

# Factors that Lead to the Immunotherapy Gap in Multiple Sclerosis Testing

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## *Introduction*

Multiple sclerosis is a disease that affects the central nervous system. Most doctors and scientists believe that it is an autoimmune disease. Simply put, the immune system attacks the nerves in a person's body, thereby causing myelin damage, inflammation, and neurodegeneration. The plaque that then builds up on the nerves is scar tissue created when the wounds made by the immune system heal. It is this plaque that inhibits communication between the axons in the body and causes the symptoms of MS, which includes problems with movement, pain, vision problems, trouble swallowing, fatigue, and heat sensitivity (Baker et al., 2011, p. 647).

There are several different types or stages of MS. The most common type of multiple sclerosis is relapsing remitting. In relapsing remitting, the patient has episodes of neurological deficits that then repair themselves. However, the patient may not gain back complete functionality of their facilities following every relapse. Another type of MS is primary progressive multiple sclerosis, which is characterized by a gradual accumulation of disability. There may be times when disability does not accumulate as quickly, but the patient never fully recovers. A third form of MS is secondary progressive multiple sclerosis, which is characterized by having two phases. The relapsing remitting phase occurs first and is followed by phase that resembles primary progressive MS (Steinman and Zamvil, 2005, p. 566).

Scientists replicate these types of MS in model systems for two primary research purposes. First, no therapies may be tested on humans before they are tested on animals first. Second, humans are often bad test subjects for MS research. The effects of multiple sclerosis can best be seen in the spinal cord, and this form of human tissue cannot be examined while the patient is still alive without causing permanent damage. There is the chance of studying the spinal cords of the deceased, but there is often a limited sample size. In addition, a sample from the deceased will most likely only provide information on the later stages of MS. Conversely, many

of the therapies for MS function best at earlier stages. Therefore, spinal cord samples of the deceased would not be of much use. Instead, animal models are used for testing (Baker et al., 2011, p. 647). Although they are more easily accessible, animal models, like human models, have both benefits and pitfalls.

### *The Animal Model for MS*

There are many different types of animal models for MS. However, the most prevalent type used by researchers is the murine (mouse or rat) experimental autoimmune encephalomyelitis (EAE) model. EAE is an induced brain inflammation disease that reflects “a spectrum of neurological disorders in laboratory animals that is used to model multiple sclerosis” (Baker et al., 2011, p. 647). This model can reflect the complexity of multiple sclerosis because the symptoms manifested depend on the strain of mice used for testing (Teixeria, 2005, p. 128). The EAE model was developed by injecting segments of inflamed spinal cord or brain tissue from one species into a different animal species. Symptoms manifested due to the animals’ genetic background and experimental parameters, such as the source and mode of application of the antigen (Mix et al., 2010, p. 387)

Scientists later improved this method through co-injection with Freund’s complete adjuvant (CFA) to boost inflammation effects of various antigens (Mix et al., 2010, p. 387). CFA was also used in combination with pertussis toxin. This allowed scientists to create fluctuating symptoms that could be used to model relapsing-remitting MS. More recently, scientists have replaced the brain proteins used to induce EAE with encephalitogenic peptides, which are short polymers of amino acids capable of producing EAE. It is interesting to note that this method of antigen application has an effect on the type of EAE induced (Mix et al., 2010, p. 387). In addition, the age, sex, antigen employed, dose, species and the commercial source of the animals also influence the type and level of EAE that is induced (Teixeria, 2005, p. 128). The varieties of EAE models that can be produced help scientist study different aspects of MS. For example, some rats are generally prone to developing an acute form of EAE with spontaneous recovery while others show predictable clinical symptoms (Teixeria, 2005, p. 128). This available variation is very important to the testing because it allows scientists to explore how to treat different phases of multiple sclerosis.

The EAE murine model and human MS share many other similarities that make the EAE model an acceptable model for MS. In both cases, genetic susceptibility is strongly linked with major histocompatibility complex class II molecules, which tell white blood cells which molecules are foreign and which belong to the body (Steinman and Zamvil, 2005, p. 568). In addition, they share similar molecular pathways. The “fundamental mechanisms underlying oligodendrocyte development and myelination [when observed] are closely conserved between mouse and human” (Miller and Fyffe-Maricich, 2010, p. 536). One of the many molecular cues that mice and humans share causes the production and proliferation of oligodendrocytes, a type of glial cell in the central nervous system that insulates axons. These cells develop from inductive signals by neural stem cells. In addition, myelination of axons in both humans and mice is done in a very specific sequence (Miller and Fyffe-Maricich, 2010, p. 536). Finally, the disease induction is similar in both EAE and MS, where similar T cells, antibodies, and complements can be found in the lesions of both diseases (Steinman and Zamvil, 2005, p. 568).

