Potential viral pathogenic mechanism for new variant inflammatory bowel disease


A new form of inflammatory bowel disease (ileocolonic lymphonodular hyperplasia) has been described in a cohort of children with developmental disorder. This study investigates the presence of persistent measles virus in the intestinal tissue of these patients (new variant inflammatory bowel disease) and a series of controls by molecular analysis.

Methods: Formalin fixed, paraffin wax embedded and fresh frozen biopsies from the terminal ileum were examined from affected children and histological normal controls. The measles virus Fusion (F) and Haemagglutinin (H) genes were detected by TaqMan reverse transcription polymerase chain reaction (RT-PCR) and the Nucleocapsid (N) gene by RT in situ PCR. Localisation of the mRNA signal was performed using a specific follicular dendritic cell antibody.

Results: Seventy five of 91 patients with a histologically confirmed diagnosis of ileal lymphonodular hyperplasia and enterocolitis were positive for measles virus in their intestinal tissue compared with five of 70 control patients. Measles virus was identified within the follicular dendritic cells and some lymphocytes in foci of reactive follicular hyperplasia. The copy number of measles virus ranged from one to 300 000 copies/ng total RNA.

Conclusions: The data confirm an association between the presence of measles virus and gut pathology in children with developmental disorder.

MATERIALS AND METHODS

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 appendicectomy 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).

Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DIG, digoxigenin; f, fusion; H, haemagglutinin; IL, interleukin; LNH, lymphonodular hyperplasia; MV, measles virus; N, nucleocapsid; NBT, nitroblue tetrazolium; PCR, polymerase chain reaction; RT, reverse transcription; SSC, saline sodium citrate; SSPE, subacute sclerosing panencephalitis; Th1, T helper cell type 1.
Analysis probes were labelled at the 3′end of the probes were dual labelled with the fluorescent molecule FAM. For TaqMan quantitative reverse transcription PCR (RT-PCR), the reactions 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); EZ buffer. The digoxigenin using a DIG end labelling kit (Roche Molecular Biochemicals).

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 amplicons for two different H gene primer sets. Lane 1 and 10, 100 base pair DNA ladder (Gibco-BRL, Paisley, Scotland, UK). Lanes 2 and 3, Fusion (F) gene cDNA amplicons for two different F gene primer sets. Lanes 4 and 5, Nucleocapsid (N) gene cDNA amplicons for two different N gene primer sets. Lanes 6 and 7, Haemagglutinin (H) gene cDNA amplicons for two different H gene primer sets. Lane 8, positive control human pyruvate dehydrogenase PCR product. Lane 9, no template control for lane 2.

50°C in 5× saline sodium citrate (SCC) with 0.2% sodium dodecyl sulfate for one hour, washed once in 5× SCC at 50°C for 10 minutes, once in 2× SCC at room temperature for 10 minutes, and once in 0.2× SCC 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. Template

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′ GAC TGA GAG CGG TGG GAC CC 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>N1 reverse</td>
<td>5′ GCC CGG TTT CTT CTG TAG CT 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>N2 forward</td>
<td>5′ GAG TCG AGG AGA AGC CAG GG 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>N2 reverse</td>
<td>5′ GCT GGA CTC CGA TGC AGT GT 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>H1 forward</td>
<td>5′ TTC ATG GGG CCA CCA TCT AC 3′</td>
<td>150 bp</td>
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<tr>
<td>H1 reverse</td>
<td>5′ CTC TGA GGT GTC CTC AGG CC 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>H2 forward</td>
<td>5′ TGG GCA CCA TTA AGG GAT AA 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>H2 reverse</td>
<td>5′ AAC CTT GTG TGA TCA ATG GC 3′</td>
<td>150 bp</td>
</tr>
<tr>
<td>F1 forward</td>
<td>5′ TGG GAT GGT GAT GGA ATT TC 3′</td>
<td>226 bp</td>
</tr>
<tr>
<td>F1 reverse</td>
<td>5′ CAA ACA GAG TCG AGG AGA AGC CAG GGA 3′</td>
<td></td>
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<tr>
<td>GAPDH 1</td>
<td>5′ GAA GTG GAA GGT CGG AGT 3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH 2</td>
<td>5′ GAA GAT GGT GAT GGG ATT TC 3′</td>
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<tr>
<td>N1 probe</td>
<td>5′ CGG CAG AGA AGA AGC CAG GGA 3′</td>
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<tr>
<td>F2 forward</td>
<td>5′ CAC CTT GCC CGA AAG ATG GT 3′</td>
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<td>F2 reverse</td>
<td>5′ CTG CAC GAG GAT AGA GAT CCC TCA GCA C 3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH probe</td>
<td>5′ CGG ACT CTT GCC GCT CAG AC 3′</td>
<td></td>
</tr>
</tbody>
</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, U03664, U03669, U03660, U03671, U03667, Z80793, AF045204, Z66517, and Z80616. Measles virus F-gene primers and probes were designed based on the following Genbank sequence entries: X16565, S58640, NC_002494, NC_002496, X01999, U03661, U03658, and U03657, U03651, U03659, AJ133108, X16567.
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 transformed into bacterial cells and the positive clones were identified by colony PCR using the primers used in the Taqman RT-PCR. The positive clones were then purified and sequenced to confirm the identity of the cloned DNA.

