

Proposed clinical and scientific study

**A new syndrome: enteritis and disintegrative disorder following measles
and measles/rubella vaccination?**

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Responsibilities

- Referral and co-ordination of patient admission for investigation.
- Clinical evaluation, procurement of blood, urine and serum samples.
- Colonoscopy, and tissue procurement/processing.

1.2 Department of Histopathology & Medicine and Department of Molecular Chemistry, University of Greenwich*

Dr Andy J Wakefield (Senior Lecturer & co-ordinating investigator)

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Responsibilities

- Evaluation and description of histopathological changes.
- RNA studies for identification of measles and rubella viruses.
- Cobalamin studies.

1.3 Department of Child Psychiatry

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Responsibilities

- Confirmation and characterisation of features of disintegrative disorder.

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Responsibilities

- Full neurological assessment and investigation.

Introduction

There are indications of the emergence of a new syndrome comprising disintegrative disorder and a possible enteritis associated with vitamin B12 deficiency. The syndrome has been linked - anecdotally but consistently - with either measles or measles/rubella vaccination. It appears to occur in previously well and developmentally normal children following exposure to these vaccines.

Disintegrative disorder (Heller's disease)

Disintegrative disorder (or Heller's disease) occurs when normally developing children suddenly, or over a period of several months, show marked behaviour changes and developmental regression after age 2, often in association with some loss of co-ordination and bowel or bladder function (Reviewed by Rutter et al (1). Behavioral changes include social withdrawal, reduced response to sounds, complete loss of communication, unusual sensory behaviours and development of simple rituals and hand and finger stereotypes, much like those of autistic children. However, disintegrative disorder differs from autism in the loss of motor and self-help skills and usually, too, in the lack of more complex stereotype behavioral patterns (although simple motor stereotypes may occur). This rare disorder can sometimes be linked to measles encephalitis, cerebral lipoidoses, leukodystrophies or other neurological conditions but in most cases no clear cause is ever identified. Even in cases where progressive neurological disorder is eventually identified, initial medical tests are often negative and sometimes diagnoses of hysterical reactions are considered. Thus, it is important to repeat medical investigations if a child's conditions does not improve.

Two different courses are typical of children with regressions occurring after the first few years. Most common are regressions that extend over several months and then plateau, resulting in a developmental and behavioral pattern that looks much like autism with severe mental handicap. In some cases, deterioration continues, with increased motor dysfunction, development of seizures and localized neurological signs.

Another disorder that overlaps in a symptomatology but that does not have quite so poor a prognosis is Landau-Kleffner syndrome of acquired aphasia with epilepsy. Children with this disorder lose receptive and expressive language usually over a period of months, typically in conjunction with the development of seizures or transient EEG

abnormalities. Some social withdrawal and unusual behaviours may occur, but usually relatively normal social relationships are maintained with parents and others known to the child. Non verbal cognitive functioning remains intact. In most cases, the outlook for these children is better than for children with disintegrative psychoses or autism, and sometimes language is eventually regained.

Evidence is accumulating for the participation of immune mechanisms in the pathogenesis of this conditions, including the presence of circulating antibodies to myelin basic protein (MBP) (2,3) and the putative brain serotonin receptors (4). A recent study identified an increase in circulating DR+ (activated) T cells in affected subjects without an increase in IL-2 receptor+ cells (5), suggesting incomplete activation of the immune system, a finding that has been observed in several established autoimmune disorders (6-9), including chronic autoimmune hepatitis, a disease in which a role for measles virus has been implicated (10).

Complement, measles and disintegrative disorder: The association between measles vaccine and disintegrative disorder has been noted independently by Warren R et al in Utah, where they have linked susceptibility to this condition which they have termed secondary autism) to a congenital deficiency of the C4B complement protein (11, 12). They identified a deficient form (null allele) of the C4B gene located in the class III region of the MHC complex, which occurred more than twice as often in affected individuals as in a matched, normal population. The C4B gene products are crucial to activation of the classical complement pathway, a vital mechanism for protection against invading microorganisms. The actual relationship of the C4B null allele to the induction of disintegrative disorder is not known. Hypothetically, individuals inheriting one or two C4B null alleles may not be able to clear certain viruses completely, include live "attenuated" vaccine strains. Persistent infection may induce the development of chronically activated T cell clones with reactivity to epitopes in the central nervous system. Immune mediated damage to, for example, myelin in the developing brain may result in clinical disease. The study will seek to identify and characterise both inherited and acquired abnormalities of complement activation and complement regulation, based upon the hypothesis that abnormal complement regulation is central to the susceptibility and subsequent expression of disease.

