Intestinal Lymphocyte Populations in Children with Regressive Autism: Evidence for Extensive Mucosal Immunopathology

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Inflammatory intestinal pathology has been reported in children with regressive autism (affected children). Detailed analysis of intestinal biopsies in these children indicates a novel lymphocytic enterocolitis with autoimmune features; however, links with cognitive function remain unclear. To characterize further, the nature and extent of this disease we examined the mucosal infiltrate using flow cytometry. Duodenal, ileal, and colonic biopsies were obtained from 52 affected children, 25 histologically normal, and 54 histologically inflamed, developmentally normal controls. Epithelial and lamina propria lymphocyte populations were isolated and examined by multicolor flow cytometry. Adjacent biopsies were assessed by semiquantitative histopathology. At all sites, $CD3^+$ and $CD3^+CD8^+$ IEL as well as $CD3^+$ LPL were significantly increased in affected children compared with developmentally normal noninflamed control groups ($p < 0.01$) reaching levels similar to inflamed controls. In addition, two populations—$CD3^+CD4^+$ IEL and LP $CD19^+$ B cells—were significantly increased in affected children compared with both noninflamed and inflamed control groups including IBD, at all sites examined ($p < 0.01$). Histologically there was a prominent mucosal eosinophil infiltrate in affected children that was significantly lower in those on a gluten- and casein-free diet, although lymphocyte populations were not influenced by diet. The data provide further evidence of a pan-enteric mucosal immunopathology in children with regressive autism that is apparently distinct from other inflammatory bowel diseases.

INTRODUCTION

Autistic spectrum disorder (ASD) is a complex developmental disorder of childhood, characterized by pervasive impairments in social interaction, deficits in verbal and nonverbal communication, and stereotyped and repetitive behavioral patterns, developing within the first 3 years of life (1). The prevalence of autism has increased substantially over the last decade in developed countries (2–6). There is recent awareness of gastrointestinal comorbidity in some affected children of uncertain pathophysiological significance (7–10). Gastrointestinal involvement has been reported in children with an apparent later onset of behavioral symptoms on validated screening tests (11, 12). A changing pattern of presentation is also suggested by lesser degrees of mental retardation in this "late onset group" compared with classical descriptions of autism (13).

We have examined a group of children with autistic spectrum disorder (affected children) associated with gastrointestinal symptoms. Affected children may suffer frank diarrhea, constipation, or alternating bowel habit; pain and abdominal bloating are common. Parents report that symptoms commonly improve when children are placed on a gluten and casein restricted diet. Often, however, the presence of gastrointestinal (GI) pathology may be difficult to infer in the presence of behavioral abnormalities and may manifest as sudden, unprovoked temper tantrums, aggression, and self-injurious behavior. Our observations that these symptoms may peak prior to bowel

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evacuation, and are relieved by the latter (observed in the clinical setting of bowel preparation prior to colonoscopy) are a clue that they may reflect visceral pain (8).

In a recent series of histopathological and immunohistochemical studies we have described an apparently novel gastrointestinal immunopathology in affected children (7–10, 14) in which chronic ileocolonic lymphoid nodular hyperplasia (LNH) and enterocolitis are characteristic. Briefly, the mucosal lesion in affected children represents a lymphocytic enterocolitis, characterized by a relatively consistent pattern of lymphocyte infiltration with a variable degree of acute inflammation. In particular, both $CD8^+$ cells and $\gamma\delta$ T cells are present at high density (9). Despite the lymphocyte infiltration, HLA-DR is not upregulated on colonic epithelium. Serum IgG of affected children colocalized with complement C1q at the epithelial basolateral membrane, raising the possibility of a low-grade autoimmune lesion (14). These changes are not seen in either histologically normal or disease controls, or children with cerebral palsy.

