Detection of persistent measles virus infection in Crohn's disease: current status of experimental work

The aetiology of Crohn's disease is unknown. Any hypothesis must take into account the continuing increase in incidence in some countries, including the UK. The increase affects the population from early teens, and specific comprehensive epidemiological studies in children and adolescents show a continuing rise in the rate of age and sex standardised incidence in Scotland.\(^1\) This suggests an environmental trigger, which may interact with underlying genetic susceptibility. A number of such environmental triggers have been proposed, including persistent infections, transient infections in a host with abnormal mucosal immunity, particulate materials, or dietary changes. Persistent infection with the measles virus after wild-type virus infections or immunisation with live attenuated measles vaccine have been proposed as important environmental triggers based on epidemiological observations.\(^2\) Measles is a single stranded RNA virus which can induce immune suppression. Measles infection is generally self limited and results in long term immunity, but rarely, persistent infection may occur after wild-type virus infection. The virus has special affinity for epithelial cells of the respiratory tract and cells of the immune system, such as lymphocytes and macrophages.\(^3\) A number of chronic diseases have been linked with persistent measles virus infection. These include multiple sclerosis, Paget's disease, a variety of autoimmune diseases, and autism, although definite evidence is lacking in each. Inflammatory bowel disease (IBD), especially Crohn's disease, has been linked to persistent measles virus infection. Ekbom \emph{et al} proposed perinatal exposure to wild-type measles virus may lead to development of Crohn's disease later in life.\(^4\) Such case control epidemiological studies are of course prone to a variety of confounding factors, especially selection and recall biases. Epidemiologically robust data from a large controlled prospective study showed absence of a link between intrauterine exposure to measles and Crohn's disease.\(^5\) However, epidemiological studies linking persistent measles virus infection to Crohn's disease generated a hypothesis\(^6\) for confirmation or rejection by searching for measles virus in Crohn's affected tissue.

Another line of epidemiological evidence linking Crohn's disease with measles implicated the vaccine strain, and the safety of measles-mumps-rubella vaccination has been a topic of lively debate both in the medical and lay press. Vaccines against measles, mumps, and rubella (MMR) are produced from live attenuated viruses which have been propagated in a variety of cell substrates, such as embryonated chicken eggs and/or human diploid cells. The genomes of all three viruses consist of a single stranded RNA molecule that has negative polarity for measles and positive polarity for rubella. MMR vaccines have been propagated in a variety of cell substrates, such as embryonated chicken eggs and/or human diploid cells. The virus has special affinity for epithelial cells of the respiratory tract and cells of the immune system, such as lymphocytes and macrophages.\(^4\) Aseptic meningitis and acute arthritis.\(^7\) Aseptic meningitis was ascribed to the Urabe strain of mumps virus, which was withdrawn in 1992. Arthritis has been related to rubella vaccination but no significant association between chronic arthropathy and rubella vaccination has been found in women. Severe allergic reactions to MMR may be related to egg allergy or allergy to gelatin.\(^8\) In 1995, the safety of measles and MMR vaccination was brought into question by the suggestion that live attenuated measles vaccine may be a risk factor for the development of IBD, especially Crohn's disease.\(^9\) This link was postulated following epidemiological observations of the temporal link between an increasing incidence of Crohn's disease and introduction of live attenuated measles vaccine in 1968 in the UK. The MMR vaccine was introduced much later in 1988. However, the increase in the incidence of Crohn's disease started earlier than 1968 and still continues.\(^1\) A case control study of 140 IBD patients (83 Crohn's disease) born after 1968 and 280 controls matched for age, sex, and general practitioner area provided no support for the hypothesis that measles vaccination in childhood predisposed to the later development of either IBD overall or Crohn's disease in particular.\(^10\) In Finland, a countrywide surveillance system to detect serious adverse events was set up in 1982 after introduction of MMR vaccination. By the end of 1996, 1.8 million individuals were immunised and three million vaccine doses were consumed. The rate of serious adverse events with possible or indeterminate causal relationship with MMR vaccination was 5.3 per 100 000 vaccinees or 3.2 per 100 000 vaccine doses. The majority of serious adverse events were neurological or allergic. No association between IBD and MMR vaccination was reported. This is currently the best prospective data on the safety of MMR vaccination.\(^11\)

Considerable interest was then generated by the report of the presence of measles virus nucleocapsid protein in intestinal tissues affected by Crohn's disease.\(^12\) This article reviews the current state of the evidence from the experimental work regarding the question of persistent measles virus in Crohn's disease. The epidemiological evidence itself has generated a lively debate\(^13\)–\(^17\) but this article deals with the experimental work aiming to confirm or reject the hypothesis formulated from the epidemiological evidence.

