Future perspectives for glycolipid research in medicine†

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Medical interest in glycolipids has been mainly directed to the rare and complex glycosphingolipid storage disorders that are principally caused by unitary deficiencies of lysosomal acid hydrolases. However, glycolipids are critical components of cell membranes and occur within newly described membrane domains known as lipid rafts. Glycolipids are components of important antigen systems and membrane receptors; they participate in intracellular signalling mechanisms and may be presented to the immune system in the context of the novel CD1 molecules present on T lymphocytes. A knowledge of their mechanism of action in the control of cell growth and survival as well as developmental pathways is likely to shed light on the pathogenesis of the glycosphingolipid storage disorders as well as the role of lipid second messengers in controlling cell mobility and in the mobilization of intracellular calcium stores (a biological role widely postulated particularly for the lysosphingolipid metabolite sphingosine 1-phosphate). Other sphingolipid metabolites such as ceramide 1-phosphate may be involved in apoptotic responses and in phagocytosis and synaptic vesicle formation. The extraordinary pharmaceutical success of enzymatic complementation for Gaucher's disease using macrophage-targeted human glucocerebrosidase has focused further commercial interest in other glycolipid storage diseases: the cost of targeted enzyme therapy and its failure to restore lysosomal enzymatic deficiencies in the brain has also stimulated interest in the concept of substrate reduction therapy using diffusible inhibitory molecules. Successful clinical trials of the iminosugar *N*-butyldeoxynojirimycin in type 1 Gaucher's disease prove the principle of substrate reduction therapy and have attracted attention to this therapeutic method. They will also foster important further experiments into the use of glycolipid synthesis inhibitors for the severe neuronopathic glycosphingolipidoses, for which no definitive treatment is otherwise available. Future glycolipid research in medicine will be directed to experiments that shed light on the role of sphingolipids in signalling pathways, and in the comprehensive characterization and their secretory products in relation to the molecular pathogenesis of the storage disorders; experiments of use to improve the efficiency of complementing enzymatic delivery to the lysosomal compartment of storage cells are also needed. Further systematic screening for inhibitory compounds with specific actions in the pathways of glycosphingolipid biosynthesis will undoubtedly lead to clinical trials in the neuronopathic storage disorders and to wider applications in the fields of immunity and cancer biology.

Keywords: glycosphingolipidoses; glycolipid storage disorders; therapeutic lysosomal targeting; glycolipid biosynthesis inhibitors; substrate reduction

1. ROLE OF GLYCOLIPIDS

Having heard the presentations, one cannot help but be struck by the inane scientific neglect that the study of glycolipids has so long endured. Despite being key molecules for cellular recognition and as integral components of receptors implicated in common diseases, they have long been relegated to a marginal position in mainstream medical science. Glycolipids are essential components of several blood group antigens (e.g. the Lewis, H, A/B and P systems) and are thus of historical importance after their discovery by Landsteiner in immunology; indeed polymorphic variation in blood groups provides the basis for our understanding of human population genetics

(Cavalli-Sforza & Bodmer 1971; Mourant *et al.* 1975; Naiki *et al.* 1975; Race & Sanger 1975). The study of blood group antigens is moreover of everyday importance in working medicine throughout the world.

As components of cellular receptors for microbes and their toxins (Karlsson 1989), glycolipids are also of world-wide importance: cholera toxin, shiga toxin and the verotoxin of *Escherichia coli*—an important cause of renal failure and the haemolytic-uraemic syndrome—immediately come to mind (Brown *et al.* 1991; Boyce *et al.* 1995). Through the systematic genetic 'knockout' approach taken by Dr Proia, however, we learned of the involvement of complex gangliosides in interleukin 2 signalling in T cells and of the role of GM3 gangliosides in the modulation of insulin receptor activity (Proia 2003). Similar modulatory properties have been suggested for the epidermal growth factor receptor and other receptors that use tyrosine kinase activity for their downstream signalling. The importance of these findings further demonstrates the

†constitutes concluding remarks.

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Table 1. A history of discovery in Gaucher's disease.

