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Animal models for human disease

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Summary

A useful animal model for disease must be similar in its pathology to disease conditions in humans. Experimental animal models of rheumatoid arthritis and multiple sclerosis are useful for a better understanding of disease mechanisms and for evaluating the therapeutic efficacy of new and emerging drugs.

Outline

The significance of animal modeling in biotechnology was described.
Different types of animal model to study the pathogenesis of rheumatoid arthritis were explained.
Various methods for the evaluation of experimental models of multiple sclerosis were discussed.
Ethical topics for using living animals in scientific research were noticed.

What you expect to know

This chapter introduces the subject of animal models both spontaneous and induced models, used in different human disease studies. The benefits of animal models are that one can study the mechanisms of diseases as well as test new and emerging drugs for their therapeutic efficacy. Rheumatoid arthritis (RA) is one of the autoimmune disorders for which different animal models are available, and each and every model has its merits and demerits. We will discuss the importance and induction of RA by collagen. Collagen-induced arthritis in animal models reflects characteristic features of RA patients. Multiple sclerosis (MS) is another debilitating disease that affects the central nervous system (CNS) of humans. The animal model used to study MS is known as Experimental Allergic Encephalomyelitis (EAE). We have given the details of how to induce EAE and also how to apply this animal model. Although various ethical issues are involved with the development and use of animal models to study human disease, the importance of animal models can neither be ignored nor be denied.

Introduction

The architecture of human body is comprised in such a manner that cells cannot be considered as a separate entity. Physiologically, homeostasis is the reason that these components live and perform their functions within that environment. Disruption of this process leads to fatal conditions and is considered a disease. To investigate the mechanism of disease and to find the means to reverse adverse conditions, various strategies are used including cell-based assays and tissue culture studies. Although these models can provide useful information, they fail to address various physiological conditions and the complex interactions among different cell types of tissues and organs. Ideally a useful animal model for any disease has to have pathology similar to the disease conditions in humans. Use of animals in research has a long history that dates

back to the fourth century B.C. In the 1600s, William Harvey used animals to describe the blood circulatory system. Many scientists, such as Louis Pasteur and Emil von Behring, have used animal models for experimental purposes to prove their hypotheses. Animal models are good for understanding disease mechanisms and treatment and for overcoming the limitations of clinical trials that use human subjects. For example, experimental animal models for diseases like rheumatoid arthritis or multiple sclerosis have been successfully employed to screen new bioengineered, chemical, or herbal therapeutics that might have the potential for the treatment of human patients. So far, more than 550,000 studies have been reported in the NCBI database; they use animal models for different diseases. Animal model studies have been the main reason for a better understanding of disease mechanisms. Animal models of disease can be divided into two categories (Kurkó et al., 2013): spontaneous disease models and (van Heemst et al., 2014) induced disease models. In the case of induced disease models, induction can occur by various agents, both chemical and biological. This chapter discusses some of the most important animal models.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disorder with progressive occurrence that preferentially affects peripheral joints. In spite of the fact that RA is severe and crippling and affects large numbers of people, very little knowledge about its etiology and pathogenesis is available in the literature.

Epidemiology and etiology

Rheumatoid arthritis affects about 1% of the population. The ratio of the prevalence of RA in males and females is 1−2.5. RA can occur at any age, but it is mainly reported to affect the 40- to 70-year-old age group. No doubt the incidence has been reported to increase with age. The etiology of RA is unknown, but it has been predicted that genetic and environmental factors play an important role in the onset of RA. Recent advances have identified genetic susceptibility markers both within and outside of the major histocompatibility complex (MHC). Human leukocyte antigen (HLA) genes located on chromosome 6p have been found to have a strong association with rheumatoid arthritis. The contribution of HLA to heritability of RA has been estimated to be 11%−37%. Individuals carrying HLA-DR4 and HLA-DR1 alleles have been shown to have a higher risk of RA. Apart from the

known shared epitope alleles (HLA-DRB1*01, DRB1*04), other HLA alleles, such as HLA-DRB1*13 and DRB1*15, have been linked to RA susceptibility (Kurkó et al., 2013). The HLA class II locus is the most important risk factor for anticitrullinated protein antibodies (ACPA)-positive RA (ACPA+ RA) (van Heemst et al., 2014). A positive correlation has been suggested for the role of HLA in terms of the severity of RA rather than the onset of the disease. The most relevant non-HLA gene single nucleotide polymorphisms (SNPs) associated with RA include PTPN22, IL23R, TRAF1, CTLA4, IRF5, STAT4, CCR6, and PADI4 (Kurkó et al., 2013; Suzuki and Yamamoto, 2015; Stanford and Bottini, 2014). Although the data regarding this conclusion are inconsistent, some of the studies have shown associations between tumor necrosis factor (TNF) alleles and rheumatoid arthritis. Other genes like those for corticotrophin-releasing hormone, interferon (IFN)-γ, and interleukin-10 (IL-10) have also been implied for RA. It can be concluded that the role of genetic components in RA is modest at the best (Viatte et al., 2013).

Epigenetics is another important factor that contributes to RA. In the case of identical twins, RA has not been shown to have 100% concordance; therefore, the role of nongenetic factors has also been implicated in the etiology of RA (Meda et al., 2011). Throughout the world, rheumatoid arthritis is more common in women than in men. This indicates that hormones may play an important role in the development of the disease. Pregnancy has also been considered as a risk factor for rheumatoid arthritis. Studies show that the onset of RA is rare during pregnancy, but the risk increases after delivery. Smoking is associated with increased incidences of RA, especially in men. On the contrary, populations that consume a diet high in omega-3 fatty acids have been reported to be protected from rheumatoid arthritis. From experimental models in animals, a large number of infectious agents such as viruses and bacteria have also been suggested to trigger or contribute to the development of rheumatoid arthritis. However, no relationship between infectious agents and the development of RA has been found.

Pathogenesis

An inflamed synovium is central to the pathophysiology of rheumatoid arthritis. Histologically, RA shows pronounced angiogenesis, cellular hyperplasia, an influx of inflammatory leukocytes, and changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors, and many cytokines. Synovial changes in rheumatoid arthritis vary with disease progression. In the first weeks of the disease,

tissue edema and fibrin deposition are prominent and can manifest clinically as joint swelling and pain. Within a short period, the synovial lining becomes hyperplastic, commonly becoming ten or more cells deep and consisting of type A (macrophage-like) and type B (fibroblast-like) synoviocytes that produce glycosaminoglycans (e.g., hyaluronan, as reported to be present in synovial tissue and synovial fluid). The sublining also undergoes alterations for its cellularity, both in cell type and in cell numbers, with prominent infiltration of mononuclear cells, including T cells, B cells, macrophages, and plasma cells.

The abundance and activation of macrophages at the inflamed synovial membrane correlates significantly with the severity of the disease. Activated macrophages over-express major histocompatibility complex (MHC) class II molecules and produce pro-inflammatory or regulatory cytokines and growth factors [IL-1, IL-6, IL-10, IL-13, IL-15, IL-18, TNF-α, and granulocyte macrophage colony stimulating factor (GM-CSF)], chemokines [IL-8, macrophage inflammatory protein 1 (MIP-1), monocyte chemoattractant protein 1 (MCP-1)], metalloproteinases, and neopterin. These biomolecules are routinely detected in inflamed joints.

Most of the T cells infiltrating the rheumatoid synovium express CD45RO and CD4, which is an indication that the T-cell subset present in the synovium is memory helper T cells. Surprisingly, 10%−15% of the T cells present in the case of the synovium have granzymes A and perforins. This 10%−15% of cells present in the synovium represents cytotoxic T-cell subsets. Therefore, it can be concluded that CD8-expressing cells are infrequent in the synovium. In the synovial fluid of rheumatoid arthritis patients, CD4 and CD8 T cells are equally represented. TCRα/TCRß is expressed on most of the T cells while only a minority of cells show TCRγ/TCRδ expression. It has, however, been found that the expression of TCRγ/TCRδ is increased in the synovium of patients with active RA. Synovial-vessel endothelial cells transform into high endothelial venules early during the course of disease. High endothelial venules are specialized post-capillary venules usually present in secondary lymphoid tissue or inflamed nonlymphoid tissues; these venules facilitate the transit of leukocytes from the bloodstream into tissues.

The cytokine-mediated events have conventionally been viewed in the milieu of the CD4 + Th1/Th2 paradigm. Nowadays, newer cytokines of the IL-17/IL-23 axis and others (IL-27, IL-33, and IL-35) have changed investigations into the immunopathogenesis of arthritis. Both the CD4 + Th17 and $\gamma\delta$-T cells secreted IL-17 which is a chemotactic for neutrophils and its response inhibited by IL-27 due to IFN-γ induction. The roles of other cytokines such as IL-18 and IL-33 in arthritis have been clarified further with inhibitors of them (Veenbergen et al., 2010; Palmer et al., 2009). Recent studies in arthritis models have revealed new aspects toward regulatory T cell (Treg) activity. In the CIA model, treatment with IL-35 induced the regression of arthritis via expansion of regulatory T cells(Kochetkova et al., 2010).