Dysregulated immunity may not be confined to the intestine in affected children; many of affected children suffer recurrent prolonged infections, particularly of the upper respiratory tract, and there is a high prevalence of dietary allergy, eczema, and adenotonsillar hypertrophy. Routine immunological investigation frequently reveals lymphopenia, affecting both CD4 and CD8 populations (15). Consistent with allergic predisposition, IgA is usually in the lower quartile of the normal range (16). Functionally the majority of children assessed showed unresponsiveness for all common recall antigens on cutaneous delayed-hypersensitivity testing, in significant contrast with age-matched controls (15). Similar findings of lymphopenia and systemic immune dysregulation have been reported and reviewed by other groups (17–19). A history of organ-specific autoimmunity in first-degree family members in 30–50% of cases was reported in a recent U.S. study (20).

With evidence of both upper and lower GI pathology in children with autism, the question arises as to whether these are distinct lesions or, alternatively, whether they represent part of a continuous pathological process, perhaps reflecting a diffuse mucosal immunoregulatory defect. The aim of this study was to test the hypothesis that there is a novel and characteristic enterocolitis in a subset of children with autism and gastrointestinal symptoms. To this end we sought to characterize the nature and extent of the mucosal lymphocytic infiltrate at different anatomical sites and compare this with histologically normal and inflamed controls. Flow cytometric data were compared with systematically scored routine histopathology. In addition, immune profiles in the mucosa and peripheral blood were compared.

PATIENTS AND METHODS

Patient Population

All specimens investigated were collected from children at a single medical center. Biopsy specimens that were obtained included those from the fourth part of the duodenum, terminal ileum, and transverse colon from a total of 131 children undergoing upper gastrointestinal endoscopy, and/or colonoscopy for the investigation of gastrointestinal symptoms. Details of these children are provided in Table I. Children were investigated consecutively to avoid selection bias. Inclusion criteria for ASD children were as follows: gastrointestinal symptoms sufficient to warrant invasive investigation, a securely diagnozed developmental disorder on the autistic spectrum by standard measures based upon the *Diagnostic and Statistical Manual* (*DSM III-R/DSM IV*) for mental disorders and ICD-10 criteria, where no other cause for their developmental disorder was identified, and there was no contraindication to anesthetic for ileocolonoscopy. Chronic

Table I. Patient Details

Patient category	No.	Age Mean and range	Sex M	Number of biopsies examined		
				Duodenum	Ileum	Colon
ASD (affected children)	52	6.8 (2–16)	39	46	46	45
Non-ASD controls						
Histologically normal controls	25	6.8 (1.17)	11	16	12	9
Histologically inflamed controls	54	10.8 (1–19)	39	27	37	31
Food allergy	7	10.3 (3–17)	6	5	3	3
Crohn's disease	19	12.9 (1–19)	14	6	17	11
Ulcerative colitis	3	11 (8–13)	3	1	3	3
Indeterminate colitis	11	13 (9–18)	6	1	10	10
Coeliac disease	7	6.3 (1–13)	4	7	1	1
Other enteropathies	7	10 (1-16)	6	7	3	3

gastrointestinal symptoms in affected children included frank diarrhea (15), constipation (37), alternating episodes of diarrhea and constipation (4), overt abdominal pain (5), weight loss (1), and nausea (1) with multiple symptoms in three children. In affected children informed consent to the procedure was not declined by parents in any case. Inclusion criteria for controls were: gastrointestinal symptoms sufficient to merit ileocolonoscopy, a history of normal development or no history of a developmental disorder on the autistic spectrum and written consent as above. Children were grouped according to the following criteria: 1) ASD (affected children), 2) non-ASD children with histologically noninflamed mucosa (including three children with cerebral palsy and two with developmental delay), and 3) non-ASD children with histologically inflamed mucosa (diagnoses are provided in Table I). Affected children were not on any specific anti-inflammatory or immunomodulatory therapy. To make valid comparisons between relatively homogenous groups when comparing flow cytometric findings in duodenal biopsies, patients with coeliac disease were analyzed separately from other disease controls. Likewise, when comparing these findings in ileal and colonic biopsies, patients with inflammatory bowel disease were analyzed separately from other disease controls.

