-specific enhancer of *Sox9* core) that lie within the gonadal specific enhancer of *Sox9* (indicated by the coloured regions on the DNA) and upregulate *Sox9* expression cooperatively. At 11.5 dpc, after initiation of *Sox9* expression, an auto-regulation system operates in which SOX9 also binds directly to TESCO with SF1 to prolong and amplify *Sox9* expression. Abbreviations: SOX9, SRY box containing gene 9; SRY, sex-determining region on the chromosome Y.

Figure 2: Cellular mechanism of SRY function. Source: http://dev.biologists.org/content/137/23/3921.full

NR5A1

NR5A1 is sometimes referred to as the SF-1 gene

because it encoded the SF-1 receptor. The *NR5A1* gene spans seven exons and is located on the ninth chromosome (Johns Hopkins University School of Medicine 2011). It functions in gonadal differentiation and in steriodogenesis. Knockout mice (mice that were genetically altered) missing the *NR5A1* gene developed with complete adrenal and gonadal agenesis. Studies show that SF-1 is involved early in embryogenesis during development of the bipotential gonad. Although it is not completely understood how SF-1 functions in the embryogenetic stage, mice missing the part of the *NR5A1* gene that encodes for SF-1 never developed a genital ridge. SF-1 is already active in humans at four weeks when the gonadal ridge starts to form, and its levels remain high in the testis even once the *SRY* shuts off. SF-1 aids in activating *SOX9* and continues to work with *SOX9* to elevate the anti-mullerian hormone transcription. As the name steriodogenic indicates, SF-1 is involved in hormone production. It is involved in multiple steroid-producing functions, such as collaborating with *SOX9* to produce AMH (anti-mullerian hormone), and activating genes involved with testosterone production in Leydig cells (Shen et al. 1994). Mice lacking the SF-1 gene lack both adrenal glands and gonads, proving that the SF-1 gene is involved in early embryogenesis of the urinary and reproductive system (Luo et al. 1994).

OVARY PATHWAY

In order to provide a complete picture of development, the molecular pathway of the ovaries is briefly discussed, although these genes are not involved in XY Gonadal Dysgenesis.

WNT4

Early on, *WNT4* is expressed in the genital ridge in both male and female embryos. However, its expression, while still maintained in the fetal ovaries, is undetectable in the fetal testes. Mice that lack the gene will fail to develop ovaries (Cotinot et al. 2002).

R-SPONDIN-1

Another critical gene is R-spondin-1, a protein expressed by RSPO1. An XX fetus with gene mutation in RSP01 will be XX male. RSPO1 acts together with *WNT4* to produce beta-catenin, which activates further ovarian development, and block *SOX9* production. An XY fetus with a duplication of the *WNT4* and RSPO1 on its chromosome will develop ovaries instead of testis (Gilbert 2010).

(There are many other genes involved in both testis development and ovarian development that are not discussed in this paper.)

SECONDARY SEX DEVELOPMENT

Once the gonads are formed, they start to produce hormones. These hormones are initiators of secondary sex development. Once these hormones begin to be secreted, secondary sex development can start. It is important to note that primary and secondary developments do overlap, as the testes and ovaries continue to develop once steroids are produced.

INTERNAL DUCT SYSTEM

The gonads are just one part of the sex differentiation process. The ducts system, like the gonads, start out in an undifferentiated state. In the undifferentiated stage, the embryo presents with mesonephric ducts, also known as the Wolffian ducts. Paramesonephronic ducts, which appear in the sixth week, form part of the urogenital cord. The Wolffian ducts form from the nephrogenic cord and develop toward the cloaca. Mesonephric tubules, another part of the mesonephros involved in the development of the internal ducts, arise from the mesenchyme of the mesonephros and eventually fuse with the mesonephric duct to create a passage to the cloaca (Schoenwolf et al. 2009). The Mullerian Duct is formed when the coelomic epithelium, near the top of the mesonephros, invaginates. Both the Mullerian and the Wolffian ducts continue to grow caudally towards the urogenital sinus (Figure 3) (Sweeney 1998).

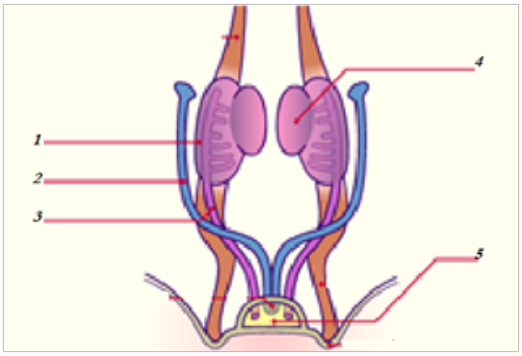


Figure 3: Internal Duct System
1-Mesonephros
2-Paramesonephric Duct
3-Mesonephric Duct
4-Indiffernt Gonads
5-Urogenital Sinus
Source: Celio et al. 1999.

MALE DUCT SYSTEM DEVELOPMENT

During early embryogenesis, the Wolffian duct serves as an excreting duct for the mesonephros. Once the metanephros becomes functional, most of the mesonephric duct atrophies, and the part that remains is dependent on the testosterone released from the Leydig cells in the fetal testis. In the eighth week of gestation, the Leydig cells start releasing testosterone and thus begin two important aspects of the internal male duct system development. One is the atrophying of the paramesonephric duct, and the other is the further development of the mesonephric duct (Celio et al. 1999). The undifferentiated state includes both sets of ducts, and with the start of duct differentiation, the paramesonephric duct starts to atrophy due to the effect of anti-mullerian hormone released by the Sertoli cells within the seminiferous tubules. It is interesting to note that a small part of the paramesonephric duct does remain and differentiates into the appendix testis (Jacob and Barteczko 2005). Meanwhile, the Leydig cells begin releasing testosterone to support the development of the mesonephric duct. Most of the top portion of the Wolffian duct atrophies, leaving only the portion which differentiates into the efferent duct, epididymis, vas deferens, and seminal vesicles.

FEMALE DUCT SYSTEM DEVELOPMENTS

In a human embryo, the absence of the anti-mullerian hormone causes the undifferentiated ducts to develop into the female duct system. The mesonephric ducts and tubules atrophy, and the paramesonephric duct further develops. In the eighth week, the two lower portions of the mullerian duct fuse. This fused portion is the site of the uterus. The upper portion of the paramesonephric duct proliferates outwards towards the gonad and develops as the fallopian tubes. The lowest portion forms the utero-vaginal canal (Celio et al. 1999).

External Genitalia Development

The external genitalia arise from the genital tubercle, the urogenital groove and sinus, and the labioscrotal fold.

As opposed to male development where testosterone plays a key role in differentiation, estrogen is not involved in female sexual differentiation. Instead, it is produced by the ovaries to function as support for follicular maturation. In fact, both male and female fetuses are exposed to high amount of estrogen from their mother. The embryo has a predisposition to develop internal female duct systems (fallopian tubes and uterus), so unless there is an active intervention by anti-mullerian hormone (secreted by Sertoli cells) and testosterone (secreted by the steroidogenic Leydig cells) to promote development of the epididymis, vas deferens, efferent ducts, and other male organs, a female system will develop. Furthermore, male external genitalia are dependent on specific hormonal activity. The undifferentiated embryo is very rich in androgen receptors. Once the Leydig cells start producing androgen, particularly dihydro testosterone, the male external genitalia develop, first into a phallus, and then further into the mature penis, phallic urethra, and scrotum. The absence of androgen causes the development of the clitoris, urethra, vagina, and labia. This further demonstrates that male external genitalia formation requires active intervention (Schoenwolf et al. 2009). This is why it is said that an embryo, if left to its own devices, will go to "default development" and develop female.

This concludes a summary of sex differentiation (Figure 4). Most discoveries of the mechanisms involved in this development were discovered when studying patients with an abnormal sex development or disorder of sex development.

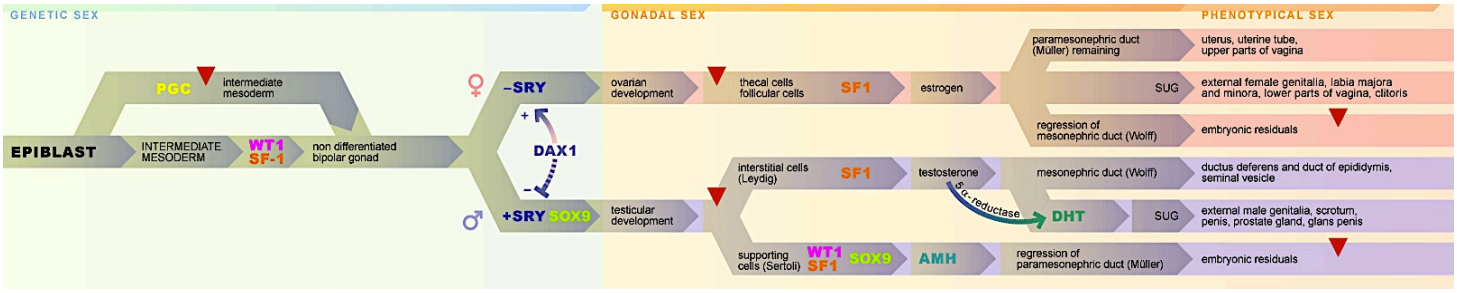


Figure 4: Timeline of the primary and secondary sex development. Source: http://www.embryology.ch/images/imagegraphe/u1e_schemdifferentEn.gif.

Disorders of Sex Development

Disorders of sex development are defined as any congenital condition in which development of chromosomal, gonadal, or anatomical sex is atypical (The Intersex Society of North America 2006). As seen earlier, sex differentiation is a complex and lengthy process, involving many different genomic pathways. As a result, there are many different variations along the developmental process which can cause atypical presentation. If a mutation exists which impedes the development of one of the necessary functions, it is expected that an abnormal phenotype will occur. Disorders of sex development is a very broad term which spans a spectrum of various different phenotypical presentations. The defining factor in determining the outcome of a variation depends on which point of development it affects. The first step of sex differentiation is genetic sex. Examples of disturbances in the proper genetic sex

development are Turners Syndrome (45 XO female), Klinefelters Syndrome (47 XXY male), XYY syndrome, and a few others. The next step in sexual determination is gonadal sex. Disorders of gonadal sex include XX Gonadal Dysgenesis and XY Gonadal Dysgenesis. The final part of sex development is anatomical sex. Variations in anatomical sex are the broadest of the three steps of sex development. Conditions range from phenotypical female, ambiguous genitalia, and phenotypical male. These conditions include congenital adrenal hyperplasia, androgen insensitivity syndrome, hypospadias, 5-alpha-reductase deficiencies, and many others (The Intersex Society of North America 2006).

XY Gonadal Dysgenesis

One of the defining moments in male or female sex differentiation is the identity of the mature undifferentiated gonad. Once the gonad has differentiated to either testis or ovary, it initiates steroidogenic activity which will define the rest of the anatomical development. If gonadal development were to halt at the stage of indifference, such an occurrence would be called XY gonadal dysgenesis. The term gonadal dysgenesis involves the broader group of conditions that would cause the dysgenesis, or failure to develop, of the gonads. In Turner Syndrome, gonadal development fails due to a monosomy of the sex chromosome; the gonads are missing crucial genes for gonadal development. In a condition known as XX gonadal dysgenesis, gonads which should develop into ovaries are missing ovarian defining genes or receptors to complete ovarian development. Perhaps the most fascinating is that of an XY fetus developing phenotypically as a female due to gonadal dysgenesis in a condition called XY gonadal dysgenesis (Sinclair and Cameron 2004).

Clinical Presentation

XY Gonadal Dysgenesis is usually diagnosed when a patient who failed to develop secondary female sex characteristic undergoes testing to determine the cause. The patient usually presents a female phenotype but no or sparse pubic and underarm hair, no or little breast tissue development, and amenorrhea. Examinations and ultrasound images reveal usual female external genitalia, fallopian tubes, and uterus but no visible ovaries. The incidence of occurrence is hard to estimate, with some papers stating numbers as frequent as 1:30,000 and others quoting numbers as low as 1:150,000 (Wilhelm 2010).

Treatment

Although XY Gonadal Dysgenesis is not a diagnosis that comes along with serious medical conditions (except in gene specific abnormalities that will be discussed later), it does come along with some medical aspects that need to be addressed.

Hormone Replacement Therapy (HRT)

One option is to substitute the hormones that the patient is missing due to the dysfunctional gonads. Patients with XY Gonadal Dysgenesis need to start hormone replacement therapy to initiate pubertal development, and they need to remain on the therapy to maintain optimal health. The doctor will usually start off the patient on a low dosage of estrogen, most often in the form of a 0.3 mg tablet of premarin, a form of estrogen. The dose will increase over a span of 2-3 years. The incremental increase is intended to mimic the hormonal activity that normally would take place at the start of

puberty. During this time, all the expected secondary sex characteristic should develop.

Because the uterus is also maturing during this time, it is important to initiate menstruation with progesterone to reduce the risk of uterine cancer. A doctor will usually recommend bringing on a menses a minimum of four times a year for the upkeep of optimal uterine health. There are a number of different forms of HRT: tablets, patches, gels, injections, and implantable pellets. Some health professionals recommend taking a combined form of estrogen and progesterone, and some add a low dose of testosterone to more accurately mimic the natural hormone production in healthy females. There are conflicting reports as to what form and dose is optimal, and most patients find that the process to find the right regimen depends on what works best for them personally (WebMD 2009).

GONADECTOMY

Patients with XY gonadal dysgenesis must undergo a gonadectomy, surgery to remove the streak gonads so that they do not develop into dysgerminomas (germ cell tumors). The risk of an XY gonadal dysgenesis patient developing cancer is estimated at around 30% occurrence rate, which is why doctors recommend that gonadectomy be preformed soon after diagnosis. Dysgerminomas can develop in adults with streak gonads due to their indifferent embryonic structures which are not intended to be mature organs. The gonadal tissue of the streak gonads contains germ cells and many pre-Sertoli/granulose cells scattered throughout the stroma. These cells can develop into gonadoblastomas, benign neoplasms, exclusive to these types of cells. These neoplasms can become malignant and form dysgerminomas and other malignant growths (Michala et al. 2008). Cases have been reported in which patients as young as nine months have developed dysgerminomas (Dumic et al. 1993); therefore doctors recommend removing the gonads as early as possible. With the advances of modern surgical techniques, most gonadectomies are done laparoscopically and involve minimal recovery with little scarring (Figure 5).

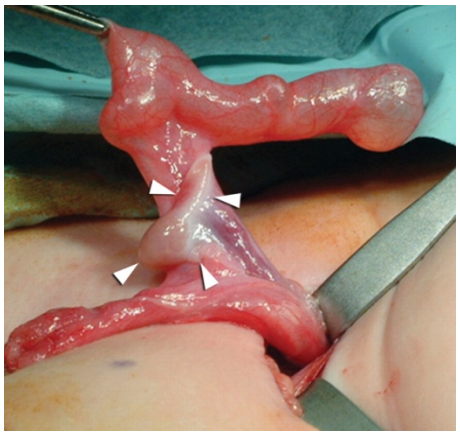


Figure 5: Laparoscopic removal of streak gonads (indicated by the white arrows) in patient with XY Gonadal Dysgenesis. Source: http://radiographics.rsna.org/content/28/7/1891/F14.large.jpg

GENETIC CAUSES OF XY GONADAL DYSGENESIS

Five genes have been identified in relation to XY gonadal dysgenesis. Because of the rarity of the condition and the constantly developing field of molecular genetics, many of their mechanisms are poorly understood.