Enteritis: mothers of those children reported to us, describe a variety of abdominal signs

and symptoms suggesting intestinal pathology. These include pain, bloating, alternating constipation and diarrhoea, steatorrhoea and failure to thrive. Behavioral symptoms parallel intestinal disturbances. Some have documented macrocytic or iron deficiency anaemias. As yet, the pathogenesis of these features have not been established. This study aims to identify and characterise any morphological and immunohistochemical changes in intestinal tissues of individuals affected by the syndrome and to seek, using established techniques, the presence of measles and rubella virus proteins and RNAs, using appropriate positive and negative controls (Appendix 1).

It is notable that the link between the major elements of this possible syndrome - CNS disease, enteritis, measles virus and complement dysregulation - has been made before. Idiopathic inflammatory bowel disease (Crohn's disease and ulcerative colitis) is associated with multiple sclerosis (13-17), an immune demyelinating disease of the central nervous system: measles virus has been linked, independently, to both conditions (18, 19). In addition, familial abnormalities of complement regulation have been described in inflammatory bowel disease (20). Although the precise nature of this link has not been established, it may provide a paradigm for co-existing and related cerebral and gut-associated immune pathologies.

B₁₂ (Cobalamin: Cbl) metabolism, enteritis and disintegrative disorder: The bulk of the vitamin B12 (Cbl) in the body is represented as its two co-enzymes methyl cobalamin (MeCbl) and adenosylcobalamin (AdoCbl). Both are required for normal cellular metabolism (12). Cbl is known to be involved in only 3 reactions in human tissues: as ado-Cbl in the isomerisation of methylmalonyl CoA to succinyl CoA and of α -leucine to β -leucine, and as Me-Cbl in the methylation of homocystine to methionine, a reaction which also requires methyltetrahydrofolate. A tissue deficiency of MeCbl leads to increased plasma homocystine levels and urinary excretion of homocystine (12). AdoCbl is co-enzyme to methylmalonyl CoA mutase required in the final step of propionate to succinate catabolism. Depletion of cellular AdoCbl leads to raised levels of methylmalonic acid (MMA) in plasma and increased urinary MMA excretion. Absorption of Cbl takes place in the terminal ileum through a process of receptor mediated endocytosis to the plasma Cbl-binding protein transcobalamin II.

The role of cobalamins and pathophysiology of cobalamin deficiency in the central nervous system.

The evidence that Cbl plays a role in the central nervous system comes from several sources. These include case reports describing neurological complications in patients with Cbl deficiency and in patients with inborn errors affecting Cbl metabolic pathways. In addition, animal models have provided experimental evidence that Cbl is necessary for normal function of the nervous system. It is notable that neurological symptoms of Cbl deficiency may be the presenting features and neurological changes frequently occur without anaemia or macrocytosis (reviewed by Green and Jacobsen: ref. 13).

Clinical features of Cbl-deficient myeloneuropathy.

The principal role of Cbl appears to be in myelin producing cells (oligodendroglia), and therefore the pathological effects of deficiency are largely as a consequence of myelopathy. The central nervous system appears to be more susceptible to Cbl-deficiency in infants and children than adults, and involvement is diffuse rather than localised. This may be due to rapid and more widespread myelinogenesis during this period of active growth and development of the nervous system: accordingly, in Cbl-deficient infants and children, a somewhat different spectrum of neurological complications is seen compared with adults. In paediatric patients Cbl-deficiency is usually associated with inborn errors of Cbl metabolism, Cbl-dependent enzymes, or binding proteins which mediate transport of Cbl (15). The more common neurological manifestations encountered in such patients include seizures, mental retardation and abnormal cerebellar and spinal cord function. In addition to metabolic errors, a syndrome of Cbl-deficiency has been described in breast-fed infants of strictly vegetarian mothers consisting of apathy, developmental regression and involuntary movements of the head, trunk and limbs (22).

Electro-physiological abnormalities associated with Cbl myelopathy slowed nerve conduction, abnormal electroencephalogram tracings, delayed visual evoked responses, and abnormal brain stem auditory evoked responses and sensory evoked responses.

Pathogenesis of Cbl-deficient myeloneuropathy.