1924	Lieb characterized the storage material (galactocerebroside)
1934	Aghion correctly identified the material as glucocerebroside
1964–1965	Patrick and Brady identified glucocerebrosidase deficiency
1968	lysosomal localization of glucocerebrosidase (Weinreb <i>et al.</i> 1968)
1971	O'Brien identified saposin C as a co-activator
1973–1977	purification of placental glucocerebroside (Furbish <i>et al.</i> 1977)
1977	Gaucher's cell shown to be of macrophage lineage (Burns <i>et al.</i> 1977)
1978	Stahl and Sly demonstrated the uptake of glycoproteins by mannose receptors on the surface of macrophages
1982	neurotoxic lysosphingolipids in Gaucher tissue (Nilsson & Svennerholm 1982)
1977–1987	Radin developed the concept of substrate deprivation
1984, 1985	cloning of human glucocerebrosidase gene
1984–2002	pathological mutations of glucocerebrosidase identified
1972–1991	trials of enzyme replacement therapy
1992	alglucerase (placental glucocerebrosidase) licensed
1993	recombinant human glucocerebrosidase (imiglucerase)
1995	gene therapy research
1998	trials of <i>N</i> -butyldeoxynojirimycin
2002	<i>N</i> -butyldeoxynojirimycin licensed for Gaucher's disease

extent to which the intellectual status of glycolipids in biological science has been marginalized.

2. THE STUDY OF RARE VARIANTS

As a physician, and one particularly interested in inborn errors of metabolism, I am struck by the unexpected revelations that repeatedly emerge from the scientific investigation of rare hereditary diseases. In fact, Sir Archibald Garrod FRS, the discoverer of the inborn errors of metabolism, was the first to popularize William Harvey's dictum about the value of 'careful investigation of rarer forms of disease' (Garrod 1928). This statement was itself a rare finding by Garrod as he was preparing to deliver the Harveian oration at the Royal College of Physicians in 1924 and as he read Harvey's correspondence (Willis 1848, p. 616). Be that as it may, it was Garrod who acknowledged 'the debt of science to medicine': a debt that is as real and apparent today (Garrod 1924).

From what we have heard at the meeting, we can see that the scientific investigation of glycolipids itself provides a beautiful example of the general applicability of Harvey's view—a view long accepted in the medical world as a result of Garrod's first incursion into the field of human biochemical genetics (Garrod 1908). For it has been the occurrence of, and needs of, patients with unitary defects in glycolipid metabolism that has driven much of the research described in the presentations. In my particular microdomain of Gaucher's disease, the chronology of discovery is instructive (table 1). From the earliest observational studies of the pathology by Gaucher himself in 1882 and the elementary biochemistry of the disease, research projects have depended on a supply of clinical (and pathological) material. Gaucher's index case was the spleen available at autopsy from a young woman with unexplained splenomegaly (Gaucher 1882). Similar material enabled pathologists to extract the stored lipid and ultimately determine its structure (Aghion 1934). Later it was the living tissue taken opportunistically during the course of clinical work that enabled Dr Brady and others to identify the metabolic defect (Brady *et al.* 1965).

Indeed, after many years, scientific access to patients became essential for clinical trials of enzyme therapy, bone marrow transplantation and, latterly, substrate reduction therapy (as described by Dr Brady, Dr Moyses and Dr Zimran (Ringdén *et al.* 1988; Brady *et al.* 1994; Brady 1997; Cox *et al.* 2000)).

3. INTERDISCIPLINARY RESEARCH INTO MAMMALIAN GLYCOLIPIDS

It is in the nature of the interdisciplinary meetings that, in the words of Sir Richard Burton: '...truth is the shattered mirror strown in myriad bits while each believes his little bit the whole to own' (Burton 1880). Can we bring these myriad bits together here? Perhaps we have had some success since, as outlined by our title, 'glycolipids in cell biology and medicine' implies that the conceptual bridges are already built.