SRY

Mutations in the *SRY* gene (the testis-determining gene) have been found in 10-15% of all XY Gonadal Dysgenesis. Most mutations are in the HMG (High Mobility Group) box on the *SRY* gene. The HMG box is capable of sequencing specific DNA binding and bending. This is crucial in the *SRY* gene, because it is the specific bending pattern which activates the *SRY* in the gonads. The bends act by changing the

chromatin structure in the regulatory region of the target gene that starts the assembly of the regulatory complex. The identification of HMG as the critical region was formed when researchers noticed that there are variations in the non-HMG portions of the *SRY* nucleotide sequences which do not have any effect on testicular development. This finding demonstrated that these regions are not involved in testis development. As previously mentioned, *SRY* is expressed in the genital ridge for a brief time, stimulating Sertoli cell and testis determination (Sinclair and Cameron 2004). A few factors have been identified as regulators for *SRY*. The WT1 may activate *SRY* through an *SRY* promoter region which it expresses. It is also hypothesized that the *SRY* gene itself might have a transcription site on it for auto regulation (Vilain et al. 1992). The roles identified in *SRY* include the induction of the mesonephric cells into the ridge to form the testis cord, proliferation of the cells, and inducement of Sertoli cell differentiation. The lack of *SRY* also causes the failure of the *SOX9* and any other gene relying on *SRY* as its regulatory "switch."

SRY related XY gonadal dysgenesis has been observed in familial lines, but most cases are de novo mutations which cause the loss of binding and its subsequent failure to initiate testis differentiation. Although *SRY* is expressed in somatic cells in different areas in the body, no other effects have been observed as a result of the *SRY* mutation (Simpson 2008).

DHH

DHH, the desert hedgehog gene, is located on the twelfth chromosome and is composed of three exons. *DHH* expression was observed in the testis of fetal mice. In the ovaries of fetal mice, however, little *DHH* expression was observed. This led to the initial belief that *DHH* is involved in testis development. The product of the *DHH* is found in Sertoli cells and in Schwann cells. Therefore, patients with *DHH* mutation usually have polyneuropathy (many nerves acting simultaneously). Unlike *SRY* which directly affects Sertoli cell development, *DHH* affects the development of different cells called peritubular myoid cells, which are cells that make a thin lining around the testis cord. Interruption of peritubular cell proliferation will cause a failure in the development of the testis cord, which will cause a failure in gonadal development. In fact, *DHH*-deficient knockout mice showed male sterility, peritubular defects, and testis-development failure. In addition to its role in testis cord development, studies suggest that the *DHH* is a regulator signal for the differentiation of Leydig cells in the fetal testis and is also involved in upregulating the SF-1 factor (Canto et al. 2004).

Patients with *DHH* mutation may have complete or partial Gonadal Dysgenesis. Partial gonadal dysgenesis usually presents itself with slightly masculinized external genitalia and some internal male ductal development. Some development does occur, a result of some androgen production, usually due to a mosaic presentation, meaning that not all cells have the mutation of the *DHH* gene. In complete gonadal dysgenesis, most patients posses homozygous mutations which do not allow any *DHH* expression to reach the threshold for testicular development.

DHH is also expressed in Schwann cells along peripheral nerves. Therefore, mutations in *DHH* can cause a condition called polyneuropathy. Polyneuropathy is a neurological disorder which causes many nerves to malfunction simultaneously. Impaired nerve transmission occurs due to reduced insulation of nerves by the affected Schwann cells (Canto et al. 2004). *DHH*-related gonadal dysgenesis is estimated to

account for 20% of all complete gonadal dysgenesis cases and 50% of all partial gonadal dysgenesis cases.

NR5A1 (SF-1)

NR5A1 encodes the SF-1 (steroidogenic factor-1) and is sometimes referred to as the SF-1 gene. It is a nuclear receptor that binds to and regulates the transcription of many target genes involved in gonadal and adrenal development. SF-1 was first studied in XY gonadal dysgenesis patients displaying complete adrenal failure. This was a direct indication of the role SF-1 plays in the formation of both gonadal and adrenal glands. These patients had homozygous mutations and did not have any SF-1 expression. In most human studies, it is rare to find a patient with complete adrenogonadal failure due to *NR5A1*. Later studies dealt with patients with heterozygous missense and frameshift mutation affecting only parts of the gene. These patients presented little or no adrenal failure. Many of these patients displayed androgen biosynthesis failure and impaired leydig cell development. Some even presented testicular tissue development (Achermann et al. 2002). This highlighted the fact that SF-1 acts within a dose-dependent function as opposed to acting as an on and off switch.

The prevalence of *NR5A1* XY gonadal dysgenesisis low.

CBX2

A fourth gene whose mutation can cause XY Gonadal Dysgenesis is the Chromobox homolog protein 2 (*CBX2*). The *CBX2* gene is part of Polycomb Recessive Group 1 on the seventeenth chromosome, which encodes polycomb group proteins. These proteins form large protein complexes capable of chromatin remodeling on primordial germ cells. Chromatin remodeling initiates the transition from mitotic division early in embryogenesis to meiosis once they are in a specific gonad. Research also indicates that the *CBX2* might have a role as a promoter of the SF-1 (Baumann and De La Fuente 2011). Knockout mice missing the *CBX2* gene expressed skeletal abnormalities and sex reversal (Lauber et al. 2009).

DMRT1/ 9P DELETION

Deletion of the tip of the 9p chromosome is associated with gonadal dysgenesis. This association, however, was discovered quite recently, so most research is still based on presumption. Because the deletion of the tip of 9p affects a number of chromosomes, patients present with complex phenotypic features including ambiguous genitalia and craniofacial abnormality. Deletion of the *DMRT1* gene, located on the ninth chromosome, has been identified as a cause of XY gonadal dysgenesis. *DMRT1* is unregulated in the genital ridge and is, therefore, linked to testis development. Research shows that *DMRT1* is critical for maintaining the activity of other male differentiation genes like *SOX9*. It is a sequence-specific transcriptional regulator, meaning that it will regulate the transcription of other genes. In this case, it is likely that it binds to *NR5A1* and promotes SF-1 production to upkeep the continued gene expression of *SOX9*. Loss of the *DMRT1* gene in mice, even after undergoing complete male development, can cause reversal of the Sertoli cells into granulose cells by promoting the production of female promoting genes. In humans, this deletion does not result in female development as is does in mice, but it does halt male development, causing XY gonadal dysgenesis (Matson et al. 2011).

CONCLUSION

With the discovery of sex chromosomes in 1921, the scientific world anticipated that the genetics of male and female differentiation would become clearer. However, it was not until the 1990s that the *SRY* gene was discovered when XY females were studied. The study of XY females also revealed other genes involved in the different aspects of sex differentiation. The connection between *DHH* and the development of the peritubular myoid cells is evident in patients with XY gonadal dysgenesis due to a mutation of the *DHH* gene. *NR5A1* plays a crucial role in encoding the steriodogenic factor-1 which plays multiple roles in gonadal cord development and further differentiation of the testis. Another gene mutation in XY gonadal dysgenesis patients, a mutation in *CBX2*, provides insight into its role in regulation of primordial germ cell development. Studying the role of *DMRT1* in regulating continued male differentiation helps scientists gain a deeper understanding of the mechanisms involved in testicular development.

Science is constantly evolving, and research conducted on patients with XY gonadal dysgenesis and other disorders of sex development will further the understanding of the different components of male differentiation and development.

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QUEST FOR VACCINES TO TREAT ADDICTION

Rachel Florence

ABSTRACT

Drug addiction is a prime example of biochemical psychology. When people use drugs such as nicotine, they trigger dopamine receptors in the brain, causing a pleasurable sensation. People want to repeat the feeling and thus get addicted to the drug. With the development of a vaccine to treat addiction, researchers attempt to prevent drugs from crossing over the blood-brain barrier and triggering the dopamine receptors. Experiments and clinical trials prove the efficacy of the nicotine vaccine. However, Phase III trials and additional research are necessary before the vaccine can be launched for public use.

INTRODUCTION

Addiction is one of the greatest issues facing our society today. Thousands of people struggle with smoking, drugs, and other addictions on a daily basis. What starts as one cigarette or one sip of alcohol often develops into a daily necessity or an ever-present need. However, it is not the cigarette or alcohol that satiates a person; it is the chemical reaction of the dopamine receptors that the addictive substance triggers in the brain that satisfies (Koob and Moal 2001). Addiction is a classic case of biochemical psychology, in which certain activities in the brain cause specific forms of behavior. In this case, the chemical reaction occurring in the brain in response to a drug causes the user to feel a certain reward. This eventually causes one to become dependent on the drug, to the extent that one is willing to forego all ethical limits to obtain more of it. Numerous researchers have attempted to find a way to help addicts recover (Koob and Moal 2001). Previous experiments as well as cutting-edge research have furthered this discovery. Researchers have developed a vaccine to aid in the treatment of nicotine addiction and restore normal brain receptor activities. However, what is the efficacy of the "nicotine vaccine" in treating smokers addicted to nicotine? Before analyzing the effectiveness of the vaccine, a complete understanding of dopamine receptors and their effects on the body and behavior must be gained.

THE BRAIN-BEHAVIOR CONNECTION: DOPAMINE RECEPTORS

Psychologists and scientists have been mystified by the brain-behavior connection in drug or alcohol abuse and have been trying to determine how to treat addiction. Drugs and other addictive substances cause a surge in levels of a brain chemical called dopamine, the neurotransmitter that is responsible for feelings of pleasure. The brain remembers this pleasure and wants to repeat it (Long 2011). Neurons containing the dopamine receptors are clustered in the substantia nigra, an area in the midbrain (Schultz 2010). The pleasure sensation creates the motivation for a person to proactively pursue activities such as eating and drinking that are crucial for survival. A person is driven to perform these vital functions because the brain is conditioned to expect the dopamine rush that accompanies them. Drugs such as methamphetamine, heroin, and cocaine produce their effects by acting on the flow of neurotransmitters and affecting the brain chemistry (Schultz 2010). They can cause profound changes in human behavior (Wise and Rompre 1998) that can have negative consequences in varying areas of an individual's life (Chandler et al. 2009).

Widely documented experimental evidence suggests that the mesolimbic dopamine system is hypofunctional in the addicted brain (Melis et al. 2005). When

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using addictive drugs, the brain is flooded with up to ten times the normal amount of dopamine. The mesolimbic dopamine system becomes hypofunctional due to down-regulation of the dopamine receptor because of excess dopamine present when certain addictive drugs are used. By decreasing the dopamine (DA) system function in addicted subjects, there will be a decreased interest in non-drug related stimuli and increased sensitivity to the drug of choice (Melis et al. 2005). When a user's brain adapts to a higher level of dopamine to get pleasure, it begins associating the addictive drug with this neurochemical reward, and eventually, the drugs create a scenario that only they can meet (Diana 2011). This process leads to addiction, in which a person is left with a drive to compulsively take the drug, conditioned to expect artificially high levels of the neurotransmitter. The brain begins to require more dopamine than it can naturally produce, and it becomes dependent on the addictive drug, which never actually satisfies the need it created (Kosten 2011).

Normally, dopamine conditions us to do what we need to do to continue surviving. Regulation of dopamine plays a crucial role in our mental and physical health. However, just as food is linked to survival in day-to-day living, addictive drugs triggering the release of dopamine begin to take on the same significance for the addict. The need to obtain and take drugs becomes more important than any other need, including truly vital behaviors like eating. Eventually, all ethical guidelines in a person's life, such as family, work, and community obligations and values, are lost to the disease of addiction (Koob and Moal 2001). When the brain's dopamine receptor is down-regulated, greater amounts of dopamine are required to induce the normal effect. Eventually, the disrupted dopamine system renders the addict incapable of feeling any pleasure even from the drugs they seek to feed their addiction. The lack of control causes people who are addicted to continue using drugs, even when the drugs have lost their power to reward (Diana 2011).

Based on the above, one form of treatment used to treat addicted patients is to block entry of the addictive drug into the brain receptor system (Kenny et al. 2006). In this way, the DA system hypofunction will eventually revert to normal functioning with time.

Nicotine Addiction and Treatment

Cigarette smoking is the most common cause of death in industrialized countries. Thirty percent of all deaths in smokers from 35-69 years of age are due to chronic smoking. Though there are many forms of medication available for the addiction, there is still an extremely low success rate for people who have tried to quit smoking. According to the American Lung Association, nearly half of U.S. smokers try to quit each year, and only 4% to 7% of the people who make the attempt are successful. Norman Edelman, the Chief Medical Officer of the American Lung Association, says that at best, only one out of three people trying to quit are successful (American Lung Association 2011).

Therefore, a new approach to treating addiction has been developed. This technique utilizes injected vaccines to block addictive substances from reaching the brain. As indicated in Figure 1, the vaccine induces the immune system to produce antibodies that bind to nicotine. This prevents the nicotine from crossing the blood-brain barrier and acting on dopamine receptors in the brain. When people smoke, the nicotine inhaled from tobacco moves from the lungs to the bloodstream, and up to the smoker's brain within seconds. There, nicotine triggers a number of chemical responses, one of which involves the dopamine receptors, creating feelings of pleasure and a variety of neural effects that initiate and maintain tobacco dependence. The sensation lasts minutes. However, as the nicotine levels drop, smokers feel agitated, a symptom of nicotine withdrawal. In order to relieve discomfort, they often light another cigarette, beginning a vicious cycle of addictive smoking. Therefore, efforts to develop treatment for people addicted to smoking have focused on targeting neural pathways involved in nicotine addiction (Hall 2005).