The developmental diagnoses in affected children (Group 1) were made prior to referral to our unit by a suitably qualified psychiatrist, psychologist, or developmental pediatrician. Children had a similar history of achieving normal developmental milestones, followed by arrest and loss of acquired skills and onset of aberrant behaviors. Diagnoses included autism (50) and Asperger's syndrome (2). All gastrointestinal diagnoses were made by experienced paediatric gastroenterologists, on the basis of established clinical, serological, endoscopic, and histological criteria. Ileal LNH was assessed visually and scored 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) according to criteria as described previously (9, 10).

All children underwent routine histological assessment of mucosal biopsies by the surgical histopathologists in the Department of Histopathology (Royal Free Hampstead NHS Trust, London, U.K.). Haematoxylin and eosin-stained histological sections were independently reviewed by a research histopathologist (AA), who was blinded to the flow cytometric data. Systematic scoring of tissue sections was performed using a standard research *pro forma* as previously described and validated (10). The *pro forma* scores the following mucosal features: (i) *neutrophil infiltration* (0 = no neutrophils, 1 = increased neutrophils in lamina propria, 2 = cryptitis, 3 = crypt abscesses), (ii) *chronic inflammatory cell infiltration* (0 = no increase in chronic inflammatory cells, 1 = increase in chronic in-

flammatory cells in upper third of lamina propria, 2 = increase in chronic inflammatory cells in upper two thirds of the lamina proria, 3 = increase in chronic inflammatory cells in entire lamina propria), (iii) *eosinophil infiltration* (0 = no increase in mucosal eosinophils, 1 = mild increase in mucosal eosinophils, 2 = moderate increase in mucosal eosinophils, 3 = severe increase in mucosal eosinophils). On the basis of other subtle pathological changes we have previously identified in autistic children the slides were also assessed for the presence or absence of an excess of intraepithelial lymphocytes, presence and reactivity of lymphoid tissue, the latter including increased size and confluence of lymphoid follicles and the presence of expanded germinal centers containing Tingible body macrophages, mucosal nuclear debris, epithelial disruption, and crypt/glandular distortion.

Routine stool and serological analyses were performed to screen for common pathogens. Serum anti-endomyseal and anti-glaidin antibody titers were measured in affected children to screen for coeliac disease.

Preparation of Single Cell Suspensions for Flow Cytometry

Single cell suspensions were isolated from the epithelial compartment of the biopsy specimens by continuous agitation for 1 h at 37°C, using three changes of an EDTA containing calcium-free Hanks' balanced salt solution (Sigma, Poole, U.K.). Single cell suspensions were washed twice in RPMI-1640 medium (Gibco, Life Technologies Ltd., U.K.) supplemented with antibiotics and 10% fetal calf serum.

Lamina propria mononuclear cell suspensions were released from the remaining tissue following treatment with RPMI-1640 medium containing (2 mg/mL) collagenase (Sigma) and supplemented with 10% FCS (21). Biopsy specimens were continuously agitated for 2 h at 37°C. The resulting crude cell suspension was washed twice in RPMI-1640 medium. Both epithelial and lamina propria cells were resuspended in 1 mL PBS containing 1% BSA and 0.02% sodium azide and fixed with 1% paraformaldehyde and stored at 4°C, until antibody staining and flow cytometric analysis, which was performed within 1 week of collection.

Following isolation, cell number and viability of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from both cases and controls were compared, using trypan blue exclusion. Confirmation of viability was performed using 7AAD (7-amino-actinomycin-D, Via-Probe^{TM}, Pharmigen, U.K.) staining. Cell viability was >95% and did not vary between affected children and control groups ($p > 0.1$). CD19$^+$ B

cells were not detected within the epithelial layer where CD8$^+$CD3$^+$ T cells were the predominant population, consistent with an IEL phenotype.