The formation of locally invasive synovial tissue (i.e. pannus) is a characteristic feature of rheumatoid arthritis. Pannus is involved in the erosion of joints in rheumatoid arthritis. Pannus is histologically distinct from other regions of the synovium and shows phases of progression. Initially, there is penetration of cartilage by synovial pannus, which is composed of mononuclear cells and fibroblasts, with a high-level expression of matrix metalloproteinases (MMPs) by synovial lining cells. In later phases of the disease, cellular pannus can be replaced by fibrous pannus comprised of a minimally vascularized layer of pannus cells and collagen overlying cartilage. The tissue derivation of pannus cells has not been fully elucidated, although they are thought to arise from fibroblast-like cells (type-B synoviocytes). *In vitro* work shows that these fibroblast-like synoviocytes have anchorage-independent proliferation and loss of contact inhibition, which a phenotype is usually found in transformed cells. However, the molecular pathogenic mechanisms driving pannus formation still remains poorly understood.

Clinical manifestations

The range of presentations of rheumatoid arthritis is broad, but the disease onset is insidious in most cases, and several months can elapse before a firm diagnosis can be ascertained. The predominant symptoms are pain, stiffness, and swelling of peripheral joints. Although articular symptoms are often dominant, rheumatoid arthritis is a systemic disease. Active rheumatoid arthritis is associated with a number of extra-articular manifestations, including fever, weight loss, malaise, anemia, osteoporosis, and lymphadenopathy.

The clinical course of the disorder is extremely variable, ranging from mild, self-limiting arthritis to rapidly progressive multisystem inflammation with a profound morbidity and mortality. Analyses of clinical course and laboratory and radiological abnormalities have been defined as negative prognostic factors for progressive joint destruction; unfortunately, none of these are reliable enough to allow therapeutic decision-making. Frequent assessment of disease symptoms and responses to therapy is crucial for a successful and long-term management of rheumatoid arthritis. Joint destruction from synovitis can occur rapidly and early in the course of the disorder; radiographic evidence is

present in more than 70% of patients within the initial 2 years. More sensitive techniques such as magnetic resonance imaging (MRI) can identify substantial synovial hypertrophy, bone edema, and early erosive changes as early as 4 months after the onset of disease. These radiographic changes predate misalignment and functional disability by years; by the time physical deformity is evident, substantial irreversible articular damage has commonly occurred. Furthermore, the biopsy analysis of clinically symptomless knee joints in patients with early rheumatoid arthritis shows active synovitis, highlighting the poor correlation between clinical assessment and disease progression, and the rapid development of polyarticular synovitis.

Treatment

The main goal of RA treatment is to stop inflammation, relieve symptoms, prevent joint damage, and reduce long-term complications. The past decade has seen a major transformation in the treatment of rheumatoid arthritis in terms of approach and choice of drugs. The previous therapeutic approach generally involved initial conservative management with nonsteroidal antiinflammatory drugs (NSAIDs) for several years; disease-modifying antirheumatic drugs (DMARDs) were withheld until a clear evidence of erosion was seen. DMARDs were then added individually in slow succession as the disease progressed. This form of treatment has been supplanted by early initiation of DMARDs and combination DMARD therapy in patients with the potential for progressive disease. The idea of early intervention with the conventional disease-modifying antirheumatic drugs (cDMARD) has been validated in several randomized trials. cDMARDs contain medications from different classes of drugs including methotrexate, gold salts, hydroxychloroquine, sulfasalazine, ciclosporin, and azathioprine. DMARDs are often partly effective and poorly tolerated for long-term therapy. In meta-analyses of dropout rates from clinical trials, 20%−40% of patients discontinued the use of DMARDs assessed as monotherapy during the duration of the trial; even in clinical practice, the median duration of DMARD monotherapy was less than 2 years for nonmethotrexate agents. Although there are many reasons for the lack of long-term adherence to treatment, poor efficacy, delayed onset of action, and toxic effects are major limitations. Additionally, DMARDs therapy requires patients to undergo frequent monitoring of blood and physical examinations for toxic effects of treatment protocol. Results from clinical trials showed that DMARD therapy decreased markers of inflammation such as erythrocyte sedimentation rate and swollen joint counts, and that improved symptoms in a selected subset of patients; however, most patients continued to show progression of irreversible joint destruction on radiography. cDMARDs is increasingly burdened by side effects or clinical inefficacy, so other immunosuppressive drugs such as tacrolimus that blocks T-cell activation by specifically inhibiting calcineurin pathway and leflunomide have been developed. A new synthetic DMARD, Iguratimod, which exerts its action by the inhibition of the inflammatory cytokines (TNF-α, interleukin (IL)-1 β, IL-6, IL-8, and IL-17), is recently developed.

The findings illustrate the consequences of progressive disease and have shown the need for the development of new and more effective therapies based on the therapeutic principles used for oncology; it means that treatment protocols for RA patients require the use of several therapeutic agents from different classes to be used in combination. Recent studies have shown that combination therapy of biological DMARDs like TNF-α inhibitors with methotrexate has clear-cut benefits with tolerable toxic effects. Treatment with agents that can block TNF-α function has proved to be highly effective against RA. Further studies reported downregulation of synovial GM-CSF, IL-6, and IL-8, suggesting that TNF-α supports the production of other pro-inflammatory cytokines. However, the mechanisms behind the clinical effect of the TNF-α-blocking treatment are not fully understood. In an animal model, TNF-α-blocking agents such as etanercept (a soluble TNF-α receptor) and infliximab (a monoclonal antibody) reduce the expression of vascular adhesion molecules and inhibit the spontaneous production of IL-1 and IL-6. Patients with a new onset of symptoms and those with diseases of several years' duration and who had failed previous DMARD therapy all benefited. These results suggest that patients in many stages of disease progression can benefit from combination therapy (Chiu et al., 2012). With the approval of TNF-α inhibitors (infliximab, etanercept, adalimumab, certolizumab, and golimumab), non-TNF biologic agents (rituximab, abatacept, tocilizumab, and anakinra), and other biologic agents, determining advances in treatment options of RA were made. Rituximab (chimeric monoclonal antibody targeted against CD 20) is a selective B-cell depleting agent for treating refractory rheumatoid arthritis. Abatacept selectively modulates T-cell co-stimulation and has shown efficacy in several clinical trials. Tocilizumab, a humanized monoclonal antiinterleukin-6 receptor antibody, has proven to be efficacious in patients who did not respond to methotrexate or other synthetic DMARDs.

Recently, several clinical trials have focused on a new class of drug: the Janus kinase (JAK) inhibitors. JAKs are a family of nonreceptor tyrosine kinases

(JAK1, JAK2, JAK3, and TYK2) involved in the intracellular signal transduction of many cytokines. Tofacitinib is a pan-JAK inhibitor that primarily inhibits JAK1 and JAK3. In addition to Tofacitinib, other JAK inhibitor molecules including baricitinib, peficitinib, and decernotinib have also been studied in RCTs. Finally, Filgotinib is a selective JAK1 inhibitor which is currently in clinical development for the treatment of RA (Calabrò et al., 2016).

As mentioned before, pro-inflammatory/regulatory cytokines and growth factors play important roles in the pathogenesis of RA. Therefore, each of them or their pathway represents an attractive therapeutic target for RA. Tocilizumab, a humanized monoclonal antibody targeting IL-6 receptor, has already been approved for the treatment of RA in patients who failed to achieve remission with cDMARDs. Another cytokine that plays an important role in the pathogenesis of RA is IL-17 in which ixekizumab and brodalumab as humanized monoclonal antibody were developed against IL17A and its receptor. A new possible therapeutic target for the treatment of RA is the GM-CSF pathway. The efficacy and safety of Mavrilimumab (an anti-GM-CSF receptor monoclonal antibody) in patients with moderate-to-severe RA has been investigated (Takeuchi et al., 2015). As an increased activation of osteoclasts contributes to bone erosions in RA, the inhibition of RANKL that is essential for the osteoclast activation by denosumab (human monoclonal antibody against RANKL) can reduce joint destruction in RA patients. Takeuchi et al. (2016), finally, do not forget that certain nutritional components interfere in the pathological inflammatory process, so that they should be considered as coadjuvant in the treatment of RA. It has been mentioned that flavonoids reduce cytokine expression and secretion. In this regard, flavonoids may have a therapeutical potential in the treatment of inflammation-related diseases as cytokine modulators (Rosillo et al., 2016; Leyva-López et al., 2016).

Experimental models

In order to study the pathogenesis of RA, one can use different animal models. There are many experimental models that resemble RA in different respects. Since RA is a heterogeneous disease, there is probably a need for different animal models that each reflect a characteristic feature of a particular subgroup of RA patients or illustrate a particular aspect of the disease.

Spontaneous models

Despite the fact that RA is not a spontaneously developing disease, spontaneously developing models for arthritis may be useful to study the role of genetics in the development of the disease. An activated immune response was reported in models such as the human tumor necrosis factor-α transgenic (hTNFtg), interleukin 1receptor-α (IL-1Ra) knockout, IL-6R-activating mutation knockin, or SKG mouse which bears the primary inflammatory response in joints (Keffer et al., 1991). In addition, arthritis can be rapidly induced with an adoptive transfer of T-helper 17 cells in the IL-6R knockin mouse. Transgenic mice expressing a TCR specific for bovine pancreas ribonuclease develop spontaneous arthritis that is mediated by antibodies (Korganow et al., 1999). This model is particularly interesting because it demonstrates that T cells specific for a ubiquitous antigen may induce an organ-specific autoimmune disease. The expression of the gene product causes an upregulation of several cytokines (IL-1, IL-6, TGF-β1, IFN-γ, and IL-2) and subsequent development of arthritis (Iwakura et al., 1995). There are some other spontaneous models for arthritis in nontransgenic mice (Bouvet et al., 1990).