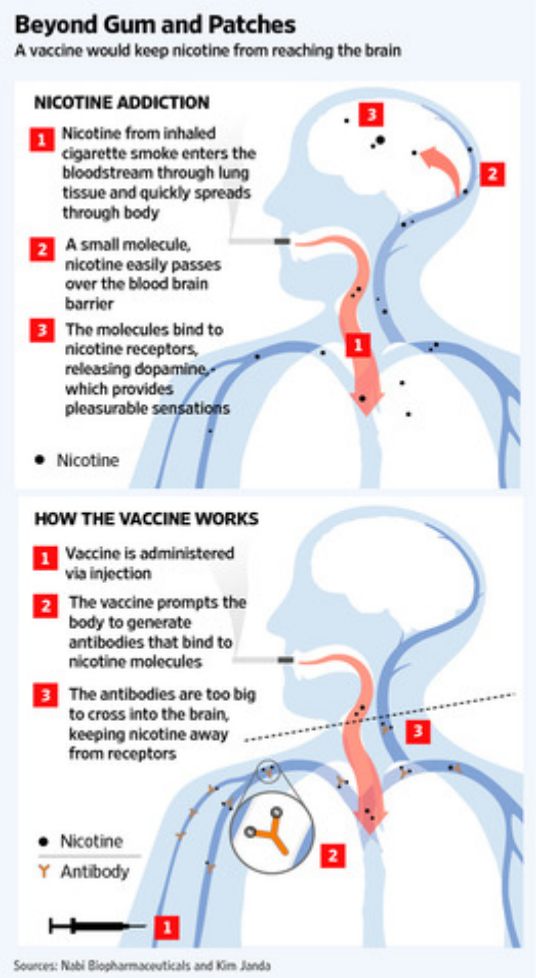

Figure 1: With the administration of the vaccine, the body produces antibodies that bind to nicotine molecules and prevent them from crossing the blood brain barrier. Source: Long 2011

When people take addictive drugs, the drug molecules travel through the bloodstream to the brain. Because addictive drugs are so small, they bypass the immune system completely. However, using the vaccine, scientists attach molecules similar to addictive drugs to much bigger antigens, such as deactivated versions of the common cold virus (Long 2011).

When injected into lab animals and people, these so-called conjugate vaccines spur the immune system to create antibodies to fight the tiny, addictive drug molecules (Kosten 2011). The antibodies attach to molecules of nicotine and cocaine before they cross the blood-brain barrier, thereby blocking them from triggering the pleasure centers in the brain (Hall 2005).

Vaccination against nicotine can reduce the risk of relapse in addicted smokers by easing the pharmacological effects of nicotine for the first few months after quitting, the period when most smokers relapse (Hall 2005). Unlike prior medications that worked via the brain, addictive-treatment vaccines work in the bloodstream (Long 2011).

Vaccines against nicotine are a promising concept in smoking cessation research. This is because it can aid current smokers attempting to quit, former smokers wanting to avoid relapse, and adolescent smokers from becoming confirmed smokers. Because nicotine is the pharmacological agent controlling the rate of cigarette smoking, by reducing the rate and extent of uptake in the brain, researchers can attempt to treat the addiction (Vocci and Chiang 2001). Even if the smoker increases the dosage of nicotine, the rewarding effect would still be circumvented by the vaccine, hopefully ensuring that the lapse would not lead to daily smoking (Khoury et al. 2003). However, the vaccine does not combat cravings. They simply trick the body to reject drugs as if they are foreign pathogens. The vaccine also has the potential to work on many drugs aside from nicotine, such as cocaine and heroin, amongst other addictive substances.

VACCINE FORMATION AND ADMINISTRATION

Since nicotine is extremely small (molecular weight =167 kD) and therefore not immunogenic, the body does not create antibodies against it. By using a linker such as succinic acid, researchers can convert nicotine to an immunogenic carrier protein to form an immunogen, a conjugate nicotine vaccine. Multiple types of carrier proteins have been used, such as keyhole limpet hemocyanine, recombinant cholera toxin B subunit, and recombinant psuedomonas exoprotein A. The latter two have been used in vaccines administered to humans before. The vaccines are then mixed with an adjuvant such as alum, to enhance the immune response (Hieda et al. 1997).

The ideal vaccine elicits antibodies that have the characteristics of immunogenicity, specificity, and affinity to bind to nicotine. Immunogenicity refers to the maximally effective serum concentration of antibody throughout the period of interest. In that way, there will be a higher ratio of antibody to nicotine to increase binding of nicotine to serum. Affinity refers to the strength of the antibodies binding to the nicotine, and specificity refers to the extent that antibodies bind to nicotine as opposed to other compounds (Hall 2005).

EXPERIMENTATION PERFORMED WITH ANIMALS

Studies performed with animals have proven that attaching nicotine to a viable antigenic protein produces antibodies that have a high affinity for nicotine (Hall 2005). A series of 2-4 injections of vaccine was given to rats over 4-8 weeks. The vaccine was aimed at eliciting higher serum concentration of nicotine specific antibodies that would not bind to nicotine metabolites (Pentel and Malin 2002). When the rats were vaccinated, they were given a single dose of nicotine, equivalent to the nicotine absorbed by a smoker from two cigarettes. The researchers then tested the serum and found that the nicotine delivered to the brain 1-3 minutes later was 60% less than that of the control group. Even when the rats received heavier doses, equivalent to that of a chronic smoker, vaccination remained effective in reducing the early distribution of each dose to the brain. Vaccination of rats reduced the nicotine-induced release of dopamine from the nucleus accumbens, a neurochemical event that is thought to be a key mediator of nicotine dependence (Pentel and Malin 2002).

These results indicate that there is potential usage for vaccines in the prevention of relapse. With the effect of the vaccine and the antibodies attacking the nicotine, the nicotine fails to pass through the blood-brain barrier and affect the dopamine receptors. By staying in the blood, there is no pleasurable response to the

nicotine. Cigarette smokers who quit often experience cravings and thus resume smoking to relieve their discomfort. However, if the vaccine renders the cigarette ineffective, the smokers will be less likely to smoke a cigarette. A downside of the experiment was that it only managed to prevent 60% of the nicotine from reaching the receptors.

Investigators at the University of Minnesota also performed experiments with a vaccine to treat addiction to nicotine (Keyler et al. 2008). They began by taking a group of rats and injecting them with different types of proteins—proteins that would bind to the nicotine and attract the antibodies, preventing them from passing through to the brain. The experiment had three experimental groups, each injected with a different type of binding protein. The control group was a group of rats who received no protein injections at all. After three series of vaccinations, the rats were anesthetized with dropiridol/fentanyl, and then injected with .03/ mg/kg of 6-CMUNic, 3-AmNic, and Bivalent over 10 sec via the jugular cannula. The rats were decapitated 3 minutes later and levels in the blood and brain were collected and stored at -20 degrees Celsius until processed. Serum and brain protein concentrations, nicotine protein binding parameters, and serum NicAb concentrations were compared among groups by one way ANOVA and individual comparisons were analyzed by t-test. As seen in Figure 2, the results indicated that each of the vaccines increased the total serum nicotine concentration, and reduced the nicotine concentration in the brain compared to the control group.

The results indicate that it is possible to design more than one immunological distinct hapten from a small molecule such as nicotine to inject via vaccine to bind to nicotine and prevent it from crossing the blood-brain barrier. The fact that the Bivalent showed evidence for antibodies in the blood, and lack thereof in the brain, shows the success of having the antibodies bind to the nicotine vaccine. The results show potential for using the vaccine to treat addiction for nicotine and ensure that the dopamine receptors are not affected by the drug (Keyler et al. 2008).

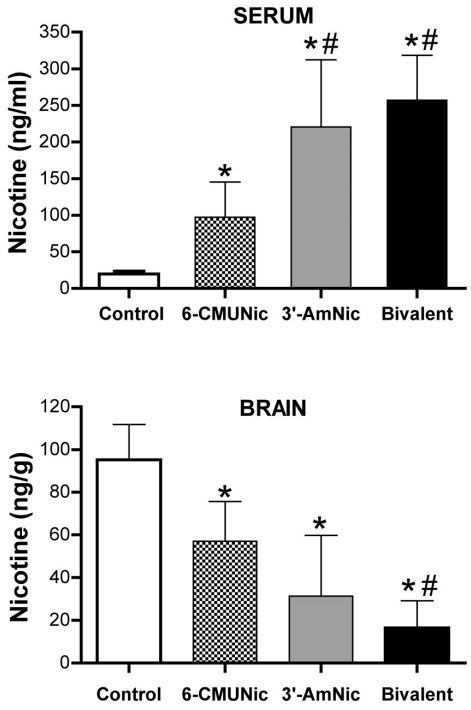

Figure 2: Results indicating the decreased nicotine levels in the blood of rats that were vaccinated, as well as the increased nicotine levels in the brains of non-vaccinated rats. Source: Keyler et al. 2008

Clinical Trials

Researchers at Maastricht University extended the studies to human subjects. In this case, the researchers evaluated the safety and immunogenicity of four doses of a nicotine vaccine in smokers and non-smokers. The subjects were in good physical and mental health. Each volunteer either received an injection of a placebo in the control group, or the vaccine in the experimental group. In this case, the

scientists were not trying to see how much nicotine reached the brain. They were attempting to measure the success of the vaccine in creating nicotine-specific antibodies. The results would lead scientists one step further in treating subjects addicted to nicotine. The subjects received a vaccination of 3'-aminomethylnicotine conjugated to detoxified Pseudomonas aeruginosa r-exoprotein A each week. At first, no difference was seen between the two groups. However, after 21 days, 7 days after the second vaccination, significant increase in the geometric mean titer (GMT) levels of nicotine-specific antibodies were observed in the smokers. Nicotine-specific antibody levels rose to a GMT of at least 8 at day 49, and at least 10.8 at day 217. With each additional vaccination, the level continuously rose. These results indicate that the immunogenicity of the vaccine was not impeded by the presence of nicotine, thus providing evidence in humans that the vaccine used may represent a feasible strategy for evoking type-specific antibodies against nicotine. With these type-specific antibodies, the body can ward off nicotine and prevent it from reaching the brain (Wagena et al. 2008).

Researchers at Yale University performed a similar experiment testing the therapeutic effects of a cocaine vaccine, using the same technique as the nicotine vaccine. The researchers used 34 former cocaine abusers as their experimental group. They were divided up and each was given a different dosage of the vaccine, 8 at 13 micrograms of the active vaccine, 10 at 82 micrograms, and 10 at 709 micrograms. Two subjects in each group represented the control group who received a placebo. Each group got an intramuscular injection for up to 2 months and was monitored for safety and antibody production for 3 months. Twenty-seven of the subjects completed the full course of three injections. However, only 24 returned for the final scheduled visit on day 84. The vaccine had no drug-related adverse effects, but three subjects at the highest dose experienced brief twitching after being injected (Martell et al. 2007).

Antibody levels were correlated with vaccine dosage and number of injections. Anti-cocaine antibodies were first detected after the second injection. The number of antibodies peaked after 3 months of treatment and then declined to baseline by 1 year. The therapeutic vaccine was well tolerated, with dose related increases in antibody levels, and a high proportion of patients recruited into the study were retained (Martell et al. 2007).

Another experiment was performed using a vaccine to treat cocaine dependence. Eighteen subjects were tested with dose-escalation over fourteen weeks. Ten subjects received four 100 mg injections over the course of eight weeks. The other eight subjects received five 400 mg injections over twelve weeks. The urine toxicologies and cocaine antibody titers were compared, three times each week.

Sixteen of the 18 subjects completed the study. The 2000 mg total dose group had significantly higher mean antibody titer response (2000 units) than the 400 mg total dose group. Despite the fact that there were relapses in both groups, the subjects said they lost the euphoric effect of cocaine at the six month follow-ups, 63% in the 400 mg and 100% in the 2000 mg groups.

The conjugated vaccine to treat cocaine addiction was well-tolerated and the antibodies were prevalent for at least six months. Additionally, the subjects who received the more intense vaccination schedule had less likelihood of using cocaine.

These experiments prove that vaccines were effective in raising the antibody level in the body, as well as preventing the drugs from reaching the dopamine

receptors. However, the experimental groups were small and the researchers did not extend the experiment to see how long the effects lasted and if the dopamine receptor levels were up-graded and normal levels restored.

Multiple pharmaceutical companies are performing vaccine clinical trials as well. The adjuncts used in the clinical trials are known as alum hydroxide or phosphate. All of the clinical trials have administered the vaccine via injection. The company Cytos has successfully completed a Phase I clinical trial involving 40 non-smoking subjects who showed no unexpected toxicities. In Phase II of the study, individuals with antibodies in the highest percentile were able to avoid relapse to cigarette consumption for longer than the subjects who received a placebo vaccine. Abstinence rates in subjects with lower antibody responses were not significantly different from those in the placebo group (Escobar-Chavez et al. 2011). This experiment proved that elevated antibody levels are effective in preventing relapse to cigarette consumption.

Cytos's nicotine vaccine program now collaborated with Novartis AG. Together they performed a double-blind placebo-controlled Phase I study evaluating immunogenicity and tolerability of the vaccine. The study contained four groups of 10 non-smoking subjects who were given different doses of the vaccine. All of the subjects evaluated responded with high levels of nicotine-specific antibodies and a long-lasting immune response. Up to half of the patients reported negative effects such as muscle aches, fever, and chills. Those symptoms disappeared within one day, and the elevated antibody level declined over time.

In Phase II of the clinical trial, a group of 341 smokers were divided: two thirds received the active vaccine and one third received a placebo. Afterwards, five injections of 100 mg of vaccine conjugate were given monthly and the subjects received counseling for the first three months. The subjects were required to abstain from smoking from week 8 to week 52 after receiving treatment. The researchers used self-reporting and biochemical markers to evaluate that the subjects were adhering to the regulations. The participants reacted as predicted. The two thirds receiving the active vaccine developed elevated levels of antibodies, while the control group did not. Though the vaccine was tolerated, there were some side effects such as flu-like symptoms. However, the effects only lasted for one day.

In May 2005, six-month results were published, and later that year, 12-month results were published. According to the results of the antibody levels, the smokers were divided into three groups: low, medium, and high responders. The high responders group had continuous abstinence after 6 to 12 months of 57% ($P=0.004$ as compared to the placebo group) and 42% respectively. The medium responders group had a result of 32% and 21% respectively, and the low responders group had 32% and 26% respectively. The relatively high continuous abstinence rate for the placebo group was 32% and 26%.

Another study Cytos performed with healthy volunteers evaluated giving 300 mg per injection as opposed to 11 mg. The higher dose induced a greater mean antibody production that was four times higher than the initial Phase II study. The company also reported new formulations reducing the incidents of fever and flu-like symptoms to about 10% as opposed to the original 60% (Escobar-Chavez et al. 2011).

The clinical trials performed by Cytos indicated the success of the vaccine in treating nicotine addiction. The subjects with increased levels of antibody production

maintained their smoking prevention and abstained from relapsing for longer periods of time. Immunization against nicotine can significantly ease some behavioral effects of nicotine. The results of these experiments suggest that immunologic intervention could have use in the treatment of tobacco dependence. However, further research and clinical trials are necessary to validate that vaccinations facilitate abstinence from nicotine use (Escobar-Chavez et al. 2011).

POTENTIAL FOR THE NICOTINE VACCINE

The nicotine vaccine certainly shows some degree of efficacy. Overall, the data indicated in the experiments and clinical trials support the vaccine in preventing nicotine from affecting the dopamine receptors. Clinically, though, the vaccine will not replace existing medication. Nicotine replacement therapy, such as bupropion and nortriptyline, will still be the main form of treatment. However, the vaccine can lessen the rewarding effects of nicotine, something which existing therapies cannot do significantly, thereby complementing current treatment (Escobar-Chavez et al. 2011).

The nicotine vaccine can also be useful in relapse prevention by blocking the effects of using a cigarette. Vaccination can also be done while the individual is still smoking, thereby preparing the individual to quit. The vaccine may also have a possible role in preventing high-risk teens from becoming completely addicted to smoking. However, that will require additional confidence and safety assurances aside from proof of efficacy.