Flow Cytometric Analysis

One hundred microliters of each cell suspension (1 × 10^5 cells) was dispensed into 96-well microtiter plates. Ten microliters of fluorochrome-conjugated monoclonal antibody, directed against one of the following human cell surface markers, was added to the wells and the plates incubated for 30 min in the dark at 4°C: CD3$^+$-FITC, CD4$^+$-PE, and CD8$^+$-PC5 (all Dako, U.K.) for T cells; CD19$^+$-PE and CD21$^+$-PC5 (CR2 complement receptor) (Coulter, U.K.) for detection of B cells; CD14$^+$-PE (LPS receptor) (Coulter) for the detection of monocytes; CD56$^+$-PE (Coulter) for detection of Natural Killer (NK) cells, and isotype matched fluorochrome-conjugated irrelevant immunoglubulin (Dako), used at the same concentration, for assessment of nonspecific binding. Finally, cells were subjected to two washes with PBS containing 1% BSA and 0.02% sodium azide.

Samples were analyzed using a multicolor Galaxy 2000 four-color flow cytometer (Dako) equipped with a 488-nm argon laser and 633-nm diode laser. Results were analyzed by Winlist Version 4.0 (Verity, U.S.A.). A gated region based upon forward and side light-scatter properties was generated around the lymphocyte population and 10,000 events per sample were collected. Specific cell staining within the lymphocyte region was analyzed, with the CD3$^+$ cell population, and the proportion of CD4$^+$ and CD8$^+$ subsets, assessed in both epithelial and lamina propria compartments. In addition, the numbers of B cells expressing CD19$^+$ was determined in the lamina propria. Further populations including monocytes/macrophages, NK cells, and plasma cells were also investigated on the basis of specific antibody staining.

Lymphocytes were detected in the gated region (R1) and analyzed for the cell surface expression of, for example, CD3$^+$, CD4$^+$, and CD8$^+$ with double-positive CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ populations in the upper right quadrants (Fig. 1). Quadrants were set on the basis of antibody staining using isotype matched fluorochrome-conjugated irrelevant immunoglubulin, used at the same concentration as the T-cell phenotype-specific antibody.

Peripheral Blood Lymphocyte Counts and Serum Immunoglobulins

Peripheral blood lymphocyte (PBL) counts and serum immunoglobulins were performed as routine analyses in the Departments of Immunology and Haematology, Royal Free Hampstead NHS Trust. This is an approved reference laboratory in which appropriate age-standardized
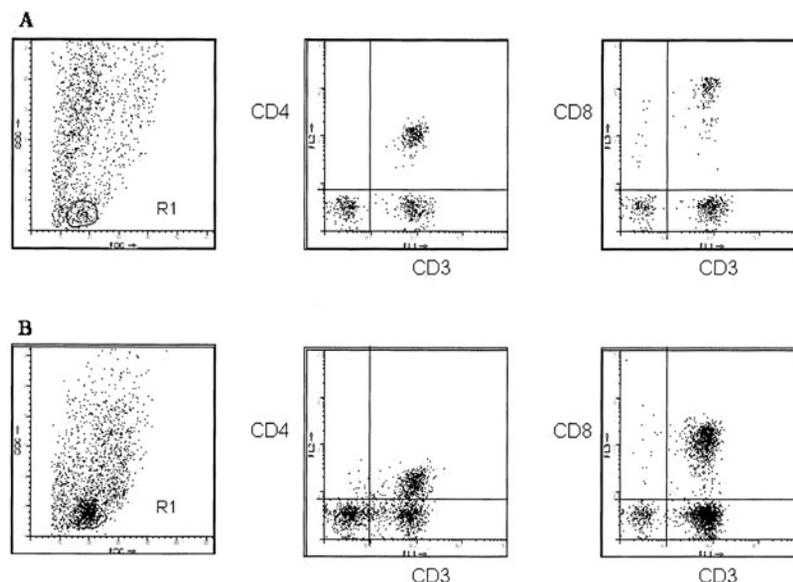

Fig. 1. Representative flow cytometry dot plots of CD3$^+$ subsets in affected children. Lymphocytes are identified on the basis of physical characteristics using forward and side light scatter measurements; a gated region (R1) that included lymphocytes but excluded debris was generated. Following fluorescent-conjugated monoclonal antibody staining CD3$^+$ and subset populations, CD4$^+$ and CD8$^+$, were detected in the epithelial (A) and lamina propria (B) compartments within this gated region (R1).

reference ranges were derived from a normal control population using identical methodology. Individual results were provided with these age-standardized upper and lower limits (95th–5th centiles) of normal for each lymphocyte subset and immunoglobulin class.