The mechanism whereby Cbl-deficiency results in neurological damage is not known. Several hypotheses have been advanced, although none fully or satisfactorily explain the

pathogenesis of neurological damage. Overall, however, aberrant myelin synthesis appears to be central to the pathological changes that are observed.

Cbl-deficiency and disintegrative disorder.

A recent, as yet, unreported pilot study of cobalamin metabolism in children with disintegrative disorder and/or attention deficit/hyperactive disorder (AD/HD) has shown that many had abnormally increased MMA excretion and other signs of impaired cobalamin function (Linnell J, personal communication). Some then received high dosage cobalamin treatment which reduced MMA excretion to normal and appeared to confer other benefits, although these were difficult to quantify. Most of these children had as yet undefined intestinal problems; the proposed investigation will include an assessment of intestinal status and function in addition to a range of metabolic tests to assess cobalamin function and metabolism.

Studies of children with inherited errors of B12 metabolism have shown that cobalamin deficiency is due either to impaired intestinal absorption, ineffective plasma transport or defective cellular metabolism. A gross defect is rare and can be life-threatening but a subtle deficiency appears more commonly and requires assessment. Functional cobalamin status is better indicated by direct measurement of the cobalamin co-enzymes or the relevant metabolites than by assaying the 'serum B12' (24). This is because most of the circulating cobalamin is firmly attached to an inactive protein binder of long half-life which does not promote tissue uptake of the vitamin (25).

The proposed studies are designed to determine whether B12 deficiency in affected children occurs as a consequence of failure of terminal ileal reabsorption due to enteropathy, or alternatively, whether there is an innate error of B12 metabolism in such individuals.

Working hypotheses

The possible link between an environmental insult (measles/rubella vaccine) in a previously healthy child, who may be genetically susceptible to responding inappropriately to the virus(es) via abnormal complement regulation, and the subsequent development of enteritis, Cbl-deficiency and disintegrative disorder, permits several working hypotheses to be proposed.

- 1) Enteritis hypothesis: the simplest model may be as follows. In the genetically susceptible individual, the measles virus does not induce an appropriate complement

response leading to persistent infection and inflammation.

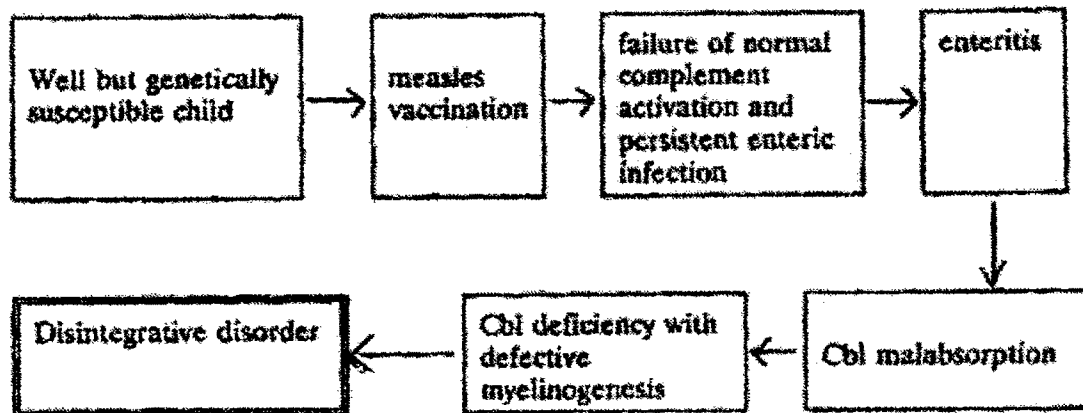


Figure 1. The "enteritis" model for disintegrative disorder

This scenario is supported, indirectly and anecdotally, by the clinical improvement in some affected children treated with Cbl replacement (Linnell J, personal communication). It also suggests that therapeutic intervention directed at controlling the enteritis and replacing Cbl, may be of benefit.

2. Encephalitis/enteritis hypothesis

This invokes that in the genetically susceptible infant, vaccination leads to persistent measles virus infection of both the gut and the brain. It is possible that ensuing inflammation may occur either as a direct result of infection, or via the immune response to persistent infection. Any Cbl deficiency that occurs as a consequence of the enteritis, may then exacerbate neurological damage, but is not its prime cause. The observation that disintegrative disorder may follow acute measles encephalitis, supports this hypothetical pathway. This scenario would be less likely to be amenable to specific therapy since persistent measles virus infection cannot, as yet, be eradicated, although Cbl replacement may still be of some benefit.

