Multiple Sclerosis, Mad Cow Disease and Acinetobacter

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To my grandchildren: Eliza, Alex, Ruby, Arlo, Thomas, Flora, Madeline and James

This book is dedicated to the memory of the late Dr. Stanley John Pirt (1923–2000), Professor of Microbiology at Queen Elizabeth College and later at King's College. During World War II, he served as a navigator in Bomber Command, and on one occasion, following a dispersal after a thousand bomber raid over Germany, he landed his Lancaster safely in Scotland with five minutes of fuel left in the tanks.

After gaining a degree in chemistry and a Ph.D. in starch structure, he worked with Sir Ernst Chain on the biosynthesis of various penicillins. From 1953 to 1961, he was Principal Scientific Officer at Porton and then moved to Queen Elizabeth College, where he set up a powerful Microbiology Department devoted to a wide variety of research interests from microbial growth dynamics and fermentation to the microbiological aspects of diseases. He strongly supported the establishment of an Immunology Unit within the college which concentrated on the study of immunological and microbiological features of ankylosing

spondylitis, rheumatoid arthritis, Crohn's disease and later the cattle disease bovine spongiform encephalopathy (BSE). These results then provided novel approaches to further investigations into the possible causes of multiple sclerosis. He was strongly supported in his work by his wife Margaret who was also a microbiologist.

He vigorously defended the work from the college and in a letter to The Times (July 25 1997) wrote:

"The fury raised by the challenge to the prion theory by the autoimmune theory of the disease reminds of Machiavelli's dictum: There is nothing more difficult to carry out, nor more dubious of success, nor more dangerous to handle than to initiate a new order of things. For the reformer has enemies in all those who profit by the old order and only lukewarm defenders in all those who would profit by the new."

Acknowledgments

First, I would like to acknowledge the support of Professor Donald Robinson and Professor John Pirt from Queen Elizabeth College and later from King's College who supported the establishment of an Immunology Unit within their departments devoted to research into autoimmune diseases, especially ankylosing spondylitis, rheumatoid arthritis, Crohn's disease and later into bovine spongiform encephalopathy and multiple sclerosis.

They encouraged us academically and financially by providing the unit with Ph.D. SRC and MRC studentships as well as with laboratory space and general financial support.

Second, I would like to acknowledge the skilled laboratory work of many research workers and Ph.D. doctoral fellows, initially from Queen Elizabeth College and later from King's College, who were responsible for the excellent results that eventually saw the light of day in refereed publications. I would like to thank Dr. C. Wilson, Dr. T. Rashid, Dr. H. Tiwana, Dr. P. Cunningham, Dr. Lucy Hughes, Dr. C. Ettelaie, Dr. A. Binder, Dr. S. Bansal and Dr. Sandra Amor.

Third, I would like to acknowledge and thank the Chief Scientist of the Ministry of Agriculture, Fisheries and Food (MAFF) Dr. David W.F. Shannon and the Government for providing funds and biological material to carry out research into the autoimmune theory of "bovine spongiform encephalopathy", or BSE.

In 1997 (CSA 4302=£18,032) and in 1999 (CSA 5115=£216,228), the Government through MAFF and now the Department of Environment, Food and Rural Affairs (DEFRA) authorized and funded two studies into the possibility that BSE could be an autoimmune disease. The combined results of these two studies, involving 157 BSE-affected animals and 229 healthy ones, form the basis of the report submitted to DEFRA and are included in this book.

Additional financial support to carry out this study was obtained from the American Friends of King's College.

I also would like to acknowledge Dr. N. Cox from Winchester who introduced us to Mr. W. Cartmell who provided sera from cattle from an organic farm which had never used feed supplements.

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Dr. John Croker and Dr. Judy Vowles from the Department of Geriatric Medicine at University College Hospital, London, provided 20 sera from patients who had sustained in the preceding 12 months a cerebrovascular accident or stroke, and Professor Gavin Giovannoni carried out a study comparing CSF and serum anti-*Acinetobacter* antibodies.

Next, I would like to thank and acknowledge the help provided by Dr. Kevin J. Towner from the Department of Microbiology and PHLS Laboratory, University Hospital, Queen's Medical Centre, Nottingham, UK, in supplying several different strains of *Acinetobacter* bacteria.

Finally, I would like to thank members of the House of Commons, especially Mr. Tam Dalyell MP, Mr. Peter Ainsworth MP and Sir Nicholas Soames MP, who supported our efforts to obtain funds from DEFRA.

This book would not have been possible without the support of all these people, but the errors of commission as well as the opinions expressed are all my own.

London, UK

Alan Ebringer B.Sc., MD, FRCP, FRACP, FRCPath, Hon FRSPH

Contents

Chapter 1
Multiple Sclerosis as a Scientific Problem

1.1 Multiple Sclerosis: An Introduction

Multiple sclerosis is a neurological demyelinating disease which has numerous clinical manifestations involving the central nervous system.

There are over 10 million people in the world who suffer from this incapacitating disease but the number of affected individuals may be higher if early stages or "formes frustes" are included.

The disease is not only a health problem for the affected individual but is also a social burden to society in the costs involved in caring and treating such patients, as well as the attendant loss of economic activity by the patients.

Multiple sclerosis was first definitely described in 1868, by Jean Martin Charcot from the Pitié-Salpetrière Hospital in Paris who identified sclerotic plaques on post-mortem examination and gave it its modern name of "sclérose en plaques". However there were previous incomplete clinical descriptions by Cruveilhier (1845) and anatomical drawings of such plaques, especially by Robert Carswell (1838) Professor of Anatomical Pathology at University College Hospital in London.

The origin of this disease is unknown but extensive research studies have been carried out over the last 100 years to try and characterise the onset and cause of this disabling condition.

It is thought that the main pathological factor is the destruction by the immune system of myelin, the covering of neurones and myelin producing cells, the oligodendrocytes.

External agents that have been suggested as setting off this disease are Epstein-Barr virus infection, but other possible triggering conditions could be measles, mumps or rubella. These viral infections occasionally give rise to acute encephalomyelitis which in some features resemble multiple sclerosis.

Multiple sclerosis is usually diagnosed on the presenting symptoms and signs, together with supporting medical tests involving both examination of the cerebrospinal fluid for oligoclonal bands and radiological investigations.

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The multiple sclerosis plaques commonly affect the white matter of the optic nerve, brain stem, basal ganglia, spinal cord and areas near the lateral ventricles. There are widespread patches of demyelination followed by gliosis.

The study of this disease has had a close relationship with attempts at treating patients who had been bitten by rabid dogs or wolves. In the 1890s Pasteur and colleagues in Paris carried out attempts at immunising patients with rabbit brain homogenates which contained the rabies virus. Although the treatment was in the majority of cases successful, in some patients it led to the development of unusual neurological complications which in some cases had a fatal outcome.

It was not until the 1930s that it was discovered immunising animals with saline brain homogenates led to a condition labelled "experimental allergic encephalomyelitis" (EAE) which was a demyelinating condition resembling multiple sclerosis. This experimental condition came to be considered as an animal model of multiple sclerosis.

It is the theme of this book that the link between rabies, attempts to treat rabies patients with brain homogenates and the discovery of "experimental allergic encephalomyelitis" provides new ways of looking at the demyelinating disease multiple sclerosis.

1.2 Clinical Features of Multiple Sclerosis

The onset of the disease is often sudden but may be insidious and the patient may complain of transient muscular pains, stiffness, tiredness, malaise and fatigue with gait incoordination, slurred speech and eye problems.

The acute lesion causes temporary functional interruption but with the neuronal fibres not being permanently damaged, so the early symptoms tend to improve. The initial clinical feature develops fairly suddenly and disappears after a few days or weeks.

In young people, optic neuritis, involving blurring or temporary loss of vision is probably the commonest symptom, whilst in elderly subjects, weakness of one or both lower limbs is usually the presenting symptom.

In general, the lower limbs are three to four times more frequently affected than the upper limbs (Brain 1962).

Weakness, heaviness and stiffness of one or both lower limbs is due to corticospinal tract demyelination and may develop fairly suddenly. Often other symptoms are incoordination of arms with intention tremor, ataxia of gait and dysarthria presenting as slurred speech. Occasional unsteadiness of arm or leg may be due to impaired proprioception. Transient disturbances of bladder functions such as urgency, precipitancy or hesitancy of micturition, with impotence in men is occasionally reported by patients.

Nystagmus is present in at least 70 % of cases. It is usually absent on central fixation and appears on conjugate deviation both laterally and vertically.

Corticospinal tract signs such as extensor plantar responses, muscle weakness and increase in flexor tone are common.

Vibration sense is frequently diminished and impairment of the sense of passive movement may also be found.

Analgesia or loss of sensation of light touch may be found but rarely persists.

In many cases early manifestations are followed by remissions. However relapses are a striking feature of this disorder and are not predictable, occurring without warning (Compston and Coles 2002).

The pathological lesion is a circumscribed patch of nervous tissue in which destruction of myelin occurs first, followed eventually by damage to the neurofilaments of the axis cylinders.

The most important method in making a diagnosis is a careful history and examination of the patient. In general, multiple sclerosis is defined as two or more neurological episodes or attacks separated in time and space. The diagnosis can be helped by examination of the cerebrospinal fluid and radiological examination of the central nervous system.

The McDonald criteria using clinical, laboratory and radiological evidence of lesions at different times and in separate areas, provide a comprehensive framework to arrive at a diagnosis (McDonald et al. 2001). Revisions of these criteria have been published emphasizing that extra information can be gleaned from radiological examinations.

Several patterns of progression of the disease have been described:

(a) relapsing-remitting,
(b) secondary progressive,
(c) primary progressive and
(d) progressive relapsing.

The primary-progressive is commoner in people in their 50s (Miller and Leary 2007). The most common length of time between disease onset and conversion from relapsing-remitting course to secondary progressive multiple sclerosis is 19 years (Rovaris et al. 2006).

The term “malignant multiple sclerosis” is sometimes used to describe multiple sclerosis patients who reach a significant level of disability in a short period of time.

1.3 Laboratory and Radiological Features

Abnormalities of the cerebrospinal fluid are found in well over half of multiple sclerosis patients. There is a mild lymphocytosis and an increased proportion of gamma globulin. On electrophoresis oligoclonal bands of IgG are found in 70–80 % of multiple sclerosis patients.

Elevated levels of autoantibodies to myelin are also found in the cerebrospinal fluid as well as in blood.

The blood-brain barrier of the capillary system prevents the entry of large molecules such as IgM or cells circulating in the vascular system to penetrate the central nervous system. However during inflammatory episodes, the blood-brain barrier may become permeable to cells or large molecules.

Gadolinium cannot cross a normal blood-brain barrier. However gadolinium enhanced magnetic resonance imaging (MRI) can show lesions and areas of increased blood-brain barrier permeability .

1.4 The Therapy of Multiple Sclerosis

The treatment of multiple sclerosis involves many parameters. One of the main aims is to reduce the frequency of neurological relapses and to prevent permanent disabilities. Some workers have also emphasized that reduction in disease progression is an important aim in the management of this condition (Compston and Coles 2002).

The therapy of multiple sclerosis involves not only the use of steroids, glatiramer acetate but also newer biological preparations which place an exorbitant financial strain on health providers and society in general. Many of these drugs have undesirable side-effects.

The general principle in such a therapy is to reduce the intensity of the inflammation once it has started in a patient.

Inflammation is the body's response to injury. The question arises "What has been responsible for the tissue injury?", in other words what is the primary cause which sets off multiple sclerosis.

A possible way as to how to answer such a question may be to look for previous successful solutions in finding the cause of a disease and no better example is provided than by rheumatic fever.

1.5 Molecular Mimicry and Rheumatic Fever

The prototype of an autoimmune disease evoked by an external agent and operating through the mechanism of "molecular mimicry" is rheumatic fever.

It usually occurs some 2–4 weeks after an upper respiratory tract infection by Lancefield group A *streptococci.* Some *streptococci* have been found to have antigens which crossreact with cardiac myosin and others resemble some molecular sequences found in the basal ganglia of the brain. When someone develops tonsillitis by this microbe the resultant antibodies will not only attack the *streptococcal* bacteria but also the heart and the brain. Thus anti-*streptococcal* antibodies produce rheumatic fever and Sydenham's chorea by acting as cytopathic autoantibodies. It would appear that rheumatic fever and Sydenham's chorea are autoimmune diseases caused by an infection.

Other diseases operate by a similar mechanism. Some 20–30 million individuals in South America, especially Brazil, are infected by the protozoan parasite *Trypanosoma cruzi.* Patients with Chaga's disease have antibodies which react with both antigens present on the surface of the parasite as well as with cardiac endothelium and myocardium giving rise to a myocarditis which pathologically resembles rheumatic fever. Thus it would appear that even parasites can be triggers or causative agents of an autoimmune disease (Ebringer et al. 2003).

Molecular mimicry has been demonstrated to operate in both ankylosing spondylitis and rheumatoid arthritis.

It is not inconceivable that a similar mechanism may operate in multiple sclerosis.

1.6 The Properties of the Multiple Sclerosis Problem

A "scientific problem" involves defining the properties of the puzzle which interests the scientist and in tackling the relevant question. It is these properties that provide possible answers for the scientific enquiry.

The philosopher of science Karl Popper has always emphasised that in trying to solve a scientific problem one must generate hypotheses which can then be tested experimentally. For such hypotheses to be labelled as scientific they must prohibit certain results. If such prohibited results are obtained then the theory is found to be invalid or has failed in explaining the "scientific problem".

We then must produce new hypotheses to tackle the problem under investigation. So scientific research proceeds by a succession of conjectures or guesses and refutations.

The properties of the multiple sclerosis problem would appear to be the following:

1. **Sex ratio:** Multiple sclerosis is found 2–3 times more frequently in women than men.
2. **Early age of onset:** The age of onset in multiple sclerosis is between the ages of 20 and 30 years. In some patients it starts even in their teens. This clearly distinguishes this autoimmune disease from rheumatoid arthritis where the age of onset is in the 40s and 50s although again occurring more frequently in women.
3. **Family studies:** It has been known for a long time that there is a familial aggregation of multiple sclerosis and this suggests somehow that there is a genetic link associated with the development of the disease.

   The probability of getting multiple sclerosis is higher in relatives of an affected person. If both parents are affected, the risk in their children is almost ten times compared to that found in the general population (Milo and Kahana 2010).
4. **Genetic links:** The most consistent finding is an association between multiple sclerosis and HLA-DR15 and HLA-DQ6. Overall it has been estimated that

HLA accounts for about 30 % of the genetic predisposition in this disease (Baranzini 2011).

5. **Geography, latitude and sunlight:** There is a striking North-to-South prevalence of the disease. It is commonly found in the northern countries of Europe. It is found frequently in Norway, Sweden and Finland compared to Italy or Spain. Multiple sclerosis is commoner in Scotland compared to England. This latitude gradient is also found in the USA (Kurtzke 1993).

   It appears that multiple sclerosis is associated with the latitude, in that it is commoner the further one goes from the equator.

   In the southern hemisphere, the reverse occurs, multiple sclerosis is seven times commoner in the southern island of New Zealand and Tasmania, compared to Queensland in populations having the same ethnic origin.

   This has led to the suggestion that the relative lack of sunlight may reduce the level of vitamin D.

   The lack of this vitamin may affect the functioning of the immune system. However in other autoimmune diseases, such as rheumatoid arthritis or ankylosing spondylitis, the possible lack of vitamin D has so far not been noticed.
6. **Upper respiratory tract infections and sinusitis:** Several studies over the last 30 years have indicated that there is an association between upper respiratory tract infections including sinusitis and the presence of multiple sclerosis. Whether this indicates some viral or bacterial trigger factor involved in this disease is not clear but requires further consideration.

   It is proposed to use these properties of the multiple sclerosis problem to investigate the possible cause of this disease by research workers associated with the King's College Immunology Unit in London.

1.7 King's College Immunology Unit[1]

The Women's Department of King's College London opened in 1885 and in 1915 moved to Campden Hill road, Kensington.

In 1953, it received a Royal Charter and was named Queen Elizabeth College after the Queen Mother.

The college distinguished itself in teaching and research in microbiology, biochemistry, physiology and nutrition. In 1972 an Immunology Unit was set up within the departments of biochemistry and microbiology with an interest in research into genetic and environmental factors in rheumatic diseases, especially ankylosing spondylitis and rheumatoid arthritis and later in Crohn's disease, as well as bovine spongiform encephalopathy and multiple sclerosis.

[1] This section appeared in an abbreviated form in *Rheumatoid Arthritis and Proteus*. Alan Ebringer. Springer, London 2012.

Many students and doctoral candidates passed through the Unit and two stayed for over 12 years, Dr. Clyde Wilson Ph.D, FRCPath and Dr. Taha Rashid MBChB, M.Phil.

In 1985 Queen Elizabeth College remerged with King's College and moved to the Waterloo Campus in Stamford street on the South Bank.

It is the aim of this book to try to answer some of the questions posed by the properties of the scientific problem and to try to examine whether current difficulties involving cattle diseases may be associated with "experimental allergic encephalomyelitis" and thereby suggest some novel approaches to the study of this disease.

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Chapter 2
History of the Attempts to Find the Origin of Multiple Sclerosis

2.1 Introduction

Multiple sclerosis would appear to be a neurological disease which has been described in various patients throughout the ages. The cause of the disease is unknown and has stimulated numerous studies over the last 150 years.

A comprehensive study of the history of multiple sclerosis and of the various eminent contributors trying to elucidate this disabling neurological disorder has appeared in the literature (Murray 2005).

2.2 Multiple Sclerosis Before Charcot

The oldest report of a patient with possible multiple sclerosis is Saint Lidwina from Schiedam in the Netherlands. She was born in 1380 and in 1396, following a fall whilst skating, Lidwina developed headaches, walking difficulties and a muscular weakness of her face with a drooping lip. She continued to deteriorate, although at times she was free of any symptoms.

She was canonised in 1890 and has become the patron saint of skaters.

Another possible sufferer from multiple sclerosis was Augustus d'Este, a grandson of George III. His symptoms consisted of blurred vision which cleared up and then relapsed over the course of his lifetime.

He later developed weakness in his legs, numbness, episodes of double vision and bladder and bowel problems. The remissions and relapses were described in his diaries. In his last year he had persistent tremors, with spasms and was confined to bed.

The diagnosis of multiple sclerosis was made after his death by neurologists reading his diaries.

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2.3 Carswell in London

Robert Carswell (1793–1857) was a Professor of Pathological Anatomy at University College Hospital. He frequently carried out post-mortem examinations which were then published in 1838 in his "Atlas of Pathology". During a post-mortem examination in 1830, he found strange lesions in the spinal cord which were later considered to resemble sclerotic plaques. However Carswell did not describe the ante-mortem clinical features of the patient.

2.4 Cruveilhier in Paris

Jean Cruveilhier (1791–1874) was Professor of Pathological Anatomy in the University of Paris. In 1841, Cruveilhier described a woman who had neurological symptoms for 6 years. Her symptoms consisted of abnormal sensations in her lower limbs. Later on she developed trembling of arms and hands. Soon afterwards a trembling of the arms and clumsiness of the hands occurred. Later she could not use her upper limbs and often grasped objects would slip from her hands. She was handicapped by frequently stumbling, dragging her feet with occasional collapsing of knees and had to give up her job as a chambermaid. During the last weeks of her life she lost all sensory feelings in both her legs. The patient died aged 38 years. No post-mortem examination was carried out on the subject. Many of her symptoms were subsequently recognised to occur in other patients with the disease which eventually became known as "multiple sclerosis".

2.5 Charcot at the Salpetrière

Jean Martin CHARCOT (1825–1893) was Professor of Neurology in the University of Paris. He is also known as the "Father of neurology".

In 1868, Charcot examined a young woman at the Salpetrière Hospital who had a tremor that he had never seen before. He noted that she had three characteristic symptoms: intention tremor, slurred speech and abnormal eye movements or nystagmus. These three characteristic symptoms later became known as "Charcot's triad", but it occurs in only 20 % of multiple sclerosis patients.

He also noted that the patient had "cognitive changes" in that she had a weak memory and could not express her ideas clearly.

When she died, he carried out a post-mortem examination and found that her brain contained characteristic "plaques" of sclerosed tissue and labelled the disease as "sclérose en plaques", the name it has nowadays.

Although Charcot gave a complete description of the disease, he could not work out the cause of this condition and found that strychnine, gold or silver injections as well as electrical stimulation were of no help in treating such patients.

Charcot diagnosed several other patients in his hospital having a similar disease pattern. In total, he studied some 30 patients with multiple sclerosis and described most of the clinical features of the disease. He clearly can be credited in having discovered or identified a new neurological disease. For this alone he deserves to be called the "Father of Neurology".

Charcot had a bitter dispute over the causes of "hysteria" with his pupil Sigmund Freud. Charcot thought that there was a neurological explanation for this condition whilst Freud felt that it was a psychiatric problem which could be resolved by psycho-analysis.

2.6 Multiple Sclerosis in Other Countries

Multiple sclerosis was first recognised in the U.K. by Dr. Walter Moxon in 1873 and in the USA by Dr. Edward Seguin in 1878.

Wilhelm Uhthoff (1853–1927), a German ophthalmologist described optic neuritis patients who had visual problems which were made worse following physical exercise.

The Swiss pathologist Georg Eduard Rindfleisch (1836–1908) noted that the inflammation associated lesions were located around blood vessels.

In Sweden, a cluster of multiple sclerosis cases suggested that the condition arose some two centuries ago and acquired the name of the "Fenno-scandian focus" (Kurtzke 1968).

One of the problems in assessing multiple sclerosis in other countries is that there are in the community patients who have minor symptoms but have not been seen or diagnosed by a neurologist. There is thus an underestimate in the number of patients with the disease in the population which complicates attempts at studying the aetiology of this condition.

For instance, in a study from Catalonia in Spain, the whole population of a small town with 72,000 inhabitants was examined over a period 5 years and many of the subjects had not been seen previously by a neurologist. The prevalence rate of multiple sclerosis was found to be 58/100,000. This is 5–10 times higher than had previously been reported from Catalonia (Sempere et al 1995).

Since many subjects suffer from viral and bacterial infections in the upper respiratory tract, the presence of such common ailments would not be included in studying neurological problems.

2.7 Microscopy of Multiple Sclerosis Lesions in Edinburgh

Dr. James Dawson (1870–1927) of the University of Edinburgh, performed careful and detailed microscopic examinations of the brains of multiple sclerosis patients. He described that the inflammation was located around blood vessels, thereby indicating that the agent or toxin came from an extra-thecal space from the blood in the

vascular system and therefore was probably coming from an infectious source outside central nervous system.

Dr. Dawson further pointed out that the damage was primarily affecting the myelin sheaths of neurones and oligodendrocytes, the cells making myelin (Dawson 1916).

2.8 Clues from Therapy

Following the microbial discoveries of Louis Pasteur and Robert Koch the possibility of infection by microbes crept into many diseases. The clear implication being that if one could identify such external agents, the severe complications of the disease could be mitigated by immunization, as occurred in the case of rabies, smallpox and polio or eliminate the microbial agent by the use of antibiotics.

Since the cause of multiple sclerosis is not known, the main principle of treatment is to reduce the level of inflammation by steroids, azathioprine, interferons, glatiramer acetate, mitoxanthrone, intravenous immunoglobulins and a variety of biologicals (Pozzilli et al. 2002).

The clear clue provided by the variety of therapeutic agents is that reducing the level of inflammation somehow reduces the severity of symptoms and signs of the disease. This suggests that inflammation itself targeted at myelin and its related products is the probable cause of the disease. Agents that attack myelin would be auto-antibodies directed against that substance. Thus multiple sclerosis could be considered as an autoimmune disease where the main external antigen has sequences or shows "molecular mimicry" with myelin.

The question arises where is the external antigen which has sequences resembling myelin?

2.9 A "Eureka Moment" in London

"Molecular mimicry" has previously been shown to operate in rheumatic fever and many other auto-immune diseases. Work from the Immunology Unit at Queen Elizabeth College and later King's College showed that in the auto-immune disease ankylosing spondylitis there was "molecular mimicry" between HLA-B27 and the bowel microbe *Klebsiella.* Subsequent studies showed that there was "molecular mimicry" between the urinary microbe *Proteus mirabilis* and HLA-DR1/4, antigens which are prevalent in rheumatoid arthritis. The conclusions from these studies were that urinary tract infection by *Proteus mirabilis* was the probable cause of rheumatoid arthritis and bowel infection by *Klebsiella* microbes was the possible cause of ankylosing spondylitis.

These studies were carried out in the Immunology Unit which was located jointly within the Departments of Microbiology and the Department of Biochemistry. Professor John Pirt (1923–2000), who was the Head of the Department of

Microbiology strongly supported the work of the Immunology Unit with funds, laboratory space and SRC and MRC Ph.D studentships.

In the early 1980s Professor Pirt retired to Wales and came across an epidemic of a cattle disease known as “bovine spongiform encephalopathy”, which had been labelled by the lay press as “mad cow disease”. It was thought to belong to a group of conditions called collectively as “transmissible spongiform encephalopathies” and included the disease scrapie which occurs in sheep and goats and the human diseases Creutzfeldt-Jakob disease and kuru which occurs in New Guinea natives. Bovine spongiform encephalopathy was thought to have passed to humans by meat consumption where it was described as new-variant Creutzfeldt-Jakob disease (nvCJD).

The situation encountered by Professor Pirt was that several cattle graziers were reported to have committed suicide when they lost their livestock which they had nurtured for years. If only one cow showed symptoms of the condition, the whole stock was then culled and samples collected by the Ministry of Agriculture (MAFF) (Ministry of Agriculture, Fisheries and Food) for further investigations.

The possibility that the disease had been passed on to humans caused great concerns with the general public and the press. Parliament was inclined to support any studies on the cause of “bovine spongiform encephalopathy”.

Professor Pirt suggested that the Immunology Unit should investigate “bovine spongiform encephalopathy” using the methods that had been used to study ankylosing spondylitis and rheumatoid arthritis.

There was an initial reluctance to study a veterinary disease but on careful review in the press and on television, it showed a remarkable behaviour by the cows affected by “bovine spongiform encephalopathy”.

The television pictures showed affected cows being led by the farmer with a rope round their necks. The affected cows, were stumbling with ataxia and falling down, especially on their hind-quarters. The cows had difficulties in going round corners. There was clearly a hind-quarters ataxia and paralysis (Fig. 2.1).

Fig. 2.1 Cow affected by bovine spongiform encephalopathy has ataxia and paralysis of hind quarters but is able to stand on its fore-quarters (With permission from ITN)

The “eureka moment” occurred when hind-quarters ataxia and paralysis was found to resemble hind-quarters ataxia and paralysis in guinea pigs injected with brain homogenates, when they had developed “experimental allergic encephalomyelitis” (EAE) (Raine et al. 1974).

Furthermore, multiple sclerosis patients suffer from lower limb ataxia and paralysis 3–4 times more frequently compared to upper limbs involvement.

Clearly a clinical similarity present between “bovine spongiform encephalopathy”, “experimental allergic encephalomyelitis” (EAE) and multiple sclerosis, raised the issue of a possible aetiological similarity as to the origin of these disparate diseases.

It appeared that further studies were required to clarify these issues.

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Chapter 3
The Problem of Bovine Spongiform Encephalopathy also Known as "Mad Cow Disease" in the United Kingdom

3.1 First Cases of Bovine Spongiform Encephalopathy in the United Kingdom

In 1984, an outbreak of disease in Pitsham Farm in West Sussex occurred in which several cows showed unusual clinical signs and then died.

Subsequently farms in Malmesbury, Wiltshire, reported cases of neurological diseases in dairy cows.

By 1986, it became apparent that histological examination of the brains from the affected cows from several farms in Kent and the Bristol area showed "spongiform changes" which resembled those previously observed in sheep affected by scrapie (Hope 1988).

The clinical features of the animal condition, together with the neurological examination suggested that a new disease had been discovered in British cattle and was given the name of "bovine spongiform encephalopathy" (BSE) (Wells et al. 1987).

The lay press quickly gave this condition the more expressive name of "Mad cow disease" which evoked some concern among the general public both in the U.K. and throughout the world.

The government then set up an exhaustive inquiry under the Chairmanship of Lord Phillips of Worth Matravers together with Mrs. June Bridgeman and Professor Malcolm Ferguson-Smith.

Their report ran to 16 volumes and was published under the aegis of the House of Commons in October 2000.

By late 1987, over 200 confirmed cases of bovine spongiform encephalopathy (BSE) had been identified in the U.K. (Wilesmith et al. 1988).

It was recognised that clinical symptoms of bovine spongiform encephalopathy had first appeared in Southern England (Sussex, Kent, Hampshire, Devon and Somerset) in 1985.

The disease then spread to the Midlands, Wales and Northern England, reaching Scotland in November 1987 and Northern Ireland in late 1988 (Wilesmith et al. 1991).

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3.2 Origin of the Disease

The origin of the disease evoked a great public interest lest consumption of the meat from the BSE affected animals entered the human food chain and caused similar diseases as observed in cattle. The possibility arose that Creutzfeldt-Jakob disease (CJD) which has similar histological features as bovine spongiform encephalopathy could have been transmitted to humans.

Several possible environmental factors were examined but no clear evidence emerged for their involvement in the onset of "bovine spongiform encephalopathy".

Although the use of toxic compounds was a possibility, it was found that 23 % of BSE farms had not used herbicides and 69 % of BSE farms had not used organophosphate pesticides (Lord Phillips of Worth Matravers et al. 2000).

The one factor common to all affected BSE farms was the use of commercial cattle feed. For many years, animal protein derived from abattoir material such as brain, spinal cord, intestines and spleen, had been incorporated in the form of "meat-and-bonemeal" (MBM) supplements into cattle feeds to provide a rich source of protein.

Further anecdotal evidence of "meat-and-bonemeal" (MBM) as the agent responsible for the appearance of "bovine spongiform encephalopathy" (BSE) were the reports of a similar disease in ruminant nyala and gembok in wildlife parks who also were fed MBM supplements.

Subsequently a scrapie like disease was reported in an Arabian oryx, greater kudu, eland, moufflon, scimitar horned oryx, ankole cow and bison (Lord Phillips of Worth Matravers et al. 2000).

Clearly the use of "meat-and-bonemeal" supplements in cattle farms also affected other animals kept in wildlife parks and zoological gardens.

The suspicion that the "meat-and-bonemeal" (MBM) supplements were somehow involved in the spread of the disease appeared to be gaining support.

A scrapie like disease was reported in carnivorous cats and then in exotic carnivorous animals kept in zoos such as cheetah, pumas, ocelots, tigers and lions. Post-mortem examinations of the brains in many of these animals showed "spongiform changes" similar to those found in sheep and goats affected by scrapie.

The use of the "meat-and-bonemal" (MBM) supplements was banned by the Government in June 1988 and since then the incidence of BSE has progressively declined in British cattle (Fig. 3.1).

3.3 Scrapie and Related Diseases

Scrapie is an endemic disease of sheep and goats which has been known in the U.K. for over 250 years. It first appeared in the USA in 1947.

It is also well known in Europe and is called "la Tremblante" in France.

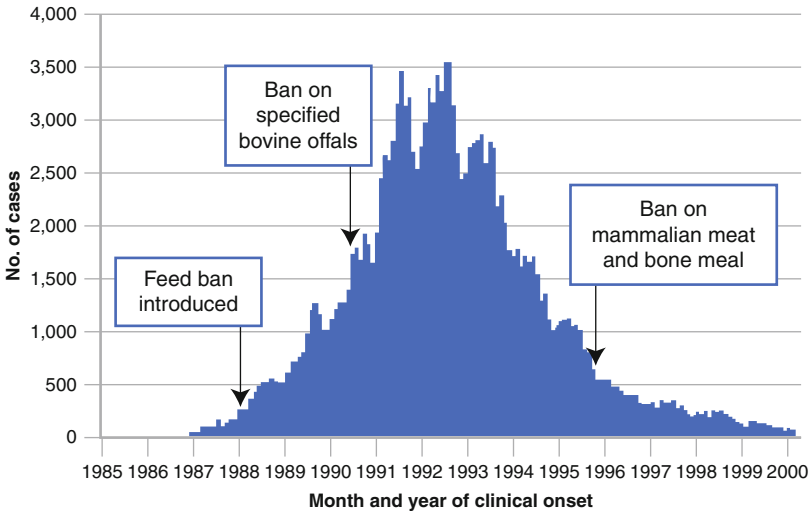

Fig. 3.1 Frequency of bovine spongiform encephalopathy in the UK

The first full description in England appeared just before the First World War (Stockman 1913).

The clinical signs include itching whereby the animal is scraping against garden posts or rocks, hence the term "scrapie".

It is characterised by incoordination, excitability of the animal and trotting movements. The disease is readily recognised by sheep farmers.

An interesting observation suggesting that a "virus" was involved was made when spinal cord homogenates from 80 animals affected by scrapie were inoculated into healthy sheep, leading to the development of the disease (Cuille and Chelle 1936). This was interpreted as the transmission of the disease by a "scrapie agent".

However injecting nervous tissue from the spinal cord, could also be explained by another mechanism, the production of a disease resembling "experimental allergic encephalomyelitis" (EAE).

Another possible suggestion that "scrapie" may have been caused by a virus, concerned the louping-ill vaccination program set up in 1937, in sheep. The vaccine was prepared as a saline solution of brain, spinal cord and spleen from sheep, which 5 days previously had been inoculated intra-cerebrally with formaldehyde inactivated louping-ill virus. The vaccine was given by subcutaneous injection and 2 years later the vaccinated sheep started developing "scrapie" (Greig 1950).

Again a homogenate of brain tissue was used but the possibility of an EAE-like autoimmune disease was not entertained as the concept of an allergic reaction to brain tissue had not yet made its entrance into medical and veterinary journals.

Louping-ill, also known as "ovine encephalomyelitis" is an acute viral disease caused by an RNA virus, belonging to the Flaviviridae. It is transmitted by the bite of the sheep tick, Ixodes ricinus.

The disease manifests itself by muscular tremors in the affected animal leading to paralysis, coma and then death.

Studies in Iceland indicated that the "scrapie" agent could be transmitted to sheep from scrapie-brain material that had been passed through filters designed to remove bacteria. It was suggested that the agent was a filterable virus (Sigurdsson 1954).

However it appeared that the transmissible agents were not viruses because no immune response could be demonstrated. The inability to demonstrate an immune response to scrapie infection was recognised in 1959 and this feature was found to be characteristic of other "transmissible infections" such as kuru, Creutzfeldt-Jakob disease and "transmissible mink encephalopathy" (Porter et al. 1973).

"Transmissible mink encephalopathy" was described in 1965 and documented several outbreaks of the disease on mink farms in Wisconsin.

Affected animals present with behavioural changes such as hyper-excitability, aggressiveness, followed by muscular incoordination and death.

A similar disease called "chronic wasting disease" of mules and elks occurs in the wildlife parks of Colorado and Wyoming (Williams and Young 1980).

The investigations on the pathogenesis of the "transmissible spongiform diseases" was based on the so-called "bio-assay", which is a method to quantify the amount of infective agent in animal tissue.

3.4 The "Bio-assay" is the Fundamental Flaw in "Transmissible Spongiform Diseases" Research

The investigation on the pathogenesis of "scrapie" like diseases is based on the "bio-assay".

For the bio-assay, tissues are ground up, usually in saline, to produce a suspension which is sequentially diluted.

A specified volume of each dilution is then injected into groups of experimental animals, usually mice, which are then observed for the development of the disease.

In this way the titre or concentration of infectivity per gram of particular tissue can then be calculated from the last dilution that was sufficient to cause disease in 50 % of a group of experimental animals.

The first assumption being made here is that the denatured tissue which contained the putative infectious agent, the "scrapie" agent will not evoke an immune response in the experimental animal.

The second assumption is being made here is that any pathological responses are due to the "scrapie" agent.

The first assumption is clearly wrong, since we know since the time of Pasteur that brain homogenates produce "experimental allergic encephalomyelitis" (EAE). Homogenates from any organ will evoke an auto-immune disease.

The second assumption is also wrong since the "scrapie" agent has not been identified.

The "prion" molecule is anyway a self-molecule encoded by the DNA of the original animal or patient, therefore it is a self-molecule.

It is certainly not "infectious" since infection implies replication of a bacterial or viral agent.

If denaturation of a self-molecule is described as "replication", then this occurs readily in patients with burns who make auto-antibodies against burnt skin.

Patients following a myocardial infarction will make antibodies against myocardial tissue and this is known as Dressler's syndrome.

3.5 Conclusions

The appearance of a neurological disease in British cattle in the 1980s has been called "bovine spongiform encephalopathy" (BSE) and was soon re-labelled by the press as "Mad cow disease".

Its clinical and histological features appeared to resemble the sheep and goat disease known as "scrapie", especially in that brain examination showed microsopic "evidence of spongiform changes".

Epidemiological studies indicated that the most likely causative factor was the exposure of cattle to "meat-and-bonemeal" supplementary feeds which were produced from offal material, including brain tissues, obtained from the abattoirs.

The use of "meat-and-bonemeal" feed supplements was banned in 1988 in the U.K. and since that time the number of cattle affected by "bovine spongiform encephalopathy" has progressively decreased.

The use of the "bio-assay" to provide a metric measurement of disease activity in "transmissible spongiform encephalopathies" is deeply flawed since it makes the unacceptable assumption that saline brain homogenates do not evoke an immune response in experimental animals which is contrary to extensive immunological evidence over the last 100 years.

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Chapter 4
Experimental Allergic Encephalomyelitis as a Model of Multiple Sclerosis

4.1 Post-rabies Vaccination Allergic Encephalomyelitis

This complication of rabies vaccination was discovered almost by accident in 1880s, by Pasteur and his colleagues in Paris.

Louis Pasteur and Emile Roux developed the first rabies vaccine in France. This vaccine was first used on 6th July 1885 on a 9 year old boy Joseph Meister (1876–1940) who had been bitten by a rabid dog.

Pasteur was trying to immunise patients who had been bitten by rabid dogs and wolves. To produce anti-rabies immunity, he had available the brains of only two rabid animals, one from a dog and the other one from a rabid wolf. In an endeavor to increase the quantity of rabies material, he injected the brains of the two rabid animals into some 60 rabbits.

He then used the rabbit brain homogenates to immunize patients who had been bitten by rabid dogs or wolves, and injected them, vaccinated them with rabbit brain homogenates. Some patients developed, like young Joseph Meister as expected anti-rabies immunity and survived but a small number of injected subjects developed a neurological disease which was characterized by ataxia and in some cases led to a fatal outcome.

Pasteur's inoculum was prepared from desiccated rabbit spinal cord, injected with fixed rabies virus and would cause a "neuroparalytic accident" in approximately 1/1,000 of injected people.

An extensive literature is present in European medical journals describing these serious complications and by the 1940s, the "World Health Organisation" (WHO) in Geneva had reported between 200 and 300 cases of patients who had died from a disease known as "post-rabies vaccination allergic encephalomyelitis".

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4.2 Rabies as a Neurological Disease

Rabies still remains a serious problem in many parts of the world and about 20,000 people die from rabies every year in India (Santhoshkumar et al. 2012).

Rabies is a viral disease that causes acute encephalitis. The disease can be transmitted to humans from another species, commonly by a bite from an infected animal.

Most cases of human infection are due to dog bites, though bites of jackals, cats and wolves are occasionally responsible. Even bites from bats in Trinidad have been reported to cause rabies.

For a human, rabies is almost invariably fatal if post-exposure prophylaxis is not administered prior to the onset of severe symptoms. The rabies virus infects the central nervous system, ultimately causing severe disease in the brain and death.

The rabies virus travels to the brain by following the peripheral nerves. The incubation period of the disease is usually a few months in humans, depending on the distance the virus must travel, from the site of the bite to reach the central nervous system. Once the rabies virus reaches the central nervous system and symptoms begin to show, the infection is virtually untreatable and usually fatal within days to weeks.

Early-stage symptoms of rabies are malaise, headache and fever, progressing to acute, violent movements, uncontrolled excitement, depression and a characteristic hydrophobia due to pharyngeal spasm brought on by attempts to drink.

There is a progressive paralysis which affects first the lower extremities and then spreads upwards. Finally, the patient may experience periods of mania and lethargy, eventually leading to coma. The primary cause of death is usually respiratory insufficiency.

On post-mortem examination there are characteristic degenerative changes in the ganglion cells of cerebrospinal and sympathetic ganglia with clumps of inflammatory and glial cells known as Babes' nodes. Acidophil inclusions, known as Negri bodies are found in the cytoplasm of affected cells and are of diagnostic importance.

Rabies causes about 55,000 human deaths annually worldwide. Over 95 % of human deaths due to rabies occur in Asia and Africa. Roughly 97 % of human rabies cases result from dog bites.

In the United States, animal control and vaccination programs have effectively eliminated domestic dogs as reservoirs of rabies. In several countries, including Australia and Japan, rabies carried by terrestrial animals has been eliminated entirely. While classical rabies has been eradicated in the United Kingdom, bats infected with a related virus have been found in the country on rare occasions.

Rabies has a predilection for the gray matter whilst "acute disseminated encephalomyelitis" (ADEM) has a predilection for white matter. Magnetic resonance imaging helps to distinguish rabies from "acute disseminated encephalomyelitis" (Santhoshkumar et al. 2012).

The human diploid cell rabies vaccine was started in 1967 and produced from the attenuated Pitman-Moore strain of the virus. As the vaccine does not contain any brain antigens it does not lead to the complications initially discovered by Pasteur and colleagues.

The question arises as to the origin of the complications following rabies immunization with extracts containing brain antigens.

4.3 "Experimental Allergic Encephalomyelitis" as an Animal Model of Multiple Sclerosis

The disease complication occurring after rabies immunisation has to some extent been reproduced when injecting brain tissues into experimental animals.

Animals injected with brain homogenates were rabbits, guinea pigs, mice, rats and monkeys and in all these different species pathological brain lesions could be demonstrated.

The source of the normal brain homogenates came from a variety of animal sources; rabbits, sheep, ox, monkeys and even man but they all gave similar pathological and histological results on ante-mortem and post-mortem examinations. The disease is characterised by severe muscle wasting, producing predominantly hind quarters paralysis eventually leading to quadriplegia and finally death.

It has acquired the name of "Experimental allergic encephalomyelitis" (EAE) and is considered as an animal model of the demyelinating disease multiple sclerosis. It can also be transmitted by sensitised lymphocytes from animals who have developed "experimental allergic encephalomyelitis" following immunisation with various brain extracts (Paterson 1966).

This complication or disease was discovered in 1880s, by Pasteur and his colleagues in Paris as a result of anti-rabies therapy.

A great controversy followed about the rare but well documented cases of neuroparalytic accidents. Pasteur was trying to immunise patients who had been bitten by rabid dogs and wolves. The controversy involved claims that the injected vaccine had been contaminated by bacteria. Pasteur vigorously denied that such contaminations had occurred.

The rabbit brain homogenates were used to immunize patients who had been bitten by rabid dogs or wolves.

Some patients developed, as expected anti-rabies immunity but a small number of injected subjects developed a neurological disease which was characterized by ataxia and in some cases led to a fatal outcome.

The cause for this unexpected and lethal response was not explained till the 1930s when it was shown that injection of foreign brain homogenates will evoke an immune response in the immunized individual or animal by the production of anti-brain autoantibodies which will damage the brain tissues of the host (Hurst 1932).

In the 1950s it became apparent that this was a general observation in immunology: immunization with any organ homogenate would produce an autoimmune disease in the target organ.

The classical work of Rose and Witebsky demonstrated that peripheral injection of homogenates of thyroid tissue produced an experimental disease in animals which was similar to the human autoimmune disease, Hashimoto's thyroiditis (Rose and Witebsky 1956).

It was only after the work of Medawar on allogeneic skin transplants when it was recognized that this phenomenon was an example of the homograft response by which the recipient recognizes foreign "transplantation antigens".

4.4 Features of Experimental Allergic Encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is an inflammatory autoimmune condition following immunization with brain tissues.

Experimental animals can be immunised into various sites, such as foot-pads, intra-muscularly or subcutaneously and after several weeks will develop clinical symptoms of experimental allergic encephalomyelitis.

Rabbits were immunised with suspensions of white matter in isotonic saline intradermally into foot pads (Prineas et al. 1969) and monkeys were injected intramuscularly with brain homogenates but a similar pathological pattern of encephalomyelitis was obtained (Rivers and Schwentker 1935) in both sets of animals.

In animals killed within 3 weeks of injection large numbers of mononuclear cells were present in the gray matter, white matter and in the meninges. Some of the inflammatory cells were seen in tightly packed cuffs around blood vessels leading eventually to the formation of fibrotic plaques similar to those observed in multiple sclerosis patients. Most of the large diameter axons had lost their myelin sheaths.

In animals killed between 3 and 10 months, widely distended, apparently myelin sheaths were relatively common throughout the brain tissues with many areas showing remyelination.

These animal models have been used for studying demyelinating diseases, such as viral post infectious encephalomyelitis occurring in humans. The disease affects the central nervous system and leads to the formation of large plaques of demyelinated gliotic scar tissue traversed by axons which also eventually become destroyed.

The pathogenesis of EAE is mediated by immune responses mounted against self antigens present in the myelin tissues. A high proportion of circulating lymphocytes in multiple sclerosis exhibit lymphoblastic transformation on contact with myelin tissues (Behan et al. 1968).

Demyelination leads to the formation of plaques which coalesce to produce vacuoles with areas of remyelination (Prineas et al. 1969).

In "acute EAE" observed 1–3 weeks, following immunization with brain homogenates, there is perivascular infiltration with inflammatory cells leading eventually to the formation of fibrotic plaques resembling those observed in MS patients. This is one of

the main reasons why EAE is considered to be an animal model of MS. The anti-myelin antibodies are produced in an extra-thecal site as occurs in Sydenham's chorea.

In "chronic EAE" observed 3–6 months following immunization, characteristic "vacuolar changes" have been described, in rabbits (Prineas et al. 1969) and in guinea pigs (Raine et al. 1974).

The widespread vacuolation that develops in chronic EAE, at least in rabbits and guinea pigs gives rise to a spongiform appearance on histological examination. It would appear that "spongiform changes" occur in EAE.

One of the main components in the central nervous system responsible for the production of EAE is a basic protein present in the white matter of the brain. In 1970, a group from San Diego, identified a highly active peptide sequence from bovine myelin which when injected in microgram quantities into guinea pigs, would produce hind legs paralysis, tremors, weight loss and eventually death. Such a sequence could be used to study the molecular properties and biological consequences leading to the induction of experimental allergic encephalomyelitis.