Induced models

Arthritis can be induced by complete Freunds' adjuvant (CFA). Pearson (Pearson, 1956) described this model for the first time. Subsequently, it was demonstrated that other adjuvants, such as IFA, pristane, or squalene, could also induce arthritis (Carlson et al., 2000). Microbially derived products such as lipopolysaccharide (LPS), muramyle dipeptide (MDP), and trehalosedimycolate (TDM) can also induce arthritis when given with mineral oil (Lorentzen, 1999; Kohashi et al., 1980).

Collagen-induced arthritis (CIA) is normally induced by the immunization of susceptible mouse (e.g., DBA/1) or rat (DA, Lewis) strains at the base of the tail. The inoculum used for immunization contains both adjuvant and collagen type II. The adjuvant has to be sufficiently strong to cause tissue destruction as well as induction of a strong pro-inflammatory immune response (Holmdahl and Kvick, 1992; Kleinau et al., 1995). Susceptibility to CIA is dependent on both MHC (class II region) and non-MHC genes (Lorentzen and Klareskog, 1996). Antibodies against collagen II are essential for the development of CIA. This fact has been demonstrated by the passive transfer of anti-CII antibodies, which results in synovitis (Svensson et al., 1998). T cells are also important for CIA development during early stages of disease progression. The dependence of both T- and B-cell responses has also been demonstrated in the same model (Seki et al., 1988).

Pathology of collagen-induced arthritis

In CIA, an immune response is being directed against a joint collagen type II (CII) antigen. Inflamed

joints in CIA are infiltrated by inflammatory cells that accumulate in the synovial membrane and fluid, similar to RA. The most frequent cell type in the synovial fluid is granulocyte. There is also a great infiltration of leukocytes into the synovial membrane. These cells have signs of an activated phenotype of RA since MHC class II molecules are expressed (Klareskog and Johnell, 1988). In addition, there is an intense production of macrophage-derived cytokines in inflamed joints (e.g., TNF-α and IL-1β) (Mussener et al., 1997; Ulfgren et al., 2000). A small number of T cells are encountered, and some of these T cells have IL-2 receptor α chain upregulated. The disease shows a thickened synovial membrane that subsequently forms a pannus on the cartilage surface (Holmdahl et al., 1988; Holmdahl et al., 1991). In both CIA and RA, cartilage and bone destruction occurs mainly at the cartilage—pannus junction. There are some features of the pathology of CIA that differ from what is usually observed in RA (e.g., extra-articular manifestation). Although the compatibility of the CIA model to human RA has been argued, many pathological features of CIA are similar to those of rheumatoid arthritis. Currently, collagen type II-induced arthritis in mice and rats is one of the most widely used arthritis models in academia and industry.

Methodology and protocols

Experimental collagen-induced arthritis was initiated by injecting bovine collagen type II at the base portion of the tail of the animal (Saadat et al., 2005). Male Lewis rats weighing about 160—180 g were used. After the induction of CIA, animals were divided randomly into four or more groups based on the experimental design. At least four different groups were needed, including a control group without arthritis, animals with collagen-induced arthritis, CIA animals with treatment, and CIA animals treated with methotrexate as a positive control.

Sample preparation: bovine collagen type II (CII) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/mL by stirring overnight at 4°C (the dissolved CII can be stored at -70°C if it has to be used at a later time). Before injecting the animals, CII was emulsified with an equal volume of complete Freund's adjuvant (CFA). For the induction of CIA, on Day 1 rats were injected intradermally at the base of the tail with 100 μL of emulsion (containing 100 μg of CII). After 12—16 days, animals showed the development of inflammation at peripheral joints (Fig. 8.1). On Day 21, a booster injection of CII in CFA was administered. This model was used to evaluate the anti-RA effect by giving intraperitoneally injections of test materials
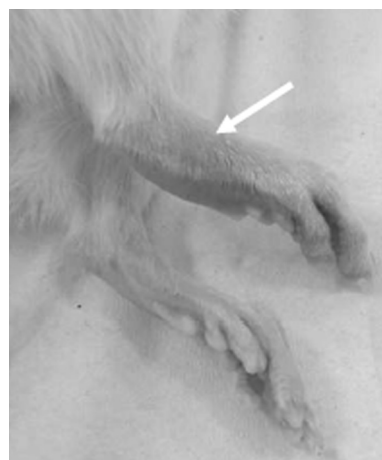

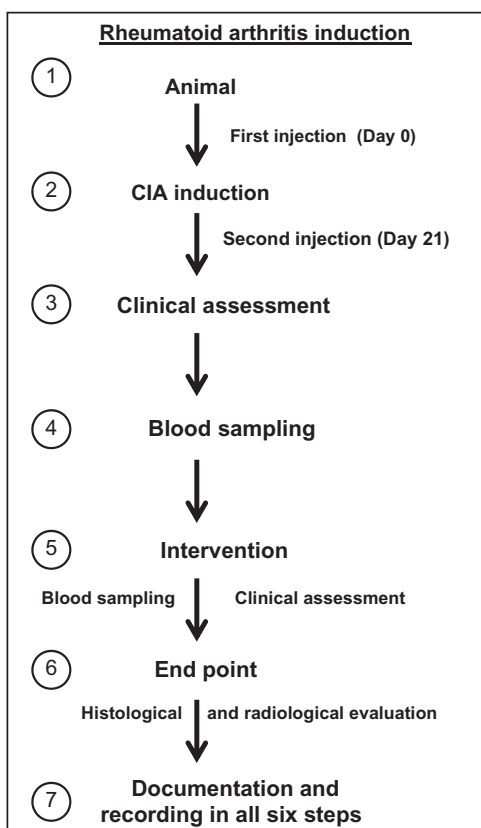FIGURE 8.1   Inflammatory edema (*white arrow*).

(e.g., chemical or herbal extracts). Methotrexate was a control used to evaluate the effect of the test compound and to compare the efficacy of the new compound with methotrexate. In this model, the test compound was given from Day 25, where the frequency, route of administration, and dose could be selected as needed. The end point and days for the evaluation of different parameters were selected; one of the most common points was Day 35 (Flow Chart 8.1). The paws and knees were then removed for the histopathological assay.

Clinical assessment of collagen-induced arthritis

The visual observation can be done by using the macroscopic system as given in Table 8.1. Moreover, rats immunized with CFA should be checked for weight gain from the first to the end of experiment at least every other day. The decline in body weight that followed on the onset of arthritis was proportional to the disease severity and, hence, can be used as a measure of disease activity. The scaling to record the observation should be from 0 to 4 for each paw (Szabó et al., 1998).

Histological assessment

On Day 35, animals were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally) and euthanized. Blood was collected by intracardiac puncture, and paws and knees were removed, trimmed, and fixed in 10% buffered formalin, decalcified, and then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for the histological examination. Joint damage was assessed based on synovial hypertrophy, pannus formation, inflammatory cell infiltration, and cartilage and subchondral bone destruction. Joint erosion was graded on a scale of 0—3 for each limb (Table 8.2), according to the severity of damage (Fig. 8.2).

Rheumatoid arthritis induction

① **Animal**

First injection (Day 0)

② **CIA induction**

Second injection (Day 21)

③ **Clinical assessment**

④ **Blood sampling**

⑤ **Intervention**

Blood sampling   Clinical assessment

⑥ **End point**

Histological and radiological evaluation

⑦ **Documentation and recording in all six steps**

FLOW CHART 8.1 CIA induction for rheumatoid athritis.

TABLE 8.1 Scaling to record the signs of collagen-induced arthritis (CIA).

Scale	Observed symptoms
0	No signs of arthritis
1	Swelling and/or redness of the paw or one digit
2	Involvement of two joints
3	Involvement of > two joints
4	Severe arthritis of the entire paw and digits

TABLE 8.2 Scoring for histological evaluation of collagen-induced arthritis (CIA).

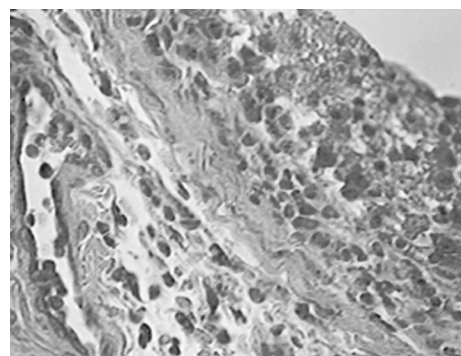| Scale	Histological evaluations
0	No signs of inflammation
1	Infiltration of inflammatory cells in the joints
2	Swelling and tissue edema in the joints
3	Bone erosion in the joints

FIGURE 8.2 Representative histopathological slides of a hind limb joint of a healthy Lewis rat.

TABLE 8.3 Scoring for radiological evaluation of collagen-induced arthritis (CIA).

