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ORIGINAL RESEARCH ARTICLE

Small intestinal enteropathy with epithelial IgG and complement deposition in children with regressive autism

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We have reported lymphocytic colitis in children with regressive autism, with epithelial damage prominent. We now compare duodenal biopsies in 25 children with regressive autism to 11 with coeliac disease, five with cerebral palsy and mental retardation and 18 histologically normal controls. Immunohistochemistry was performed for lymphocyte and epithelial lineage and functional markers. We determined the density of intraepithelial and lamina propria lymphocyte populations, and studied mucosal immunoglobulin and complement C1q localisation. Standard histopathology showed increased enterocyte and Paneth cell numbers in the autistic children. Immunohistochemistry demonstrated increased lymphocyte infiltration in both epithelium and lamina propria with upregulated crypt cell proliferation, compared to normal and cerebral palsy controls. Intraepithelial lymphocytes and lamina propria plasma cells were lower than in coeliac disease, but lamina propria T cell populations were higher and crypt proliferation similar. Most strikingly, IgG deposition was seen on the basolateral epithelial surface in 23/25 autistic children, co-localising with complement C1q. This was not seen in the other conditions. These findings demonstrate a novel form of enteropathy in autistic children, in which increases in mucosal lymphocyte density and crypt cell proliferation occur with epithelial IgG deposition. The features are suggestive of an autoimmune lesion.
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Background

We have previously reported findings of ileal lymphoid hyperplasia, in children with autistic spectrum disorders associated with regression, together with a novel form of lymphocytic colitis with striking infiltration of CD8 and $\gamma\delta$ T cells.[1–3] Epithelial damage appeared disproportionate for the extent of mucosal inflammation, and an autoimmune mechanism was speculated. The concept that unexpected intestinal dysfunction may occur in at least some children with autism has been supported by reports from other groups of small intestinal enteropathy with reduced disaccharidase expression,[4] defective sulphation of ingested phenolic amines such as paracetamol,[5] excess paracellular permeability,[6] and cognitive and behavioural response to exclusion diets.[7] In an open-label study, the antibiotic vancomycin, given to reduce putative intestinal dysbiosis, induced striking cognitive responses in children with regressive autism, maintained only during the period of administration.[8]

Whether such abnormalities may be seen in the majority of children with autism, or are restricted to a subgroup with clear regression, remains uncertain. Focused assessment of an unselected population of autistic children in Arizona showed that gastrointestinal symptoms occurred significantly more commonly than in the general childhood population, and affected about half of the children.[9] However the presence of symptoms alone does not necessarily equate with a common immune-mediated pathogenesis, and further characterisation of the intestinal lesion is clearly necessary.

Our immunohistochemical assessment of the colonic lesion, in comparison to both normal and disease control groups, showed lymphocytic infiltration and epithelial abnormalities to an extent much greater than was evident on conventional histological assessment.[3] We considered that the clinical response to exclusion

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diets seen in many of the children referred to us was also suggestive of small intestinal pathology. This was formally demonstrated by Horvath and colleagues, who found histological duodenitis in 24 of 36 autistic children studied, with Paneth cell hyperplasia in 27 children and reduction of brush-border hydrolase activity in 21.[4] Following this report, we have performed upper endoscopy in addition to colonoscopy in those children with gastrointestinal symptoms sufficient to warrant endoscopic assessment.

We now examine systematically the small intestinal lesion in our first 25 children with autism who underwent upper endoscopy, in an extensive immunohistochemical analysis. We compare their findings to 18 children investigated for possible gastrointestinal disease whose small intestinal mucosa proved to be histologically normal (histologically normal controls), 11 children with coeliac disease and five children with severe mental retardation with cerebral palsy who required endoscopy because of gastro-oesophageal reflux and poor weight gain. We report findings of a novel form of enteropathy in the children with autism, characterised by lymphocytic infiltration, increased crypt cell proliferation and enterocyte numbers, with co-localisation of IgG and complement C1q on the enterocyte basolateral membrane.