The animal model of "experimental allergic encephalomyelitis" would appear to resemble multiple sclerosis and therefore could be used to study that disease.

However this concept is disputed by some workers (Sriram and Steiner 2005) who pointed out that it has failed to provide or propose a meaningful therapy or therapeutic approach for treatment of multiple sclerosis. The spectrum of agents and approaches that showed promising results in EAE ranges from turmeric to manipulation of the immune system with cytokines but the majority of these failed to provide a viable answer to the treatment of multiple sclerosis.

Glatiramer acetate represents the only drug currently in use whose application in a clinical setting was first useful in EAE (Lisak et al. 1983). It is modestly effective in reducing relapses but has not prevented the progression of multiple sclerosis.

4.5 Conclusions

The severe and fatal neurological disease of rabies, following a bite from a rabid animal, has been brought under control by immunisation with the vaccine developed by Pasteur and Roux over a century ago in France.

However an undesirable but rare complication of the immunisation with the vaccine led to the occurrence of a frequently fatal complication of post-vaccinial encephalomyelitis.

The original vaccine contained both xenogeneic brain tissues and viral antigens. Although the viral antigens stimulated anti-rabies immunity but the presence of foreign brain antigens led to the production of cells and anti-brain autoantibodies leading to brain pathology and allergic encephalomyelitis.

Some of the features of rabies post-vaccinial encephalomyelitis can be reproduced by injecting brain tissues into healthy animals and it is considered that this experimental allergic encephalomyelitis could be a model of the human disease multiple sclerosis.

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Chapter 5
Bovine Spongiform Encephalopathy: Comparison Between the "Prion" Hypothesis and the Autoimmune Theory

5.1 Bovine Spongiform Encephalopathy as an Environmental and Nutritional Problem Involving Cattle

The first cases of bovine spongiform encephalopathy (BSE), a neurological disorder, were identified in the late 1980s in cattle raised to produce beef for human consumption and in dairy herds especially in southern England (Kimberlin 1993).

Changes in the composition of cattle supplementary feeds especially the use of "green offal" were suggested to be involved in the origin of this disease (Anderson et al. 1996).

"Green offal" in abattoirs, consisting of intestines and their contents, as well brain and spinal cord were then extensively used to produce these supplementary cattle feeds as "meat-and-bonemeal" (MBM).

The occurrence of BSE appeared to be associated with the consumption of feeds which may have contained scrapie "infected" products leading to the "recycling" of the infection (Crawford et al. 1991).

The possibility of infection was further enhanced by changes in the rendering process whereby the heating processes were significantly reduced because of financial considerations, thereby possibly increasing the quantity of the "scrapie" infectant in the feeds.

It has also been proposed that BSE-affected cattle might enter the human food chain and cause Creutzfeldt-Jakob disease (CJD) or a related neurological disorder in humans (Anderson et al. 1996).

Other groups suggested that organo-phosphate might be involved. Anticholinesterase "organophosphates" (Ops) (Phosmet) are used primarily as pesticides in cattle and sheep are often added to grains and nuts which go into cattle feeds. The organophosphates are mutagenic compounds and were thought to induce

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mutations in the cellular "prion protein" (PrP), resulting in the formation of the infectious "prion agent" (Purdey 1994). Cattle which have been exposed to high organophosphate concentrations appeared to exhibit classic BSE symptoms such as hind leg weakness, stumbling and a staggering gait. However geographical studies on the use of organo-phosphates did not correlate with the distribution of BSE in England.

5.2 Features of Bovine Spongiform Encephalopathy

The clinical features of bovine spongiform encephalopathy (BSE) vary daily during the early stages and it can take 1–6 months for the disease to develop and the diagnosis to be made.

Behavioral changes that frequently occur include a change in the milking order and a reluctance to pass through narrow passages.

Affected cattle become disorientated and are often found staring for long periods of time.

They avoid other cows in open areas and can attack humans or other cows especially when located in confined spaces.

The most noticeable characteristic features are changes on movements.

Shortened strides, swaying and difficulty in moving around corners occur early but later there is progressive weakness and ataxia with weight loss.

There is a characteristic hind quarters weakness and ataxia with affected cows readily falling down whilst still standing on their fore-quarters.

The inability of affected cattle to coordinate movements implies that irreversible nervous damage has occurred.

Histopathological examination of the brain and medulla in affected animals shows the presence of spongiform changes owing to the presence of vacuoles of varying sizes. Vacuolation of brain tissue is frequently found as the main feature of BSE.

Vacuoles are formed as a result of degeneration of myelinated axons and swelling of dendritic cells (Jeffrey et al. 1992).

The most prominent changes affect the solitary tract nucleus, the spinal tract nucleus of the trigeminal nerve and the vestibular nuclei.

As the disease progresses the vacuoles increase in size and number, often coalescing together thereby giving rise to the characteristic spongiform appearance.

It has been suggested that vacuolation may be due to the presence of prion (PrP) molecules. The site of most intense vacuolation is also the site of highest prion concentration and this observation has been used to support the "prion hypothesis" which implies that the prions are the "infectious agents" in BSE (Prusiner et al. 1993).

5.3 The Prion Agent as the Cause of BSE

One of the main features observed in BSE is the accumulation of prions (PrP's) in brain tissues.

Prions have unique biological properties: they have been defined as small proteinaceous particles that resist inactivation by procedures which modify or destroy nucleic acids. They have a molecular weight of 27–30 kDa and exist in different polymorphic forms which include rods with a diameter of 10–20 nm and 100–200 nm in length (Prusiner 1994).

There are two forms of prions: the host cellular form PrP^{c} and the infectious form PrP^{sc} also known as the scrapie infectious agent. It has been proposed that prions are the "transmissible pathogens" causing degenerative diseases which affect the central nervous system in both humans (CJD, kuru and Gerstmann-Sträussler-Scheinker syndrome) and animals (scrapie, BSE, transmissible mink encephalopathy).

Prions are resistant to many processes that would denature or inactivate viruses. Prions isolated from CJD and kuru-infected humans were found to be protease resistant and this was also a characteristic of prions obtained from scrapie infected sheep.

The native prion (PrP) is located on the cell surface and could possibly be involved in processes of cell interaction.

Prion proteins do not contain nucleic acids and therefore are unable to undergo self-replication in a normal host. They are encoded in the host genome and are found in both cattle and humans.

Both native and "infectious" forms are believed to be produced by the host cells but the difference in structure is obtained by some form of post-transcriptional modification.

The prion agent appears to have the following features (Prusiner 1993):

1. PrP and the scrapie agent co-purify in detergent extractions.
   Limited proteolysis leads to the aggregation of prions into amyloid rods.
2. PrP^{sc} is absent in normal uninfected animals.
3. Separation by immunoaffinity techniques using PrP antibodies coupled to protein-A Sepharose results in the co-purification of PrP and the infectious agent suggesting similar biochemical composition.
4. Purification involving hydrolysis or selective modification decreases the prion titre probably through denaturation.
5. The prion agent is resistant to heating at 100 °C as are in general all proteins.

It has been suggested that the prion agent appears to be a novel type of "infectious particle" not containing nucleic acids and is different from parasites, bacteria or viruses which all require nucleic acids for their replication.

Although the prion protein is somehow involved in these neurological disorders there could be another mechanism by which it could cause pathological damage to explain its great ability to cross species barrier and induce neuronal disease (Lasmezas et al. 1997).

5.4 Difficulties Associated with the Prion Hypothesis

There are several problems associated with the concept that prions are the causative agents of BSE:

1. The prion hypothesis has not been able to account for the different types of PrP^{sc} which have been detected in scrapie brains. Since PrPs do not contain any nucleic acids, genetic mutations cannot explain the occurrence of these different forms of prions which have different incubation times and also various types of rod shapes.
2. The mode of replication proposed by the prion hypothesis does not conform to current concepts of molecular biology. Replication of this proteinaceous molecule is somehow dependent on cell division. However it is difficult to explain how it leads to exponential increase in the numbers of PrP^{sc} molecules without some form of ribosomal processing.
3. The two isoforms PrP^{c} and PrP^{sc} are believed to have the same amino acid sequence. Current knowledge of protein structure suggests that identical amino acid sequences would have similar secondary and tertiary structures that exist in the most stable energy state.
4. It has been suggested that replication occurs, first, by the "infectious prion" forming a homotypic $PrP^{c} - PrP^{sc}$ complex. The "infectious prion" then transforms the cellular isoform PrP^{c} into the "infectious form" PrP^{sc}. Such a mode of replication requires the protein molecules to overcome a high activation energy to transform the dominant alpha helix PrP^{c} to the beta pleated sheet PrP^{sc} structure. The mechanism of how this occurs independently without the involvement of an energy source is not clear.
5. The "infectious protein" PrP^{sc} has not been individually isolated, there is no immunological evidence of its presence in affected animals and there are no microbial methods of culturing the agent.
6. The histological evidence of damaged brain tissues is not evidence of the presence of a replicating "infectious agent".
7. Immunodeficient mice such as SCID mice, do not develop scrapie following inoculation with affected brain tissue. If "infectious prions" (PrP^{sc}) were the cause of spongiform encephalopathies, SCID mice should be extremely susceptible to the development of scrapie following exposure to the "infectious prion", since such animals readily succumb to infection by viruses and bacteria. Another possible explanation is that the immune system itself is responsible for the scrapie lesions and in animals devoid of an adequate immune system, scrapie does not arise.

These serious molecular and microbiological problems associated with the prion concept, indicates that another hypothesis is required to explain the BSE phenomena.

In the absence of a clear virological theory to explain BSE and scrapie or a chemical theory such as the exposure to organo-phosphates there is another possible

mechanism involving immunological pathways leading to the development of autoimmune diseases.

Autoimmune diseases have been clearly described over the last 50 years and could provide an explanation for the origin of BSE.

5.5 General Theory of Autoimmune Diseases

Autoimmune diseases occur when the host mounts an immune response against antigens found in external agents or the environment which contain sequences resembling self tissues.

In 2009, at the "Autoimmunity Symposium" held in Dresden, Professor Yehuda Shoenfeld, from Israel proposed his famous "Conjecture" that "All autoimmune diseases are produced by external agents, unless proved otherwise".

The molecular mimicry theory states that the self antigen has a sequence which is biochemically and immunologically similar to agents or microorganisms present in the environment (Ebringer 1978).

The question arises whether spongiform encephalopathies could be explained by an autoimmune mechanism.

The autoimmune theory has provided aetiopathological models which demonstrate how *Streptococcus, Klebsiella* and *Proteus* bacteria are involved in the development of rheumatic fever, rheumatoid arthritis (Wilson et al. 1995) and ankylosing spondylitis (Fielder et al. 1995):

1. Rheumatic fever is the classical prototype of an autoimmune disease. A bacterial infection of the tonsils by group A *Streptococci* evokes antibodies which bind to heart tissue resulting in acute rheumatic fever because there is molecular mimicry between cardiac tissues and *streptococcal* antigens.
2. Rheumatoid arthritis is a systemic disorder which affects preferentially the peripheral joints. The majority of rheumatoid arthritis patients carry either HLA-DR1 or HLA-DR4 antigens.

   Injection of HLA-DR4 lymphocytes into rabbits produced antibodies to *Proteus* bacteria, a common urinary microbe. A urinary tract infection readily explains the increased frequency of rheumatoid arthritis in women. Furthermore *Proteus* haemolysin shows molecular mimicry or crossreacts with antigens present in HLA-DR1 and HLA-DR4 lymphocytes. Antibodies to *Proteus* bacteria have been found in rheumatoid arthritis patients from 17 different countries.
3. Ankylosing spondylitis is an arthritic disorder of the spine and 96 % of patients with the disease possess the HLA-B27 antigen whilst it is only present in 8 % of the general population in the UK or the USA. Injection of HLA-B27 lymphocytes into rabbits produces antibodies against the bowel microbe *Klebsiella.* The hexamer amino acid sequence QTDRED was found to be present in both the HLA-B27 molecule and *Klebsiella pneumoniae* nitrogenase reductase enzyme (Schwimmbeck et al. 1987). Ankylosing spondylitis patients from 16

different countries have elevated levels of antibodies against the bowel microbe *Klebsiella.*

If molecular mimicry operates in these three human diseases it is possible that a similar mechanism might operate in BSE.

A possible model to explain BSE in terms of molecular mimicry is provided by the immunological responses observed following immunization of human subjects with extracts of rabies viruses.

5.6 Experimental Allergic Encephalomyelitis as a Model of BSE

Pasteur and his colleagues, in 1890s, developed a therapeutic model to stop the paralytic and lethal effects of rabies in individuals who had been bitten by rabietic dogs or wolves. They injected saline extracts of brain tissues from animals infected by the rabies virus into humans and the majority of patients survived and recovered. However a small proportion of such injected patients developed a neurological disease involving ataxia, lower limb paralysis, inability to swallow liquids, hence the term "hydrophobia" and ultimately death.

It was only in the 1930s that it was discovered that saline injection of normal brain tissues leads to a condition which was called "experimental allergic encephalomyelitis (EAE).

EAE is an inflammatory autoimmune condition following immunization with brain tissues.

It has been used as an animal model for studying demyelinating diseases occurring in humans such as multiple sclerosis. This experimental disease affects the central nervous system and leads to the formation of large plaques of demyelinated gliotic scar tissue traversed by axons which also eventually become destroyed.

The pathogenesis of EAE is mediated by immune responses mounted against self antigens present in the myelin tissues.

Demyelination leads to the formation of plaques which coalesce to produce vacuoles and then a characteristic spongiform appearance.

The question arises whether BSE could be a form of EAE?

5.7 Growth Hormone Injections as a Cause of CJD or EAE

Growth hormone replacement therapy has resulted in some patients developing a form of Creutzfeldt-Jakob Disease (CJD) which is a progressive spongiform encephalopathy, resembling BSE and manifested by presenile dementia, ataxia and myoclonus tremors, leading eventually to a fatal outcome (Richard et al. 1994).

Prior to 1985, cadaveric growth hormone extracts were used in the treatment of children having a small height. This practice has been discontinued as this treat-

ment resulted in some patients developing Creutzfeldt-Jakob disease (CJD) (Ellis et al. 1992).

Since then recombinant DNA has been used to produce growth hormones which do not contain the putative "infectious agent", namely "denatured normal brain" tissue.

The administration of growth hormone preparations probably contaminated with nervous tissue antigens led to the development of pathological brain features similar to those observed in CJD or animals with EAE. The growth hormone preparations which contained such denatured brain tissue antigens may have been responsible for the development of this fatal spongiform condition in the injected children.

5.8 The Autoimmune Theory of BSE

The hypothesis is proposed that BSE is caused by an autoimmune response similar to that which occurs in EAE or in patients treated with growth hormone preparations.

Demyelination and neuronal damage resulting in spongiform formation are the main characteristics of BSE affected cattle.

When applied to BSE, the autoimmune theory proposes that cross-reactive auto-antibodies target bovine self antigens, following exposure to food borne microbiological material and in this it could resemble other autoimmune diseases such as rheumatic fever, ankylosing spondylitis and rheumatoid arthritis.

The autoimmune response could arise as a result of molecular mimicry between biological agents present in the winter feedstuffs, the meat-and-bonemeal (MBM) materials and myelin proteins.

Proteonomic analysis is required to assess whether encephalitogenic peptides producing EAE and environmental bacteria, possess sequences showing molecular mimicry to myelin and other brain tissues.

5.9 Comparisons Between the Prion Hypothesis and the Autoimmune Theory

Scientific theories cannot be proved, since they cannot overcome David Hume's paradox that it is impossible to examine all instances of the problem. However they can readily be disproved and this was first pointed out by the philosopher of science Karl Popper (Popper 1963).

Popper stated that "All good scientific theories should predict possible facts that are incompatible with the proposed scientific theory or hypothesis".

If such a fact is found to occur, the proposed theory has been disproved or is no longer tenable and has to be replaced by a new hypothesis or theory to explain the problem.

The theory or generalization that "All tigers are carnivorous" is incompatible with the observation of a vegetarian tiger.

Table 5.1 Comparison of the experimental and clinical predictions of the prion hypothesis and the "autoimmune theory"

Prion hypothesis	Autoimmune theory
Biological activity of agent retained at 100 °C	Biological activity of bacterial myelin peptides retained at 100 °C
Increased antibody levels to cross-reacting bacteria in sera of affected animals not present	Increased antibody levels to cross-reacting bacteria in sera of affected animals present
Prion proteins are 'infectious particles'	Prion proteins are not 'infectious particles' but breakdown products of damaged nervous tissue
Autonomous 'infectious prions' exist in the environment	Autonomous 'infectious prions' do not exist in the environment
Brain and muscle tissue arc infected by 'prions' in affected animals	Brain and muscle tissues are not infected, but crossreacting autoantibodies binding to nervous tissue are present and cause neurological damage
The agent causing BSE is in the brain and spinal cord of offal material	The agent causing BSE is not in the brain and spinal cord, but in the bacteria present in the 'green offal' material
Consumption of BSE-affected meat is dangerous	Consumption of BSE affected meat is not dangerous
CJD epidemic is expected in the human population	No CJD epidermic is expected since humans do not consume 'green offal' material
'Growth hormone preparations' were contaminated with prions which caused CJD in some patients	'Growth hormone preparations' were contaminated by denatured human brain tissue which caused an EAE-like syndrome in some patients
The 'prion hypothesis' is not compatible with current concepts of molecular biology and postulates the existence of novel particles which cause neurological damage	The 'autoimmune hypothesis' is compatible with current concepts of molecular biology and proposes that BSE/scrapie arc produced by a mechanism involving molecular mimicry between common bacteria and nervous tissue

The "autoimmune theory" provides a new model to explain BSE and scrapie. Furthermore it makes experimental or observational predictions that distinguish it from the "prion hypothesis".

The predictions made by the two theories in relation to BSE are compared and experiments should be carried out to distinguish between these two models (Table 5.1):

1. The two theories have significantly different economic implications.

   The "prion hypothesis" implies that the only method of eliminating BSE or scrapie is by culling all affected animals The "autoimmune theory", however proposes that the removal of the cross-reactive bacteria present, if any, in the supplementary "meat-and-bonemeal" preparations would prevent the development of these neurological diseases and therefore the culling of cattle is and was unnecessary.
2. The "autoimmune theory" predicts increased levels of antibodies against common bacteria which exhibit molecular mimicry with myelin and other nervous tissues.

   Experiments should therefore be carried out to determine if elevated levels of antibodies to these bacteria exist in bovine BSE serum.

3. Moreover, sporadic cases of scrapie infected sheep should have increased levels of antibodies to some of these microbes (Ebringer et al. 1998).
4. BSE is probably an autoimmune disease caused by bacteria which carry antigens resembling or showing molecular mimicry with brain tissues.
5. The BSE epidemic occurred as a result of producing animal feeds containing high concentrations of bacteria showing molecular mimicry with myelin and other brain antigens.
6. Experiments are required to determine if immune responses to these bacteria have occurred in BSE or scrapie affected animals.

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Chapter 6
Molecular Sequences in EAE and BSE Point to *Acinetobacter* Bacteria

6.1 Introduction

The relative increase in the 1980's of bovine spongiform encephalopathy (BSE), in cattle in the United Kingdom has evoked a general and world wide public interest in the relative safety of meat products for human consumption. Bovine spongiform encephalopathy was quickly labelled by the press as "mad cow disease".

These cases of BSE occurred after feeding animals with food preparations that had been produced using reject biological materials from slaughter houses. This practice was legally prohibited by the government and since then the number of BSE cases has steadily declined (Anderson et al. 1996).

Several theories have been proposed to explain this phenomenon, the most prominent being the prion hypothesis (Prusiner 1982).

The prion hypothesis suggests that there is an infectious particle of a virus/prion nature which is transmitted from sheep (scrapie) and cows (BSE) and maybe even to humans to produce Creutzfeldt-Jakob Disease (CJD).

Several groups of workers have suggested that there are several problems with this theory:

1. If there was an infection one should be able to demonstrate an immunological response to the agent. Yet no such immune response can be demonstrated.
2. Furthermore there are no suitable methods of culturing the agent or virus/prion (Weissmann 1996).
3. The prion sequence is actually part of the DNA of the host (Chesebro et al. 1985) and is therefore a self-protein.
4. Italian research workers have shown that the human prion sequence that accumulates in brain lesions, KTNMKHAGAAAAGAVVGGLG consists mostly of aliphatic amino acids. These protein chains readily polymerize into amyloid like fibrils (Forloni et al. 1993). Such aliphatic chains are relatively resistant to hydrolysis by enzymes found in macrophages.

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Therefore it is not surprising that such fibrils would accumulate in neurological lesions

5. The suggestion that the prion consists only of self-replicating proteins, also known as the "protein only hypothesis"(Griffith 1967) is incompatible with current concepts of molecular biology (Watson and Crick 1953).
6. Furthermore animals without an adequate or deficient immune system, such as SCID mice do not develop scrapie following immunization with affected tissues (Taylor et al. 1966). Here we encounter a most unusual paradox; absence of immune reactivity is protective since SCID mice readily succumb to viral and bacterial infections.

6.2 Experimental Allergic Encephalomyelitis (EAE) as a Model of an Autoimmune Disease Produced by a Mechanism Involving Molecular Mimicry

The development of an antirabies vaccine by Pasteur some one hundred years ago led to some patients developing severe neurological disorders. It took a few decades to identify the cause of these neurological complications but eventually they were ascribed to the presence of contaminating brain antigens evoking immune responses in the host.

In the 1930's several models of "experimental allergic encephalomyelitis" (EAE) were described in which injections of brain tissue led to immune responses producing a variety of neurological disorders in the injected test animals (Patterson 1966).

It is possible that bacteria may carry antigens cross-reacting or resembling brain tissue, which if present in sufficiently high quantities could evoke an immunological response in affected animals or humans resembling EAE.

A similar pathological sequence occurs in rheumatic fever where anti-streptococcal antibodies target cardiac tissue because of "molecular mimicry" or molecular similarity between heart tissue and *streptococcal* antigens.

A somewhat similar mechanism occurs in ankylosing spondylitis where 95 % of patients possess the HLA-B27 molecule but is only present in 8 % of the general population in the UK and the USA. However the bowel microbe *Klebsiella* has sequences resembling HLA-B27 and spinal collagens. Anti-*Klebsiella* antibodies target HLA-B27 bearing chondrocytes and spinal collagens thereby causing pathological damage and explaining the origin of the lesions found in this disease. Elevated levels of antibodies to *Klebsiella* microbes have been found in ankylosing spondylitis from 16 different countries (Ebringer 2013).

Molecular mimicry also occurs in rheumatoid arthritis where the HLA-DR1/4 antigen is found in over 90 % of rheumatoid arthritis patients but in only 35 % of the general population. However the upper urinary microbe *Proteus mirabilis* has sequences which resemble hyaline cartilage and HLA-DR1/4 antigens. Elevated levels of antibodies to *Proteus* bacteria are found in active rheumatoid arthritis patients from 14 different countries.

The possibility arises that BSE could have occurred by a similar mechanism as the one described for ankylosing spondylitis and rheumatoid arthritis. The supplementary feeds given to cattle were known to contain "green offal" from the abattoirs. Such "green offal" may have contained or been inadvertently contaminated by soil and animal environmental bacteria.

Some of these bacteria could have contained antigens cross-reacting with brain tissues thereby leading to the production of anti-bacterial antibodies with activity against various brain components, similar to the situation described for ankylosing spondylitis and rheumatoid arthritis.

Cases of BSE were first described in the early 1980's reaching a peak in 1988 when the statutory prohibition of the use of supplementary feeds containing bonemeal was introduced by the Ministry of Agriculture, Fisheries and Food (MAFF). This led to a dramatic reduction in the number of BSE cases.

The question still remains as to why this ban has been successful.

Was it due to the elimination of animals infected by prions or could another explanation account for these results.

6.3 The Hypothesis That BSE Is an Autoimmune Disease

It is suggested that BSE is caused by cross-reactive autoantibodies evoked following exposure of cattle to biological material obtained from abattoirs containing bacterial antigens that cross-react or resemble bovine brain tissues. Since neurological damage is the main feature of BSE it is proposed that damage to nerve tissues occurs probably in two stages:

Firstly, the outer covering of neurones, namely the myelin sheath is damaged by bacterial antibodies having anti-myelin specificity because of molecular mimicry between myelin tissue and bacterial antigens. The question here is which bacteria show molecular mimicry to myelin tissues.

In the second stage, nerves are damaged and denatured brain and prion proteins accumulate at sites of neuronal damage. Such denatured prion proteins may consist of amyloid like fibrils previously described by the Italian group (Forloni et al. 1993).

Injection of brain tissue into experimental animals causes an autoimmune disorder, experimental allergic encephalomyelitis (EAE) which is associated with the development of neurological symptoms.

On histological examination there is an extensive vacuolar formation due to destruction of myelin and followed by subsequent degeneration of neuronal tissues. Coalescing vacuoles form larger cavities and give rise to a sponge-like or spongiform appearance.

6.4 Molecular Analysis of Myelin Sequences Point to *Acinetobacter* Bacteria

Injection of myelin preparations into experimental animals have been found to give rise to experimental allergic encephalomyelitis (EAE).

Table 6.1 Comparison of amino acids of bovine myelin and prion to microorganisms from Genbank and SwissProt, which have similar sequences in other proteins

Source	Amino acids	Positions	Locations
Bovine myelin comparisons			
Bovine myelin	LSRFSWGAE	110–118	
Acinetobacter calcoaceticus	ISRFAWGEV	41–49	4-carboxy-mucono lactone decarboxylase
Agrobacterium tumefaciens	YTRFTWGAP	693–701	Beta-glucosidase
Ruminococcus albus	YTQFEISAE	274–282	Beta-glucosidase
Prion protein comparisons			
Bovine prion	NMKHVAG	119–125	
Human prion	NMKHMAG	108–114	
Escherichia coli	QMKHMAG	340–346	*E. coli* signal recognition protein
Escherichia coli	NMKQMSG	118–124	*E. coli* colicin M

Adapted from Ebringer et al. (1997)

Abbreviations: *A* alanine, *E* glutamic acid, *F* phenylalanine, *G* glycine, *H* histidine, *I* isoleucine, *L* leucine, *M* methionine, *N* asparagine, *P* proline, *Q* glutamine, *R* arginine, *S* serine, *T* threonine, *V* valine, *W* tryptophan, *Y* tyrosine

Eylar's group from Los Angeles have identified a highly encephalitogenic peptide from bovine myelin with the following sequence FSWGAEQK (Eylar et al. 1970).

This short amino acid sequence was used to search the Genbank and SwissProt databases for similar sequences allowing for mismatches. The results of this genetic analysis identified three microbes which show partial molecular mimicry to bovine myelin.

The three microbes were: *Acinetobacter calcoaceticus, Ruminococcus albus* and *Agrobacterium tumefaciens* (Table 6.1).

Acinetobacter is a microbe found extensively in soil and water supplies. It is also found on human skin and in the nasal cavities.

Ruminococcus is found in the bowel flora of ruminants.

Agrobacterium is a plant pathogen causing galls which appear as excrescences on trees and shrubs. Scrapie occurs in sheep and goats in animals which are essentially nibblers of grass and shrubs. It is possible that such animals may have been exposed to large quantities of the *Agrobacteria* found in such plants.

The amino acid sequence of *Acinetobacter* contains a positively charged arginine (R) and a negatively charged glutamic acid (E). The two charged amino acids present in the sequence form a powerful immunogenic epitope which evokes antibodies with high binding affinities (Fig. 6.1).

The host protein consisting of arginine-phenyl alanine-serine and tryptophan (RFSW) would then readily bind antibodies evoked against the antigens of *Acinetobacter* and in presence of complement, produce damage to nervous tissues.

The sequences in both *Acinetobacter* and *Agrobacterium* contain tryptophan (W), an amino acid found to be important in producing experimental allergic encephalomyelitis (EAE). If the tryptophan (W) amino acid is modified this leads to loss of encephalitogenic activity (Eylar et al. 1970).

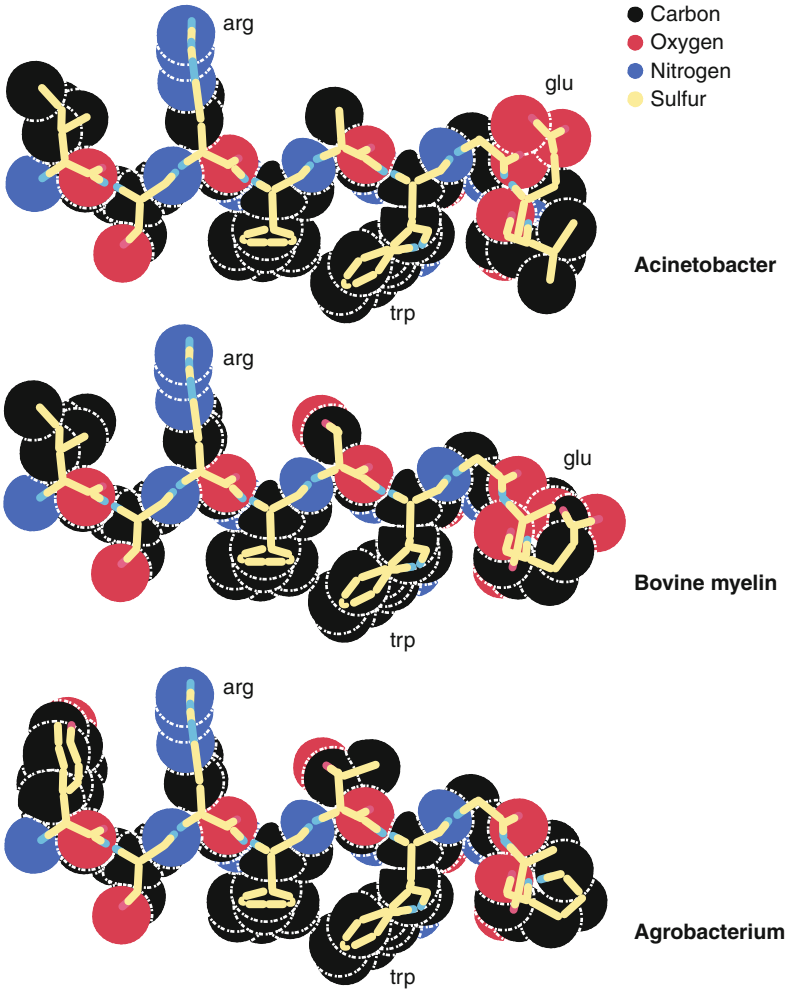

Fig. 6.1 Comparison of space filling models, using Alchemy III (Tripos ASSOC Inc, St. Louis, MO) of *Acinetobacter calcoaceticus*, bovine myelin and *Agrobacterium tumefaciens* which shows molecular mimicry between myelin and bacterial antigens. The immune response to these bacteria, over time, may cause spongiform changes characteristic of chronic experimental allergic encephalomyelitis and neurological symptoms of bovine spongiform encephalopathy. **Abbreviations**: *arg* arginine, *glu* glutamic acid, *trp* tryptophan (Adapted from Ebringer et al. (1997))

The immunological and biological activity of the encephalitogenic peptide remains after it is heated to 100 °C for one hour or treated with 8 M urea. Resistance to heat at 100 °C and to 8 M urea are also, properties found in other proteins such as prions (Weissmann 1996).

The bovine prion sequence NMKHVAG was used to search for similar sequences in microbes. Three sequences were found, all in the same microbe: NMKQMSG in *Escherichia coli* colicin M, QMKNGG in *Escherichia coli* signal recognition

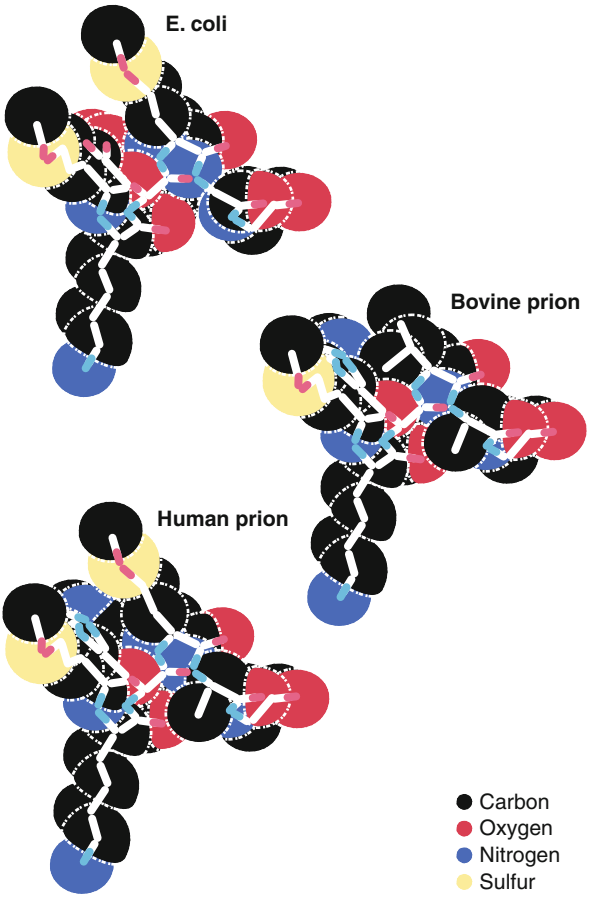

Fig. 6.2 Comparison of space filling models of *Escherichia coli* signal recognition protein, bovine prion and human prion. The nonaliphatic sequence of the prion molecule shows molecular mimicry with the *E. coli* recognition protein. The immune response to *E. coli* may contribute to the neuronal lesions found in spongiform diseases (Adapted from Ebringer et al. (1997))

protein (Fig. 6.2) and NMQHVAG in *Escherichia coli* maltodextrin glucosidase (Ebringer et al. 1997). The question arises whether this microbe is involved in BSE. It can only be answered if elevated levels of antibodies are found against this microbe in BSE animals.

6.5 Discussion and Conclusions

If BSE is an autoimmune disease, caused by bacteria showing molecular mimicry to brain antigens, then elevated levels of anti-bacterial antibodies should be present during active phases of the disease. Evidence of inflammatory disease activity could be demonstrated by elevated levels of C-reactive protein, as occurs in humans (Cowling et al. 1980).

The pathological mechanism could be similar to that found in other autoimmune diseases such as rheumatic fever, ankylosing spondylitis and rheumatoid arthritis.

The possibility arises that feeding of cattle with supplementary foods containing meat and bonemeal could have exposed these animals to such common soil and animal bacterial fragments.

Continuous exposure to such antigenic materials may have evoked autoimmune responses leading to a disease like BSE.

The two theories have different economic implications: the prion-virus hypothesis proposes that cows/sheep (BSE/scrapie) are infected by the prion-virus agent. Therefore such animals should be culled, the farmers compensated and meat production greatly curtailed but leading to huge financial costs, to the farmer, customer and taxpayer.

The autoimmune hypothesis by contrast proposes that neuronal damage is caused by immune processes similar to those found in experimental allergic encephalomyelitis (EAE) following exposure in the gut to bacterial protein sequences resembling myelin and other nervous tissues.

In this situation, the tissue damage is caused by self-proteins, namely antibacterial antibodies acting as autoantibodies. The affected animals are not infected and the treatment is to remove the offending cross-reactive antigenic bacterial fragments from the bowel flora.

Maternal transmission of BSE has occurred from dam to calf. However a similar situation occurs in human pathology in which pregnant women suffering from myasthenia gravis or thyrotoxicosis can transmit the disease via transplacental transfer of maternal IgG to their offspring.

After birth, these neonates with thyroid diseases progressively recover as the level of trans-placental maternal IgG autoantibodies subside over time.

The autoimmune hypothesis predicts that BSE affected animals should have elevated levels of antibodies to bacteria which carry antigens cross-reacting or resembling brain tissues.

The autoimmune hypothesis is a new theory that explains BSE by molecular mimicry between bacteria and brain tissues. However the theory does not conflict with the existing tenets of molecular biology as does the prion theory.

The theory could be readily tested by examining sera from BSE affected animals for antibodies to these bacteria cross-reacting with brain tissues.

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Chapter 7
Autoantibodies to Brain Components and Antibodies to *Acinetobacter* Are Present in Bovine Spongiform Encephalopathy

7.1 Introduction: Bovine Spongiform Encephalopathy or "Mad Cow Disease" Could Be Due to Environmental Factors

Bovine spongiform encephalopathy (BSE) or "mad cow disease" is a recently discovered neurological disorder of cattle which was first reported in the United Kingdom in 1985, after changes were introduced in the preparation of "meat and bone meal" (MBM) feeds. These supplementary feeds were used especially in the winter months.

It had been suggested that the bovine disease could be transmitted to humans (Will et al. 1996). This suggestion evoked widespread public concern because of its implications in possibly causing a similar disease in humans. There were several explanations proposed for the origin of this disease. One suggestion was that it was caused by abnormal prions (PrP^{SC}) and another one was that it was caused by exposure to organophosphates (Purdey 1994).

The disease belongs to a group of conditions labelled as transmissible spongiform encephalopathies which include kuru, Creutzfeldt-Jakob disease and scrapie. In these diseases both in affected animals and humans, autoantibodies to brain neurofilaments have been described by Gajdusek's group (Aoki et al. 1982).

A characteristic feature of BSE is a vacuolar appearance labelled as "spongiform changes" but a similar coalescence of vacuoles occurs in chronic experimental allergic encephalomyelitis (Raine et al. 1974). In experimental allergic encephalomyelitis autoantibodies to brain neurofilaments and myelin also occur.

Another possibility to explain the disease is that environmental bacteria may contain sequences which cross-react with brain tissues and following infection antibodies will be produced which will attack brain tissues.

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7.2 Computer Analysis of a Short Sequence of Bovine Myelin Suggests "Molecular Mimicry" with 3 Common Bacteria: *Acinetobacter, Agrobacterium* and *Escherichia*

Workers from San Diego have identified a short sequence of bovine myelin (RFSWGAEGQK) which is resistant to denaturation by heating to 100 °C or by treatment with 8 M urea, These are properties which have also been described for prions.

It was reported over 40 years ago, that injection of this short peptide into experimental animals, such as guinea pigs will lead to ataxia, hind quarters paralysis, tremors and eventually death (Eylar et al. 1970).

These features resemble to some extent, those observed in cattle affected by BSE.

An analysis of proteins found in the GenBank and SwissProt databases was carried out and it showed that there were sequences in three microbes which exhibited molecular mimicry with some brain tissues.

The best sequence was found in 4-carboxy-muconolactone decarboxylase of *Acinetobacter calcoaceticus*, a common saprophytic microbe found in soil and water supplies and which also possesses sequences resembling bovine neurofilaments (Table 7.1).

Another common environmental microbe *Agrobacterium tumefaciens* also showed some similarities to bovine myelin although not to the same extent as *Acinetobacter calcoaceticus.* Further molecular analysis revealed similarities with

Table 7.1 Comparison of similar sequences in bovine neurofilaments compared with *Acinetobacter calcoaceticus* molecular sequences

Sequence	Source (amino acid positions in brackets)
NEALEK	Neurofilament (326–331)
KEALEK	Mercuric reductase (24–29)
LKKVHEE	Neurofilament (222–228)
IEKVEEE	RNA polymerase sigma-S4 factor (54–60)
EALEKQL	Neurofilament (327–333)
EALEYGL	Lysyl tRNA synthetase (471–477)
ELEDKQN	Neurofilament (335–341)
ALEDKSN	Protocatechuate 3,4-dioxygenase (212–218)
EALEKQL	Neurofilament (327–333)
EAYAKQL	β-carboxy-*cis*-muconate cyclomerase (218–224)
KKVHEE	Neurofilament (223–228)
KKVKEE	Regulatory protein (13–18)
EIRDLR	Neurofilament (141–146)
EIRDLE	Secretion protein (279–284)
EQEIRDLR	Neurofilament (139–146)
EQIVRDAR	Acyl coenzyme A dehydrogenase (174–181)

Sequences were retrieved from the Protein Information Resource Database release 44. Identical amino acids are shown in boldface

prion proteins found in three molecules of *Escherichia coli*: the three molecules were: recognition protein, colicin M and maltodextrose-glucosidase (Ebringer et al. 1997).

7.3 Sera from Animals with and Without BSE and from Healthy Animals from an Organic Farm

Sera from BSE affected cattle and healthy controls have been tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to the three common microorganisms as well as autoantibodies to bovine neurofilaments and myelin.

Since BSE was thought to be caused by consumption of MBM winter feeds, it was considered that the "mucosal associated lymphoid system" present in the mucosal tissues of the respiratory and gastrointestinal tract would encounter such dietary antigens. Therefore mucosal immunoglobulin A (IgA) was more likely to show any possible differences in the titre of autoantibodies to brain components.

Molecular modelling suggested three possible microbes which showed cross-reactivity to brain tissues. Therefore a total Ig (IgG + IgA + IgM) assay was developed in an attempt to detect any immunological signal. The investigation was authorised and sponsored by the government, especially by the Ministry of Agriculture, Fisheries and Food (MAFF) of the U.K.

Sera from Animals with and Without BSE Supplied by the Central Veterinary Laboratory of the Ministry of Agriculture, Fisheries and Food (MAFF)

Animal sera were obtained from 29 animals (mean age 74.4 months, range 44–122 months) which were found at post-mortem to satisfy the criteria of having BSE and 18 animals which did have the disorder. These sera were supplied by the Central Veterinary Laboratory (CVL) (New Haw, Addlestone, Surrey, England), which is an executive agency of the Ministry of Agriculture, Fisheries and Food.

The 18 animals which did not have BSE had been referred to CVL because of abnormal behaviour involving ataxia and suggesting a neurological disease. The attending veterinarian decided that the limping and ataxic animal should be referred to government agencies and a post-mortem examination was carried out to exclude BSE.

The BSE and control sera provided by the CVL were obtained from animals raised on farms in different parts of England, each having its own water supply and belonging to separate herds. The majority of the BSE positive animals came from dairy Friesian herds.

Specifically there was no genetic or breeder link between the various animals that had developed BSE or the controls.

Control Sera from an Organic Farm Not Associated with MAFF

Another set of control sera were also investigated which came from animals unconnected with government departments.

These consisted of an additional 58 healthy animals to act as extra controls. This group consisted of 30 serum samples from animals aged less than 30 months (8 Friesians and 21 Hereford-Friesian and 1 Charolais-Friesian crossbreed, the crossbreed being raised for meat production).

There were also 28 serum samples from animals aged more than 30 months, all of which were dairy Friesians.

The animals were raised on a farm in Hampshire where no case of BSE had been reported and were kept under organic farming conditions, with winter feeds consisting of hay and grains but no MBM supplements.

Serum samples were obtained during yearly annual herd testing for brucellosis and were provided by the attending veterinarian.

7.4 Bacterial Cultures, ELISA and Absorption Studies

Bacterial Cultures

The microbes *Acinetobacter calcoaceticus* (NCIMB 10694) and *Agrobacterium tumefaciens* (NCIMB 9036) were obtained from the National Collections of Industrial and Murine Bacteria Ltd. (Aberdeen, Scotland) and *Escherichia coli* (NCTC 9002) was provided by the Department of Microbiology at King's College.

IgA and total Ig (IgG + IgA + IgM) antibodies were measured by ELISA.

Cultures were grown in 2 l flasks on an orbital shaker for 16 h at 37 °C for *Escherichia coli* and for 2 days at 30 °C for *Acinetobacter calcoaceticus* and *Agrobacterium tumefaciens* in 200 ml of nutrient broth (Oxoid 25 g/l).

Flasks were inoculated with 10 ml of the corresponding starter culture and were left shaking at 37 °C for 6 h. Batch culture cells were harvested by centrifugation at 6,000 rpm for 20 min at 4 °C.

The pellets of cells were then washed with 0.15 M phosphate-buffered saline (PBS; pH 7.4) before being finally resuspended in 20 ml of PBS.

A stock solution of the suspension was prepared by diluting in 0.05 M carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 (10^6 bacterial cells/ml) on the spectrophotometer (Corning Model 258).

ELISA

The ELISA method was carried out as follows: ELISA plates were coated (5 µg/well) with neurofilaments prepared from bovine spinal cord (Sigma), myelin basic protein obtained from bovine brain (Sigma) or bacterial suspension (200 µl/well) overnight at 4 °C.

Non-specific sites were blocked with PBS containing 0.1 % Tween and 0.2 % ovalbumin, plates were washed and 1/200 dilution of test or control serum was added.

The plates were incubated at 37 °C for 2 h, washed and rabbit antibovine alpha-chain-specific horseradish peroxidase conjugate (1/3,000) (Bethyl Laboratories Ltd.) or rabbit anti-cow Ig (IgG + IgA + IgM) horseradish peroxidase (1,4000) (Dako Ltd.) was added.

The plates were re-incubated for 2 h, washed and a substrate solution of 0.5 mg of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) per ml in citrate-phosphate buffer (pH 4.1) containing 0.98 mM H_2O_2 (Sigma) was added to each well.

The reaction was stopped with a 2 mg/ml solution of sodium fluoride (Sigma), the plates were read at 630 nm on a microtitre plate reader (Dynatech MR 600). Results were expressed as OD units ± standard errors (SE). Each serum sample was tested in duplicate All studies were done blind in that the tester did not know which were test or control sera.

The mean OD units of IgA or total Ig antibodies in serum samples from BSE positive animals resulting from tests against the two autoantigens and three different microorganisms were compared to the corresponding control groups by using Student's *t*-test.

Furthermore, triplicate ELISA studies were carried out in serial doubling dilutions of three selected BSE serum samples which had high, medium and low reactivities to the respective antigens bovine neurofilaments, bovine myelin and *Acinetobacter calcoaceticus.*

Absorption Studies

Serum samples from six animals with BSE and high antibody levels to *Acinetobacter calcoaceticus,* bovine myelin and neurofilaments were selected for absorption studies.

A suspension of *Acinetobacter calcoaceticus,* OD 1.60 at 540 nm, was sonicated using an MSE Soni prep 150 with ½-in probe, amplitude 10–14, for five 1-min bursts. Serum samples (200 µl) were absorbed with sonicated bacteria (25 µl) in a plastic tube and rotated gently overnight at 4 °C.

The absorption was repeated until the antibacterial antibodies levels for each sample were below the mean value for healthy controls when measured by ELISA (mean OD ± SE). Absorbed sera were then retested for reactivity against bovine myelin and neurofilaments, as previously described.