Scale	Radiological evaluations
0	No alteration of joint
1	Swelling of soft tissue
2	Joint space narrowing
3	New bone formation and bone destruction

Radiographic evaluation

Radiological scoring was performed by an investigator who was blind to the treatment protocol (on Day 35). Radiographical analysis of affected joints in control rats typically showed soft tissue swelling, joint space narrowing, reduced lucency due to demineralization, and areas of recalcification indicative of new bone formation. A score was assigned to each joint on the basis of the information as listed in Table 8.3. Scores were 0−3 per joint (0, normal; 3, maximum joint destruction).

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the central nervous system (CNS) that affects over 2.3 million individuals worldwide. Similar to the affected population in other autoimmune diseases, twice as many women as men have MS. Multiple sclerosis, like many other diseases, has existed as long as human life. In the 1860s, the first report by Dr. Jean-Martin Charcot certified MS as a disease. A patient of him who suffered an unusual symptom died. After dissection, brain lesions were discovered. He called the disease *scleroseen plaques*. Myelin was subsequently discovered, although its exact role was not recognized. About one century of research resulted in the discovery

of MS as an autoimmune disease. Since this finding, extensive studies on MS have revealed some aspects of disease pathogenesis and etiology. Steroids and disease-modifying agents were used. However, this debilitating disease is not completely understood and MS remains an incurable neurological disorder.

Epidemiology and etiology

Multiple sclerosis is the most common inflammatory demyelinating disease of the CNS in Europe and North America. The prevalence of MS in North America and Europe is $\sim 80-100$ per 100,000 people. However, the prevalence is not globally uniform, geographically decreases in latitudes, and has been observed in only $\sim 1-2$ per 100,000 individuals in Africa and Asia. The etiology of MS is unknown. Both genetic involvement and environmental factors have been indicated in MS. The only consistent correlation of involvement of the MHC locus is the MHC class II allele HLA-DR2, which reflects a linkage with MS. In addition to associations within the major histocompatibility complex (MHC) region, other non-MHC loci reached a genome-wide significance. They map to the genes *L3MBTL3*, *MAZ*, *ERG*, and *SHMT1*. Products of the genes *L3MBTL3*, *MAZ*, and *ERG* play important roles in immune cell regulation. *SHMT1* encodes a serine hydroxymethyl transferase catalyzing the transfer of a carbon unit to the folate cycle, which is important for establishment and maintenance of epigenetic signatures (Andlauer et al., 2016). Some other factors like dietary components (e.g., milk), pathogens like human herpes virus 6 (HHV-6), measles virus, Epstein—Barr virus, and chlamydia have been implied as etiological factors. However, the association between any of these agents with MS is debatable.

Pathogenesis

The presence of CNS inflammation is a hallmark of MS. This inflammatory process greatly increases in the CNS by the activation and deregulation of different cell types of the immune system. Activation and entry of myelin-specific lymphocytes into the CNS cause damage to oligodendrocytes, leading to demyelination. Most of the cells from the immune system can contribute toward demyelination, but the main process of demylation is mediated by antibody and complement. So far, it has been noticed that antibody and complement are responsible for lesions in 40%—50% of MS patients.

Myeloid cells may cause axonal damage by releasing molecules, such as glutamate, reactive oxy-gen species, and reactive nitrogen species. Besides, these cells decreased the expression of glutamate clearance. As a result of increased glutamate in the cerebrospinal fluid, MS patients would be vulnerable to degeneration (Yandamuri and Lane, 2016).

In addition, CD8 + T cells play an important role in MS pathogenesis. CD8 + T cells make up the largest percentage of lymphocytes found in the brain of MS patients. All neuroectodermal cells in MS lesions express MHC class I molecules, making them an excellent target for CD8 + T cells. In addition to their pro-inflammatory properties, CD8 + T cells can also suppress the immune system and down-regulate inflammation. However, experiments with perforin, an important regulator of cytotoxic damage to immune cells, have made it clear that CD8 + T cells present at MS lesions cause cytotoxicity, which could be the main source for demyelination and axonal damage (Sinha et al., 2015). Bystander CD4 + T cells do not contribute to the demyelinating process, but once CD4 + T cells move into the CNS and become activated against myelin antigen, these CD4 + T cells could be contributing directly toward the demyelination of CNS (Basdeo et al., 2016). Moreover, CD4 TH17 effector T cells are postulated to play a crucial role in the pathogenesis of MS (Bettelli et al., 2006). In addition to autoreactive immune cells against myelin and nerves, a progressive loss of the structure and function of neurons occurs. It has been reported that the alterations in the expression of miRNAs may play a crucial role in MS pathogenesis (Huang et al., 2016).

After demyelination, remyelination is possible, which could further damage the CNS. The ratio of demyelination to remyelination determines whether a patient will develop secondary progressive MS (SPMS) or relapse remitting MS (RRMS). If remyelination occurs before axonal damage, irreversible physiological damage can be prevented. None of the FDA-approved therapies target oligodendrocytes to stimulate remyelination, but it is a very interesting possibility for future therapeutic intervention (Huang et al., 2016).

Clinical manifestations

The majority of symptoms associated with MS can be directly attributed to inflammation, edema, demyelination, and/or axonal damage within the brain, spinal cord, and optic nerves. Clinical motor manifestations include weakness, stiffness, and/or pain in arms or legs, abnormal reflex activity, and spasticity. Often, the earliest symptoms of MS are somatosensory, including numbness and tingling. In MS, cerebral involvement is often accompanied by symptoms such as ataxia and intention tremor. Many individuals with

MS complain of increased urinary frequency, urgency, and incontinence. Bladder and bowel disturbances remain among the most disabling and embarrassing symptoms experienced by MS patients. Sexual symptoms are also very common among both men and women. Fatigue, sleep disturbances, depression, and deficits in cognitive functioning are also common. The clinical course of MS is often highly variable and is generally characterized by relapses or exacerbations and deterioration of neurologic function, which entitle relapsing remitting MS. The features of relapsing remitting MS are defined as "episodes of acute worsening of neurologic function followed by a variable degree of recovery, with a stable course between attacks." Approximately 80%−85% of patients are initially diagnosed with RRMS that evolves from an isolated demyelinating attack, which is characterized by multifocal inflammation along with varying degrees of axonal injury. A patient may experience disease progression with or without relapses and minor remissions; that clinical condition is defined as secondary progressive MS. It has been seen that 75% of RRMA patients will eventually develop the SPMS state. Primary progressive MS (PPMS) affects ∼10%−15% of MS patients. PPMS is defined as "disease progression from onset, with occasional plateaus and temporary minor improvements in clinical condition." The duration of MS varies significantly among MS patients. Some patients will live with MS for several decades, while about 10% will develop an acute, fulminant form of MS. Patients with an acute and fulminant form of MS show a rapid deterioration in their clinical signs and symptoms that have fatal consequences; these patients usually die within 1−3 years after the onset of disease. In general, the clinical spectrums among MS patients represent a benign disease and a low relapse rate, and these may never develop into secondary progressive disease. The heterogeneity of the clinical course of MS is shown to have a similar variation in its pathology.

Multiple Sclerosis lesions were recently segregated into four distinct subtypes. The general pathology of MS, the formation of demyelinating lesions in the CNS associated with infiltrating CD3+ T cells, activated macrophages, and microglia-containing myelin debris, and infiltrating B cells, is common to all forms of the disease. It is thought that MS lesions are mediated by soluble factors such as TNF-α and immunoglobulin deposition on the myelin sheath, and the local activation of the complement cascade. The diagnostic criteria for clinically definite MS (CDMS) include factors such as clinical history, MRI imaging, and CSF abnormalities. At present, there are no identifiable biomarkers that can predict the clinical subtype of MS. Similarly, there are no factors that can assist in predicting whether a patient diagnosed with MS will develop either a progressive or a benign version of the disease. The clinical and pathological heterogeneity in MS has made it important to either develop or identify reliable biomarkers. Several cytokines, immunoglobulins, MMPs, markers of axonal/neuronal injury, and apoptotic markers have been suggested to have potential as biomarkers, but these biomarkers need validation by rigorous durability trials.

Treatment

In MS, treatment strategies can be either acute or long term. During a relapse, the goal of acute treatment is to reverse neurological disability as well as to delay further neurological dysfunction, so that the normal function can be restored. This type of treatment for MS patients is in contrast to the goals of long-term treatments. The main objective of long-term treatment for MS patients is to decrease relapses (both severity and frequency), which could lend support to stopping the progression of disability. Patients experiencing a relapse, such as optic neuritis or transverse myelitis, are often administered high-dose corticosteroid first-line therapy. During progressive phases of the disease, patients may be prescribed immunosuppressive agents such as cyclophosphamide or mitoxantrone because the progressive phase is often accompanied by worsening inflammatory demyelination and axonal degeneration (Rommer et al., 2019).