Most importantly, vaccination can play a role in aiding individuals who are taking proactive steps to a complete recovery. Although research suggests that immunologic intervention can play an important role in treatment of tobacco addicts, the patients first need to be motivated to quit. The vaccine does not treat the non-pharmacological factors that maintain tobacco dependence. By giving the vaccine in conjunction with behavioral intervention, patients can maximize the results and may quit their nicotine-dependence.

More reports on the vaccine will be publicized within the coming year. Phase III trials and marketing launch dates for nicotine vaccines have yet to be announced. However, the results of the experiments and clinical trials performed thus far are indicative of the possibility of successfully distributing a vaccine to induce type-specific antibodies to prevent nicotine from entering the brain (Long 2011). These experiments provide a basis for creating vaccines to treat other substances as well, using the same strategy. Scientists are merely steps away from succeeding in creating an effective vaccine against nicotine, and perhaps with that success, they will attempt to create further vaccines type-specific for antibodies to bond to other addictive substances.

DISADVANTAGES/ETHICAL ISSUES

There are numerous ethical issues that arise with the possibility of producing and distributing a nicotine vaccine. Firstly, misconceptions may arise that the vaccine will give lifelong immunity against nicotine and may cause parents to vaccinate their children, and as minors, children will be unable to dissent. Some may say that parents have a right to protect their children, while others may fight this view, namely, tobacco producers. Additionally, there is a danger that people may overdose on drugs after receiving the vaccine due to their inability to feel pleasure from the drugs.

On the other hand, the vaccine used for treatment of addiction has many advantages. Most importantly, though not eliminating the cravings, the vaccine will

help the addict stick to his recovery, in that after his injection he will be free of the drug induced chemical reactions that he relied on so heavily (Kosten 2011). With this-once-a-month vaccine, the recovering addict has a stronger chance of maintaining his treatment plan, as it only takes a monthly injection to ensure that he does not feel the affects of succumbing to his desire to relapse, and thus can overcome the initial most difficult moths of addiction treatment (Long 2011). The most crucial part of any drug treatment is the prevention of relapse. With the antibodies from the vaccine remaining within the user's system for an entire month, the user will be protected from relapses throughout that time period, and hopefully remain drug-free by choice after that.

Additionally, the vaccine poses minimal danger to the subject's health and normal brain functioning. By blocking the brain from receiving the chemicals from the drugs, the user will be able to stop his behavior and control his addiction. The vaccine can also be used alongside psychological therapy because it does not affect normal brain functioning; it prevents both the vaccine and the drug from entering the brain (Long 2011). Another advantage of the vaccine is that it saves time, since it only has to be given once a month. It saves money as well, as other forms of pills, compresses and treatments may be minimized or eliminated. Though it is advisable for subjects of addiction to see a psychologist continuously, the vaccine helps prevent a relapse because they will stop using the drug once they realize it has no effect on them, thus eliminating relapse costs.

Another major advantage is that the addictive substance is not being treated with another addictive substance, drug for drug. With the vaccine, the effect of the addictive substance is being eliminated, while refraining from adding additional potentially addictive substances to the body. Traditional addiction treatments typically involve medications that mimic a drug in the brain. For example, methadone will stand in for heroine and a nicotine patch will substitute for cigarettes. Other medications block activity in the brain's reward system, such as Vivitrol injections for alcoholics and Pfizer Inc.'s Chantix pills that block the brain's pleasure receptors from being activated when people smoke. Some of these drugs function inside the brain and thus can pose potential damage to the brain. Warnings include depression and suicidal thoughts (Long 2011). On the other hand, vaccines pose no risk to normal brain functioning.

CONCLUSION

Addiction is a clear indicator of biological and biochemical psychology and the way the brain controls behavior. The brain plays a strong role in human behavior in that ultimately, addicts are craving the chemical reaction that the drug causes. Though there are many medications for addicts, psychologists have noted that the success rate of treatment for drug-abuse is unfortunately low (Koob and Moal 2001). The fact that the development of a vaccine may be able to treat addiction poses a new hope for addicts' recovery.

The idea of using the body's innate immune system functions to treat addictions is brilliant. By using antibodies, no risk is posed to the brain or normal functioning. This technique may also be implemented for other health issues that involve chemicals reaching the brain. The development of such a vaccine can be the basis for further development and treatment for other dangerous chemicals that reach the brain. Using vaccines to treat addiction is just one step in using the antibodies already in our bodies

to treat illnesses. Antibodies are G-d's army to fight disease; why not utilize them to their complete capacity?

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FUNCTIONAL ELECTRICAL STIMULATION IN SPINAL CORD INJURY REHABILITATION

Meir Hildeshaim

INTRODUCTION

Spinal cord injury is defined as a "disconnection syndrome" that results in a loss of ability of the spinal cord to communicate ascending and/or descending impulses (Hamid and Hayak 2008). Due to its role as the primary conduit of motor and sensory impulses, spinal cord injury is widely regarded as one of the most catastrophic, survivable injuries a person can suffer. Depending on the severity and placement of the injury, the patient can experience a wide range of disability or death. A mild injury may result in the patient lacking strength in one limb, while a severe injury can place the patient on a ventilator for life (Field-Fote 2009).

Before World War II, treatment for a spinal cord injury was very limited and rehabilitation was almost non-existent. Life expectancy for a patient with a spinal cord injury (SCI) was very short. In most cases, secondary renal, cardiovascular, and pulmonary conditions took the life of the individual shortly following the injury. Advances in the past forty years have improved care to the extent that individuals living with a spinal cord injury can now expect to live nearly as long as able-bodied individuals (Hamid and Hayak 2008). The increased use of intermittent bladder catheterization dramatically cut down the chances of an individual developing renal complications, and advances in emergency medical care resulted in fewer incomplete spinal injuries turning into complete spinal cord injuries during stabilization and transport of the patient (Field-Fote 2009).

According to the Spinal Cord Injury Statistical Center, there are roughly 250,000 individuals living with spinal cord injury, with approximately 11,000 new injuries happening yearly. Between the 1970s and 2000, the average age of an individual with a spinal cord injury has risen from 28.7 years to 38 years. The rise in average age indicates that people are living longer with spinal cord injuries.

As people live longer with spinal cord injuries and the population of spinal cord disabled people increases, secondary conditions that SCI patients suffer become more apparent. The question of how the medical community can best service them becomes of more pressing importance. While the possibility of regaining the body's natural conduction system of sensory and motor impulses is far off in the future, there are numerous rehabilitative measures that can be employed to maximize the remaining healthy neural pathways and maintain optimal health.

While the central nervous system has suffered a cataclysmic injury from which it may never recover, the peripheral nervous system emerges mostly intact. This being the case, it is possible to generate muscle contractions in spinal cord patients using an external device to generate the impulse that would have otherwise descended from the brain via the spinal cord. Since as early as the eighteenth century, clinicians were using electrical impulses to generate muscle contractions (Hamid and Hayak 2008). In the 1960s, researchers began systematically applying electrical stimulation with the hope of helping patients recover. Muscle contractions were generated by stimulation that

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was delivered via electrodes placed proximal to the nerve that innervated the desired muscle.

As technology advanced, stimulation patterns became increasingly sophisticated and useful. Therapists and doctors began using this system, called Functional Electronic Stimulation (FES), to assist spinal cord patients with their rehabilitation and daily functioning needs (Prochazka 2009).

While the initial use of Functional Electronic Stimulation was simply to make the muscle contract and to apply the contraction to a functional motion, researchers began to notice that the technology may have other positive physiological effects on the users.

Aside from the obvious sensory and motor deficits that arise from a spinal cord injury, spinal cord injury patients typically suffer from a variety of secondary conditions caused by the injury itself and the sedentary lifestyle imposed on them by the injury. Muscular spasticity, muscular atrophy, cardiovascular and cardiopulmonary deficits are all common conditions amongst spinal cord injury patients (National Spinal Cord Injury Statistical Center 2009).

Researchers hypothesized that if they could utilize FES to keep spinal cord patients reasonably active, there is a good possibility that they can stop, slow, or even reverse the secondary conditions arising from the injury.

Care must be taken to distinguish between "neurotherapeutic" achievements and "neuroprosthetic" effects. The former refers to rehabilitative methods that result in a lasting therapeutic benefit that persists after the intervention is removed. The latter refers to the application of an external stimulus that allows for functional movement only as long as the external device is in use (Nogan-Bailey et al. 2010).

While many spinal cord patients regard walking again as the ultimate goal of rehabilitation, there are a number of issues that must be resolved before ambulation can be safely considered. This paper will follow the logical sequence of recovery that the patient and therapist must follow if he or she is to regain locomotion capacity. Spasticity management, atrophy reduction, and cardiovascular/cardiopulmonary fitness are all preconditions to successful, safe ambulation. For each of these, this paper discusses what it is, how it arises in spinal cord patients, how it affects spinal cord patients, and how FES may reduce its severity. Finally, an extensive look is taken at post-spinal cord injury locomotion.

To do this, published, peer-reviewed research on Functional Electronic Stimulation is reviewed and an attempt is made to state what, if any, are the therapeutic, rehabilitative, and functional improvements that patients experience when using Functional Electronic Stimulation as part of their rehabilitative regimen.

In preparing for this paper, twenty-five published peer-reviewed papers, eighteen of which are cited in the paper, were critically reviewed. A comprehensive textbook, *Spinal Cord Injury Rehabilitation*, written by Edelle C. Field-Fote, PT, PhD, a leading researcher in the field, provided much of the introductory material in each section of this paper. Data from the National Spinal Cord injury Statistical Center was also utilized.

SPASTICITY

While the descending excitatory impulses the spinal cord transmits may be the most noticeable, the inhibitory impulses are no less important. When these are

disrupted by a spinal cord injury, the inhibitory functions of the spinal cord are affected. The lack of inhibition is most noticeable in the symptoms of spasticity. Spasticity is a hyper-reflexive response of a muscle to an outside stimulus (Field-Fote 2009). Spasticity is described as the fourth and final stage of spinal shock (Ditunno et al. 2004). Spinal shock is a condition immediately following a spinal cord injury that progresses from a period of absent reflexes, or hypo-reflexia, to the eventual emergence of hyper-reflexia. The reflexes emerge in a predictable pattern. The polysynaptic reflexes occur first, followed by the monosynaptic reflexes some weeks later. When the monosynaptic, deep-tendon reflex emerges, it is often highly sensitive to stimulation. The response is inappropriate in relation to the stimulus received and interferes with many activities the patient performs. Occasionally, the patient is able to anticipate the stimulus that causes the spasm and actually use the spasm for functional movement. More often, though, the spasm is an impediment. For example, some paraplegic patients are able to drive vehicles modified specially to accommodate their disability. For others, muscle spasms are triggered by passive stimulation as mild as the pressure the seat exerts on the driver when the driver executes a turn. The resulting spasm makes driving unsafe (Hamelburg 2009).

FES has been applied in an effort to reduce spasticity. Krause, et al. (2008) performed a crossover study of five patients with acute T3-T7 spinal cord injury. The patients performed both passive and FES activated leg-cycling movements on an ergometer. The FES activated muscles were the quadriceps, hamstrings, and gluteal groups. The results showed a consistent decrease in spastic muscle tone following the FES applied exercise, which was not always present following the passive muscle movements. Whatever reduction was experienced was gone by a week after the exercise. The reduction in spasticity can be explained by something as simple as muscle fatigue following the exercise, with the greater fatigue following active movement of the muscles involved. This study, however, is limited in a number of ways. The sample group was very small, and because the study was performed in an outpatient setting, the clinicians only personally tested the spasticity immediately before and after each session. All other data was subjectively reported by the participants themselves.

The Ashworth scale is a test often used to measure spasticity. Researchers question the validity of the test, because spasticity is an issue that can be more or less severe depending on the time of day, prior muscle activation, and patient fatigue. The test only scores spasticity at a single point in time. In this regard, subjective assessment by the patients themselves may actually be more useful than the Ashworth scale (Johnston et al. 2007).

Other studies have shown reductions in spastic muscle tone, but these, too, have been small studies. The physiological explanation of such reduction is also unclear. The use of FES in spastic muscle reduction thus seems limited (Thomas and Field-Fote 2009).

ATROPHY

In the months following a spinal cord injury, the individual undergoes a dramatic amount of musculoskeletal atrophy. The atrophy carries with it a higher risk for secondary complications of SCI, such as pressure sores, deep vein thrombosis, and bone fractures (Baldi et al. 1998).

Muscles undergo two distinct types of atrophy following a spinal cord injury. The first is "disuse atrophy" and the second is "denervation atrophy." Disuse atrophy results from damage to the central spinal pathway. By interfering with the transmission of upper motor control, the injury prevents the patient from voluntarily initiating a contraction. The muscle remains physiologically capable of contracting, yet undergoes atrophy because the patient is unable to use it. Denervation atrophy results from damage to the lower motor neuron itself. The ability to conduct an impulse to the muscle is lost. Following this type of injury, an FES contraction is much harder to generate because the lower motor neuron is affected. The amount of muscles that undergo denervation atrophy is usually very small; the injury will only directly affect a small number of lower motor neurons. The majority of atrophy spinal cord injury patients experience is disuse atrophy (Gordon and Mao 1994). Even regarding denervation atrophy, the ability to contract the muscle is not lost completely. Because most muscles are innervated by more than one motor neuron, the intact remaining motor neurons can still generate a contraction. However, as the ratio of motor neurons to muscle decreases, the ability to grade contractions is compromised (Field-Fote 2009).

Reducing atrophy is crucial for the patient who wishes to walk again. If the muscle is unable to bear the weight of the patient, walking will remain impossible. It is, therefore, essential that the occurrence of atrophy be reduced as much as possible (Janssen and Pringle 2008).

Many studies have substantiated the claim that FES is useful in stopping atrophy. The increased muscle use of the activated muscles directly reduces the incidence of muscular atrophy (Nogan et al. 2007; Hamid and Hayak 2008; Johnston et al. 2007; Field-Fote et al. 2005). In fact, the use of resistance FES has been documented to prevent atrophy in weightless non-disabled individuals (i.e. astronauts), giving reason to believe that FES may also benefit neurologically deficient individuals (Baldi et al. 1998).

In using FES to reduce muscle atrophy, it is important to determine the best method of applying the stimulation to achieve the desired outcome (Gordon and Mao 1994). In this instance, the desired outcome is sufficient muscle strength and endurance to allow the patient to walk. The therapy is designed to enhance the muscle's ability to bear weight, as well as make the muscle less prone to fatigue. Generally speaking, exercises which are of a small load and long duration are best for increasing endurance, while exercises that place the maximum safe stress on the muscle, with fewer repetitions, will increase strength (Gordon and Mao 1994).

In targeting the muscles that need intervention most, studies have shown that weight-bearing muscles, such as the soleus (plantarflexion), undergo significant atrophy, while non weight-bearing muscles, such as the tibialis anterior (dorsiflexion), undergo little atrophy (Gordon and Mao 1994).