7.5 Measurement of Autoantibodies to Brain Components

Elevated levels of IgA autoantibodies to bovine neurofilaments (Fig. 7.1a) and bovine myelin (Fig. 7.1b) were found in the 29 animals with BSE (respective mean ODs ± SEs, 0.451 ± 0.029 and 0.260 ± 0.019) when compared to 18 animals free of BSE (0.149 ± 0.009; $p < 0.001$) (0.100 ± 0.0012; $p < 0.001$), 30 organically raised

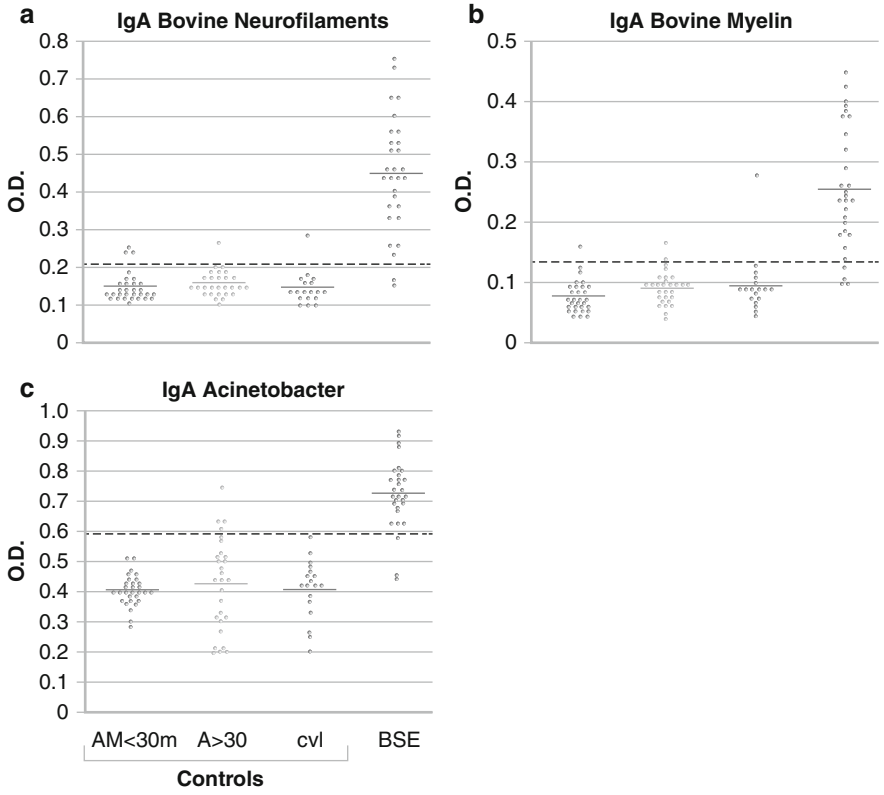

Fig. 7.1 IgA antibody titres (bar = mean) for 30 control serum samples from cows aged less than 30 months (A <30 m), 28 control serum samples from cows aged more than 30 months (A >30 m) and 18 control serum samples from cows not having BSE at post-mortem examination compared to 29 serum samples from cows with BSE at post-mortem examination against bovine neurofilaments (**a**), bovine myelin (**b**) and *Acinetobacter calcoaceticus* bacteria (**c**). *Dashed line* represents 95 % confidence limits for mean of control as given by A<30 M + A>30 M –results of the one tailed test (Copyright © American Society for Microbiology; Tiwana et al. (1999))

cows less than 30 months of age (0.149 ± 0.007; $p < 0.001$) (0.078 ± 0.005; $p < 0.001$) and 28 organically raised cows greater than 30 months of age (0.157 ± 0.006; $p < 0.001$) (0.078 ± 0.005, $p < 0.001$).

Elevated levels of IgA antibodies to whole *Acinetobacter calcoaceticus* bacteria (Fig. 7.1c) were found in the 29 BSE affected cattle (0.737 ± 0.022) when compared to 18 animals free of BSE (0.416 ± 0.024; $p < 0.001$), 30 organically raised cows less than 30 months of age (0.409 ± 0.0.009; $p < 0.001$) and 28 organically raised animals greater than 30 months of age (0.432 ± 0.029; $p < 0.001$).

Absorption of BSE sera with sonicated *Acinetobacter calcoaceticus* reduced autoantibodies to bovine myelin and neurofilaments almost to the levels found in control sera (Table 7.2), although some activity to neurofilaments remained.

Table 7.2 Levels of IgA before and after ELISA absorption with bacteria (mean OD ± SE)

Source	IgA levels	
	Pre-absorption	Post-absorption
Acinetobacter calcoaceticus	0.71 ± 0.02	0.13 ± 0.01
Bovine myelin	0.41 ± 0.01	0.22 ± 0.02
Bovine neurofilament	0.54 ± 0.06	0.28 ± 0.03

7.6 Measurement of Anti-Bacterial Antibodies

Antibodies to *Acinetobacter calcoaceticus* of total Ig (IgG + IgA + IgM) were significantly elevated in the sera from animals with BSE (0.99 ± 0.05) (Fig. 7.2a) compared to CVL controls (0.65 ± 0.06; $p < 0.001$) and organic farming controls, either in animals greater than 30 months of age (0.57 ± 0.03; $p < 0.001$) or in animals less than 30 months of age (0.53 ± 0.02; $p < 0.001$).

There was no significant difference between the CVL controls and the organic farming controls, but there was a small, statistically significant difference when compared with the sera from animals aged less than 30 months ($p < 0.05$).

However there was no significant difference in the level of anti-*Acinetobacter calcoaceticus* antibodies between organic farming animals aged more than 30 months when these animals were compared to those aged less than 30 months.

There was no significant difference between the BSE sera and the three control groups in the levels of either anti-*Agrobacterium tumefaciens* (Fig. 7.2b) and anti-Escherichia *coli* antibodies (Fig. 7.2c).

7.7 Measurement of Antibodies by Serial Dilutions

ELISA estimations of three BSE serum samples which had high, medium and low respective reactivities to the following antigens are shown: bovine neurofilaments (Fig. 7.3a), bovine myelin (Fig.7.3b) and *Acinetobacter calcoaceticus* (Fig. 7.3c).

In each case, the high titre serum reacted with a dilution of up to 1/6,400 of its respective antigen, whereas the medium and low titre sera gave lower readings (Tiwana et al. 1999).

7.8 Discussion and Conclusions

In these studies significantly elevated levels of autoantibodies to bovine neurofilaments and myelin were detected.

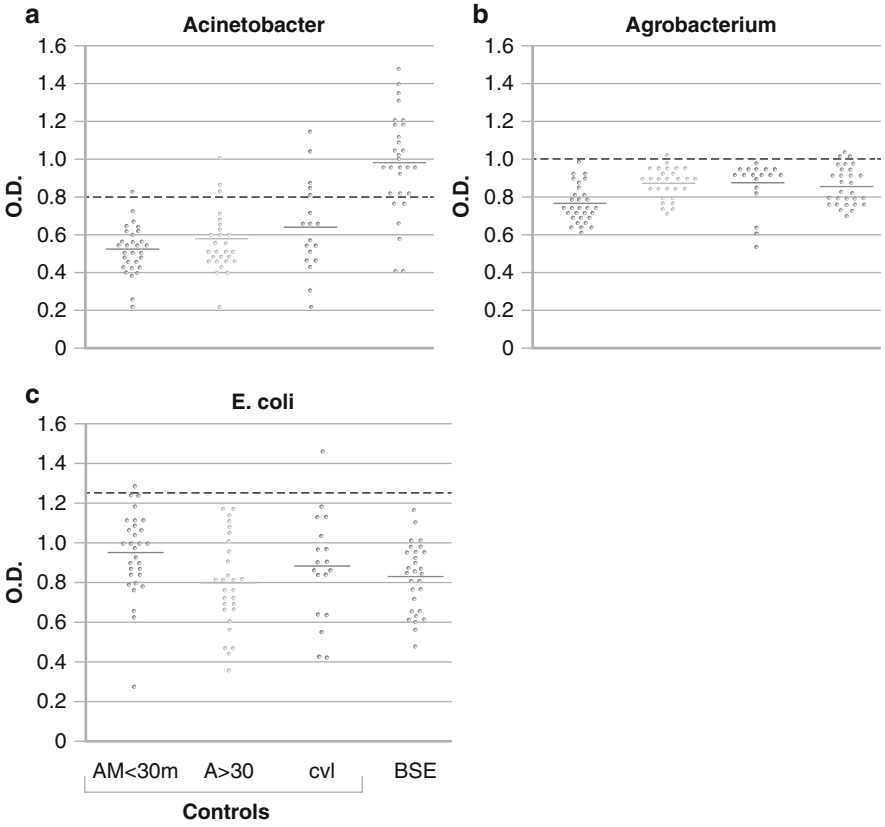

Fig. 7.2 Total antibody titres (bar = mean) for 30 control samples from cows aged less than 30 months (A < 30 m), 28 control samples from cows aged more than 30 months (A > 30 m) and 18 control samples from cows not having BSE at post-mortem examination compared to 29 serum samples from cows with BSE at post-mortem. Total antibody titres were measured against *Acinetobacter calcoaceticus* bacteria (**a**), *Agrobacterium tumefaciens* bacteria (**b**) and *Escherichia coli* bacteria (**c**). *Dashed line* represents 95 % confidence limits for mean of controls by the same formula as in the legend of Fig. 7.1 (Copyright © American Society for Microbiology; Tiwana et al. (1999))

Furthermore elevated levels of specific antibodies to *Acinetobacter calcoaceticus* have been shown to be present in BSE affected cattle when compared to the three different groups of controls. These elevations were statistically highly significant.

By contrast, no such antibody elevations were observed against either *Escherichia coli* or *Agrobacterium tumefaciens.* This is clearly a specific observation in that only one species of bacteria namely *Acinetobacter* would appear to have antibodies in BSE affected animals.

Clearly, the other two microbes, despite having cross-reacting sequences, did not evoke such specific elevations in their antibody levels and therefore were not involved in the causation of this disease.

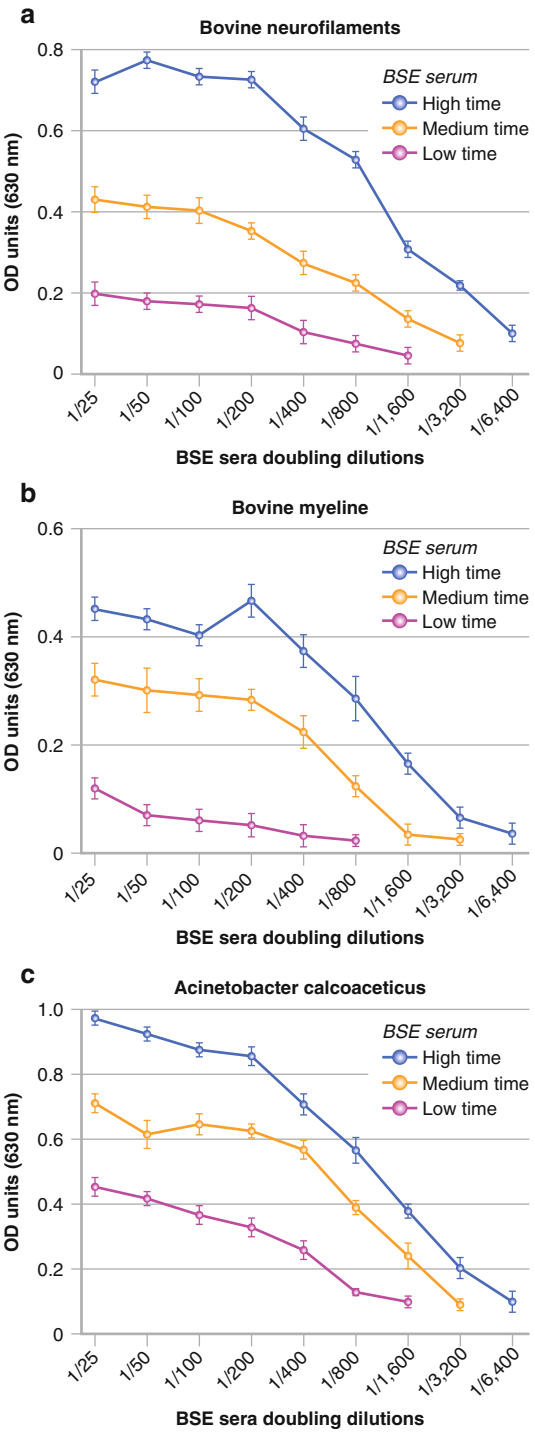

Fig. 7.3 Serial doubling dilutions (mean ± SE) of high, medium and low reactivity BSE sera against bovine neurofilaments (**a**), bovine myelin (**b**) and *Acinetobacter calcoaceticus* bacteria (**c**) (Copyright © American Society for Microbiology; Tiwana et al. (1999))

The agent responsible for the production of these specific autoantibodies is unclear but it would seem that BSE cattle have been exposed to *Acinetobacter calcoaceticus* bacteria, microbes which possess or carry antigens cross-reacting with brain tissues. Whether these bacteria are responsible for the neurological features of the disease remains to be determined.

This interesting observation requires confirmation with a larger sample of sera from animals with BSE selected from different parts of the United Kingdom and the analysis carried out with different species of *Acinetobacter.*

Furthermore, such sera should be tested against other bacteria commonly present in the bowel flora of ruminants, as well as against peptides derived from the cross-reacting sequences resembling bovine neurofilaments, myelin and other brain tissues.

Acinetobacter calcoaceticus is a species of saprophytic and aerobic Gram-negative bacteria that is widely distributed in soil and water supplies but can also be cultured from skin, mucous membranes, nasal cavities and body secretions from both animals and humans.

It is relevant to note that *Agrobacterium tumefaciens* antibodies are not elevated in animals with BSE. This microbe does not have glutamic acid in the cross-reacting epitope when compared to either *Acinetobacter* or bovine myelin (Ebringer et al. 1997). Furthermore, it is a plant pathogen of small trees and shrubs, which makes it unlikely that grass eating animals like cows would have been exposed to it.

It can be concluded from these investigations that in at least one "transmissible spongiform encephalopathy" disease, namely BSE, specific immune responses, involving predominantly the IgA system, would suggest that bacteria and bacterial antigens are acting across a mucosal surface of the gut. The microbe appears to be *Acinetobacter calcoaceticus,* one that is readily found in the environment of cattle. This microbe also happens to possess molecular sequences resembling bovine neurofilaments and bovine myelin. It is unclear why BSE animals have antibodies to this microbe but the possibility arises that it could have been introduced into the food chain of cattle, especially after changes were made in the preparation of winter feeds. Whether this has any pathological significance in the development of BSE awaits further studies.

Autoantibodies to neuronal components have previously been reported in patients with kuru and Creutzfeldt-Jakob disease (Sotello et al. 1980) and in animals with natural scrapie (Aoki et al. 1982). The pathological significance of these autoantibodies remains unclear but there are three human autoimmune diseases in which molecular mimicry occurs between bacterial antigens and self tissues: rheumatic fever, ankylosing spondylitis and rheumatoid arthritis. In rheumatic fever anti-streptococcal antibodies bind to the basal ganglia of the brain, thereby producing abnormal gait movements and the syndrome is described as Sydenham's chorea (Husby et al. 1976).

A similar neurological disorder could occur in cattle with BSE following production of anti-*Acinetobacter calcoaceticus* antibodies, since this microbe possesses antigens resembling brain tissues. The mechanism responsible for the neurological lesions remains unclear but at least these results confirm and extend the observations of Gajdusek's group that autoantibodies to neurofilaments and other brain components are present in BSE, a disease that belongs to the group of "transmissible spongiform encephalopathies".

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Chapter 8
Antibodies to *Acinetobacter* Bacteria But Not to Other Microbes Are Present in Animals with Bovine Spongiform Encephalopathy

8.1 Introduction: Bovine Spongiform Encephalopathy Could Be Caused by Environmental Bacteria

Bovine spongiform encephalopathy or "mad cow disease" is a recently discovered neurological disorder of cattle which was first reported in the United Kingdom in the early 1980's. It belongs to a group of diseases called "transmissible spongiform encephalopathies" together with scrapie, kuru and Creutzfeldt-Jakob disease.

The disorder has attracted public concern in case it could be transmitted to humans. It has been suggested that BSE is caused by either abnormal prions (PrP^{SC}) or exposure to common environmental bacteria such as *Acinetobacter.*

Encephalitogenic sequences in bovine myelin have been identified which resemble similar proteins known to occur in environmental bacteria. Such a chemical similarity or molecular mimicry is especially associated with the enzyme 4-carboxy-muconolactone decarboxylase of *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa.*

These bacteria, especially *Acinetobacter* microorganisms occur frequently as components of the commensal flora of man and animals. They can frequently be identified in soil, sewage samples and muddy water (Baumann 1968). Biological detritus and muddy water is frequently encountered in abattoirs from which offal material is collected to make "meat-and-bonemeal" (MBM) preparations. It is possible that the source of biological contamination with microbial agents may have occurred during the collection of offal material.

A preliminary study involving 29 BSE positive animals showed elevated levels of antibodies to *Acinetobacter calcoaceticus* but not to *Escherichia coli* or *Agrobacterium tumefaciens* (Tiwana et al. 1999).

A study involving a larger number of BSE animals was indicated and the project was approved and sponsored by the Ministry of Agriculture, Fisheries and Food (MAFF).

The microbe *Acinetobacter radioresistens* is a ubiquitous microorganism found in many different sites. It can be isolated from many different animals but because it is a saprophyte it can also be found in a variety of soil samples (Nishimura et al. 1988).

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
DOI 10.1007/978-3-319-02735-7_8

The microbe can also be obtained from a variety of clinical samples in human patients (Tjernberg and Ursing 1989). This *Acinetobacter* microbe was used in this extensive and comprehensive study.

8.2 Sera from Animals with and Without BSE and Test Bacteria

This second study involved a much larger number of animals to see if the previous observations could be confirmed in a more comprehensive investigation. The sera collected for this survey involved sera from 128 BSE positive animals, 63 BSE negative cows and 64 healthy control animals. Furthermore in this study a greater number of bacteria were investigated to determine how specific the previous observation in relation to *Acinetobacter* was in BSE affected animals.

In this more extensive study the bacteria studied were *Acinetobacter radioresistens, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Serratia marcescens, Proteus mirabilis* and *Klebsiella pneumoniae.*

Again the ELISA method was used to measure class specific antibodies (IgA, IgG or IgM) to *Acinetobacter radioresistens,* bovine myelin basic protein and bovine neurofilaments.

Since BSE is a notifiable disease the only centres that have access to such materials are government institutions and academic centres whose research studiers have been approved by the ministry. The samples came from 128 animals found to fulfil the criteria of having BSE at post-mortem and 63 animals that did not have the neurological disease and were supplied by the Veterinary Laboratories Agency (VLA) (New Haw, Addlestone, Surrey, England), an executive agency of the Ministry of Agriculture, Fisheries and Food. The 63 BSE negative animals were referred to the VLA due to abnormal behaviour and ataxia and underwent post-mortem examination to exclude BSE as the cause of their neurological symptoms. The VLA also supplied 64 healthy control bovine sera.

8.3 Bacterial Cultures, Peptides and ELISA

Bacterial Cultures

Bacterial cultures were provided by the Department of Microbiology at King's College, London (Table 8.1).

Cultures were grown in flasks on an orbital shaker for two days at 30 °C for *Acinetobacter resistens* and at 37 °C for *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Serratia marcescens, Proteus mirabilis* and *Klebsiella pneumoniae* in 200 ml nutrient broth (Oxoid 25 g/l), as previously described.

Table 8.1 Source and reference number of bacterial cultures

Source of bacterial culture	Name	Catalogue number
King's College London	*Pseudomonas aeruginosa*	NCTC 8203
	Bacillus subtilis	NCTC 10662
	Escherichia coli	NCTC 9002
	Serratia marcescens	NCTC 1377
	Proteus mirabilis	Clinical isolate
	Klebsiella pneumoniae	Clinical isolate

Wilson et al. (2003)
NCTC National Collection of Type Cultures

Peptides

Peptides were prepared by solid phase synthesis and analysed for purity by high performance liquid chromatography.

The first test 15-mer peptide was "RGSLS(RFSWGAE)GQK", which represents amino acid residues 107–121 of bovine myelin basic protein (MBP) This peptide shows molecular mimicry with the bacterial sequence "QNFIS(RFAWGE)VNSR" which represents amino acid residues 38–53 in 4-carboxy-muconolactone decarboxylase of *Acinetobacter calcoaceticus.*

The second brain 15-mer peptide studied was "KQLQ(ELEDK)QNADIS" which refers to amino acid residues 331–345 of bovine neurofilament.

This peptide shows molecular mimicry with the bacterial sequence "RALI(ALEDK)SNFIEA" which represents amino acid residues 208–222 of protocatechuate 3,4 dioxygenase of *Acinetobacter calcoaceticus.*

The sequences were derived from the "Protein Information Resource" database (release 44).

ELISA

ELISA's were carried out as previously described.

8.4 Measurement of Anti-Bacterial Total Immunoglobulins

Significantly elevated levels of antibodies to *Acinetobacter radioresistens* were found in BSE positive animals (0.645 ± 0.022) (mean OD ± SE) when compared to BSE negative animals (0.328 ± 0.016) ($t = 9.38$, $p < 0.0001$) and healthy control cows (0.362 ± 0.017) ($t = 8.36$, $p < 0.0001$) (Fig. 8.1).

Antibodies to *Pseudomonas aeruginosa* were shown to be elevated in BSE positive cows (0.370 ± 0.010) compared to BSE negative animals (0.311 ± 0.016) ($t = 9.26$, $p < 0.01$) and control animals (0.309 ± 0.014) ($t = 3.50$, $p < 0.001$).

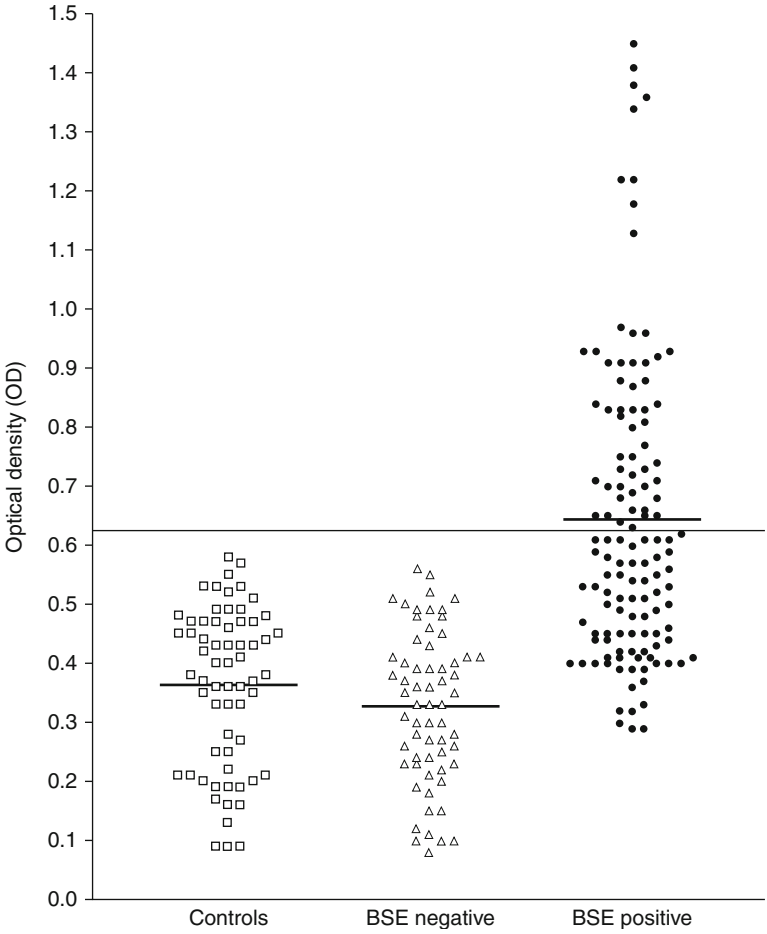

Fig. 8.1 Total (IgA + IgM + IgG) antibody levels (*OD*) to *Acinetobacter radioresistens* in 128 BSE positive animals, 63 BSE negative animals, and 64 healthy control animals (bar = mean OD) (Data from Wilson et al. (2003))

There were no significant differences in the levels of antibodies against *Bacillus subtilis, Escherichia coli, Proteus mirabilis* or *Klebsiella pneumoniae* between BSE positive animals and either BSE negative cows or healthy control animals.

Anti-*Serratia* antibodies were shown to be significantly lower in BSE positive animals (0.247 ± 0.009) ($t = 2.31$, $p < 0.05$) than in healthy control cows (0.278 ± 0.007) but no difference was seen with the BSE negative group.

No significant difference in the levels of antibodies to any of the bacteria tested were seen between the BSE negative and healthy control animals (Fig. 8.2).

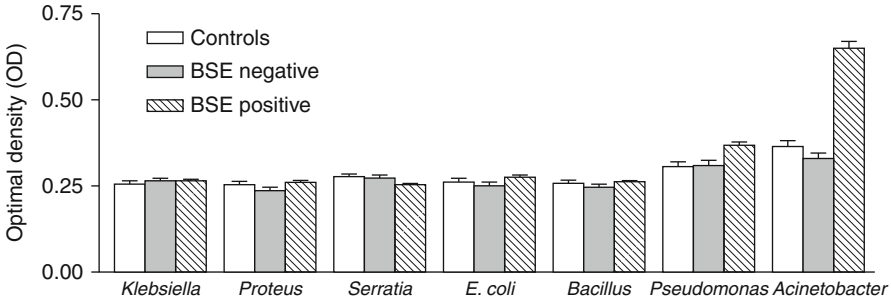

Fig. 8.2 Total (IgA + IgG + IgM) antibody levels (mean ± SE) to *Acinetobacter radioresistens, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Serratia marcescens, Proteus mirabilis* and *Klebsiella pneumoniae* in 128 BSE positive animals, 63 BSE negative animals and 64 healthy control animals (Data from Wilson et al. (2003))

Table 8.2 Class specific antibody responses (Mean ± SE) to *Acinetobacter resistens* in 128 BSE positive, 63 BSE negative and 64 healthy control animals

Source	BSE positive	BSE negative	Controls
Acinetobacter Radioresistens			
IgA	0.521 ± 0.019	0.215 ± 0.012	0.255 ± 0.013
IgG	0.280 ± 0.010	0.153 ± 0.008	0.172 ± 0.009
IgM	0.145 ± 0.004	0.099 ± 0.004	0.098 ± 0.004

Wilson et al. (2003)
SE standard error

8.5 Measurement of Class Specific Anti-*Acinetobacter* Antibodies

Significantly elevated levels of IgA antibodies to *Acinetobacter radioresistens* were found in BSE positive animals when compared to BSE negative cows ($t = 10.51$, $p < 0.0001$) and healthy control animals ($t = 9.18$, $p < 0.0001$) (Table 8.2).

IgG anti-*Acinetobacter* antibodies were shown to be significantly raised in BSE positive cows compared to BSE negative cows ($t = 8.29$, $p < 0.001$) and control animals ($t = 7.00$, $p < 0.0001$).

IgM antibodies to *Acinetobacter* were shown to be elevated in BSE positive cows when compared to BSE negative cows ($t = 7.82$, $p < 0.0001$) and control animals ($t = 7.93$, $p < 0.0001$).

IgA anti-*Acinetobacter* antibodies were also shown to be slightly elevated in control animals compared to BSE negative cows ($t = 2.25$, $p < 0.05$).

No significant differences were seen in the levels of IgG or IgM anti-Acinetobacter antibodies between the BSE negative cows or control animals.

8.6 Measurement of Autoantibodies to Brain Components

Significantly elevated levels of IgA autoantibodies to bovine myelin basic protein were found in BSE positive animals when compared to BSE negative cows ($t = 10.30$, $p < 0.0001$) and healthy control animals ($t = 8.97$, $p < 0.0001$).

There were also significantly elevated levels of IgA autoantibodies to neurofilaments in BSE positive animals when compared to BSE negative cows ($t = 11.85$, $p < 0.0001$) and healthy control animals ($t = 11.80$, $p < 0.0001$) (Table 8.3).

IgG anti-myelin autoantibodies were significantly raised in BSE positive animals compared to BSE negative cows ($t = 5.89$, $p < 0.0001$) and control animals ($t = 5.70$, $p < 0.0001$).

IgG anti-neurofilament autoantibodies were significantly raised in BSE positive animals when compared to BSE negative cows ($t = 8.62$, $p < 0.0001$) and control animals ($t = 9.09$, $p < 0.0001$).

Significantly elevated levels of IgM autoantibodies to bovine myelin were found in BSE positive animals when compared to healthy control cows ($t = 2.02$, $p < 0.05$).

There were also significantly elevated levels of IgM autoantibodies to neurofilaments in BSE positive animals when compared to healthy control cows ($t = 6.06$, $p < 0.0001$).

IgM anti-neurofilament antibodies were raised in BSE positive animals when compared to BSE negative cows ($t = 5.47$, $p < 0.0001$).

However there was no significant difference in the levels of IgM anti-myelin antibodies between BSE positive and BSE negative animals.

Furthermore no significant differences in anti-myelin or anti-neurofilament antibodies were seen between the BSE negative cows and healthy control animals.

8.7 Relative Comparison of Immunoglobulin Isotypes

The relative increase in antibody levels against *Acinetobacter radioresistens,* myelin basic protein and neurofilaments between BSE positive animals and healthy control cows was found to be greatest for the IgA isotype (Fig. 8.3).

Table 8.3 Class specific antibody responses (Mean ± SE) to bovine myelin and bovine neurofilaments in 128 BSE positive, 63 BSE negative and 64 healthy control animals

Source	BSE positive	BSE negative	Controls
Bovine myelin			
IgA	0.429 ± 0.013	0.221 ± 0.012	0.245 ± 0.013
IgG	0.273 ± 0.009	0.184 ± 0.011	0.187 ± 0.011
IgM	0.104 ± 0.004	0.099 ± 0.005	0.091 ± 0.005
Bovine neurofilaments			
IgA	0.527 ± 0.014	0.274 ± 0.013	0.278 ± 0.012
IgG	0.320 ± 0.010	0.186 ± 0.010	0.183 ± 0.009
IgM	0.132 ± 0.004	0.095 ± 0.005	0.090 ± 0.006

Wilson et al. (2003)
SE standard error

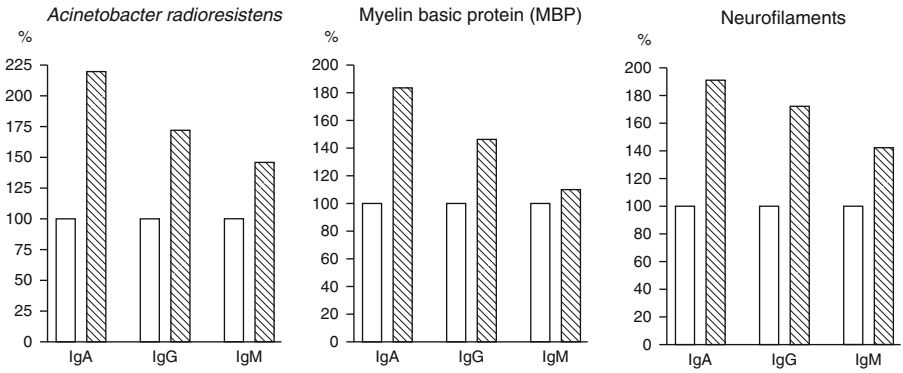

Fig. 8.3 Relative increase in isotype antibody levels to *Acinetobacter radioresistens,* myelin basic protein and neurofilaments in BSE positive animals.
Controls = healthy animals + BSE negative animals = 100 % (Data from Wilson et al. (2003))

This suggests that formation of these antibodies and autoantibodies occurred predominantly across a mucosal surface such as the gut and were probably antigens, either prions or *Acinetobacter* fragments present in the feed stuffs consumed by these animals.

8.8 Correlation Coefficient Analysis

Pearson's correlation coefficient (r) was calculated using the statistical package Prism 3.0 (GraphPad Software). The correlation coefficient (r) was calculated between *Acinetobacter radioresistens* antibodies and autoantibodies to bovine myelin and bovine neurofilaments.

There was a significant positive correlation between the IgA levels of *Acinetobacter radioresistens* and both myelin ($r = +0.915$, $p < 0.0001$) and neurofilaments ($r = +0.872$, $p < 0.0001$).

A significant positive correlation between bovine myelin and bovine neurofilaments was also observed, with regards to IgA ($r = +0.931$, $p < 0.0001$), IgG ($r = +0.439$, $p < 0.0001$) and IgM ($r = +0.611$, $p < 0.0001$) antibody levels.

8.9 Discussion and Conclusions

Elevated levels of antibodies to *Acinetobacter radioresistens* and to a lesser extent to *Pseudomonas aeruginosa* have been shown to be present in BSE affected cattle when compared to BSE negative and healthy control animals.

No such elevations were observed against *Bacillus subtilis, Escherichia coli, Serratia marcescens, Proteus mirabilis* or *Klebsiella pneumoniae.*

This confirms a previous study that demonstrated elevations in IgA antibodies to *Acinetobacter calcoaceticus* but not to *Escherichia coli* or *Agrobacterium tumefaciens* in 29 BSE positive cattle compared to three control groups (Tiwana et al. 1999).

This larger study involved 128 BSE affected cattle and compared them to 127 control cows, involving altogether 255 animals.

This is the first observation of slightly elevated anti-*Pseudomonas* in BSE cattle, although it is not surprising as *Pseudomonas* species share many antigens with *Acinetobacter* species.

This clearly suggests that BSE positive cattle have previously been exposed to *Acinetobacter* and possibly *Pseudomonas* bacteria.

The presence of specific anti-*Acinetobacter* antibodies, but not to six other bacteria thus defines a new property of BSE affected animals, which could be used either to detect early cases of BSE or assess whether the transmission of the disease has been produced in experimental animals.

Significantly raised levels of IgA, IgG and IgM autoantibodies to bovine neurofilaments and bovine myelin basic protein have been shown to be present in BSE positive animals when compared to BSE negative and control cows. This confirms previous reports of autoantibodies to brain components in transmissible spongiform encephalopathies (Sotello et al. 1980).

Acinetobacter bacteria possess amino acid sequences that mimic both bovine myelin basic protein and bovine neurofilaments. The activity of such autoantibodies can be absorbed out with *Acinetobacter* bacteria.

It is therefore possible that exposure to such *Acinetobacter* bacteria which carry epitopes resembling brain antigens could lead to production of autoantibodies which may then produce neurological damage, thus leading to BSE.

It is not clear whether prion particles initiate such damage or contribute to further damage once autoantibodies have been produced following bacterial contamination across a mucosal surface such as the gut, thereby explaining the higher relative prevalence of antibodies belonging to the IgA isotype, compared to the IgG and IgM isotypes.

However the presence of such antibodies could be used to detect BSE affected animals before they are transported to a slaughterhouse.

Acinetobacter bacteria are widely distributed in nature and can be isolated from soil samples, as well as raw sewage effluent and rural drinking water supplies (Bifulco et al. 1989).

Acinetobacter microbes also form part of the bacterial flora of the skin of man and animals.

In humans, *Acinetobacter* species usually produce respiratory tract infections, especially in patients lying in intensive care units (Gerner-Smidt 1987).

Clearly an assay is required which could detect such infections during the early stages of the disease in cattle.

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Chapter 9
An Ante-Mortem Test for Bovine Spongiform Encephalopathy Involving "Myelin-Acinetobacter-Neurofilaments" (MAN) Tested in 12 Strains of *Acinetobacter* Bacteria

9.1 Introduction: Bovine Spongiform Encephalopathy and Environmental Bacteria

Previous studies have shown that animals afflicted by bovine spongiform encephalopathy (BSE) have elevated levels of specific antibodies to *Acinetobacter* bacteria but not to six other common environmental microbes.

Furthermore such animals have specific autoantibodies to bovine myelin and to bovine neurofilaments.

The possibility arises that such observations could be used to develop an ante-mortem test for the presence of bovine spongiform encephalopathy in the early stages of the disease.

When the bovine spongiform encephalopathy was first described in the 1980's, it was feared that consumption of meat from such animals might be transmitted to humans and cause a neurological disorder resembling Creutzfeldt-Jakob disease.

Acinetobacter bacteria are found usually in the biological environment of man and animals. These bacteria can be isolated from soil, sewage and also from muddy water. In the case of the feeds provided to the BSE animals, contamination with *Acinetobacter* bacteria may have occurred during collection of animal brain and other offal material from abattoirs where muddy waters are frequently encountered. This was probably the most likely way that *Acinetobacter* components were incorporated in "meat-and-bonemeal" (MBM) preparations.

Clearly MBM supplements were involved since the statutory ban on their use has led to a significant decrease in BSE in the UK, although not to zero levels.

Early detection of the disease was considered of paramount importance in providing safe meat for human consumption.

An attempt to develop an ante-mortem test for bovine spongiform encephalopathy was initially approved and sponsored by the Ministry of Agriculture, Fisheries and Food (MAFF) and subsequently endorsed by the newly baptised Department of the Environment, Food and Rural Affairs (DEFRA).

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
DOI 10.1007/978-3-319-02735-7_9

Table 9.1 Source and reference number of bacterial cultures

Source of bacterial culture	Name	Species number	Catalogue number
Public health laboratory	*Acinetobacter calcoaceticus*	sp1	ATCC 23055
	Acinetobacter baumannii	sp2	ATCC 19606
	Acinetobacter	sp3	ATCC 19004
	Acinetobacter haemolyticus	sp4	ATCC 17906
	Acinetobacter junii	sp5	ATCC 17908
	Acinetobacter	sp6	ATCC 17909
	Acinetobacter johnsonii	sp7	ATCC 17909
	Acinetobacter lwoffii	sp8	NTCC 5866
	Acinetobacter	sp9	ATCC 9957
	Acinetobacter	sp10	ATCC 14924
	Acinetobacter radioresistens	sp12	
	Acinetobacter	sp16	ATCC 17988
	Acinetobacter	sp17	

Wilson et al. (2003)
NCTC National Collection of Type Cultures, *ATCC* American Type Culture Collection

9.2 Sera from Animals with and Without BSE, Test Bacteria and Peptides

This study involved a much larger number of sera from 189 BSE positive animals and compared to 127 BSE negative cows and 64 healthy control cows.

The study was carried out on sera obtained from 380 cows, each test was done blind and in duplicate, with the 3 different isotypes (IgA, IgG and IgM) and 4 separate peptides, involving altogether 9,672 separate estimations.

Acinetobacter bacterial cultures were provided by Dr. Kevin Towner from the Public Health Laboratory, Nottingham, UK (Table 9.1).

Peptides and ELISA studies were carried out as previously described.

The first test 15-mer peptide was "RGSLS(RFSWGAE)GQK", which represents amino acid residues 107–121 of bovine myelin basic protein (MBP).

The second brain 15-mer peptide studied was "KQLQ(ELEDK)QNADIS" which refers to amino acid residues 331–345 of bovine neurofilament.

This peptide shows molecular mimicry with the bacterial sequence "RALI(ALEDK)SNFIEA" which represents amino acid residues 208–222 of protocatechuate 3,4 dioxygenase of *Acinetobacter calcoaceticus*.

Further analysis showed a sequence identity involving bovine prion (RPVDQ) (arginine-proline-valine-aspartic acid-glutamine) spanning residues 175–179 which was also present in uridine-di-phosphate-N-acetyl-glucosamine-l-carboxy-vinyl transferase (RPVDQ) spanning residues 121–125 of *Acinetobacter calcoaceticus*.

The question arose whether BSE affected animals had produced antibodies against these crossreacting sequences found in bovine prions and also in *Acinetobacter* bacteria.

Further peptides were prepared by the methods previously described. The 14-mer test peptides used were QVYY(RPVDQ)YSNQN, which represents amino acid residues 171–184 of bovine prion and AIGS(RPVDQ)HLKAL, which represents amino acid residues 117–130 of UDP-N-acetyl-glucosamine-l-carboxy-vinyl transferase, a molecule present in *Acinetobacter calcoaceticus*.

9.3 Antibodies to *Acinetobacter* Peptides and Autoantibodies to Corresponding Bovine Brain Peptides

Significant levels of antibodies to the 3 different isotypes (IgA, IgG and IgM) were found against both the *Acinetobacter* peptides and bovine brain peptides involving altogether 24 separate comparisons.

The t-values were calculated, all of which were significant at a p-value of $p<0.001$ (Table 9.2).

Significantly elevated levels of IgA autoantibodies against the bovine prion peptide were found in 189 BSE positive animals when compared to 127 BSE negative animals ($t=10.44$, $p<0.001$) and 87 healthy controls ($t=9.94$, $p<0.001$).

Furthermore significantly elevated levels of IgA antibodies against the corresponding *Acinetobacter* peptide were found in the BSE positive animals when compared to BSE negative animals ($t=13.20$, $p<0.001$) or healthy animals ($t=13.61$, $p<0.001$).

These results would seem to indicate that prions, at least over the sequences studied, can be the subject of immune activity. Therefore the suggestion that prions are not involved in immune responses would appear to require revision.

9.4 An Ante-Mortem Test for BSE

In this study an attempt was made to develop an ante-mortem test for BSE using ELISA.

The ELISA study was carried out with 12 different species of *Acinetobacter,* as well as bovine brain peptides, in each case involving 28 BSE positive and 18 BSE negative animals, in an attempt to determine which bacteria gave optimal results in a M.A.N. (Myelin-*Acinetobacter*-Neurofilament) assay.

The 12 *Acinetobacter* strains were provided by Dr. Kevin Towner from the Public Health Laboratory in Nottingham (Table 9.1).

To improve the reliability of the assay, short synthetic peptide sequences of bovine brain molecules showing molecular mimicry to *Acinetobacter* bacteria were used, rather than total extracts of bovine myelin basic protein or bovine neurofilaments, in an endeavour to reduce non-specific antibody bindings. Total immunoglobulin (Ig) levels to myelin, *Acinetobacter* and neurofilaments were used in the M.A.N. assay.

Table 9.2 Statistical results (t-values and significance) of anti-peptide antibody levels in the sera of 189 BSE positive, 127 BSE negative and 87 healthy control cows

	IgA	IgG	IgM						
	BSE+ve vs controls	BSE+ vc vs BSE -vc	BSE -vc vs controls	BSE+ vc vs controls	BSE+ vc vs BSE -vc	BSE -vc vs controls	BSE+ vc vs controls	BSE+ vc vs BSE -vc	BSE -vc vs controls
Acinetobacter-myelin	16.06	15.89	NS	15.41	15.85	NS	11.16	9.92	2.33
	<.001	<.001		<.001	<.001		<.001	<.001	<.005
Acinetobacter-neurofilament	16.07	16.27	NS	13.75	14.51	NS	6.72	8.62	NS
	<.001	<.001		<.001	<.001		<.001	<.001	
Bovine-myclin	18.11	17.58	NS	17.55	17.87	NS	16.38	15.23	NS
	<.001	<.001		<.001	<.001		<.001	<.001	
Bovinc-neurofilament	16.56	17.20	NS	15.62	13.32	NS	5.72	6.48	NS
	<.001	<.001		<.001	<.001		<.001	<.001	

NS not significant

The 15-mer test peptides were prepared as previously described and involved "RGSLSRFSWGAEGKQ" which represents amino acid residues 107–121 of bovine myelin basic protein (MBP) and "KQLQELEDKQNADIS" spanning amino acid residues 331–345 of bovine neurofilaments.

The M.A.N. (Myelin-*Acinetobacter*-Neurofilament) index was calculated in both BSE positive and BSE negative cows as follows:

$$\text{M.A.N. Index} = (\text{Ig MBP} \times 10) \times (\text{Ig Acinetobacter} \times 10) \times (\text{Ig Neurofilament} \times 10)$$

The 99.9 % confidence limits (CL) of the controls were calculated as follows: = Mean ± 3 SD (standard deviations)

The sensitivity and specificity were determined by the method of Anderson (1976).

$$\text{Sensitivity} = \frac{\text{Number of BSE positive animals above 99.9\% CL}}{\text{Total number of BSE positive animals}} \times 100(\%)$$

$$\text{Specificity} = \frac{\text{Number of BSE negative animals below 99.9\%CL}}{\text{Total number of BSE negative animals}} \times 100(\%)$$

9.5 Sensitivity and Specificity of Ante-Mortem Test

Total antibody (IgA, IgG and IgM) measured against the 12 different strains of *Acinetobacter* in the BSE positive animals showed that all had significantly elevated levels ($p < 0.001$) when compared to the 18 controls, the highest difference being given by *Acinetobacter johnsonii* (Table 9.3).

Table 9.3 Antibody responses (mean ± SE) to different strains of *Acinetobacter*

Name	BSE positive	Controls	t-value	Statistical significance
A. calcoaceticus (spl)	0.668 ± 0.031	0.298 + 0.098	8.66	$p < 0.001$
A. baumannii (sp2)	0.452 ± 0.013	0.251 ± 0.030	7.02	$p < 0.001$
Acinetobacter (sp3)	0.402 + 0.011	0.230 + 0.015	9.27	$p < 0.001$
A. haemolyticus (sp4)	0.376 ± 0.012	0.237 + 0.013	7.79	$p < 0.001$
A. junii (sp5)	0.245 ± 0.011	0.145 ± 0.011	5.95	$p < 0.001$
Acinetobacter (sp6)	0.399 ± 0.016	0.222 ± 0.021	6.74	$p < 0.001$
A. johnsonii (sp7)	0.627 ± 0.014	0.340 + 0.014	13.52	$p < 0.001$
A. lwoffii (sp8)	0.494 + 0.024	0.228 + 0.016	8.07	$p < 0.001$
Acinetobacter (sp9)	0.506 ± 0.016	0.268 + 0.023	8.63	$p < 0.001$
Acinetobacter (sp10)	0.383 + 0.010	0.266 + 0.017	6.34	$p < 0.001$
Acinetobacter (sp16)	0.425 ± 0.015	0.254IO.022	6.65	$p < 0.001$
Acinetobacter (sp17)	0.415 + 0.020	0.223 ± 0.026	5.94	$p < 0.001$

Wilson et al. (2003)

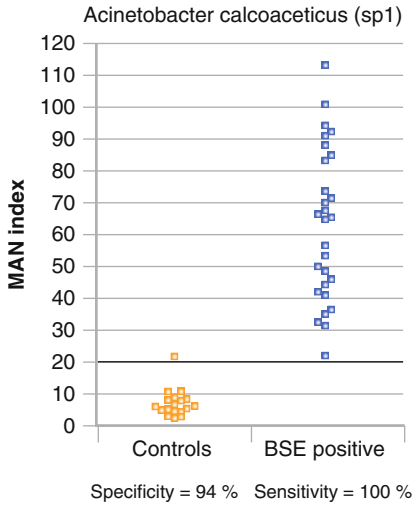

Fig. 9.1 Sensitivity and specificity of *Acinetobacter calcoaceticus (sp 1)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

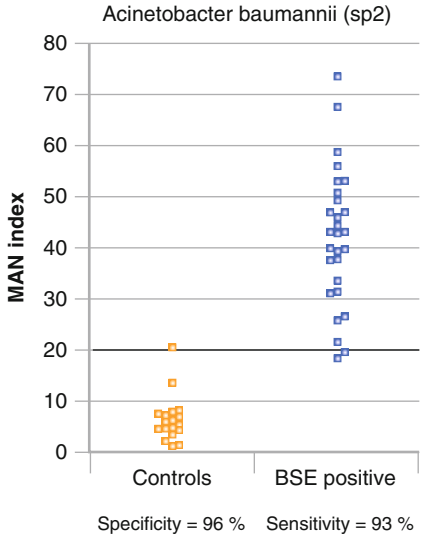

Fig. 9.2 Sensitivity and specificity of *Acinetobacter baumannii (sp2)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

Furthermore, significantly elevated levels of antibodies to bovine myelin basic protein peptides ($t = 6.93$, $p < 0.001$) and neurofilament peptides ($t = 10.09$, $p < 0.001$) were present in the BSE affected animals when compared to the 18 controls.