In 1993, IFN-β was the first agent to demonstrate the significant clinical efficacy among patients suffering with RRMS. Although the exact disease-modifying effects of IFN-β in MS are unknown, several immunomodulatory mechanisms have been suggested. Presently, two forms of IFN-β, including IFN-β1a (Avonex and Rebif) and IFN-β1b (Betaseron and Extavia), have been prescribed. Glatiramer acetate (Copaxone) is a synthetic mixture of polypeptides that has been approved to treat RRMS. Similar to IFN-β, glatiramer acetate is found to be not effective for progressive forms of MS. Natalizumab (Tysabri) is an alpha-4 integrin antagonist and is the first drug of an entirely new class of immune-directed therapies that has been approved by the FDA to treat relapsing MS. Natalizumab is a humanized recombinant monoclonal antibody that blocks leukocyte migration into the CNS by binding to α-4 integrins; these are components of the very late antigen-4 (VLA-4) complex constitutively expressed on the leukocyte surface. In monotherapy trials, natalizumab has been reported to reduce the risk for sustained progression of disability as well as decrease the frequency of relapses. Based on the current literature, natalizumab appears to be one of the most

effective agents to prevent relapses as well as to stop disease progression. Other monoclonal antibodies administered by intravenous (IV) infusion include lemtrada (alemtuzumab) and novantrone (mitoxantrone).

Currently, numerous other monoclonal antibodies are under investigation as potential therapies for MS; for example, anti-CD25 (daclizumab), anti-CD 20 (rituximab), and so on. A number of other agents are under investigation for possible future use in MS including secukinumab (a humanized monoclonal antibody to IL-17), RTL1000 (inhibitor of the activation of myelin-reactive T cells), firategrast (affect on the VLA-4 system) and Aimspro (neuropeptide stabilizer). Moreover, stem cell-based therapy might be considered as another approach for attenuating MS through regulating the immune system, although several challenges should be resolved. More investigations and clinical trials should be designed to assess the effectiveness of several drugs and approaches that can target both inflammatory and degenerative components of MS. These kinds of approaches may offer hope for individuals who are suffering from this debilitating disease (Mansoor et al., 2019; Agrawal and Yong, 2007; Hart and Bainbridge, 2016).

Experimental models

To gain ideas about MS mechanisms, a number of models have been developed. These experimental models fall into two categories: spontaneous models and induced models. Each model reflects characteristic features of MS patients and has its own merits and demerits.

Spontaneous models

Myelin basic protein mutant (taiep rat), proteolipid protein mutants (Rumpshaker and Jimpy mice), as well as gene-knockout animals (the myelin-associated glycoprotein (MAG) knockout, Thy1-EB3-YFP mice, and Thy1-XFP mice) show dysmyelination, altered neurotransmission and, in some instances, clinical disease. These models have frequently been used to study myelination.

Induced models

With chemically induced lesions, viral and autoimmune models are developed to show some evidence of demyelination, which is considered a pathological hallmark of MS. Direct injection of ethidium bromide or lysolecithin into the CNS produces demyelination. These induced models are usually effectively repaired once macrophages clear the myelin debris. For this reason, these models are rarely used at the present time. Besides, local administration of glutamate or nitric
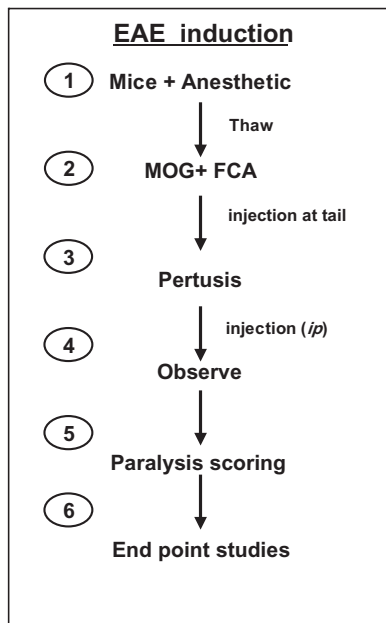
oxide donors induces axonopathy in mice and have also been used to understand mechanisms of axonal degeneration and regeneration (Luchtman et al., 2016). A number of viruses, including Semliki Forest Virus, Theiler's Murine Encephalomyelitis Virus, and a murine coronavirus have been found to induce disease by neurotrophic infection of the CNS, specifically oligodendrocytes (Lane and Hosking, 2010). Moreover, studies using immunodeficient RAG1 − / − mice have indicated that CD4 + and CD8 + T lymphocytes as well as macrophages are key contributors to demyelination in coronavirus-infected mice (Dandekar et al., 2001). Finally, experimental allergic encephalomyelitis (EAE) has received the most attention as a model for MS; this animal model is routinely used for testing different therapeutic strategies. Today, EAE as the most commonly used preclinical murine model of MS induced actively by the injection of defined encephalitogenic myelin protein epitopes plus CFA, or passively by the transfer of encephalitogenic myelin-sensitized T lymphocytes. Some of these EAE models also require the administration of the microbial-based immunologic adjuvant pertussis toxin (PT) (Yandamuri and Lane, 2016). EAE exhibits many clinical and histological features of MS and is caused by autoimmunity induced against antigens that are expressed either naturally or artificially in CNS (Denic et al., 2011).

Methodology and protocol

The method for EAE induction and preparation of antigens to induce EAE in C57BL/6 mice was adapted from the method described by Kafami et al. (2010). It is important for the successful induction of EAE to follow standard precautions for the use of animals. Female C57BL/6 mice that are 4- to 6-week old are used for the induction of EAE. Animals must adhere to the normal laboratory animal maintenance guide.

Protocol

Animals were immunized with the Hooke kits (Hooke labs, EK-0115, Lawrence, MA, USA). It is recommended to follow the manufacturer's instructions. A mesh was dampened in ether and put in a desiccator. The mouse was kept in the desiccator and observed until breathing slowed down to ascertain whether the mouse had been anesthetized. The mouse was removed from the anesthetic chamber and laid on its side. Two syringes were filled with 1 mL of myelin oligodendrocyte glycoprotein (MOG) emulsion with complete Freund's adjuvant. Each animal was given an injection of 200 μL. The needle was gently inserted into the subcutaneous space at the base of the tail, and 200 μL of emulsion was injected into the site. Since it

EAE induction

① Mice + Anesthetic

Thaw

② MOG+ FCA

injection at tail

③ Pertusis

injection (*ip*)

④ Observe

⑤ Paralysis scoring

⑥ End point studies

FLOW CHART 8.2   Important steps for the induction of experimental allergic encephalomyelitis (EAE).

TABLE 8.4   Scoring criteria for paralysis in case of experimental allergic encephalomyelitis (EAE).

Scale	Clinical evaluations
0	No clinical disease
0.5	Partial tail paralysis
1.0	Complete tail paralysis
1.5	Complete tail paralysis and discrete hind limb weakness
2.0	Complete tail paralysis and strong hind limb weakness
2.5	Unilateral hind limb paralysis
3.0	Complete hind limb paralysis
3.5	Hind limb paralysis and forelimb weakness
4.0	Complete paralysis (tetraplegia)
5.0	Moribund or dead

was difficult to give the mouse a 200-μL injection, every mouse was given a 100-μL injection at two different sites on the same day. Immediately, and after 24 hours from the first injection, each mouse was given an intraperitoneal injection of pertussis toxin (100 μL/animal). The animal was observed until complete recovery, and it could move without a floppy gate. This procedure was repeated for all animals. After 2−3 days, the flanks were bulging in response to the subcutaneous injection (Flow Chart 8.2).

Clinical evaluation

One day before immunization, and from the 7th to the 35th day post-immunization, the animals were evaluated on a daily basis for signs of EAE following the 10-point score system (Table 8.4).

Three different clinical parameters were analyzed to compare the course of EAE (Fig. 8.3): (1) Severity of disease as the cumulative disease index (CDI) was the mean of the clinical scores of the animals; (2) disease onset, calculated as the mean of the first-day animals showed the signs of the disease in experimental animals; and (3) peak of disease score, which represented the mean of the highest clinical score of disease for all animals in each group. Tonicity of the tail and the distal part of the tail was ascertained by touching the tip of the tail. If the distal part of the tail was flaccid, the animal was removed from the base and observed to see if its tail remained erect or fell down (examined with the touch of the finger).

After ascertaining tonicity of the tail, the gate of the animal was observed by keeping it in an open area (like a tabletop) and allowing it to walk. After checking the gate, the hind limb was observed by grabbing its tail. After that, the paralysis score was recorded for unilateral paralysis. By holding the animal in the palm of the hand, it was easy to evaluate the type of paralysis (unilateral or bilateral). It was noted whether the mouse rolled spontaneously in its cage or was dead with complete paralysis.