#### *Weaknesses of the EAE Murine Model*

This is not to say that the EAE model is without flaws. It is important to note that EAE is not an animal version of multiple sclerosis; rather it is only a model for the human disease. First and foremost, MS is a spontaneously occurring disease with an unknown trigger. On the other hand, EAE is an induced disease with a very specific cause (‘t Hart et al., 2011, p. 120). It is also a short term model, thus experiments are often conducted within a few weeks or months. In contrast, human MS takes much longer to develop (Baker et al., 2011, p. 649-653). For these reasons, the EAE murine model may not accurately correspond with the pathology of MS or its presentation over time.

There are also some inherent differences in the way that MS and EAE operate. One of the biggest challenges is a basic difference in pathology between human MS and rat EAE. Because the symptoms in EAE rats arise mainly due to inflammation and edemas rather than an autoimmune response, demyelination, a major component of MS, is rarely observed in rats (Teixeria, 2005, p. 128). Demyelination is sometimes noted in long term studies of the model (Baker et al., 2011, p. 649-653), but most studies are not of long duration. Additionally, in many cases EAE murine models

are unlikely to develop brain lesions, an important factor used in diagnosing the depth of human MS, due to the fact that brain scans can be done on a living patient. Instead, the spinal cords of rats are used to determine the depth of EAE present as they provide more definitive results. These variations between murine EAE and human MS enlarge the immunotherapy gap.

Although some of the inherent differences in the various EAE murine models do not reflect MS progression, changing the experimental process might lead to more relevant results. One of the important differences rests in the type of T-cells that are active in the lesion areas. T-cells are responsible for engaging macrophages or destroying virally infected cells. By doing this, they are responsible for much of the damage that occurs to the axons in the body. In the lesion areas of the murine model, the predominant type of T-cells found is CD4+. However, in human MS patients, CD8+ T-cells are more abundant (‘t Hart et al., 2011, p.120). This means that, at times, drugs that work in the murine model because they utilize CD4+ T-cells do not work as well in human patients. Glatiramer acetate, which was approved for use by the FDA in 1996, is one of the few drugs that has successfully translated from the EAE murine model to human patients because it utilizes both CD4+ and CD8+ T-cells (Lalive et al., 2011, p. 401-411) as targets. Therefore, it is able to work in both body systems. It is important that researchers acknowledge these differences and subsequently test therapies to ensure that they will, in fact, work with both immune systems, rather than just target a single type of T-cell.

### *The Immunotherapy Gap*

This brings about the question, what is it that causes the immunotherapy gap? There are several parameters including the model’s poor ability to indicate toxicity, the use of CFA in studies, the genetic background of knockout mice, the experimental conditions (environment) and the testing practices (timing, sample size) involved in experimentation with the murine model. However, by implementing certain changes, it is possible to reduce the immunotherapy gap.

### *Toxicity*

At times, the murine EAE model is a poor indicator of a therapy’s toxicity in humans due to a general lack of detailed toxicology testing in EAE

experiments (Steinman and Zamvil, 2005, p. 565-269). Since many experiments with the murine model are done over the span of a few weeks and experiments that are considered long term only last several months, toxic effects that often show up in studies that span years are not accounted for.

Natalizumab is an example where drug toxicity (and risk of infection) was not noticed in the EAE model. The drug was at first approved by the FDA, but later taken off the shelves because it caused progressive multifocal leukoencephalopathy (PML), which may be fatal in some cases. The fact that Natalizumab increases the risk for PML could not have been known using just the EAE murine model, as the virus that causes PML does not affect most mice species (Steinman and Zamvil, 2005, p. 569). One change in experimental design that might help overcome this inadequacy would be to test drugs on multiple animal models, such as monkeys or marmosets, rather than just on the murine model.