The sensitivity and specificity of the M.A.N. assay was calculated for each of the 12 *Acinetobacter* strains and summarized in the following figures (Figs. 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 9.10, 9.11 and 9.12).

Five out of these 12 species had a sensitivity of 100 % and specificity of 100 % in detecting BSE affected animals.

These were *Acinetobacter* (sp.3), *Acinetobacter haemolyticus, Acinetobacter* (sp.9), *Acinetobacter lwoffii* and *Acinetobacter johnsonii* (sp.7).

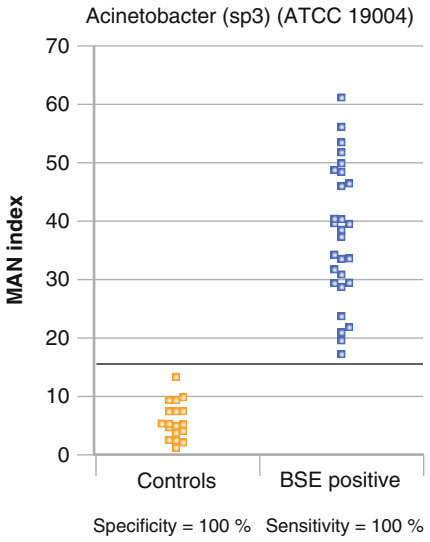

Fig. 9.3 Sensitivity and specificity of *Acinetobacter (sp 3) (ATCC 19004)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

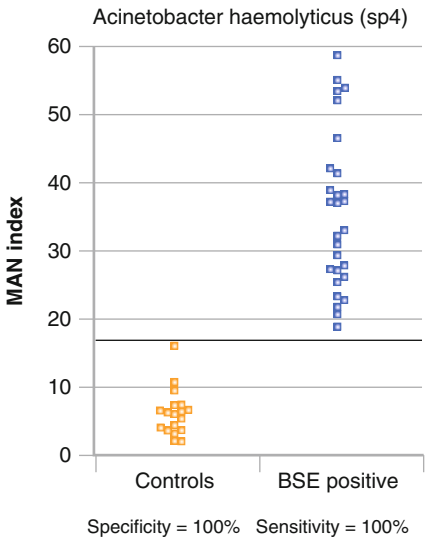

Fig. 9.4 Sensitivity and specificity of *Acinetobacter haemolyticus (sp 4)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

The remaining seven *Acinetobacter* strains also gave high readings close to 100 % in sensitivity and specificity (Wilson et al. 2003).

Acinetobacter calcoaceticus can survive on dry surfaces for up to 60 h (Musa et al. 1990).

Acinetobacter (sp.3) is found in soil and clinical specimens.

Acinetobacter baumannii is the commonest clinical isolate, especially from tracheal aspirates and wound swabs (Towner 1996).

Acinetobacter haemolyticus (sp.4) has been isolated occasionally from patients, the hospital environment and activated sludge samples.

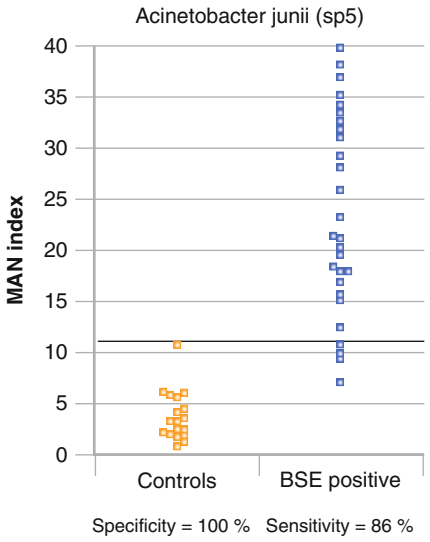

Fig. 9.5 Sensitivity and specificity of *Acinetobacter junii (sp 5)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

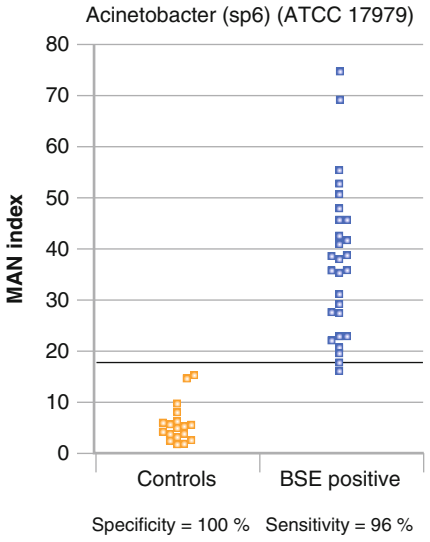

Fig. 9.6 Sensitivity and specificity of *Acinetobacter (sp 6) (ATCC 17979)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

Acinetobacter johnsonii (sp.7) and *Acinetobacter lwoffii* (sp.8) have been isolated from animals and animal products, soil and activated sludge samples but rarely from human clinical specimens.

However *Acinetobacter johnsonii* (sp.7) and *Acinetobacter lwoffii* (sp.8) can be isolated from various food sources, including fresh and spoiled meat, fish, vegetables, raw milk and cheese (Gennari and Lombardi 1993).

The ecological niche of *Acinetobacter* (sp.9) has not been clearly defined.

It is interesting to note that most of these *Acinetobacter* strains are found in soil and sludge materials. Whether the MBM feed preparations had been contaminated by such materials during collections from abattoirs is at the moment unknown.

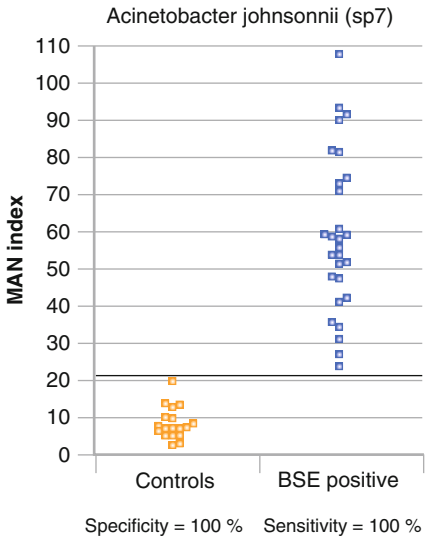

Fig. 9.7 Sensitivity and specificity of *Acinetobacter johnsonii (sp7)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

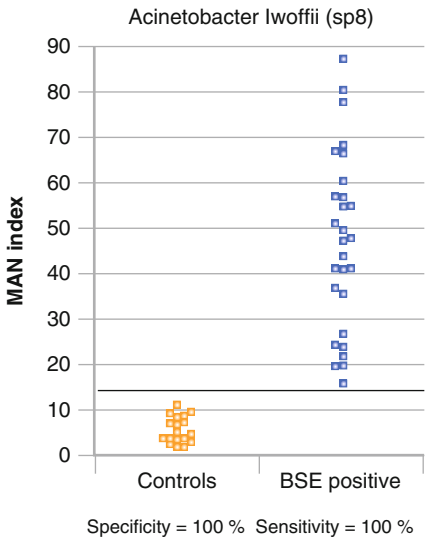

Fig. 9.8 Sensitivity and specificity of *Acinetobacter lwoffii (sp 8)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

9.6 Discussion and Conclusions

The M.A.N. assay has been used in this study to compare the ability of 12 different species of *Acinetobacter* to detect BSE affected cattle.

Short synthetic peptide sequences of bovine brain molecules, showing molecular mimicry to *Acinetobacter* bacteria have been used, rather than total extracts of bovine myelin basic protein or bovine neurofilaments in an attempt to improve the sensitivity and accuracy of the test assay.

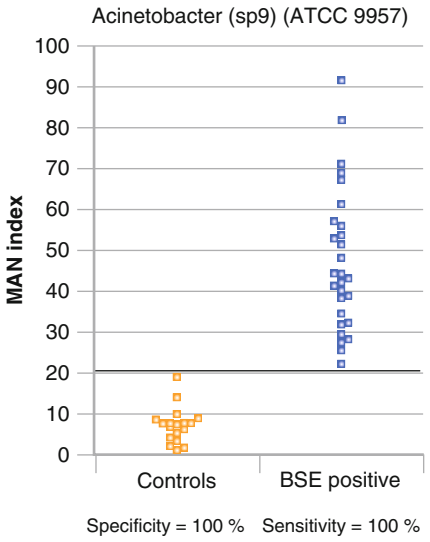

Fig. 9.9 Sensitivity and specificity of *Acinetobacter (sp9) (ATCC 9957)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

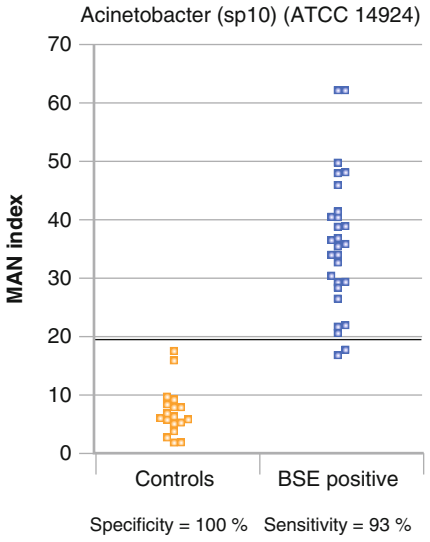

Fig. 9.10 Sensitivity and specificity of *Acinetobacter (sp10) (ATCC 14924)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

Five of the twelve *Acinetobacter* species studied had a sensitivity of 100 % and a sensitivity of 100 % in detecting BSE affected cattle and the remaining seven gave also high readings close to 100 %.

The important question arises how damage to brain tissues occurs with *Acinetobacter* or even *Pseudomonas* infection. Clearly antibody cytotoxic activity occurs with complement activation and the onset of inflammatory cascades leading to production of neurological damage.

The measurement of anti-*Acinetobacter* antibodies could be used to identify animals affected by BSE so that they could be excluded from the abattoirs and entry into the human food chain.

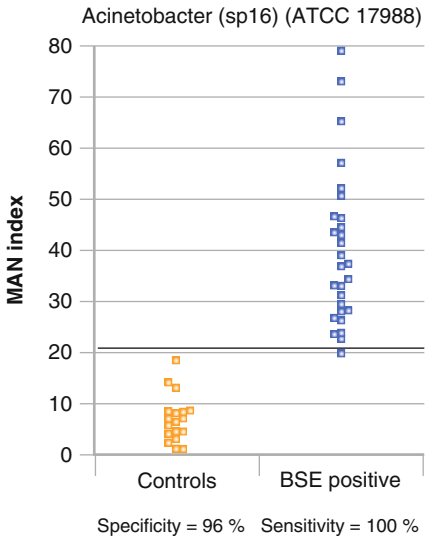

Fig. 9.11 Sensitivity and specificity of *Acinetobacter (sp16)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

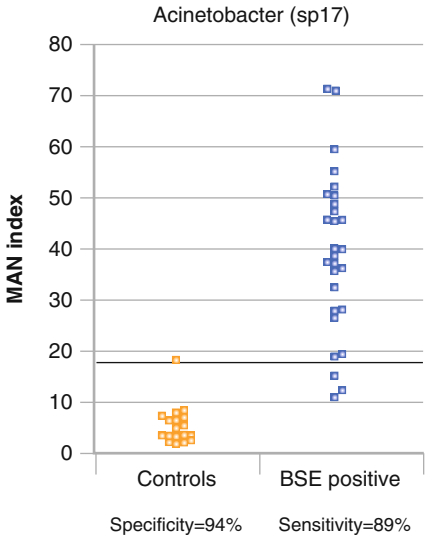

Fig. 9.12 Sensitivity and specificity of *Acinetobacter (sp 17)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003)

More extensive investigations are required to determine the value of measuring anti-*Acinetobacter* antibodies in dairy herds as well as in animals used for meat production.

The M.A.N. assay appears to provide reliable results but more field studies are clearly necessary to determine its value for general veterinary services.

The pathology of BSE and its onset in animals remains unresolved but at least the M.A.N. assay provides a new way of identifying animals that could be carrying the disease. Suspect or compromised animals need to be excluded from human consumption and this test could assist in early identification.

It is to be noted that the test can be carried out on 1 ml of blood.

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Chapter 10
Antibodies to Prion and *Acinetobacter* Peptide Sequences in Bovine Spongiform Encephalopathy

10.1 Introduction: The Possible Link Between Prions and *Acinetobacter* Bacteria

Transmissible spongiform encephalopathies (TSE's) are neurological diseases characterized by related pathological factors and include BSE and scrapie in animals as well as Creutzfeldt-Jakob disease (CJD) and kuru in humans.

The main theory to explain these diseases has been the "prion hypothesis". However other possible explanations are exposure to organophosphate pesticides or soil bacteria carrying antigens resembling brain tissues and evoking an autoimmune pathology (Ebringer et al. 1998).

The gene for cellular prion protein (PrP^c) is located on chromosome 20. The molecule is a sialoglycoprotein that is expressed predominantly in neurones of normal animals and man (Chesebro et al. 1985).

The pathological process in transmissible spongiform encephalopathies is thought to involve the change of a normal prion (PrP^c) protein into an abnormal molecular form (PrP^{SC}) which is relatively resistant to hydrolysis by the protease enzyme (Bolton et al. 1982).

These abnormal denatured proteins ($PrP^{SC)}$ then accumulate in nervous tissues (McKinley et al. 1983) and probably provide the driving force for pathological changes (De Armond and Bouzamondo 2002).

Some 30 years ago, Gajdusek's group had reported that autoantibodies to neurofilaments were present in patients with kuru and Creutzfeldt-Jakob disease and in sheep affected by scrapie.

The observation that antibodies against *Acinetobacter calcoaceticus*, as well as autoantibodies against bovine myelin basic protein and neurofilaments suggested the possibility that a diagnostic test could be developed which would indicate or possibly propose the presence of BSE in suspect animals.

The results appear to indicate that an ante-mortem diagnostic test with a high sensitivity and specificity in detecting BSE in affected animals can be used in field studies.

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However these observations need to be confirmed in other centres. In a study by a Canadian group it was shown that antibodies to lipopolysaccharide (LPS) of *Acinetobacter* were elevated in BSE affected animals but not to whole cell preparations of bacteria. There was a considerable overlap with sera obtained from BSE and control animals and the authors suggested further studies were required before a link of *Acinetobacter* to BSE could be considered to have been established (Nielsen et al. 2002).

An identity has been found between the bovine prion and the enzyme UDP-N-acetylglucosamine-1-carboxy-vinyl-transferase of *Acinetobacter calcoaceticus*.

This enzyme, which is also produced by other microbes such as *Pseudomonas putida,* has thermostable characteristics. It allows these bacteria to resist high temperatures and this could be relevant in the production of "meat-and-bone-meal" supplements used for feeding cattle (Ornston and Stanier 1966).

A study was undertaken to determine whether antibodies to the sequences found in prions and *Acinetobacter* enzymes could be detected in BSE affected cattle.

10.2 Materials and Methods: Serum Samples, Peptides and ELISA

Serum Samples

Sera from 189 cattle, as previously described, found to fulfil the criteria of having BSE at post-mortem and 127 animals that did not have these features were supplied by the Veterinary Laboratory Agency, New Haw, Surrey.

The BSE negative animals had been referred because of limping or some other locomotor problem and the attending veterinarian deemed that BSE had to be excluded from the differential diagnosis.

A further 87 serum samples were supplied from healthy cattle who did not have any clinical signs of a neurological disease.

The samples were tested against the bovine prion and *Acinetobacter* peptides by ELISA.

Peptides

Peptides were prepared, as previously described by solid phase synthesis and analyzed for purity by high performance liquid chromatography.

The test peptides were QVYY(RPVDQ)YSNQN which represents amino acid residues 171–184 of bovine prion and AIGS(RPVDQ)HLKAL which represents amino acid residues 117–184 of *Acinetobacter calcoaceticus* UDP-*N*-acetyl glucosamine-L-carboxy-vinyl-transferase.

ELISA

ELISA studies were carried out as previously described.

Statistical Studies

Statistical analyses were carried out using a one-tail Student's t-test and 95 % confidence limits of control group were calculated.

Pearson's correlation coefficient (r) was also calculated using the statistical package Prism 9.0 (Graph Pad Software).

10.3 The Presence of Molecular Mimicry Between Bovine Prion and *Acinetobacter* Sequences

An analysis was carried out to identify if molecular mimicry was present between the bovine prion molecule and published sequences of proteins found in *Acinetobacter* bacteria (Wilson et al. 2004).

A sequence identity was found between bovine prion (RPVDQ) spanning amino acid residues 175–179 and the enzyme UDP-*N*-acetylglucosamine-l-carboxy-vinyl-transferase (RPVDQ) residues 121–125 of *Acinetobacter calcoaceticus* (Fig. 10.1)

This sequence is also present in a number of other micro-organisms (Table 10.1).

10.4 Antibodies to Bovine Prion QVYY(RPVDQ)YSNQN Peptide Sequences

There were significantly elevated levels of IgA antibodies against the bovine prion peptide in the 189 BSE positive animals (mean ± SE) (0.123 ± 0.004) when compared to 127 BSE negative animals (0.092 ± 0.007; $p < 0.001$) and 87 healthy control animals (0.055 ± 0.005; $p < 0.001$) (Fig. 10.2). Levels of IgG antibodies against the bovine prion peptide were also significantly elevated in the BSE positive animals (0.070 ± 0.002; $p < 0.001$) when compared to BSE negative cattle (0.040 ± 0.002; $p < 0.001$) and healthy control animals (0.043 ± 0.003; $p < 0.001$) (Fig. 10.3).

Significantly elevated levels of IgM antibodies against the bovine prion peptide were also observed in the BSE positive animals (0.037 ± 0.001) when compared to BSE negative animals (0.032 ± 0.001; $p < 0.01$) and healthy control animals (0.030 ± 0.001; $p < 0.01$) (Fig. 10.4).

There were no significant differences in levels of any antibodies against the bovine prion peptide between BSE negative animals and healthy control animals.

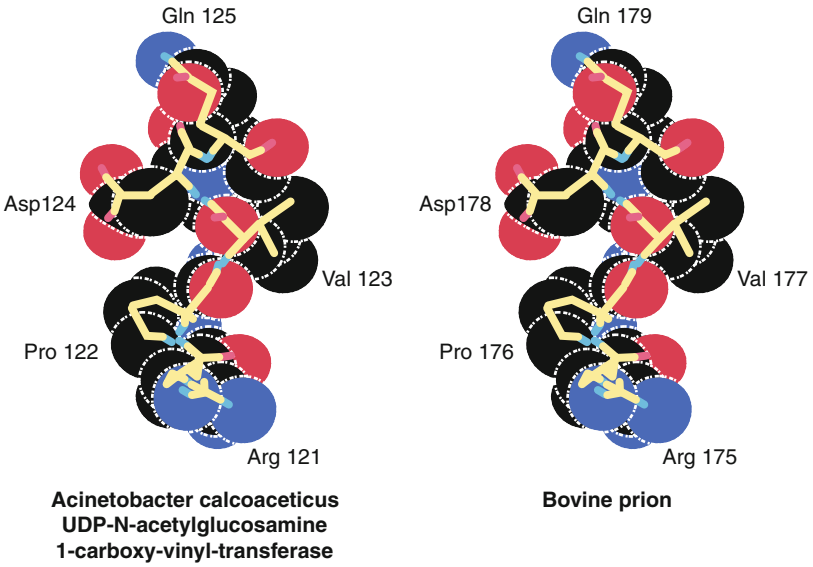

Fig. 10.1 Molecular similarities between the bovine prion protein peptide sequences and the enzyme UDP-*N*-acetyl glucosamine-1-carboxy-vinyl-transferase of *Acinetobacter calcoaceticus* bacteria

This would appear to suggest that elevations in antibodies in BSE positive animals were linked to possible exposure to *Acinetobacter* bacteria.

10.5 Antibodies to *Acinetobacter* AIGS(RPVDQ)HLKAL Peptide Sequences

Elevated levels of IgA antibodies against *Acinetobacter calcoaceticus* UDP-N-acetyl glucosamine-1-carboxy-vinyl-transferase peptide were detected in the BSE positive cattle (0.211 ± 0.006) when compared to BSE negative animals (0.092 ± 0.007; $p < 0.001$) and to healthy controls (0.076 ± 0.007; $p < 0.001$) (Fig. 10.2).

Similarly elevated levels of IgG antibody levels against the same enzyme peptide of *Acinetobacter* were also found to be significantly elevated in BSE positive animals (0.113 ± 0.003) when compared to BSE negative animals (0.051 ± 0.003; $p < 0.001$) and to healthy controls (0.043 ± 0.003; $p < 0.001$) (Fig. 10.3).

Also significantly increased levels of IgM antibodies against the same *Acinetobacter* peptide were observed in the BSE positive animals (0.051 ± 0.002) when compared to BSE negative animals (0.031 ± 0.002; $p < 0.001$) and to healthy controls (0.030 ± 0.002; $p < 0.001$) (Fig. 10.4).

Table 10.1 Comparison of amino acid sequences from bovine prion, spanning residues 175–179 (RPVDQ) and bacteria from Swissprot (release 41; 6 March 2003)

Source	Protein
Bovine	Prion
A. calcoaceticus	UDP-*N*-acetyl glucosamine-1-carboxy-vinyl-transferase
Pseudomonas aeruginosa	Hypothetical Protein—unknown function
Pseudomonas syringae	*Putative dihydropteroate synthase*
Pseudomonas fluorescens	Unknown function
P. putida	Exodeoxyribonuclease I
Bacteriodes thetaiotaomicron	Cation efflux system protein
Nitrosomonas europaea	Unknown function
Lactobacillus delbrueckii	ArbX
Rhizobium rhizogenes	VirD5
Bradyrhizobium japonicum	Blr3995
Ralstonia solanacearum	UDP-*N*-acetyl glucosamine-1 -carboxy-vinyl-transferase
Agrobacterium tumefaciens	Oxygen-independent coproporphyrinogen III oxidase
Agrobacterium rhizogenes	*VirD5*
Mycobacterium tuberculosis	Probable transcriptional regulatory protein
Xanthomonas campestris	UDP-*N*-acetyl glucosamine-1 -carboxy-vinyl-transferase
Xanthomonas axonopodis	UDP-*N*-acetyl glucosamine-1 -carboxy-vinyl-transferase
Neisseria meningitides	UDP-*N*-acetyl glucosamine-1 -carboxy-vinyl-transferase
Rodobacter capsulatus	Unknown function
Schizosaccharomyces pombe	Putative nuclei acid bi
Burkholderia cenocepacia	Hypothetical protein
Escherichia coli O 157:H7	Putative cytoplasmic membrane export protein
Clostridium acetobutylicum	Hypothetical protein
Caulobacter crescentus	Hypothetical protein

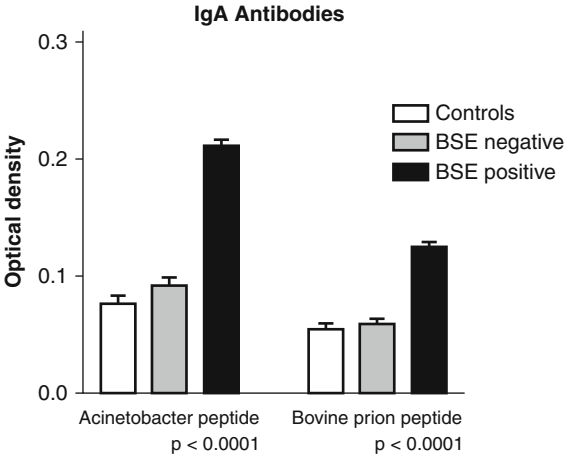

Fig. 10.2 IgA antibody levels (mean ± SE) (bar = mean) for 87 healthy control serum samples, 127 BSE negative serum samples and 189 BSE positive serum samples when tested against *Acinetobacter* peptide and bovine prion peptide

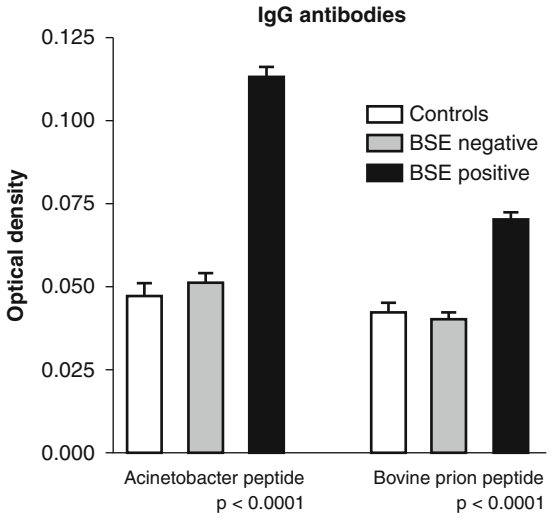

Fig. 10.3 IgG antibody levels (mean ± SE) (bar = mean) for 87 healthy control serum samples, 127 BSE negative serum samples and 189 BSE positive serum samples when tested against *Acinetobacter* peptide and bovine prion peptide

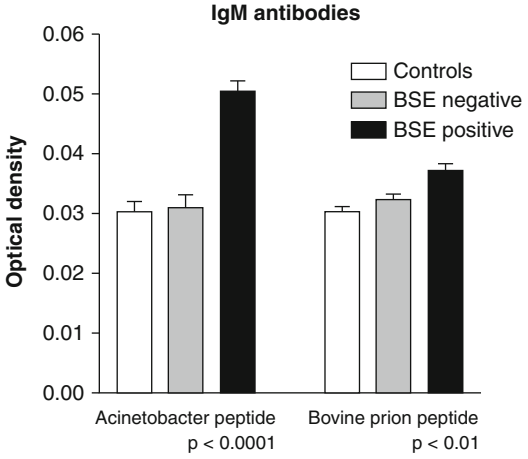

Fig. 10.4 IgM antibody levels (mean ± SE) (bar = mean) for 87 healthy control serum samples, 127 BSE negative serum samples and 189 BSE positive serum samples when tested against *Acinetobacter* peptide and bovine prion peptide

However, no significant differences were detected within the antibody isotypes between BSE negative animals and control animals.

10.6 Correlation Coefficient Studies

The correlation coefficient (r) was calculated between the antibodies to bovine prion and *Acinetobacter calcoaceticus* peptides.

There was a significant positive correlation between IgA antibody levels to bovine prion and *Acinetobacter calcoaceticus* peptides ($r = +0.855$; $p < 0.0001$).

Furthermore significant positive correlations were also observed between IgG ($r=+0.862$; $p<0.0001$) and IgM ($r=+0.637$; $p<0.0001$) antibodies against bovine prion and *Acinetobacter calcoaceticus* peptides, respectively.

10.7 Discussion and Conclusions

This study, showed that autoantibodies to a bovine prion sequence can be detected in cattle affected by BSE.

The detection of these autoantibodies occurred following the discovery of a shared amino acid sequence between the bovine prion and the enzyme UDP-N-acetyl-glucosamine-l-carboxy-vinyl transferase of *Acinetobacter calcoaceticus.*

Elevated levels of IgA, IgG and IgM antibodies against the bovine prion and *Acinetobacter* peptides were found in the sera from BSE positive cattle compared to BSE negative cows or healthy control animals. These findings are compatible with a previous study that demonstrated elevations in IgA antibodies to *Acinetobacter calcoaceticus* in BSE positive cattle compared to BSE negative animals and healthy control cows (Tiwana et al. 1999).

Furthermore, significantly raised levels of class-specific IgA, IgG and IgM autoantibodies to bovine myelin and neurofilaments have been shown to be present in BSE positive animals compared to BSE negative and control animals. These results are compatible with the observations that the microbe *Acinetobacter calcoaceticus* possesses amino acid sequences that are structurally related to both bovine myelin and neurofilament proteins.

The theoretical possibility arises that exposure to *Acinetobacter calcoaceticus* and related bacteria, especially if they carry epitopes which resemble brain antigens such as bovine myelin, neurofilaments and prions, could lead to the production of autoantibodies which may cause or exacerbate BSE.

Further studies examining cattle in the pre-clinical phase of BSE are required to establish the pathological significance of these observations.

Autoantibodies against neurofilaments have previously been reported in patients with kuru and Creutzfeldt-Jakob disease, as well as in sheep with scrapie.

In a related study it was shown that human IgG autoantibodies are present in the serum of patients with kuru and Creutzfeldt-Jakob disease which reacted most frequently with a 200 kDa singly or in combination with a 150 kDa protein of neurofilament (Toh et al. 1985).

It was suggested that the increased incidence of such autoantibodies in patients with chronic neurological diseases over that in control subjects, might indicate the activation of pre-existing clones of antibody producing B cells. The activation of such clones of B-cells may have been triggered by a viral or microbial infection.

Such a situation occurs not infrequently in mumps and measles where infection by the causative virus leads to autoantibody production to vimentin (Toh et al. 1979).

The possibility that the immune system of the host may be involved in BSE is suggested by the demonstration by McConnell's group that immunocompetent but

not immunodeficient mice develop a neurological disease when scrapie brain homogenates were used to immunise these animals by skin scarification.

Clearly the presence of autoantibodies to denatured prion sequences is similar to the situation of rheumatic fever patients having autoantibodies to cardiac antigens evoked by *streptococcal* upper respiratory infections. In the case of BSE affected animals these autoantibodies are evoked by exposure to *Acinetobacter* antigens probably contained within the MBM feeds.

The presence of autoantibodies to brain components and *Acinetobacter* bacteria in BSE animals raises the issue whether similar observations could occur in some human neurological diseases such as multiple sclerosis.

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Chapter 11
Antibodies to *Acinetobacter* and *Pseudomonas* Bacteria in Multiple Sclerosis Patients

11.1 Introduction: Possible Immune Responses to *Acinetobacter* and *Pseudomonas* Bacteria in Multiple Sclerosis Patients

Multiple sclerosis is the most common demyelinating disease of the central nervous system, affecting, when formes frustes are included, almost 100,000 individuals in the U.K and over 500,000 in the USA with the condition having characteristic immunological features. Over two million individuals in the world are thought to suffer from multiple sclerosis.

It is generally considered that an autoimmune process is involved, triggered by an infectious agent and possibly through a process of molecular mimicry (Albert and Inman 1999). It involves production of anti-neuronal antibodies which lead to pathological and neurological complications.

A similar process was previously described for rheumatic fever (Kaplan and Meyeserian 1962) and more recently for rheumatoid arthritis (Wilson et al. 1995) and ankylosing spondylitis (Fielder et al. 1995).

Sydenham's chorea is a neurological disease associated with rheumatic fever and which is caused by anti-*streptococcal* antibodies that bind to basal ganglia. In this condition autoantibodies against brain tissues are evoked outside the nervous system but any IgG antibodies produced will cross the blood-brain barrier and if present in high enough concentrations, they will activate the complement cascade and cause tissue damage. Such tissue damage will manifest itself with a variety of neurological signs and symptoms.

It is possible that multiple sclerosis is produced by a similar mechanism and the demonstration that *Acinetobacter* bacteria are involved in BSE raises the question whether active multiple sclerosis patients also possess elevated levels of antibodies to these or related microorganisms (Wilson et al. 2003).

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It was decided to investigate multiple sclerosis patients for possible immune responses to bacterial pathogens that carry sequences crossreacting with brain sequences such as *Acinetobacter* species and *Pseudomonas aeruginosa.*

These two microbes had previously been identified to have sequences crossreacting with bovine myelin basic protein and myelin destruction is a characteristic feature of multiple sclerosis in both cows with BSE and in human patients affected by multiple sclerosis.

11.2 Materials and Methods: Serum Samples, Bacteria and ELISA

Serum Samples

Sera from 26 multiple sclerosis patients (9 males and 17 females having a mean age 42 years, range: 29–55 years) were obtained from the Institute of Neurology at the Hospital for Nervous Diseases, Queen Square, London. Diagnosis was made according to the Poser criteria (Poser et al. 1983).

"Benign" multiple sclerosis patients are characterised by infrequent exacerbations but leading to full recovery.

"Relapsing remitting" multiple sclerosis patients have more frequent exacerbations. However they are followed by partial or complete remission in their clinical status. For this study, serum samples were obtained from "relapsing remitting" patients during exacerbations.

"Secondary progressive" multiple sclerosis patients are considered as those who continue to deteriorate following an initial relapsing remitting course of disease.

Primary progressive" multiple sclerosis patients are those who have a continuous deterioration without remission from the start of the disease

In addition serum samples were obtained from 20 patients in the Department of Geriatric Medicine at University College Hospital who had suffered from unilateral hemiplegia due to a "cerebro-vascular accident" (CVA) or stroke (10 males and 10 females having a mean age of 80.5 years, range 69–94 years).

Furthermore, sera were also obtained from 10 patients with viral encephalitis (8 males and 2 females, mean age 38 years, range: 3–66 years), attending the National Hospital for Neurology and Neurosurgery.

Sera from 25 subjects attending the London Blood Donor services were used as healthy controls (12 males and 13 females, mean age 40.6 years, range 22–67 years).

A further set of sera from 29 healthy control subjects attending the London Blood Donor services was used in the viral encephalitis study (15 males and 14 females, mean age 43 years; range 19–66 years).

Bacterial Cultures

Cultures *Acinetobacter sp. strain 11171*, *Acinetobacter sp. strain 19004*, *Acinetobacter junii 17908*, *Acinetobacter lwoffii 5866* and *Acinetobacter radioresistens (sp.12)* were provided by the Public Health Laboratory, Nottingham, United Kingdom.

Acinetobacter calcoaceticus (NCIMB 16904) was obtained from the National Collections of Industrial and Murine Bacteria Ltd. (Aberdeen, Scotland).

The Department of Microbiology at King's College provided *Pseudomonas aeruginosa* (NCTC 8203) and *Escherichia coli* (NCTC 9002) bacterial samples.

Cultures were grown as previously described.

ELISA

ELISA studies were carried out as previously described.

Statistical Analysis

The mean OD units of control groups (CVA and healthy blood donors) were compared with the mean OD of the 26 multiple sclerosis patients, using a one-tail Student's t-test and 95 % confidence limits of control groups were calculated.

Pearson's correlation coefficient (r) was also calculated using the statistical package Prism 9.0 (GraphPad Software).

11.3 IgA Anti-*Acinetobacter* Antibodies in Multiple Sclerosis and CVA Patients

Elevated levels of IgA antibodies occur when the initiating antigen or microbe is crossing a mucosal surface such as the "gut associated lymphoid tissue" (GALT).

Levels of IgA antibodies to *Acinetobacter sp. strain 11171* ($p<0.0001$) (Fig. 11.1), *Acinetobacter sp. strain 19004* ($p<0.0001$) (Fig. 11.2), *Acinetobacter junii 17908* ($p<0.01$) (Fig. 11.3), *Acinetobacter lwoffii 5866* ($p<0.0001$) (Fig. 11.4) and *Acinetobacter radioresistens* ($p<0.0001$) (Fig. 11.5) in multiple sclerosis patients were significantly higher than those in the healthy control group.

Levels of IgA antibodies to *Acinetobacter sp. strain 11171* ($p<0.0001$) (Fig. 11.1), *Acinetobacter lwoffii 5866* ($p<0.001$) (Fig. 11.4) and *Acinetobacter radioresistens* ($p<0.0001$) (Fig. 11.5) were also shown to be significantly elevated in multiple sclerosis compared to CVA patients.

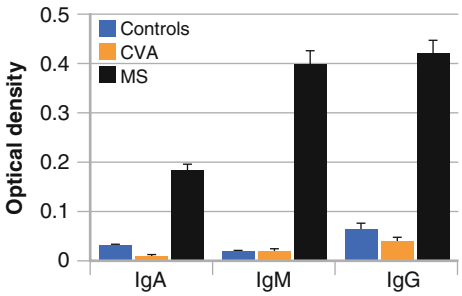

Fig. 11.1 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter sp. strain 11171* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

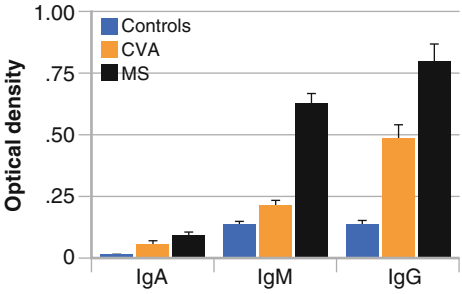

Fig. 11.2 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter sp. strain 19004* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

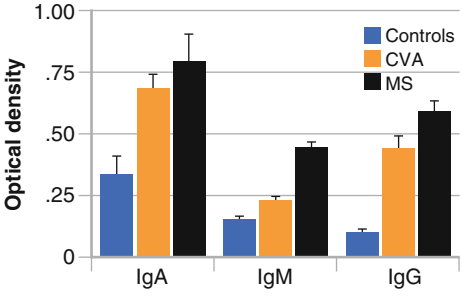

Fig. 11.3 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter junii sp. strain 17908* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

No significant difference was seen between multiple sclerosis patients and CVA patients for IgA antibodies to either *Acinetobacter sp. strain 19004* (Fig. 11.2) and *Acinetobacter sp. strain 17908* (Fig. 11.3).

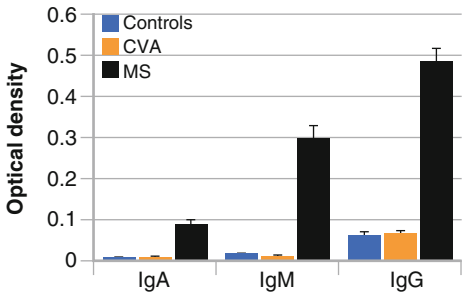

Fig. 11.4 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter lwoffii strain 5866* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

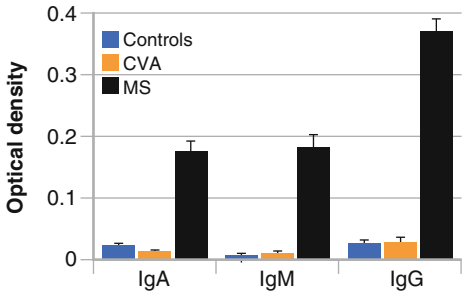

Fig. 11.5 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter radioresistens* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

Significantly elevated levels of IgA antibodies to *Acinetobacter sp. strain 19004* ($p < 0.0001$) and *Acinetobacter junii 17908* ($p < 0.001$) were observed in CVA patients compared to controls.

Levels of IgA antibodies to *Acinetobacter sp. strain 11171* were shown to be significantly higher in controls ($p < 0.001$) than in CVA patients.

No other significant difference were seen in the levels of IgA antibodies to *Acinetobacter lwoffii* 5866 (Fig. 11.4) or *Acinetobacter radioresistens* (Fig. 11.5) between the control and CVA groups.

11.4 IgG Anti-*Acinetobacter* Antibodies in Multiple Sclerosis and CVA Patients

Circulating monomeric IgG antibodies, because of their relatively low molecular weight compared to dimeric IgA or pentameric IgM antibodies can readily cross the blood-brain barrier.

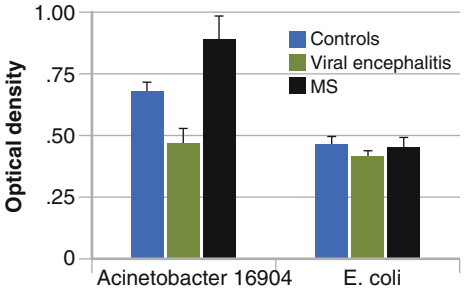

Fig. 11.6 IgG antibody levels (mean ± SE with error bars) to *Acinetobacter calcoaceticus 16904* and *Escherichia coli* in sera from 26 multiple sclerosis patients, 10 viral encephalitis patients and 29 healthy blood donors. Copyright © American Society for Microbiology, Hughes et al. (2001))

Monomeric IgG autoantibodies thus can penetrate to brain tissues such as myelin of nervous tissues and if present in sufficiently high concentrations produce pathological damage, as is observed in Sydenham's chorea patients.

Levels of IgG antibodies to *Acinetobacter sp. strain 11171* ($p < 0.0001$), (Fig. 11.1), *Acinetobacter sp. strain 19004* ($p < 0.0001$) (Fig. 11.2), *Acinetobacter junii 17908* ($p < 0.0001$) (Fig. 11.3), *Acinetobacter lwoffii 5866* ($p < 0.0001$) (Fig. 11.4) and *Acinetobacter radioresistens* ($p < 0.0001$) (Fig. 11.5) were significantly elevated in multiple sclerosis patients compared to healthy controls.

Levels of IgG antibodies to *Acinetobacter sp. strain 11171* ($p < 0.0001$) (Fig. 11.1), *Acinetobacter sp. strain 19004* ($p < 0.01$) (Fig. 11.2), *Acinetobacter junii 17908* ($p < 0.05$) (Fig. 11.3), *Acinetobacter lwoffii 58666* ($p < 0.0001$) (Fig. 11.4) and *Acinetobacter radioresistens* ($p < 0.0001$) (Fig. 11.5) were significantly higher in multiple sclerosis patients compared to CVA patients.

Significantly elevated levels of IgG antibodies to *Acinetobacter sp. strain 19004* ($p < 0.0001$) (Fig. 11.2) and *Acinetobacter junii 17908* ($p < 0.0001$) (Fig. 11.3) were found in CVA patients compared to healthy controls.

However there was no significant difference in the levels of IgG antibodies to *Acinetobacter sp. strain 11171*, *Acinetobacter lwoffii 5866* and *Acinetobacter radioresistens* between the CVA patients and healthy controls.

A further study, measuring IgG antibody levels to *Acinetobacter calcoaceticus 16904* demonstrated a slight elevation in sera from multiple sclerosis patients compared to levels in sera from viral encephalitis patients ($p < 0.02$) and healthy blood donor controls ($p < 0.05$) (Fig. 11.6).

11.5 IgM Anti-*Acinetobacter* Antibodies in Multiple Sclerosis and CVA Patients

Circulating IgM antibodies cannot cross the blood-brain barrier.

Levels of IgM antibodies to *Acinetobacter sp. strain 11171* ($p < 0.0001$) (Fig. 11.1), *Acinetobacter sp. strain 19004* ($p < 0.0001$) (Fig. 11.2), *Acinetobacter junii 17908* ($p < 0.0001$) (Fig. 11.3), *Acinetobacter lwoffii 5866* ($p < 0.0001$)

(Fig. 11.4) and *Acinetobacter radioresistens* ($p<0.0001$) (Fig. 11.5) were significantly higher in multiple sclerosis patients than in the healthy control group.

Levels of IgM antibody to *Acinetobacter sp. strain 11171* ($p<0.0001$) (Fig. 11.1), *Acinetobacter sp.strain 19004* ($p<0.0001$) (Fig. 11.2), *Acinetobacter junii 17908* ($p<0.0001$) (Fig. 11.3), *Acinetobacter lwoffii 5866* ($p<0.0001$) (Fig. 11.4) and *Acinetobacter radioresistens* ($p<0.0001$) (Fig. 11.5) were also shown to be significantly elevated in multiple sclerosis patients compared to CVA patients.

Significantly elevated levels of IgM antibodies to *Acinetobacter sp strain 19004* ($p<0.001$) and *Acinetobacter sp. strain 17908* ($p<0.001$) were found in CVA patients compared to healthy controls.

No significant differences were seen in the level of IgM antibodies to *Acinetobacter sp. strain 11171*, *Acinetobacter lwoffii 5866* and *Acinetobacter radioresistens* between the CVA patients and the control groups.

11.6 Antibodies to *Pseudomonas aeruginosa*

Levels of IgA antibodies to *Pseudomonas aeruginosa* were shown to be significantly higher in multiple sclerosis patients than in the control group ($p<0.001$) (Fig. 11.6) and CVA patients ($p<0.05$) (Fig. 11.7).

There was also a slight elevation in the level of IgA anti-*Pseudomonas* antibodies in the CVA patients ($p<0.05$) when compared to healthy controls.

Elevated levels of IgG antibodies to *Pseudomonas* were also observed in multiple sclerosis sera compared to controls ($p<0.0001$) and also to CVA patients ($p<0.05$).

Furthermore, there was also a slight elevation in the level of IgG antibodies to *Pseudomonas* ($p<0.05$) in the CVA group when compared to controls.

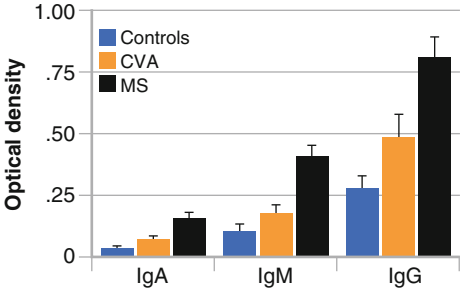

Fig. 11.7 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Pseudomonas aeruginosa* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

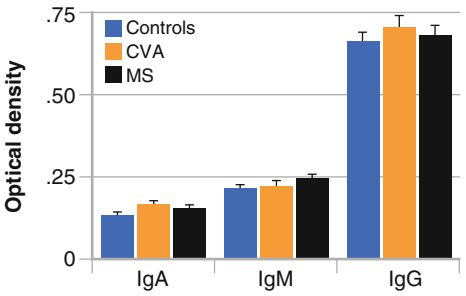

Fig. 11.8 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Escherichia coli* (Symbols: controls, CVA, MS) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

IgM antibody levels to *Pseudomonas* were shown to be significantly elevated in multiple sclerosis patients compared to controls ($p < 0.0001$) and also to CVA patients ($p < 0.001$).

There was no significant difference in the level of IgM antibodies to *Pseudomonas* between the CVA patients and the healthy control group.

