A potential viral pathogenic mechanism for new variant inflammatory bowel disease


Patients and RNA extraction

All patient samples were provided by the department of gastroenterology, Royal Free Hospital, London, UK. Ileal lymphoid tissues from 91 affected children were examined (median age, 7 years; range, 3–14; 77 boys). Developmentally normal paediatric controls (n = 70; range, 0–17 years; 47 boys) included: 19 children with normal ileal biopsies, 13 children with mild non-specific chronic inflammatory changes, three children with ileal lymphonodular hyperplasia (LNH) investigated for abdominal pain, eight children with Crohn’s disease, one child with ulcerative colitis, and 26 children who had undergone appendectomy for abdominal pain including appendicitis.

MV positive control material included two cases of SSPE and MV infected Vero cells. Negative control material included uninfected Vero cells, and human tissues, control RNA extracted from Raji cells (Applied Biosystems, Foster City, California, USA) and normal peripheral blood mononuclear cells.

Total RNA was extracted from fresh frozen biopsies, peripheral blood mononuclear cells, and MV infected and uninfected Vero cell lines using the Ultraspec-11 RNA isolation system (Biotex Laboratories, Houston, Texas, USA). Total RNA was extracted from formalin fixed, paraffin wax embedded tissues using the Purescript® RNA isolation kit (Gentra Systems, Minneapolis, Minnesota, USA).

**Materials and Methods**

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**Abbreviations:** AP, alkaline phosphatase; BCIP, bromochloroindoylphosphate; DIG, digoxigenin; F, fusion; H, haemagglutinin; IL, interleukin; LNH, lymphonodular hyperplasia; MV, measles virus; N, nucleocapsid; NBT, nitrobluetetrazolium; PCR, polymerase chain reaction; RT, reverse transcription; SSC, sodium citrate; SSPE, subacute sclerosing panencephalitis; Th1, T helper cell type 1.
Table 1  Measles virus primer and probe sequences

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence 5′−3′</th>
<th>Amplicon size</th>
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<tr>
<td>N1 forward</td>
<td>5′ TCA GTA GAG CGG TGG GAC CC 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>N1 reverse</td>
<td>5′ GCC CGG GTT CTT CGT TAG CT 3′</td>
<td></td>
</tr>
<tr>
<td>N2 forward</td>
<td>5′ GAG TCG AGG AGA AGC CAG GG 3′</td>
<td></td>
</tr>
<tr>
<td>N2 reverse</td>
<td>5′ GCT GGA CTC CGA TGC AGT GT 3′</td>
<td>120 bp</td>
</tr>
<tr>
<td>H1 forward</td>
<td>5′ TTC ATG GGG CAG CCA TCT AC 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>H1 reverse</td>
<td>5′ CTG TCA GGT GTT CTC AGG CC 3′</td>
<td></td>
</tr>
<tr>
<td>H2 forward</td>
<td>5′ TGG GCA CCA TTA AGG GAT AA 3′</td>
<td>120 bp</td>
</tr>
<tr>
<td>H2 reverse</td>
<td>5′ AAC GGT GTG TTA TCA ATG GC 3′</td>
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<tr>
<td>F1 forward</td>
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<td>F1 reverse</td>
<td>5′ CCC ACC GGT CAA ATC GAT T 3′</td>
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<tr>
<td>F2 forward</td>
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<tr>
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<tr>
<td>GAPDH 1</td>
<td>5′ GAA GAT GGT GAT GGG ATT TC 3′</td>
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<tr>
<td>GAPDH 2</td>
<td>5′ GAA GAT GGT GAT GGG ATT TC 3′</td>
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<tr>
<td>H1 probe</td>
<td>5′ CCC CAG AGA AGA GGA ATA AAA GCC TCA GCA C 3′</td>
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</tr>
<tr>
<td>F1 probe</td>
<td>5′ CTG CAC GAG GGT AGA GAT CCG AGC ATA CAG 3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH probe</td>
<td>5′ CCG ACT CTT GTC CTT CGA AC 3′</td>
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</table>

Measles virus N gene primers and probes were designed based on the following Genbank sequence entries (accession numbers); X16565, S58435, NC_002494, NC_002496, X01999, U03661, U03658, and U03656. Measles virus H gene primers and probes were designed based on the following GenBank sequence entries; X16565, U03649, U03654, U03669, U03650, U03677, U03678, 270073, AF045204, Z66517, and Z80816. Measles virus F gene primers and probes were designed based on the following Genbank sequence entries; X16565, U03655, U03666, U03648, U03662, U00146, U03657, U03651, U03659, AJ133108, X16567.