Measles serology

The hypothesis that persistent measles virus infection may result in Crohn's disease prompted serological studies for measles antibody. Sera from 14 affected members of two French families with a high incidence of Crohn's disease and from unaffected family members as well as healthy age and sex matched controls were tested for measles IgG and IgM antibodies.\(^18\) Commercially available indirect enzyme immunoassay kits (Whittaker Bioproducts Inc., Walkersville, Maryland, USA) were used to detect measles IgM and IgG antibodies. There were no differences in measles antibody levels between affected and unaffected individuals.\(^12\)

Abbreviations used in this paper: IBD, inflammatory bowel disease; PCR, polymerase chain reaction; RT, reverse transcriptase; SSPE, subacute sclerosing panencephalitis; NIBSC, National Institute for Biological Standards and Control; MMR, measles-mumps-rubella vaccination; pfu, plaque forming units.
IgM or IgG antibody levels between patients and controls, or between patients and unaffected family members. The authors concluded that their data did not support the hypothesis that persistent measles virus infection causes Crohn’s disease. Fisher and colleagues reported that the median measles virus IgG antibody titre in Crohn’s disease patients determined by an inhouse complement fixation test was actually lower than the median titre in controls. There was also a trend towards lower median measles virus IgM antibody titre determined by a commercial ELISA in Crohn’s disease patients compared with controls. In a study from the National Institute for Biological Standards and Control (NIBSC), all patients with Crohn’s disease, ulcerative colitis, and non-IBD controls had detectable levels of serum neutralisation antibodies against measles virus. The average antibody titre for all patients and controls (n=30) was 1:197, suggesting that all patients had either been exposed to measles wild-type or vaccine strains previously. The antibody titres of the non-IBD control group were similar to the titres of the Crohn’s disease and ulcerative colitis groups. A characteristic feature of serological studies in subacute sclerosing panencephalitis (SSPE) cases, where measles persistence is well established, is the significantly elevated concentrations of measles specific antibodies both in serum and cerebrospinal fluid. Such an increase in antibody concentrations has not been detected in serum samples from IBD patients by any research group. On serological grounds therefore it is unlikely that Crohn’s disease shares with SSPE the characteristics of measles virus infection in early life followed by a latent illness with persistent measles virus particles in the target organ.

Non-polymerase chain reaction (PCR) based methods
The initial methods that demonstrated the presence of measles virus in Crohn’s affected tissues included immunocytochemistry, in situ hybridisation, and transmission electron microscopy. The specificity and methodology used in these studies are open to question. Non-specific reactivity of measles antibody can be problematic and the used in these studies are open to question. Non-specific measles virus in Crohn’s disease is unlikely that Crohn’s disease shares with SSPE the characteristics of measles virus infection in early life followed by a latent illness with persistent measles virus particles in the target organ.

PCR based methods
Measles specific PCR enables amplification of measles virus RNA to allow definitive molecular characterisation. Several laboratories developed their own RNA based reverse transcription (RT) PCR assays to detect measles virus genomic RNA in tissues derived from Crohn’s disease patients. Most groups used resected materials which provide large amounts of tissues involving all layers of the intestine but often represented end stage disease after intensive medical therapy, including immunosuppressives. The sensitivity limits of these RT-PCR assays varied considerably between laboratories but a N, F gene specific RT-PCR nested PCR is reported to have an assay sensitivity of a single genome copy.