FES generated contractions do little to reduce existing atrophy in chronic spinal cord injury patients. Baldi et al. (1998) suggests that perhaps FES would be more successful in stopping or slowing atrophy than in reversing it. Until twenty years ago, there was no research that studied the effect of FES induced contractions on slowing the atrophic progress of spinal cord injury patients. Most of the research had been done on chronic SCI patients (>1 year post-injury), attempting to reverse existing atrophy. Baldi et al. cites animal studies that indicate that more muscle mass is lost

during the first eleven months following injury than during the next eight years. While previous attempts at reversing atrophy in patients had been largely unsuccessful, researchers concluded that these disappointing results were because the muscle had reached a new "steady state" from which it was nearly impossible to be removed. By the time the patient received the FES, it was corrective, as opposed to prophylactic, in nature. Addressing this concern, Baldi et al. designed a study of six spinal cord injury patients in the acute stage of the injury to determine if preventive FES is more successful than the current model. He hypothesizes that if FES would be applied early enough, it would ward off the muscle atrophy, thereby reducing the degree of secondary complications the SCI patient suffers.

The study had two goals. One goal was to identify the amount of atrophy that occurs in the six months, starting not less than 4 weeks and not more than 15 weeks, after the injury. The second objective was to study the differences between cycle ergometer-load bearing (aka resistance training) and isometric FES.

Twenty-six subjects were randomly assigned to the FES-cycle ergometer load-bearing group, isometric FES group, or control group. All subjects were 4-15 weeks post a cervical or thoracic spinal cord injury. The FES-cycle ergometer group used the cycle ergometer three times a week for 30 minutes each session. Each participant wore a fitted garment over the surface electrodes to minimize slipping of the electrodes. The device stimulated the hip extensors, knee extensors, and knee flexors. The FES isometric contraction group received similar stimulation for one hour, five times weekly.

Six months following the start of the study, the participants were assessed to determine the lower-limb lean body mass (LL-LBM). The results were as follows: The control group lost 21.4% of LL-LBM. The cycle ergometer-load bearing group gained 9.3% LL-LBM. The isometric FES group lost muscle mass, but far less than the control group.

The finding that the isometric FES group experienced minimal amounts of atrophy is consistent with earlier findings that non-load bearing contractions are not capable of building muscle mass.

The results show that starting FES as soon as possible after the injury is beneficial in preventing or diminishing the degree of atrophy the individual will suffer. Safety of the patient must be taken into account, however. Following injury, most SCI patients experience "spinal shock" in which the muscles do not respond with a contraction to any stimulation at all. Patients also frequently experience hypotension, necessitating bed-rest. Therapists must also watch for dangerous conditions that are specific to spinal cord injury patients, such as autonomic dysreflexia, at all times.

CARDIOVASCULAR/CARDIOPULMONARY

The sedentary lifestyle that follows a spinal cord injury puts SCI individuals at a higher risk for conditions associated with lower fitness levels. Obesity, diabetes, and cardiovascular disease are all far more prevalent amongst spinal cord patients than the general population. While the normal resting heart rate for an able-bodied person is between 60-100 beats per minute, a spinal cord injury patient has a normal resting heart rate of only 50 beats per minute (Perret et al. 2010). According to the National Spinal Cord Injury Statistical Center, renal failure was the leading cause of death in

spinal cord injury patients until the 1970s. With the increased use of intermittent catheterization, renal failure has ceded its top spot to cardiovascular problems.

Besides for the obvious difficulty in getting enough exercise if one is motor deficient, there are other cardiovascular problems that contribute to the overall reduction in cardiac health. If the injury is above T1, sympathetic activation of the heart is compromised and a low resting blood pressure is the result. Lower blood pressure causes atrophy of the left ventricle and further compromises the circulatory system. The lower blood pressure can increase the likelihood of heart disease or a deep vein thrombosis (Nash 2009). Decreased circulation coupled with muscle atrophy results in lower systemic O_2 consumption and, consequently, a lower CO_2 production (Janssen and Pringle 2008).

Typically, SCI individuals are limited to upper body exercises, neglecting the greater mass of the lower body. The need for safe methods for spinal cord patients to achieve their daily exercise needs is great (Field-Fote 2009).

Since the early 1980s, it has been well documented that FES is a relatively easy way for a spinal cord injury patient to maintain heart health (Janssen and Pringle 2008; Nash 2009). As the technology becomes increasingly convenient and affordable, FES is becoming a popular method of cardiovascular health maintenance for spinal cord compromised individuals. There is one device that has been the focus of a significant amount of research. The FES leg-cycle ergometer is a machine that activates the major muscle groups of the lower body and moves them around a stationary bicycle. The leg-cycle ergometer is a safe way for many spinal cord patients to maintain cardiovascular health.

The benefits of FES to a tetraplegic are obvious. Lacking motor capability in all limbs, the only means of cardiovascular benefits is an electronically stimulated contraction. Even for paraplegics, the FES leg-cycle ergometer is a useful way to reduce reliance on the possibly overburdened upper limbs (Perret et al. 2010).

Nogan et al. (2007) conducted an exhaustive case study of a participant with a C6-C7 injury. The participant received an implanted 8-channel system that allowed limited community ambulation once mastered. While the main focus of the study was the ambulation of the participant, the participant also underwent a thorough cardio evaluation pre and post FES training. Following the twelve weeks of training, the participant presented a reduced resting and working heart rate. The patient showed greater oxygen consumption, attributed to the increased walking speed achieved from the FES.

It was noted that after a period of several weeks of training, the patient reaches a plateau of cardiac activity that is hard to pass. This discourages the patient from maintaining the exercise schedule. Janssen and Pringle (2008) hypothesized that the plateau observed in patients using the leg-cycle ergometry training could be due to the design of the regimen of stimulation they use. Perhaps by modifying the stimulation pattern to generate greater overload of the muscles, better cardiac and muscular results would be observed. In effect, shorter, more intense sessions may prove better for those purposes. This would be accomplished by maximizing the current amplitude used to generate contractions and by modifying the duration of the sessions. To address this, they developed a modified method of applying the stimulation.

They tested the effects of the modified stimulation patterns on 12 patients, six tetraplegics and six paraplegics. The patients used the system 18 times over 6 weeks.

As stated earlier, the sessions were designed to apply shorter, more intense exercise periods on the participants. The results indicate that the maximum possible gain is observed after training on a system that is designed to produce more intense contractions for shorter duration. The significant findings are summarized in Table 1.

Table 1: Effects of Modified Leg-Cycle Ergometry.

	Standard	Modified	After Training
Peak O_2 Consumption (mL/min)	670 ± 208	818 ± 287	1065 ± 264
Peak CO_2 Production (mL/min)	765 ± 228	1154 ± 390	1405 ± 363
Peak Pulmonary Ventilation (L/min)	30.1 ± 9.0	41.3 ± 12.3	49.1 ± 9.1
Max Cardiac Output (L/min)	6.5 ± 1.4	8.6 ± 1.9	9.5 ± 2.3
Stroke Volume (mL)	82.6 ± 20.6	91.7 ± 23.5	91.2 ± 28.7
Max Heart Rate (bpm)	81.9 ± 17.3	97.4 ± 11.2	113.3 ± 23.0

Source: Janssen and Pringle 2008

There are findings in reviewing the data that highlight some interesting cardiac occurrences in the participants. While heart rate increased, cardiac output and stroke volume did not. This can be contributed to dilation of lower-limb blood vessels resulting in lower venous return, indicated by the lightheadedness reported by some of the participants following the treatments (Janssen and Pringle 2008).

Another interesting result was the increase in oxygen consumption that was not accompanied by any concurring increase of cardiac output. This could be explained by stating that there was improved blood distribution. The oxygen differential between the arteries and veins is, thus, improved while the cardiac output does not change. As noted earlier, better circulation through the body tissues reduces the atrophy that muscles undergo and the occurrence of pressure sores.

Generally, the recommendation for SCI patients is to use 1000-2200 kcal per week to maintain heart health. Perret et al. (2010) conducted a study to determine how much activity is needed to achieve this goal and whether or not this is practical for the general SCI community. The study looked at eight otherwise healthy individuals who had sustained a T3-T9 injury more than three years earlier. They conclude that 4-8 hours of intense FES cycling is enough to generate the 1000-2200 recommended kcal. Considering the normal variation of responses between different individuals, this is fairly consistent with the 30 minutes daily recommended by most therapists who work with FES.

While clearly beneficial, the system still has downsides. It is very time consuming to set up, and, often, the individual needs assistance in properly setting up the equipment. Given these facts, it may be better to use it fewer times a week for longer sessions (Perret et al. 2010). This conclusion does not accommodate the previous recommendation of shorter, more intense bursts of FES to maximize muscle overload and cardiopulmonary benefits. It disregards the benefit of the combination of short-intense and long-intense sessions which maximize strength as well as endurance and reduce occurrence of muscle atrophy. Reasonable disagreement in this regard is expected. One must keep in mind that the SCI patient has a disability that presents a logistical transportation obstacle that must be overcome each time he or she is to participate in therapy away from home. The benefits of frequent sessions are of little use if the patient cannot practically maintain the exercise schedule. The therapist must design an exercise schedule on a patient-by-patient basis, making sure to factor all considerations when recommending what the session duration and intensity should be.

Finally, Perret et al. (2010) suggest that the use of a rowing type machine for paraplegics, where the lower limbs are stimulated electronically and the upper-limb use is voluntary, could provide a good combination of upper and lower body exercise while maximizing cardiopulmonary advantages. This combination needs to be studied more before a recommendation can be made.

LOCOMOTION/AMBULATION

The use of FES to assist spinal cord injury patients in standing, sitting and walking started in the early 1980s. In 1982, a device was introduced by researchers at Wright State University in Ohio that could stimulate a spinal cord patient's muscles to allow for standing and level ground walking. The disadvantages of this early technology were obvious. The battery pack needed to operate this device weighed nearly eight pounds and was worn on the user's back. This was the lightweight option and was for walking only. A heavier battery was needed when the user wished to make use of the stand and sit feature. An updated device introduced in 1989 had its disadvantages too. Putting on and removing the system took around an hour. Phillips (1989) outlines in agonizing detail the procedure for generating the necessary pattern of stimulation and positioning of the patient to allow rudimentary locomotion. Clearly this was not a practical option for the average disabled individual.

In a case study, Nogan-Bailey et al. (2007) reports that some non-ambulatory individuals are able to combine their remaining volitional motor, sensory, and proprioceptive abilities with an FES device to allow limited ambulation.

For FES-assisted walking, the stimulation was formulated to accomplish three goals. The first goal was to "augment" existing volitional contraction. The second objective was to initiate contraction of paralyzed muscle. The third purpose was to reduce extensor tone for easier walking; for example, stimulation of hip flexors (iliopsoas) reduced tone in the hip extensor (biceps femoris) dramatically, making hip flexion easier for stepping. "Stimulation was the means to reducing extensor tone during standing to allow stepping."

The goal was to generate the strongest contraction that would not hurt the patient or overflow to a neighboring muscle group. Once this was accomplished, the maximum threshold has been reached.

The variable used as the baseline index for the study was voluntary walking following aggressive pre-study rehab using robotic-assisted body-weight-supported treadmill training. They hypothesized that "exercise and gait training with FES would improve voluntary motor control and baseline volitional walking ability. It would also increase the strength, endurance and repeatability of muscle contraction over maximal pre-implant levels."

This hypothesis was tested with pre and post implant assessments of gait function (speed, distance, symmetry, and physiological cost) and isokinetic muscle contractile properties (strength, endurance, and repeatability) of the knee extensors on a dynometer. The goal was to improve a nonambulatory patient's function to that of independent household ambulation or limited community ambulation. The patient selected for the study was unable to voluntarily initiate a single step with either leg. The patient was evaluated at the following points: after the pre-study therapy, after the implant, six weeks into FES training, and 12 weeks into FES training.

The participant used a hand trigger to manually initiate the impulse for each step. The patient needed to be trained in this device with a specific sequence of switches at different points in the gait.

While there were some small improvements attributable to the pre implant therapy, the patient remained functionally non-ambulatory prior to the implant (Nogan-Bailey et al. 2007).

At the 12-week assessment, the following results were obtained: Walking distance improved 20x (14m in 11 min to 309m in 30 min). Walking speed increased 10x (0.02m/s to 0.20m/s). The patient needed less standby assistance and a smaller walking aid than before.

The FES did not improve volitional ambulation or motor control at all. The results signified that the device is useful for household or limited community use.

While walking speed and cadence improved from pre to post implant, it peaked at 6 weeks and did not get any better at the 12-week checkup. The reason for this was a technical limitation of the system. Speed is largely a function of plantarflexor strength. The primary muscle of plantarflexion is the gastrocnemius. The gastrocnemius was not implanted due to a limited number of channels available on the system; priority was given to muscle groups needed for ambulation. The participant "thus relied on voluntary plantarflexion strength during walking … and this strength was lacking" (Nogan-Bailey et al. 2007).

In 2010, researchers published a single-subject study on the therapeutic effects of FES. The hypothesis was that it would seem reasonable to expect increases in volitional motor control following therapy which utilizes FES. "Neuroprosthetic interventions may have neurotherapeutic value" (Nogan-Bailey et al. 2010).

The subject was C6 incomplete. He was unable to stand without support and able to walk only limited distances (<30m) using both a wheeled walker and a left ankle-foot orthotic. The limiting factor in his ambulation was upper body exhaustion due to the use of his trunk and hip to elevate his weak left leg during the swing phase of gait. He presented with significant left side weakness as well as weakness in his trunk and upper limbs. Because the main deficit was on his left side, only the left leg was implanted. The muscles implanted were the iliopsoas (hip flexion), tensor fasciae latae (hip flexion and abduction), gluteus medius (hip abduction), posterior portion of adductor magnus (hip extension), gluteus maximus (hip extension), vastus lateralis

(knee extension), tibialis anterior (ankle dorsiflexion), and peroneus longus (foot eversion). The patient followed a home exercise routine to build strength in the muscle groups that were stimulated. This included exercising the extensors as a group (standing), the flexors as a group (swing phase of gait), the ankle dorsiflexor, and the knee extensor. The patient self-reported participation in the home portion of the program.

At first, the therapist triggered the stimulation of the left leg as needed to initiate and continue the walking. As proficiency increased, the patient himself took over that function. Eventually, the patient was able to start a "continuous cycling stimulation" for locomotion (Nogan-Bailey et al. 2010).

Data was collected at the start of the program and after 36 sessions of FES training. The testing schedule was staggered to avoid any fatigue factors that could interfere with the results.

The data was statistically analyzed to determine the therapeutic effect of FES, how much the voluntary control of the muscles in question improved, and how useful the neuroprosthetic effect of FES was in restoring function

The patient experienced significant improvements in volitional walking ability. The max distance he could walk in six minutes increased to 80 meters from 28 meters, indicating a "strong neurotherapeutic effect." With the use of FES, his maximum walk distance in the six-minute test jumped to 248 meters, sufficient to allow limited community ambulation for the user. Similar results were obtained for the walking speed test. The baseline speed of 0.17 m/s increased to a volitional speed of 0.22 m/s, with a further increase to 0.27 m/s while employing the FES system. The neuroprosthetic effect here is an additional 20% walking speed. The gait analysis revealed a reduction of double support time, indicating a more dynamic gait.