Patients and methods

Clinical details of the 25 autistic children studied are given in Table 1. All had been referred to the Royal Free Hospital for gastroenterological assessment by local developmental paediatricians or general practitioners. All had a history of a regressive developmental disorder, with a median age of regression of 18 months. In 23/25 there was no history of developmental abnormality or parental concerns within the first year of life, while 2/25 had shown some developmental delay but further regressed in the second year. Prior to referral, all had been diagnosed within the autistic spectrum following formal assessment by consultant child psychiatrists. Twenty four of the 25 children showed features of core autism, while 1/25 had previously been diagnosed with core autism but had improved with input from local therapists and the institution of a gluten and case in-free diet and had been reassessed as Asperger's syndrome. All had gastrointestinal symptoms, of constipation and/or diarrhoea, abdominal pain or distension. Plain abdominal X-ray was performed in all cases at initial assessment and showed severe faecal retention with acquired megarectum in all 25, regardless of any history of diarrhoea. No pathogens were detected on routine stool and serological analysis. The patients were selected for the study simply on the basis of having undergone upper GI endoscopy and having frozen tissue available for staining at the time of the study (patients enrolled 1999–2000). Thus no patients were excluded. Additional biopsies were obtained from all children, with informed parental consent, as approved by the local Research Ethics Committee. Biopsies from 18 histologically normal control patients were examined similarly. All had been investigated to exclude gastrointestinal pathology and no final gastroenterological or other diagnosis made. In addition we studied biopsies from 11 children with active coeliac disease, all of whom had positive endomysial antibodies and evidence of crypt hyperplastic villous atrophy. We additionally included biopsies from five children with severe mental retardation and cerebral palsy, endoscoped during the study period for probable oesophagitis. All five children suffered from spastic quadriplegia, had major feeding difficulties causing a highly restricted diet due to the need for tube feeding, and had clinical and radiological evidence of severe chronic constipation. However none were on formal exclusion diets, and none had evidence of micronutrient deficiency.

Table 1 Clinical characteristics of autistic patients

Numbers	25—24 core autism, 1 Asperger syndrome
GI symptoms	Present in all—diarrhoea and/or constipation, pain
Age	Mean 6.4 years (SE 0.5, range 2.5–12.6)
Sex ratio	21 M, 4 F
CRP	Mean 2.3 mg dl^{-1} (SE 0.5)
ESR	Mean 7.5 mm h^{-1} (SE 0.9)
Serum IgA	Mean 0.97 g l^{-1} (SE 0.09)
Serum IgE	Mean 601 kIU l^{-1} (SE 231)—available in 11
Antigliadin IgG	Increased in 3/25
Antiendomyseal antibody	Negative in all 25

Immunohistochemical and histochemical analysis

Biopsies were taken endoscopically from the 4th part of the duodenum in all cases and immediately snap-frozen. Biotin/avidin immunohistochemistry (Vectastain Elite, Vector Laboratories, Peterborough, UK) was used for peroxidase immunohistochemistry, with inactivation of endogenous peroxidase using hydrogen peroxide as previously described.[3] Primary antibodies included anti-human T cell CD3 (dilution 1:40), CD4 (1:20), CD8 (1:25), the cell proliferation marker Ki-67 (1:40), HLA-DR (1:40), laminin (1:100) and cytokeratin (1/60), all from Dako, UK and $\gamma\delta$ T cells (TCR 1, T cell Sciences, USA, 1:25). FITC-conjugated anti-human immunoglobulin antibodies were used to study distribution of IgA, IgG and IgM within the mucosa (1:40, Dako), while complement C1q was localised with a rabbit polyclonal antibody (1:200, Dako) followed by TRITC-conjugated swine anti-rabbit antibody. Co-localisation of fluorescent antibodies was assessed by double exposure and by confocal microscopy. Matched biopsies fixed in formalin were immunostained for heparan sulphate proteoglycan (1:50, Seikagaku, UK), E-cadherin (1:50, Serotec, UK) and syndecan-1 (1:50, Serotec) and stained by specific

histochemistry for sulphated glycosaminoglycans (GAGs) as previously described.[3]

Quantitation
Intraepithelial lymphocyte density was determined per 100 epithelial cells by manual counting. The numbers of enterocytes per mm of villous epithelium and the density of lymphocyte subtypes within the lamina propria were assessed using a computerised image analysis programme (Leica, UK). For lymphocyte density, immunoreactive cells were identified within a defined area which excluded all crypt tissue, taking a mean of three representative areas. Reproducibility of counting was within 8%. The epithelial expression of e-cadherin, syndecan-1, heparan sulphate proteoglycan and sulphated GAGs, as well as the lamina propria density of heparan sulphate proteoglycan and sulphated GAGs was assessed semiquantitatively on a scale from 0 (absent) through 3 (normal) to 4 (enhanced expression).

