No evidence of persistent mumps virus infection in inflammatory bowel disease

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Abstract

Background and aim—There is controversy regarding whether paramyxovirus infection is causally associated with inflammatory bowel disease (IBD). The latest cohort study claimed that atypical measles and mumps infections in childhood may be risk factors for later IBD. This study was conducted to clarify the validity of a causal link between persistent mumps virus infection and IBD.

Subjects and methods—(1) Amplification of the mumps virus genome was performed in both intestinal specimens (ulcerative colitis 15, Crohn's disease 15, control 10) and peripheral blood lymphocytes (PBL) (ulcerative colitis seven, Crohn's disease six, control three) by reverse transcription-polymerase chain reaction (RT-PCR) followed by Southern hybridisation using primers specific to the viral genome encoding phosphoprotein or haemagglutinin-neuraminidase. (2) Titre of serum antimumps IgG was measured in 16 patients with ulcerative colitis, in 16 patients with Crohn's disease, and in 16 normal controls using an enzyme linked immunosorbent assay.

Results—(1) The mumps virus genome was not detected by RT-PCR in intestinal specimens or PBL in any case. (2) Anti-mumps IgG titre was positive in 7/16 ulcerative colitis, 10/16 Crohn's disease, and 11/16 control specimens. The mean (SEM) titre of antimumps IgG was 12.281 (7.831) in ulcerative colitis, 7.675 (1.608) in Crohn's disease, and 8.637 (1.969) in controls, with no significant difference between the three groups.

Conclusion—We could not find any evidence to support a causal link between persistent mumps virus infection and IBD.

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Keywords: inflammatory bowel disease; ulcerative colitis; Crohn disease; mumps virus; measles virus; paramyxovirus

Although the pathogenesis of inflammatory bowel disease (IBD) is unknown, it is suggested that IBD occurs in susceptible hosts with some unknown exogenous agents in the environment; one of the most likely candidates is thought to be infections.¹ In this regard, a series of studies by Wakefield and colleagues²⁻⁶ claimed a close link between measles virus and Crohn's disease on the basis of epidemiological and immunohistochemical findings, but this measles hypothesis is controversial⁷ because the measles virus genome was not detected in

patients with Crohn's disease,⁸⁻¹¹ and the measles antibody recognised the host protein as well as the measles virus.^{12,13}

The latest cohort study¹⁴ from the same group (Montgomery *et al*) put forward another possibility: a close relationship between mumps virus and IBD—namely, that mumps virus infection before the age of two years was a risk factor for ulcerative colitis, and mumps and measles virus infections in the same year of life were significantly associated with IBD. However, this cohort study did not include cases who had received measles, mumps, and rubella (MMR) vaccination but it was concerned that the results of the study might provoke reduced uptake of MMR vaccination.¹⁵ Further, it is known that mumps virus establishes persistent infection in mammalian cells.¹⁶ Based on these findings, we wished to clarify the validity of a causal link between persistent mumps virus infection and IBD.

As far as we are aware, only one study¹⁷ tried to detect mumps virus genome in intestinal specimens from patients with IBD but none was detected. However, reverse transcription-polymerase chain reaction (RT-PCR) was performed using only one primer set to the mumps virus genome and the sensitivity of RT-PCR was unknown.¹⁷ Hence it is difficult to reach the conclusion of no causal link between persistent mumps virus infection and IBD from their results alone. Our study was conducted to clarify this issue using RT-PCR followed by Southern hybridisation with high sensitivity, and a serological test for mumps virus.

Materials and methods

AMPLIFICATION OF THE MUMPS VIRUS GENOME

Amplification of the mumps virus genome was performed in both intestinal specimens and peripheral blood lymphocytes (PBL) by RT-PCR. Intestinal specimens were obtained by endoscopic biopsy or surgical resection from 15 patients with ulcerative colitis, 15 patients with Crohn's disease, and 10 controls. The endoscopic biopsies contained mucosa and submucosa of the intestine, and the surgical biopsies contained the full thickness of the intestine. In each case the diagnosis was established using standard clinical, radiological, endoscopic, and histological criteria. Clinical details of the patients are shown in table 1. PBL

Abbreviations used in this paper: ag, atto gram; ELISA, enzyme linked immunosorbent assay; fg, femto gram; IBD, inflammatory bowel disease; MMR, measles, mumps, and rubella; PBL, peripheral blood lymphocytes; P, phosphoprotein; HN, haemagglutinin-neuraminidase; RT-PCR, reverse transcription-polymerase chain reaction; SSPE, subacute sclerosing panencephalitis.