On the other hand, there are some drugs whose toxicity might have been predicted using the EAE murine model had experimental procedures been different. Quinoline carboxamide or Linomide is another drug that worked effectively in reducing disease in the EAE murine model. In the beginning, this drug helped MS patients by reducing MR activity in MRI scans, but it was withdrawn due to cardiotoxicity in the human trials. Only after years of testing could other drugs like Linomide be administered to humans without cardiotoxic effects (Steinman and Zamvil, 2005, p. 569). Had these EAE studies been done on more animals over longer periods of time, with detailed toxicology studies done in each trial, the toxic effects may have been noticed before the therapies reached humans.

### *The Blood Brain Barrier*

The blood brain barrier (BBB) is a system within the human body that helps the central nervous system maintain homeostasis (Bennett et al., 2010, p. 180). Many drugs, including drugs targeting MS, must be permeable to the blood brain barrier in order to be effective. As a result, the therapies most affected by a difference in permeability would be those targeting cytokines, which are cell-signaling protein molecules that work by infiltrating the CNS (Mix et al., 2010, p. 395). There is a difference in blood brain barrier permeability between the murine and human models and some scientists feel that the difference in blood brain barrier permeability between these two models causes problems (Zhang et al., 2011,

p. 6). However, Bennett et al. stated that the permeabilities are actually comparable (Bennett et al., 2010, p. 190). In addition, scientists from the University of British Columbia studied the adaptor protein zonula occludens (ZO-1) in the tight junctions of MS patients and EAE models. Tight junctions help control the flow of elements between circulating blood and the brain's fluids in the CNS. They chose to study the ZO-1 protein because it is abnormally disrupted due to varying BBB permeabilities. They found that these proteins reorganized themselves before EAE was induced. Overall, their study showed that junction pathology between MS patients and EAE rats are very similar (Bennett et al., 2010, p. 180-190), which means that the BBB permeabilities are also similar. Nonetheless, it is important to note that the use of certain experimental conditions increase BBB permeability in animals, which influences data interpretation (Teixeira et al., 2005, p. 129).

It is most likely the use of adjuvants that causes the differences (Teixeira et al., 2005, p. 129). In the murine EAE model, certain adjuvants are used to increase the blood brain barrier's permeability, affecting the interpretation of data. If the BBB in the murine model has greater permeability than the BBB in humans, medicines would more easily reach target areas in the murine model. Therefore, when the dose is converted for humans, the therapy dose would be incorrect, or inefficient. Simply giving humans patients a larger dose may not always be an option, as more medicine may mean an increase in detrimental side effects and toxicity.

#### *Complete Freund's Adjuvant*

It may be the use of Freund's adjuvant and not the actual BBB permeability that is the problem. Most experiments dealing with the EAE murine model call for the use of Complete Freund's adjuvant (CFA) to boost the inflammation reaction. This solution is simply an antigen emulsified in mineral oil, which includes inactivated and dried mycobacteria. CFA works by causing an antigen to exist in the body longer as it changes immunological tolerance, which in turn allows EAE to manifest itself in the murine model more clearly. CFA is used in certain murine models that are, at times, more resistant to EAE (Teixeira, 2005, p. 132). CFA is known to affect the induction of EAE in the murine model, thereby changing how the CNS reacts. When testing instances that involve an autoimmune response, this model is farther from the true MS than a murine EAE model

without the use of CFA. This may, for example, affect testing with human MS since it is an autoimmune disease (Tiexeira, 2005, p. 129-130). CFA is also known to increase BBB permeability (Tiexeira, 2005, p. 129). Therefore, CFA may be the cause for the difference in therapy absorption between the murine model and human MS that is seen in some tests.

There are ways to avoid the use of CFA by making some simple changes to the way the experiment is conducted. For example, in mice with a high tolerance against EAE, passive induction can be used to eliminate the need for CFA. In this process, clones of epitope-specific encephalytogenic T cells from immunized animals, which can cause brain inflammation, are used to induce EAE (Tiexeira, 2005, p. 130). By reducing the use of CFA, it is possible to produce comparable BBB permeabilities between EAE and MS thus providing more accurate results in EAE testing.