Statistical analysis
Data are expressed as mean ± Standard Error except where stated. Comparison was made between the autistic group and the normal controls, children with cerebral palsy with metal retardation and the coeliac disease group using the Kruskal–Wallis test. A P value of less than 0.05 was considered significant.

Results

Routine histopathology was reported within normal limits or showing non-specific increase of mononuclear cells in 23/25 autistic children, with two biopsies showing villous blunting and 14/25 an increase in intraepithelial lymphocytes. The biopsies from the histologically normal controls and 4/5 of the children with cerebral palsy were reported normal, while the fifth showed a minor increase in lamina propria cellularity. All children with coeliac disease showed crypt hyperplastic villous atrophy.

Formal quantitation showed pericryptal lymphocyte aggregates in 16/25 of the children with autism, 7/11 with coeliac disease, 2/18 controls and 0/5 with cerebral palsy. Eosinophil infiltration was noted in five controls, one with cerebral palsy, 12 autistic children and seven with coeliac disease. Paneth cell numbers were increased in the group with autism, as previously reported,[4] with a mean of 2.9 ± 0.1 Paneth cells per crypt compared to 2.1 ± 0.2 in the controls ($P < 0.01$), 2.0 ± 0.3 in the children with cerebral palsy ($P < 0.05$) and 1.4 ± 0.2 in the coeliac disease group ($P < 0.001$). Morphometry demonstrated a mean crypt-villus ratio in the autistic group of 2.4 (± SE 0.3), similar to the controls (2.5 ± 0.2) and the cerebral palsy group (2.6 ± 0.2) but significantly greater than coeliac disease (0.6 ± 0.2). However there was an increase in the density of enterocytes along the villus in the autistic group, with the cells frequently close-packed and the nuclei thinned and elongated. The mean enterocyte count per mm of villous epithelium of 240.7 ± 6.5 in the autistic children was significantly greater than in the normal controls (201.4 ± 8.3, $P < 0.003$) and the group with cerebral palsy (184.5 ± 8.1, $P < 0.01$), or within the surface epithelium in coeliac disease (172.6 ± 6.5, $P < 0.001$).

Immunohistochemistry
By contrast to the rather mild findings on routine staining, immunohistochemistry demonstrated marked abnormalities in the group with autism. The density of CD8 intraepithelial lymphocytes in the autistic children (32.4 ± SE 1.9 per 100 enterocytes) was significantly greater than normal controls (17.7 ± 1.4) or the cerebral palsy children (16.7 ± 2.5) but less than in coeliac disease (46.3 ± 2.7, see Figure 1).

Within the lamina propria, there was evidence of excess lymphocyte infiltration (CD3, CD4 and CD8), with the autistic children showing overall higher density than all the other groups (Figure 1). There was often evidence of pericryptal aggregation of CD3 and CD8 T cells (Figure 2). The mean density of CD3 cells in the autistic children (836 ± SE 34 mm^{-2}) was significantly higher than in controls (355 ± 24.2), cerebral palsy (473 ± 48) and coeliac disease (514 ± 43) groups (Figure 1). Similar findings were seen for CD4 cell density (708 ± 37 mm^{-2} in autism, 346 ± 24 in controls, 399 ± 45 in cerebral palsy, 438 ± 37 in coeliac disease) and CD8 cell density (454 ± 27 mm^{-2} in autism, 218 ± 18 in controls, 282 ± 38 in cerebral palsy, 208 ± 16 in coeliac disease, see Figure 1). In coeliac disease, the T lymphocyte infiltration was focally dense in the upper lamina propria (Figure 2), but more scanty in the deep lamina propria, where the dominant lymphocyte population was syndecan-1^+ plasma cells. Thus in contrast to the T cell infiltrate, the mean plasma cell density was significantly lower in autism than coeliac disease (1366 ± 141 compared to 2578 ± 127 mm^{-2}), although still higher than the control and cerebral palsy groups (764 ± 83 and 613 ± 104 mm^{-2} respectively, Figure 1). We did not find significant infiltration of cells in 23/25 of the autistic children studied, in contrast to findings within the colon[3] and in the children with coeliac disease. HLA-DR expression was however increased within the lamina propria and the surface, but not crypt, epithelium was frequently HLA-DR positive.