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Table 1 Clinical details of patients examined for mumps RNA in colonic mucosa

Patient No	Disease	Sex	Age (y)	Disease activity	Site of specimen	Method of sampling
1	UC	M	37	Remission	Colon	Endoscopy
2	UC	M	48	Remission	Colon	Endoscopy
3	UC	F	21	Active	Colon	Surgery
4	UC	F	31	Active	Colon	Surgery
5	UC	M	36	Active	Colon	Surgery
6	UC	M	25	Active	Colon	Surgery
7	UC	F	26	Active	Colon	Surgery
8	UC	M	23	Active	Colon	Endoscopy
9	UC	M	28	Active	Colon	Endoscopy
10	UC	F	49	Active	Colon	Endoscopy
11	UC	F	42	Active	Colon	Surgery
12	UC	F	43	Active	Colon	Surgery
13	UC	F	23	Active	Colon	Surgery
14	UC	F	45	Remission	Colon	Endoscopy
15	UC	M	16	Active	Colon	Endoscopy
16	CD	M	22	Remission	Colon	Endoscopy
17	CD	M	32	Remission	Colon	Endoscopy
18	CD	M	33	Remission	Colon	Endoscopy
19	CD	F	18	Active	Colon	Endoscopy
20	CD	F	31	Remission	Colon	Endoscopy
21	CD	F	10	Remission	Colon	Endoscopy
22	CD	M	21	Active	Colon	Endoscopy
23	CD	M	31	Active	Ileum	Endoscopy
24	CD	F	26	Active	Ileum	Surgery
25	CD	F	20	Active	Ileum	Surgery
26	CD	M	13	Active	Colon	Endoscopy
27	CD	F	44	Active	Ileum	Surgery
28	CD	M	28	Active	Ileum	Surgery
29	CD	F	29	Active	Ileum	Surgery
30	CD	M	23	Active	Colon	Endoscopy
31	Colon cancer	M	53		Colon	Endoscopy
32	Colon cancer	F	55		Colon	Endoscopy
33	Colon polyp	F	55		Colon	Endoscopy
34	Normal	M	69		Colon	Endoscopy
35	Normal	F	59		Colon	Endoscopy
36	Colon cancer	M	64		Colon	Surgery
37	Colon cancer	M	68		Colon	Endoscopy
38	Colon polyp	F	65		Colon	Endoscopy
39	Normal	M	56		Colon	Endoscopy
40	Normal	F	70		Colon	Endoscopy

UC, ulcerative colitis; CD, Crohn's disease.

were obtained from seven patients with ulcerative colitis, six patients with Crohn's disease, and three normal controls. All samples were taken with informed consent. Isolation of lymphocytes from peripheral blood cells was performed using a Lymphoprep kit (Nycomed Pharmaas, Oslo, Norway) according to the manufacturer's instructions.

RNA was extracted from intestinal specimens or PBL using the acid guanidinium-phenol-chloroform method with a RNaaid Plus kit (BIO 101, California, USA). Reverse transcription of RNA was carried out by incubation at 42°C for 50 minutes using 3 µg of random hexamer (Takara, Otsu, Japan), 10 units of reverse transcriptase (RAV-2; Takara), and 10 nmol of dNTP in reverse transcription buffer containing 50 mM Tris HCl, pH 8.3, 75 mM KCl, 8 mM MgCl₂, and 10 mM DTT. Converted DNA was amplified with 1.25 units of Taq DNA polymerase (Takara Taq; Takara) in the presence of sets of primers (10 pmol), and 10 nmol of dNTP in