Statistical Analysis

All data are expressed as a percentage of positive cells per 10,000 recorded events, and shown as mean $\pm$ SEM. Statistical analyses using unequal Student's t tests (Bonferroni-corrected) were performed using SPSS software and results were considered significant if $p < 0.05$. In using parametric statistics the assumption was made that lymphocyte numbers are continuously distributed. The relationship between quantitative changes in mucosal and peripheral blood lymphocyte populations was examined by Spearman rank correlation, and between mucosal or peripheral lymphocytes counts and histology scoring by Pearson's correlations. Odds ratios are provided for histological comparisons of eosinophil and IEL changes and the effect of diet upon mucosal eosinophil infiltration, using Epi info 6 version 6.04b software (obtained from CDC, U.S.A. and WHO Switzerland).

Ethical Approval

These studies were approved by the Ethical Practises Committee of the Royal Free Hampstead NHS Trust.

RESULTS

Upper Gastrointestinal Pathology

Routine Histopathology. Upper GI biopsies were available from 46 affected children (Table I). Twenty-nine of 46 (63%) showed acute and/or chronic inflammation in the oesophagus (8 of 46; 17%), stomach (10 of 46; 22%), and duodenum (17 of 46; 37%). Twenty-five of 46 (54%) showed chronic inflammatory changes and 11 of 46 (24%) showed acute inflammation, including 7 (15%) with both acute and chronic changes. An excess of IEL was present in 14 of 46 (30%) and a raised mucosal eosinophil infiltrate was present in 13 of 46 (28%) of affected children. There was no villous atrophy detected and no serological or histological evidence of coeliac disease in the affected children.

Upper GI biopsies for histology were available from 27 developmentally normal control children who had an inflammatory pathology of the intestinal mucosa (Table I). Ten of 27 (37%) showed acute and/or chronic inflammation; in the oesophagus (2 of 27; 7%), stomach (7 of 27; 26%), and duodenum (5 of 27; 19%). These differences were not statistically significant compared with affected children ($p > 0.05$). Nine of these 27 children (33%) showed chronic inflammatory changes and 6 of 27 (22%) showed acute inflammation, including 5 (19%) with both acute and chronic changes. An excess of IELs was present in 11 of 27 (41%) and a raised mucosal eosinophil infiltrate was present in 4 of 27 (15%).

Systematic histological review of all 16 available noninflamed controls, using the same pro forma, confirmed the absence of acute and chronic inflammation, with no detectable increase in either eosinophils or IEL in the mucosa.

Flow Cytometry

Duodenum—Epithelium. $CD3^+$ cells were statistically significantly elevated within the epithelial compartment of the duodenum in affected children compared with noninflamed controls ($31 \pm 3\%$ vs. $11 \pm 3\%$, $p < 0.0001$). Statistically significant increases were seen in the affected children for both $CD4^+$ ($10 \pm 2\%$ vs. $1 \pm 0.2\%$, $p < 0.0001$) and $CD8^+$ ($19 \pm 3\%$ vs. $8 \pm 2\%$, $p < 0.004$) subsets. In addition, significant differences were seen between affected children and the inflamed, noncoeliac disease controls when comparing $CD3^+$ ($31 \pm 3\%$ vs. $19 \pm 3\%$, $p < 0.009$) and $CD4^+$ ($10 \pm 2\%$ vs. $5 \pm 1\%$, $p < 0.01$) but not $CD8^+$ ($19 \pm 3\%$ vs. $10 \pm 2\%$, $p < 0.06$) subsets (Fig. 2B). The differences between affected children and children with coeliac disease were not statistically significant for these lymphocyte subsets ($p < 0.05$; Fig. 2B).