#### *Knock Out Mice*

Some problems that create the immunotherapy gap stem from the types of mice used for testing. One variety of mice and rats that scientists use for experiments are “knock out” mice. Knock out mice are a variety of genetically modified animals that have had one or more segment of their DNA removed. The pieces of removed DNA often turn off certain functions in the mice, sometimes for their entire lives. Due to this, knock out mice can have abnormal body structures such as “abnormal spleen architecture, blood lymphocytosis, absence of lymph nodes, and functional defects in T cell physiology” (Steinman, 1997, p. 2039). These deficiencies affect their body functions when tests are conducted, convoluting data interpretation when comparing to human MS.

The genetic code has many redundancies. Consequently, when one portion is removed, other pieces take over the function and fill in the gap. This is especially a problem in experiments dealing with cytokines, which are cell-signaling molecules, because cytokines often have diverse functions. Redundancy of TNF- $\alpha$  is another important factor when considering the use of knock out mice. TNF- $\alpha$  is a tumor necrosis factor whose blockade may or may not help MS patients’ symptoms (Steinman and Zamvil, 2005, p. 567). Knock out mice were used to show that TNF- $\alpha$  has a redundant function, meaning that the removal of the loci that creates TNF- $\alpha$  does not change how the mouse functions (Steinman, 1997, p. 2039-2040).. Although this is a very important discovery, scientists are

unable to use knock out mice to study the effects of TNF- $\alpha$  because they have no way to work around the redundancy in the code.

Although knock out mice have their pitfalls, their flaws can be overcome and therefore they may be useful for certain types of testing (Mix et al., 2010, p. 389). Perhaps it is best to use knock out mice to test pathways in MS, rather than using them to test the efficiency of a therapy. By finding pathways through which the disease works, researchers can find other possible ways to treat MS.

### *Timing*

Many of the practices that scientists use when testing with animals cannot possibly be replicated in humans, thereby creating a large gap in results between the two groups. These practices involve measuring the effectiveness of therapies on induction versus progression of the disease. For example, several successful therapies worked best to block induction of the disease when administered to mice before EAE symptoms arose. However, this cannot be done with humans, as MS cannot be diagnosed before symptoms appear. Yet still, approximately forty-eight percent of therapies are administered before EAE induction, twenty-two percent on the day of induction, and thirty percent right after induction in many murine model testing. Only about four percent of studies are started two weeks after induction and less than one percent are started three weeks after induction (Vesterinen et al., 2010, p. 1052). Many of the therapies are tested in the early stages of EAE but are often used in later time periods for MS patients. This detrimentally affects translation (Baker et al., 2011, p. 648-653). In order to better reflect real life circumstances, more tested therapies should be administered in the later stages of EAE.

There are also differences in how the efficiency of a therapy is measured in each instance. For human patients, success is based on relapse frequency and the slowing of disease progression. Yet in the EAE murine models, success is often based on the severity of the initial illness, as drugs are given before EAE is induced. This means that some of the EAE therapies that are a success actually work by blocking the induction of the disease. Experiments conducted after the induction of EAE had lower efficiency rates, meaning they were overall less successful. (Vesterinen et al., 2010, p. 1052). Therefore, only the results of trials started after induction should be employed in a clinical setting. When this is done in combination with

testing later, after EAE induction, results will be more in line with possible results in human MS testing.

### *Environment*

The environments that the mice and rats live in contribute to the immunotherapy gap since the life of a lab animal does not reflect the human condition. Although the EAE model can exhibit the complexity of MS, there is very little genetic variety within a murine population being tested due to inbred strains (Steinman and Zamvil, 2005, p. 565). This poses a problem as EAE shows itself in different manners based on genetics. In addition to a lack of genetic variation within a population, a controlled environment also often means a very clean and sterile environment ('t Hart et al., 2011, p. 120). As a result, the murine EAE models are often poor indicators of both the risk of infection and the toxicity of the therapy in humans. In addition, most lab mice and rats are kept in a very controlled environment. This is done specifically to ward off infection and other contaminants that might affect the end results of an experiment. Consequently, this also means that scientist cannot test if and how a therapy increases the risk of infection for a human with MS who cannot be kept in a sterile environment. For example, PML, which caused Natalizumab to be pulled from the shelves, generally only affects patients whose immune systems are compromised, such as those with AIDS. Since the lab rats are kept in a sterile environment and are healthy except for the induced disease, their risk of contracting infection would not be registered nor would these animals truly have compromised immune systems. This, in combination with other experimental practices, would make side effects such as infection nearly impossible to detect in the murine EAE model.

