LETTERS TO THE EDITOR

Immunohistochemical analysis of measles related antigen in IBD

EDITOR,—What is one to make of the immunohistochemical study of Iizuka *et al* (*Gut* 2000;**46**:163–169)? Before addressing the possible scientific implications of their findings, it is worth clarifying a few points with respect to our own research. Iizuka *et al* state that "the measles hypothesis is based on the theory that measles antibody recognises measles virus itself and the measles virus antigen is uniquely present in Crohn's disease." Is this the authors' hypothesis? It is certainly not ours. That the measles antibody used in our studies (not that used by the authors in the present study) recognises the measles virus is not in doubt. The appropriate question is "does the antibody detect human antigenic epitopes in addition to measles virus N-protein?" Specificity studies in our own laboratory, including application to mixed preparations of measles virus infected and uninfected cells, application to cell lines infected with other viruses, and tissue studies incorporating in situ hybridisation (ISH) and combined ISH-reverse transcription-polymerase chain reaction (RT-PCR)-ISH for measles virus N-gene in serial tissue sections of infected human tissues suggest that the antibody, when used appropriately, is specific for measles virus.

Our own hypothesis relates not to the unique presence of measles in Crohn's disease but rather to the specific localisation of the virus to the hallmark lesions of this disease—granulomas and secondary lymphoid reactions—that are themselves a likely response to persistent and potentially causative antigen(s). While the focus of our studies has been the exclusive presence of viral antigen in these foci in Crohn's disease, Iizuka *et al* have assiduously avoided these pathological structures altogether. Instead, they have identified a pattern of non-specific staining in inflamed mucosal biopsies that has never been observed in our own laboratory using the measles virus monoclonal antibody. We have particular anxieties about their statement regarding the cellular localisation of the positive signal. The authors state that, "As Wakefield and colleagues reported, positive cells comprised macrophages, lymphocytes, vascular endothelial cells, fibroblasts, and neutrophils". This is clearly misleading. We described positive staining in "endothelium, macrophage-like cells, and occasional lymphocytes in foci of granulomatous inflammation".[1] It was not seen elsewhere in tissue sections, either in the presence or absence of inflammation. Iizuka *et al* have abbreviated this specifically worded statement, removing any reference to granulomatous inflammation and substituting their own findings of signal in fibroblasts and neutrophils. Moreover, the signal that they identified was predominantly cytoplasmic. The signal localisation that we reported was exclusively nuclear, except in rare syncytial giant cells where it was seen additionally in cytoplasm (fig 1). We are also concerned by the implication that we would consider a weakly positive signal to be negative thus potentially biasing the results. Signal strength in Iizuka's studies was evidently on a continuum. In contrast, we found that it could be readily dichotomised into present (discrete and punctate; fig 1) or absent.

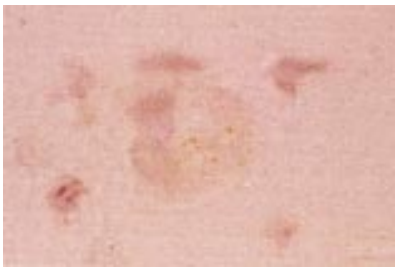

Figure 1 Multinucleate giant cell in a Crohn's disease granuloma. Rare cytoplasmic signal for measles virus N-protein using monoclonal antibody immunohistochemistry (Seralab, Crawley Down, Sussex, UK). An extremely discrete punctate signal is seen. Original magnification ×1000 (oil immersion).

A crucial experiment that has been omitted from their study is application of their antibody to primate cell lines either infected or stimulated with something other than measles virus. The indication from the observations in gut tissues is that expression of the epitope recognised by their antibody is a function of inflammation. Is this elicited by specific or non-specific processes?

It is worth emphasising the differences between the study of Iizuka *et al* and our own. They used a different antibody on tissues processed in an entirely different way (periodate/lysine/4% paraformaldehyde fixed, cryostat sections versus 10% neutral buffered formalin fixed, paraffin sections). They identified markedly different signal characteristics, both in the lineage of positive cells and the subcellular distribution (cytoplasmic versus nuclear). Most importantly, they excluded from analysis the specific foci that were identified as exclusively positive in our studies.