Histology

After 35 days, animals that had an EAE score of 5 and did not change for 3 more days were euthanized by Chloral hydrate injection (0.3 mL, ip). For histopathlogical evaluations, different tissues were harvested after dissecting the animals. Animals were placed appropriately in the dissection tray. A midline incision was made on the abdomen; the diaphragm was opened while ribbons were cut to expose the beating heart. The needle was inserted into the left ventricle of the heart while a phosphate-buffered saline (PBS) tap was allowed to fill the heart for 2 seconds. The right aorta was cut with small scissors to allow the PBS and PFA to circulate to exit. PBS allowed perfusion until the liver turned from red to yellow (∼2−3 minutes). The best sign was when the liquid flowed out of the incised left aorta and turned from red to clear. Another indicator was when PBS entered the pulmonary system and emerged through the nose of the animal. Then, the PBS tap was closed and the tap was turned on for 4% paraformaldehyde (PFA, pH 7.4 at 37°C) to allow PFA to flow and perfuse the circulatory system for 3 minutes. Perfusion was evaluated by involuntary hind limb movement and tail shivering. When the mouse became stiff, it was time to stop PFA perfusion.
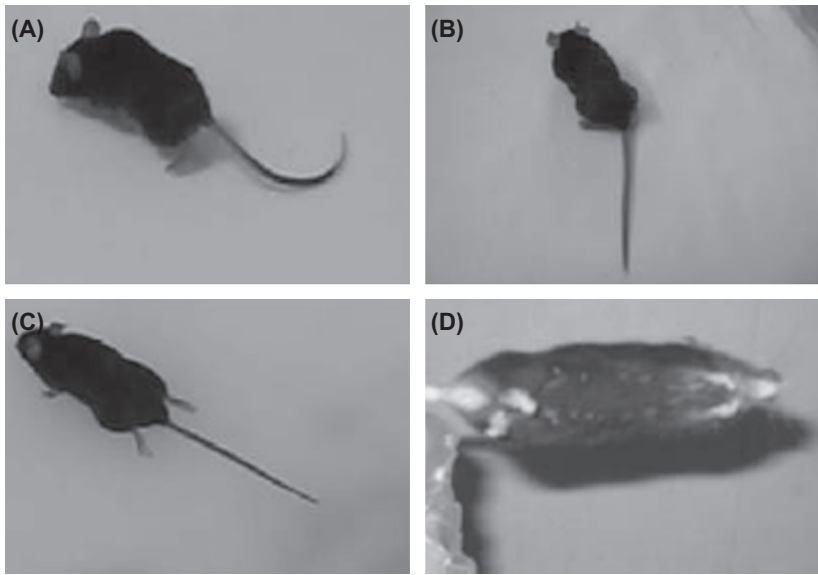
FIGURE 8.3   Clinical signs of EAE: (A) Loop tail; (B) flaccid tail and paralyzed limbs; (C) flaccid tail and paralyzed hind and front limbs; and (D) flaccid tail and weakness of hind limbs.
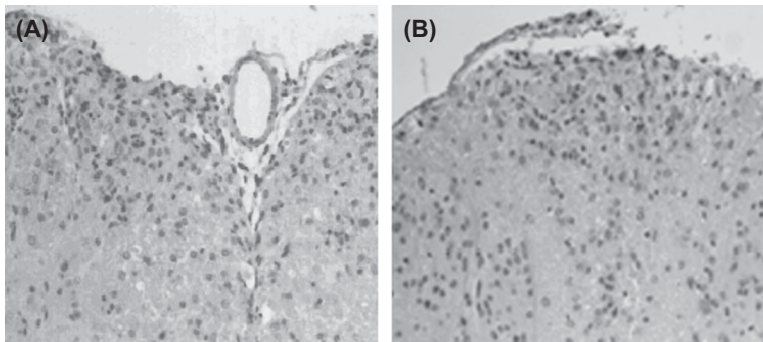

FIGURE 8.4   (A) Immunostaining for CD3 for T cells and (B) immunostaining for APP to assess acute axonal damage.

After the perfusion was complete with PFA, the system was washed with PBS to remove residual PFA. After perfusion, the various tissues of interest were harvested and stored in fresh 4% PFA for 3 days at 4°C. Then, these tissues were washed with PBS and the PFA-fixed tissue could be stored in PBS for a few months. These tissues were then available for sectioning and staining (Fig. 8.4).

Immunohistochemistry

For immunohistochemistry, the three sections showing the highest infiltrations were studied. An area $\geq 1.5 \times 107 \, \mu m^2$ from the brain/spinal cord was selected and analyzed under $200 \times$ magnification to assess the average number of positive cells per millimeter square and to quantify it on a computerized imaging system [BX51 microscope (Olympus, Hamburg, Germany) with AnalySIS software (Special SIS Docu; Soft ImagingSystem)] by planimetry. The inflammatory index had to be calculated as a percentage determined by dividing the number of visual fields with >10 CD3 T cells by the total number of visual fields examined. Detection of amyloid precursor protein (APP) was performed for acute axonal damage.

Enzyme-linked immunosorbent assay

To assess the content of circulating proinflammatory cytokines like IL-6, IL-4, IL-12, IL-10, TNF-α, and IFN-γ, enzyme-linked immunosorbent assay (ELISA) was employed. To evaluate the levels of different cytokines, blood was collected into tubes by a retro-orbital plexus method. The collected blood was kept in the tube to clot. After the clotting of the blood serum, it was separated and stored at $-20°C$. These serum samples were then used for the evaluation of different cytokines using the ELISA.

Real-time polymerase chain reaction

In order to quantify the mRNA of different proinflammatory cytokines such as TNF-α and IFN-γ, anti-inflammatory cytokines like IL-10, myelin-deteriorating matrix metalloproteinase MMP-9, and the content of

myelin basic protein (MBP 3–4), samples from animals had to be analyzed by real-time PCR. Animals were sacrificed with lethal injection and perfused with cold PBS. Then, the limbs and muscles were removed with scissors and the skin removed from these organs. A transverse cut was made at the base of the skull and vertebral column to separate them. The nasal bridge was broken with a small scalpel and the eyeballs removed. Very thin forceps were used under the skull bones to break it into pieces from the frontal to occipital lobes. The bony connection under the cerebellum was broken to expose the cerebellum. The broken bones of the skull needed to be removed. The nerve root connection with the brain was cut. The brain was removed and stored in liquid nitrogen. For the removal of the spinal cord, an oblique cut was made from the lateral side of the spinal cord (started from the cervical part) to the furthest part of the vertebral column (both sides). The spinal cord was then exposed by cutting the boney flap. The steps above were repeated to get to the coda aquina. The spinal cord was taken out by cutting its adhesion to the base. It was then stored in liquid nitrogen.

The frozen tissue sample was used for RNA extraction. First, the sample was homogenized by pushing and rotating it with a sterile glass homogenizer. Next, the homogenate sample was left on the bench top at room temperature (15°C–25°C) for 5 minutes to promote the dissociation of nucleoprotein complexes. Then, 200 μL of chloroform was added to the tube and the tube was shaken vigorously for 15 seconds. The tube containing the homogenate was placed on the bench top at room temperature for 2–3 minutes and then centrifuged again at 15,000 rpm for 15 minutes at 4°C. After centrifugation, the sample separated into three phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The upper, aqueous phase was transferred to a new sterile Eppendorf tube. One volume (usually 600 μL) of 70% ethanol was added to the tube containing the aqueous phase and mixed thoroughly by vortexing. Visible precipitates after the addition of ethanol could then be noticed. Up to 700 μL of the sample was processed for total RNA extraction by using an RNeasy Mini spin column (Roche Germany) according to the kit instructions. After RNA extraction, RNA was quantified spectrophotometrically and the purity of RNA was ascertained by taking out a ration between the OD at 260 and 280 nm. A quantitative real-time reverse transcriptase PCR was performed to analyze the levels of mRNA of different cytokines using cytokine-specific primers. The first step was to perform cDNA synthesis by using a cDNA synthesis kit (TaKaRa, Japan), which was followed by a Syber Green I real-time PCR master mix kit (TaKaRa, Japan).

A house-keeping gene (like the β-actin gene) was included in the study to compare the results.

Ethical issues

The use of laboratory animals in research is of major ethical concern. Much of the argument revolves around moral values. Today, there is a wide spectrum of views on animal rights. This has prompted the establishment of guidelines on the care and use of experimental animal models. The guidelines endorse some essential principles for the care and use of animals for scientific projects. The basis of these principles is to replace animals with other methods such as mathematical models, computer simulations, and in vitro biological systems, thus reducing the number of animals used in order to obtain valid results without unnecessary duplication, and finally, refining projects by selecting appropriate species and techniques to minimize pain or distress to animals using appropriate sedation or anesthesia.

As a researcher, one must always assume that procedures that cause pain to humans will cause pain in such situations in animals. Surgical procedures should be performed on anaesthetized animals. It should be kept in mind that if the animal would suffer severe pain during a procedure, or if at the end point cannot be alleviated swiftly, the animals must be killed humanely.

The transportation, housing, feeding, and handling of animals are also important. Housing facilities should be compatible with the needs of the species and equipped to achieve a high standard of animal care. The place should be designed to facilitate control of environmental factors. Cages should be comfortable and should fulfill behavioral requirements such as free movement and activity, bedding, contact with others of the same species, lighting, temperature, air quality, appropriate day/night cycles, and protection from excessive noise.

The population density of animals within cages should also be considered from an ethical standpoint. This statement refers to the need for the reader to operate in accordance with the guidelines at her/his academy.

Translational significance

The concept of translational research is to try to convert the results derived in animal models into a new understanding of disease mechanisms and therapeutics in human beings. It is a bridge from experimental models to clinical medicine. Over recent years, the

importance of this kind or research has progressively increased. Consequently, translational research is considered a key component to finding practical applications, especially within medicine.

With the improvement of technologies, significant progress has been made in producing various types of engineered experimental animal models based on a better understanding of the molecular and genetic principles of disease. As a result, any interventions in experimental models are more practical and repeatable when compared to patient-oriented research.