The patient was unable to extend the knee voluntarily pre implantation. Post implantation, the patient was able to consciously generate 8.78±2.59 Nm of knee extension moment on the implanted side. When FES assisted, the patient was able to generate 30.22±1.07 Nm. The improvements in volitional abilities post training in walking speed, walking distance, and double support time demonstrate the neurotherapeutic effects of FES. These benefits can potentially increase the mobility of an individual to the level of limited community ambulation while using FES. Even without being attached to the FES system, the use of FES in rehabilitation seems to have led to significant improvements in walking speed, distance, and gait quality (Nogan-Bailey et al. 2010).

While there were therapeutic gains, it is difficult to determine which of these gains are results of FES and which would have happened with traditional gait training alone. Further studies are needed to determine the effect of FES that cannot be replicated by extensive traditional overground training and body-weight-supported treadmill training. In any case, this study demonstrated that FES is a viable therapeutic tool (Nogan-Bailey et al. 2010).

There are various methods available for locomotor training of SCI patients. A study was designed in 2005 to collect data on the various advantages and disadvantages each method has to offer (Field-Fote et al. 2005). The study looked at 27 patients with motor incomplete injuries at spinal level T10 or above, who were able to initiate a step with at least one leg. The methods tested were treadmill training with

manual assistance, treadmill training with stimulation, over ground training with stimulation, and treadmill training with robotic assistance

While researchers agree that sensory input from locomotor training is an important aspect that contributes to the patient's improvements, there is disagreement as to the best way to provide that sensory input. Manual assistance has the advantage of the physical therapist being "hands on" and thereby able to provide very precise levels of assistance based on a moment-to-moment assessment of the patient's condition. The disadvantages of manual assistance are that the trainer cannot assist as consistently as an electronic stimulator and that therapist fatigue can also limit the duration of a session.

FES, likewise, has a number of advantages. The FES uses a spinal reflex that is thought to be important in healthy locomotion. "As such, repeated activation of this reflex may be associated with beneficial neural changes and may improve the synaptic efficiency of this circuit." The disadvantage of FES is that patients display a wide variety of therapeutic responses to the treatment. Thus, FES cannot be generalized as being advantageous and must instead be evaluated on a patient-to-patient basis (Field-Fote et al. 2005).

Overall, the various techniques resulted in a 37% increase in walking speed for a "long-bout" walking test (2 minutes) and a 55% increase in walking speed for a "short-bout" walking test (6 meters).

Detailed statistical analysis of the data shows a trend toward better walking improvements for the groups that had FES assisted locomotor training. While it is tempting to deduce from this result that FES training works best, the authors of the paper warn that as their research team works primarily with FES, it is possible that their practitioners are simply better acquainted with FES therapy and, therefore, obtain better results. Other rehab venues may get better results as well with their preferred method of gait training.

While all subjects in the study got better to some degree, none even came close to returning to community ambulation.

Volitional locomotion benefits for individuals with SCI were only observed in incomplete SCI patients. Patients with complete SCI may have been able to generate locomotion like movement on a treadmill but were not able to accomplish this over ground (Field-Fote et al. 2005).

THEORY OF GAIT TRAINING

Why does gait training in general and FES-assisted gait training in particular have a therapeutic effect following a spinal cord injury? The following theory has been proposed. After an injury to the spinal cord, the loss of descending neural control results in a massive and ongoing reorganization of the cerebral and spinal pathways. This evolution continues for years following the injury. The reorganization includes the formation of many new synapses and connections. The new synapses are largely abnormal and interfere with normal transmission of impulses. The result of these abnormal connections is uncoordinated movements and spasticity. Fine movements are impossible to generate. For example, attempting to flex the ankle often results in the entire leg flexing from the hip down (Fong et al. 2009).

The spinal cord can be retrained in the use of its walking patterns with locomotion training, with FES providing afferent input of the sensory patterns

associated with walking. It has been theorized that when the descending motor impulse is generated at the same time that there are incoming sensory impulses that approximate normal ambulation impulses, this may retrain the spinal walking programs to allow for functionally useful synapses to form. Experiments on SCI cats demonstrate that the spinal cord has the ability to perform locomotion pattern behavior without upper nervous input. For many standard motor tasks, the spinal cord is, in large part, autonomous from the brain. The implications of these experiments in spinal cord rehab are enormous. If the spinal cord can generate walking patterns without being connected to the brain, there should be a way to rehabilitate SCI patients. This can help explain the therapeutic effects researchers have observed in patients who have used FES (Fong et al. 2009).

Researchers suggest that when intact lower spinal motor neurons lose the neurotransmitter input from upper motor neurons following a spinal cord injury, the now inactive synapse sprouts new "collateral" dendrites. The emergence of these new synapses can cause unwanted motor activity. If FES is applied to generate functional movements while the sprouting is in progress, this afferent input can direct the sprouting dendrites toward pathways that are functionally useful (Ditunno et al. 2004).

ELECTRODE TYPE AND SPILLOVER

There are two other general discussions related to FES induced walking. The first discussion is what type of electrode is used to generate the contraction, and the second is the concept of "spillover."

To generating contractions, the FES device can employ surface electrodes, percutaneous electrodes, or fully implanted electrodes. Surface electrodes have the advantage of no risk of infection and only a mild risk of skin irritation. The disadvantages of surface electrodes are threefold: first, they cannot be very precise; second, they cannot stimulate deep muscles; and third, when large muscles contract (i.e. the quadriceps) the trigger point can move two or more centimeters under the skin, thereby limiting the effectiveness of the impulse. Percutaneous electrodes have the advantage of precision but a very high risk of infection. Fully implantable electrodes are precise, long lasting, and only have a very small risk of infection. The disadvantages of fully implantable electrodes are that the procedure is invasive and that any repair to the equipment necessitates further surgery (Nogan-Bailey et al. 2007).

"Spillover" in FES refers to an unwanted contraction generated by the impulse. When the impulse reaches the minimum threshold of an unwanted muscle before the maximum useable contraction of the targeted muscle is reached, an unwanted contraction results. The rate of spillover was studied in 10 patients from 1988 to 1998 (Triolo et al. 2001). The total number of electrodes studied was just over 600. The purpose of the study was to map the most frequent sites of spillover in order to help surgeons place the electrodes better, as well as to help the therapist understand and anticipate movements a patient may make during therapy.

A common location of contraction spillover is where the desired contraction of the vasti muscles (vastus lateralis, medialis, and intermedius) to assist standing unintentionally generates contractions of the rector femoris and sartorius muscle, which leads to hip flexion that is counterproductive to standing. This happens because

the electrode is placed proximal to the femoral nerve and can easily produce the undesired contraction. Where the desired effect of the vasti contraction is standing still, activation of the unintended muscles flex the hip or tilt the pelvis anteriority. The hip flexion can cause the patient to adopt a lordotic posture. Additionally, if the hip is flexed, the sartorius can rotate laterally and abduct the thigh. The disadvantages of walking in this manner become clear when considering the fact that all of these patients must walk with an assistive device.

LIMITATIONS

The applications of FES are numerous, but so are the limitations. First, and perhaps most serious, a study done by Bickel et al. (2004) indicates that there is a very real risk of muscle damage when load-bearing FES is applied to muscles that have been inactive for some amount of time. The muscles of SCI patients after an injury undergo structural changes in which the percentage of fast twitch fibers goes up as the percentage of slow twitch fibers goes down. The muscle fatigues quicker and is more susceptible to damage. This hypothesis was confirmed with MRI imaging of eight subjects who had suffered C5-T9 injuries years earlier. The risk of further injury can prevent patients from participating in the treatment. An actual injury can set the patient back months or years in treatment.

Getting up and walking around with a deficient spinal cord always carries greater risk than able-bodied walking. Muscle weakness and coordination difficulties make a fall more likely. Furthermore, bone density is typically compromised in SCI patients. This puts them at greater risk for fracture if they do fall.

Another limitation of FES-assisted walking is that the patients need significant upper-body strength to manage the system. Many patients exhibit varying degrees of weakness or a lack of coordination in their upper body following a spinal cord injury, preventing them from making use of FES for walking.

If sensation has been spared in the lower limbs, some patients will find the feeling of stimulation intolerable (Hamid and Hayek 2008). This presents another limitation to the use of FES.

There are also limitations in the design of the studies on FES. For the most part, studies of FES have been of small sample size and only included short follow up time (Hamid and Hayek 2008). Patients displayed a wide variety of therapeutic responses to the treatment. It is, therefore, difficult to predict what the benefit may be for a particular patient (Field-Fote et al. 2005).

One study suggested that the ability for the patient to self-administer the therapy at home is beneficial because it cuts out the need to arrange transportation to and from therapy (Johnston et al. 2007). A second study suggested that compliance to the therapy session suffers if patients are trusted to administer the therapy themselves. Therapy with FES, according to this study, is most beneficial if administered in a monitored setting (Field-Fote et al. 2005).

Finally, and perhaps most importantly, it is very difficult to generalize from any study of spinal cord rehabilitation for the rest of the "extremely heterogeneous" population of incomplete and complete spinal cord injuries. Because each patient has a unique degree of sensory and motor sensation loss, the effects of FES vary widely from patient to patient (Nogan-Bailey et al. 2010).

THE FUTURE OF FES

Technological advances in the past ten years have made FES more accessible, portable, cosmetically appealing, and more therapeutically helpful. As computers get more powerful, the microprocessors in portable FES systems are better able to process a host of factors that give greater control to the user. Computers can calculate, in real time, the variable muscle forces, fatigue, joint position, and other data available. It can then make instant dynamic adjustments to allow for smoother and safer ambulation (Hamid and Hayek 2008). For tetraplegic patients who lack upper-limb strength, walking may still be out of reach, but there are FES systems that can facilitate hand movements. Using vibrations generated by tooth clicks and detected by a Bluetooth like device worn behind the ear, patients are able to initiate FES impulses to grasp, squeeze, pinch, pull, twist, and execute other hand motions (Harvey et al. 2011).

In the future, FES systems may use more "natural" methods of activation. The descending motor commands would be intercepted, interpreted, and forwarded past the site of the injury to the limb. This would be particularly useful for tetraplegics who, due to their lack of upper-limb strength, are unable to utilize traditional FES to facilitate walking (Nogan-Bailey et al. 2010).

CONCLUSION

The uses of FES in spinal cord rehabilitation are numerous. Over the past thirty years, study after study has demonstrated the gains patients make in reduction of spastic muscle tone, attenuation of muscle atrophy, cardiovascular health, cardiopulmonary health, and volitional or FES-assisted walking.

Spastic muscle tone is reduced by FES. This reduction is only temporary, but, nevertheless, proves an important point about functional electrical stimulation. There are enough real, demonstrable, repeatable benefits of FES that the application of the therapy is recommended. The temporary reduction in spasticity can be considered a side perk of the primary reason for therapy.

Muscle atrophy is slowed by the application of FES. Research indicates that although the reversal of atrophy is not likely, nevertheless, FES slows the progress of atrophy. The earlier FES is applied, the better off the patient's muscles will be. Healthy muscle mass reduces pressure sores and is a precondition for safe standing, sitting, and walking. FES thus aids in retention of healthy muscle mass.

Spinal cord injury patients have a reduced resting and working heart rate. Vasodilation due to low smooth muscle tone causes low blood pressure that reduces venous return. Fewer skeletal muscle contractions means a further reduction in venous return. When applied as part of a structured routine that reaches the recommended level of weekly activity, FES serves the vital function of helping the patient maintain healthy cardiac performance.

The instantly recognizable disability of many spinal cord injury patients is the inability to walk. FES has been able to return a small number of patients to limited community ambulation. Can activation of spinal walking patterns using FES help the injured spinal cord redevelop its ability to generate useful reflexive or volitional contractions? That remains unclear. Some studies have shown improvement in volitional abilities in patients with less severe injuries, while others indicate that volitional abilities remain unchanged. These differing results indicate that the use of FES does not carry the same level of benefit for all patients. Existing research does not

point to a conclusive recommendation regarding gait training using FES. Because the spinal cord population is "extremely heterogeneous," risk of further muscle damage or falls must be weighed against the potential gains made possible by the therapy. Aside from the functional and therapeutic applications of FES, there is an undoubted psychological benefit for patients to be able to "walk" again. Patients reported better self-esteem and lower incidence of depression (Hamid and Hayek 2008). Many subjects reported great improvements in their mental state. The ability to use bathrooms not compliant with ADA (Americans with Disabilities Act), the ability to move around the kitchen using the counters for support, and the ability to climb a flight of stairs all contributed to the patient's sense of purpose and functional well-being (Field-Fote et al. 2005). Regarding walking with FES, the decision whether or not to use FES must be made only after carefully considering all risks and benefits on a patient-by-patient basis.

FES definitely helps patients regain function. Exactly how FES achieves this and how to best use FES to achieve maximum function remains unclear. The studies cited in this paper and the majority of studies conducted overall are small and not well suited for generalization to the spinal cord injury population. There is a great need for large scale, long-term studies with control groups to further assess the role FES can play in spinal cord rehabilitation and to assess the methods of application that can elicit maximum recovery. Better understanding of the pathophysiology of the spinal cord disability can better guide research in the field. If the phenomenon of lower neurons stopping to communicate with each other following an injury is better understood, better treatments can be designed.

It may be some time, if ever, before the medical community is able to cure spinal cord injury paralysis. It was once thought that when FES became sophisticated enough, disabled individuals would be able to simply plug their damaged bodies into the system and walk again. This is not yet the case. Walking with FES is still too risky and inefficient to be the used on a large-scale basis. In the meantime, the goal of patients and therapists is to prevent spinal cord injury patients from developing conditions secondary to the spinal cord injury. While FES can only help a very limited number of patients walk, many patients can, and do, derive crucial health benefits with regard to atrophy reduction and cardiac health maintenance from a carefully structured use of the FES systems currently available.

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How Biological and Non-biological Disease Modifying Drugs are Used in the Treatment of Rheumatoid Arthritis

Esther Mantel

Introduction

Rheumatoid arthritis is a long-term disease that leads to chronic inflammation of the joints and the surrounding tissue. Effects of the inflammation are pain and destruction of the bone and cartilage, which leads to severe disability and, possibly, shorter life expectancy. That is why early diagnosis and aggressive treatment is a fundamental strategy to stop the progression of the disease and suppress the inflammation before the damage is irreversible.