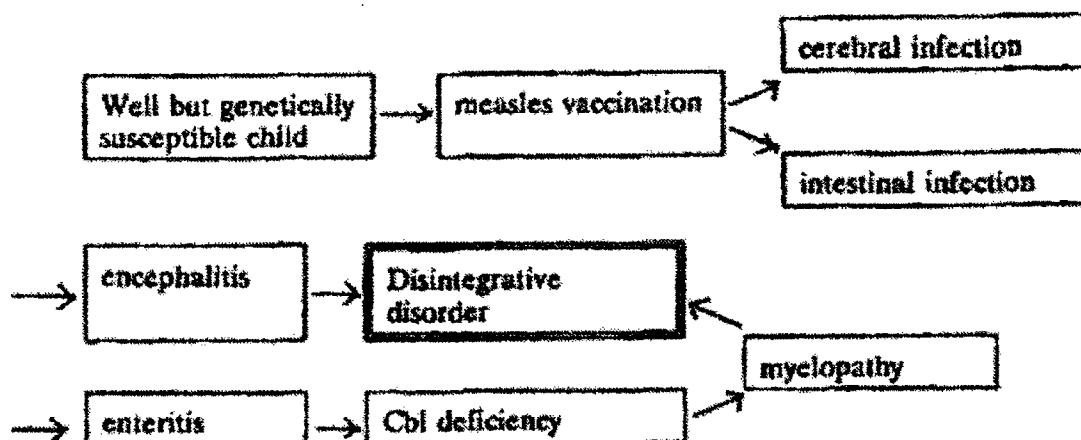


Figure 2. The "encephalitis/enteritis" model for disintegrative disorder.

The proposed studies will involve:

1. Identification and characterisation of any intestinal pathology in affected children by:

- full history/clinical examination (see appendix 2)
- FBC, ESR, U & Es, LFTs, TFT, CRP, Factor XIII, antiendomysial antibodies IgE, IgG subsets, IgA, IgM (total)
- Measles and rubella IgG and IgM immunoreactivity (ELISA & RIA)
- Colonoscopy/ileoscopy and biopsy (formalin + frozen) to include both normal and lesional mucosa
- Histology and immunohistochemistry for measles virus N-protein, and rubella virus as described previously (24). Other studies will be performed as indicated (eg GAGs, T cell markers etc)
- Ultrastructural analysis of mucosal biopsies for more subtle changes
- RNA extraction from frozen biopsies and amplification of measles and rubella virus RNAs (appendix 1).
- Exclusion of other GI infections - shigella, campylobacter, cytomegalovirus, rotavirus, salmonella.

2. Neuropsychiatric studies (Dr Mark Berelowitz)

Confirmation and characterisation of disintegrative disorder features of

CAPA - Child and Adolescent psychiatric assessment.

ADI - Autism diagnostic interview.

3. Neurological and neuroradiological studies (Dr P. Harvey)

Full clinical examination: Magnetic Resonance Imaging (MRI), Lumbar puncture and CSF antibody profile (measles: rubella), cytokine measurement, Electroencephalography (EEG) with visual, somatosensory and brain stem auditory evoked potentials.

4. Cobalamin studies (Dr J Linnell)

4.1 A modified Schilling test will be carried out to assess intestinal cobalamin absorption from food (25). MMA and creatinine will be estimated in random urine specimens (26). Blood samples will be required for plasma total homocystine and total cobalamin assays (27). The blood will need to be protected from exposure to white light if plasma cobalamin coenzymes are also to be estimated (28).

• Modified Schilling Test

Approx. 1mCi Co-Cyanocobalamin (CNCbl) to cooked egg is ingested, a flushing dose of 1mg CNCbl is administered i.m. and the total urine output collected for 24 hrs.

4.2 Urinary MMA excretion

A random urine specimen (10-20ml) is required. MMA is estimated by a thin layer chromatography (TLC) method and creatinine by a routine chemical analyser method. This is effectively a screening method which can be repeated on a 24-hr urine collection if indicated.

4.3 Plasma total homocystine

Plasma (0.5-1.0ml) is extracted and derivatised and the homocystine-derivative separated by high pressure liquid chromatography (HPLC) and detected fluorimetrically.