Solution phase RT-PCR
Polymerase chain reaction (PCR) primers and probes to conserved regions of the MV Nucleocapsid (N), Haemagglutinin (H), and Fusion (F) genes were designed using Primer Express Software Version 1.5 (ABI Prism; Applied Biosystems). The specificity of selected sequences was checked using the NCBI Blast program (www.ncbi.nlm.nih.gov/blast). Table 1 shows the MV primer and probe sequences, amplicon sizes, and GenBank accession numbers used for designing PCR primers and oligonucleotide probes. In some instances primer sets overlap with each other (for example, the sequence of amplicon N1 overlaps partially with the N2 PCR amplicon).

In situ PCR, oligonucleotide probes were modified at the 5′ end by the addition of a biotin moiety and for Southern blot analysis probes were labelled at the 3′ end with digoxigenin. For TaqMan quantitative reverse transcription PCR (RT-PCR), probes were dual labelled with the fluorescent molecule FAM at the 5′ end and the quencher TAMRA at the 3′ end.

Purified MV RNA (HU2) was used as a positive control to optimise PCR assays. The following optimal reaction conditions were used for each 25 µl reaction: 0.4mM dNTPs, 0.4µM forward and reverse primers, 2.5mM magnesium acetate, 5 U rTth DNA polymerase, 0.01 U AmpErase, and 1× EZ buffer. The EZ buffer consisted of 50mM bicine, 125mM potassium acetate, 40% (wt/vol) glycerol (pH 8.2) (Applied Biosystems); 50 ng of extracted RNA was used for each reaction. The following RT-PCR thermal cycling conditions were used on a 9700 PCR thermocycler (Applied Biosystems); 50°C for two minutes, 58°C for 30 minutes, 95°C for five minutes, then 40 cycles of 94°C for 20 seconds, 59°C for 20 seconds, and 72°C for 20 seconds, followed by an extension step at 72°C for 10 minutes.

Southern blot analysis
To confirm reaction specificity, solution phase RT-PCR was performed, as described above, on four affected children positive for MV by TaqMan RT-PCR (see below). MV F and H gene amplicons from MV infected Vero cells, SSPE brain, and ileal lymphoid tissues from four affected children, together with a no template control, were examined by Southern blotting using sequence specific probes (Table 1). MV specific oligonucleotide probes were labelled at the 3′ end with digoxigenin using a DIG oligonucleotide 3′ end labelling kit (Roche Molecular Biochemicals, Mannheim, Germany), hybridised to the Southern blot at 50°C in 5× saline sodium citrate (SSC) with 0.2% sodium dodecyl sulfate for one hour, washed once in 5× SSC at 50°C for 10 minutes, once in 2× SSC at room temperature for 10 minutes, and once in 0.2× SSC at room temperature for 10 minutes. Hybrids were detected using the DIG luminescent detection kit (Roche Molecular Biochemicals).

TaqMan RT-PCR
Real time quantitative RT-PCR based on the 5′ nuclease assay was performed on an ABI 7700 Sequence detector (Applied Biosystems). Sequence specific PCR primers and TaqMan probes were designed using Primer Express software as described above. All quantitative PCRs were prepared in a dedicated facility in a class 2 laminar flow bench hood using dedicated pipettors and aerosol resistant pipette tips.
RNA was prepared and added to the PCR mastermix in a separate facility.

TaqMan RT-PCR was performed using EZ TaqMan RT PCR reagents according to the manufacturer’s instructions (Applied Biosystems). RT-PCR reactions were performed in duplicate under the following conditions for each 25 µl reaction: 1× EZ buffer, 3mM MnOAc₂, 200 nmol of each primer, 100 nmol of TaqMan probe, 0.01 U of AmpErase, 0.1 U of rTth polymerase, and 3 µl (5–50 ng) of total RNA. The thermal cycling conditions on the 7700 were as follows: 50°C for two minutes, 58°C for 30 minutes, 95°C for five minutes, followed by 40 cycles of 94°C for 20 seconds and 60°C for one minute.