11.7 Antibodies to *Escherichia coli* in Multiple Sclerosis Patients

No significant differences in the IgA, IgG and IgM antibody levels to *Escherichia coli* were observed between any of the groups tested (Figs. 11.6 and 11.8).

11.8 Correlation Coefficient Analysis

The correlation coefficient (r) was calculated between all strains of *Acinetobacter* tested and *Pseudomonas aeruginosa.*

There was a significant positive correlation between the IgM levels of *Acinetobacter junii 17908* and *Pseudomonas aeruginosa* ($r = +0.831$; $p < 0.0001$).

A significant positive correlation was also seen between the IgM levels of *Acinetobacter sp. strain 19004* and *Pseudomonas aeruginosa* ($r = +0.819$; $p < 0001$).

There was also a significant correlation between IgA antibodies to *Acinetobacter sp. strain 19004* and *Pseudomonas aeruginosa* ($r = +0.407$, $p < 0.01$).

11.9 Discussion and Pathological Implications

Elevated levels of antibodies directed against several strains of *Acinetobacter* bacteria have been found in multiple sclerosis patients when compared to CVA patients or healthy control subjects.

This would appear to be the first report that multiple sclerosis patients have antibodies against microbial species, such as *Acinetobacter* which are readily found in the environment, for instance on skin, in soil samples and in nasal cavities. The pathological implications would appear to be unclear but it is relevant to note that *Acinetobacter* bacteria possess molecular sequences which resemble those found in myelin and nerve filaments.

Previous studies have suggested that respiratory infections may be involved in the onset of multiple sclerosis. In this study we have looked at potential respiratory pathogens such as *Acinetobacter* bacteria and *Pseudomonas aeruginosa.*

A sequence homology has been found between a known encephalitogenic myelin peptide and the enzyme 4-carboxymucono lactone decarboxylase in both *Acinetobacter* species and *Pseudomonas aeruginosa.*

Another approach has been to suggest that molecular mimicry may operate through variable T-cell recognition (Gran et al. 1999). Several viral and bacterial peptides have been found to activate three of seven T-cell clones isolated from multiple sclerosis patients, specific against myelin basic protein especially involving amino acid positions 85-99.

The bacterial peptide identified was phospho-mannomutase protein in *Pseudomonas aeruginosa* (Wucherpfennig and Strominger 1995).

These results would appear to suggest that antibodies to *Acinetobacter* bacteria are present in multiple sclerosis patients but whether such antibodies have activity against human myelin or human neurofilaments and therefore behave as autoantibodies awaits further studies.

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Chapter 12
Antibodies to *Acinetobacter* Peptide Sequences Resembling Myelin and Neurofilaments in Multiple Sclerosis Patients

12.1 Introduction: The Role of Antibodies to Myelin in Multiple Sclerosis Patients

Multiple sclerosis is the most common demyelinating disease of the central nervous system and antibodies to myelin in such patients have been described by several groups.

Antibody to myelin basic protein extract, obtained from human brain, was measured by radioimmunoassay. Anti-myelin basic protein activity in brain extracts was detected from all 11 patients with multiple sclerosis and was mediated by IgG antibodies (Bernard et al. 1981).

Peptide sequence analysis suggested that the most likely epitope of anti-myelin basic protein was located between residues 84 and 95 of human basic myelin protein, just proximal to the tri-proline sequence located at positions 99–101 (Warren and Catz 1997).

Whether antibodies to specific myelin peptides are useful predictors of progression to clinical multiple sclerosis remains controversial.

Some have suggested antibodies against myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) have been shown to be present more frequently in patients who after a first neurological event, have relapses and go on to develop overt clinical multiple sclerosis (Berger et al. 2003).

However other workers, have suggested, in a multi-centre study involving 462 patients with a first clinical neurological event, that antibodies against myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) as detected by Western blot analysis are not associated with an increased risk of progression to clinically definite multiple sclerosis (Kuhle et al. 2007).

It has been previously suggested that the association of antibodies to *Acinetobacter* bacteria in multiple sclerosis patients may operate by a similar mechanism as that found in patients with Sydenham's chorea where extra-thecal, anti-*streptococcal*

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
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antibodies cross the blood-brain barrier and cause damage to basal ganglia. The damage is caused by IgG1 and IgG3 antibodies which preferentially bind complement and readily cross the blood-brain barrier.

The question arises whether anti-myelin and anti-neurofilament antibodies in multiple sclerosis patients can be linked to the environmental exposure of various *Acinetobacter* bacteria.

12.2 Materials and Methods: Serum Samples, Bacteria and ELISA

Serum Samples

Sera from 26 multiple sclerosis patients (9 males and 17 females having a mean age 42 years, range: 29–55 years) were obtained from the Institute of Neurology at the Hospital for Nervous Diseases, Queen Square, London, as previously described and were diagnosed by the attending neurologists.

Diagnosis was made according to the Poser criteria (Poser et al. 1983) and clinical data of patients are summarised (Table 12.1):

"Benign multiple sclerosis" patients are characterised by infrequent exacerbations but leading to complete recovery. It is mostly seen in young females.

"Acute relapsing remitting multiple sclerosis" patients are those who have more frequent exacerbations followed by partial or complete remission. Some 80 % of multiple sclerosis patients begin with a "relapsing-remitting" course. Many years after an initial onset, a number of "relapsing-remitting" patients will go on to develop "secondary progressive" multiple sclerosis.

Serum samples were obtained from "relapsing remitting" patients during exacerbation and remission.

"Secondary progressive multiple sclerosis" patients are defined as those who continue to deteriorate without remission following an initial "relapsing remitting" course of disease.

"Primary progressive multiple sclerosis" is characterised as continuous deterioration of symptoms without remission from the onset of disease and usually occurs in an older age group.

"Transitional multiple sclerosis" patients are those who are deemed to be between "relapsing remitting" and "secondary progressive" stages.

Serum samples were also obtained from 20 patients in the Department of Geriatric Medicine at University College Hospital, London who had suffered from unilateral hemiplegia due to a "cerebro-vascular accident" (CVA) or stroke (10 males and 10 females having a mean age of 80.5 years, range 69–94 years).

Sera from 25 subjects attending the London Blood Donor services were used as healthy controls (12 males and 13 females, mean age 40.6 years, range 22–67 years).

Table 12.1 Clinical data of multiple sclerosis patients

Laboratory no.	Sex	Age (yr)	Diagnosis	EDSS	Disease duration (Yr)
MS 006	M	53	B	3.0	20
MS 028	F	54	B	NA	NA
MS 033	F	NA	B	NA	NA
MS 051	F	55	B	1.0	25
MS 054	F	49	B	3.0	17
MS 080	F	49	B	1.0	20
MS 007	F	38	RR	2.5	10
MS 042	F	31	RR	3.5	NA
MS 084	F	50	RR	5.5	8
MS 095	F	32	RR	6.5	3
MS 017	F	53	ARR	4.0	10
MS 019	F	29	ARR	4.0	1
MS 030	F	31	ARR	4.0	7
MS 037	F	34	ARR	5.0	7
MS 087	M	40	Transitional	5.0	9
MS 016	M	49	PP	5.5	5
MS 056	M	41	PP	8.0	1
MS 062	M	31	PP	7.0	10
MS 065	M	43	PP	8.0	8
MS 091	M	47	PP	7.5	23
MS 092	F	44	PP	8.5	6
MS 102	F	47	PP	8.0	26
MS 020	M	31	2P	8.0	5
MS 040	F	33	2P	8.0	2
MS 055	M	51	2P	8.0	10
MS 104	F	38	2P	6.5	4

Abbreviations: *M* male, *F* female, *B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *PP* primary progressive, *2P* secondary progressive, *EDSS* expanded disability status scale, *NA* not available

Bacterial Cultures

Cultures of *Acinetobacter sp. strain 11171* and other strains, were provided by the Public Health Laboratory, Nottingham, United Kingdom.

The Department of Microbiology at King's College provided *Pseudomonas aeruginosa* (NCTC 8203).

Cultures were grown as previously described.

ELISA

ELISA studies were carried out as previously described.

For measurement of IgG subclasses by ELISA, purified mouse anti-human IgG1, IgG2, IgG3 or IgG4 (BD Biosciences, Pharmingen), diluted 1/400 and biotin conjugated goat anti-mouse immunoglobulin specific polyclonal antibody (BD Biosciences, Pharmingen), diluted 1/100 was used.

Statistical Analysis

The statistical analysis was carried out as previously described. The mean OD units of control groups which consisted of the CVA group and the healthy blood donors were compared with the mean OD of the 26 multiple sclerosis patients, using a one-tail Student's *t*-test.

Pearson's correlation coefficient (r) was also calculated using the statistical package Prism 9.0 (GraphPad Software).

12.3 Antibodies to *Acinetobacter 11171* in Different Multiple Sclerosis Groups

The multiple sclerosis patients were divided into different clinical groups and their antibody responses investigated.

It was found that "secondary progressive" patients were shown to have significantly elevated levels of IgA antibodies to *Acinetobacter sp. strain 11171* compared to "relapsing remitting" multiple sclerosis ($p<0.001$), "benign" multiple sclerosis ($p<0.02$), "acute relapsing remitting" multiple sclerosis ($p<0.02$) and "primary progressive" multiple sclerosis ($p<0.05$) patients (Fig. 12.1).

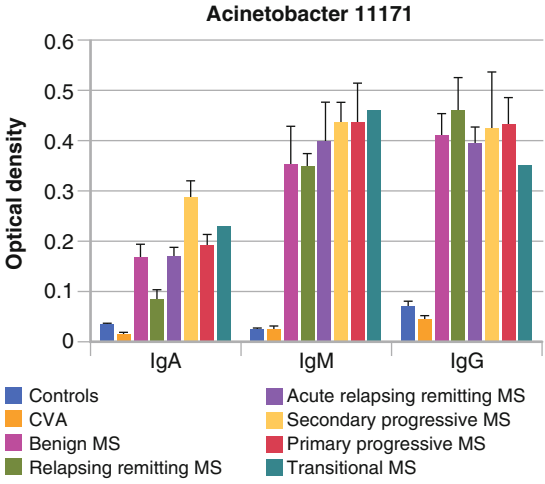

Fig. 12.1 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter sp. strain 11171* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

The multiple sclerosis patients described as "primary progressive" were also shown to have significantly elevated levels of IgA antibodies to *Acinetobacter sp, strain 11171* compared to "relapsing remitting" patients ($p < 0.01$) (Fig. 12.1).

12.4 Antibodies to Myelin Basic Protein in Multiple Sclerosis and CVA Patients

Significantly elevated levels of IgA antibodies to myelin basic protein were observed in multiple sclerosis sera compared to controls ($p < 0.0001$) and CVA sera ($p < 0.001$) (Fig. 12.2).

However no significant difference was observed between the control and CVA groups, when measuring IgA antibody levels.

Increased levels of anti-myelin basic protein IgG antibodies were also seen in multiple sclerosis sera compared to controls ($p < 0.0001$) and CVA sera ($p < 0.01$) (Fig. 12.2).

Multiple sclerosis patients also showed significantly higher levels of IgM antibodies to myelin basic protein than those seen in controls ($p < 0.0001$) and CVA patients ($p < 0.0001$) (Fig. 12.2).

There were also increased levels of anti-myelin basic protein IgM and IgG antibodies in CVA patients compared to controls ($p < 0.0001$) (Fig. 12.2).

However there were no significant differences in the levels of IgA, IgM or IgG antibodies to myelin basic protein between the different groups of multiple sclerosis patients.

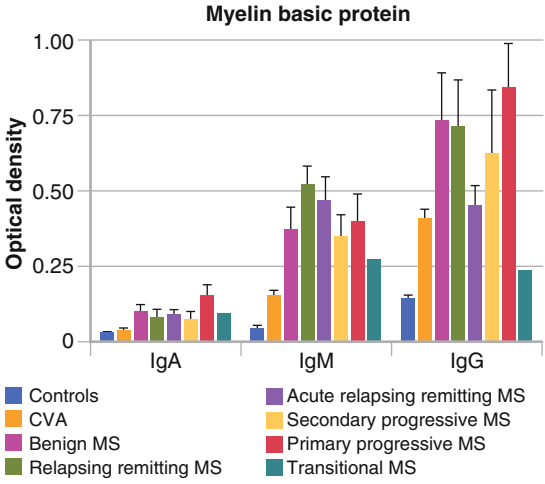

Fig. 12.2 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to myelin basic protein in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

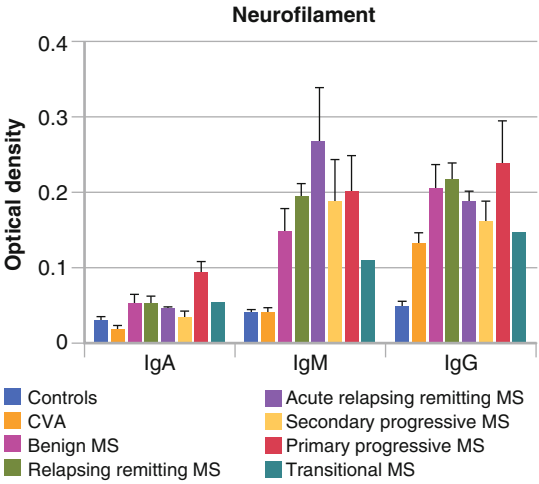

Fig. 12.3 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to neurofilaments in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Copyright © American Society for Microbiology, Hughes et al. (2001))

12.5 Antibodies to Neurofilaments in Multiple Sclerosis and CVA Patients

Levels of IgA antibody to neurofilaments were shown to be significantly higher in multiple sclerosis patients than in controls ($p<0.01$) and CVA patients ($p<0.0001$) (Fig. 12.3).

No significant difference between the control and CVA groups was seen in the levels of anti-neurofilament IgA antibodies.

Multiple sclerosis patients also showed significantly elevated IgG anti-neurofilament antibodies compared to the controls ($p<0.0001$) and CVA patients ($p<0.01$) (Fig. 12.3).

The levels of IgG antibodies to neurofilaments was significantly increased in CVA patients compared to controls ($p<0.0001$).

Significantly elevated levels of IgM antibodies to neurofilaments were observed in multiple sclerosis sera compared to those from healthy controls ($p<0.0001$) and CVA patients ($p<0.0001$).

There was no significant difference between the control and CVA groups in the levels of anti-neurofilament IgM antibodies.

No significant differences in the levels of IgA, IgM and IgG antibodies to neurofilaments were found between the different groups of multiple sclerosis patients.

12.6 IgG Subclass Antibodies to *Acinetobacter* Species

Significantly elevated levels of IgG1 and IgG3 antibodies to all strains of *Acinetobacter* species were observed in multiple sclerosis sera when compared to controls ($p<0.0001$) and CVA sera ($p<0.0001$) (Figs. 12.4, 12.5, 12.6, 12.7, 12.8 and 12.9).

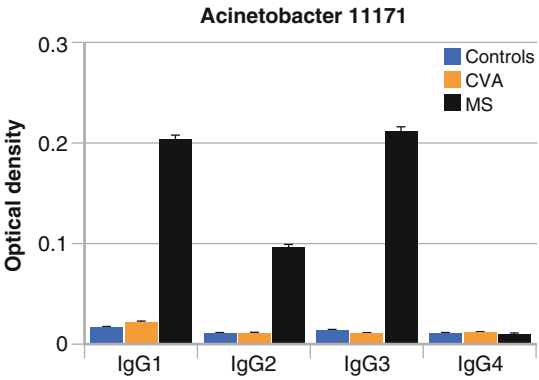

Fig. 12.4 Levels of IgG1, IgG2, IgG3 and IgG4 antibody levels to *Acinetobacter 11171* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Reprinted with permission from Dr. L. Hughes)

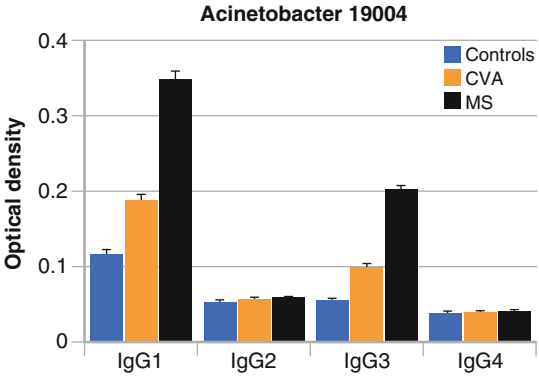

Fig. 12.5 Levels of IgG1, IgG2, IgG3 and IgG4 antibody levels to *Acinetobacter 19004* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Reprinted with permission from Dr. L. Hughes)

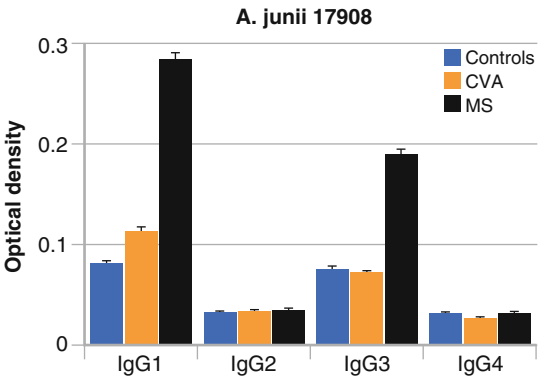

Fig. 12.6 Levels of IgG1, IgG2, IgG3 and IgG4 antibody levels to *Acinetobacter junii 17908* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Reprinted with permission from Dr. L. Hughes)

Significantly higher levels of IgG1 to *Acinetobacter 11171* ($p<0.05$) (Fig. 12.4), *Acinetobacter 19004* ($p<0.001$) (Fig. 12.5) and *Acinetobacter junii 17908* ($p<0.0001$) (Fig. 12.6) and IgG3 to *Acinetobacter 19004* ($p<0.0001$) (Fig. 12.5) were found in CVA patients compared to healthy controls.

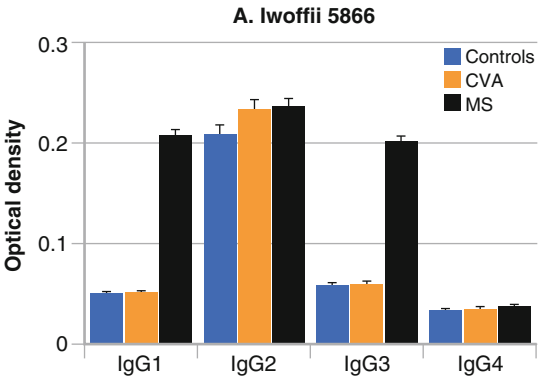

Fig. 12.7 Levels of IgG1, IgG2, IgG3 and IgG4 antibody levels to *Acinetobacter lwoffii 5866* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Reprinted with permission from Dr. L. Hughes)

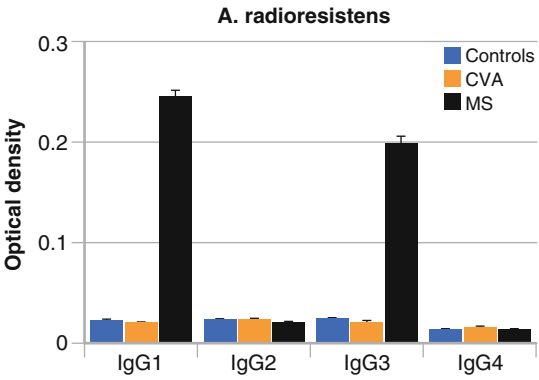

Fig. 12.8 Levels of IgG1, IgG2, IgG3 and IgG4 antibody levels to *Acinetobacter radioresistens* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Reprinted with permission from Dr. L. Hughes)

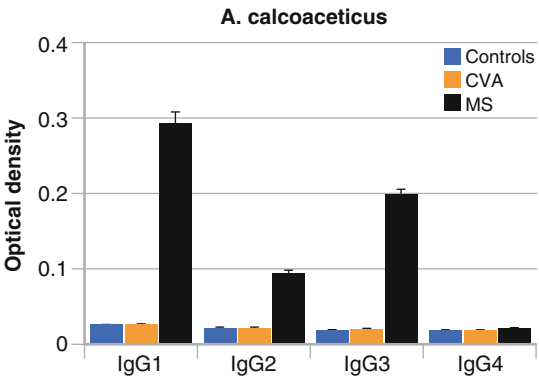

Fig. 12.9 Levels of IgG1, IgG2, IgG3 and IgG4 antibody levels to *Acinetobacter calcoaceticus* in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Reprinted with permission from Dr. L. Hughes)

No significant differences in the levels of IgG2 antibodies to *Acinetobacter 19004*, *Acinetobacter junii 17908* or *Acinetobacter radioresistens* or IgG4 antibodies to all strains of *Acinetobacter* tested were observed in multiple sclerosis patients compared to either CVA patients or healthy controls.

Furthermore when patients were divided into different multiple sclerosis, "primary progressive" multiple sclerosis patients were shown to have elevated levels of IgG3 and IgG1 antibodies to *Acinetobacter junii 11171* compared to "secondary progressive" multiple sclerosis patients ($p<0.01$).

IgG1 antibodies to *Acinetobacter 11171* were demonstrated to be higher in "primary progressive" multiple sclerosis patients when compared to "relapsing remitting" multiple sclerosis patients ($p<0.01$).

12.7 Correlation Coefficient Analysis

Values previously obtained for the different species of *Acinetobacter* and *Pseudomonas* bacteria were used in the correlation coefficient calculations.

Antibodies to *Acinetobacter sp. strain 19004* showed also a significant positive correlation with IgA ($r=+0.795$, $p<0.0001$), IgG ($r=+0.868$, $p<0.0001$) and IgM antibodies to myelin basic protein ($r=+0.784$, $p<0.0001$).

Antibodies to *Acinetobacter sp. strain 19004* showed also a significant positive correlation with IgM ($r=+0.784$, $p<0.0001$) and IgG ($r=+0.615$, $p<0.001$) antibodies to neurofilaments.

Antibodies to *Acinetobacter junii 17908* showed a significant positive correlation with IgM ($r=+0.865$, $p<0.0001$) and IgG ($r=+0.708$, $p<0.0001$) antibodies to basic myelin protein.

Antibodies to *Acinetobacter junii 17908* showed a significant positive correlation with IgM ($r=+0.769$, $p<0.0001$) and IgG ($r=+0.765$, $p<0.0001$) antibodies to neurofilaments.

A significant positive correlation was seen between the IgM levels of *Pseudomonas aeruginosa* and antibodies to myelin basic protein ($r=+0.851$, $p<0.0001$).

Also there was a significant positive correlation between the IgM levels of *Pseudomonas aeruginosa* and antibodies to neurofilaments ($r=+0.693$, $p<0.0001$) but there was no significant correlation with IgG or IgA antibodies.

A significant positive correlation was also seen between the IgA ($r=+0.399$, $p<0.05$), IgM ($r=+0.663$, $p<0.001$) and IgG ($r=+0.772$, $p<0.0001$) levels of myelin basic protein and neurofilaments.

12.8 Pathological Implications: *Acinetobacter* as a Possible Aetiological Agent in Multiple Sclerosis

Elevated levels of antibodies directed against several strains of *Acinetobacter* bacteria as well as against myelin basic protein and neurofilaments have been found in multiple sclerosis patients when compared to CVA patients or healthy control subjects.

Furthermore elevated levels of IgG1 and IgG3 antibodies to various *Acinetobacter* species were demonstrated to be present in the multiple sclerosis patients. Such antibodies can cross the blood brain barrier and if present in sufficiently high concentrations, could activate the complement cascade and cause damage to myelin tissues and associated neurofilaments.

The antigenic targets of basic myelin protein and neurofilaments in this study came from bovine material, as elevated levels of antibodies to *Acinetobacter* species had previously been shown in animals affected by bovine spongiform encephalopathy (BSE).

Whether such animals can be considered as experimental animal models of multiple sclerosis awaits further studies.

However the antibodies described here against myelin basic protein and neurofilaments cannot be considered as autoantibodies until such studies are carried out with antigens derived from human sources.

The pathological implications would appear to be unclear but it is relevant to note that *Acinetobacter* bacteria possess molecular sequences which resemble those found in myelin and nerve filaments and therefore such bacteria need to be excluded as possible aetiological agents involved in the onset of multiple sclerosis.

Previous studies have suggested that various respiratory childhood infections may be involved in the onset of multiple sclerosis in over 50 % of patients. Such patients with respiratory infections have more severe neurological symptoms as well as frequent periods of exacerbations of the disease (Lamoureux et al. 1983).

Other groups have emphasized an increased incidence of viral infections preceding the onset of multiple sclerosis (Andersen et al. 1993).

Longitudinal prospective studies would appear to suggest that exacerbations in multiple sclerosis are associated with various infections (Buljevac et al. 2002).

In this study we have looked at potential respiratory pathogens such as *Acinetobacter* bacteria and *Pseudomonas aeruginosa* who possess sequences cross-reacting with brain antigens, especially myelin and neurofilaments as possible agents triggering the disease.

The description of molecular mimicry or an amino acid sequence homology between a known encephalitogenic myelin peptide and the enzyme 4-carboxymucono lactone decarboxylase found in both *Acinetobacter* species and *Pseudomonas aeruginosa* could be relevant in the pathology of the disease.

However not only bacterial but also many viral antigens cross-react with brain components. Precise peptide studies are required to identify such sequences which are relevant in the pathology of multiple sclerosis (Wucherpfennig and Strominger 1995).

These results would appear to suggest that definite antibodies against *Acinetobacter* bacteria are present in multiple sclerosis patients as well as antibodies to myelin basic protein and neurofilaments.

The question arises whether such objective measurements could assist in the identification of multiple sclerosis patients, in a similar manner as the Myelin-Acinetobacter-Neurofilament (MAN) index has been used in the study of animals affected by bovine spongiform encephalopathy (Wilson et al. 2003).

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Chapter 13
The Myelin-*Acinetobacter*-Neurofilament Index in an Attempt to Diagnose Multiple Sclerosis

13.1 Introduction: The Need for a Laboratory Test of Multiple Sclerosis

The diagnosis of multiple sclerosis is difficult and depends on clinical criteria because the pathological process may affect many different nerves which lead to various and protean neurological signs and symptoms.

Some mimicking diseases can be excluded such as Lyme disease, but there is no simple blood test for multiple sclerosis.

However over 80 % of multiple sclerosis patients have detectable abnormalities in their cerebro-spinal fluid (CSF), such as oligoclonal bands (Link and Huang 2006).

The presence of two or more oligoclonal bands suggests the presence of disease activity in multiple sclerosis patients (Davies et al. 2003).

Other groups have suggested that antibodies to myelin or "myelin oligodendrocyte glycoprotein" (MOG) could be used to detect patients who will go on to develop multiple sclerosis following an initial neurological event (Klawiter et al. 2010).

Antibodies to MOG have been reported to be present in 38 % of multiple sclerosis patients compared to antibodies to myelin basic protein being present in only 28 % of such patients (Reindl et al. 1999).

Neurodegeneration with leakage of axonal components such as neurofilaments would appear to be a component of pathological damage in multiple sclerosis (Teunissen and Khalil 2012).

Antibodies to neurofilaments may also be used in the assessment of disease activity (Gahan et al. 1985) and release of axonal proteins would appear to be associated with progressive disease in multiple sclerosis patients as well as with clinical disability (Semra et al. 2002).

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Previous studies have shown that animals affected by bovine spongiform encephalopathy could be detected in many cases by using a composite parameter, the "Myelin-*Acinetobacter*-Neurofilament" antibody index.

Since antibodies to these three components have been investigated, it is proposed to assess whether a similar composite index the "myelin-*Acinetobacter*-neurofilament" assay could be calculated for multiple sclerosis patients.

13.2 Materials and Methods: Serum Samples, Bacteria and ELISA

Serum Samples

Sera from 26 multiple sclerosis patients were obtained from the Institute of Neurology at the Hospital for Nervous Diseases, Queen Square, London, as previously described.

Diagnosis was made according to the Poser criteria and the following abbreviations have been used to describe the patients:

CVA: Serum samples were obtained from 20 patients in the Department of Geriatric Medicine at University College Hospital, London who had suffered from a unilateral hemiplegia due to a "cerebro-vascular accident" or stroke.

B: "Benign multiple sclerosis" patients are characterised by infrequent exacerbations but leading to complete recovery.

RR: "Relapsing remitting" course are patients who have relapses followed by remissions.

ARR: "Acute relapsing remitting multiple sclerosis" patients are those who have more frequent exacerbations followed by partial or complete remission.

Secondary P: "Secondary progressive multiple sclerosis" patients are defined as those who continue to deteriorate without remission following an initial "relapsing remitting" course of disease.

PP: "Primary progressive multiple sclerosis" are patients who are characterised by a continuous deterioration of symptoms without remission from the onset of disease.

Trans: "Transitional multiple sclerosis" patients are those who are deemed to be between "relapsing remitting" and "secondary progressive" stages.

Sera from 25 subjects attending the London Blood Donor services were used as healthy controls.

Bacterial Cultures and ELISA

Bacterial cultures and ELISA studies were carried out as previously described. The coefficient of variation was less than 10 % for all ELISA tests carried out.

Statistical Analysis

Statistical calculations were carried out as previously described.

13.3 Calculation of the Myelin.*Acinetobacter.*Neurofilament Index in Multiple Sclerosis Groups

The MAN (Myelin-*Acinetobacter*-Neurofilament) index was calculated in both multiple sclerosis patients and healthy controls, as previously described for "bovine spongiform encephalopathy":

$$\text{M.A.N. Index} = (\text{Ig MBP} \quad 10) \times (\text{Ig } \textit{Acinetobacter} \times 10) \times (\text{Ig Neurofilament} \times 10)$$

The 99.9 % confidence limits (CL) of the controls were calculated as follows: = Mean ± 3 SD (standard deviations)

The MAN index was expressed as $\log_{10}$ in sera and controls.

13.4 Results of the "Myelin.*Acinetobacter.*Neurofilament" Index Calculations in Multiple Sclerosis Patients

The "myelin.*Acinetobacter.*neurofilament" index was calculated for five strains of *Acinetobacter* species and once with *Pseudomonas* bacteria.

Since only IgG antibodies are likely to cross the blood-brain barrier, the MAN index was calculated using this immunoglobulin isotype.

All multiple sclerosis patients had values above the 99.9 % confidence limits of the controls, when the MAN index was calculated with *Acinetobacter lwoffii 5866* (Fig. 13.1), *Acinetobacter sp, strain 11171* (Fig. 13.2), *Acinetobacter radioresistens* (Fig. 13.3), *Acinetobacter sp. strain 19004* (Fig. 13.4) and *Acinetobacter junii 17908* (Fig. 13.5).

Only 88.5 % of multiple sclerosis were shown to have a MAN index above the 99.9 % confidence limit, when calculating with *Pseudomonas aeruginosa* antibodies (Fig. 13.6).

In all cases only one control was shown to lie above the 99.9 % confidence limit.

Seven CVA patients were shown to have values above the 99.9 % confidence limits when the MAN index was calculated using the *Acinetobacter sp. strain 11171* (Fig. 13.2).

For all other strains of *Acinetobacter* and *Pseudomonas aeruginosa* more than 12 CVA patients had MAN indices above the 99.9 % confidence limits.

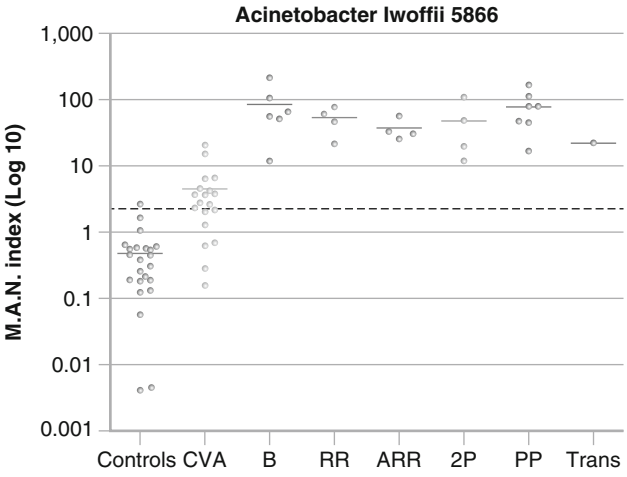

Fig. 13.1 MAN index calculated for *Acinetobacter lwoffii 5866*, expressed as $\log_{10}$ in sera from 26 multiple sclerosis patients (*B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *2P* secondary progressive, *PP* primary progressive, *Trans* transitional), 20 CVA patients and 25 healthy blood donors. *Dashed line* represents 99.9 % confidence limit of controls (Copyright © American Society for Microbiology, Hughes et al. (2001))

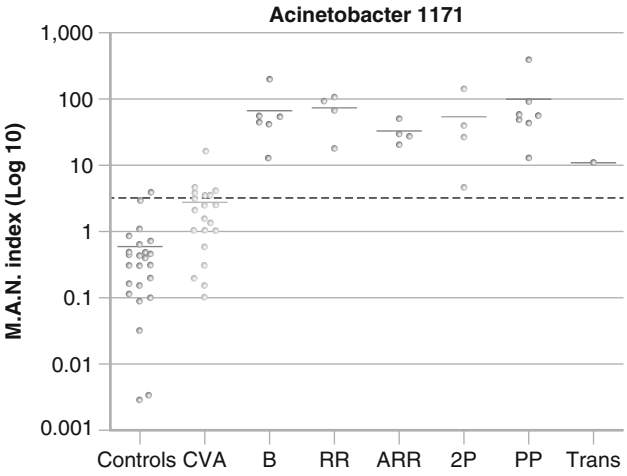

Fig. 13.2 MAN index calculated for *Acinetobacter 11171*, expressed as $\log_{10}$ in sera from 26 multiple sclerosis patients (*B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *2P* secondary progressive, *PP* primary progressive, *Trans* transitional), 20 CVA patients and 25 healthy blood donors. *Dashed line* represents 99.9 % confidence limit of controls (Copyright © American Society for Microbiology, Hughes et al. (2001))

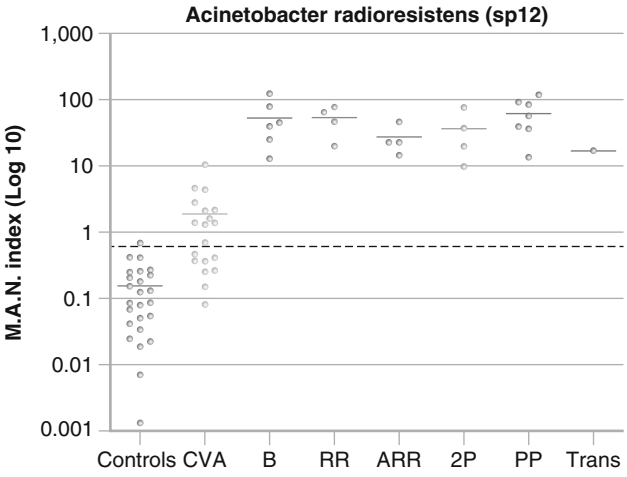

Fig. 13.3 MAN index calculated for *Acinetobacter radioresistens (sp 12)*, expressed as $\log_{10}$ in sera from 26 multiple sclerosis patients (*B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *2P* secondary progressive, *PP* primary progressive, *Trans* transitional), 20 CVA patients and 25 healthy blood donors. *Dashed line* represents 99.9 % confidence limit of controls (Copyright © American Society for Microbiology, Hughes et al. (2001))

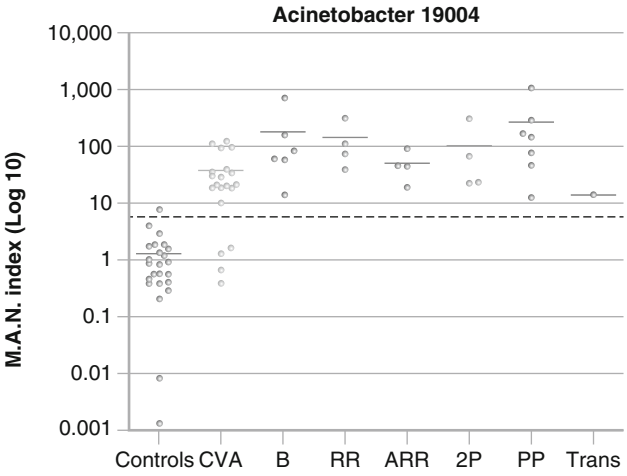

Fig. 13.4 MAN index calculated for *Acinetobacter 19004*, expressed as $\log_{10}$ in sera from 26 multiple sclerosis patients (*B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *2P* secondary progressive, *PP* primary progressive, *Trans* transitional), 20 CVA patients and 25 healthy blood donors. *Dashed line* represents 99.9 % confidence limit of controls (Copyright © American Society for Microbiology, Hughes et al. (2001))

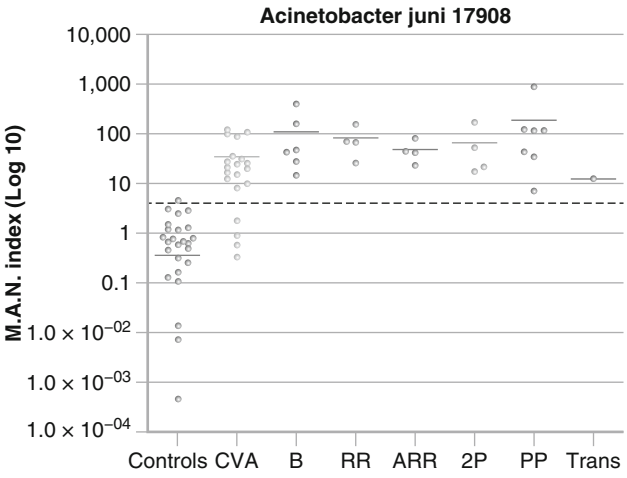

Fig. 13.5 MAN index calculated for *Acinetobacter junii 17908*, expressed as $\log_{10}$ in sera from 26 multiple sclerosis patients (*B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *2P* secondary progressive, *PP* primary progressive, *Trans* transitional), 20 CVA patients and 25 healthy blood donors. *Dashed line* represents 99.9 % confidence limit of controls (Copyright © American Society for Microbiology, Hughes et al. (2001))

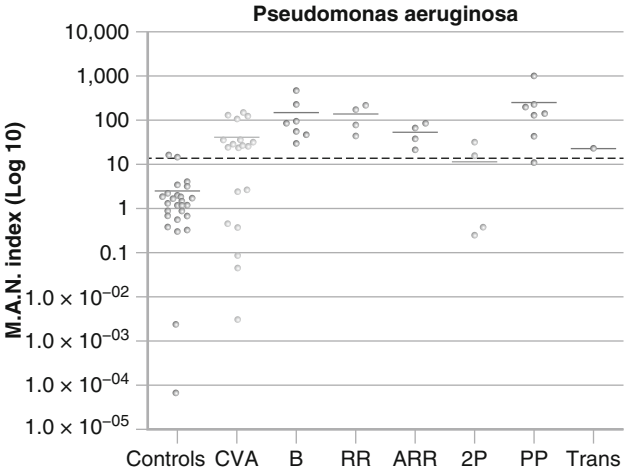

Fig. 13.6 MAN index calculated for *Pseudomonas aeruginosa*, expressed as $\log_{10}$ in sera from 26 multiple sclerosis patients (*B* benign, *RR* relapsing remitting, *ARR* acute relapsing remitting, *2P* secondary progressive, *PP* primary progressive, *Trans* transitional), 20 CVA patients and 25 healthy blood donors. *Dashed line* represents 99.9 % confidence limit of controls (Copyright © American Society for Microbiology, Hughes et al. (2001))

No significant differences were seen in the MAN indices between the different multiple sclerosis groups.

However when calculating the MAN index using *Acinetobacter radioresistens* all multiple sclerosis patients could be distinguished from the two control groups, the healthy individuals and the CVA patients acting as a "disease control" group (Fig. 13.3).

When removing the *Acinetobacter* parameter, the MAN index could distinguish multiple sclerosis patients from healthy controls but not from the CVA group.

When the MAN index was calculated with IgA and IgM antibodies, immunoglobulin isotypes that do not cross readily the blood-brain barrier, the differences between the multiple sclerosis patients and control groups were not as significant, when compared to the calculations carried out with the IgG isotype.

Whether the MAN index can be useful in the diagnosis, progression and follow-up of multiple sclerosis patients requires further studies involving many neurological centres.

13.5 Intrathecal Production of Antibodies and *Acinetobacter*

The origin of antibodies to environmental agents such as viruses or bacteria in causing a neurological disease poses the question as to where is the site of production of such immunological markers.

For instance anti-*streptococcal* antibodies in Sydenham's chorea are produced in the tonsils and related lymph nodes. Then the IgG isotype antibodies cross the blood-brain barrier and affect the basal ganglia with consequent clinical and neurological complications.

Auto-antibodies specific for myelin and myelin oligodendrocyte glycoprotein (MOG) were bound to disintegrating myelin around axons in lesions of acute multiple sclerosis and also in the marmoset model of experimental allergic encephalomyelitis (Genain et al. 1999).

Intrathecal anti-MOG antibody production is elevated in patients with multiple sclerosis. The recombinant myelin oligodendrocyte glycoprotein (rMOG) consists of the 120 amino acid of the extracellular domain of MOG. The rMOG index was elevated in multiple sclerosis patients compared to controls.

Patients with progressive multiple sclerosis had higher indices than patients with relapsing-remitting multiple sclerosis (Klawiter et al. 2010).

Previous studies have shown that over 90 % of patients with clinically definite or probable multiple sclerosis showed abnormal polyacrylamide gel electrophersis patterns in the forms of oligoclonal bands together with cytological presence in the cerebro spinal fluid (CSF) of atypical large lymphocytes and plasma cells (Thompson et al. 1979).

The origin of these antibodies was ascribed to intrathecal production by immune cells which had been stimulated by agents present within the nervous tissues or by autoimmune activity (Newcombe et al. 1985).

Studies on intrathecal synthesis of antibodies to a number of viruses, such as measles, rubella, para-influenza type 2, respiratory syncytial virus, mumps, influenza A, influenza B, adeno and herpes simplex virus showed fluctuations which did not correlate with the clinical course of the disease (Arnadottir et al. 1982). The authors concluded that the viral antibodies studied were not relevant to the aetiology and pathogenesis of multiple sclerosis.

Multiple sclerosis is a disease with extensive heterogeneity in the clinical course as well as in pathological lesions. Some post-mortem studies have shown that the patterns of demyelination are heterogeneous between patients (Lucchinetti et al. 2000).

13.6 Pathological Implications: *Acinetobacter* as an Extra-thecal Aetiological Agent in Multiple Sclerosis

The question arises where were the anti-*Acinetobacter* antibodies produced which had been observed in patients with multiple sclerosis. Paired serum and cerebrospinal fluid samples from multiple sclerosis were compared to other neurological diseases. There was no greater incidence of high affinity antibodies in the CSF compared to serum in multiple sclerosis patients compared to patients suffering from other neurological diseases. This suggests that there is no intrathecal production of antibodies to *Acinetobacter* bacteria (Chapman et al. 2005).

Clearly it would appear that antibodies to *Acinetobacter* behave in the same way as anti-*streptococcal* antibodies in rheumatic fever and Sydenham's chorea.

The antibodies are produced in an extra-thecal space, in the case of Sydenham's chorea in the tonsils and related lymph nodes and in the case of multiple sclerosis probably in the lymph nodes associated with the upper respiratory tract and nasal sinuses.

The antibodies then cross from the serum across the blood-brain barrier to the cerebro-spinal fluid and if present in sufficiently high concentrations, then IgG1 and IgG3 antibodies will activate the complement cascade and cause myelin and neurofilament pathology.

The *Acinetobacter* microbe is frequently isolated from nasal sinuses (Casiano et al. 2001) and sinusitis has been described to be present in many multiple sclerosis patients (Gay et al. 1986).

These observations would appear to be consistent with the previous reports that respiratory infections are somehow associated with the onset or progression of multiple sclerosis.

Autoantibodies occur following tissue damage, such as those observed after myocardial infarctions or burns. A similar situation would appear to occur in CVA patients following damage to brain tissues with production of autoantibodies binding to myelin basic protein or neurofilaments.

Further studies are clearly required to assess whether particular sequences of *Acinetobacter* would provide better antigenic epitopes to study and follow the progression of patients affected by multiple sclerosis.

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Chapter 14
Antibodies to Short Synthetic *Acinetobacter* and *Pseudomonas* Peptide Sequences Resembling Myelin and Neurofilaments in Multiple Sclerosis Patients

14.1 Introduction: The Use of Synthetic Peptide Sequences of Myelin and Neurofilaments to Study the Role of Antibodies in Multiple Sclerosis Patients

Peptide sequences of myelin basic protein and neurofilaments are relevant in the study of these epitopes for their role in the aetiology of multiple sclerosis.

Proteonomic investigations have identified an amino acid homology between a sequence present in 4-carboxy-muconolactone decarboxylase of *Acinetobacter calcoaceticus,* γ-carboxymuconolactone decarboxylase of *Pseudomonas aeruginosa* and a sequence of myelin basic protein (residues 110–124).

A peptide sequence of myelin basic protein (residues 110–124) was found to produce experimental allergic encephalomyelitis (EAE) in guinea pigs (Ben-Nun et al. 1981).

A similar epitope of myelin basic protein (residues 101–120) also induces experimental allergic encephalomyelitis in DA rats (Stepaniak et al. 1997).

Myelin basic protein (residues 82–100) have been demonstrated to be immunodominant in multiple sclerosis patients for both T cells and autoantibodies (Salvetti et al. 1993).

Generation of T cell clones from multiple sclerosis patients defined the specific peptide of myelin basic protein (residues 110–124) in their induction (Mazza et al. 2002).

Another sequence has been identified involving an epitope of myelin oligodendrocyte glycoprotein (MOG 43–57) which is known to induce experimental allergic encephalomyelitis in many mouse strains including Biozzi mice (Amor et al. 1994).