Despite its limitations, this work may contribute to our understanding of why measles has been linked with inflammatory bowel disease (IBD). It is our hypothesis that *atypical* exposure to the measles virus in early life increases the risk of subsequent IBD among genetically susceptible individuals. Parallels can be drawn from the rare neurological disease subacute sclerosing panencephalitis (SSPE), a delayed sequelae to persistent measles virus infection with a long natural history where the chronic disease manifests several years after the acute measles infection. In SSPE, several atypical characteristics of acute measles infection increase the risk of disease. This is likely to be because elements of the host immune response to the virus are established at the time of acute measles infection and atypical infection may alter the characteristics of this response, increasing the risk of inappropriate immune reactivity.

Measles (or at least components of measles viral material) may persist at very low copy number concentrations, making it difficult to detect using conventional RT-PCR, even with hybrid capture.[2] Indeed, it has been argued that such retention of viral material may be important in maintaining functional immunity.[3] Therefore, two important questions are: (1) What patterns of acute infection increase the risk of inappropriate immune programming and subsequent IBD? (2) What are the likely mechanisms that may result in IBD? The answer to the first question is beginning to be answered by epidemiological studies that identify complex patterns of exposure to measles virus. This includes a close temporal relationship of measles with another paramyxovirus infection taking place during the first five or six years of life.[4]

It is possible that the work of Iizuka *et al* may provide a clue to a potential mechanism linking atypical measles infection with IBD. This group reported the presence in inflamed tissue of "measles related antigen" and speculated that this is a human protein as yet unidentified. An atypical measles infection and viral persistence could increase the risk of inappropriate immune reactivity to "measles related antigen"; therefore, molecular mimicry could be one mechanism to explain chronic inflammation in IBD patients. The presence of this protein in other inflammatory disorders of the bowel may indicate that it is produced as part on the inflammatory cascade. This may shed some light on why the onset of IBD appears to be triggered by transient enteric infections. If gut inflammation results in expression of "measles related antigen" in an individual who has been primed by an earlier atypical measles infection, this could trigger an ongoing immune response. Clearly this is highly speculative but if this group has identified a human protein that shares a high degree of homology with measles virus N-protein, it may be a useful tool to investigate the relationship between atypical exposure to measles virus and IBD.

A J WAKEFIELD
S M MONTGOMERY
Centre for Gastroenterology, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, UK

Correspondence to: A J Wakefield.
awakefield@rfc.ucl.ac.uk

1 Wakefield AJ, Pittilo RM, Sim R, *et al*. Evidence of persistent measles virus infection in Crohn's disease. *J Med Virol* 1993;**39**:345–53.
2 Chadwick N, Bruce IJ, Schepelmann S, *et al* Measles virus RNA is not detected in inflammatory bowel disease using hybrid capture and reverse transcription followed by the polymerase chain reaction. *J Med Virol* 1998;**55**:305–11.
3 Zinkernagel R. Immunology taught by viruses. *Science* 1997;**271**:173–8.
4 Montgomery SM, Morris DL, Pounder RE, *et al*. Paramyxovirus infections in childhood and subsequent inflammatory bowel disease. *Gastroenterology* 1999;**116**:796–803.

Reply

EDITOR,—We thank Dr Wakefield and colleagues for their interest in our work and we acknowledge that we had initially pursued the same hypothesis that they proposed. However, we have come to a different conclusion through a series of studies[1–3] revealing that the antimeasles monoclonal antibody (Seralab, Crawley Down, Sussex, UK) that Wakefield *et al* used in their study[4] and in fig 1 above recognises a host antigen as well as measles virus. Furthermore, we proved this cross reaction by identifying a clone with this antibody in a lambda-phage expression library constructed from a typical Crohn's disease patient, by subsequently obtaining a monoclonal antibody to this clone (which we found to be unrelated to measles virus), and finally, by demonstrating that this monoclonal antibody (4F12) reacted with both measles virus and the antigen used for immunisation (the host protein).

Further to our observation that no part of the measles virus genome was detected by reverse transcription-polymerase chain

reaction,[1] our reasons for concluding that what was stained with 4F12 (and the monoclonal antibody that we purchased from Seralab) was not the measles antigen but the host protein unrelated to the measles virus were given in detail in our paper (*Gut* 2000;**46**:163–169) and we see no need to reiterate them here. One final word with regard to the comment of Wakefield *et al* on our description of molecular mimicry as a possible mechanism for pathogenesis, let us be clear that our report should not be interpreted as support for the hypothesis of measles virus or measles vaccination triggering Crohn's disease.