Controls for TaqMan RT-PCR included the following: no template control (water added as template), no amplification control (omission of rTth polymerase), irrelevant target primers and specific TaqMan probe (human papillomavirus 16, human herpes virus 8 primers), probe only control (omit PCR primers), human RNA control, spiked RNA control, and asymmetric TaqMan PCR (TaqMan PCR with one or other primer and specific TaqMan probe).

A gene dosage correction was carried out using glyceraldehyde phosphate dehydrogenase as a housekeeping gene. Measles virus quantitative TaqMan RT-PCR was performed by generating standard curves for the F and H genes. Taqman RT-PCR standards were generated by cloning the F and H gene specific PCR products into a vector using the TOPO TA cloning® system (Invitrogen, Groningen, the Netherlands), according to the manufacturer’s instructions. Plasmids containing the PCR products were used to generate standard curves.

Figure 2  [A] Agarose gel electrophoresis of measles virus Fusion (F) gene and haemagglutinin (H) gene reverse transcription polymerase chain reaction (RT-PCR) amplicons generated using RNA extracted from fresh frozen terminal ileum biopsies from affected children and subacute sclerosing panencephalitis (SSPE) brain tissue and measles infected Vero cells. M denotes 100 base pair molecular weight marker; lanes 1–6 show F gene PCR amplicons from the following RNA samples: measles infected Vero cells, SSPE brain, affected children 1–4, respectively; lane 7, no template control. Lanes 8–14 show H gene PCR amplicons generated from RNA samples in the same order as above. For one sample, RT-PCR for the F gene failed (lane 5). (B) Southern blot of the agarose gel described in (A) probed with a digoxigenin (DIG) labelled F gene specific probe as described in materials and methods. (C) Southern blot of the agarose gel described in (A) stripped and reprobed with a DIG labelled H gene specific probe.
controls were examined. Biopsies from 73 affected children and five normal patients. RT in situ PCR facilitates low copy gene detection and permits cellular localisation within tissues, with a reported sensitivity of one viral genome copy/cell.\(^1\) Serial dilutions of this cRNA were used to generate standard curves.

RT in situ PCR

RT in situ PCR was amplified using the following protocol: 58°C for 45 minutes and 94°C for five minutes, followed by 25 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. After amplification, sections were fixed in 100% ethanol and air dried.

Hybridisation was carried out with a 5′ biotinylated oligonucleotide probe using previously published protocols.\(^4\) Hybrid detection was achieved using a three step immunocytochemical method\(^1\) or dinitrophenol tyramide signal amplification.\(^2,3\) Alkaline phosphatase was detected with nitroblue tetrazolium (NBT) and bromochloroindoylphosphate (BCIP) as chromogen. Endogenous alkaline phosphatase was blocked using levamisole, an endogenous AP inhibitor (Dako), during chromogenic detection.

Reaction optimisation experiments were initially carried out using formalin fixed, paraffin wax embedded, measles infected Vero cells, and a variety of probe concentrations (1 µg/ml, 1.5 µg/ml, 2 µg/ml). A concentration of 1 µg/ml yielded optimal signals and was used in subsequent experiments. Biopsies from 73 affected children and five normal controls were examined.

Controls for RT in situ PCR included the following: MV infected and uninfected Vero cells, a mixed population of infected and uninfected Vero cells, MV N gene primers and an irrelevant probe (nonsense pyruvate dehydrogenase probe), and irrelevant primers and N gene specific probe. Hybridisation control experiments were performed using a histone mRNA probe. Other control experiments included RNase digestion of MV infected Vero cells before RT in situ PCR.

Combined RT in situ PCR and immunohistochemistry

To examine MV signal localisation, RT in situ PCR for the MV N gene was performed as described above on tissue sections following immunohistochemistry using the follicular dendritic cell CNA 42 monoclonal antibody\(^1\) (Dako). For in situ hybridisation a 5′ biotin labelled oligonucleotide probe was used as above.