Epithelial changes
E-cadherin was expressed normally on the basolateral epithelial surface in the autistic group, with staining intensity similar to controls and the cerebral palsy group, in contrast to reduced expression in coeliac disease (mean staining intensity 2.5 in coeliac disease, 3 in the other groups). Immunohistochemistry for heparan sulphate proteoglycan showed reduced epithelial expression in the autistic group compared to controls (1.9 ± SE 0.2 *vs* 2.5 ± 0.2, $P < 0.05$), although greater than coeliac disease (1.6 ± 0.15, $P < 0.05$). No significant differences were seen between groups in epithelial syndecan-1 expression. Staining for sulphated GAGs showed similar reduction compared to controls but did not reach statistical significance, while

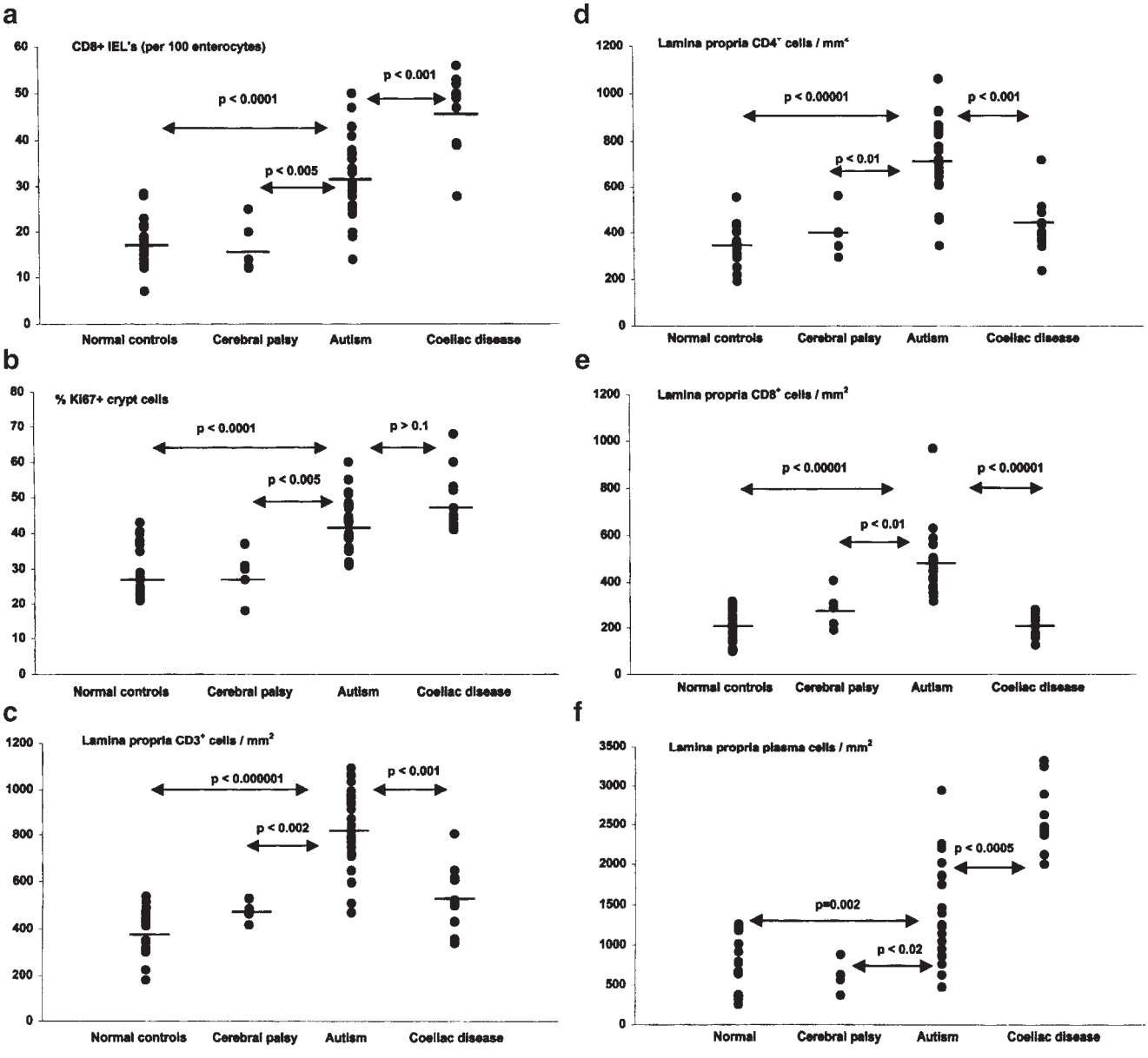

Figure 1 Quantitation of mucosal density of lymphocyte populations in the group with autism ($n = 25$) in comparison with normal controls ($n = 18$), cerebral palsy and mental retardation ($n = 5$) and coeliac disease ($n = 11$). The staining for syndecan-1 plasma cells (F) was performed on formalin-fixed specimens, the other markers on frozen specimens. The autistic group showed significant increase above controls and the cerebral palsy group for CD8 intraepithelial lymphocytes (a), proliferating crypt cells (b), lamina propria T cells (c–e), and plasma cells (f). The coeliac disease group showed higher CD8 intraepithelial lymphocytes (a) and lamina propria plasma cells (f) than the autistic group, but similar crypt proliferation (b) and lower lamina propria T cell densities (c–e). All comparisons were made with the Kruskall–Wallis test.

epithelial GAG expression was significantly lower in coeliac disease. As in the colon,[3] there was no degradation of sulphated GAGs within the lamina propria, and expression of heparan sulphate proteoglycan was unchanged. There was evidence of basement membrane thickening in the groups with autism and coeliac disease, on staining for both laminin and heparan sulphate proteoglycan. Despite the normal morphometry, there was marked increase of crypt cell proliferation (Ki67+ cells) in the autism group (43.7 ± 1.7%), similar to coeliac disease (49.5 ± 2.8), and significantly greater than normal controls (29.4 ± 1.8) and the cerebral palsy group (28.6 ± 3.1, see Figure 1).

Immunoglobulin deposition

No significant abnormalities were seen in the distribution of IgG or IgM in the autistic group, and normal uptake by crypt epithelium was seen. By contrast, the most striking finding was the deposition of IgG on the basolateral enterocyte membrane and the subepithelial basement membrane in 23/25 of the autistic children studied, particularly IgG1 and IgG4, not seen in the