Table 2 Oligonucleotide primers used for nested polymerase chain reaction

Gene region amplified	Primer sequence	Position	Product size
Phosphoprotein	Outer	5' AACCAACTCGT/TGAGCAAGG 3'	94–113
		5'TGAACCTGTTGGTTGGATAG 3'	345–364
	Inner	5'TACTCATTGGCAATCCAGAG 3'	141–160
		5'TCCTGATAAACGGTGGTGAC 3'	320–339
Haemagglutinin-neuraminidase	Outer	5'GATCCTAGTTACAAATTGCG 3'	1248–1267
		5'AGTGATAGTCAATCTGGTTAG 3'	1816–1836
	Inner	5'ATCAGACACTGATGGGTG 3'	1289–1306
		5'AGAATTTGGAATTCTCAA 3'	1790–1808

the PCR buffer containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. The mumps virus genome was amplified by nested PCR using primers specific to the portion of the viral genome encoding phosphoprotein (P)¹⁸ and haemagglutinin-neuraminidase (HN)¹⁹; both primers can also amplify vaccine strains. Nucleotide sequences of these primers are shown in table 2. Amplification of the P gene was performed as follows. After an initial four minutes of denaturation at 94°C, 35 PCR cycles (one minute at 94°C, 1.5 minutes at 44°C, and one minute at 72°C), followed by a seven minute extension step at 72°C using a thermal cycler (GeneAmp PCR system 9600; Perkin Elmer, Applied Biosystems Division, New Jersey, USA). For nested PCR, 1 µl of the first PCR product was mixed and the samples were subjected to PCR using the same thermal cycle programme.

Amplification of the HN gene was performed as follows. The first three cycles were two minutes at 92°C, three minutes at 55°C, and two minutes at 72°C. These were followed by 30 cycles (one minute at 93°C, one minute at 58°C, and two minutes at 72°C), with a final extension period of five minutes at 72°C. Nested PCR was performed using the same thermal cycle programme. The PCR products (5 µl) were electrophoresed on 2% agarose gel and visualised by staining with ethidium bromide and exposure to UV light. PCR products, which were amplified with P gene primer sets, were transferred subsequently to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. Then, Southern hybridisation was performed with an alkaline phosphatase labelled oligonucleotide probe of the mumps virus P gene, the same region of the nested PCR product, using an AlkPhos Direct kit (Amersham Pharmacia Biotech).

SEROLOGICAL TEST

Antimumps IgG titre was measured in 16 patients with ulcerative colitis, in 16 patients with Crohn's disease, and in 16 normal controls by enzyme linked immunosorbent assay (ELISA) using the mumps IgG (II)-EIA Seiken kit (Denka Seiken, Tokyo, Japan). Briefly, a plastic plate was coated with purified whole virion of mumps virus and covered with patient serum. The plate was then incubated with a peroxidase labelled antihuman IgG polyclonal antibody. Enzyme activity was measured spectrophotometrically after addition of 3, 3', 5, 5'-tetramethylbenzidine as the specific chromogenic substrate. Clinical details of the patients are shown in table 3. None of the cases received mumps vaccination.

Statistical analysis was done using the Mann-Whitney U test, and p<0.05 was considered significant.

Results

AMPLIFICATION OF THE MUMPS VIRUS GENOME Sensitivity of RT-PCR

We detected the mumps virus P gene in 120 atto grams (ag) of RNA (fig 1A) and the HN gene in 12 femto grams (fg) of RNA (fig 1B)