A closely related sequence to myelin oligodendrocyte glycoprotein (MOG) is found in 3-oxoadipate CoA transferase sub-unit in *Acinetobacter calcoaceticus* (residues 83–97) (Table 14.1). This sequence can also be found in the same enzyme of *Pseudomonas putida.*

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Table 14.1 15-mer peptides used in ELISA and EAE studies comparing similar sequences in mouse myelin basic protein and myelin oligodendrocyte glycoprotein to *Acinetobacter* sp. and *Pseudomonas aeruginosa* (identical amino acids in bold)

Peptide	Source	Amino acid sequence	Amino acid position	Location
1	*Acinetobacter sp.*	QNFIS**RF**A**WG**EVNSR	38–52	4-carboxy-muconolactone decarboxylase
2	*Pseudomonas aeruginosa*	QEMIT**R**H**AWG**D1WTR	38–52	γ-carboxy-muconolactone decarboxylase
3	MBP	GLSL**SRF**S**WG**AEGQR	110–124	
4	*Acinetobacter sp.*	DSYVFDE**LYR**A**GK**IB	83–97	3-oxoadipate Co A-transferase subunit A
5	MOG	PFSRVVH**LYR**N**GK**DQ	43–57	
6	Human papilloma virus (type 16)	TVIQDGDMVHTGFGA	219–233	Major capsid protein LI

Retrieved from Swissprot
Reprinted from Hughes et al. (2003), with permission from Elsevier
The human papilloma virus sequence was used as a control peptide
A alanine, *D* aspartic acid, *E* glutamic acid, *F* phenylalanine, *G* glycine, *H* histidine, *I* isoleucine, *K* lysine, *L* leucine, *M* methionine, *N* asparagine, *P* proline, *Q* glutamine, *R* arginine, *S* serine, *T* threonine, *V* valine, *W* tryptophan, *Y* tyrosine

Recombinant myelin oligodendrocyte glycoprotein (MOG) T cells from multiple sclerosis patients have been shown to respond to three main regions of myelin oligodendrocyte glycoprotein (MOG) including MOG (residues 34–56) (Kerlero de Rosbo et al. 1997).

Similar observations were made by other workers, linking the responses to the clinical course of the disease (Correla and de los Milagros 2003).

Anti-MOG antibodies in multiple sclerosis patients have a highly variable epitope specificity depending on the stage of the multiple sclerosis disease (Haase et al. 2001).

Some groups have demonstrated increased autoantibodies to MOG (residues 35–55) especially in secondary progressive multiple sclerosis (Kennel de March et al. 2003).

Clearly elevated levels of autoantibodies to myelin basic protein and myelin oligodendrocyte glycoprotein have been reported by several groups to be present in patients with multiple sclerosis.

The aims of the present study were to determine whether multiple sclerosis patients have elevated levels of antibodies to the mimicry peptides found in *Acinetobacter* and *Pseudomonads aeruginosa* species, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) when compared to stroke patients or healthy blood donors.

14.2 Materials and Methods: Serum Samples, Peptides and ELISA

Serum Samples

Sera from 26 multiple sclerosis patients (9 males and 17 females having a mean age 42 years, range: 29–55 years) were obtained from the Institute of Neurology at the Hospital for Nervous Diseases, Queen Square, London, as previously described.

Serum samples were also obtained from 20 patients in the Department of Geriatric Medicine at University College Hospital, London who had suffered from unilateral hemiplegia due to a "cerebro-vascular accident" (CVA) or stroke (10 males and 10 females having a mean age of 80.5 years, range 69–94 years).

Sera from 25 subjects attending the London Blood Donor services were used as healthy controls (12 males and 13 females, mean age 40.6 years, range 22–67 years).

Peptides

Myelin basic protein (MBP) (residues 110–124) with amino acid sequences according to mouse myelin basic protein was synthesized which resembles the sequence found in human myelin basic protein. The only difference is that MBP differs from the human sequence at position 122 (Lys to Arg).

Myelin oligodendrocyte glycoprotein (MOG) peptide (residues 43–57) was synthesized according to mouse MOG and closely resembles the amino acid sequence found in human MOG.

Bacterial mimicry peptides, *Acinetobacter* 4-carboxymuconolactone decarboxylase, *Pseudomonas* γ-carboxy-mucono-lactone decarboxylase and *Acinetobacter* 3-oxoadipate CoA-transferase subunit A were also synthesized (Table 14.1).

The 15-mer peptides incorporated a C-terminal amide and were purified via High-performance liquid chromatography (HPLC) (ABC Biotechnology, UK).

Preparation of MOG and MBP

Recombinant MOG (rmMOG) (amino acid residues 1–116) was synthesized as follows.

Escherichia coli strain JM109 was transfected with the cDNA encoding murine MOG N-terminal 1–116 amino acids sequence ligated to pRSET A (Invitrogen) expression vector.

The pRSET A vector encodes an N-terminal fusion peptide, upstream and in-frame of the MOG DNA insert.

The culture was grown in SOB media containing 100 mg/ml ampicillin and 17 mg/ml chloramphenicol (temperature 37 °C, 95 % O_2/5 % CO_2; 220 rpm).

RmMOG expression was initiated by adding 1 mM isopropyl-b-thiogalactopyranoside (IPTG) (Sigma).

Affinity purification of the soluble rmMOG was achieved by TALON metal affinity resin (Clontech), which binds the 6-histidine residues present in the fusion protein.

The protein-resin complex was added to the column and non-bound proteins released from the column by repeated washing.

The rmMOG protein was prepared by elution from columns and fractions containing the rmMOG collected, pooled and tested by UV spectrophotometer.

The eluted protein was dyalised for 16 h and concentrated via Centricon Biomax-5 K concentrating tubes at 4,000 g.

Myelin basic protein (MBP) from guinea pig brain was obtained from Sigma-Aldrich.

ELISA

ELISA studies were carried out as previously described.

Statistical Analysis

The mean OD units of control groups (CVA and healthy blood donors) were compared with the mean OD of the 26 multiple sclerosis patients, using a one-tail Student's t-test and 95 % confidence limits of control groups were calculated.

14.3 Molecular Mimicry Between MOG and *Acinetobacter*

The Swissprot database was used to identify any amino acid sequence that showed similarities or homologies between *Acinetobacter* and myelin oligodendrocyte glycoprotein (MOG).

A sequence similarity was found between MOG (residues 50–55), **LYRNGK** and *Acinetobacter* 3-oxoadipate CoA-transferase unit (residues 90–95) **LYRAGK.**

14.4 Multiple Sclerosis Patients Respond to Bacterial Peptide Sequences

The antibody responses to bacterial peptides was investigated by ELISA in sera collected from multiple sclerosis patients.

Significantly elevated levels of antibodies to the mimicry peptides were found to be present in multiple sclerosis patients when compared to CVA patients and healthy controls.

However no elevation was seen to an irrelevant peptide from Human Papilloma virus (HPV) in multiple sclerosis patients compared to the control groups.

Furthermore no correlations were seen between antibody responses to these peptides with either age or sex of the multiple sclerosis patients.

14.5 Results to Peptide 1: Antibodies to *Acinetobacter* 4-carboxy Muconolactone Decarboxylase (QNFISRFAWGEVNSR)

Levels of IgA antibody to **SRF**A**WG** peptide were shown to be significantly elevated in multiple sclerosis sera (0.044 ± 0.004) when tested against control sera (0.013 ± 0.001) ($t = 6.51$, $p < 0.0001$) and CVA patients (0.008 ± 0.002) ($t = 6.96$, $p < 0.0001$) (Fig. 14.1).

Increased levels of IgG antibody to anti-**SRF**A**WG** peptide (0.10 ± 0.007) were also seen in multiple sclerosis patients in comparison to the control group IgG (0.012 ± 0.003) ($t = 9.58$, $p < 0.0001$) and CVA patients IgG (0.02 ± 0.006) ($t = 7.26$, $p < 0.0001$).

Increased levels of IgM antibody to anti-**SRF**A**WG** peptide (0.059 ± 0.007) were also seen in multiple sclerosis in comparison to the control group IgM (0.011 ± 0.003) ($t = 5.66$, $p < 0.0001$) and CVA patients IgM (0.0005 ± 0.0004) ($t = 6.44$, $p < 0.0001$).

No significant differences were seen in the levels of either IgA or IgG anti-**SR**AWG peptide between control subjects and CVA patients.

Furthermore there was a significant difference between the CVA and the healthy blood donor groups when observing IgM antibody levels ($t = 3.26$, $p < 0.01$).

14.6 Results to Peptide 2: Antibodies to *Pseudomonas aeruginosa* γ-carboxy Muconolactone Decarboxylase (QEMITRHAWGDIWTR)

Levels of IgA antibody to **TR**H**AWG** peptide were shown to be significantly elevated in multiple sclerosis sera (0.047 ± 0.003) when tested against control sera (0.009 ± 0.001) ($t = 12.07$, $p < 0.0001$) and CVA patients (0.008 ± 0.001) ($t = 11.78$, $p < 0.0001$) (Fig. 14.2).

Increased levels of IgG antibody to anti-**TR**H**AWG** peptide (0.104 ± 0.009) were also seen in multiple sclerosis patients in comparison to the control group IgG (0.010 ± 0.002) ($t = 10.35$, $p < 0.0001$) and CVA patients IgG (0.012 ± 0.002) ($t = 1035$, $p < 0.0001$).

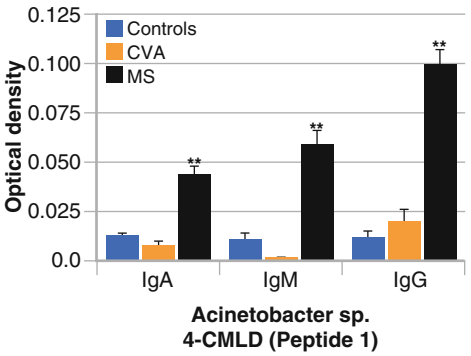

Fig. 14.1 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Acinetobacter* 4-carboxy muconolactone decarboxylase (Peptide 1) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors. (Symbols are indicated:) (Reprinted from Hughes et al. (2003), with permission from Elsevier)

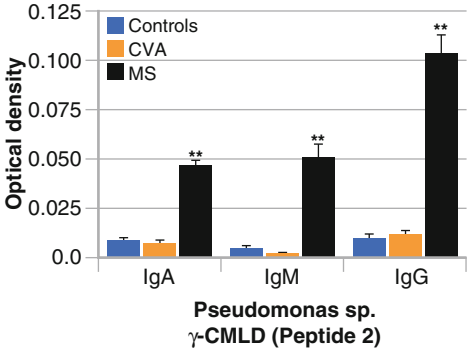

Fig. 14.2 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to *Pseudomonas aeruginosa* γ-carboxy-muconolactone decarboxylase (Peptide 2) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors. (Symbols are indicated:) (Reprinted from Hughes et al. (2003), with permission from Elsevier)

Increased levels of IgM antibody to anti-**TR**HAWG peptide (0.051 ± 0.007) were also seen in multiple sclerosis patients in comparison to the control group IgM (0.005 ± 0.001) ($t=6.03$, $p<0.0001$) and CVA patients IgM (0.001 ± 0.001) ($t=5.88$, $p<0.0001$).

No significant differences were seen in the levels of either IgA or IgG anti-**TR**HAWG peptide between control subjects and CVA patients.

However there was a significant difference between the CVA and the healthy blood donor groups when observing IgM antibody levels ($t=2.58$, $p<0.01$).

14.7 Results to Peptide 3: Antibodies to Myelin Basic Protein (MBP Residues 110–124) (GLSLSRFSWGAGQR)

Levels of IgA antibody to **SRFSWG** peptide were shown to be significantly elevated in multiple sclerosis sera (0.037 ± 0.005) when tested against control sera (0.010 ± 0.004) ($t=4.08$, $p<0.0001$) and CVA patients (0.003 ± 0.002) ($t=5.33$, $p<0.0001$) (Fig. 14.3).

Increased levels of IgG antibody to anti-**SRFSWG** peptide (0.080 ± 0.010) were also seen in multiple sclerosis patients in comparison to the control group IgG (0.004 ± 0.002) ($t=5.79$, $p<0.0001$) and CVA patients IgG (0.001 ± 0.001) ($t=5.41$, $p<0.0001$).

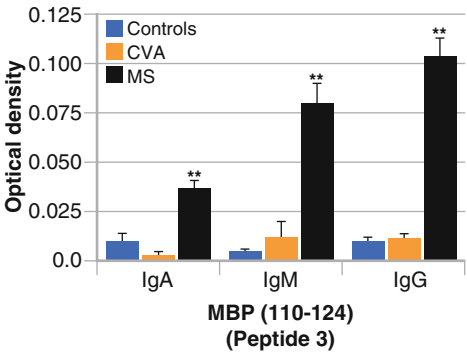

Fig. 14.3 Levels of IgA, IgM and IgG antibodies (mean ± SE with error bars) to myelin basic protein (residues 110–124) (Peptide 3) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors. (Symbols are indicated:) (Reprinted from Hughes et al. (2003), with permission from Elsevier)

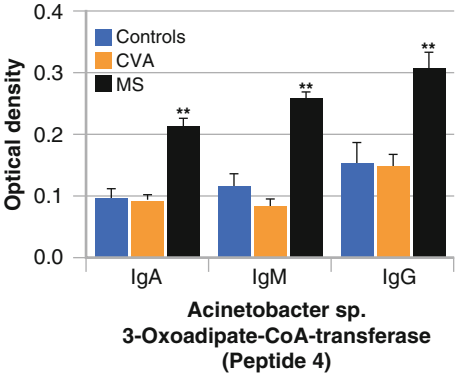

Fig. 14.4 Levels of IgA, IgM and IgG antibody levels to *Acinetobacter* 3-oxoadipate-CoA-transferase (Peptide 4) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Symbols are indicated:) (Reprinted from Hughes et al. (2003), with permission from Elsevier)

Increased levels of IgM antibody to anti-**SRFSWG** peptide (0.080 ± 0.010) were also seen in multiple sclerosis patients in comparison to the control group IgM (0.004 ± 0.002) ($t = 6.15$, $p < 0.0001$) and CVA patients IgM (0.012 ± 0.008) ($t = 4.53$, $p < 0.0001$).

No significant differences were seen in the levels of either IgA, IgG or IgM anti-**SRF**SWG peptide between control subjects and CVA patients.

14.8 Results to Peptide 4: Antibodies to *Acinetobacter* sp 3-oxoadipate CoA-transferase Subunit A. (DSYVFDE LYRAGKIE)

Levels of IgA antibody to **LYRAGK** peptide were shown to be significantly elevated in multiple sclerosis sera (0.212 ± 0.014) when tested against control sera (0.098 ± 0.015) ($t = 4.66$, $p < 0.0001$) and CVA patients (0.094 ± 0.010) ($t = 5.46$, $p < 0.0001$) (Fig. 14.4).

Increased levels of IgG antibody to anti-**LYRAGK** peptide (0.306 ± 0.027) were also seen in multiple sclerosis patients in comparison to the control group IgG (0.155 ± 0.032) ($t = 3.25$, $p < 0.01$) and CVA patients IgG (0.149 ± 0.019) ($t = 3.91$, $p < 0.001$).

Increased levels of IgM antibody to anti-**LYRAGK** peptide (0.257 ± 0.012) were also seen in multiple sclerosis patients in comparison to the control group IgM (0.117 ± 0.020) ($t = 6.26$, $p < 0.0001$) and CVA patients IgM (0.085 ± 0.012) ($t = 9.31$, $p < 0.0001$).

No significant differences were seen in the levels of either IgA, IgG or IgM anti-**LYR**AGK peptide between control subjects and CVA patients.

14.9 Results to Peptide 5: Antibodies to Myelin Oligodendrocyte Glycoprotein (MOG) (Residues 43–57) (PFSRVVH LYRNGKDQ)

Levels of IgA antibody to **LYRNGK** peptide were shown to be significantly elevated in multiple sclerosis sera (0.245 ± 0.016) when tested against control sera (0.083 ± 0.019) ($t = 5.65$, $p < 0.0001$) and CVA patients (0.123 ± 0.018) ($t = 4.54$, $p < 0.0001$) (Fig. 14.5).

Increased levels of IgG antibody to anti-**LYRNGK** peptide (0.272 ± 0.019) were also seen in multiple sclerosis patients in comparison to the control group IgG (0.170 ± 0.020) ($t = 3.29$, $p < 0.001$) and CVA patients IgG (0.145 ± 0.019) ($t = 4.34$, $p < 0.0001$).

Increased levels of IgM antibody to anti-**LYRNGK** peptide (0.285 ± 0.024) were also seen in multiple sclerosis in comparison to the control group IgM (0.078 ± 0.015) ($t = 5.54$, $p < 0.0001$) and CVA patients IgM (0.150 ± 0.018) ($t = 3.83$, $p < 0.001$).

No significant differences were seen in the levels of either IgA or IgG anti-**LYR**NGK peptide between control subjects and CVA patients.

However there was a significant difference between the CVA and the healthy blood donor groups when observing IgM antibody levels ($t = 2.97$, $p < 0.01$).

14.10 Multiple Sclerosis Patients have Antibodies to Bacterial *Acinetobacter/Pseudomonas* Peptides

Elevated levels of IgA, IgM and IgG antibodies directed against peptide sequences from *Acinetobacter* 4-carboxy-muconolactone decarboxylase, *Acinetobacter* 3-oxo-adipate-CoA-transferase subunit A and *Pseudomonas aeruginosa*

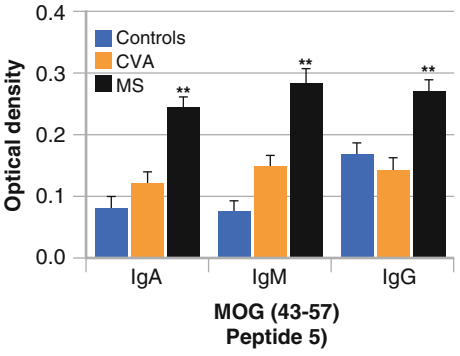

Fig. 14.5 Levels of IgA, IgM and IgG antibody levels to myelin oligodendrocyte glycoprotein (MOG) (residues 43–57) (Peptide 5) in sera from 26 multiple sclerosis patients, 20 CVA patients and 25 healthy blood donors (Symbols are indicated:) (Reprinted from Hughes et al. (2003), with permission from Elsevier)

γ-carboxy-muconolactone decarboxylase were found in the sera from multiple sclerosis patients compared to CVA patients or healthy controls (Hughes et al. 2003). Dilution studies showed that antibody activity against the peptides could be detected up to a dilution of 1/6,400.

Both genetic and environmental factors would appear to be implicated in the aetiology of multiple sclerosis and any infectious agent must be ubiquitous within the risk groups (Granieri et al. 2001).

Acinetobacter and *Pseudomonas* bacteria are found frequently in the environment of multiple sclerosis patients and it would not be inconceivable to consider these bacteria as potential pathological agents.

Acinetobacter is a ubiquitous, common microbe found in soil, water and on the skin of many animals. It is also frequently encountered in the mucous membranes of animals and man, especially the nasal sinuses.

Acinetobacter is an opportunistic pathogen and frequently described as being associated with nosocomial infections, especially in hospital environments, resulting in bacteraemia and pneumonia (Villers et al. 1998) and sometimes leading to chest and sinus infections.

Pseudomonas microbes are also opportunistic pathogens of man and causing wound, urinary tract and even upper respiratory tract infections. They are widely distributed throughout the environment of man and can cause transient colonization of skin and intestinal tract.

The search for an aetiological agent in multiple sclerosis has been focussed on viruses but no microorganisms have so far been implicated.

It has been shown that 52 % of multiple sclerosis patients have a history of repeated upper respiratory tract infections. Increased risk of clinical relapses in multiple sclerosis have suggested links with upper respiratory tract viral infections (Edwards et al. 1998) although 41 % of multiple sclerosis patients also have a concomitant bacterial infection (Rapp et al. 1995).

Several mechanisms have been suggested as to how an infection could initiate autoimmune disease, including molecular mimicry, determinant spreading and bystander activation (Talbot et al. 2001).

Molecular mimicry has been suggested as a potential pathogenic mechanism leading to the development of multiple sclerosis (Steinman 2001) probably through a sequence or structural homology (Kohm et al. 2003).

Some microorganisms have been identified to possess amino acid sequences with homology to myelin antigens including Herpes virus 6 (HHV-6) (Tejada-Simon et al. 2003), Epstein-Barr virus, Herpes simplex virus, influenza and *Pseudomonas aeruginosa* however it is unclear whether these agents are involved in the pathogenesis of multiple sclerosdis.

Our studies have previously identified potential molecular mimicry sequences between *Acinetobacter* and *Pseudomonas* and brain components.

Elevated levels of antibodies to these microorganisms as well as against these bacterial peptides were found in multiple sclerosis patients.

Class specific immune responses to peptide sequences from *Acinetobacter* and *Pseudomonas* bacteria, which mimic the brain components of myelin basic protein and myelin oligodendrocyte glycoprotein (MOG) were found in multiple sclerosis patients.

Autoantibodies to myelin antigens, including MOG, myelin basic protein (Warren and Catz 1997), myelin associated glycoproteins (Wajgt and Gorny 1983) and gray matter neurofilaments are well recognised in multiple sclerosis patients.

Our studies indicate that amino acid sequences can also function as a B cell epitope as increased levels of IgA, IgM and IgG antibodies were observed to myelin basic protein (MBP) (residues 110–224) in multiple sclerosis patients when compared to healthy controls or CVA patients.

Both T cell and antibody responses to MOG (residues 35–55) have been shown in multiple sclerosis patients (Kennel de March et al. 2003).

We show that IgA, IgM and IgG antibodies to a similar epitope, namely MOG (residues 43–57) are elevated in multiple sclerosis patients compared to CVA patients or healthy controls.

The results suggest that the mimicry sequences identified between *Acinetobacter, Pseudomonas,* myelin basic protein and myelin oligodendrocyte glycoprotein (MOG) could be potentially cross-reactive.

It is therefore suggested that the antibody responses to the specific bacterial peptide antigens seen in multiple sclerosis patients could cross-react and bind to myelin constituents within the brain, thereby enhancing the pathological lesions seen in multiple sclerosis.

Further studies are required to evaluate the role of *Acinetobacter* and *Pseudomonas* bacteria in multiple sclerosis.

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Chapter 15
Antibodies to *Acinetobacter* and Myelin in Multiple Sclerosis and Creutzfeldt-Jakob Disease Patients

15.1 Introduction: Comparison of Antibodies to *Acinetobacter* and Myelin in Multiple Sclerosis and Creutzfeldt-Jakob Disease Patients Compared to Other Neurological and Arthritic Conditions

Previous studies have shown that elevated levels of antibodies to the upper respiratory tract and nasal microbe *Acinetobacter* are present in patients with multiple sclerosis (Hughes et al. 2001).

In an endeavour to extend these observations, further studies were carried out on a different set of multiple sclerosis patients and compared them to non-neurological arthritic patients, having ankylosing spondylitis and rheumatoid arthritis as well as the previously described control groups.

Creutzfeldt-Jakob disease is another debilitating and fatal disease which is classified into various types such as sporadic, familial, iatrogenic and variant forms (Irani 2003).

Only sera from two patients with sporadic Creutzfeldt-Jakob disease (sCJD) were available for analysis in the sera obtained from the Institute of Neurology, Queen Square Hospital, London (Courtesy of Prof. Edward Thompson).

Some autoimmune diseases are triggered by external environmental agents, especially microorganisms. Rheumatic fever is a disease that is produced by an environmental factor. When antibodies are produced against group A haemolytic *Streptococci* which cause an upper respiratory tract infection, the resulting antibodies bind to the cross-reactive antigens in myocardial tissues, synovial joints and the basal ganglia of the brain causing carditis, arthritis and Sydenham's chorea respectively.

Rheumatoid arthritis, is a disease that operates by a similar mechanism. It is a relatively common disabling arthritic disorder and appears to be triggered by the urinary pathogen *Proteus mirabilis* which shares cross-reactive epitopes with HLA-DR4 genetic markers as well as with collagen type XI molecules.

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
DOI 10.1007/978-3-319-02735-7_15

Type XI collagen is present in the synovial tissues of small joints of the hands and feet. Patients with rheumatoid arthritis from 14 different countries worldwide were shown to have elevated levels of antibodies to *Proteus* (Ebringer 2012).

Ankylosing spondylitis, a chronic disease manifesting itself predominantly as persistent backache was shown to have significantly elevated levels of antibodies to the bowel microbe *Klebsiella* from 16 different countries. *Klebsiella* microbes possess epitopes which cross-react with HLA-B27 and with collagen types I, III and IV (Fielder et al. 1995).

15.2 Materials and Methods: Serum Samples, Myelin Basic Protein and ELISA

Using an ELISA method, a coded study was carried out on serum samples from different multiple sclerosis patients and healthy subjects (Table 15.1) in which IgA antibody levels against *Acinetobacter calcoaceticus, Klebsiella pneumoniae, Proteus mirabilis* and *Escherichia coli* were determined. The preparation of these bacteria and the ELISA used in this study have been previously described.

Myelin basic protein (MBP) from bovine brain was obtained from Sigma-Aldrich.

Table 15.1 Sources of the serum samples and demographic characteristics of the patient and control groups included in the study

	Number	Age (years): mean (range)	Male/female	Source of sera
Multiple sclerosis	53	46 (29–68)	26/27	National Hospital for Neurology and Neurosurgery, London
Encephalitis	10	38 (3–66)	8/2	National Hospital for Neurology and Neurosurgery, London
Creutzfeldt-Jakob disease	2	66 (58–74)	1/1	National Hospital for Neurology and Neurosurgery, London
Cerebrovascular accident	18	82 (58–94)	7/11	Department of Geriatric Medicine, UCL, London
Ankylosing spondylitis	20	49 (32–71)	16/4	AS Research Clinic. Middlesex Hospital, London
Rheumatoid arthritis	20	68 (47–88)	4/16	Department of Rheumatology, Lister Hospital, Stevenage
Healthy controls	29	43 (19–66)	15/14	Blood Donor Bank, London

Serum Samples

Serum samples were obtained from a total of 123 patients (53 multiple sclerosis, two sporadic Creutzfeldt-Jakob disease, 18 cerebro vascular accident, 20 rheumatoid arthritis, 10 ankylosing spondylitis and 10 encephalitis) and 29 healthy controls were investigated. The age and sex distributions of these individuals, as well as their recruitment source are summarised (Table 15.1).

The selection of each group of patients was defined according to the following diagnostic criteria: New York criteria for ankylosing spondylitis (Van der Linden et al. 1984), American College of Rheumatology criteria for rheumatoid arthritis (Arnett et al. 1988) and the Poser criteria for the diagnosis of multiple sclerosis. The diagnosis of sporadic Creutzfeldt-Jakob disease (sCJD) was based on the characteristic pathological appearance involving spongiform changes at post-mortem examination.

Sera from 29 subjects attending the London Blood Donor services were used as healthy controls.

Bacterial Preparations

Acinetobacter calcoaceticus microbes were obtained, as previously described from the National Collections of Industrial and Murine Bacteria (Aberdeen, UK), while *Klebsiella pneumoniae, Proteus mirabilis* and *Escherichia coli* were provided by the Department of Microbiology, at King's College London.

All these bacteria were cultured, as previously described.

The pellets of bacterial cells were then washed three times with 0.15 M phosphate-buffered saline (PBS, pH 7.4) before being finally resuspended in 20 ml PBS and used in the ELISA studies.

ELISA

An optimal concentration of each bacterial sample was prepared by dilution in 0.05 M carbonate buffer (pH 9.6) to give an optical density reading of 0.25 (10^6 bacterial cells/ml) on the spectrophotometer (Corning, model 258).

The ELISA method was carried out as previously described by Khalafour et al. (1988).

Each serum sample was tested in duplicate and all studies were carried out under code, so that the tester was blind to whether the sample was a test or a control.

The same method was also used to determine the level of autoantibodies against bovine basic protein (MBM; Sigma) with an optimal antigen concentration of 25 μg/ml.

Statistical Analysis

The mean optical density (OD) units of patients with multiple sclerosis or other disease control groups were compared with the mean OD values of healthy controls using Student's *t*-test. The 95 % confidence limits of the control group were calculated. Pearson's correlation coefficient "r" was calculated using the statistical package Prism 3.0 (GraphPad software).

15.3 IgA Anti-Bacterial Antibodies

IgA antibodies to *Acinetobacter calcoaceticus* were significantly elevated in serum samples from multiple sclerosis patients when compared with healthy controls ($t = 4.12$, $p < 0.0001$) (Fig. 15.1).

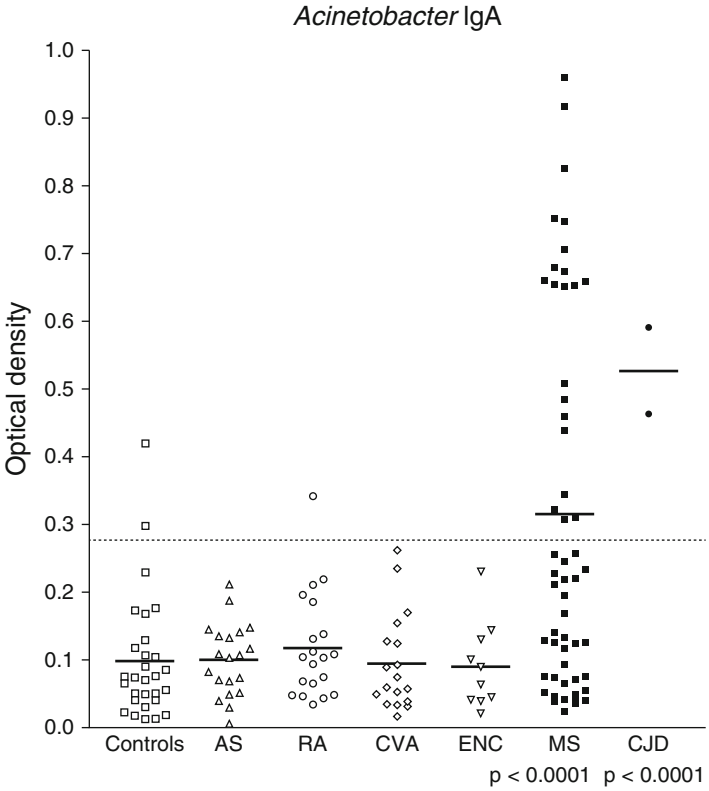

Fig. 15.1 Anti-*Acinetobacter* IgA levels (bars = means) in sera of 29 healthy controls and 123 patients with different diseases: 20 with ankylosing spondylitis (*AS*), 20 with rheumatoid arthritis (*RA*), 18 with cerebro-vascular accident (*CVA*), 10 with encephalitis, 53 with multiple sclerosis and two with sporadic Creutzfeldt-Jakob disease (*CJD*). The *dashed line* represents the 95 % confidence limits for the means of healthy controls (two-tailed test)

IgA antibodies in multiple sclerosis were also significantly elevated when compared to ankylosing spondylitis patients ($t=3.46$, $p<0.001$), rheumatoid arthritis patients ($t=3.12$, $p<0.005$), cerebro-vascular accident (CVA) patients ($t=3.84$; $p<0.005$), and those with encephalitis ($t=2.56$, $p<0.02$) (Ebringer et al. 2004).

IgA anti- *Acinetobacter* antibodies were also significantly elevated in two sporadic Creutzfeldt-Jakob disease (sCJD) patients when compared to healthy controls ($t=6.25$, $p<0.0001$).

IgA anti-*Acinetobacter* antibodies were also significantly elevated in the two sporadic Creutzfeldt-Jakob disease (sCJD) when compared to ankylosing spondylitis patients ($t=10.24$, $p<0.0001$), rheumatoid arthritis patients ($t=6.97$, $p<0.0001$), cerebro-vascular accident (CVA) patients ($t=7.99$, $p<0.0001$) and those patients with encephalitis ($t=8.39$, $p<0.0001$).

However there was no significant difference between the IgA antibodies to *Acinetobacter* in the two sporadic Creutzfeldt-Jakob disease (sCJD) compared to the level found in multiple sclerosis patients.

There was no significant difference in the IgA anti-*Acinetobacter* antibodies between healthy controls and the other disease groups.

IgA antibodies to *Klebsiella* were significantly elevated in ankylosing spondylitis patients when compared to healthy controls ($t=4.22$, $p<0.001$), rheumatoid arthritis patients ($t=3.49$, $p<0.005$), cerebro-vascular accidents patients (CVA) ($t=3.44$, $p<0.005$), encephalitis patients ($t=2.54$, $p<0.02$) and multiple sclerosis patients ($t=5.89$, $p<0.001$).

There was no significant difference in the level of IgA anti-*Klebsiella* antibodies between the other disease groups.

Furthermore, there was no difference in the level of anti-IgA antibodies to *Escherichia coli* between all disease groups and healthy controls.

15.4 IgG Antibacterial Antibodies

IgG antibodies to *Acinetobacter calcoaceticus* were significantly elevated in multiple sclerosis patients when compared to healthy controls ($t=2.65$, $p<0.01$).

IgG antibodies to *Acinetobacter calcoaceticus* were also significantly elevated in multiple sclerosis patients when compared to ankylosing spondylitis patients ($t=2.57$, $p<0.02$), rheumatoid arthritis patients ($t=2.86$, $p<0.01$), cerebro-vascular accidents (CVA) patients ($t=9.08$, $p<0.005$) and encephalitis patients ($t=2.96$, $p<0.005$).

IgG antibodies to *Acinetobacter calcoaceticus* were significantly elevated in the two sporadic Creutzfeldt-Jakob disease (sCJD) when compared with those patients with cerebro-vascular accident (CVA) ($t=2.40$, $p<0.05$) or encephalitis patients ($t=9.62$, $p<0.01$).

IgG antibodies to *Proteus mirabilis* were significantly elevated in rheumatoid arthritis patients when compared with healthy controls ($t=3.93$, $p<0.001$); patients with ankylosing spondylitis ($t=3.31$, $p<0.005$); cerebro-vascular accident (CVA) patients ($t=3.15$, $p<0.005$); encephalitis patients ($t=2.30$, $p<0.05$) and multiple sclerosis patients ($t=5.17$, $p<0.001$).

There was no significant difference in the level of IgG anti-*Proteus* antibodies between the other disease groups.

Furthermore, there was no significant difference in the level of IgG anti-*Escherichia coli* antibodies between all disease groups and healthy controls.

15.5 IgA Anti-Myelin Basic Protein Antibodies

IgA antibodies to myelin basic protein were significantly elevated in multiple sclerosis patients when compared with healthy controls ($t = 4.88$, $p < 0.001$) (Fig. 15.2).

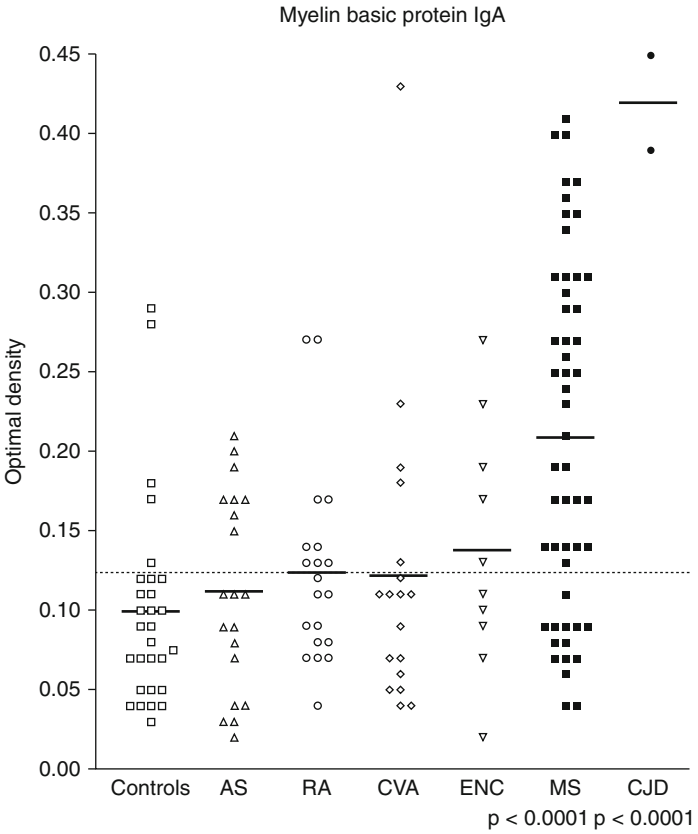

Fig. 15.2 IgA anti-basic myelin protein levels (bars = means) in sera of 29 healthy controls and 123 patients with different diseases: 20 with ankylosing spondylitis (*AS*), 20 with rheumatoid arthritis (*RA*), 18 with cerebro-vascular accident (*CVA*), 10 with encephalitis, 53 with multiple sclerosis and two with sporadic Creutzfeldt-Jakob disease (*CJD*). The *dashed line* represents the 95 % confidence limits for the means of healthy controls (two-tailed test)

IgA antibodies to myelin basic protein were significantly elevated in multiple sclerosis patients when compared to patients with ankylosing spondylitis ($t=3.75$, $p<0.001$); patients with rheumatoid arthritis ($t=3.10$, $p<0.005$) but not when compared to patients with encephalitis.

Anti-myelin basic protein antibodies were also significantly elevated in the two sporadic Creutzfeldt-Jakob disease (sCJD) patients when compared to healthy controls ($t=6.82$, $p<0.0001$).

Furthermore anti-myelin basic protein antibodies were also significantly elevated in the two sporadic Creutzfeldt-Jakob disease (sCJD) when compared to patients with ankylosing spondylitis ($t=6.78$, $p<0.0001$), rheumatoid arthritis patients ($t=6.67$, $p<0.0001$), cerebro-vascular accident (CVA) patients ($t=4.37$, $p<0.001$), encephalitis patients ($t=4.93$, $p<0.001$) and multiple sclerosis patients ($t=2.71$, $p<0.01$).

15.6 Longitudinal Assessment of IgA Antibacterial Antibodies

A longitudinal study of one 38 year old female patient with multiple sclerosis showed three peaks of IgA anti-*Acinetobacter calcoaceticus* antibody levels during weekly measurements, over a period of 10 weeks but no such elevations were observed with IgA anti-*Klebsiella* or anti-*Escherichia coli* antibodies. The peaks of anti-*Acinetobacter calcoaceticus* antibody activity appeared to be preceded either by a urinary tract or upper respiratory tract infection (Fig. 15.3).

Further cases of multiple sclerosis patients should be examined longitudinally for antibodies to *Acinetobacter* bacteria.

15.7 Correlation Coefficient Analysis

The correlation coefficient "r" was calculated between IgA antibody to *Acinetobacter calcoaceticus* and myelin basic protein in 53 multiple sclerosis patients and 87 non-multiple sclerosis individuals (20 with ankylosing spondylitis, 20 with rheumatoid arthritis, 18 cerebro-vascular accident (CVA) patients and 29 healthy controls).

There was a significant positive correlation between antibodies to *Acinetobacter calcoaceticus* and antibodies to myelin basic protein in multiple sclerosis ($r=+0.20$, $p<0.01$), whilst no such significant correlation was seen when each of the groups were studied separately.

These studies indicate that multiple sclerosis patients and maybe sporadic Creutzfeldt-Jakob disease patients have elevated levels of antibodies to *Acinetobacter* bacteria.

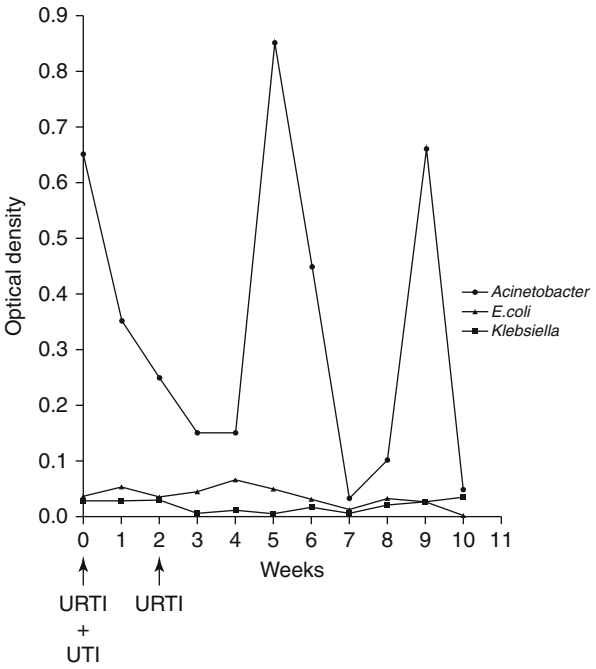

Fig. 15.3 Serial IgA antibody levels to *Acinetobacter, Klebsiella* and *Escherichia coli* in one patient with multiple sclerosis followed at weekly intervals for over 2 months. *URTI* upper respiratory tract infection, *UTI* urinary tract infection

15.8 Discussion

Significantly elevated levels of IgA and IgG antibodies to *Acinetobacter calcoaceticus* and IgA autoantibodies to myelin basic protein (MBP) were found in the majority of multiple sclerosis patients and two sporadic Creutzfeldt-Jakob disease (sCJD) patients compared to healthy controls. The antibody levels were also elevated when compared to other disease controls such as sera from ankylosing spondylitis, rheumatoid arthritis, cerebro-vascular accidents (CVA) patients as well as those with encephalitis.

Furthermore, one multiple sclerosis patient had shown episodes of antibody elevations against *Acinetobacter calcoaceticus* but not against *Klebsiella pneumoniae* or *Escherichia coli* microbes following attacks of upper respiratory tract infections.

Moreover, a significant positive correlation was observed between anti-Acinetobacter and anti-myelin basic protein (MBP) antibodies.

These results confirm previous studies on multiple sclerosis patients (Hughes et al. 2003) in that elevated levels of antibodies are present in such patients against *Acinetobacter,* myelin basic protein and maybe *Pseudomonas* but not against *Klebsiella, Proteus* or *Escherichia* microbes. In this study patients with rheumatoid arthritis had specific antibodies against *Proteus* and ankylosing spondylitis patients had specific antibodies against *Klebsiella* as reported in many previous studies and were acting as disease controls for the multiple sclerosis patients.

Multiple sclerosis is the most common immune mediated, demyelinating disorder of the central nervous system. Patients with multiple sclerosis show relatively high morbidity and mortality rates, especially in chronic progressive forms of the disease.

The disease appears to be prevalent in countries having high latitudes, such as Sweden, Norway or Canada.

Multiple sclerosis is also found more frequently in Scotland compared with southern England (Rothwell and Charlton 1998).

Possible environmental factors suggested in the onset of multiple sclerosis have been viruses (Gilden 2001) and smoking (Riise et al. 2003).

However these studies suggest that another environmental factor, namely, upper respiratory tract infection by *Acinetobacter* bacteria could also be considered as a possible aetiological trigger for the onset of multiple sclerosis.

Whether such a microbe is also involved in Creutzfeldt-Jakob disease (CJD) raises relevant considerations.

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Chapter 16
Creutzfeldt-Jakob Disease and its Variants

16.1 Introduction: The First Descriptions of Creutzfeldt-Jakob Disease

Creutzfeldt-Jakob disease or spastic pseudosclerosis was a term applied by Jakob to a set of patients first described by Creutzfeldt (1920).

The course of the disease is usually rapid, death occurring within a few months or rarely years from of onset of symptoms. The disease is characterised by progressive dementia with memory loss, personality changes and hallucinations. Clinically there is dysarthria, spastic weakness of limbs, extra-pyramidal symptoms such as a Parkinsonian tremor, athetosis and pronounced muscular wasting. There is difficulty in talking normally made worse by the concomitant dysarthria.

On post-mortem examination there is global atrophy of cerebral gyri from frontal to parietal regions, widespread demyelination with degeneration of cortico-spinal tracts and destruction of anterior horn cells.

In the 1920's Creutzfeldt-Jakob disease was first described and identified as a debilitating and fatal neurological disease. It is classified into various types such as sporadic, genetic or familial.

Some of these forms are iatrogenic or acquired and more recently variant forms of Creutzfeldt-Jakob disease have been described (Irani 2003).

Only sera from two patients with sporadic Creutzfeldt-Jakob disease (sCJD) were available for analysis in the sera obtained from the Institute of Neurology, Queen Square Hospital, London (Courtesy of Prof. Edward Thompson) which showed elevated levels of antibodies to *Acinetobacter* bacteria. The relevance of this observation must await more extensive studies with a greater number of Creutzfeldt-Jakob disease patients.

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
DOI 10.1007/978-3-319-02735-7_16

16.2 Transmissible Spongiform Encephalopathies

Transmissible spongiform encephalopathies include Creutzfeldt-Jakob disease and its variants, the genetic disorders Gerstmann-Sträussler-Scheinker (GSS) syndrome, Fatal Familial Insomnia (FFI) and kuru in New Guinea natives.

Other diseases also thought to belong to this group is bovine spongiform encephalopathy (BSE), chronic wasting disease of elk and deer, and scrapie in sheep and goats.

All of these diseases are thought to be caused by misfolded or aberrant prions.

16.3 Sporadic-Creutzfeldt-Jakob Disease

The incidence of sporadic-Creutzfeldt-Jakob disease is about one case per million, per year in the world. Sporadic Creutzfeldt-Jakob (s-CJD) disease accounts for about 85 % of cases.

In the USA there are about 200 cases per year.

In the UK, Creutzfeldt-Jakob disease is monitored by the CJD Surveillance Unit in Edinburgh. Since May 1990, when the surveillance of Creutzfeldt-Jakob disease was started, there have been, till 2013, 1,454 cases of sporadic-CJD.

In 2012, 5 New Zealanders were confirmed to have died from sporadic-Creutzfeldt-Jakob disease, in a country where no cases of bovine spongiform encephalopathy had been reported.

The onset of sporadic-CJD usually appears later in life, around the age of 60 years and it has a rapid course with death within months to years. In some individuals it gives a characteristic pattern on electro-encephalogram examination involving periodic sharp and slow wave complexes.

Symptoms can vary from dementia to disturbance of balance and movement. Early non-specific symptoms include headaches, dizziness, fatigue, sleep disturbances and behavioural changes with mood swings. Visual hallucinations occur frequently in sporadic-CJD (Zerr and Poser 2002).

In Japanese patients with sporadic-CJD there is elevation of neuron-specific enolase in both serum and cerebro-spinal fluid (Kohira et al. 2000).

16.4 Acquired or Iatrogenic Creutzfeldt-Jakob Disease

Acquired Creutzfeldt-Jakob disease is transmitted by exposure to brain or nervous tissue usually by a medical procedure, such as injection of growth hormone extracted from pituitary glands or dura mater grafts.

Since 1990, there have been 74 cases of acquired or iatrogenic cases of Creutzfeldt-Jakob disease in the U.K.

Between 1970 and 2003, seven cases of human dura mater associated Creutzfeldt-Jakob disease were identified in the U.K. One of these was a porcine dura mater graft. The latent period between surgery and onset of neurological symptoms was 6–7 years.

Up to 2012, some 200 cases of dura mater associated Creutzfeldt-Jakob disease have been reported world wide. Autopsy carried out on some of these cases showed widespread spongiform changes with variable neuronal loss and extensive gliosis.

16.5 Genetic Creutzfeldt-Jakob Disease

Approximately 5–10 % of Creutzfeldt-Jakob disease are hereditary and occur in families having a genetic predisposition for developing the condition.