The NIBSC evaluated both a two step approach (RT followed by PCR and nested PCR) and a single step approach (both RT and primary PCR step combined using EZ RTth RNA PCR kit). The latter was at least 100- to 1000-fold more sensitive than the two step approach. The NIBSC assay was able to identify a measles virus genome sequence in control samples that had measles virus correspondence to as little as 5.5x10⁻¹ plaque forming units (pfu). Measles N gene primers used in this study were highly sensitive and could amplify target sequence from all known strains of measles virus. Partially homogenised brain material from a patient with SSPE was examined as a positive control, and measles specific nucleic acid was detected from a sample equivalent to 18 cells. In contrast, no tissue sample from IBD patients derived from endoscopic biopsies was positive for measles specific nucleic acid. Biopsies from all control patients were also negative. A total of 93 colonoscopic biopsies were examined and both steroid naïve and steroid treated patients were included. About one million cells were examined per biopsy. All
patients had detectable neutralisation antibodies against measles virus in serum and 5/30 patients had confirmed measles vaccination.

Thirty one peripheral blood lymphocyte preparations (20 patients with IBD, 11 non-IBD controls) were also examined by the rTth RT-PCR nested PCR method, as described above, at the NIBSC.\cite{20} None of the amplifications of the nucleic acids extracted from the lymphocyte preparations produced DNA fragments of the size expected for measles specific sequences.\cite{20}

Colonoscopic biopsies are superficial and may miss virus persistence in deeper layers within the granulomas. Hence full thickness resection specimens of seven Crohn’s disease patients taken from macroscopically inflamed and healthy areas of the gut as well as mesenteric lymph nodes were further investigated.\cite{35,36} Resected specimens had been snap frozen in liquid nitrogen immediately after removal. Despite using oligonucleotide primers corresponding to three different portions of the measles virus genome (N, M, and H gene regions, full details of primer positions and sequences reported elsewhere\cite{34}), none of the surgically resected specimens gave any positive signals that could suggest the presence of measles virus RNA sequences in Crohn’s tissue. The extraction mixtures derived from 3–4 slices of intestinal tissue had about 6–8 million cells, and that derived from 2–3 slices of mesenteric lymph node tissue had 4–6 million cells. The assay sensitivity limits for the wild-type strain of the N gene primer was $5.5 \times 10^{-1}$ to $5.5 \times 10^{-3}$ pfu, of the M gene primer was $5.5 \times 10^{-1}$ to $5.5 \times 10^{-2}$ pfu, and of the H gene primer was $5.5 \times 10^{-1}$ to $5.5 \times 10^{-2}$ pfu.

The negative results obtained in all attempts to detect measles virus genome in Crohn’s affected tissue using RT-PCR have failed, a recent study based on a Japanese-Royal Free Hospital group collaboration have claimed detection of the presence of wild-type and vaccine-type measles virus genome in peripheral blood mononuclear cells from IBD patients (as well as patients with autism).\cite{37} However, the NIBSC study also attempted to detect measles virus genome in lymphocyte preparations from IBD and control patients with paired tissue specimens and all lymphocyte preparations were negative. The experience with clinical specimens at NIBSC has shown that even with the most meticulous technique, cross contamination of specimens can occasionally occur.\cite{20} PCR contamination has also been reported by the Royal Free Hospital group.\cite{35} A range of methods and consideratory techniques are not applied. The issue of cross contamination during PCR based diagnosis is well documented and reviewed recently in context with human

<table>
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<tr>
<td>Royal Free Hospital, London, UK\cite{20}</td>
<td>CD, UC, IC, controls</td>
<td>Resected material + PBMC</td>
<td>Hybrid capture + N, H gene specific RT-PCR</td>
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<td>Resected intestine + biopsies</td>
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<td>CD, UC, controls</td>
<td>Resected intestine</td>
<td>N, M, F, H gene specific RT-PCR</td>
<td>Not reported</td>
<td>Not reported</td>
<td>–ve</td>
</tr>
<tr>
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<td>CD, UC, controls</td>
<td>PBMC</td>
<td>F, H gene specific RT-PCR nested PCR</td>
<td>Not reported</td>
<td>SSPE (brain); SSPE (PBMC), vaccine and wild strains</td>
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| Table 1 Summary of polymerase chain reaction (PCR) studies performed to identify measles virus in inflammatory bowel disease

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| Table 2 Oligonucleotide primer sequences of three measles virus genes used in the NIBSC study