In an attempt to avoid invasive treatments like arthroscopies and surgeries, the orthopedist's first choice of non-pharmacological treatments includes physical and occupation therapies. Pharmaceutical treatments such as non-steroidal anti-inflammatory drugs (NSAIDs) and non-opioid analgesics (pain medication such as acetaminophen and aspirin) work on reducing the inflammation caused by rheumatoid arthritis, which often results in pain relief. Glucocorticoids, a class of steroid hormones, also possess anti-inflammatory effects and were once considered the most powerful treatment of inflammatory arthritis, but their use was virtually abandoned due to their association with toxicity; they are only used nowadays in controlling acute flare-ups joint disease. While these therapeutic strategies reduce inflammation and pain caused by rheumatoid arthritis, they are not that beneficial in slowing down the joint and bone damage and the progression of the disease. Rheumatologists didn't realize that while the pain was being covered by the medications and anti-inflammatory drugs, the inflammation and pannus (an abnormal layer of tissue) were continuing to cultivate inside the patients joints and articular tissue. For this reason, a new and very important group of agents called disease modifying anti-rheumatic drugs, or DMARDs, have become a major interest as a potential new therapy in the treatment of rheumatoid arthritis.

While most treatments focus on reducing the inflammation already present in the bone tissue and joints, DMARDs work on slowing down occurring bone damage and the progression of the disease by actually modifying the disease itself (Katzung 2001). They are different than other rheumatoid arthritis treatments because they work by suppressing the underlying factors that result in synovitis, tissue reactivity, erosions, ligament and tendon laxity, subluxations and other complications caused by rheumatoid arthritis (Johnson 2011). Since there is no presently known cure for rheumatoid arthritis, a lot of research is being done in finding a treatment that will stop or at least slow the progression of the bone damage caused by the disease, so that the patient can be in remission for a long period of time.

Discussion

Rheumatoid arthritis is an autoimmune disease that affects the synovia of joints and, eventually, the healthy surrounding tissue and bone, resulting in symmetric and erosive polyarthritis. According to Shah and Clair (2011), rheumatoid arthritis affects

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approximately 0.5-1% of the adult population worldwide. The ratio of rheumatoid-arthritis-affected women to rheumatoid-arthritis-affected men at premenopausal age is 4:1 while the same ratio at postmenopausal age is 1:1; this is attributed to the role that estrogen has in stimulating tumor necrosis factor-α, a major cytokine in the rheumatoid arthritis pathogenesis (Shah and Clair 2011). The exact etiology of rheumatoid arthritis is still unknown. It is known, however, that genetics plays some role in development and severity in certain patients. It remains a matter of debate whether the trigger of the disease is an exogenous infectious agent, a break in immune tolerance leading to classical autoimmunity, or simply random proceedings that accumulate with age (Klippel 2001).

While the auto-antigen that triggers rheumatoid arthritis has not been identified yet, the progression and evolution of the disease can be blamed on immune cells and mediators that contribute to the inflammation response that occurs. The process of how inflammation and erosion develop in synovial tissue and periarticular bone has been studied and researched in patients with rheumatoid arthritis. The primary agents involved in the immune response in rheumatoid arthritis patients are T-cells, which mainly function in stimulating other cells in the joint to produce and secrete cytokines. The most important cytokines involved in rheumatoid arthritis are tumor necrosis factor (TNF) and interleukin-1 (IL-1), both produced by macrophages and synovial lining cells that were activated by the T-cells in the joints. Once released, TNF and IL-1 stimulate the synovial cells to proliferate and produce factors contributing to the destruction of cartilage, such as inflammatory mediators and matrix metalloproteinases, which are endopeptidases. Eventually, bone destruction is caused by osteoclasts activated by a TNF ligand called RANKL (Receptor activator of nuclear factor kappa-B ligand), which is produced by T-cells and synovial fibroblasts. As the hyperplastic and hypertrophy synovium grows over the articular surface, pannus develops, which stimulates the resorption of surrounding cartilage (Kumar et al. 2005).

In addition to T-cells acting up, activated B-cells produce inflammatory-contributing autoantibodies. Some rheumatoid arthritis patients possessor develop rheumatoid factors, auto-antibodies that bind to the Fc fragment of Immunoglobulin G to form immune complexes that lead to the recruitment of polymorphonuclear leukocytes, further exacerbating the ongoing inflammation. The increasing pannus and inflamed synovium that spread over the articular cartilage produce large amounts of degradative enzymes (e.g. collagenase and stromelysin) that assist in irreversible cartilage destruction and subchondral bone erosion (Heaverstock and Jorizzo 2008).

Since it is a systemic disease, rheumatoid arthritis can affect internal organs as well, eventually leading to early death if left untreated. Rheumatoid arthritis typically affects joints of the hands and feet first, but can spring up in larger joints at any time. One of the essential factors of diagnosing rheumatoid arthritis is stiffness and soreness in the mornings after an extended lack of movement. Other clinical findings of rheumatoid arthritis are morning pain and swelling in areas such as the phalanges and on the balls of the feet. Routine morning activities, such as brushing ones teeth or hair, might become difficult due to the clinical manifestations. If left untreated, the disease will progress and result in increasing pain, swelling and stiffness caused by the destruction of the joints and healthy bones. Figure 1 shows irreversible bone and cartilage loss due to untreated rheumatoid arthritis.

DISEASE MODIFYING ANTI-RHEUMATOID DRUGS (DMARDS)

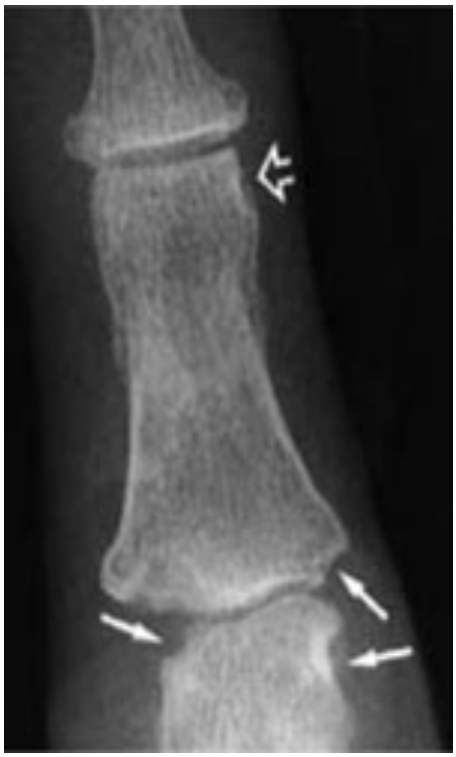

Figure 1:
Interphalangeal joint abnormalities. Osseous erosions are evident at the radial and ulnar aspects of the PIP joint of the second finger (arrows). Soft-tissue swelling and loss of interosseous space are additional findings. Marginal erosion is also seen on the middle phalanx at the distal interphalangeal joint (open arrow) . Source: Kountz and Von Feldt 2007

DMARDs are a class of drugs that include a diverse group of non-biological and biological agents. Although both work on suppressing the underlying cause of the inflammation in the disease, biological DMARDs are protein therapeutics that are designed mainly to target cytokines and cell-surface molecules that promote the inflammation response (Shah and Clair 2011). It may take 6 weeks to 6 months for the effects of the disease-modifying therapies to become evident since they are slow acting. It is necessary to start the use of DMARDs very early in the progression of the disease, since they work by slowing the progression and not reversing the damage already done. A large number of rheumatoid arthritis patients can reach remission or at least a low disease activity with the use of a single non-biological DMARD. However, for those patients with moderate or high disease activity or for those who failed to respond to a single agent due to prolonged disease duration, combinations of non-biological DMARDs are used.

Clinicians realized inadequate response was being achieved by patients being treated with monotherapy DMARDs, and a more aggressive treatment with DMARDs was essential for improving rheumatoid arthritis symptoms and slowing the progression of the disease. The use of biological DMARDs is reserved for those who indicate poor prognosis of the disease and do not respond to non-biological DMARDs treatment. Many patients who don't achieve sufficient results from either non-biological or biological DMARDS have treatment plans that include combining a synthetic DMARD with a biological DMARD in order to reach optimal responses from both agent types. DMARD agents are also commonly used in combination with non-steroidal anti-inflammatory drugs to reduce present inflammation and relieve pain.

Rheumatologists are trying to achieve early and sustained suppression of the disease activity with DMARDs. They believe that early detection of the disease and treatment with DMARDs might negate the need for NSAIDs and corticosteroids. Each rheumatoid arthritis patient's treatment is personalized, taking into account the severity of the disease and the potential adverse effects of the drugs. Since toxicity is a major concern with DMARDs, the effects of the drugs must be closely monitored, which can cost as much as the drug itself (Johnson 2011). Therefore, a large amount of effort and research is being put in to find the right combination of DMARDs that will work best on slowing the onset of the disease with reduction of the inconvenience of high costs and close monitoring. The latest research being done on the productivity and effectiveness of single, dual, and triple combination of synthetic DMARDs and biological DMARDs will be discussed in this paper, along with the safety monitoring that is necessary with the use of these drugs.

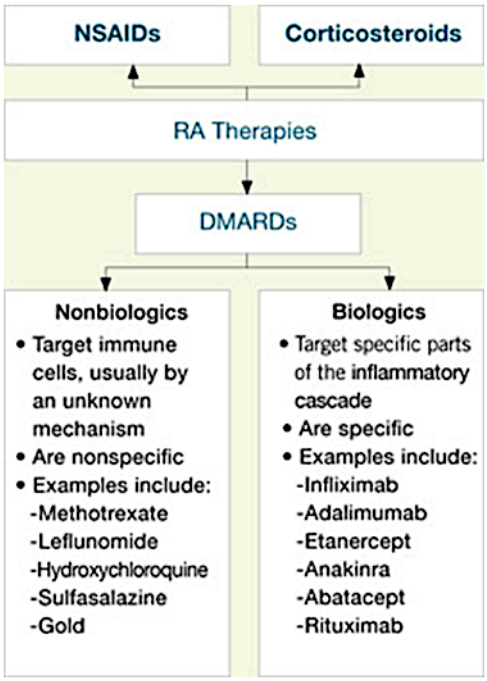

Figure 2: The three major rheumatoid arthritis therapies.
Source: http://stg.jfponline.com/ccp_article.asp?a=1&ref=5610ACCP_Supplement5#5610ACCP_Supplement5-fig7

It is important to understanding the mechanism of action of each individual drug, because DMARDs work by modifying the disease through inhibiting specific parts and pathways of the inflammatory response that occurs in rheumatoid arthritis. Knowing the mechanism also helps researchers decide which combination of drugs might work well together and which ones to experiment with, resulting in the finding of the most productive and effective treatment for the broadest variety of people.

COMMONLY USED SYNTHETIC DMARDS

Methotrexate, an analog of folic acid and of aminopterin, is the most commonly prescribed DMARD against rheumatoid arthritis in the United States and is usually the initial choice when using disease-modifying drugs in rheumatoid arthritis treatment. While its mechanism of action when used at a low dose in rheumatic diseases is unclear, it may relate to the polyglutamates metabolized from the methotrexate that cause extracellular adenosine to be released, which has anti-inflammatory and immunotherapy properties (Imboden et al. 2007). According to two meta-analyses, methotrexate has the best efficacy/toxicity ratio.

The most important action shown in studies of methotrexate against rheumatoid arthritis is its effects in increasing adenosine (anti-inflammatory agent) levels, lowering the pro-inflammatory cytokine levels, and increasing the anti-inflammatory cytokine levels (Swierkot and Szechinski 2006). Intensive treatment and observation while taking methotrexate is recommended in order to attain the most benefit from the drug. While patients tend to remain on methotrexate longer than any

other DMARD because of better clinical responses and less toxicity, a significant number of patients do not achieve premium control over the disease when taking the drug alone. As a result, methotrexate can either be used as a monotherapy or in combination with other synthetic DMARDs or anti-tumor necrosis factor agents, a class of biological DMARDs (Imboden et al.2007).

A study was done in Japan to evaluate the effectiveness of the government recommended 8mg/week dose of methotrexate given to people with rheumatoid arthritis. One hundred seventy-six patients with active rheumatoid arthritis at Konan Kakagowa Hospital and Kobe University Hospital participated in the study. The effects of methotrexate were evaluated by the American College of Rheumatology (ACR) core set, which showed maintained improvements in the clinical signs and symptoms of rheumatoid arthritis for 24 months. However, according to European League Against Rheumatism (EULAR) response criteria, 63.5% of the patients were found nonresponsive at 24 months from the methotrexate therapy. Despite the treatment, x-rays showed the progression of joint destruction. This study is important because it verifies as mentioned before that many patients do not achieve sufficient disease control when using methotrexate as a monotherapy (Hashiramoto et al. 2009). Most combination therapies involve using another DMARD with methotrexate, to enhance the methotrexate clinical response.

Leflunomide is another very important synthetic DMARD that is widely used. Once administered, leflunomide is well absorbed and quickly metabolized in vivo into A771726, which is the active form of the drug. At its molecular level, leflunomide is a pyrimidine synthesis inhibitor that inhibits dihydroorotatedehydrogenase, an enzyme involved in the synthesis of pyrimidines. Unlike other cells during proliferation, lymphocytes increase their pool of pyrimidines much more than their increase in purines, therefore synthesizing them from both salvage and de novo pathways. By inhibiting dihydoorotatedehydrogenase, A771726 prevents the damaging lymphocytes from accumulating enough pyrimidines to support DNA synthesis, which is why leflunomide is considered an immunosuppressive agent.

In addition to being a pyrimidine synthesis inhibitor, current research is being done to investigate A771726's effect on inhibiting the over-expression ofCD147, thereby resulting in the down-regulation of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in active macrophages. CD147 is a member of the immunoglobulin superfamily, which consists of autoantibodies that are linked to autoimmune diseases such as rheumatoid arthritis. Pro-inflammatory cytokines, such as TNF, interleukin 1 and interleukin 17 are released in patients with rheumatoid arthritis and work synergistically to release matrix metalloproteinase-3's (MMP3) from fibroblast-like synoviocytes and macrophages. MMP3's are connected to pathologic tissue destruction, making them a vital interest to the research being done to find a cure for rheumatoid arthritis.

Since CD147 is known to induce several MMP3's and its expression levels have been found elevated in the synovial membranes of rheumatoid arthritis patients, research is being done that focuses on CD147 as a novel target in the treatment of rheumatoid arthritis. A study was done on phorbol myristate acetate differentiated THP-1 cells line, a monocyte-macrophage, to observe the effects of leflunomide's active metabolite on CD147 levels. As MMP3's are the major MMPs secreted by activated inflammatory macrophages and markers of progression of joint damage in

early rheumatoid arthritis, the effects of A771726 on MMP3 gelatinases were also evaluated in this study.

In the macrophage cell model used in the study, an increased mRNA expression of MMP-2 and MMP-9 (both of which can be activated by MMP3) occurred in addition to the up-regulation of CD147, once the cells differentiated. The results found in the study showed that A771726 did not affect the mRNA expression of CD147, but did inhibit CD147 protein expression on the cell surface in a dosage dependent manner, which demonstrates that A771726 only has post-transcriptional effect on CD147 production in THP-1 cells. The authors go on to suggest that future studies should be conducted on the effect of A771726 on the glycosylation of CD147, since abnormal glycosylation was the cause of the instability of the CD147 proteins in their experiment. The study also showed that A771726 inhibited the induced increase of gelatinolytic activity of MMP-2 and MMP-9. The authors conclude by saying that their study indicates that A771726 inhibits the production of CD147 and the gelatinolytic activity of MMP-2 and MMP-9 in THP-1 cells, and they suggest that serum concentration of the metabolite should be monitored in rheumatoid arthritis patients so that sufficient concentration is maintained to allow the patient to achieve remission (Juang et al. 2011).