Lamina propria. Within the lamina propria, $CD3^+$ cells were statistically significantly greater in affected children than in with noninflamed controls ($42 \pm 3\%$ vs. $20 \pm 4\%$, $p < 0.0006$). The $CD4^+$ ($24 \pm 3\%$ vs. $7 \pm 3\%$, $p < 0.0001$), but not $CD8^+$ subset ($15 \pm 2\%$ vs. $9 \pm 3\%$, $p < 0.08$), was statistically significantly raised compared with noninflamed, but not inflamed or coeliac disease controls ($p < 0.05$; Fig. 2A). Furthermore, statistically significantly increased numbers of $CD19^+$ B cells were present in the lamina propria of affected children ($10 \pm 2\%$) compared with noninflamed ($3 \pm 1\%$, $p < 0.001$), inflamed ($2 \pm 1\%$, $p < 0.001$), and coeliac disease ($1 \pm 1\%$, $p < 0.001$) control groups (Fig. 5). Similarly, $CD21^+$ cells were increased in affected children compared with noninflamed (9 ± 3 vs. $2 \pm 1\%$, $p < 0.05$) and coeliac disease controls ($9 \pm 3\%$ vs. $1 \pm 1\%$, $p < 0.03$), but not inflamed controls (9 ± 3 vs. $6 \pm 2\%$, $p > 0.05$). In addition, $CD14^+$ monocytes were increased in the lamina propria of affected children compared with noninflamed controls ($8 \pm 2\%$ vs. $1 \pm 1\%$, $p < 0.006$), and with children with coeliac disease ($8 \pm 2\%$ vs. $1 \pm 1\%$,
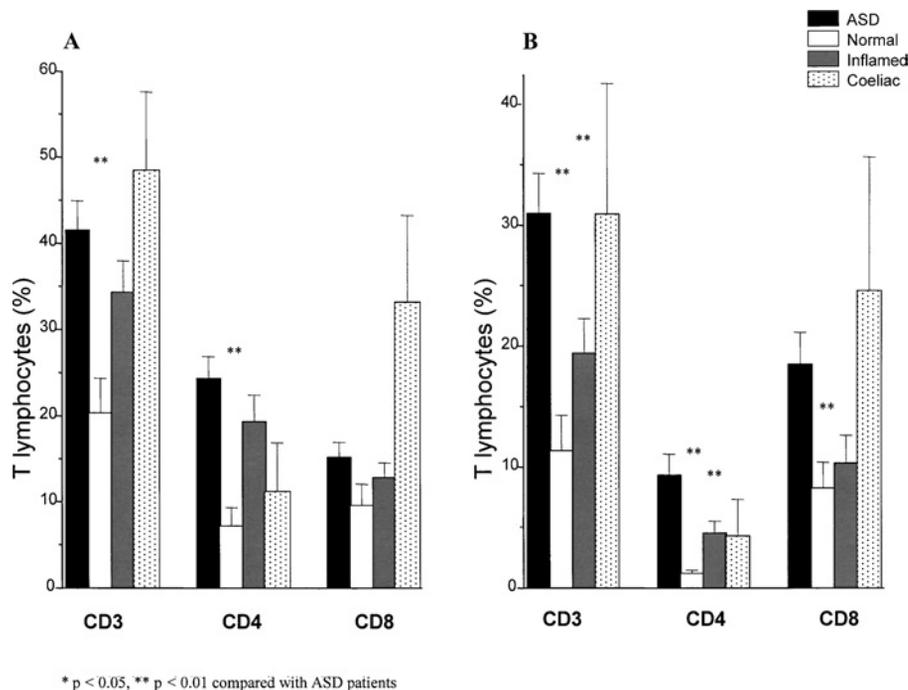
* p < 0.05, ** p < 0.01 compared with ASD patients

Fig. 2. Comparison of T-cell populations within the lamina propria (A) and epithelial (B) compartments of duodenal biopsy specimens obtained from affected children (blue), noninflamed controls (white), inflamed controls (yellow), and celiac disease controls (red). Increased CD3$^+$, CD4$^+$, and CD8$^+$ T-cell subsets were identified for affected children compared with noninflamed controls, $^*p < 0.05$ and $^{**}p < 0.01$.