The MV hybridisation signal was developed with horseradish peroxidase and aminoethyl carbazole (AEC; Vector Laboratories, Burlingame, California, USA), and the dendritic cell signal was developed using a three step detection method with AP (Dako) and NBT and BCIP (Roche Molecular Biochemicals) as substrate.

Ethical approval

Approval for these studies was obtained from the ethical practice committee of the Royal Free, Hampstead, NHS Trust. Fully informed, written parental consent was obtained from all trust patients including controls.

RESULTS

Overall, 75 of 91 affected children had MV RNA in their ileal lymphoid tissue compared with five of 70 in the control patient cohort (Fisher exact test, \(p < 0.0001\); Analyse-it Software, General 1.62). A total of six different PCR primer sets were optimised by solution phase RT-PCR to amplify the MV F, H, and N genes from RNA extracted from MV infected Vero cells (fig 1).

The specificity of the primer/probe sets to detect MV F and H genes was established using RNA extracted from: (1) cryopreserved ileal biopsy material from four affected children, (2) SSPE brain, and (3) MV infected Vero cells (fig 2A). All four patient samples were positive for MV F and H genes by TaqMan RT-PCR. Amplicon specificity was confirmed by Southern blot analysis using F and H gene specific probes (fig 2B,C). No template controls run in parallel were negative.

Seventy of 91 affected children were positive for MV compared with four of 70 controls as analysed by TaqMan RT-PCR (table 2). MV copy number in positive biopsies was generally low, but ranged from 1 to 3 × 10⁵ copies of MV/µg of total RNA. Of the paediatric control group, MV was not detected in normal children or children with isolated ileal LNH. However, four of 26 appendicectomy samples harboured the MV genome (table 2).

![Figure 3](http://www.molpath.com)

**Figure 3.** Reverse transcription (RT) in situ polymerase chain reaction (PCR) experiment using a single stranded biotinylated oligonucleotide probe for the detection of the measles virus nucleocapsid (N) gene in infected Vero cells. Optimal cytoplasmic staining is achieved in (A) (1 µg/ml), higher probe concentrations in panels (B) (1.5 µg/ml) and (C) (2 µg/ml) showed increasing non-specific nuclear staining.

<table>
<thead>
<tr>
<th>Table 2 Summary of TaqMan RT-PCR and RT in situ PCR results</th>
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<tr>
<td><strong>Affected patients</strong></td>
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<tr>
<td>Controls</td>
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<tr>
<td>Normal controls</td>
</tr>
<tr>
<td>Mild non-specific changes</td>
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<td>Lymphomalous hyperplasia</td>
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<td>Appendicectomies</td>
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<td>Ulcerative colitis</td>
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<td>Crohn’s disease</td>
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<td>Total number of patients tested in parentheses</td>
</tr>
<tr>
<td>NT not tested, RT-PCR, reverse transcription polymerase chain reaction</td>
</tr>
</tbody>
</table>

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RT in situ PCR optimisation experiments were performed as described (fig 3). A probe concentration of 1 µg/ml yielded optimal signals and was used in subsequent experiments.

In MV infected Vero cells, MV amplicons were identified as a cytoplasmic signal (fig 3). In SSPE brain material, discrete, intense foci of MV amplicons were detected in grey matter using RT in situ PCR (fig 4A). Signal was not detected in similarly processed normal brain, or when irrelevant PCR primers were used on sections of SSPE brain (fig 4B).

Of the 57 tissue biopsies from affected children, 42 were positive for the MV N gene by RT in situ PCR and amplicons could be detected in serial sections of ileal biopsies. Four samples were inconclusive for the presence of MV because of high background staining, and 11 biopsies were negative for MV. Of the control group, one of five children with histologically normal small and large bowel mucosa had detectable MV N gene RNA, present in a distribution that was identical to that seen in biopsies from affected children (table 2). MV amplicon localised to reactive follicle centres, and was associated with cells possessing dendritic processes (fig 4C,E) and some lymphocytes (fig 4F). No MV signal was seen in serial control sections where irrelevant PCR primers were used (fig 4D). Additional RT in situ PCR control experiments were performed on measles infected Vero cells after RNase digestion (fig 5).