Table 3 Clinical details of patients for measured antimumps IgG

Patient No	Disease	Sex	Age (y)	Birth year	Disease activity
1	UC	M	25	1973	Active
2	UC	M	41	1956	Active
3	UC	F	24	1971	Active
4	UC	F	46	1949	Active
5	UC	M	21	1974	Active
6	UC	F	36	1960	Active
7	UC	M	17	1978	Active
8	UC	F	32	1958	Active
9	UC	M	36	1953	Active
10	UC	F	29	1962	Active
11	UC	F	27	1971	Active
12	UC	F	32	1966	Active
13	UC	F	27	1965	Active
14	UC	F	28	1960	Remission
15	UC	F	20	1972	Active
16	UC	F	29	1969	Active
17	CD	M	34	1961	Active
18	CD	F	23	1972	Active
19	CD	F	24	1973	Active
20	CD	M	22	1973	Active
21	CD	M	34	1963	Active
22	CD	M	26	1966	Active
23	CD	M	23	1973	Active
24	CD	F	46	1949	Active
25	CD	F	23	1972	Active
26	CD	F	29	1962	Remission
27	CD	M	26	1972	Remission
28	CD	M	24	1969	Active
29	CD	F	21	1976	Active
30	CD	M	17	1980	Active
31	CD	M	15	1981	Active
32	CD	F	17	1979	Active
33	Control	M	46	1949	
34	Control	F	35	1960	
35	Control	F	30	1965	
36	Control	M	27	1968	
37	Control	M	21	1974	
38	Control	M	19	1977	
39	Control	F	27	1971	
40	Control	F	30	1967	
41	Control	F	29	1968	
42	Control	F	20	1977	
43	Control	M	39	1969	
44	Control	M	28	1956	
45	Control	M	43	1954	
46	Control	F	18	1980	
47	Control	F	20	1974	
48	Control	M	20	1976	

UC, ulcerative colitis; CD, Crohn's disease.

extracted from mumps virus infected Vero cells. We were also able to detect both P and HN genes of the mumps virus in a throat swab collected from a patient with mumps parotitis.

Amplification of P gene

Single or multiple bands were observed in all samples encompassing intestinal specimens and PBL, but the sizes of these bands were not consistent with the expected band of mumps virus (199 bp). Eleven PCR products are shown in fig 2A. No hybrids were observed between these bands and labelled probe constructed of the mumps virus P gene (lanes 6–13; fig 2B) after Southern hybridisation. Strong hybrids were observed in positive controls of mumps virus (lanes 2, 3; fig 2B) and in the throat swab from the mumps parotitis patient (lane 15; fig 2B).

Amplification of HN gene

The expected band was not observed in any colonic mucosa or PBL sample. Four PCR products are shown in fig 3.

SEROLOGICAL TEST

Mean (SEM) age of the patients was 28.9 (2.0) years for those with ulcerative colitis, 25.3 (1.9) for those with Crohn's disease, and 28.3 (2.2)

Figure 1 Nested reverse transcription-polymerase chain reaction (RT-PCR) products from mumps virus infected cells. (A) Phosphoprotein (P) gene: molecular weight marker (lane 1), PCR product from 120 fg (lane 2), 12 fg (lane 3), 1.2 fg (lane 4), 120 ag (lane 5), and 12 ag (lane 6) of template RNA, and PCR product from a throat swab of mumps parotitis (lane 7). (B) Haemagglutinin-neuraminidase (HN) gene: molecular weight marker (lane 1), PCR product from 12 pg (lane 2), 1.2 pg (lane 3), 120 fg (lane 4), 12 fg (lane 5), and 1.2 fg (lane 6) of template RNA, and PCR product from a throat swab of mumps parotitis (lane 7).

Figure 2 (A) Nested polymerase chain reaction (PCR) products using primers to the phosphoprotein (P) gene. Molecular weight marker (lane 1), PCR products from intestinal specimens of ulcerative colitis (lanes 2–4), Crohn's disease (lanes 5–7), controls (lanes 8–10), PCR products from peripheral blood lymphocytes (PBL) of ulcerative colitis (lanes 11, 12), and PCR product without template DNA (lane 13), and from the throat swab of the patient with mumps parotitis (lane 14). (B) Southern hybridisation of PCR products using primers to P gene. Molecular weight marker (lane 1), PCR products from 1.2 fg (lane 2), 120 ag (lane 3), 12 ag (lane 4) of template RNA from mumps virus infected cells, blank (lane 5), PCR products from intestinal specimens of ulcerative colitis (lanes 6–8), Crohn's disease (lanes 9–11), PCR products from PBL of ulcerative colitis (lanes 12, 13), blank (lane 14), and PCR product from the throat swab of mumps parotitis (lane 15).

for the controls. Mean (SEM) birth year of the patients was 1965 (2.1) for ulcerative colitis, 1970 (2.1) for Crohn's disease, and 1968 (2.3) for the controls. There was no significant difference in age or birth year between the three groups. In the ELISA system, antimumps IgG titres showing over 4.0 EIA were judged positive, below 2.0 as negative, and between 2.0 and 4.0 as indeterminate. According to these criteria, 7/16 ulcerative colitis patients, 10/16 Crohn's disease patients, and 11/16 controls were judged to be positive for antimumps IgG. The mean (SEM) titre of antimumps IgG was 12.281 (7.831) EIA for ulcerative colitis, 7.675 (1.608) for Crohn's disease, and 8.637