$p < 0.001$), but not inflamed controls ($8 \pm 2\%$ vs. $3 \pm 1\%$, $p < 0.06$). When histopathology scores were compared with flow cytometric data, there was no correlation between duodenal CD3$^+$, CD4$^+$, and CD8$^+$ lymphocytes in either the lamina propria or epithelium and the degree of acute and chronic inflammation. However, there was a statistically significant positive correlation between the extent of eosinophil infiltration and the extent of chronic inflammation in upper gastrointestinal biopsies ($r = 0.39$; $p < 0.004$).

Ileal Pathology Endoscopic Grading of Ileal LNH and Histopathology. Macroscopic evidence of LNH, as described previously (9, 10), was observed in 93% of affected children. Graded visual assessment showed that 16 (35%) of these children had LNH scores of mild (grade 1), and 27 (59%) had a score of moderate to severe (grades 2–3). Histologically, lymphoid follicles were present in ileal biopsy sections from 42 of 46 cases (91%), of which 30 (71%) showed evidence of reactive follicular hyperplasia. The latter were characterized by expanded, confluent follicles, loss of margination between reactive follicle centers and their mantle zone, and the presence of Tingible body macrophages. Ileal changes in the noninflamed group were minimal by endoscopy, with lymph follicles present in 3 of 12 (25%) children as assessed by histology, of which no

follicle showed reactive changes. The difference between affected children and noninflamed controls for the presence of lymphoid follicles in ileal biopsies was statistically significant (OR = 31.5; 4.8–234.99, $p < 0.00001$). Acute ileitis was observed in 6 of 46 (13%) affected children, consisting of focal peri-follicular cryptitis. Increased ileal IEL were present in 2 (8%) affected children and none of the noninflamed controls ($p > 0.5$). Raised mucosal seosinophils were seen in 13 of 46 (28%) affected children compared with 2 (17%) noninflamed controls. Routine screening revealed no evidence of enteric infection.

In the inflammatory control group ($n = 37$), six (16%) had acute inflammation, eight (22%) had chronic inflammation, and five (14%) had both. This was not significantly different compared with affected children ($p > 0.05$). Mucosal ulceration and granulomas were present in six children with Crohn's disease.

Flow Cytometry

Epithelium. Epithelial CD3$^+$ lymphocytes were statistically significantly increased in the terminal ileum of affected children compared with noninflamed controls ($27 \pm 3\%$ vs. $6 \pm 1\%$, $p < 0.0001$). Statistically significant increases were seen in the affected children, compared with noninflamed controls, for both CD4$^+$ ($9 \pm 1\%$
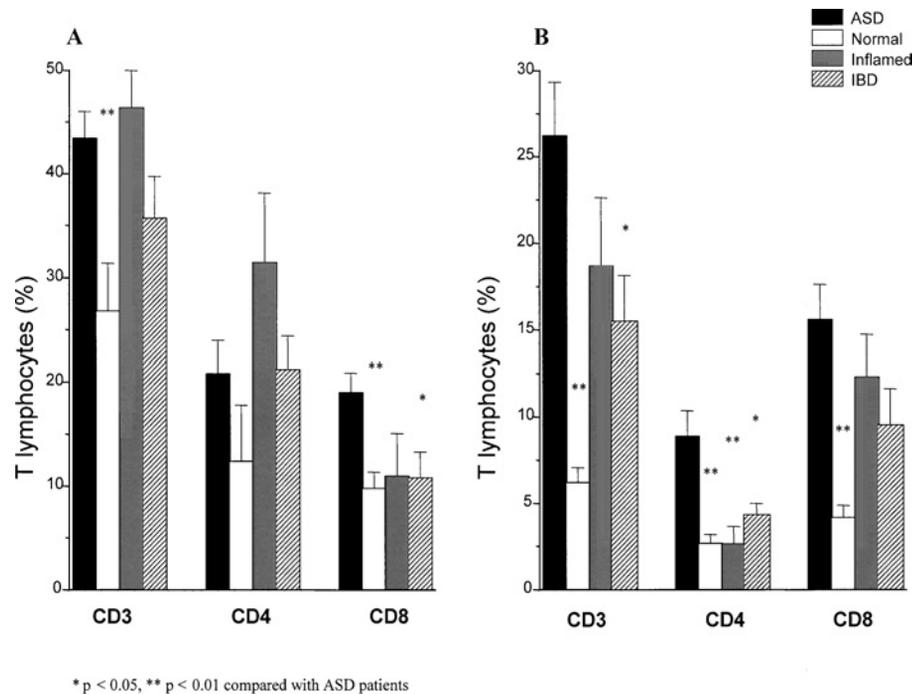
Fig. 3. Detection of lymphocyte populations within lamina propria (A) and epithelial (B) compartments from terminal ileum biopsy specimens obtained from affected children (blue) compared with noninflamed controls (white), inflamed controls (yellow), and IBD patients (red). Increased CD3$^+$ and CD8$^+$ T-cell subsets were identified for affected children compared with noninflamed controls, $^*p < 0.05$ and $^{**}p < 0.01$.