M IIZUKA
S WATANABE
M CHIBA
First Department of Internal Medicine, Akita University School of Medicine, Akita, Japan

O NAKAGOMI
Department of Microbiology, Akita University School of Medicine, Akita, Japan

Correspondence to: M Iizuka, First Department of Internal Medicine, Akita University School of Medicine, 1–1–1 Hondo, Akita 010–8543, Japan. iizuka@med.akita-u.ac.jp

1 Iizuka M, Nakagomi O, Chiba M, *et al*. Absence of measles virus in Crohn's disease. *Lancet* 1995;**345**:199.
2 Nakagomi O, Iizuka M. Measles virus in Crohn's disease. *Lancet* 1995;**345**:600.
3 Iizuka M, Masamune O. Measles vaccination and inflammatory bowel disease. *Lancet* 1997; **350**:1775.
4 Wakefield AJ, Pittilo RM, Sim R, *et al*. Evidence of persistent measles virus infection in Crohn's disease. *J Med Virol* 1993;**39**:345–53.

Survivin gene expression and prognosis in recurrent colorectal cancer

EDITOR,—Sarela and colleagues (*Gut* 2000;**46**:645–50) report on the association of *Survivin* gene expression and prognosis in recurrent colorectal cancer. The methods described for detecting *Survivin* mRNA relied on reverse transcription-polymerase chain reaction (RT-PCR), an exquisitely sensitive technique that has not previously been validated for this gene. We wish to point out three areas of technical difficulty in the methodology.

(A) The fidelity of mRNA extraction and RT was tested using oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a "housekeeping" gene. However, this may give rise to false positives by amplification of pseudogenes from contaminating genomic DNA.[1] The β-actin primers (as described by Raff and colleagues[1]) do not amplify genomic DNA and therefore provide absolute evidence that RT has been successful. Alternatively, this problem could be corrected either by DNase digestion of RNA before RT or by having negative RT controls for each sample.

(B) The process of RT using an oligo dT nucleotide as the RT primer results in the creation of cDNA templates for all mRNAs in the sample. This may be a problem if the gene for effector cell protease receptor 1 (EPR-1) is expressed. This gene codes for a cellular receptor of blood clotting factor Xa.[2] The DNA sequence for this gene is highly homologous to that of *Survivin* and differs by only five nucleotide changes and six nucleotide insertions.[3] The reverse primer described recognises the EPR-1 sequences (as ascertained by searching of the basic local alignment search tool of the National Cell Biology Institute (BLAST)). The forward primer does not produce a match on BLAST searching but only 1011 bases of the sequence for EPR-1 have been published on Genebank (GeneBank Accession No. L26245. Human effector cell protease receptor-1 (EPR-1) mRNA, partial CDs). Implicit in the description is that this sequence is incomplete. Given the close similarity between the probable sequences of the two genes it is not impossible that this homology continues and could provide a recognition site for the forward primer in EPR-1. This problem has been alluded to by Mahotka and colleagues[4] who used a sequence specific RT primer to eliminate it but was not taken into account elsewhere in work on survival in small cell lung cancers.[5] This may explain the detection of "*Survivin*" mRNA in normal colorectal mucosa.

(C) The PCR primers as published are in the first and fourth exons. The amplified sequence would be expected to include the published splice variants caused by deletion of the third exon or insertion of the 2B exon, as described by Mahotka and colleagues.[4] This would result in multiple bands detected on agarose gel. We would be interested to know whether these points were taken into account.