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<th>Gene Designation</th>
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<th>Sequence (5’→3’)</th>
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<tr>
<td>M MV1/outer</td>
<td>1198–1218</td>
<td>TTAGGGCAAGAGATGGTAAAGG</td>
</tr>
<tr>
<td>M MV2/outer</td>
<td>1609–1630</td>
<td>GTTCTTCCGGAGATCCGCGCA</td>
</tr>
<tr>
<td>M MV3/inner</td>
<td>1248–1268</td>
<td>AGGACTCTGAACCTCCTGAC</td>
</tr>
<tr>
<td>M MV4/outer</td>
<td>1480–1500</td>
<td>AGGCCCTGCAGCATTCTGTCT</td>
</tr>
<tr>
<td>M MV13/outer</td>
<td>3565–3584</td>
<td>GGGACAGGAGGATGAAATGC</td>
</tr>
<tr>
<td>M MV14/outer</td>
<td>3832–3851</td>
<td>GGGTGGGCTTTGAACAGCTC</td>
</tr>
<tr>
<td>M MV15/inner</td>
<td>3587–3603</td>
<td>TATGTAACGTTTCTGCC</td>
</tr>
<tr>
<td>M MV16/inner</td>
<td>3811–3829</td>
<td>TGTGTAGCCATCTTCTCC</td>
</tr>
<tr>
<td>M MV16/outer</td>
<td>8014–8125</td>
<td>CAGTCGTAATGCTCTGCAC</td>
</tr>
<tr>
<td>M MV17/outer</td>
<td>8670–8701</td>
<td>GCACTGTAAATTGGAGAATA</td>
</tr>
<tr>
<td>M MV18/outer</td>
<td>8147–8171</td>
<td>GAACCTCAACATCGACCGTTC</td>
</tr>
<tr>
<td>M MV19/outer</td>
<td>8457–8482</td>
<td>ATCCTTCAATGGTGCCCACTCGGGA</td>
</tr>
<tr>
<td>M MV20/outer</td>
<td>8101–8125</td>
<td>CAGTCGTAATGCTCTGCAC</td>
</tr>
<tr>
<td>M MV21/outer</td>
<td>8147–8171</td>
<td>GAACCTCAACATCGACCGTTC</td>
</tr>
<tr>
<td>M MV22/outer</td>
<td>8457–8482</td>
<td>ATCCTTCAATGGTGCCCACTCGGGA</td>
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The nucleotide positions are in relation to the sequence reported for the measles virus genome in the EMBL-Genbank database under accession number K01711. N, nucleocapsid protein gene; M, matrix protein gene; H, haemagglutinin protein gene.\cite{35,36,20}
Persistent measles virus infection in Crohn’s disease

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viral diagnosis. The exquisite sensitivity of the PCR reaction has led to applications in medical diagnostics, population genetics, and forensic analysis. Obviously, the fewer the number of molecules we are trying to detect, the more is the need to guard against false positives or mistyping, a caveat well known to forensic molecular biologists. The use of PCR for amplification of a few molecules of nucleic acids requires rigorous adherence to a strict set of protocols as the product of amplification serves as the substrate for generation of more product. A single PCR cycle generates very large numbers of amplifiable molecules that have the potential to contaminate subsequent amplifications of the same target sequence. To control contamination, one must prevent the transfer of nucleic acid molecules between amplified samples, and between positive and negative experimental controls. A number of precautions are generally adopted to reduce false positive rates in laboratories, but false positives may still occur. Indeed, if there is any reasonable doubt about a critical experimental result, it is wisest to repeat the experiment. The negative controls may eliminate reagent contamination but cannot guarantee against sporadic contamination; it is however very unlikely that a sporadic contamination event would occur twice in exactly the same way. It is possible that the sequences reported by Kawashima and colleagues have emerged as a result of cross contamination of the samples with measles virus controls or pre-existing DNA templates during nested PCR amplification. This is supported by the fact that the sequences derived from cases of IBD and autism could not be grouped equally in relation to their clinical histories and established phylogenetic relationships of wild-type and vaccine strains. In a recent correspondence, we have presented evidence that the results of Kawashima et al are not internally consistent and not consistent with the findings of others. Based on the sequence data provided by Kawashima et al, the sequence derived from a Crohn’s disease patient differed by only two nucleotides from the vaccine strain while the sequences derived from cases of autism, which theoretically should be identical to the vaccine strains, differed by 3–5 nucleotides. There is also evidence to suggest the possibility that mixed DNA fragments were present in the PCR products that are usually produced by cross contamination with more than one template.