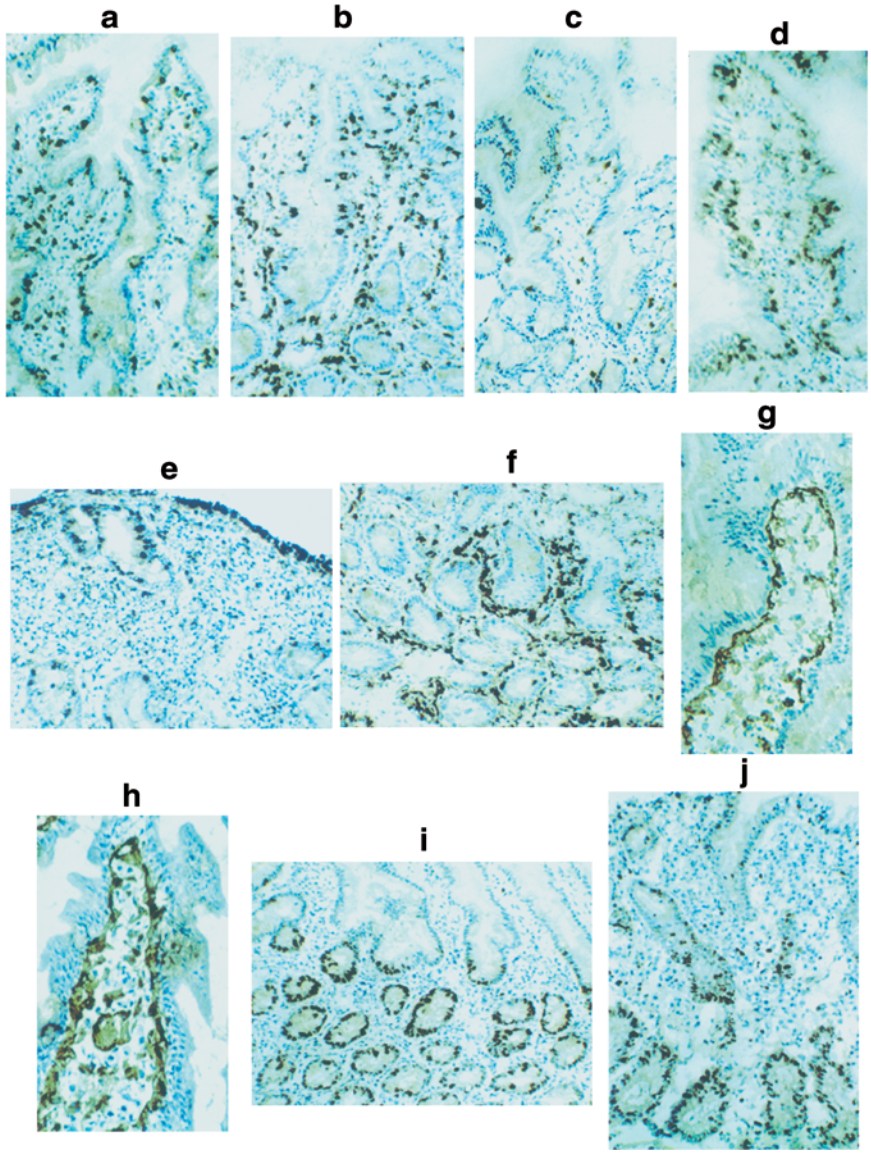

Figure 2 Peroxidase immunohistochemistry of snap-frozen mucosal biopsies. Positively stained cells are brown, while negatively stained cells show blue counterstaining. (a) Distribution of $CD3^+$ T cells in biopsy from child with cerebral palsy and mental retardation. (b) Increased density of $CD3^+$ cells in epithelial and lamina propria compartments of child with regressive autism. (c) $CD8^+$ cells in villus of normal control. (d) Dense infiltration of $CD8^+$ cells in villus of autistic child. (e) Distribution of $CD3^+$ T cells in case of coeliac disease, showing dense infiltrate within epithelial compartment, moderate density in subepithelial region and more scanty distribution in deep lamina propria. (f) Dense pericryptal aggregates of $CD3^+$ T cells within deep lamina propria of child with autism. (g) Laminin distribution within the subepithelial basement membrane and lamina propria of a normal control. (h) Laminin distribution in child with autism, showing marked thickening of the basement membrane. (i) Proliferating cells expressing Ki67 within the crypts of autistic child. The density of proliferating cells is significantly higher than normal, and similar to that seen in coeliac disease, shown in (j).

normal or cerebral palsy group (Figure 3). While several coeliac children showed some epithelial IgG deposition, this was not localised to the basolateral surface (Figure 3). Both the autistic and coeliac patients showed marked increase of IgG deposition within the subepithelial basement membrane compared to controls and the cerebral palsy group. In some autistic children the epithelial IgG deposition was patchy and incomplete, while in others it was striking and extensive. Double staining for complement C1q was performed in 18 patients with sufficient remaining tissue, who showed a similar pattern of deposition within the basement membrane and on the basolateral enterocyte surface, with clear co-localisation identified on double exposure or confocal microscopy (Figure 3).

Discussion

These findings confirm and extend Horvath's findings of significant duodenitis in autistic children.[4] This