Figure 3 Nested polymerase chain reaction (PCR) products using primers to the haemagglutinin-neuraminidase (HN) gene. Molecular weight marker (lane 1), PCR product from intestinal specimens of ulcerative colitis (lane 2), Crohn's disease (lane 3), control (lane 4), PCR product from peripheral blood lymphocytes (PBL) of ulcerative colitis (lane 5), PCR product without template DNA (lane 6), and PCR product from a throat swab of mumps parotitis (lane 7).

(1.969) for the controls; there was no significant difference between the three groups.

Discussion

Our study failed to detect mumps virus genome in intestinal specimens or PBL in any patient with IBD. We attempted to detect the mumps virus genome in PBL as well as intestinal specimens as the mumps virus preferentially infects human lymphocytes, especially activated T lymphocytes.²⁰ Amplification of the mumps virus genome was performed by RT-PCR using two reliable primers sets to P¹⁸ and HN¹⁹ genes of the mumps virus. We detected samples as small as 120 ag of virus RNA using RT-PCR, which corresponds to approximately 10–20 copies of the mumps virus RNA; hence the sensitivity of RT-PCR for detection of the mumps virus was high and higher than that of mumps virus isolation.¹⁹ We extracted RNA from gut tissue using glass powder (RNaid) because some PCR inhibitors can be eliminated by its use and better qualitative results were obtained.²¹ Further, the sensitivity of RT-PCR was not changed when RNA extracted from gut tissue was added in the reaction mixtures of RT-PCR (data not shown) which suggests that extracts from gut tissue contained no compound which could inhibit the PCR primers used in the study. Therefore, we consider that our results showed absence of mumps virus in patients with IBD.

Subacute sclerosing panencephalitis (SSPE) is caused by persistent measles virus infection and is most likely to occur if measles virus infection occurs at a young age.²² Antibody response to the measles virus in such patients is abnormal and tends to be increased and maintained in both serum and the central nervous system.^{23–24} In view of these observations, our serological results of no increase in antimumps IgG is regarded as support for the finding of absence of persistent mumps virus infection in IBD.

In this study we did not examine mumps virus antigen in the colonic mucosa by immunohistochemistry using antimumps monoclonal antibody as monoclonal antibodies against virus antigens sometimes cross react with host proteins.^{25–26} We also demonstrated in our previous reports^{12–13} that the antimeasles monoclonal antibody recognised host protein in the colonic mucosa as well as measles virus; this phenomenon led us to the false conclusion that persistent measles virus infection occurs in the colonic mucosa of Crohn's disease.

Our study, combined with the previous investigation of Folwaczny and colleagues,¹⁷ showed absence of mumps virus genome encoding three structural proteins of the mumps virus (phosphoprotein, haemagglutinin-neuraminidase, and a small hydrophobic protein) in patients with IBD, indicating that persistent mumps virus infection in ulcerative colitis or Crohn's disease is unlikely. While Montgomery and colleagues¹⁴ postulated a close link between atypical paramyxovirus infection in childhood and later onset of IBD, our study together with previous investigations^{8–13} suggesting no evidence of persistent measles virus infection in IBD showed little evidence that IBD is caused by persistent paramyxovirus infection, even if atypical paramyxovirus infection has occurred. With regard to MMR vaccination and IBD, a cohort study of Montgomery and colleagues¹⁴ did not include patients who had received MMR vaccination. Further, we performed RT-PCR using primer sets that can also detect vaccine strains but we could not detect any vaccine strains or non-vaccine strains of either measles or mumps virus in patients with IBD. Hence it is worth emphasising that there has been no epidemiological or experimental evidence directly showing an association between MMR vaccination and IBD. In contrast, it is true that our studies did not exclude the possibility that mumps virus infection works as a triggering event for later onset of IBD, but we believe that further epidemiological investigations with larger populations are needed to confirm this.

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