Various risk factors that are linked to, or even responsible for, differences in clinical results should also be considered as significant for the development of experimental models; this will enhance the translational value of experimental models. These risk factors can be categorized into genetic factors, acquired factors, and health conditions, which can be studied in models in a controlled manner. In medicine, the performance of successful translational research requires data from hospitals.

Clinical correlations

As we mentioned before, rheumatoid arthritis as a progressive debilitating disease is characterized by hyperplasia of synoviocytes leading to joint destruction and permanent deformity. Although the definite pathophysiology of RA is ambiguous, some evidence suggests that telomerase is also involved in the pathogenesis of this disease. Nobel laureates in physiology/medicine in 2009, Elizabeth Blackburn, Jack Szostak and Carol Greider, have solved a major problem of the chromosomal protection against degradation during cell divisions. They identified telomerase and a unique DNA sequence in the telomeres. Telomerase is a ribonucleoprotein enzyme that adds repeated units of TTAGGG to the ends of chromosomes. This enzyme is composed of an RNA component, called hTERT which serves as a template for addition of telomeric repeats. Although it is now known that the DNA sequence in the telomere attracts proteins that form a protective cap around the fragile ends of the DNA strands, a number of reports have mentioned a link between the increased telomerase activity of human tumor samples and degree of invasiveness. In patients with RA, an impaired telomerase enzyme and premature cellular ageing (senescence) of thymic naïve and memory T cells was reported. Moreover, transfection of rheumatoid arthritis synovial fibroblasts with vectors expressing antisense oligonucleotide against the hTERT component of telomerase enzyme has led to cytolysis of these cells that exhibit high telomerase activity. Taken together, their discoveries have shed light on disease mechanisms and stimulated the development of potential new therapies in experimental models.

There are many methods to evaluate telomerase activity, but we measured it by telomere repeat amplification protocol using TRAPeze telomerase detection kit (Intergen, Inc., USA) in animals treated with *Camellia sinensis* stew. In detailed, biopsies of synovial tissue were obtained aseptically from the knee joints of rat after the induction of CIA. Synovial tissue specimens were rinsed, minced, and digested with 0.2% collagenase in high-glucose DMEM containing 10% FBS and antibiotics. Following overnight incubation at 37°C, cells were collected, plated in culture flask, and allowed to reach confluency at 37°C in a humidified atmosphere of 5% CO2. After the lysis of equal number of cells which harvested from synovial tissue with the CHAPS lysis buffer, the telomerase was first extended for 30 minutes at 30₀C and then amplified by30 cycles of PCR. The products of PCR were detected by polyacrylamide gels and revealed by silver nitrate staining. Telomerase activity was calculated as the ratio of the intensity of telomerase ladders to the intensity of the 36-bp internal standard. In conclusion, we show that *C. sinensis* stew effectively suppresses collagen arthritis and a potent inhibitory effect on telomerase activity. So, natural products should continue to provide innovative lead compounds currently entering clinical trials. Recently, the circular plant peptide kalata B1 (cyclotide) was investigated by Thell et al. using the MS mouse model experimental autoimmune encephalomyelitis. According to their findings, treatment of mice with the cyclotide resulted in a significant delay and diminished symptoms of EAE by oral administration. Taken together, natural product should be considered as a candidate for the future investigations to possible implication for human health.

Conclusion

Using these above-mentioned models associated with other experimental models gives us such an opportunity to accomplish many findings in human medicine. Until now, many progresses in medical sciences have been achieved. The discovery of numerous types of antibiotics for controlling infectious disease and elimination some viral disease like smallpox might be considered as one of researcher and indeed experimental animals honor. Also, blood transfusions, open heart surgery, and other life-saving techniques have all been developed. Nevertheless, there are many unsolved subjects included cancer, aging, Alzheimer's disease, and acquired immunodeficiency syndrome in front of the society. Without no doubt, until to find another means for answering human beings dilemma,

the use of living animals in scientific research would be the best and applicable procedure. With all those valuable function, it is pivotal to consider ethical concerns over the quality of life of animals when you as a young researcher start to write a proposal.

World Wide Web resources

http://www.niams.nih.gov/

National Institutes of Health (NIH) site for the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

http://www.ncbi.nlm.nih.gov/pubmed/

PubMed comprises over 22 million citations for biomedical literature from MEDLINE, life science journals, and online books. PubMed citations and abstracts include the fields of biomedicine and health, and cover portions of the life sciences, behavioral sciences, chemical sciences, and bioengineering. PubMed also provides access to additional relevant web sites and links to other NCBI molecular biology resources.

http://www.nlm.nih.gov/medlineplus/multiple-sclerosis.html

MedlinePlus is the National Institutes of Health (NIH) site for patients and their families and friends. Produced by the National Library of Medicine, it brings you information about diseases, conditions, and wellness issues in easy-to-understand language. MedlinePlus offers reliable, up-to-date health information, anytime, anywhere, for free.

http://www.ebi.ac.uk/ipd/imgt/hla/

The IMGT/HLA Database provides a specialist database for sequences of the human major histocompatibility complex (HLA) and includes the official sequences for the WHO Nomenclature Committee for Factors of the HLA System. The IMGT/HLA Database is part of the international ImMunoGeneTics project.

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Glossary

Adhesion molecule A cell surface molecule (e.g., selectin, integrin, and member of the Ig superfamily) whose function is to promote adhesive interactions with other cells or the extracellular matrix. These molecules play crucial roles in cell migration and cellular activation in innate and adaptive immune responses.

Adjuvant A substance such as complete Freund's adjuvant (CFA) that enhances T- and B-cell activation, mainly by promoting the accumulation and activation of antigen-presenting cells at the site of antigen exposure. Adjuvants stimulate the expression of T-cell-activating co-stimulators and cytokines by antigen-presenting cells and may also prolong the expression of peptide—MHC complexes on the surface of these cells.

Autoimmune disease A disease caused by a breakdown of self-tolerance such that the adaptive immune system responds to self-antigens and mediates cell and tissue damage. Autoimmune diseases can be organ specific (e.g., thyroiditis or diabetes) or systemic (e.g., systemic lupus erythematosus).

CD molecules Cell surface molecules expressed on various cell types in the immune system that are designated by the "cluster of differentiation (CD) number."

Disease-modifying antirheumatic drugs (DMARDs) They contain medications from different classes including methotrexate, gold salts, hydroxychloroquine, sulfasalazine, ciclosporin, and azathioprine. DMARDs were often only partly effective and poorly tolerated in long-term therapy of autoimmune diseases.

Enzyme-linked immunosorbent assay (ELISA) A method of quantifying an antigen immobilized on a solid surface by use of a specific antibody with a covalently coupled enzyme. The amount of antibody that binds the antigen is proportional to the amount of antigen present and is determined by spectrophotometrically measuring the conversion of a clear substrate to a colored product by the coupled enzyme.

Experimental autoimmune encephalomyelitis (EAE) This is an animal model of multiple sclerosis, an autoimmune demyelinating disease of the central nervous system. EAE is induced in rodents by immunization with components of the myelin sheath (e.g., myelin basic protein) of nerves, mixed with an adjuvant. The disease is mediated in large part by cytokine-secreting CD4 + T cells specific for the myelin sheath proteins.

Granulocyte-monocyte colony-stimulating factor (GM-CSF) A cytokine made by activated T cells, macrophages, endothelial cells, and stromal fibroblasts that acts on bone marrow to increase the production of neutrophils and monocytes. GM-CSF is also a macrophage-activating factor and promotes the differentiation of Langerhans cells into mature dendritic cells.

Granuloma A nodule of inflammatory tissue composed of clusters of activated macrophages and T lymphocytes, often associated with necrosis and fibrosis. Granulomatous inflammation is a form of chronic delayed-type hypersensitivity, often in response to persistent microbes or to particulate antigens that are not readily phagocytosed.

Granzyme A serine protease enzyme found in the granules of CTLs and NK cells is released by exocytosis, enters target cells, and proteolytically cleaves and activates caspases and induces target cell apoptosis.

Homeostasis In the adaptive immune system, the maintenance of a constant number and diverse repertoire of lymphocytes, despite the emergence of new lymphocytes and the tremendous expansion of individual clones that may occur during responses to immunogenic antigens. Homeostasis is achieved by several regulated pathways of lymphocyte death and inactivation.

Human leukocyte antigens (HLA) MHC molecules expressed on the surface of human cells. Human MHC molecules were first identified as alloantigens on the surface of white blood cells (leukocytes) that bound serum antibodies from individuals previously exposed to other individuals' cells.

Interferons A subgroup of cytokines originally named for their ability to interfere with viral infections, but that have other important immunomodulatory functions. Type I interferons include interferon-α and interferon-β, whose main functions are antiviral; type II interferon, also called interferon-γ, activates macrophages and various other cell types.

Interleukins Any of a large number of cytokines named with a numerical suffix roughly sequentially in order of discovery or molecular characterization (e.g., interleukin-1 and interleukin-2). Some cytokines were originally named for their biological activities and do not have an interleukin designation.

Lipopolysaccharide (LPS) A component of the cell wall of gram-negative bacteria that is released from dying bacteria and stimulates many innate immune responses, including the secretion of cytokines, induction of microbicidal activities of macrophages, and expression of leukocyte adhesion molecules on endothelium. LPS contains both lipid components and carbohydrate moieties.