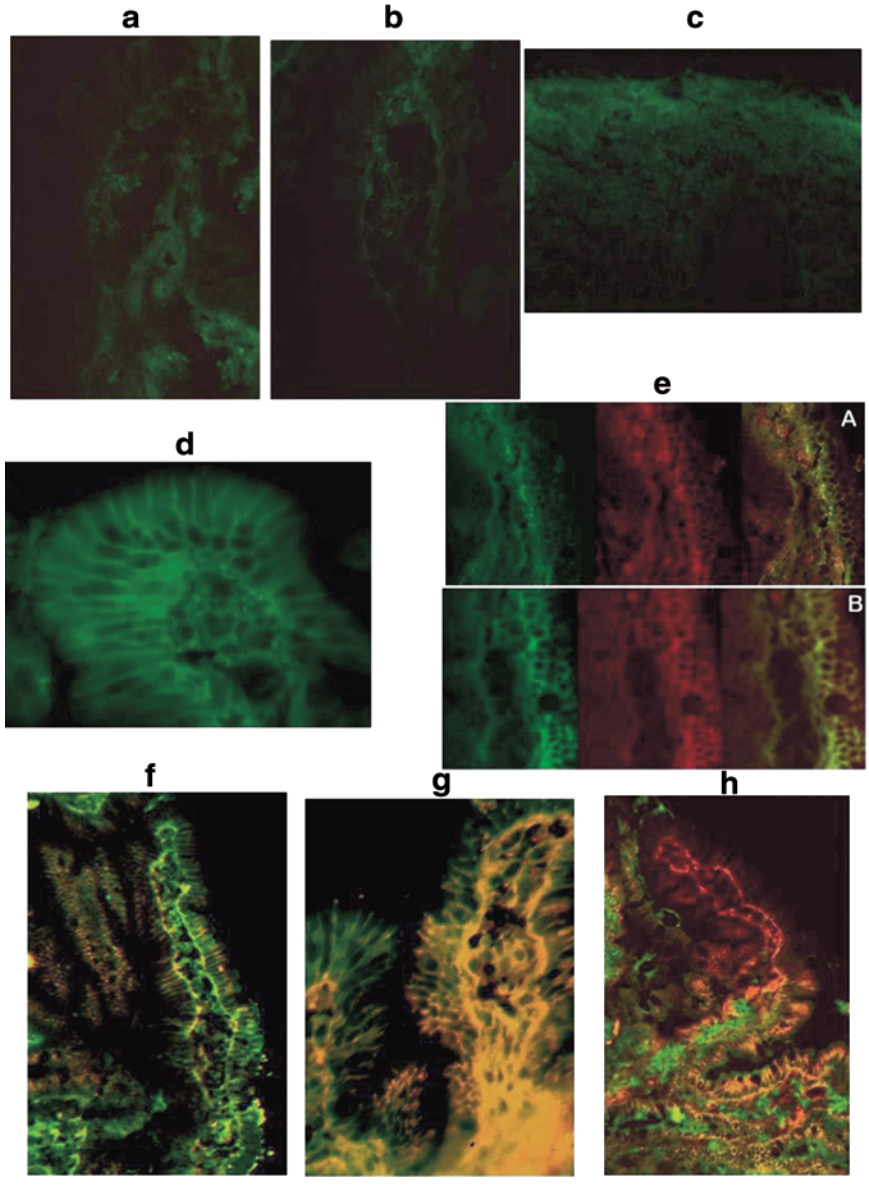

Figure 3 Immunofluorescence of immunoglogulin expression. FITC-conjugated antibodies show as green, TRITC-conjugated as red. Co-localised antibodies show a yellow colour. Staining from five autistic children is shown. (a) Single villus in normal control stained with IgG-FITC. There is very slight staining outlining the subepithelial basement membrane but the epithelium is unstained. (b) Similar findings in a child with cerebral palsy. There is some epithelial IgG deposition just at the villus tip. (c) IgG deposition subjacent to the flattened surface epithelium in coeliac disease. (d) Strong expression of IgG in the basement membrane and on the basolateral enterocyte membrane in a child with autism. These appearances were not seen in any of the other groups. (e) Confocal microscopy of double staining in another two children with autism. In A, IgG-FITC (green) and C1q TRITC (red) can be seen to co-localise extensively within the basement membrane and on the basolateral enterocyte surface. In B, IgG-FITC (green) co-localises with IgG4-TRITC (red) in the same distribution. IgG1 and IgG4 were the dominant isotypes, with much stronger staining than IgG2 and IgG3. (f) IgG-FITC and C1q-TRITC show extensive epithelial co-localisation (yellow) in another child with autism. (g) High power view of IgG-FITC and C1q-TRITC co-localising in the basement membrane and epithelium of another autistic child. (h) No evidence of co-localisation between IgA-FITC and C1q-TRITC in the autistic child whose IgG expression is shown in (g). The IgA plasma cells show normal distribution in the deep lamina propria and crypt epithelium, the C1q in the basement membrane and villus epithelium.

lesion appears distinct from all previously reported forms of childhood enteropathy.[10] The children showed overall a marked increase in mucosal lymphocyte density and numbers of CD8 intraepithelial lymphocytes, not seen in controls or children with cerebral palsy. However, in contrast to findings in the colon, intraepithelial $\gamma\delta$ cells were not increased, suggesting either a different pattern of epithelial pathology or impairment of the expression of obligatory cell ligands such as MICA[10] on small bowel epithelium. Despite the normal mucosal architecture, the autistic children showed marked increase of crypt cell proliferation. As

crypt cell proliferation is the major determinant of Paneth cell numbers,[11] this finding potentially explains their increase in both Horvath's[4] and our own studies. This is supported by the finding of increased overall villous enterocyte density in the autistic group. The most striking finding, that clearly differentiated the autistic group, was the deposition of IgG on the basolateral enterocyte membrane. This largely co-localised on the epithelium with complement C1q, to confirm pathological significance and likely autoimmune basis. However the lesion appears histologically subtle, with relatively minor features on routine staining that would preclude using standard histology in diagnosis.