The two main genetic or familial conditions which produce a clinical and pathological pattern similar to Creutzfeldt-Jakob disease are Gerstmann-Sträussler-Scheinker (GSS) disease and Fatal Familial Insomnia (FFI).

Since 1990, there have been 155 cases of genetic or familial cases of Creutzfeldt-Jakob disease in the U.K.

Gerstmann-Sträussler-Scheinker syndrome is a rare autosomal dominant genetic disorder characterized by dysarthria and cerebellar ataxia. A change in codon 102 from proline to leucine on chromosome 20 has been found in the prion protein gene of most affected individuals.

Fatal Familial Insomnia is a rare autosomal inherited disease of the brain. It involves progressively worsening insomnia which leads to hallucinations, delusions and dementia. Death occurs within 2 years of onset of symptoms.

It also occurs as a rare mutation of the PrP^C protein gene which is found on the short arm of chromosome 20.

The disease has been described in 40 families, involving some 200 individuals throughout the world.

These are inherited neurological disorders similar to Huntingdon's chorea. The question arises to what extent do environmental factors contribute to the causation of these genetic disorders.

16.6 Variant Creutzfeldt-Jakob Disease

The median age for variant-CJD is 28 years whilst for sporadic-CJD is 60 years.

Up to 2013, there have been 177 cases of variant-CJD reported by the CJD Unit in Edinburgh. These consisted of 122 confirmed cases and 55 probable variant-CJD, awaiting neuro-pathological confirmation.

The neuropathological features comprise at post-mortem examination, spongiform changes, neuronal loss, astrocytic and microglial proliferation with accumulation of abnormal prion proteins.

The proposed link of Creutzfeldt-Jakob disease, particularly the variant form (v-CJD), with bovine spongiform encephalopathy (BSE), especially in young people below the age of 40 years, was a source of considerable concern to the government and public in the U.K., as well as throughout the world (Will et al. 1996). This young age of occurrence of variant-CJD suggested that it might be due to the consumption of meat derived from BSE affected animals.

As a consequence of these observations, the use of British beef for human consumption was drastically curtailed. This led to a widespread decline in meat consumption in the mid 1990's, in the U.K. and to a lesser throughout the world.

The suggested maximum incubation period of more than 20 years for variant-Creutzfeldt-Jakob disease has passed since the possible entrance of "infected" beef into the human food chain in the 1980's. However this expected epidemic never occurred. The incidence rate of Creutzfeldt-Jakob disease are fairly similar in many European countries and no such epidemic located mainly in the U.K. has been observed (Will et al. 1998).

The distribution of variant-CJD in the UK shows the highest incidence in Scotland but this does not tally with the distribution of BSE affected cattle which was highest in the south of England.

Furthermore, a meta-analysis of control studies, in the UK, Japan and USA failed to show an increased frequency of CJD among professional groups involved in handling animal and cattle products such as farmers, butchers and veterinary workers (Wientjens et al. 1996).

In 2004, a report appeared that a person had died from a non-neurological condition but had preclinical markers of variant-Creutzfeldt-Jakob disease after receiving a blood transfusion from a person who subsequently developed Creutzfeldt-Jakob disease (Peden et al. 2004).

This led to a comprehensive restriction on the use of individuals who had spent some time in the U.K. from donating blood in the USA, Canada, New Zealand, Australia, Switzerland, Poland and the Czech republic.

It was presumed that such potential blood donors may have been exposed to the agent involved in variant-CJD whilst visiting the UK.

The number of patients who have died from variant-CJD has continued to increase over the years. Although it had been suggested that consumption of meat from BSE-affected animals may have caused the disease but nutritional studies from the "CJD Surveillance Unit" in Edinburgh failed to show a higher consumption of meat by variant-CJD patients when compared to controls (Table 16.1).

However a subsequent study showed a higher consumption of meat products. Ward et al. 2006.

The fact that some variant-CJD patients had been vegetarians for a number of years would appear not to be compatible with the hypothesis that the disease was started by the consumption of "infected meat".

Venters who investigated the *E.coli 0157* epidemic in Lanarkshie has cast some doubt that there was a link between bovine spongiform encephalopathy and variant-CJD (Venters 2001).

The scientific problem relating to the origin of variant-CJD is the question to what neurological group did the variant-CJD patients who died from the disease belong?

Table 16.1 Meat consumption in 51 cases of patients with variant-CJD compared to 27 controls

	% of cases (n = 51)	% of controls (n = 27)
Beef	98	96
Sausages	88	93
Burgers	88	88
Meat pies	86	87
Venison	25	22
Veal	18	35
Brain	0	4

See Ward et al. (2006)
Eighth Annual Report 1999, the National CJD Surveillance Unit, Western General Hospital, Edinburgh

Is variant-CJD a separate autonomous disease, "sui generic" or does it belong to a larger group?

Since both patients with sporadic-CJD in our studies had antibodies to *Acinetobacter,* the hypothesis is proposed that variant-CJD patients may also have antibodies to this microbe. If they do have such antibodies, the probability arises that variant-CJD as well as sporadic-CJD could be a severe form of multiple sclerosis. A preliminary study with a small number of sera from variant-CJD patients gave an inconclusive result and it should be repeated with a larger number of patients.

Approximately, 700 persons per year die in England and Wales from multiple sclerosis, which is about 2 persons per day (Department of Health Statistics, UK; 1995; 685, 1996; 712, 1997; 703, 1998; 801, 1999; 758, 2000; 696).

Some 7 % of multiple sclerosis patients die before the age of 40 years, which is approximately one person per week (Fig. 16.1). However the 7 % of young patients who died from "multiple sclerosis" did not have a post-mortem examination to determine whether they had similar pathological features to patients who died from Creutzfeldt-Jakob disease.

Since the majority of variant-CJD patients were aged below 40 years, could they have belonged to the group of multiple sclerosis patients who died before the age of 40 years?

Professor Scholz from Munich has pointed out that the distribution of variant-CJD in the UK shows an unequal regional prevalence.

The highest incidence of variant-CJD would appear to be in Scotland which does not tally with the distribution of BSE-affected cattle which occurred predominantly in the south of England (Fig. 16.2).

However the distribution of variant-CJD does fit quite well the distribution of multiple sclerosis in the UK. It is well known that there is more multiple sclerosis in Scotland compared to England.

The observation that multiple sclerosis is commoner in countries further away from the equator, also known as the "latitude effect", would appear to explain the higher prevalence of multiple sclerosis in Scotland and this could be related to the observation that there is a higher frequency of variant-CJD in Scotland.

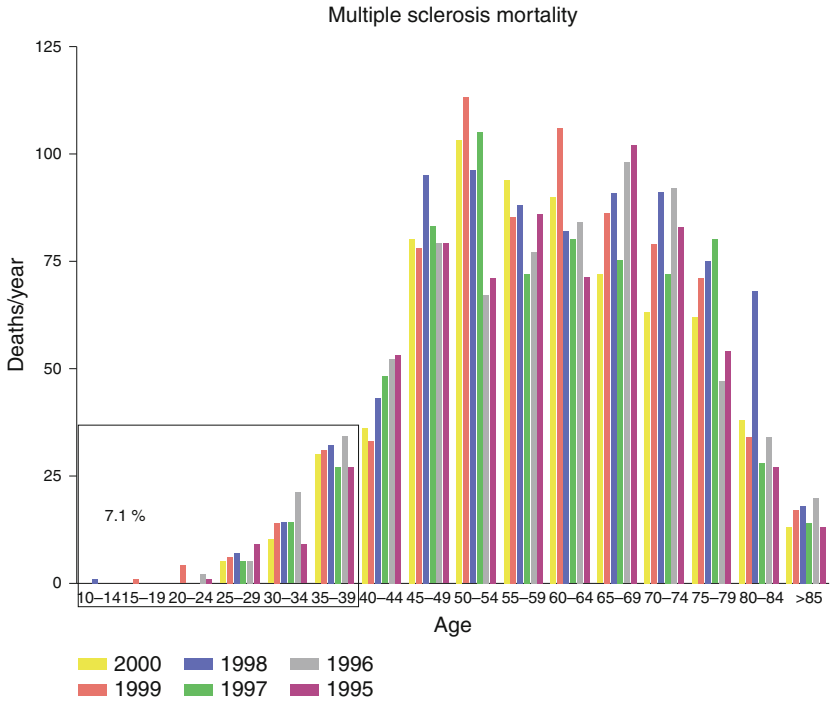

Fig. 16.1 Age and frequency distribution of patients who died from multiple sclerosis in England and Wales over the years 1995–2000 (Department of Health Statistics. Courtesy Dr. Lucy Hughes)

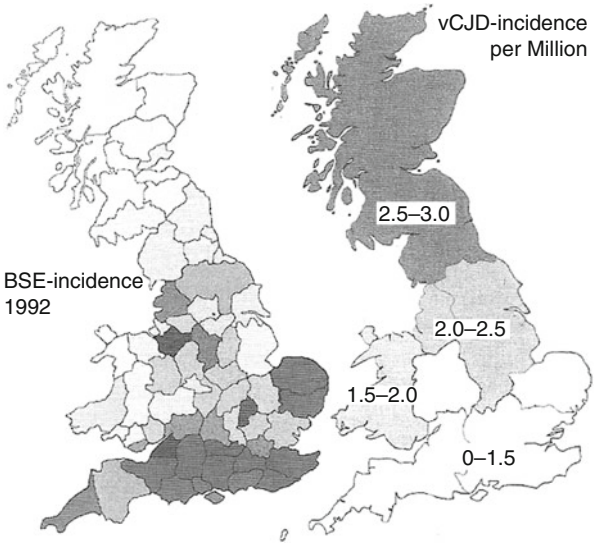

Fig. 16.2 Left-side of figure shows incidence of BSE in the U.K. according to Anderson et al. 1996. Right-side of figure shows incidence of variant-CJD per million, according to Cousens et al. (2001) (From "Phantom-BSE Gefahr" with permission)

Furthermore the reverse is found in the Southern Hemisphere. Multiple sclerosis is seven times more common in Tasmania and southern New Zealand than in tropical Queensland, in populations coming from predominantly Anglo-Celtic stock.

It is possible that the latitudinal effect could be linked to greater prevalence of respiratory infections during winter months with super-infection by environmental bacteria present in nasal sinuses such as *Acinetobacter/Pseudomonas.* Only further epidemiological and field studies can resolve these questions.

16.7 Kuru in the Fore Tribe of New Guinea and Gajdusek

Kuru was a neurological disease, also known as the "laughing disease" because of "risus sardonicus" among some patients and was prevalent among the Fore tribe people of New Guinea in the 1950's and 1960's.

Daniel Carleton Gajdusek, an American paediatrician and neurologist connected the disease to the spread of funerary cannibalism involving the consumption of the brains of diseased relatives.

He then transmitted the disease to primates by drilling holes in the skulls of chimpanzees and injecting homogenized brain matter from the affected dead individuals into the cerebellum. The animals then developed a disease resembling kuru and it was claimed that this proved the transmission of an infectious agent (Gajdusek et al. 1967).

The possibility that Gajdusek's group were repeating Pasteur's mistake of half a century earlier and producing "experimental allergic encephalomyelitis" or EAE in the chimpanzees was not considered.

Further studies showed that heterogeneic autoantibodies against axonal neurofilament proteins were detected by indirect immunofluorescence in 13 % of chimpanzees infected with kuru material and in 35 % of sera from sheep affected by scrapie (Aoki et al. 1982).

Gajdusek obtained the Nobel Prize in medicine in 1976.

16.8 The Role of Inflammation in Creutzfeldt-Jakob Disease

Although there have been extensive studies into the aetiology of multiple sclerosis, few similar investigations into the aetiology and pathogenesis of transmissible spongiform encephalopathies have been carried out, despite the fact that diseases like scrapie were known for several centuries, especially in Europe.

Unlike multiple sclerosis, the search for the involvement of autoimmunity in the pathogenesis of transmissible spongiform encephalopathies is surprisingly lacking.

However several groups have shown evidence of immune responses in a number of "transmissible spongiform encephalopathies":

1. Scrapie infectivity accumulates in lymphoid tissues (Mabbott and Bruce 2001).
2. Sera from scrapie infected sheep were found to react with a 62 kDa neurofilament preparation obtained from mouse brain (Toh et al. 1985).
3. Immuno-deficient mice injected with 1 % homogenate of brain tissues prepared from mice with scrapie, failed to produce a clinical disease when B lymphocytes were not present (Klein et al. 1987).
4. There have been increased values of total IgG, IgA and C3 complement in the CSF and serum of patients with Creutzfeldt-Jakob disease when compared to healthy controls (Galvez et al. 1979).
5. Patients with Creutzfeldt-Jakob disease and kuru have high titres of anti-neurofilament antibodies (Sotelo et al. 1980).
6. Microglial cells have been observed to accumulate in and around the brain lesions of animals affected by transmissible spongiform encephalopathies (Giese et al. 1998) and in patients with Creutzfeldt-Jakob disease (Van Everbroeck et al. 2002).
7. Elevated plasma levels of C-reactive proteins and Il-6 cytokines, which are indicative of ongoing inflammation somewhere in the body have been found in Creutzfeldt-Jakob disease patients (Volkel et al. 2001).
8. In a study from California, brain tissues from 5 out of 6 patients with sporadic-Creutzfeldt-Jakob disease, as well as from scrapie infected mice were found to be infiltrated with inflammatory cells and lymphocytes (Lewicki et al. 2003).
9. Active components C1q and C3b of the complement proteins have been detected in the lesions of patients with sporadic and variant Creutzfeldt-Jakob disease (Kovacs et al. 2004).

Clearly the proposal that there is limited inflammation in transmissible spongiform encephalopathies can no longer be sustained in view of these extensive reports demonstrating inflammatory and immunological processes in these diseases.

16.9 Problems Associated with Variant-Creutzfeldt-Jakob Disease

The "mad cow disease" crisis which erupted in England in the 1980's and 1990's has become notorious for the controversial science used to investigate this problem.

The German book by Scholz and Lorenzen (2005) entitled "Phantom-BSE Gefahr" (Phantom-BSE Danger) describes the inadequate and faulty advice given to government which led to worse political decisions that almost destroyed the meat industry and caused a world hysteria about meat consumption which continues to this day.

Some six million cattle were slaughtered and approximately one million cows deemed to have "bovine spongiform encephalopathy" or BSE entered the human food chain in the U.K.

Millions of people throughout the world have become concerned that they might develop some neurological disease such as variant-Creutzfeldt-Jakob disease (v-CJD).

However there is no valid scientific evidence that patients with variant-CJD consumed more meat products than the rest of the population when examined by careful nutritional studies. In their Eigth Annual Report 1999, the National CJD Surveillance Unit investigated 51 patients with variant-CJD and compared them to 27 controls (Table 1). Almost all cases and controls reported to have eaten beef, sausages, burgers and meat pies. Their careful conclusion was that these findings were consistent with there being no association between meat consumption and variant-CJD, but "we cannot exclude the possibility that such an association exists".

Some 150 patients have died from variant-CJD over the last 10 years. However over this same period of time some 7,000 patients in the U.K. have died from multiple sclerosis but the majority have not undergone a post-mortem examination. Whether there are post-mortem pathological similarities between v-CJD patients and those who have died from multiple sclerosis is at the moment unknown.

Scholz emphasises that "Zweifel ist das Salz der Wissenschaft" (Doubt is the salt of science). The book by Scholz and Lorensen discusses the limited scientific evidence which is proposed to describe where "bovine spongiform encephalopathy" or BSE came from and forcefully criticise the "prion theory".

According to Scholz and Lorensen the "prion hypothesis" which has received wide publicity is incompatible with the laws of biochemistry and immunology, as we know them today.

Furthermore Scholz points out there is a significant difference in the distribution of BSE in the U.K. and the regional distribution of v-CJD cases. There are more v-CJD cases in Scotland compared to the south of England (Cousens et al. 2001) whilst the majority of BSE cases occurred in the south of England (Anderson et al. 1996) (Fig. 16.2).

This distribution however fits well with the known prevalence of multiple sclerosis which is found more frequently in Scotland compared to England (Pugliatti et al. 2002).

Although Prusiner obtained the Nobel Prize for his theory in 1997, it is still quite controversial and other explanations may be found to explain "transmissible spongiform encephalopathies" as happened with "prefrontal lobotomy".

Previously the operation of "prefrontal lobotomy" in the 1940's was considered as the solution for some difficult psychiatric problems.

However the operation irreversibly damaged thousands of psychiatric patients unfortunate to fall under the scalpel of neurosurgeons.

Egaz Moniz, the surgeon who introduced the operation obtained the Nobel Prize in Medicine in 1949 but today it is banned in many countries and very few doctors favour its use since powerful drugs are available to treat many psychiatric conditions.

16.10 The Autoimmune Theory as an Alternative Hypothesis to the Prion Theory

The first clinical case of "bovine spongiform encephalopathy" was detected in April 1985. The disease peaked in 1992 and has resulted in the slaughter of thousands of infected and unifected cows when one case was present in the herd and cost the British taxpayer millions of pounds.

The cause of the origin of this epidemic has been investigated by several groups. The origin of this epidemic is to some extent unknown but it would appear to be associated with a change in the processing and preparation of the animal feed. A new form of "meat-and-bonemeal" (MBM) preparation consisting of abattoir materials from brain and other offals including as well as the intestinal contents was introduced to increase the protein content of the feeds to provide an extra source of protein.

The abattoir material was also called "green offal" due to the high content of grass found in the gut which also included large quantities of saprophytic microbes and their breakdown products.

The use of this "meat-and-bonemeal" was prohibited in 1988, and from early 1993 onwards the number of "bovine spongiform encephalopathy" cases in the U.K. has sharply declined.

One cause for this epidemic was thought to be due to "prions". Other groups suggested that the use of organo-phosphates may have been responsible.

A viral theory was another possibility (Manuelidis et al. 2007).

The role of the organo-phosphate theory in the development of "transmissible spongiform encephalopathies "is difficult to sustain, since scrapie has been known in England for 300 years, well before the discovery of organo-phosphates by the chemical industry.

The autoimmune theory implies that "bovine spongiform encephalopathy" and other "transmissible spongiform encephalopathies" are caused by external agents showing molecular mimicry to neuronal tissues similar to the situation of Sydenham's chorea and its link to *streptococcal* antigens.

Repeated exposures to such triggering agents, probably through an upper respiratory infection, will cause the production of cross-reactive bacterial antibodies against the targeted auto-antigens. Thus the development of inflammatory and degenerative lesions in the central nervous system could occur through exposure to bacteria showing molecular mimicry with brain antigens and especially with myelin. The microbes *Acinetobacter* and possibly *Pseudomonas* possess chemical sequences which resemble myelin. Consumption of bacterial fragments containing these molecular mimicry sequences may have evoked immune responses involving IgG1 and IgG3 antibodies which can cross the blood-brain barrier and caused damage to the nervous tissues containing myelin. The elevated levels of antibodies to *Acinetobacter* bacteria in BSE affected animals and in two patients with sporadic-Creutzfeldt-Jakob Disease is compatible with this hypothesis.

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Chapter 17
Sinusitis in Multiple Sclerosis and *Acinetobacter*

17.1 Introduction: The First Descriptions of Upper Respiratory Tract Infections in Multiple Sclerosis

Previous studies have shown that the onset of multiple sclerosis had been associated with the presence of upper respiratory tract infections involving both viral and bacterial agents. The relevance of these reports requires further consideration.

17.2 Tonsillectomy and Multiple Sclerosis

In 1965, an extensive study involving 240 patients with multiple sclerosis was carried by Poskanzer in the Harvard Medical School.

The patients came from multiple sclerosis clinics attending the Massachusetts General Hospital and the Tufts New England Medical Center.

In the study, there were 210 patients with a living sibling from whom information was obtained and 190 with a spouse. There were also 167 patients who had both a spouse and sibling.

There were 133 patients (63 %) with a sibling, who had a tonsillectomy versus 77 patients (37 %) who had no tonsillectomy and this difference was statistically significant ($p < 0.01$).

There was no significant difference in siblings who had a tonsillectomy (50.5 %) versus siblings who had not had a tonsillectomy (49.9 %).

There were 121 patients (64 %) with a spouse who had a tonsillectomy versus 69 patients (36 %) who had no tonsillectomy and again this difference was statistically significant ($p < 0.01$).

There was no significant difference between spouses who had a tonsillectomy (49.5 %) versus spouses who had not had a tonsillectomy (50.5 %).

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Investigations were carried out to ascertain the frequency of appendicectomy in multiple sclerosis patients and their siblings as well as their spouses. When the multiple sclerosis patients were compared to their nearest siblings, no significant difference in the rate of appendicectomy could be shown.

However when multiple sclerosis patients were compared to their spouses, significantly more operations had been carried out in the patients ($p < 0.05$).

The risk of a person acquiring multiple sclerosis was found to be about 1.7 times higher if he or she had had a tonsillectomy than if the subject had not undergone the operation. It was concluded that these operations suggested an exogenous, environmental factor was playing a role in the aetiology of multiple sclerosis (Poskanzer 1965).

17.3 Multiple Sclerosis and Infections in Canadian Patients

In 1983, an extensive study was carried out on 251 Canadian multiple sclerosis patients in Quebec. The aim of the investigation was to determine the existence of repeated respiratory tract infections in such patients before the onset of neurological disease.

The 251 multiple sclerosis patients were divided into two groups, those with upper respiratory tract infections before the onset of the disease (group A) and those without a past history of such infections (group B).

The mean number of attacks in the first 5 years of the disease group which suffered from upper respiratory infections was 6.2 attacks compared to 2.9 attacks in the group that had not reported such respiratory infections ($p < 0.02$).

The multiple sclerosis patients with an antecedent respiratory tract infections (group A) when compared to those without such a history (group B), it was found that group A had a significantly higher percentage of visual problems ($p < 0.01$), paresthesiae ($p < 0.01$), pain ($p < 0.004$), motor problems ($p < 0.016$) and sexual dysfunction in males ($p < 0.02$).

These results indicate that upper respiratory tract infections may have a role to play in the onset and progression of the disease in multiple sclerosis patients (Lamoureux et al. 1983).

17.4 Multiple Sclerosis Associated with Sinusitis in England

In 1986, the records of 150 multiple sclerosis patients from Essex, Hampshire and Surrey in England, from ten different practices, were examined for possible environmental factors involved in the onset of the disease.

There were 92 patients who had been seen by a consultant neurologist and were diagnosed, according to the McAlpine criteria as “definite” or “probable” multiple sclerosis.

The sample conformed to the classical pattern of multiple sclerosis in that the mean age of first attack was around the age of 30 years (men 32.4 years, women 29.7 years).

These patients were compared to two groups of matched controls who were obtained from practice registers. The first group A consisted of 92 patients matched with the multiple sclerosis patients for age and sex. The second group B were the spouses or cohabitants of the multiple sclerosis patients.

A third group of controls, group C, consisted of 92 patients, who were matched for sex but not for age, who also had a history of recurrent upper respiratory tract allergies, including seasonal rhinitis.

Finally, a further group D of 92 patients, matched for sex and as closely as possible for age were interviewed and their clinical records examined.

The authors stated that "To our surprise it became clear early in the analysis that multiple sclerosis patients had a very much higher recorded rate of chronic and relapsing nasopharyngeal infections than their controls" (Gay et al. 1986).

The mean of recorded sinusitis in the multiple sclerosis group was 69.5 % whilst in control group A it was 17.4 % and in control group B it was 16.3 %. The overall rate of recorded sinusitis in the multiple sclerosis group was over four times that found in control group A and control group B and this difference was highly significant ($p < 0.0001$).

A high rate of recorded sinus infection was found in the allergic group C (45.6 %) but even that rate was significantly lower than the rate found in the multiple sclerosis patients ($p < 0.0001$).

Direct questioning of group D patients gave a rate of 38.0 % which was still lower than the rate found in the multiple sclerosis patients.

The mean age (±standard deviation) of first attack of multiple sclerosis was 30.4 ± 9.9 years and for attacks of sinusitis was 32.5 ± 10.7 years. It is interesting to note the similarity in ages of the first attack in sinusitis and multiple sclerosis. Whether this implies a link between these two conditions awaits further studies.

The authors pointed out that "If the infectious hypothesis has any validity we would expect to find seasonal fluctuations in multiple sclerosis patients. We found significant seasonal fluctuations in both first attacks and exacerbations of multiple sclerosis".

When the seasonal behaviour of multiple sclerosis and sinusitis were compared, it appeared that sinusitis preceded multiple sclerosis by approximately 1 month.

A similar association of frequency between multiple sclerosis and sinusitis appeared to be present in all the ten GP practices that were examined in this study.

The authors concluded that "a commensal bacterium with growth favoured by the conditions in a diseased sinus might elicit an immune reaction against central nervous system antigens due to cross-reacting antigenic groups".

17.5 Sinusitis in Scottish Multiple Sclerosis Patients

In 1986, a study involving 32 multiple sclerosis patients from the county of Angus in Scotland was carried out for attacks of sinusitis. As controls an equal number of spouses or cohabitants were examined and assessed from the case notes.

No cases of radiologically confirmed sinusitis were found in the controls but 6 were noted in the multiple sclerosis patients (19 %). Furthermore, 22 multiple

sclerosis patients (69 %) were recorded to have had sinusitis whilst sinusitis was also recorded in five controls (16 %) and this difference was statistically significant ($p < 0.0001$).

The mean age of the first attacks in the Scottish investigation were similar to the English study: The mean age of first attack of multiple sclerosis was 29.7 years and the mean age of first attack of sinusitis was 25.6 years. Overall the mean age during which attacks of sinusitis occurred in the multiple sclerosis patients was 29.2 years. It would appear that sinusitis and multiple sclerosis are correlated, at least in these Scottish patients (Callaghan 1986).

17.6 Reversible Optic Neuritis Following Paranasal Sinusitis

In 1989, three patients were described in whom optic neuritis, a disease which precedes overt multiple sclerosis was presumably caused by concurrent sinus infection. Aggressive treatment of the underlying sinus condition led to prompt visual improvement

The three patients were seen in the Tucson Veterans Administration Medical Center in Arizona. The antibiotic treatment in two patients was oral ampicillin whilst the third patient received trimethoprim and methoxazole.

In two patients the maxillary sinus was drained and bacteriological cultures of the drained fluid grew *Haemophilis influenzae.* Since the maxillary sinus does not have a contiguous physical relationship with the optic nerve, it was inferred that autoimmune demyelination was the most likely mechanism for the onset of optic neuritis (Awerbuch et al. 1989).

17.7 Incidence of Sinusitis in Multiple Sclerosis as Measured by Magnetic Resonance Imaging

In 1997, a retrospective study was carried out on the incidence of sinus disease in multiple sclerosis patients attending the City Hospital in Birmingham, UK.

Magnetic resonance imaging was carried out on 108 multiple sclerosis patients (71 females and 37 males) with an age range of 22–67 years (Mean age: 39.7 years).

Fifty seven patients (53 %) had evidence of disease, the most common sinus involved was the maxillary, followed by the ethmoid, frontal and sphenoid. Three patients had fluid levels and four patients had retention cysts.

The authors concluded that incidence of sinus disease in multiple sclerosis is higher compared to studies of normal populations (Jones et al. 1997).

17.8 Viral and Bacterial Infections Associated with Onset of Multiple Sclerosis

In 1998, a study from Nottingham involving 41 patients with definite multiple sclerosis (28 women and 13 men) (Median age 35 years; range 21–50 years) was carried out. The multiple sclerosis patients consisted of 21 having relapsing-remitting and 20 were labelled as having secondary-progressive disease. They were investigated for the relation between symptomatic and serologically confirmed upper respiratory tract infections (URTI) and neurological disease which was monitored by magnetic resonance imaging (Edwards et al. 1998).

A total of 114 "upper respiratory tract infections" (URTI's) were reported by 34 of 41 patients (83 %) during the 15 month period of observation, giving an average annual infection rate of 2.4 respiratory tract infections or episodes per patient.

In 8 out of 64 (12.5 %) clinical "upper respiratory tract infections" were accompanied by a rise in antibody titre to 1 of 2 viruses, namely influenza B.

Many viruses have been suggested to be implicated in the pathogenesis of multiple sclerosis, including measles, parainfluenza, canine distemper, Epstein-Barr viruses, corona viruses, adenoviruses, herpes simplex and reoviruses. However no single virus has been consistently linked to multiple sclerosis. This implies that there may not be a specific viral multiple sclerosis agent.

However many upper respiratory viral infections are followed by secondary bacterial infections especially by common and ubiquitous microbes such as *Acinetobacter* or *Pseudomonas* which could be involved in the onset of neurological disease.

17.9 Inflammatory Changes in Acute Optic Neuritis Associated with Paranasal Changes

In 2000, magnetic resonance imaging studies were carried out on 23 patients with acute onset optic neuritis attending the University of New Mexico Hospital and the Veterans Medical Center in Albuquerque (New Mexico) and compared to 48 control subjects.

Optic neuritis is an acute, sporadic inflammatory neuropathy where patients experience impairment of vision as a result of demyelinating inflammation of the optic nerves. Optic neuritis is a common presenting feature of multiple sclerosis. Long-term follow-up studies have indicated that multiple sclerosis develops in as many as 75 % of patients who initially have optic neuritis.

It was found that 83 % of patients had paranasal sinus inflammatory changes compared to a frequency of 54 % in controls.

The distribution of paranasal inflammatory changes was highest in the maxillary sinuses (83 % versus 52 % in controls). The inflammatory changes in the ethmoid

was 4 % versus 2 %, in the frontal sinuses it was 9 % versus 14 % and in the sphenoid it was 4 % versus 10 %.

In the maxillary sinuses there was thickened mucosal lining and mucous retention cysts. A higher prevalence of bilateral sinus inflammatory changes was seen in patients with optic neuritis.

The authors concluded that optic neuritis may be associated with sinus inflammatory changes (Rupp et al. 2000).

17.10 Infections and Multiple Sclerosis in Italy

In 2002, in a wide ranging review of the prevalence of multiple sclerosis it was shown that the mountainous island of Sardinia in Italy has one of the highest prevalence rates of the disease in the world.

The authors speculated that it could be due to distant genetic links to the Samis of northern Europe. However the possibility that the physical geography of the island with increased episodes of winter sinusitis, may have been involved in an environmental predisposition to the disease was not considered (Pugliatti et al. 2002).

17.11 Prospective Studies in Dutch Multiple Sclerosis Patients

In 2002, a longitudinal study involving 73 multiple sclerosis patients (56 women and 17 men) aged between 18 and 55 years was carried out in the Netherlands. The patients participated in the "Rotterdam Study on Exacerbations" (ROSE) in multiple sclerosis.

Multiple sclerosis starts in 80–85 % of patients usually with a relapsing-remitting course and exacerbations are affected by clinically manifest infections.

In this carefully controlled study, a total of 167 infections were recorded in 86 % of patients: 77 % were described as upper respiratory tract infections, 16 % were characterized by gastrointestinal symptoms and 7 % by urinary tract symptoms. In 25 % of the infections patients reported fever at some point during the course of the illness (Buljevac et al. 2002).

It would appear that upper respiratory tract infections are associated with exacerbations, at least in these Dutch multiple sclerosis patients.

17.12 The Latitude Problem in Multiple Sclerosis and Sinusitis

Multiple sclerosis is distributed throughout the world within three zones of high, medium and low frequency (Kurtzke 1993).

The high frequency countries comprise northern and central Europe, northern USA, Canada, New Zealand and south-eastern Australia.

In the U.K. there is more multiple sclerosis in northern Ireland and Scotland compared to England.

In general, the higher the latitude, the greater the prevalence of multiple sclerosis and the reverse occurs in the southern hemisphere. There is seven times more multiple sclerosis in the southern island of New Zealand and Tasmania compared to Queensland in populations coming from Anglo-Celtic stock.

The clear question arises, if sinusitis is commoner in cold countries then this could explain the latitude prevalence observed in multiple sclerosis.

Another possible explanation for the latitude effect that has been proposed is that decreased exposure to sunlight leads to decreased vitamin D production (Ascherio et al. 2010). However it is not clear how vitamin D exerts its protective effect in multiple sclerosis.

17.13 *Acinetobacter* Microbes in Nasal Sinuses and Multiple Sclerosis

These extensive and wide ranging studies from many different parts of the world indicate that an upper respiratory tract infection is associated with the onset and continuation of clinical disease in multiple sclerosis patients.

The main site of an upper respiratory tract infection are the tonsils and the nasal sinuses where the disease is known as simply "rhino-sinusitis".

This raises the question of which microbes or viruses are linked to these anatomical areas.

Acinetobacter species form part of the bacterial flora of skin and can be grown from the oral cavity and respiratory tract of some healthy adults.

Acinetobacter baumannii causes 90 % of clinical infections and is also present in soil.

Acinetobacter calcoaceticus is present in soil and is occasionally associated with clinical infections.

The diagnosis and treatment of bacterial rhino-sinusitis is a challenge to the otolaryngologist. A computer tomography (CT) scan is usually performed and this may show mucoperiosteal thickening and air fluid levels especially in the maxillary sinus.

An "antral tap" has some disadvantages in that it can result in trauma such as bleeding within the maxillary sinus or in the soft tissues of the cheek.

A prospective study of 20 patients with acute rhino-sinusitis admitted to the Trauma Intensive Care Unit of the Jackson Memorial Hospital, University of Miami (Florida) was carried to compare two methods of examining microbial cultures of nasal sinuses over a period of 18 months.

The mean age of the patients was 40 years (Range: 23–77 years and 17 (85 %) were men).

The two methods of comparison were: "Endoscopically directed tissue culture" (ETC) which is easily performed with a nasal telescope and "antral tap" which is usually carried out under local anaesthesia.

A positive culture was defined as greater than 10^3 organisms per ml of fluid.

All patients showed mucoperiosteal thickening and variable degrees of opacification in the osteo-meatal complex. Furthermore they all had muco-periosteal thickening in the maxillary, frontal and sphenoid sinuses.

The total number of micro-organisms cultured was not significantly different between the two methods: 49 organisms in "antral tap" with 52 microrganisms in "endoscopically directed tissue culture".

Gram negative bacteria represented almost 60 % of the organisms. The most common microbe identified was *Acinetobacter baumannii.*

The second most common microbes were *Pseudomonas aeruginosa* and the Gram positive microbe *Staphylococcus aureus.*

Acinetobacter species accounted for 37 % of Gram-negative bacteria in the "antral tap" cultures and 33 % of the Gram-negative bacteria from the "endoscopically directed tissue culture" (Casiano et al. 2001).

It is clear that *Acinetobacter* species can be readily grown from nasal sinuses.

It would appear that examination of the nasal sinuses during onset and exacerbations in multiple sclerosis patients may require further neurological and otolaryngological attention.

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Chapter 18
The Theory That Multiple Sclerosis, CJD and BSE are Caused by *Acinetobacter*

18.1 Introduction to the Problem of Multiple Sclerosis, Creutzfeldt-Jakob Disease and Bovine Spongiform Encephalopathy

Multiple sclerosis affects the central nervous system and previous studies have shown that possible upper respiratory tract infections may be involved in the onset and continuation of the disease.

Proteonomic analysis has shown that a common bacterium found in the upper respiratory tract and nasal cavity, namely *Acinetobacter* contains peptide sequences which cross-react or resemble myelin.

Antibodies to *Acinetobacter* bacteria have been found in multiple sclerosis patients from the U.K (Hughes et al. 2001).

Furthermore patients with Creutzfeldt-Jakob disease in England were found to have elevated levels of antibodies to *Acinetobacter* microbes.

These observations raise important issues in the study of the origin of these two diseases.

These results involving *Acinetobacter* bacteria were started by studying cattle affected by bovine spongiform encephalopathy, also known as "mad cow disease". Animals affected by "bovine spongiform encephalopathy" appeared to have hind quarters ataxia and then paralysis.

This veterinary condition belongs to a group of neurological disorders grouped together under the name of "transmissible spongiform encephalopathies".

Other conditions that belong to this group include the animal diseases scrapie in sheep and goats, wasting disease of elk and moose, feline spongiform encephalopathy and similar conditions that have been reported in other animals kept in zoos, including carnivores like tigers, who were also fed with "meat-and-bonemeal" preparations.

In humans the diseases kuru and Creutzfeldt-Jakob disease are also considered to belong to "transmissible spongiform encephalopathies".

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In animals, these conditions are characterized by hind quarters ataxia followed by paralysis, features that are found in laboratory animals injected with brain homogenates who then develop a neurological disease labelled as "experimental allergic encephalomyelitis" (EAE).

"Experimental allergic encephalomyelitis" has been described as an animal model of multiple sclerosis.

18.2 Neurological Complications Following Rabies Vaccination

Rabies is a disease that has been known for centuries. It is caused by a single stranded neurotropic virus and it can be transmitted to birds and canines by infected saliva from a rabid animal (Fu 1997).

Early in the nineteenth century Zinke first transmitted rabies from a rabid dog to a normal one, and then from a dog to a rabbit and a hen by injection of saliva This clearly established that the disease could be passed on to other animals and humans and therefore it was a highly infectious condition (Pearce 2002).

In 1885, Pasteur and his collaborators proposed that subjects bitten by animals like rabid dogs, could be treated by using a rabies brain vaccine. The suggested proposal was that brain and spinal cord tissue obtained from infected animals could be used to immunise subjects who had been bitten by rabid animals before they developed the late and lethal stages of the disease. The vaccine was obtained from rabbits who had been injected with brain and spinal cord tissue from rabid animals.

An unexpected complication was observed in that a few years later, neuroparalytic accidents following Pasteur's post exposure rabies vaccination, were recognized, involving both white and gray matter tissues (Babes and Mironesco 1908). The origin of these puzzling neurological complications were later suggested to be the consequence of an allergic reaction to some component of the brain tissue preparation.

In the 1930's it was found that saline brain homogenates injected into healthy animals would reproduce the complications observed in Pasteur's patients.

Workers in the USA found that monkeys repeatedly inoculated with saline homogenates of brain and spinal cord materials could develop an inflammatory demyelinating encephalomyelitis which became known as "experimental allergic encephalomyelitis" (EAE) or "experimental autoimmune encephalomyelitis" (Rivers et al. 1935).

This animal model of EAE is readily reproducible in many different animals like rats, guinea pigs, rabbits and monkeys.

Antibodies to myelin proteins were thought to be involved in the pathogenesis of "experimental allergic encephalomyelitis" as immunoglobulin deficient rats failed to develop the neurological features seen in EAE.

18.3 From EAE to Multiple Sclerosis via Autoimmunity

The neurological complications resulting from injection of brain tissues from healthy or rabies infected animals indicates that an autoimmune reaction can be produced through the injection of heterologous or even homologous antigens (Kabat et al. 1947).

These homologous and heterologous brain antigens possess amino acid sequences which are shared with the test animal being assessed for the possible development of EAE (Gold et al. 2006).

In the 1960's, research workers from Los Angeles discovered that the most active myelin constituent in the induction of EAE is myelin basic protein (Eylar et al. 1969).

EAE can be produced in a variety of animals by inoculation of brain components that in chronic cases leads to development of vacuoles in which the epineurium is intact, a form of axonotmesis and then leads to coalescence of such demyelinated vacuoles, forming large spaces where the neurones no longer have the possibility of recovery, a form of neurotmesis. Such large vacuoles give rise to a spongiform appearance.

In some of these vacuoles remnants of neurofilaments can be readily detected (Prineas et al. 1969).

Over half a century ago, it was suggested that the humoral immune response has a pivotal role in the pathogenesis of multiple sclerosis.

It has been observed that elevated levels of immunoglobulins were found in the cerebrospinal fluid of more than 90 % of patients with multiple sclerosis (Kabat et al. 1948).

In some patients with multiple sclerosis there is intrathecal synthesis of IgM, as detected by cerebrospinal fluid oligoclonal bands. The detection of such bands correlates well with disease activity and relapse in multiple sclerosis patients (Sharief and Thompson 1991).

Since elevated levels of anti-*Acinetobacter* antibodies have been found in multiple sclerosis patients from the UK, the question arises "Where have these antibodies been produced?". The fact that the preponderance of these antibodies are found in the IgA class, indicates that they have been produced by the "mucosal associated lymphoid tissues" (MALT) present in the respiratory and gut mucosal system.

The antigens responsible for these immune responses have crossed a mucosal surface and some of these antibodies especially those in the IgG class can cross the blood brain barrier.

They are clearly produced in an extrathecal space, outside the central nervous system, with bacteria present in the upper respiratory tract stimulating an immune response.

In this they are similar to anti-*streptococcal* antibodies found in Sydenham's chorea which cross the blood brain barrier and give rise to neurological complications by binding to basal ganglia.

The experiments involving EAE are also other examples of extrathecal production of antibodies which produce neurological lesions, probably through IgG1 and IgG3 complement binding auto-antibodies crossing the blood brain barrier.

Various brain antigens could be targeted in the immune mediated pathogenesis of multiple sclerosis. For example the levels of antibodies to myelin basic protein (Warren and Catz 1987), myelin oligodendrocyte glycoprotein (Hughes et al. 2003) and neurofilament (Silber et al. 2002) were all found to be significantly elevated in patients with multiple sclerosis compared to other disease or control groups.

The pathological sequence is probably as follows:

Sinusitis ► Anti-bacterial antibodies ► IgG1 and IgG3 complement dependent antibodies

Whether such a pathological sequence exists in patients with multiple sclerosis awaits further experimental and clinical investigations.

However the origin of such autoantibodies can be ascribed to an infection in the upper respiratory tract.

18.4 The Central Immunological Problem of "Transmissible Spongiform Encephalopathies"

The "transmissible spongiform encephalopathies" in both animals and humans suffer from a serious defect in that an aetiological external agent has not been identified.

Initially Gajdusek and later Prusiner proposed a new agent, a "slow virus" or a "prion" as the aetiological culprit. However all animals including all humans have the gene for the "prion" molecule encoded in their heritable DNA. Others favour a virus (Manuelidis 2012).

In an attempt to demonstrate this aetiological agent, brain injections were used in experimental animals and when they developed Pasteur's complication, namely "experimental allergic encephalomyelitis" this was interpreted as the transmission of the disease.

This is the fundamental flaw in using the "bio-assay", by injecting brain homogenates into experimental animals, as a method of demonstrating the presence of an "infectious agent" in "transmissible spongiform encephalopathies".

Many different centres have demonstrated that inflammatory changes are present in scrapie, Creutzfeldt-Jakob disease, kuru and bovine spongiform encephalopathy (Sotelo et al. 1980). The external agent however in these diseases remains to be discovered.

Furthermore, the demonstration that "immuno-deficient" animals do not develop scrapie or other "transmissible spongiform" disease is a compelling demonstration that the absence of an immune system is "protective". This is another way of saying that "the immune system is causing the disease". When the immune system is not present or deficient there is no disease.

Immuno-deficient animals and humans, especially when exposed to drugs which reduce the immune response such as cyclophosphamide, azathioprine, methotrexate and the many numerous "biologicals", they are readily susceptible to infections by viruses, bacteria, fungi and parasites yet here, they are clearly protected from such extraneous invasions and pathological consequences.

Clearly, the conclusion is inescapable, when the immune system is present and healthy, injecting brain homogenates leads to the production of anti-brain antibodies and "experimental allergic encephalomyelitis" or more accurately "experimental autoimmune encephalomyelitis".

This will lead to destruction of brain tissues, cytolysis of brain cells and denaturation of normal self "prion" (PrP) molecules into aliphatic abnormal "prion" sequences (PrP^{sc}).

Italian workers have suggested that the human prion sequence that accumulates in brain lesions, KTNMKHAGAAAAGAVVGGLG consists mostly of aliphatic amino acids which readily polymerize into amyloid like fibrils (Forloni et al. 1993).

The presence of such aliphatic amino acid sequences could explain why these self-proteins are relatively resistant to hydrolysis by macrophage enzymes and therefore why they accumulate in neurological lesions.

Damaged, denatured autologous tissue is well known to be antigenic as occurs in patients with burns who develop anti-skin autoantibodies or patients following a myocardial infarction produce autoantibodies to myocardial tissue and this is known as Dressler's syndrome (Dressler 1956).

However once the damaged tissue is removed, there is no further autoimmune activity.

Thus "transmissible spongiform encephalopathies" require an external agent to produce these diseases, as suggested by the "Yehuda Shoenfeld conjecture".

18.5 The Yehuda Shoenfeld Conjecture

At the 2009, Autoimmune Symposium held in Dresden, Professor Yehuda Shoenfeld, from Tel Aviv, expressed his famous conjecture about autoimmune diseases.

The conjecture states:

> All autoimmune diseases are caused by external agents, until proved otherwise.

This conjecture is a direct challenge to the proposal made by Sir Macfarlane Burnet that "All autoimmune diseases are caused by mutations" (Mackay and Burnet 1963).

The Burnetian theory proposes that rheumatoid arthritis is caused by a mutation in lymphocytes and this will involve a clone of cells that produce antibodies which will attack synovial tissues of joints.

No such mutations have so far been discovered and there is extensive evidence that rheumatoid arthritis is caused by an upper urinary tract infection by *Proteus* bacteria (Ebringer 2012) and ankylosing spondylitis is caused by *Klebsiella* bacteria in the large bowel.

The Yehuda Shoenfeld question is "Which external agent is involved in the autoimmune disease multiple sclerosis?" Molecular similarity or molecular mimicry has been identified between a number of amino acid sequences from *Acinetobacter* and maybe even *Pseudomonas* bacteria with brain components. Such brain components are found in myelin basic protein, myelin oligodendrocyte glycoprotein, neurofilaments and prions (Fig. 18.1).

These molecular similarities suggest that *Acinetobacter* and maybe even *Pseudomonas* bacteria could be the trigger antigens in multiple sclerosis.

The pathological sequence would then appear to be as follows:

Acinetobacter sinusitis ► Anti-Acinetobacter antibodies ► IgG1 and IgG3 complement dependent myelin and neurofilament damage

18.6 *Acinetobacter* as the Causative Agent in Multiple Sclerosis

It is proposed that multiple sclerosis could result from exposure to *Acinetobacter* infection (Fig. 18.2)

The majority of such infections may be sub-clinical, occurring in the upper respiratory tract, especially the nasal sinuses.