As Professor Konrad Sandhoff has demonstrated, the understanding of glycolipid turnover was principally the realm of biochemistry but it is now clearly a matter of integrative cell biology (Sandhoff & Kolter 1996, 2003). Indeed it is through the study of cell science that we now understand bulk flow and trafficking of glycolipid-rich cell membranes through intracellular compartments (Kolter & Sandhoff 1998). There are mysteries concerning the flip-flop mechanisms and transport process needed for the correct topological delivery of glycolipid intermediates during the synthesis in endoplasmic reticulum and Golgi apparatus of mature glycosphingolipids (Van Echten & Sandhoff 1993). We have learned from Dr Harder that glycolipids are the key to a new concept of cellular membranes. They are integral components of the controversial lipid rafts, which are becoming increasingly credible as anatomical entities and diverse functional microdomains on the cell surface (Harder 2003). Professor Van Meer and Dr Pagano told us of the fate, functioning and pathological trafficking of membrane glycolipids, especially in the storage disorders (Van Helvoort & Van Meer 1995; Pagano *et al.* 2000; Pagano 2003; Van Meer *et al.* 2003). Dr Proia and Dr Walkley have revealed much about the

cellular pathology of storage diseases, particularly in the brain (Walkley 1998). They showed us how electrical system failures may be induced by aberrant connections and of the unexpected cellular infiltrates and inflammatory changes that occur in response to pathological storage of lipids in the diseased brain (Mizukami *et al.* 2002; Myerowitz *et al.* 2002; Proia 2003; Walkley 2003).

4. PATHOPHYSIOLOGY

Claims for success in pathophysiological understanding, however, would be premature. We have an incomplete picture in molecular terms of how disturbances in glycolipid turnover cause illness or even initiate disease. This matter is tackled by Professor Hans Aerts and others who have shown that the primary deficiency in Gaucher's disease induces release of inflammatory and anti-inflammatory components including cytokines (Michelakakis *et al.* 1996; Aerts & Hollak 1997; Allen *et al.* 1997; Hollak *et al.* 1997; Moran *et al.* 2000; Aerts *et al.* 2003). The Gaucher's cell expresses abundant acid hydrolases (and especially proteinases) that are implicated in tissue injury (Moran *et al.* 2000; Cox 2001; Mizukami *et al.* 2002) but may affect the turnover, shedding and fate of cell surface receptors, including the mannose receptor (Ezekowitz & Stahl 1988; Martinez-Pomares & Gordon 1999), that are the putative targets of enzymatic complementation (Brady *et al.* 1994; Brady 1997). If this principle is borne out in practice, there will be a diminishing availability of mannose receptors during the course of the disease, especially when mannosylated proteins are administered as treatment.

The loss of functional receptors for enzyme delivery to disabled tissues may have implications for the administration and dosage of enzyme replacement therapy. Expression of the mannose receptor is itself tightly regulated and an important aspect of the innate pathway of microbial defence. The receptor is found on dendritic cells as well as differentiated macrophages (Ezekowitz & Gordon 1984; Jordens *et al.* 1999; Martinez-Pomares & Gordon 1999; Martinez-Pomares *et al.* 2001). Mice lacking the receptor have increased concentrations of several plasma mannosylated glycoproteins including those with exposed *N*-acetylglucosamine residues; in addition the activities of numerous lysosomal hydrolases are enhanced in the tissues. The receptor is thus implicated in the regulation of serum glycoproteins and their turnover (Lee *et al.* 2002). We remain unable to determine how the storage phenotype is induced in the macrophage that becomes the Gaucher's cell. This ignorance applies equally to the Niemann–Pick cell, the Krabbe (globoid) cell or indeed any other of the phenotypic storage cells that are characteristic of the human glycosphingolipidoses (Cox 2001).