Overall, 91 biopsies have been examined, 56 of which were analysed by a combination of in cell RT-PCR (MV N gene) and TaqMan RT-PCR. Thirty seven of these biopsies were positive for MV and five were negative for MV using both techniques, six were positive by TaqMan RT-PCR but negative by in situ PCR, and five were positive by in situ PCR only (table 2).

DISCUSSION
We describe an association between persistent MV infection and ileocolonic lymphnodular hyperplasia and ileocolitis in children with developmental disorder. The molecular data indicate the presence of MV genomes in 75 of 91 affected children with the disorder compared with five of 70 control children. In addition, there appears to be a strong segregation of
the disease phenotype with male children, in keeping with the reported male predominance of the developmental disorder. MV was not detected in three children with isolated LNH or in biopsies from normal children. Of the 26 children who underwent appendicectomy, only four harboured MV RNA. The presence of measles virus in the intestine of a small minority of apparently healthy children is not surprising in light of the findings of Warthin-Finkeldey giant cells in inflamed appendices in children with measles infection. However, the prevalence of persistent MV infection in the general population is not known and warrants further investigation.

**Take home messages**

- Of 91 patients with a histologically confirmed diagnosis of ileal lymphoid nodular hyperplasia and enterocolitis, 75 were positive for measles virus in their intestinal tissue compared with five of 70 controls.
- Measles virus was found within the follicular dendritic cells and some lymphocytes in foci of reactive follicular hyperplasia.
- These data confirm an association between the presence of measles virus and gut pathology in children with developmental disorder.

These preliminary studies have focused principally on MV. We have not excluded the presence of alternative infections. Viruses may persist elsewhere, or exert a transient effect not requiring subsequent persistence. One such transient risk may be that concurrent exposure to measles and another infection may increase the risk of MV establishing persistent infection: this atypical pattern of exposure has been identified as a risk factor for both SSPE (chicken pox and encephalitic enterovirus).

Our study raises many questions—most importantly, does MV play an aetiological role in intestinal inflammation in developmental disorder? The study reports for the first time an association between MV and ileocolonic lympho- nodular hyperplasia and ileocolitis in children with developmental disorder.

**ACKNOWLEDGEMENTS**

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**Authors’ affiliations**

V Uhlmann, L Pilkington, I Silva, A Killalea, J J O’Leary, Department of Pathology, Coombe Women’s Hospital, Dublin 8, Ireland
C M Martin, O Shields, Department of Histopathology, Trinity College, Dublin, Ireland
S B Murch, Department of Paediatric Gastroenterology, the Royal Free Hospital, London, UK
J Walker-Smith, M Thomson, A J Wakefield, Department of Medicine, the Royal Free Hospital and University College Medical School, London, UK

**REFERENCES**

What turns neutrophils on

Insoluble and soluble immune complexes in synovial fluid in rheumatoid arthritis activate neutrophils differently, researchers in Liverpool, UK, have found. Immune complexes and neutrophils are abundant in synovial fluid in rheumatoid arthritis, as are cytokines, which can prime neutrophils making them more responsive. Understanding how immune complexes and neutrophils interact, leading to production of tissue damaging enzymes and oxidants, and the molecular control of the interactions are potential keys to new therapeutic treatments.

Fossati et al used in vitro chemiluminescence to detect production of oxidants by washed blood neutrophils in their unprimed state or primed with granulocyte-macrophage colony stimulating factor—a cytokine in synovial fluid in rheumatoid arthritis—when incubated with synthetic insoluble or soluble immune complexes. By comparing the reaction kinetics of chemiluminescence with substrates available to the neutrophils either extracellularly or intracellularly and with different specific scavengers of oxidants, they were able to unravel the processes. Insoluble complexes activated unprimed or primed neutrophils to produce oxidants that remained intracellular whereas soluble complexes reacted only with primed neutrophils but did so rapidly and transiently, the oxidants being secreted. Insoluble complexes also required FcyRIIIb but not FcyRII function whereas soluble complexes required both. In tests to detect granule enzymes insoluble complexes activated secretion with unprimed or primed neutrophils, although the reaction kinetics differed from those for the oxidants. Soluble complexes activated secretion only with primed neutrophils, leading the researchers to conclude that neutrophil activation occurs by different pathways for insoluble or soluble immune complexes and can be significantly affected by cytokines.

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