4.4 Plasma total cobalamin

Plasma (1.0ml) is extracted in an acid/cyanide buffer and total cobalamin assayed by a radioisotopic method using serum as binding agent.

4.5 Cobalamin coenzymes

Plasma (2.0ml) is extracted with hot ethanol and concentrated in a darkroom. Coenzyme and other forms of cobalamin in the extract are separated by TLC and detected bioautographically, overlaying the chromatogram with an agar medium containing a cobalamin-sensitive *E.coli*.

5. Complement studies

(to be completed) DNA/C4B genotyping

Practical issues

i. Firstly, and significantly, this is a demanding protocol both for the children, and for those clinicians carrying out invasive procedures in particular. Due consideration should be given to this when planning the details of the admission. However, it is essential that we characterise as comprehensively as possible, the pathogenesis of this condition - control of any underlying intestinal immunopathology may open up new therapeutic avenues for the treatment of affected children. Our ability to confirm or exclude a role of measles or measles/rubella vaccine also has major implications for public health.

ii. Referrals will be co-ordinated by AJW, JW-S and SM, such that they will be admitted for colonoscopy preparation on a Sunday afternoon. Patients and their parent(s) will remain in hospital for one week. A plan of investigation is shown in Appendix 3.

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APPENDIX 1

DETECTION OF MEASLES VIRUS RNA

1. RNA EXTRACTION ON INTESTINAL BIOPSIES

Using a DEPC-treated, autoclaved dispersing shaft, homogenise ~500g of snap frozen tissue in 3 ml of Total RNA Isolation Reagent (Applied Biotechnologies). Alternatively, use 1ml of reagent per biopsy specimen or 10^6 cultured cells.

1. Leave homogenate at 4°C for 5 min. to allow dissociation of nucleoprotein complexes from RNA.
2. Split homogenate into 3 large Eppendorf tubes (1ml per tube) and add 200 μ l of chloroform to each tube. Vortex and leave at 4°C for 5 min.
3. Centrifuge homogenate at 12,000g for 15 min. at 4°C.
4. Transfer the upper aqueous phase to a fresh Eppendorf, add an equal volume of Isopropanol and leave for 10 min. at 4°C.
5. Centrifuge at 12,000g for 10 min. at 4°C, remove the supernatant and wash pellet twice with 75% ethanol.
6. Resuspend pellet in DEPC-treated water and aliquot RNA solution.

Estimate RNA concentration via OD_{260} . Estimate RNA purity via OD_{260}/OD_{280} .
Examine RNA integrity via agarose gel electrophoresis.

2. HYBRID CAPTURE

Aliquot 10 µl of magnetic solid phase supports containing 40 mer oligonucleotides complementary and internal to the virus target sequence into 1.5 ml Eppendorf tubs. Hybrid capture for measles RNA sequences uses the oligonucleotides HC1 & HC2 listed below

1. Wash supports 3 times with 100 µl of binding buffer.
2. Add 50 µl of binding buffer up to 50 µl of RNA sample (containing up to 50 µg of RNA) and allow to hybridise at room temperature for 10 min.
3. Wash supports twice with 100 µl of wash buffer then add 50 µl of DEPC-treated water and elute captured sequences at 65°C for 15 min.
4. Use 10 µl of elutant for RT-PCR / NASBA amplification.

Hybrid Capture Sequences

HC1 AGA AAT GAT ACT TGG GCT TGT CTG GGT CCA ACC GCT CAT C

HC2 GTT TCA GAG ATT GCA ATG CAA CTG CTG AGG ACA AGA TCA G

- HC1 is a downstream primer binding to positions 1328-1368 of the MV genome.
- HC2 is an upstream primer binding to positions 1288-1322 of the MV anigenome.

3. *rTth* RT-PCR AMPLIFICATION

1. Add 10 μ l of hybrid capture eluant to 40 μ l of an *rTth* polymerase (Perkin Elmer) reaction mixture (prepared in a clean room) containing 1x EZ buffer, 2.5 mM Mn(OAc)₂, 300 μ M each dNTP, 0.45 μ M each PCR primer and 5U *rTth* polymerase.
2. Overlay with 60 μ l of mineral oil and incubate reaction mixture at 60°C for 30 min. followed by 95°C for 2 min.
3. Thermal cycle the reaction mixture 40 times at 94°C for 60 sec. and 56°C for 60 sec. followed by a final extension of 56°C for 7 min.
4. Use 10 μ l of mixture for gel electrophoresis and Southern blot analysis, using an internal probe (for instance MV6) for hybridization.