5. GLYCOLIPIDS IN SIGNALLING AND PATHOLOGY

The recent discovery that T lymphocytes specifically recognize glycolipid antigens has allowed definition of the molecular mechanism for lipid presentation to CD1, as described by Professor Vincenzo Cerundolo (Gadola *et al.* 2003). Furthermore, signalling pathways that involve sphingolipids are implicated in immune regulation and

apoptosis (Hannum & Bell 1987; Kolesnick & Golde 1994; Hannum & Obeid 1995; Zhang *et al.* 1997). Like lipid rafts, this is a controversial field but we know for example that a single acid sphingomyelinase is responsible for the apoptotic response to irradiation by X-rays. There is evidence that cells harvested from patients with Niemann–Pick disease and mice lacking the acid sphingomyelinase gene are resistant to this defensive response (Otterbach & Stoffel 1995). However, the ceramide/sphingomyelin pathway, much discussed in the fields of immunology and cell biology, remains a somewhat enigmatic phenomenon. Professor Cerundolo has shown that glycolipids are presented to the immune system uniquely through the agency of the novel CD1 class of immunoregulatory molecules expressed on lymphocytes of the natural killer class. It is tempting to speculate that they may thus participate actively in the inflammatory responses directed against tissues with pathological storage, and a potential role of glycolipid antigens in the mystifying granulomatous disorder, sarcoidosis, has been considered but as yet remains unproven.

There is an emerging body of research indicating that the lysosphingolipids previously proposed as mediators of neuronal death in Krabbe disease and in neuronopathic Gaucher's disease by Svennerholm and colleagues in the 1970s remain important candidates in the molecular pathology of the glycosphingolipidoses (Miyataki & Suzuki 1972; Nilsson & Svennerholm 1982). Sphingolipids and their intermediates are clearly important molecules for the normal development of axonal connections in cultured neurons and they are likely to serve similar functions in neuronal outgrowth of the developing brain *in vivo* (Harel & Futerman 1993).

Hirabayashi and colleagues have shown that soluble ceramide promote cerebellar Purkinje cell growth and differentiation, and that ceramide itself modulates kinase pathways, including the mitogen-activated kinase system (Furuya *et al.* 1998). Im and colleagues have made the striking recent observation that the lysosphingolipid psychosine can transform macrophages into the pathognomic globoid cells of Krabbe disease; they have moreover shown that selected lysosphingolipids bring about this transformation by interacting with a G-protein-coupled receptor (T-cell death-associated gene 8, TDAG8) that is expressed on, for example, the human U937 monocytic cell line (Im *et al.* 2001). In any event, despite these deficiencies in our coverage, Professor Aerts has produced a fine survey of the state of knowledge about the storage diseases and intracellular targets for treatment including, as it were, the moving target of the mannose receptor in Gaucher's disease. We should also not forget the existence of mannose-binding proteins and other plasma lectin-like proteins that may contribute to this system.

6. TREATMENT OF GLYCOLIPID STORAGE DISEASES

We have had a broad conspectus of the different treatments that are available by Professor Gieselmann, particularly in relation to bone marrow transplantation and gene therapy for neurological disorders (Matzner *et al.* 2002; Gieselmann *et al.* 2003). Dr Roscoe Brady has

mentioned his classical studies over many years leading to the successful delivery of enzymatic replacement for Gaucher's disease and, latterly, Fabry's disease (Brady 2003). Dr Terry Butters and Dr Frances Platt have given a very complete account in their extensive articles and presentations about the discovery of the iminosugars (Platt & Butters 1995) and other agents including the so-called P4 compounds developed by Norman Radin (Vunnam & Radin 1980; Inokuchi & Radin 1987; Radin 1996) that modulate the amount of incoming glycosphingolipid substrate by impeding its biosynthesis (Butters *et al.* 2003; Platt *et al.* 2003). It was Radin who first clearly identified this potential mechanism for the treatment of glycosphingolipid storage disorders (Radin & Vunnam 1981; Radin 2000). However, in practice it turned out that the experimental research of Dr Platt and Dr Butters working with Professor Raymond Dwek led ultimately to the design of the first clinical trials of substrate reduction therapy using the iminosugar *N*-butyldeoxynojirimycin in patients with adult Gaucher's disease (Cox *et al.* 2000; Platt *et al.* 2001).

The results of these trials, now extending to 36 months, have been ably summarized by Dr Moyses and Dr Ari Zimran; they confirm early findings of efficacy, now several years into use (Zimran & Elstein 2003; Moyses 2003). Recently, on the basis of an assessment of the existing efficacy and safety profile of *N*-butyldeoxynojirimycin (miglustat), marketing authorization has been granted by the European Commission, for the treatment of mild-to-moderate Gaucher's disease in adults. The use of iminosugars and other inhibitors is at various stages of clinical development and clearly other glycosphingolipid disorders represent credible targets for therapeutic exploration using this strategy.