Leflunomide can also be given as a combo-therapy. The thought of combining leflunomide with methotrexate as a double combination therapy was inspired by the idea that combining methotrexate with an agent whose mechanism of action was different than its own might produce better results than methotrexate monotherapy. In 1999, a study was done on the safety and efficacy of treating active rheumatoid arthritis with a combination treatment of methotrexate and leflunomide. It was a 52-week open-label study in which 30 patients who had active rheumatoid arthritis despite previous methotrexate treatment participated. Adverse effects and clinical response, as judged by the American College of Rheumatology 20% response criteria, were assessed as end point results. Of the patients, 53% met the ACR 20% response criteria and 2 patients met the ACR remission criteria after 1 year. While the study was only done on 30 patients and only a little more than half the patients met the ACR criteria, the study did introduce methotrexate and leflunomide combination therapy as a potential rheumatoid arthritis treatment (Mroczkowski et al. 1999).

In 2004, another study was done on the safety and efficacy of the combination therapy of leflunomide with methotrexate. After a 24-week, randomized, double blind trial of taking leflunomide or a placebo with methotrexate, the patients could enter a 24-week extension to continue the study. Results showed a 48-week maintained response to therapy for those patients who continued to receive leflunomide plus methotrexate. ACR 20% responder rates improved in the patients who switched from taking placebos to leflunomide. Similar ACR 20% response rates were found between patients who switched from placebo to leflunomide without a loading dose to those who received a randomized loading dose of leflunomide. However, fewer adverse events of diarrhea and nausea were found in those who did not receive the extra dose. In addition, patients who switched from placebo to leflunomide in the extension exhibited a lower incidence of elevated transaminases compared to the patients who were initially taking leflunomide throughout the 48-week trial, which may indicate possible hepatotoxicity caused by leflunomide (Kremer et al. 2004). While leflunomide might slow the progression of the disease, patients must discuss with their doctors

required monitoring and possible combination therapies that will yield the best efficacy/toxicity ratio while taking leflunomide.

Besides for leflunomide and methotrexate, there are other synthetic DMARDs used to treat rheumatoid arthritis. Both sulfasalazine and hydroxychloroquine were initially developed for other disease like inflammatory bowel disease and malaria, but they were coincidentally found to be effective in rheumatoid arthritis. They are weak DMARDs, which is why they are usually only used as monotherapy in the early stages of rheumatoid arthritis or used in combination with other DMARDs such as methotrexate. With the use of hydroxychloroquine, ophthalmologic examinations are required every six to twelve months to detect color change or evidence of drug in the retina. Sulfasalazine, the most common used DMARD in Europe, is usually combined with methotrexate, hydroxychloroquine, or both. It is recommended that blood cell counts, especially white blood cell counts, be monitored in the first six months of taking sulfasalazine.

Required monitoring while taking these drugs is evidence of how expensive and time-consuming DMARD treatments can be. This is why such an abundant amount of research is being done to find the most effective and convenient DMARD treatment against the autoimmune disease.

A study was done to compare the efficacy of double or triple combination therapies involving methotrexate, sulfasalazine and hydroxychloroquine in patients with rheumatoid arthritis. Combinations of the different therapies were either methotrexate (MTX) with hydroxychloroquine (HCQ), MTX with sulfasalazine (SSZ), or the triple combination of MTX, HCQ and SSZ. One hundred seventy-one rheumatoid arthritis patients who were not previously treated with the medications were randomized to receive one of the three treatment combinations in this 2-year, double blind, and placebo controlled trial. The end point goal was to find the percentage of patients after 2 years who had a 20% response to their assigned therapy according to the American College of Rheumatology. While all combination treatments were well-tolerated, patients receiving the triple treatment responded best with 78% of them achieving the 20% ACR response required, compared to the 60% percent of those receiving MTX and HCQ and only 49% of those receiving MTX and SSZ (O'Dell et al. 2002).

Commonly used biological DMARDs

Pro-inflammatory cytokines, especially tumor necrosis factor-α and interleukin-1, have vital roles in the pathophysiology of rheumatoid arthritis. This fact led to the development of biological agents that target TNF-α and interleukin-1 cytokines. In addition, recent research has been done that shows promise for therapies that block T-cell co-stimulation and those that target B-cells. Since biological disease modifying anti-rheumatoid drugs have only recently been studied, and possible long-term adverse effects are still unknown, they are usually saved for use in combination therapies with other DMARDs such as methotrexate and leflunomide, for those rheumatoid arthritis patients who did not respond to synthetic DMARD monotherapy.

DMARDs that are anti TNF-α agents include etanercept, infliximab, and adalimumab. Etanercept, a protein genetically engineered from a fusion gene, consists of two soluble TNF p75 receptor functional groups linked to the F_c portion of human

immunoglobulin-1. It binds to TNF-α molecules, thereby preventing the activation of the inflammatory cascade, in addition to inhibiting lymphotoxin-α (O'Dell 2007).

A study was done on the efficacy of etanercept combination therapy with methotrexate, where 89 patients previously treated with methotrexate, who still showed signs of active rheumatoid arthritis symptoms, were randomly assigned to receive either etanercept or placebo subcutaneously, while continuing methotrexate therapy. At 24 weeks ACR response criteria was used to measure clinical response in improvements. Results showed that at 24 weeks 71% of the patients receiving etanercept-MTX combination therapy met the ACR 20% response criteria, compared to 27% of the group receiving placebo plus MTX. Thirty-nine percent of the etanercept group reached ACR 50% response criteria compared to the 3% of the placebo group. Significantly better outcomes, according to all measures of disease activity, were present in the patients receiving the etanercept-MTX combination therapy. Adverse effects associated with etanercept in this trial included only mild injection-site reactions, showing etanercept as a safe and potential combination therapy in patients with active rheumatoid arthritis who didn't respond sufficiently enough to methotrexate therapy alone (Weinblatt et al. 1999).

Infliximab is a 25% mouse and 75% human monoclonal antibody that bind to soluble and membrane bound TNF-α cytokines with high affinity, preventing them from interacting with their receptors, resulting in the down-regulation of macrophage and T cell function.

Adalimumab is a recombinant human anti-TNF antibody. By combining with TNF-α, it prevents its interaction with its p77 and p75 cell surface receptors, resulting in the down-regulation of macrophage and T-cell function, which is similar to infliximab's mechanism of action (Furst et al. 2009). A 24-week, randomized, double-blind, placebo-controlled study was done in 2002 to test the efficacy and safety of adalimumab in combination with MTX given to patients with active rheumatoid arthritis who have not responded adequately to previous MTX mono treatment. The results showed that an ACR 20%, 50% and 70% response were all achieved by a significantly greater proportion of patients in the adalimumab plus MTX administered group than in the groups given placebos with MTX. The greater the dose of adalimumab was given, increasing from 20-mg to 40-mg, to 80-mg, respectively, the higher the response rate appeared. Response seemed rapid, as the greatest proportion of adalimumab-treated patients achieving an ACR 20% response occurred at the first scheduled visit of one week. Adverse events were similar in both control groups, indicating that adalimumab was well tolerated (Weinblatt et al. 2003).

While this clinical trial did demonstrate that adalimumab with combined MTX therapy has been effective in reducing signs and symptoms of active rheumatoid arthritis, and does give hope for potential combination therapy, the study was only held for 24 weeks. While this might be sufficient to show improvements caused by adalimumab in rheumatoid arthritis symptoms, it does not show how effective adalimumab is in the long run at stopping the progression of the disease, or pushing the patient into remission (Kremer et al. 2008).

Abatacept, a recombinant protein, acts by blocking T-cell co-stimulation and preventing the autoimmune response caused by rheumatoid arthritis. A study was done on 652 patients who had active rheumatoid arthritis, despite previously being

treated with methotrexate, to see the efficacy of abatacept. 433 patients were randomly assigned to be given an infusion of a fixed dose of abatacept once a month, while 219 received placebos. Results showed at one year, progression of structural joint damage was statistically slowed by abatacept. Physical function significantly improved in 63.7% of the patients. While these results seem to be very promising for abatacept therapy, the study only involved 1 group of patients over 1 year and therefore is very limited in its evidence of the efficacy of the drug. Longer treatment in different populations is needed to establish its effectiveness against the progression of rheumatoid arthritis (Kremer et al. 2008).

Rituximab is a genetically engineered humanized mouse monoclonal antibody that works against CD20 molecules on the B-cell surfaces, thereby depleting the B-cells, stopping their immune response and thereby reducing inflammation (Furst et al. 2009). The advantage of rituximab is that it works on B-cells rather than inhibiting TNF cytokines, which is a relief to patients who do not benefit from anti-TNF agents, either at the start of treatment or after receiving some treatment. A study was done in Finland to examine the effectiveness of rituximab on rheumatoid arthritis patients who failed to respond to TNF antagonists, or had a contraindication to these drugs. Data was collected from five rheumatology clinics and examined 81 patients in total who were treated with rituximab from April 2005 to June 2008, since previous therapies were unsuccessful in reaching adequate responses. Treatment response was defined according to EULAR response criteria and disease activity score using 28 joint counts (DAS28). The results of the trial showed adequate EULAR response in 77% of the patients and a suppressed DAS28 score of 2.08 units. Since the percentage of good responses of patients taking DMARDs other than methotrexate with rituximab, was somewhat higher than those taking methotrexate alone with rituximab, it's obvious that rituximab is equally effective when combined with methotrexate and other DMARDs. The study concludes that rituximab was effective in controlling disease activity in patients who did not show adequate results taking other DMARDs alone (Valleala et al. 2009).

Tocilizumab is the first of its kind as an anti-interleukin 6 receptor monoclonal antibody. Interleukin 6, a pro-inflammatory cytokine that is released by immune, endothelial and synovial cells, induces osteoclast differentiation, therefore contributing to the joint and bone destruction occurring in rheumatoid arthritis patients. The drug is typically given with or without methotrexate, for patients who did not respond to single or multiple anti-TNF therapies. Similarly to those associated with other monoclonal immune suppressors, adverse effects include infusion reactions, development of neutralizing antibodies, hypersensitivity reactions, and increased risk of serious infection (Murri 2010).

Studies done on rheumatoid arthritis patients who took tocilizumab with methotrexate revealed positive results. One study was done on 499 patients who had an inadequate response to one or more anti-TNF agents. Results after the 24 weeks showed that 50.0% of those who received 8mg of tocilizumab achieved ACR 20% response criteria, compared to the 30.4% in the 4mg group and the 10.1% in the placebo group. At week four, ACR 20% response criteria was reached by more patients receiving 8mg of tocilizumab than those in the control groups, as was DAS28 remission rates achieved at week 24. The most common adverse events reported in the trial were infections, gastrointestinal symptoms, rash and headaches; however, most

were mild and moderate. This study demonstrates the potential benefit of tocilizumab given with methotrexate as an effective therapy against rheumatoid arthritis (Emery et al 2008). Results showed rapid and sustained improvements of rheumatoid arthritis symptoms for those who failed to respond well to TNF antagonists and reported mild adverse effects.

Even though the biological DMARDs mentioned above did show promising trial results when given with methotrexate, biological DMARDs are so fresh and new in research that efficacy and adverse effects are unable to be studied in the long run. Most of these trials are over a 1 to 2 year period, which is not an adequate amount of time to measure achievable long-term remission induced by these therapies. While they definitely show great potential in slowing down the progression of bone destruction and active symptoms caused by rheumatoid arthritis, more research and long-term studies must be done to evaluate the lasting effects and possible negative side effects of these young progressing therapies.

ADVERSE EFFECTS AND DISADVANTAGES

Potential increased risk of serious infection is one of the major side effects of biological DMARDs. TNF inhibitors in particular have been noticed to increase the risk of developing reactivation of dormant tuberculosis. This is why it is important for a patient who is about to start on anti-TNF agents to undergo tuberculin skin testing and even chest radiographs, if needed. A national prospective observational study was done on data collected from the British Society for Rheumatology Biologics Register (BSRBR) to test if different anti-TNF agents increase the risk of tuberculosis reactivity. A comparison of TB rates in 10712 patients who were either treated with etanercept, infliximab, or adalimumab showed three-to four-fold higher TB rates in patients taking infliximab and adalimumab, than those receiving etanercept (Dixon et al. 2010).

Another prospective observational study was done from the BSRBR, where 11,881 patients treated with anti-TNF agents were evaluated to research an increased risk of septic arthritis. While the results did not show that anti-TNF therapy was a significant cause of Septic Arthritis, they did find that it was associated with doubling the risk of developing SA.

Both studies were done on an enormous number of subjects who might have had different contributing factors in developing tuberculosis or septic arthritis (Galloway et al. 2011). While these studies do not positively prove that anti-TNF agents used in rheumatoid arthritis patients increase the risk of infection and tuberculosis, they do show a probable basis for the fact that TNF inhibitors might contribute to these risks. For this reason, physicians and surgeons should be aware of these potentially life-threatening complications, and instruct their patients on how to manage and prevent these adverse outcomes.

The cost of DMARDs and the necessary monitoring required while being treated with these drugs can be extremely expensive and burdensome, especially for elderly patients with severe rheumatoid arthritis. A large amount of monitoring is needed while on DMARD treatment, since complications such as infection and toxicity can occur. Tests such as CBCs and platelet counts are necessary periodically to rule out infection, and yearly ophthalmologic tests are needed for patients on

hydroxychloroquine. All in all, sometimes the excessive expense and adverse effects might prevent patients from benefiting from these new and promising treatments.

CONCLUSION

Rheumatoid arthritis can be a crippling and incapacitating disease, if left untreated. Disease modifying anti-rheumatic drugs work in the unconventional way of modifying the disease and inhibiting the underlying cause of inflammation present in rheumatoid arthritis, in order to reach sustainable remission. While it is a relatively new group of drugs, an abundant amount of research and effort has been put in to find the most suitable and effective treatment when using these agents. From the studies mentioned above, it is obvious that DMARDs has a tremendous potential of becoming the leading treatment in autoimmune inflammatory disease, such as rheumatoid arthritis. According to Tak et al. (2011), the complex and varied mechanism of actions of these drugs make it necessary for researchers to study the different mechanism and contemplate which combinations are effective and safe. What's more, rheumatologists should put effort in predicting clinical responses of individual patients who they prescribe DMARDs to. By doing so, the physician may very possibly maximize the patient's outcome, minimize safety concerns and reduce treatment costs caused by complications (Tak et al. 2011). Even though there is a long way to go, DMARDs are thriving at helping people overcome rheumatoid arthritis, and show great potential in some day reaching the ultimate goal of causing rheumatoid arthritis patients to go into permanent remission, thus becoming the cure for rheumatoid arthritis.

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