EZ Buffer

250 mM Bicine, 575 mM Potassium Acetate, 40% Glycerol, pH 8.2.

Measles PCR Primers

MV3 (Upstream)	AGC ATC TGA ACT CGG TAT CAG
MV4 (Downstream)	AGC TCT CGC ATC ACT TGC TCT

- MV3 binds to positions 1248- 1269 of the measles virus antigenome.
- MV4 binds to positions 1480-1501 of the measles virus genome.
- PCR product = 253bp.

Internal Probe

MV6	ATC AGT AGA GCA GTT GGA CC
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- MV6 binds to positions 1324 - 1344 of the measles virus antigenome.

4. NUCLEIC ACID SEQUENCE BASED AMPLIFICATION

1. In a clean room, make up the following reaction mixture:

REAGENT	VOLUME(μ l)
DEPC-dH ₂ O	2.0
5xNN Buffer	4.0
5x Primer mix	<u>4.0</u>
	10.0

2. Vortex master mix and aliquot.
3. In a separate room, add 5 μ l of hybrid capture elutant to each tube, mix and incubate at 65°C for 5 min.
4. Transfer samples to a 41°C waterbath for 5 min, then add 5 μ l of 4x enzyme mix to each tube. Mix using pipette. Do not vortex.
5. Incubate samples at 41°C for 5 min, then pulse and incubate at 41°C for a further 90 min.
6. Nucleic acid reaction products are analysed via Northern hybridization for specific measles products using an internal ³²P-end labelled oligonucleotide (for instance MV6).

5xNN Buffer

REAGENT	VOLUME(μ l)	FINAL
CONCENTRATION		
1 M Tris, pH8.5	200.0	40 mM
1 M MgCl ₂	60.0	12 mM
4 M KCl	87.5	70 mM
1 M DTT	25.0	5 mM
20 mM Each dNTP	50.0	1 mM
20 mM ATP,UTP,CTP	100.0	2 mM
20 mM GTP	75.0	1.5 mM
100 mM ITP	25.0	0.5 mM
DEPC-dH ₂ O	<u>27.5</u>	

5xPrimer Mix

DMSO to a final concentration of 75%.

Primer 1 and primer 2 to final concentrations of 1.0 μ M.

4xEnzyme Mix

REAGENT	VOLUME(μ l)		FINAL
CONCENTRATION			
DEPC-dH ₂ O	969.0	-	
4.5 M Sorbitol	668.0	1.5 M	
20 mg/ml BSA	42.0	0.4 mg/ml	
0.87 U/ μ l RNase H	36.0	0.016 U/ μ l	
70 U/ μ l T7 Polymerase	184.0	6.4 U/ μ l	
25.3 U/ μ l AMV-RT	101.0	1.3 U/l	

Aliquot and store at -70°C. DO NOT VORTEX.

Measles Primers for NASBA

AB20 (Upstream) AGG GCA AGA GAT GGT AAG GA
AB22 (Downstream) AAT TCT AAT ACG ACT CAC TAT AGG G
GA TCA CCG TGT AGA AAT GAC A

- Bold type indicates T7 Polymerase binding site.
- AB20 binds to positions 1200-1219 of the measles virus antigenome.
- AB22 binds to positions 1358-1379 of the measles virus genome.
- NASBA product = 179b.

Internal Probe

MV6 ATC AGT AGA GCA GTT GGA CC

- MV6 binds to positions 1324 - 1344 of the measles virus antigenome.

Nick

Sequencing Studies

Investigation	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Gastro/ General	Consent	Bloods Colonoscopy Bx		Barium* Follow through X-ray		
	History/ Examination	Upper GI Endoscopy Bx				
Neuro		*MRI (Radiology) Lumbar puncture Malcolm Ward	*EEG (Neurology) Evoked responses		Dr Harvey Malcolm Ward	
Psychol				Dr Berelowitz Dept of Adolescent and Child Psychiatry		
B ₁₂					Schilling test 24-hour urine	collection
Immunol/ Complement Studies	*White cells isolated for DNA extraction		Serum Stored at -70°C			

Please note times of investigations may change according to availability.

* Dr Wakefield to arrange.

+ Paediatric Gastroenterology to arrange, as indicated.