### *Experimental Design*

In order to decrease the immunotherapy gap, several more changes to the experimental design need to be made in projects that deal with the EAE murine model. Scientists and researchers should use blinding, randomization, conflict of interest statements, power analysis, and ethical review in all EAE testing (Baker et al., 2011, p. 648). Blinding is a procedure in which certain researchers are prevented from knowing which subjects are given which treatment. This prevents subconscious prejudices from affecting the results. Randomization is a method where treatments are

randomly given across a test group, which helps reduce bias. Conflict of interest statements are often already listed and provide information about any factors that could cause undue bias in an experiment. Power analysis is a statistical calculation that informs scientists about the minimum sample size that they will need in an experiment in order to detect the effect of the experiment correctly. Ethical review of experiments is also very important since it depends on ethics guidelines that frequently inform scientists how to administer adjuvants, such as CFA, or how to treat animals. Ethics may also deal with various stressors introduced to animals, which can often affect the outcome of an experiment. All of these processes are required when conducting experiments on humans, but are not currently required when researching solely with animals (Baker et al., 2011, p. 652). Thus experimental design procedures should be reformed to decrease the immunotherapy gap.

Vesterinen et al. (2010) completed a thorough review of experiments done with EAE. The scientists researched the frequency of certain practices in EAE experiments. Of the papers studied, randomization was present in only nine percent, blinding in sixteen percent, power calculation in less than one percent, compliance with animal welfare regulation in thirty-two percent, and potential conflicts were listed in six percent of the publications (Vesterinen et al., 2010, p.1046). However, it is important to note that compliance with animal regulations has been on the rise and that studies that were randomized and blinded had lower rates of efficacy than those that were not (Vesterinen et al., 2010, p. 1052). Specifically, not blinding an experiment can lead to a thirty percent overestimation of efficiency.

Of all the studies Vesterinen et al. looked at, only two of them stated that they had conducted a power analysis calculation in order to determine sample size. Vesterinen et al. (2010) found that most of the test groups in the experiments were at least sixty-three percent smaller than power calculations indicated that they should be. Furthermore, most of the studies had test groups that only had five test subjects and control groups that only had eight subjects.

Some of the most important steps researchers can take to close the immunotherapy gap are “randomization, allocation concealment and the blinded assessment of outcome; ... sample size calculations and...preclinical testing... focus[ing] on testing efficacy under clinically relevant conditions including the initiation of treatment at some time after the induction

of injury, using models which are specifically designed to reflect the complexities of the human disease” (Vesterinen et al., 2010, p. 1054). It may also be pertinent to study the murine EAE subjects for longer periods of time in order to gauge the long term effects of drugs as sometimes potential therapies may only postpone the onset of EAE for a few days (Baker et al, 2011, p. 653).

#### Conclusion

The EAE model is a very useful tool in research for multiple sclerosis. It has produced several drugs that have been of much use to multiple sclerosis patients. It has also provided insightful information on the pathways and chemical reactions at work in MS. In addition, the EAE murine models have often been used to test combinations of drugs in order to search for side effects. For example, the models helped find that Natalizumab, when combined with IFN- $\beta$ , could be fatal (Teixeria, 2005, p. 128).

However, there are some discrepancies between human multiple sclerosis and the murine EAE model, such as the abundance of CD4+ T cells in EAE models and CD8+ T cells in human lesions.

Much of the immunotherapy gap is created by experimental procedure. Whether it is giving murine test subjects a dose that is known to be too high to be replicated or providing them with the therapy before EAE has been induced, research practices with the murine model do not parallel research practices used in human MS patients. Therefore, it is not expected that the results of such experiments will be the same in both the EAE models and MS patients.

In order to shrink the immunotherapy gap, EAE testing and MS testing must become more aligned in practice. Blinding, randomization, power calculations, toxicology reports, conflict of interest statements, proper medicating periods, and ethics reviews must be made standard for murine model testing when dealing with MS testing, just as it is required for human patients. Without these experimental procedures, the efficiency rates will be highly disproportional to the actual truth.

These procedures will also decrease the chance that false positives will reach the clinical stage of testing. In addition, the current inadequacies of testing force money and time to be funneled to experiments that will never go anywhere. Some of the therapies may even be harmful to the health of multiple sclerosis patients. By changing experimental practices, scientists

could reduce the immunotherapy gap that exists between the murine EAE model and human MS.

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