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

yields optimal signals and was used in subsequent experi-

(1 infected Vero cells, and a variety of probe concentrations

out using formalin fixed, paraffin wax embedded, measles

phatase was blocked using levamisole, an endogenous AP

nitrobluetetrazolium (NBT) and bromochloroindoylphos-

inhibitor (Dako), during chromogenic detection.

phosphate (BCIP) as chromogen. Endogenous alkaline phos-

status. Reaction optimisation experiments were initially carried

in sections. Other control experiments included RNase

to examine MV signal localisation, RT in situ PCR for the MV

inserts were then in vitro transcribed into cRNA using the

in vitro transcription system (Promega, Madison, Wisconsin, USA). Serial dilutions of this cRNA were used to

to cellular localisation within tissues, with a reported sensitivity

sections were fixed in 100% ethanol and air dried.

After pretreatment, MV RNA was amplified using the

sections were fixed in 100% ethanol and air dried.

Hybridisation was carried out with a 5′ biotinylated

or dinitrophenol tyramide signal

fully informed, written parental consent was obtained from

cals) as substrate.

Alkaline phosphatase was blocked using AP (Dako) and NBT and BCIP (Roche Molecular Biochemicals) as substrate.

Ethical approval

Approval for these studies was obtained from the ethical prac-
ticles committee of the Royal Free, Hampstead, NHS Trust.

RESULTS

Overall, 75 of 91 affected children had MV RNA in their ileal

lymphoid tissue compared with five of 70 in the control

population (Fisher exact test, p < 0.0001; Analyse-it Soft-

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) cryo-

preserved 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 × 10³ copies of MV/ng 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).

Table 2 Summary of TaqMan RT-PCR and RT in situ

PCR results

<table>
<thead>
<tr>
<th></th>
<th>TaqMan RT-PCR positive</th>
<th>In situ PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected patients</td>
<td>70 (91)</td>
<td>42 (57)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal controls</td>
<td>0 (19)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Mild non-specific</td>
<td>0 (13)</td>
<td>NT</td>
</tr>
<tr>
<td>changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphonular hyperplasia</td>
<td>0 (3)</td>
<td>NT</td>
</tr>
<tr>
<td>Appendicatomies</td>
<td>4 (26)</td>
<td>NT</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>0 (11)</td>
<td>NT</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>0 (8)</td>
<td>NT</td>
</tr>
</tbody>
</table>

Total number of patients tested in parentheses.
NT not tested, RT-PCR, reverse transcription polymerase chain reaction.

RT in situ PCR

RT in situ PCR facilitates low copy gene detection and permits

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.

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 den-

dritic cell CNA 42 monoclonal antibody (Dako). For in situ

hybridisation a 5′ biotin labelled oligonucleotide probe was

used as above.

The MV hybridisation signal was developed with horserad-

ish peroxidase and aminoethyl carbazole (AEC; Vector Labora-

tories, 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.

www.molpath.com
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. No signal was obtained on MV 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 lymphonodular 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 predominantly detected in dendritic cells in reactive follicular hyperplastic centres in ileal biopsies from affected children (fig 4C,E), but was also identified in mature lymphocytes in these hyperplastic areas (fig 4F). This result indicates a possible interaction between MV and the immune response in the pathogenesis of ileocolitis in these children. MV may be a potential “immunological trigger” in the pathogenesis of lymphoid hyperplasia and ileocolitis. Potential initiators for this type of immunological response are dendritic cells. Dendritic cells capture and process viral antigens in the periphery, express costimulatory molecules, and serve as vehicles for viral antigens to the mediators of immunity (B and T cells) in lymphoid tissue. As a result, cytokines are released and initiate an immune response.

MV localisation in follicular dendritic cells mirrors the human immunodeficiency virus type 1 (HIV-1) infection patterns seen in HIV-1 enteropathy. The presence of MV antigen in follicular dendritic cells may reflect a transient stage in the progression from latent to persistent MV infection. The hypothetical parallel with HIV infection is interesting: HIV, like measles virus, potentially disrupts cellular immunity, and induces follicular hyperplasia and lymphadenopathy in the early stage of infection. This is associated with expansion of the follicular dendritic cell network and trapping of HIV within germinal centres. 

During the early latent phase of infection, HIV antigens are detectable upon the surface of follicular dendritic cells in a pattern similar to that reported here for MV. Such a location may favour the induction of immunological tolerance and failure of viral clearance. The mechanisms by which MV, immunological abnormalities, and chronic intestinal pathology may be linked are currently not known. Precedents for delayed intestinal and immunological sequelae to MV exposure include chronic immunodeficiency, diarrhoeal disease, and death following early natural measles exposure. Natural measles infection induces an initial Th1 helper cell type 1 (Th1) response and protective cytotoxic immunity (characterised by the classic measles rash and gastrointestinal upset), followed by a prolonged Th2 response with antibody production. In most individuals, this leads to lifelong immunity without delayed pathological sequelae. Nonetheless, immune activation during the measles attack is set against a profound reduction in non-specific cellular immune responsiveness, reflecting the potent immunosuppressive properties of MV. Recent studies suggest that this may be achieved through impaired interleukin 12 (IL-12) production by infected dendritic cells, and blocking of IL-2 receptor expression by activated T cells. In such circumstances, impaired Th1 immunity, with a shift towards a dominant Th2 response, might occasionally contribute not only to establishing persistent infection, but also to delayed immunopathology.

“Measles virus may be a potential immunological trigger in the pathogenesis of lymphoid hyperplasia and ileocolitis”

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 lymphonodular 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 encephalitogenic 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 infection and ileocolonic lymphonodular hyperplasia and ileocolitis in children with developmental disorder.

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**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 FcγRIIIb but not FcγRII 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.