Much more clinical research will be needed to understand the therapeutic position of the iminosugars in Gaucher's disease and the other glycosphingolipidoses—an intensive pharmacovigilance programme has been established to assist with this—especially to ensure appropriate long-term safety for their use, which is at present highly restricted. However, the progress made so far is encouraging and especially so for those who work in the long-neglected orphan disease area. Clearly, there are concerns about the use of inhibitors of initial committed reactions in the biosynthesis of essential metabolic intermediates for macromolecules that play a central role in cell structure and function such as the glycolipids. There is an interesting analogy here, however, with the pharmaceutical use of the statins that serve as highly selective inhibitors of the pathway of cholesterol biosynthesis. Like the glycolipids and glycosphingolipids, cholesterol is an integral component of the cell membrane and without it the life of animal cells would be unconscionable. None the less the statins are widely prescribed as life-enhancing drugs for patients suffering the consequences in their vasculature of disordered biosynthesis and disposal of cholesterol-containing macromolecules. The partial inhibition of cholesterol biosynthesis through these 3-hydroxy-3-methylglutaryl CoA reductase inhibitors thus represents an excellent example of the benefits of modulating a highly regulated essential biosynthetic pathway for therapeutic purposes.

7. DIRECTIONS FOR FUTURE RESEARCH INTO GLYCOLIPID DISORDERS

At this point I will use a speaker's privilege to set out a personal view of research priorities for the future (table 2).

Experiments of 'scientific light' must surely focus principally on the molecular pathogenesis of the glycosphingolipid storage diseases. This will require the comprehensive characterization of glycolipid storage cells and their secretory products in the context of the disease tissue. We will inevitably require a systematic study of the abnormal signalling pathways in cell proliferation, apoptosis and inflammatory responses—including angiogenesis. Now, with the techniques of cDNA microanalysis, proteomics and the emerging glycomic methodologies employing sensitive tandem mass spectroscopy, we surely have most of the tools necessary for the task. All we need is an interested set of confident investigators and appropriate recognition of the significance of the field by the funding agencies. We have at our disposal authentic models of many of the glycosphingolipidoses in experimental animals. These occur either spontaneously or as a result of experimental design by targeted gene disruption technology. The use of conditional knockouts may also prove to be valuable for studying the evolution of the storage phenotype and its downstream effects.

In the table, I have also listed 'experiments of use'. Clearly enzyme replacement therapy in Gaucher's disease has been an enormous pharmaceutical (and commercial) success. Perhaps because of this there has hitherto been little additional industrial effort to improve the means for enzyme delivery and enhance its selectivity. There is, however, a need to further characterize receptor pathways *in vivo*, especially to optimize competition between tissues expressing different surface proteins that mediate uptake of glycoprotein enzymes into the lysosomal compartment, for example on macrophages.

Such studies in Gaucher's disease will clearly serve as a model for other conditions. It is salutary to consider whether or not enzyme replacement therapy would have been as successful had a target other than Gaucher's disease been chosen. In relation to substrate reduction there is a need for comprehensive knowledge about useful inhibitory compounds with their specific actions on the biosynthetic pathway for glycosphingolipids. There is also *inter alia* an urgent need to find a selective and non-toxic inhibitor of the parallel UDP-galactosylceramide transferase that would serve as a point of therapeutic attack in Krabbe disease and metachromatic leucodystrophy (Platt *et al.* 2001). We also wish to have results of clinical trials of substrate reduction in the neuronopathic storage diseases. These diseases are notoriously difficult to study given the complexity of their effects and the difficulties in assessing changes in neuropsychological, cognitive and neurological function. In relation to gene therapy the identification and development of a non-toxic long-term vector system for abundant expression of therapeutic proteins with effective delivery to specific regions of the brain remain an immense challenge. One is encouraged, however, by the potential value of lentiviral vectors, such as the feline immunodeficiency virus (Brooks *et al.* 2002), and by more recent studies on the use of herpes simplex type-1 viral vectors that have many advantages for use in non-mitotic brain cells