M MILLER
D SMITH
A WINDSOR
A KESSLING
St Mark's Hospital and Department of Medical and Community Genetics, Northwest London Hospitals NHS Trust, Watford Road, Harrow, Middlesex HA1 3UJ, UK

1 Raff T, van der Giet M, Endemann D, *et al.* Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. *Biotechniques* 1997;**23**:456–60.
2 Adida C, Ambrosini G, Plescia J, *et al.* Protease receptors in Hodgkin's disease: expression of the factor Xa receptor, effector cell protease receptor-1, in Reed-Sternberg cells. *Blood* 1996;**88**:1457–64.
3 Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997;**3**:917–21.
4 Mahotka C, Wenzel M, Springer E, *et al. Survivin*-ΔEx3 and *Survivin*-2B: two novel splice variants of the apoptosis inhibitor *Survivin* with different anti-apoptotic properties. *Cancer Res* 1999;**59**:6097–102

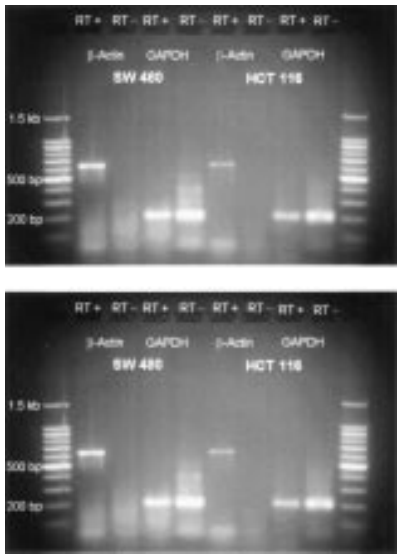

Figure 1 β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse transcription-polymerase chain reaction (RT-PCR) on two colorectal cell lines, demonstrating amplification of the GAPDH pseudogene in the RT negative controls.

5 Monzo M, Rosell R, Felip E, *et al*. A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. *J Clin Oncol* 1999;**17**: 2100–104.

Reply

EDITOR,—We thank Miller and colleagues for their interest in our study, and for pointing out the areas of technical difficulty with reverse transcription-polymerase chain reaction (RT-PCR) based projects.

(A) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification is well established as a control for the fidelity of RT and has been used as such in numerous studies, including that of Mahotka and colleagues[1] quoted by Miller *et al*. In our cell culture experiments, the intron spanning GAPDH primers used in the present investigation yielded more consistent results than β-actin primers. While GAPDH pseudogenes may occasionally be problematic, the modified Catrimox RNA isolation technique used in the present and other studies from our laboratory[2] results in minimal genomic DNA contamination, as confirmed by RT negative controls.

(B) Miller *et al* fail to recognise that although the genomic sequence of effector cell protease receptor 1 (EPR-1) is highly homologous to *Survivin*, northern hybridisation with single strand specific probes has identified distinct and mutually exclusive transcripts for *Survivin* (1.9 kb) and EPR-1 (1.3 kb).[3] Consequently, even if we were to accept Miller *et al*'s unsupported hypothesis regarding a recognition site for the *Survivin* forward primer in EPR-1, it is highly unlikely that an EPR-1 product of the same size and sequence as *Survivin* would be amplified. The specificity of our RT-PCR data is further confirmed by immunohistochemical analysis (using a monoclonal antibody kindly provided by the Yale group) that demonstrates a similar prevalence of *Survivin* protein expression, and a strong degree of concordance between protein and mRNA expression, in colorectal cancer.[4]

(C) *Survivin* splice variants, which were described in renal cell carcinoma cell lines[1] after our paper was accepted for publication, are certainly intriguing. On agarose gel electrophoresis we noted the expected *Survivin* amplification product of 338 bp (confirmed by direct sequencing) as the prominent band in all cases that were scored *Survivin* positive. In a small proportion of cases, additional minor bands, which may have resulted from alternative splicing, were noted. As discussed by Mahotka and colleagues,[1] alternative splicing adds considerably to the complexity of systems controlling apoptosis. Further investigation of the significance of this phenomenon in colorectal cancer is underway.

A I SARELA
S M FARMERY
P J GUILLOU
Professional Surgical Unit, University of Leeds, St James's University Hospital, Leeds, UK

Correspondence to: Professor P J Guillou, Professional Surgical Unit, Level 8, Clinical Sciences Building, St James's University Hospital, Leeds LS9 7TF, UK. p.j.guillou@leeds.ac.uk

1 Mahotka C, Wenzel M, Springer E, *et al. Survivin*-ΔEx3 and *Survivin*-2B: two novel splice variants of the apoptosis inhibitor *Survivin* with different anti-apoptotic properties. *Cancer Res* 1999;**59**:6097–102.