The significance of these results for autism in general remains uncertain. Firstly there is the problem faced by all tertiary centres, of excluding referral bias towards exceptional patients with atypical symptoms, particularly when encountering a disorder novel to the specialty. While the study by Melmed and colleagues suggests that about half of the children have gastroenterological symptoms when formal assessment is made,[9] we recognise that investigation of autistic children without gastrointestinal symptoms may be required to establish a denominator for our findings. Secondly, the detection of such an apparently autoimmune condition could equally well be explained as either a contributory or even causal condition, or one that is a completely separate consequence of a underlying primary genetic abnormality, manifesting primarily through its cognitive effects. In the first instance, the gut dysfunction would develop as a consequence of focal autoimmunity and contribute to altered cognitive functioning by failure to detoxify neuroactive substances originating from the flora, as is seen in hepatic encephalopathy or other constipation-associated depressive or confusional states.[12–14] The target for an autoimmune response could also be expressed in both brain and gut, with pathological abnormalities in both sites. In the second instance, the immunological and gastrointestinal abnormalities could develop because of genetic abnormality causing multisystem impairment of cellular proliferation and differentiation, but with extracranial consequences less striking than the neurodevelopmental. In Rett's syndrome, which shows similar features of cognitive regression, mutations in methyl-CpG-binding protein 2 (MeCP2) disrupt the basic mechanism of gene regulation via DNA methylation throughout the body,[15,16] but abnormal immune function is apparent only on specific testing.[17] Of potential importance, the only report of colonoscopy in Rett's syndrome has identified extensive colonic lymphoid hyperplasia with melanosis coli,[18] essentially similar to our macroscopic findings in many cases of regressive autism.[1–3]

There is undoubtedly a major genetic predisposition to autism, which is clearly polygenic. Regions on chromosomes 7q and 15 have been particularly prominent on linkage analysis studies, and several candidate genes uncovered.[19,20] Studies using linkage analysis alone have not identified the major histocompatibility complex (MHC) region on chromosome 6 as closely linked to the development of autism.[19–21] However this technique is much less powerful when immunologically-mediated disease of polygenic inheritance is not linked to an individual MHC type, as it is in coeliac disease,[10] but to several genes affecting the control of immunological reactivity. Thus neither Crohn's disease nor ulcerative colitis show significant linkage to the MHC on genome screening.[22] However, focused analysis of individual areas within the MHC has identified associations in both diseases,[23] and indeed for autism.[24] The strongest associations detected within the MHC in autism, for the null allele of complement C4B within the class III region,[24] the extended haplotype B44-S30-DR4[25] and the third hypervariable region of HLA-DR 1,[26] are all known to predispose to the development of autoimmunity. There is a significant increase in autoimmune disorders within families of autistic children[27] and circulating autoantibodies to brain components have been reported by two groups.[28,29] Several inbred strains of spontaneously autoimmune mice show early neurobehavioural abnormalities, particularly in testing of emotional reactivity domains, which are associated with focal changes in neuronal cellularity and brain-reactive autoantibodies.[30,31] Importantly, this autoimmunity-associated behavioural syndrome (AABS) can be reversed by cyclophosphamide if treated early in the course of disease.[30]

Evidence that the incidence of autism is truly increasing—in common with other immunopathologies and autoimmune conditions—would point to an acquired immunopathology causing developmental regression and the onset of autism in many children. A major difference in incidence between children from developing world countries and their relatives born in the allergy-prone developed world would also argue for environmental factors. However the epidemiological findings are frustratingly contradictory. Studies suggest either a 5–15 fold increase in the incidence of autistic spectrum disorders within the last two decades,[32–35] consistent only with a cohort of children suffering environmentally triggered or autoimmune-induced disease, or that the apparent increase is an artefact because of increased awareness and altered diagnostic criteria,[36] in which case the aetiology is entirely genetic but a remarkably high proportion of previous cases went undiagnosed. There is probably no other condition bedevilled by such fundamental uncertainty. Our findings may at least partly explain the similar clinical response to intravenous immunoglobulins[37] and to vancomycin[8] in children with regressive autism. The impressive cognitive improvement seen in autoimmune animal models, providing effective immunosuppression is commenced early in the course of disease,[30] suggests that resolution of the role of autoimmunity within the spectrum of autistic disorders is of potentially major importance.

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