Extensive published evidence is compatible with this suggestion:

1. Sinusitis was observed in 70 % of multiple sclerosis patients compared to a frequency of 15 % in controls (Gay et al. 1986).
2. The rate of exacerbations in multiple sclerosis was doubled during attacks of sinusitis.
3. Magnetic resonance imaging scans of nasal sinuses in 108 patients with multiple sclerosis showed that 53 % of patients were found to have evidence of associated sinus infections (Jones et al. 1997).
4. The microbes *Acinetobacter, Pseudomonas* and *Staphylococcus aureus* have been reported as causative microbes in nosocomial sinusitis (Birt and Lambert-Zechowsky 1996).
5. *Acinetobacter* bacteria were found to be the main bacteria isolated from acute sinusitis patients when examined by antral tap and endoscopically directed tissue culture (Casiano et al. 2001).
6. An association with upper respiratory tract infections has been found in Canadian multiple sclerosis patients (Lamoureux et al. 1983).
7. Patients suffering from optic neuritis usually go on to develop multiple sclerosis. In studies from Albuquerque in New Mexico it was shown that over 80 % of patients with optic neuritis have paranasal sinus inflammatory changes when examined by magnetic resonance imaging techniques (Ergene et al. 2000).

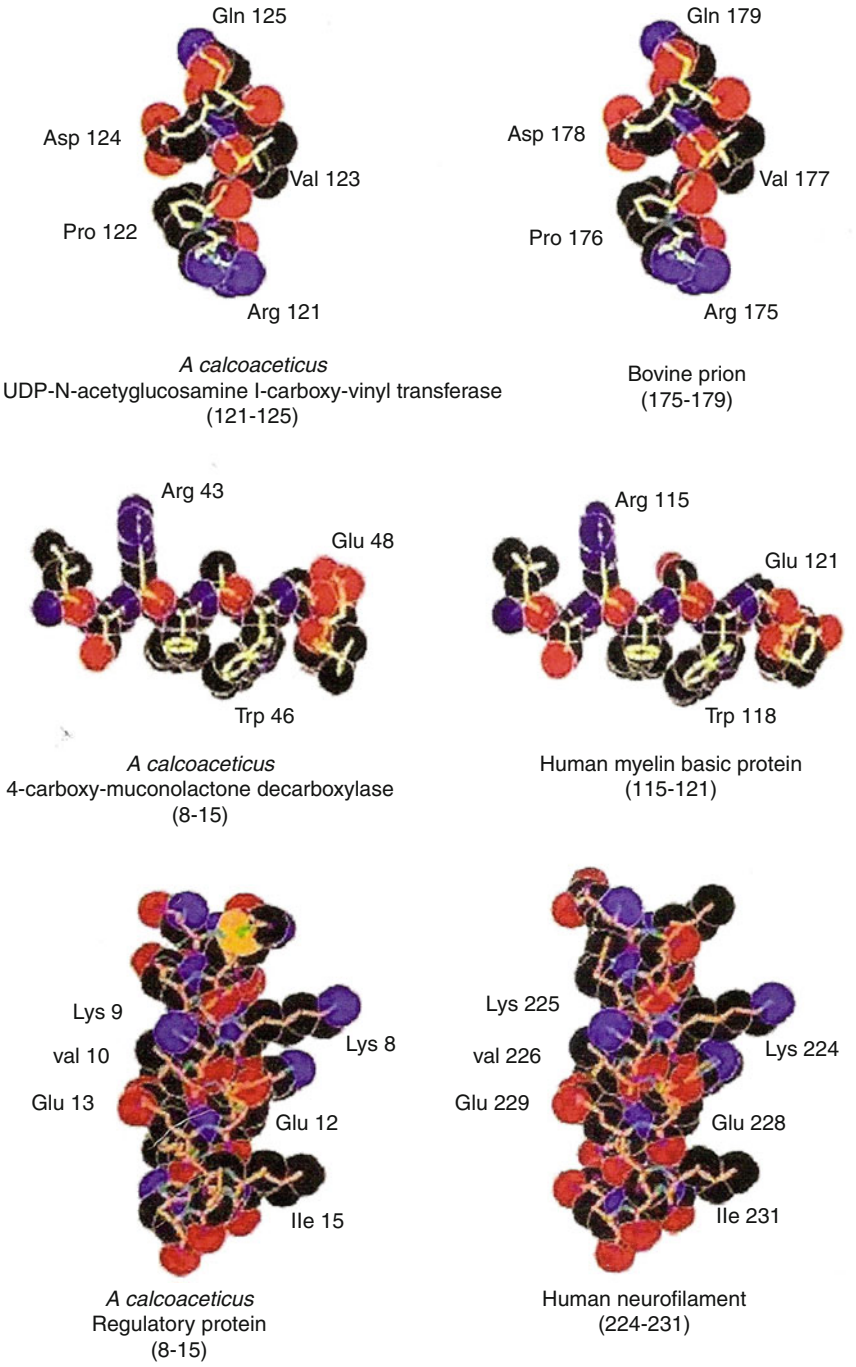

Fig. 18.1 Molecular similarities between self-neuronal antigens (prion, myelin basic protein and neurofilament) and *Acinetobacter calcoaceticus* bacterial antigens (With permission from Ebringer et al. (2005), Elsevier)

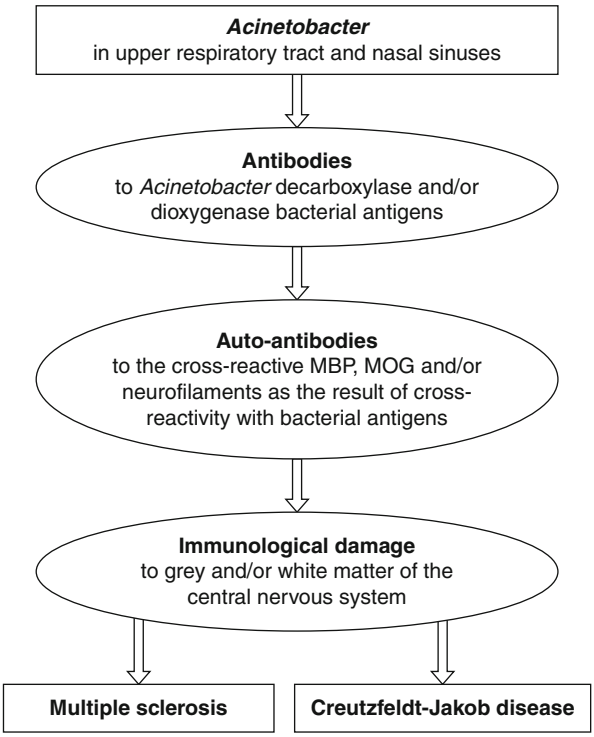

Fig. 18.2 Proposed pathogenetic mechanism for the development of multiple sclerosis and Creutzfeldt-Jakob disease (With permission from Ebringer et al. (2005), Elsevier)

These results from many different centres, as well as our observations on patients with multiple sclerosis in England indicate that further studies are required to establish whether *Acinetobacter* upper respiratory tract infections are involved in the onset and exacerbations of multiple sclerosis.

18.7 Creutzfeldt-Jakob Disease and *Acinetobacter*

The hypothesis is proposed that sporadic Creutzfeldt Jakob disease could also occur as a result of infection with *Acinetobacter* microbes. The infection would occur in the nasal sinuses and then the IgG antibodies produced would cause neurological damage (Ebringer et al. 2005).

To evaluate such a possibility further investigations need to be carried out in patients with multiple sclerosis and also in patients with sporadic Creutzfeldt Jacob disease, involving both immunological and microbiological criteria linked to *Acinetobacter* bacteria.

18.8 BSE, Scrapie and Chronic Wasting Disease of Deer and Elks

The possibility of the "bovine spongiform" epidemics being triggered by the introduction of "meat-and-bonemeal" preparations in the feed stuffs of cattle in the early

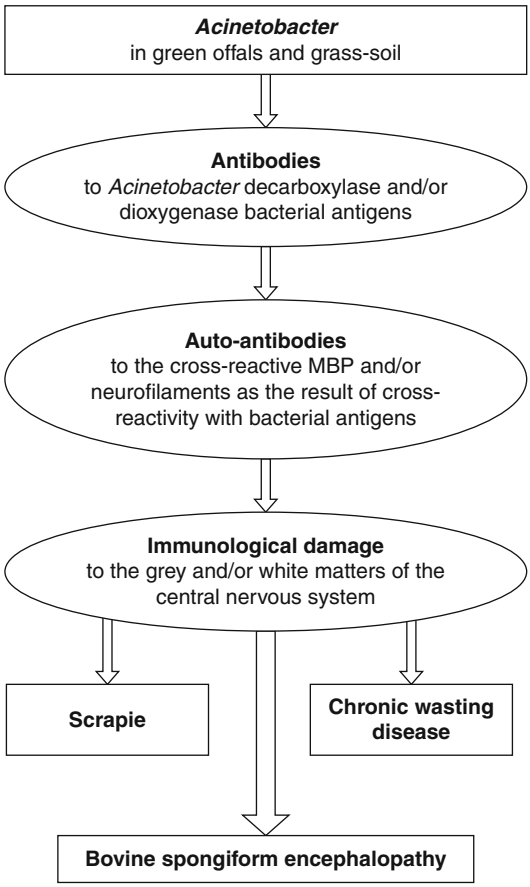

Fig. 18.3 Proposed pathogenetic mechanism for the development of bovine spongiform encephalopathy, scrapie and chronic wasting disease (With permission from Ebringer et al. (2005), Elsevier)

1980's cannot be disputed, since their statutory prohibition led to the disappearance of the disease in UK animals (Tiwana et al. 1999).

The inclusion of intestinal contents in the "green offal" material used in the preparation of "meat-and-bonemeal" feed stuffs is probably the source of the many microorganisms and their breakdown products which are found in the soil reservoir, especially since *Acinetobacter* species are well represented in that ecological niche (Fig. 18.3).

A similar explanation could apply to scrapie. This disease has been affecting sheep and goats for centuries and it is still encountered in many countries The reason for such a broad geographical distribution is that these animals are herbivores, eating mainly grass, especially in a wet environment. The possiblility arises that these herbivores are consuming large amounts of soil microbes such as *Acinetobacter.*

A similar explanation can be proposed to explain the "chronic wasting disease of deer and elks". Antibodies to *Acinetobacter* bacteria should be measured in such animals and the levels of these and other saprophytic microbes in the soils of the various farms should be compared to other areas having different prevalence rates of scrapie or "chronic wasting disease" (Ebringer et al. 2007).

18.9 Further Studies Are Required

The link between post-rabies vaccination encephalomyelitis and "experimental allergic encephalomyelitis" (EAE) has unveiled the possibility that the induction of "transmissible spongiform encephalopathies" in animals immunised with brain antigens, including normal prions, could result from autoimmune reactions to the injected tissues.

Acinetobacter is likely to be an important bacterial agent in the induction of autoimmunity in both multiple sclerosis and also in "transmissible spongiform encephalopathies (Wilson et al. 2003).

Further studies are clearly required to examine these hypotheses. However the use of saline brain homogenates in the transmission of neurological diseases has to be interpreted with caution.

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Chapter 19
The Scientific Method of Sir Karl Popper

19.1 Sir Karl Popper, the Philosopher of Science

Sir Karl Popper (1902–1994) was one of the most influential philosophers of science in the twentieth century and probably of all time. His main contribution to scientific research was that a scientific theory could not be proved but could be readily disproved or falsified if there were experimental observations that were incompatible with the proposed theory.

He claimed that "It must be possible for a scientific system to be refuted by experience. A theory that is not refutable by any conceivable event is non-scientific. Every 'good' scientific theory is a prohibition: it forbids certain things to happen. The more a theory forbids, the better it is" (Popper 1963).

A simple example of his falsification method is the proposed theory that "All tigers are carnivorous". This theory would be refuted or falsified by the observation of one vegetarian tiger.

The logical basis of scientific research is the method of proposing bold conjectures and then demonstrating attempted refutations. The process can be illustrated by the following oversimplified sequence:

Problem ▶ Theory ▶ Experiment ▶ New problem

His proposal that scientific studies should be based on rational analysis of existing theories and then submitted to severe tests by logical criticisms and experimental investigations is the basis of modern science.

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A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
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19.2 A Biography of Karl Popper

The Austrian-British philosopher of science, Sir Karl Popper (1902–1994) was born in Vienna into a middle-class family. His father was a lawyer and his mother was a talented musician.

After the First World War he attended the University of Vienna, reading mathematics, psychology and physics. He graduated in 1928 and qualified as a secondary school teacher in mathematics.

In 1934, he published his first book, "Logik der Forschung" (Logic of Scientific Research) a seminal study which established Popper's reputation as a philosopher of science and scientific methods (Popper 1959).

In December 1936 he accepted a lectureship at Canterbury College in Christchurch, New Zealand and in January 1937, he and his wife left Austria for the Antipodes.

In New Zealand, he wrote "The Poverty of Historicism" and the 2-volume "The Open Society and its enemies". He claimed these works were his contribution to the war effort. They were a powerful and critical, intellectual attack on totalitarian societies of both the Right and the Left. He stayed in New Zealand during the duration of the Second World War.

After the war he obtained a Readership at the London School of Economics and Political Science. In the succeeding 23 years as Professor of Logic and Scientific Method, he had a world-wide impact in many fields from politics, science, philosophy, biology and sociology.

19.3 The Problem of Words and Their Meanings

In his autobiography "The unended quest" Popper mentions a debate he had with his father about the Swedish dramatist Strindberg's autobiography where the writer was trying to extract the "true" meanings of certain words. Popper continues:

> When I tried to press my objections that there was no such thing as a 'true' meaning, I was disturbed, indeed shocked that my father did not see the point. The issue seemed obvious to me. When we broke off, late at night I realized that I had failed to make much impact.
>
> There was a real gulf between us on an issue of importance. I tried strongly to impress on myself that I must always remember the principle of never arguing about words and their meanings.
>
> The quest for linguistic precision is analogous to the quest for certainty and both should be abandoned. It is always undesirable to make an effort to increase precision for its own sake since this leads to loss of clarity. (Popper 1976)

It was the great merit of Popper to point out that "science" starts with "problems" and not with linguistic puzzles. It is the logical properties of the "problem" that starts a research worker speculating as to how to arrive at a solution.

The aim is to explain or throw some light on the problem, puzzle or question the scientist is trying to answer. Without "problems to resolve", without "puzzles to elucidate" there would be no science.

Popper makes the statement that "once we realize all scientific statements or hypotheses are guesses or conjectures and that the vast majority have turned to be eventually mistaken, we can proceed to new ways of looking at scientific problems."

In a famous passage Karl Popper offers a way as how to handle this situation:

> Assume a young scientist meets a problem which he does not understand. What can he do? I suggest that even though he does not understand the problem, he can try to solve it and criticise his solution. Since he does not understand the problem, his solution will be a failure, a fact which will be brought out by criticism. In this way, a first step will be made towards pinpointing where the difficulty lies. This means precisely, that a first step will be made towards understanding the problem, for a problem is a difficulty and understanding a problem consists in finding out where the difficulty lies. And this can only be done by finding out why certain solutions do not work. So we learn to understand a problem by trying to solve it and by failing. When we have failed a hundred times, we may become even experts with respect to this particular problem. That is, if anybody proposes a solution we may see at once, whether there is any prospect of success for this proposal or the proposal will fail because of the difficulties which we know only too well from our own past failures. (Popper 1972)

The question "What kind of explanation may be satisfactory?" leads to the reply, "An explanation in terms of testable theories and falsifiable universal laws and critical conditions." A solution to the scientific problem will be found to be satisfactory if it involves highly testable propositions which thereby lead to better theories.

19.4 The Scientific Problem and Its Explanation

The aim of science is to find satisfactory explanations of whatever problem strikes us as being in need of an explanation. By explanation is meant a set of statements by which one describes the state of affairs to be explained, the 'explicandum'.

The explanatory statement or 'explicans' is the object of our search and as a rule will not be known, thus it will have to be discovered. The cause of the problem has to be found involving observations and experiments.

Thus a scientific explanation, the "explicans", whenever it is a discovery will be the explanation of the known by the unknown.

The "explicans", in order to be satisfactory must fulfil a number of conditions:

1. The solution to the problem, the "explicans" must logically entail the "explicandum", which is the purpose of our search.
2. The "explicans" ought to be true, although in general it will not be known if it is true. It must not be known to be false even after the most critical examination.
3. There must be independent evidence for the existence of the "explicans". In other words it must be independent and avoid ad hoc or circular arguments.

Consider the following dialogue:

"Why is the sea so rough today?"
"Because Neptune is very angry."
"How do you know Neptune is very angry?"
"Oh, don't you see how very rough the sea is!"

The explanation is unsatisfactory because the only evidence for the "explicans" is the "explicandum", the problem itself.

4. In order that the "explicans" should not be ad hoc, it must be rich in content. It must have a variety of testable consequences, which are different from the "explicandum", the problem itself (Popper 1972). It must lead to many "Popper sequences" which can be tested experimentally and verified by many different research workers.

19.5 Evolutionary Theory of Knowledge

On the 9th June 1989, Popper was asked to give his belated Inaugural Lecture at the London School of Economics (Popper 1999).

The title he chose was "Towards an evolutionary theory of knowledge".

The lecture is relevant to all scientists or medical research workers who are grappling with problems involving studies in physics, medicine or biology. They are certainly relevant to the study of multiple sclerosis. He made the following points in describing the search for knowledge:

(1) Knowledge has the character of predictions or expectations.
(2)These predictions or expectations have usually the property of hypotheses, of conjectural or hypothetical knowledge: they are uncertain. The research workers studying the problem, may be quite unaware of this uncertainty or weakness in the predictions or expectations of this hypothetical knowledge.
(3) Most kinds of knowledge are hypothetical or conjectural.
(4) Despite this uncertainty, much of our knowledge will be objectively true. It will correspond to the observed, objective facts.
(5) Therefore we must clearly distinguish between the truth of an expectation or a hypothesis and its certainty. There are two competing ideas here: the idea of truth and the idea of certainty.
(6) Although there is much truth in our knowledge, it is clear there is little certainty. Existing knowledge may be superseded by new and unpredictable discoveries. We must approach our hypotheses critically. We must test these hypotheses as severely as we can, in order to find out whether they can be shown to be false after all.
(7) Truth is objective: it is correspondence to the facts of the problem.
(8) Certainty is rarely objective: it is usually no more than a feeling of trust, of conviction, usually based on insufficient knowledge. Such feelings are dangerous since they are rarely founded on reproducible facts or observations. They may turn us into hysterical fanatics who try to convince themselves of a certainty but often such protagonists unconsciously know convincing or irrefutable evidence is not available.
(9) The issue of social relativism is widely held, often by sociologists who study the ways of scientists and who think thereby they study science and scientific knowledge. Many of these sociologists do not believe in objective truth but think of truth as a sociological concept.

This view is a great mistake. Some sociologists think that truth is what the experts believe to be true. But in all science the experts are sometimes mistaken. Whenever there is a breakthrough, it means that the experts have been proved wrong. The objective facts were found to be different from what the experts expected them to be.

It is our suppressed sense of our fallibility that is responsible for the despicable tendency to form cliques and go along with whatever seems to be fashionable, even when the evidence is not available to support such convictions. Popper maintained that science ought to strive for objective truth that depends only on the facts; on truth that is above human authority and above arbitration, and certainly above scientific fashions. Some sociologists fail to understand that this objectivity is a possibility towards which science should aim. Yet science has aimed at truth for at least for the last 2,500 years.

(10) Philosophers and some scientists often assume that all our knowledge stems from our senses, from "sense data" which our senses deliver. Some believe that the question: "How do you know?" is in every case equivalent to the question "What are the observations that entitle you to your assertion?".

But seen from a biological point of view this kind of approach is a colossal mistake. For our senses tell us nothing without prior knowledge. This prior knowledge cannot in turn be the result of observation, it must be the result of evolution by trial and error as a solution or an attempt at a solution of a biological problem. This solution has been incorporated in our genes through evolution.

(11) Observations or data may lead in science to the abandonment of a scientific theory and thereby induce some of us to think up a new tentative theory – a new trial. But the new theory is our product, our thought, our invention and a new theory is only rarely thought by more than a few people, even when there are many who agree on the refutation of the old theory. The few are those who see the new problem. Seeing a new problem may well be the most difficult step in creating a new theory (Popper 1999).

19.6 The "World 3" Universe

Popper divided knowledge into three distinct epistemological divisions.

There is "world 1" which consists of the physical world described by physics and chemistry.

There is "world 2" which consists of our conscious and unconscious subjective experiences, of our feelings and sensations.

However there is another world, "world 3" which consists of the logical contents of books, librairies, computer memories and even symphonies. They are the discoveries and creations of the human mind.

We can discover new problems in "world 3" which were there before they were discovered and before they ever became conscious.

For example we discover mathematical problems such as prime numbers, Euler's formula linking π to the square root of -1 and Euclid's problem of whether the sequence of prime numbers is an infinite series, arises as a consequence of such a discovery of the existence of "world 3".

Thus there is a sense in which "world 3" is autonomous. In this world we can make discoveries involving real problems in a similar way to that in which we can make new geographical discoveries in "world 1". The question whether Columbus discovered a new continent falls into this category of problems.

Almost all of our subjective world, that is "world 2", depends upon "world 3" constructs, that is on linguistically formulated theories.

Thus our immediate self consciousness, our knowledge of "self" depends on "world 3" theories, which influence how we look at our environment.

Science which is essentially critical, is also more conjectural and less certain of itself than ordinary life because we have consciously raised it to the level of a problem which may have been part of our background knowledge.

This does not mean that we ever reach the stage where an ingenious scientific thinker may not detect loopholes in our arguments or possibilities which nobody has thought of so far, and which therefore nobody has attempted to exclude or include in the debate about the scientific problem under discussion.

From the point of view of objective knowledge, all theories therefore remain conjectural.

The self-correcting method by which science proceeds is the method of bold conjectures and ingenious and severe attempts to refute them.

The problem of multiple sclerosis existed before the disease was identified by Cruveilhier and Charcot.

Subsequent attempts at solution of the problem, went through the stages of direct infection by viruses, then more sophisticated solutions were proposed such as a "slow virus" and more recently the prion hypothesis.

The final solution will occur when the causative agent has been identified as in the case of Sydenham's chorea and appropriate steps can be undertaken to treat the patient as early as possible.

19.7 Bacon, Hume and Popper

Sir Francis Bacon (1561–1626) proposed that science consists of making observations about natural phenomena which then lead to theories. Repeatable observations lead to theories by the mechanism of "induction". David Hume (1711–1776) claimed that because B follows A, today, we cannot make the prediction that the same will happen tomorrow. In other words, Bacon's method of "induction" does not exist.

Thus the assumption A causes B is based on mere habit or belief and this severely undermined the belief in empiricism and empirical observations. Bertrand Russell claimed that "The growth of unreason and romantic philosophies in the nineteenth and twentieth centuries is a natural sequel to Hume's destruction of empiricism" (Russell 1946).

Popper was a great critic of the Baconian myth that all science starts with observations and then slowly and cautiously proceeds to theories. It was the great merit of Popper to point out that "science" starts with "problems" or questions about some observed phenomena such as "What is the cause of bovine spongiform encephalopathy?". It is the identification of the "problem" that starts a research worker speculating as to how to arrive at a solution which will throw some light on the puzzle or question he is trying to answer. Without "problems to resolve", without "puzzles to elucidate" there is no science.

Popper goes on to suggest that "once we realize all scientific statements or hypotheses are guesses or conjectures and that the vast majority have turned out to be false, the Baconian myth becomes irrelevant". This leads to the realization that attempts to find the truth are not final, but open to improvement; that knowledge is conjectural, that it consists of guesses or hypotheses rather than final and certain truths.

Criticism and critical discussion with the help of observations and experiments are our only means of getting nearer to the truth. It thus leads to the tradition of bold conjectures and free criticisms, the tradition which created the rational and scientific attitude of Western civilization. The problem of multiple sclerosis falls into this category of problems and we require bold but testable hypotheses which can be experimentally verified and in that sense advance the subject and maybe even help patients suffering from the disease.

Popper insisted that "The task of science is the search for truth, that is for true theories. Yet it is not the only aim of science. We want more than mere truth, what we are looking is for interesting and "deep" truth which has a high degree of explanatory power and at the same time provide answers to our problems". The final test is whether the solution of the problem allows a better way of tackling the situation that started the debate. In the debate here, the question is "Can these solutions suggest new ways of looking at multiple sclerosis?"

19.8 Popper's Scientific Method

The fundamental procedure of the growth of knowledge remains that of conjectures and refutation, of the elimination of unfit explanations.

A scientific result cannot be justified. It can only be criticized and tested.

Theories about how knowledge grows involves methods of trial and error.

Here is a tetradic scheme for the description of the growth of knowledge:

$$P(1) \blacktriangleright TT \blacktriangleright EE \blacktriangleright P(2)$$

Popper proposed a powerful analytical method to investigate scientific problems.

The sequence can be described by the following simple schematic outline of how to tackle a scientific problem.

We start from a simple scientific "problem 1" (P1) and try to solve it by a "tentative theory" (TT1) which may or may not be correct. The theory will then be subjected to "error elimination" (EE1) either by logical criticism or experimental studies. As a result of these investigations a new fact will appear, "problem 2" (P2) which in turn will require a scientific explanation. This is a "Popper sequence".

Every "Popper sequence" provides a new way of looking at the scientific problem. If each "Popper sequence" generates new facts, then the original problem becomes richer in that it has more questions to resolve but at the same time the

investigation gets closer to the truth of the inquiry, to the centre of the problem. The new facts uncovered by this "Second Popper Sequence" are different from the "First Popper Sequence" because they are related to the logical properties of the hypothesis or "tentative theory" and the facts that follow from the "error elimination" steps.

The tetradic schema is an attempt to show that the results of criticism, of "error elimination" (EE) applied to a "tentative theory" (TT) leads as a rule to the emergence of a "new problem" (P2).

Problems, after they have been solved and their solutions examined, tend to widen the scope of the inquiry. Each "Popper sequence" begets problem-children, new problems, often of greater depth and even greater fertility than the old ones (Popper 1972).

The best tentative theories, and all theories are tentative, are those which give rise to the deepest and unexpected results in relation to the problem under investigation.

If the new problem (P2) turns out to be merely the old problem (P1) in disguise, then we say that one theory only manages to shift the problem of the scientific investigation.

The decisive point is, of course always, how well does one theory solve our original problem. At any rate, one of the things we wish to achieve is to learn something new.

19.9 The Hippocratic Oath, Popper and Medicine

The centre piece of the "Hippocratic oath" states "…The regimen I shall adopt for the benefit of the patients according to my ability and judgement and not for their hurt or for any wrong" (Singer and Underwood 1962).

Popperian analysis of a scientific problem, such as multiple sclerosis may provide new clues or ideas for treatment. However it is only when the therapeutic proposals arising from Popperian analysis are actually provided to the patients that will we know if the scientific problem has been solved.

If no therapeutic benefits accrue to multiple sclerosis patients from anti-*Acinetobacter* treatment, then the hypothesis that these microbes are involved in this disease will have been disproved and the question of the origin of this condition will have to await new and better theories.

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Chapter 20
Multiple Sclerosis and "Popper Sequences"

20.1 Introduction to "Popper Sequences"

It is proposed to investigate the possible cause of multiple sclerosis (MS) by the methods of Sir Karl Popper who claimed that a theory could not be proved by induction or any other method but could be disproved.

A fact which was incompatible with a theory would thereby disprove it and this approach has provided a powerful new method to investigate scientific problems.

The theory that "All swans are white" is disproved by the observation of one black swan.

The theory that "All tigers are carnivorous" is incompatible with the observation of one vegetarian tiger.

The "Popper sequences" consist of four stages:

First is the "scientific problem" (P1 = Problem).
Secondly is the attempted or tentative solution or "theory" (TT = Tentative Theory).
Thirdly is the attempted falsification of the theory by "experiments or observations" (EE = Error Elimination).
Finally there is the generation of new knowledge which creates "new problems" and "new facts" (P2 = New problem).

Preliminary investigations have shown that "Popper sequences" provide new ways of looking at scientific problems such as rheumatoid arthritis (Ebringer et al. 2010) or ankylosing spondylitis.

If the cause of multiple sclerosis can be found, then treatment of the disease can be started in the early stages and thereby prevent or at least reduce the severe neurological complications. Such early treatment would also reduce the financial burden that this condition imposes on both the patient and society.

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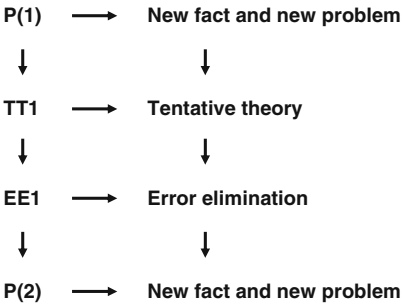
20.2 Components of a "Popper Sequence"

Each "Popper sequence" generates new facts about the original problem. These new "Popper sequences" provide novel and different views of the original problem thereby assisting in solving the scientific puzzle or question under investigation.

The Popperian method has the great advantage of increasing the probable truth content of the problem being investigated.

Any new theory proposed to explain the problem, in this case multiple sclerosis, must also provide a justification or logical explanation of the new facts uncovered by Popperian analysis.

The sequence of a "Popper Sequence" could be summarised as follows:



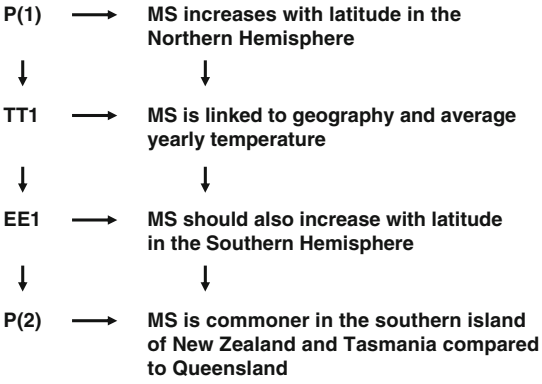
The second problem (P2) stemming from this "Popper sequence" is different from the original first problem. This new situation or condition has arisen because of the tentative theories (TT1) and the error elimination (EE1) which have provided logical arguments, novel criticisms and experimental investigations relating to the original problem.

20.3 First Popper Sequence

The link between multiple sclerosis (MS) and latitudes, especially in northern Europe has been well described in the European and American literature (Kurtzke 1993).

In the UK multiple sclerosis is commoner in Scotland than in England.

This leads to the first Popper sequence:



It is clear that multiple sclerosis is commoner in countries that are located further away from the equator in the Northern hemisphere (Simpson et al. 2011). By an argument of similarity the "First Popper Sequence" suggests that multiple sclerosis should be commoner in areas of the Southern Hemisphere further away from the equator and this would appear to be consistent with the published literature.

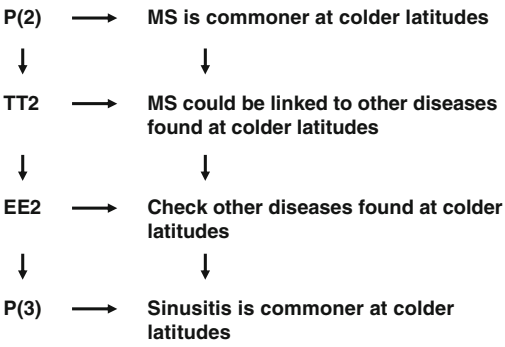
This observation is clearly independent of any genetic factors, since multiple sclerosis is seven times commoner in the southern island of New Zealand and Tasmania when compared to Queensland where the local population is descended from the same Anglo-Celtic stock (Miller et al. 1990).

It appears that multiple sclerosis is more prevalent among people living in regions with cold weather (Rosati 2001). Whether this is related to sunlight exposure and vitamin D production remains unclear.

Another possibility arises in that the geographical location is linked to cold weather according to latitude and this is the subject of the "Second Popper sequence".

20.4 Second Popper Sequence

The occurrence of multiple sclerosis throughout the world would appear to be also linked to cold weather. Previously several groups have suggested that the onset of the disease was somehow linked or associated with the occurrence of upper respiratory tract infection.

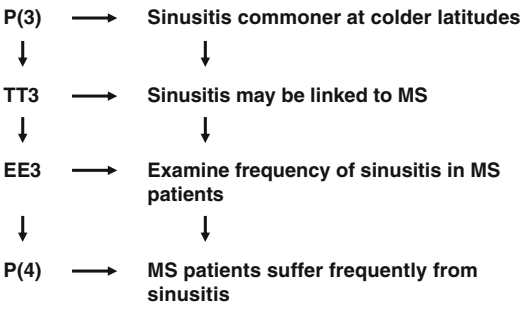


Sinusitis has been reported to be found more frequently in winter months (Pessey et al. 2000). The question arises whether sinusitis occurs more frequently in multiple sclerosis patients. Several studies have suggested that optic neuritis is associated with the presence of paranasal sinusitis.

This relevant query leads to the next Popper sequence.

20.5 Third Popper Sequence

Sinusitis is common in winter but the question is whether multiple sclerosis patients also suffer from this condition.



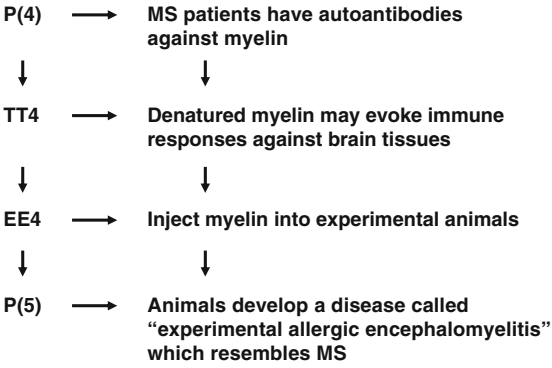
In an extensive study from Colchester, London and West Sussex, involving patients and related controls it was observed that some 70 % of multiple sclerosis patients had an episode of sinusitis compared to a frequency of 16 % in controls selected from the same clinics (Gay et al. 1986). Similar results have been published from other centres.

Antibodies to myelin have been reported in multiple sclerosis patients over the last 50 years. Such antibodies have been suggested to act as autoantibodies to myelin tissues and cause cytological damage. This poses the question whether denatured

brain tissue can cause immunological damage. This leads to the next Popper sequence.

20.6 Fourth Popper Sequence

Antibodies to myelin have been widely reported in multiple sclerosis patients. The origin of such autoantibodies is highly controversial. Are these autoantibodies produced as a result of brain tissue damage or is it the cause of the disease. Answers to such question can only be provided by animal studies. Such queries lead to the next Popper sequence.



Neurological symptoms observed in patients injected with brain tissues as a consequence of immunization with rabietic tissues was made by the French immunologist Pasteur and his colleagues in the 1880s.

Pasteur and Roux were trying to treat patients who had been bitten by rabid dogs before they developed the lethal complications of the disease. The vaccine was prepared by injecting homogenates of brain tissue from rabid dogs into a large number of rabbits. The rabbit brain homogenates were then used as a vaccine in humans bitten by rabid dogs in an attempt to develop anti-rabies immunity and prevent the lethal complications of the disease.

Pasteur and his colleagues were successful in curing patients affected by rabies but unfortunately a small number of injected subjects developed a neurological disease which in some cases had a fatal outcome.

An acrimonious debate developed between Pasteur and his medical colleagues whether the vaccine was contaminated by some external bacterial or allergenic agents.

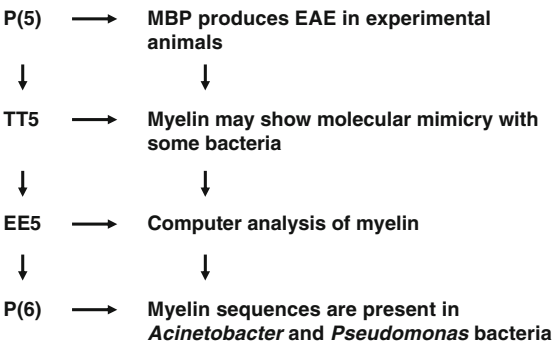
It was only in the 1930s, some 40 years later, that "experimental allergic encephalomyelitis" (EAE) was described in experimental animals injected with saline brain homogenates (Hurst 1932).

Later it was suggested that EAE could be considered as an experimental model of multiple sclerosis.

This brings us to the "Fifth Popper sequence".

20.7 Fifth Popper Sequence

The possibility arises that myelin basic protein (MBP) shares molecular similarities to certain bacterial antigens.



The common soil and skin bacteria *Acinetobacter* and *Pseudomonas* have a sequence in one of their enzymes, mucono-lactone decarboxylase which resembles or shows molecular mimicry to a similar sequence found in myelin. Antigenic moieties present in brain tissues such as myelin basic protein, myelin oligodendrocyte glycoprotein and neurofilament (Fig. 20.1) were found to share molecular similarities with antigens from *Acinetobacter* bacteria.

These two bacteria, *Acinetobacter* and *Pseudomonas* occur in the clinical environment and cause frequent infections in humans. The question then arises whether multiple sclerosis patients have been exposed to these bacteria and this brings us to the next Popper sequence.

20.8 Sixth Popper Sequence

The presence of molecular mimicry between *Acinetobacter* antigens and brain self molecules raises the possibility that in addition to auto-antibodies to myelin, multiple sclerosis patients should have elevated levels of antibodies to these bacteria.

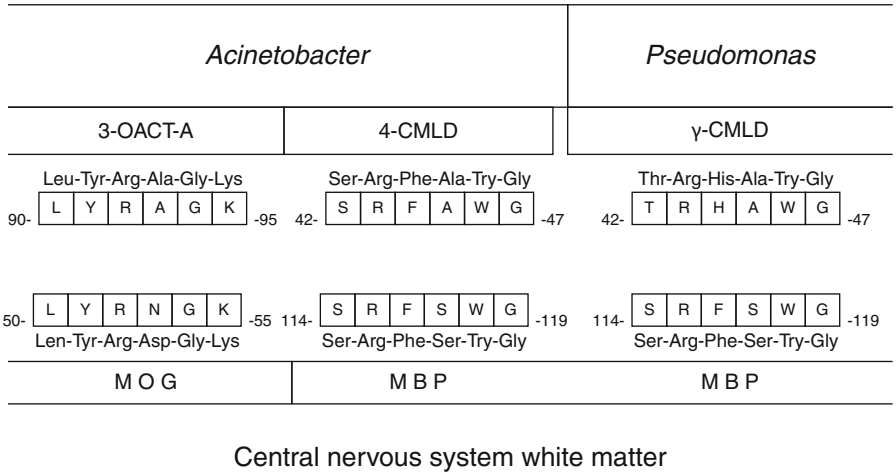
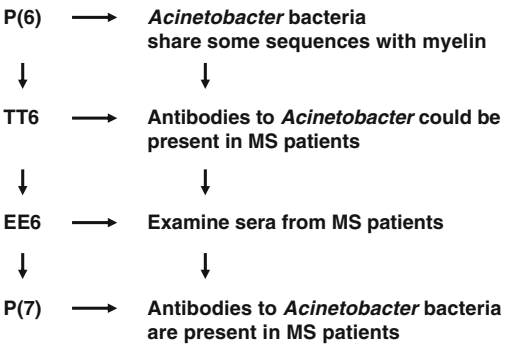


Fig. 20.1 Schematic picture depicting molecular similarities between *Acinetobacter* and *Pseudomonas* bacteria with brain antigens: *MOG* myelin oligodendrocyte glycoprotein and *MBP* myelin basic protein (*4-CMLD* 4-carboxy-muconolactone decarboxylase, *3-OACT-A* 3-oxoadipate CoA transferase, **γ**-*CMLD* **γ**-carboxy muconolactone decarboxylase). Locations and abbreviations names of amino acids are also shown (*L* leucine, *Y* tyrosine, *A* alanine, *G* glycine, *K* lysine, *N* asparagine, *S* serine, *F* phenylalanine, *W* tryptophan, *T* threonine, *H* histidine) (Reproduced from Ebringer et al. (2012), with permission from Elsevier)

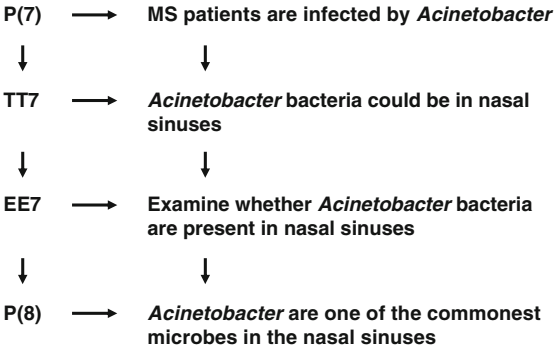


Antibody levels against five strains of *Acinetobacter* bacteria and to a lesser extent to *Pseudomonas* were found to be elevated in multiple sclerosis patients (Hughes et al. 2001).

The possible location of the *Acinetobacter* bacteria in active multiple sclerosis patients has so far not been resolved.

The fact that elevated levels of IgA antibodies to these bacteria are present in multiple sclerosis patients suggests that they are found either in the respiratory tract or the gastro-intestinal tract. These two sites possess mucosal surfaces which evoke high titres of IgA antibodies. It is possible that *Acinetobacter* bacteria are acting across a mucosal surface and this brings us to the next "Popper sequence".

20.9 Seventh Popper Sequence



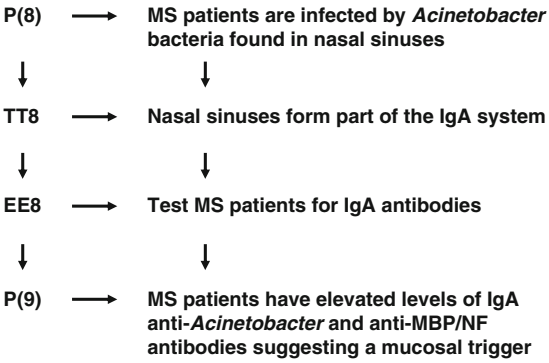
In a study of acute sinusitis it was found that *Acinetobacter* bacteria are one of the commonest microbes, isolated and cultured from the nasal sinuses (Casiano et al. 2001). Moreover, *Acinetobacter* microbes were also detected in the upper respiratory epithelial mucosa (Lee et al. 2006).

Although *Acinetobacter* bacteria are present in nasal sinuses it is not clear whether they have a pathological role in the onset or induction of neurological problems in multiple sclerosis patients.

The question is whether a mucosal surface is involved in the onset of multiple sclerosis and this brings us to the next Popper sequence.

20.10 Eighth Popper Sequence

Biological antigens when acting across a mucosal surface usually evoke predominantly IgA but also IgG antibodies. The IgG1 and IgG3 antibodies readily cross the blood-brain barrier and therefore could cause tissue damage in an intra-thecal space. The important question is whether mucosal infection occurs in multiple sclerosis patients and this is considered in the "Eighth Popper Sequence".



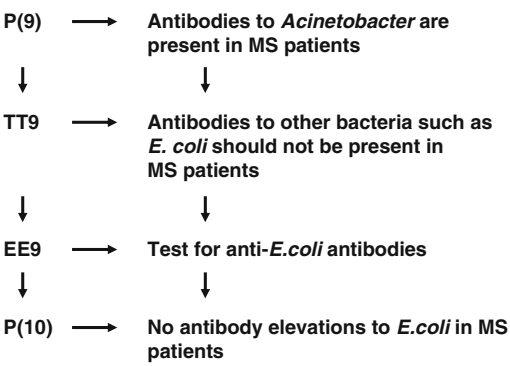
Elevated levels of predominantly IgA antibodies against *Acinetobacter* and against myelin basic protein and neurofilament antigens were found in English multiple sclerosis patients (Hughes et al. 2001).

Levels of IgG antibodies were also elevated and those components belonging to the IgG1 and IgG3 subclasses can readily cross the blood-brain barrier.

If these IgG1 and IgG3 antibodies are present in sufficiently high concentrations, they could cause tissue damage through complement mediated cytotoxicity. It is relevant to note that the pathological lesions of multiple sclerosis have a perivascular distribution suggesting a blood borne cytotoxic agent compatible with an extrathecal origin.

The enzyme mucono-decarboxylase is present in *Acinetobacter* and *Pseudomonas* bacteria but not in *Escherichia coli.* However the common and ubiquitous microbe *Escherichia coli* contains sequences which resemble or crossreact with the prion molecule and this is considered in the next Popper sequence.

20.11 Ninth Popper Sequence



The absence of specific antibodies to *Escherichia coli* would tend to suggest that this microbe is unlikely to be involved in the pathogenesis of multiple sclerosis despite having sequences cross-reacting with prion proteins.

It is proposed that multiple sclerosis could result from exposure to *Acinetobacter* infections. The majority of such infections could be nosocomial, probably subclinical and the likely site to be in the upper respiratory tract.

An upper respiratory tract viral infection may provide a suitable inflammatory exudate for secondary bacterial infection by a microbe already present in this ecological niche.

If that is the case, then this opens up a new therapeutic dimension in the treatment of multiple sclerosis.

Current therapy in multiple sclerosis uses a variety of anti-inflammatory drugs from steroids to the numerous and expensive "biologicals". These agents "dampen down" the inflammatory response but do not treat the cause of the disease.

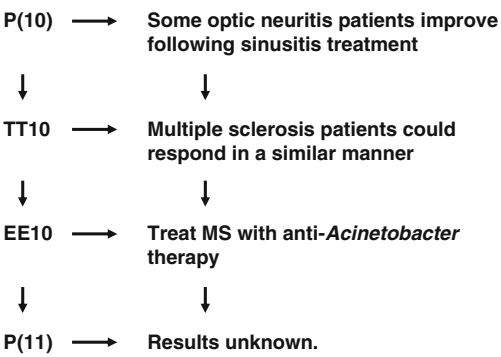
However the possibility arises of treating the disease, at source, so to speak, by removing the aetiological and triggering agent. This brings us to the next Popper sequence.

20.12 Tenth Popper Sequence

Optic neuritis or Devic's disease is a demyelinating disease involving the optic nerve. Some 70 % of optic neuritis patients eventually develop multiple sclerosis.

Some optic neuritis patients are associated with nasal sinusitis. A group from Tucson in Arizona has shown that treatment of the underlining paranasal sinusitis led to improvements in optic neuritis (Averbuch et al. 1989).

These results lead to another Popper sequence (Ebringer et al. 2012).



20.13 General Conclusions

The occurrence of a cattle disease in the 1980s in the UK led to a new way of looking at multiple sclerosis.

"Transmissible spongiform encephalopathies" appeared to have a serious immunological flaw in that it repeated Pasteur's mistake of injecting saline brain homogenates into normal animals and this led to the production of "experimental allergic encephalomyelitis", a complication which in some patients led to a fatal outcome.

Molecular mimicry between myelin and the nasal and skin microbe *Acinetobacter* indicated that such microbes could be involved in multiple sclerosis.

Elevated levels of antibodies to *Acinetobacter* were consistently found in multiple sclerosis patients.

The interesting possibility arises that bovine spongiform encephalopathy could be an animal model of multiple sclerosis.

These issues require further academic and medical attention.

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