Molecular mimicry
As persistence of measles virus genomic sequences in Crohn’s tissue has not been demonstrated by a number of researchers using state of the art techniques, we now have to re-examine the original immunohistochemical demonstration of signal for measles virus N protein using monoclonal antibody immunohistochemistry (Sereab, Crawley Down, Sussex, UK). Iizuka et al have presented evidence that this monoclonal antibody recognises a host antigen as well as measles virus. The host antigen recognised by the monoclonal antibody was unrelated to the measles virus. Serum neutralisation antibodies against measles virus is ubiquitous, both in IBD patients and in non-IBD controls. The measles related antigen has not been identified, as no homologous proteins have been found in the protein databases. However, the nucleotide sequence of the positive clone was 99% homologous with the human gene AA449055 deposited in DNA databases. A Southern blot analysis confirmed the human origin of the measles related antigen. The functional role of the measles related antigen is unclear but it may be associated with a subpopulation of macrophages. Molecular mimicry, the concept that antigenic determinants of micro-organisms resemble antigenic determinants of the host, is frequently cited as a plausible mechanism to account for the association of infection and autoimmune disease. Although an impressive number of such mimicry have been reported, there are no clear examples of a human disease caused by molecular mimicry. Epitope homology of a viral and self determinant is not in itself adequate evidence for mimicry as a pathogenetic mechanism. The mimicking determinant must also be capable of inducing disease in the absence of replicative virus, and there is no such evidence in Crohn’s disease. Molecular mimicry at the T cell level may be important in the development of systemic autoimmunity. Studies in multiple sclerosis found no evidence of a multiple sclerosis specific pattern of T cell responses to recombinant measles virus structural proteins but similar studies have not been performed in Crohn’s disease. Liu and colleagues found that almost all immunolabelling by polyclonal measles virus antibody occurs in the nucleus, and measles virus phosphoprotein is known to have immunological cross reactivity with an intermediate filament protein of human cells, probably vimentin. Antimeasles monoclonal antibodies may also cross react with mouse anterior pituitary, gastric mucosa, salivary gland, and neurons in the brain. A comparison of the temporal profile of the epidemiology of measles and Crohn’s disease makes it unlikely that molecular mimicry might be a plausible hypothesis to explain the onset of Crohn’s disease after an outbreak of measles. The widespread use of measles vaccine in children older than one year in developed countries has greatly reduced the prevalence of measles in all age groups, including infants under one year. Exposure to wild measles virus in infancy when the infant is immunologically immature (as suggested by Van damme and colleagues) in developed countries (including Scotland) has therefore dramatically reduced over a period in which the incidence of juvenile onset Crohn’s disease has increased, especially in the 12–16 year old age group. Prior to measles immunisation, 30% of measles cases in urban Africa occurred in infants under one year. This burden of wild measles infection should have resulted in an epidemic of Crohn’s disease in the survivors long ago.

Summary
Epidemiological studies may generate a hypothesis regarding the aetiology of chronic diseases that may be tested directly. In the case of persistent infections, demonstration of a putative organism by a sensitive and specific test provides essential evidence in favour of such a hypothesis. The measles virus hypothesis had generated considerable excitement, attracted resources, and spurred Crohn’s disease researchers to perform corroborative tests. It also however had undesirable effects on uptake of immunisation, a potential public health hazard. As a number of well conducted studies to demonstrate measles virus genome in Crohn’s disease tissues using state of the art RT-PCR based assays have proved to be negative, it must be concluded that the persistent measles virus infection hypothesis in the aetiology of Crohn’s disease lacks confirmatory evidence. There is now enough experimental evidence to conclude that failure to detect measles virus genome in IBD tissues is not due to the inefficiencies of the PCR based detection systems but to the absence of measles virus particles. The challenge facing the medical profession is to convince the public that there is no evidence of measles virus persistence in the intestine of Crohn’s disease patients.

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