Table 2. Directions for future glycolipid research in medicine.

experiments of light

molecular pathogenesis of glycolipid storage disorders:

comprehensive characterization of the storage cells and their secretory products in the context of the diseased tissue; signalling pathways.

genomic expression, proteomic and *glycomic* technology

authentic disease models in animals

experiments of use

improve enzyme delivery:

characterize receptor pathways and selectivity; optimize competition between tissues *in vivo*

substrate reduction:

comprehensive screening for inhibitory compounds with specific actions in glycolipid biosynthesis

clinical trials in neuronopathic storage disorders

gene therapy:

delivery to specific tissues (brain)

low toxicity

abundant, long-term expression

but whose biology in latency has yet to be fully understood.

8. GAUCHER'S DISEASE: AN EXEMPLARY GLYCOLIPIDOSIS

Gaucher's disease, then, the most frequent glycolipid storage disorder, has served investigators well—a lucky chance? Although chance favours the prepared mind we should not forget that enzymatic complementation in Gaucher's disease was theoretically facile, but proved to be a technically difficult problem to solve. Indeed it required 25 years of committed clinical research and considerable financial investment before a credible drug emerged for clinical use.

The conquest of this most conspicuous glycosphingolipidosis by enzymatic complementation none the less brought together multi-disciplinary studies in diverse fields of biology, including the discovery of the lysosome, membrane receptors, glycobiology and, later, the use of genetic engineering for the pharmaceutical manufacture of a safe treatment that has been a worldwide success. At the same time we must not forget that, were it not for this work, and the ultimate success of the Genzyme Corporation, which adroitly exploited the incoming Orphan Drug legislation, the industrial path for pharmaceutical treatment of rare diseases would never have been beaten. Aspects of this meeting have in reality been occasioned by the signal success of enzyme replacement in Gaucher's disease.

Enzyme therapy has inspired the pharmaceutical substrate-reducing 'movement' and, in the most explicit terms, has provided the strong investment and commitment needed for any therapeutic development. There are other 'small molecules' under scientific development including the iminosugars in the provenance of Oxford GlycoSciences and other inhibitors under investigation by other companies including Genzyme. For this healthy industrial interest those of us who work with otherwise untreatable glycolipid storage diseases should be eternally grateful. We are not here only recognizing the immediate debt to The Royal Society and Oxford GlycoSciences for supporting this meeting in an unrestricted manner, but

also for the excellent academic networking that has resulted. The meeting clearly illustrates the immense scientific stimulation that results from the introduction of a new therapeutic idea; this has given impetus to a whole field of glycolipid research.

9. CLINICAL RESEARCH AND THE DEBT OF SCIENCE TO MEDICINE

As earlier discussed, none of the research carried out on the treatment or molecular cell pathology of glycolipid storage diseases would have been possible without the intense involvement of those who suffer from them. It well behoves us all to remember this: for it is the patients and their families who take the greatest risk for a better world for themselves and others affected by these diseases, and it is only they who can participate meaningfully in clinical trials. Above all, it is the patients who encourage us, the scientists and doctors, to behave ourselves and put squabbles behind us for a 'higher medicine' and proper scientific fulfilment. This, then, is the real debt all of us have: 'the debt of science to medicine'. The debt naturally can be repaid and reciprocated, thereby establishing the most productive amplification of all that is good in the humanity of medicine.

Clearly, opportunities for the future—as illustrated by this meeting—are vast. It is then appropriate to end with the fine words of Archibald Garrod in his Harveian oration: 'obviously clinical medicine presents immense fields of scientific research and those who cultivate them have the added satisfaction that every advance of medical science will, sooner or later, bring in its train some forward movement of the healing art' (Garrod 1924, p. 752).

The most authentic point now to make at the end of this discussion meeting is to marvel at the sheer breadth of what we have heard, so freely communicated, in the presentations over these scientifically enriching days. I join my co-organizers in expressing immense gratitude to all the speakers and participants for their contributions.

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