Major histocompatibility complex (MHC) molecule A heterodimeric membrane protein encoded in the MHC locus that serves as a peptide display molecule for recognition by T lymphocytes. Two structurally distinct types of MHC molecules exist. Class I MHC molecules are present on most nucleated cells, bind peptides derived from cytosolic proteins, and are recognized by CD8 + T cells. Class II MHC molecules are restricted largely to dendritic cells, macrophages, and B lymphocytes, bind peptides derived from endocytosed proteins, and are recognized by CD4 + T cells.

Matrix metalloproteinase (MMP) MMPs are a family of highly conserved endopeptidases dependent on $Zn2 +$ ions for activity. MMPs can collectively cleave most extracellular matrix. At present, 25 vertebrate MMPs and 22 human homologs have been identified and characterized. MMPs participate in many physiological processes, such as embryonic development, organ morphogenesis, blastocyst implantation, ovulation, nerve growth, cervical dilatation, postpartum uterine involution, mammary development, endometrial cycling, hair follicle cycling, angiogenesis, inflammatory cell function, apoptosis, tooth eruption, bone remodeling, and wound healing.

Myelin oligodendrocyte glycoprotein (MOG) MOG is a CNS-specific type I membrane glycoprotein of the immunoglobulin superfamily expressed mainly on the outermost layer of the myelin sheath, making it an ideal target for antibody-mediated demyelination. It is highly immunogenic, and unlike other myelin proteins used to induce EAE, is unique in inducing both an encephalitogenic T-cell response and a demyelinating response in EAE.

Multiple sclerosis (MS) A chronic inflammatory demyelinating disorder of the central nervous system. The majority of symptoms associated with MS can be directly attributed to inflammation, edema, demyelination, and/or axonal damage within the brain, spinal cord, and optic nerves.

Nitric oxide (NO) A biologic effector molecule with a broad range of activities that in macrophages functions as a potent microbicidal agent to kill ingested organisms.

Pannus Formation of locally invasive synovial tissue is a characteristic feature of rheumatoid arthritis.

Perforin A protein that is homologous to the C9 complement protein and is present in the granules of CTLs and NK cells. When perforin is released from the granules of activated CTLs or NK cells, it promotes the entry of granzymes into the target cell, leading to apoptotic death of the cell.

Rheumatoid arthritis (RA) An autoimmune disease characterized primarily by inflammatory damage to joints and sometimes inflammation of blood vessels, lungs, and other tissues. CD4 + T cells, activated B lymphocytes, and plasma cells are found in the inflamed joint lining (synovium), and numerous pro-inflammatory cytokines, including IL-1 and TNF, are present in the synovial (joint) fluid.

Reverse transcriptase (RT) An enzyme encoded by retroviruses, such as HIV, that synthesizes a DNA copy of the viral genome from the RNA genomic template. Purified reverse transcriptase is

used widely in molecular biology research for purposes of cloning complementary DNAs encoding a gene of interest from messenger RNA.

TH1 cells Subset of CD4 + helper T cells whose principal function is to stimulate phagocyte-mediated defense against infections via secretion of a group of cytokines, including IFN-γ.

TH2 cells Subset of CD4 + helper T cells whose principal functions are to stimulate IgE and eosinophil/mast cell-mediated immune reactions via a particular set of cytokines, including IL-4 and IL-5.

TH17 cells Subset of CD4 + helper T cells that are protective against certain bacterial infections and also mediate pathogenic responses in autoimmune diseases.

Tumor necrosis factor (TNF) A cytokine produced mainly by activated mononuclear phagocytes that stimulates the recruitment of neutrophils to sites of inflammation.

TNF-α blocking agents A group of biological disease-modifying antirheumatic drugs such as Etanercept (a soluble TNF-α receptor) and infliximab (a monoclonal antibody).

Very late antigen (VLA) The set of integrins that shares a common beta-1 chain.

NSAIDs	nonsteroidal antiinflammatory drugs
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PLP	proteolipid protein
PP-MS	primary progressive multiple sclerosis
PR-MS	progressive relapsing multiple sclerosis
RA	rheumatoid arthritis
RNA	ribonucleic acid
RPM	revolutions per minute
RR-MS	relapsing-remitting multiple sclerosis
RT	reverse transcriptase
RT-PCR	real-time polymerase chain reaction
SNPs	single nucleotide polymorphisms
SPMS	secondary progressive MS
TDM	trehalosedimycolate
TNF	tumor necrosis factor
Treg	regulatory T cell
VLA-4	very late antigen-4

Abbreviations

μg	microgram
μL	microliter
μm	micrometer
ACPA + RA	anticitrullinated protein antibodies-positive RA
APP	amyloid precursor protein
CD	cluster of differentiation
CDI	cumulative disease index
cDMARD	conventional disease-modifying antirheumatic drugs
CDMS	clinically definite MS
CFA	complete Freund's adjuvant
CNS	central nervous system
CSF	cerebrospinal fluid
DMARDs	disease-modifying antirheumatic drugs
DNA	deoxy ribonucleic acid
EAE	experimental allergic encephalomyelitis
EDTA	ethylene diamide tetra acetic acid
ELISA	enzyme-linked immune sorbent assay
FDA	food and drug administration
Fig	figure
GM-CSF	granulocyte macrophage colony stimulating factor
H&E	hematoxyline and eosin
HHV-6	human herpes virus 6
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein
MDP	muramyle dipeptide
mg	milligram
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory
ml	milliliter
mm	millimeter
MMP	matrix metalloproteinase
MMPs	matrix metalloproteinases
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
ng	nanogram
NO	nitric oxide

Long-answer questions

1. Describe the significance of animal modeling in biotechnology?
2. How CIA is induced and how the ability of medications is evaluated in mice?
3. Discuss about different types of animal model to study the pathogenesis of rheumatoid arthritis?
4. Why the presence of inflammation in the CNS is considered as a hallmark of Multiple Sclerosis?
5. Explain the various methods for evaluation of experimental models of Multiple sclerosis?

Short answer questions

1. What is the reason of reportedly experiencing different animal models for studying the pathogenesis of Rheumatoid arthritis?
2. Give an example which shows the impact of the epigenetics in the initiation of RA?
3. Which types of evaluation should be performed after "collagen-induced arthritis" aroused?
4. What are the "intervening factors" in experimental model of multiple sclerosis?
5. After the activation of the immune system, which type of lymphocytes enters into central nervous system (CNS)?

Answers to short answer questions

1. There are many experimental models that resemble RA in different respects. Since RA is a heterogeneous disease there is probably a need for different animal models that each reflect a characteristic feature of a particular subgroup of RA patients or illustrate particular aspect of the disease.

2. The epigenetics of RA have also been responsible in the initiation of RA. Since the concordance of rheumatoid arthritis in identical twins is not 100% other nongenetic factors also play a role in the disease etiology.

3. Daily clinical assessment according to a macroscopic scoring system, histological processing and assessment of arthritis damage, radiographic evaluation by an investigator blinded to the treatment protocol on day 35.

4. Age, weight, and possible infectious disease in animals should be considered as the intervening factors.

5. Following activation, myelin-specific lymphocytes enter into the CNS and oligodendrocytes are damaged.

Yes/no type questions

1. The HLA class II locus is the most important risk factor for anticitrullinated protein antibodies (ACPA)-positive RA (ACPA + RA).

2. In the CIA model, treatment with IL-35 induced regression of arthritis via expansion of cytotoxic T cells

3. Tacrolimus induces T-cell activation by specifically inhibiting calcineurin pathway.

4. Adoptive transfer of T-helper 17 cells in the IL-6R knockin mouse induced arthritis.

5. The most widely used arthritis models in academia is CIA model in mice and rats.

6. Among genetic factor, both MHC region and non-MHC loci have been indicated in MS.

7. Increased glutamate in the cerebrospinal fluid of MS patients would protect them from the axonal degeneration.

8. Natalizumab blocks leukocyte migration into the CNS by binding to ICAM.

9. EAE induced actively by injection of the microbial-based immunologic adjuvant pertussis toxin.

10. Housing facilities should be compatible with the needs of the species and equipped to achieve a high standard of animal care.

Answers to yes/no type questions

1. Yes—The most important risk factor for ACPA + RA is the HLA class II locus.

2. No—IL-35 induced regression of arthritis via expansion of Treg cells.

3. No—Tacrolimus blocks T cell activation by specifically inhibiting calcineurin pathway.

4. Yes—Arthritis can be rapidly induced with adoptive transfer of T-helper 17 cells in the IL-6R knockin mouse.

5. Yes—currently, collagen type II-induced arthritis in mice is one of the most widely used arthritis models in academia and industry.

6. Yes—In addition to associations within the MHC region, other non-MHC loci reached genome-wide significance in MS.

7. No—As a result of increased glutamate in the cerebrospinal fluid, MS patients would be vulnerable to degeneration

8. No—Natalizumab blocks leukocyte migration into the CNS by binding to α-4 integrins

9. No—EAE model of MS induced actively by injection of defined encephalitogenic myelin protein epitopes plus CFA.

10. Yes—Housing facilities should be compatible with the needs of the species and equipped to achieve a high standard of animal care.