vs. $3 \pm 1\%$, $p < 0.0002$) and CD8$^+$ ($16 \pm 2\%$ vs. $4 \pm 1\%$, p<0.0001) subsets. In addition, CD4$^+$ cells were statistically significantly increased compared with inflamed controls ($9 \pm 1\%$ vs. $3 \pm 1\%$, $p < 0.0009$) but no difference was seen between these groups for either CD3$^+$ or CD8$^+$ populations (inflamed controls = $19 \pm 4\%$ and $12 \pm 2\%$, respectively; $p > 0.05$). However, in affected children CD3$^+$ and CD4$^+$ populations were statistically significantly greater than for the IBD control group ($27 \pm 3\%$ vs. $16 \pm 3\%$, $p < 0.03$, and $9 \pm 1\%$ vs. $4 \pm 1\%$, $p < 0.02$, respectively) (Fig. 3B). In a further subanalysis, CD4$^+$ cells were statistically significantly greater in affected children than in children with food allergy ($9 \pm 1\%$ vs $1 \pm 1\%$, $p < 0.0002$).

Lamina Propria. Lamina propria CD3$^+$ cells were statistically significantly raised in affected children compared with noninflamed controls ($44 \pm 3\%$ vs. $27 \pm 5\%$, $p < 0.005$). CD4$^+$ coexpression in these cells was not different between affected children and noninflamed controls ($21 \pm 3\%$ vs. $12 \pm 5\%$, $p > 0.05$), whereas the CD8$^+$ subset was statistically significantly greater ($19 \pm 2\%$ vs. $10 \pm 1\%$, $p < 0.002$) (Fig. 3A). No differences were seen in CD3$^+$, CD4$^+$, or CD8$^+$ populations when affected children were compared with inflamed con-

trols. However, CD8$^+$ cells are statistically significantly increased in affected children compared with IBD controls ($19 \pm 2\%$ vs. $11 \pm 2\%$, $p < 0.03$) and the subgroup of children with food allergy ($19 \pm 2\%$ vs. $3 \pm 1\%$, $p < 0.0006$). A substantial increase in CD19$^+$ B cells was seen in the lamina propria of affected children ($26 \pm 5\%$) compared with noninflamed ($3 \pm 1\%$, $p < 0.00001$) and inflamed ($10 \pm 2\%$, $p < 0.02$) controls (Fig. 5). Within the affected group, children with grade 3 LNH had significantly more CD19$^+$ cells than those with grade 0 (5% vs. 32%, $p < 0.004$).

In those affected children with acute ileitis, CD3$^+$ and CD19$^+$ lymphocytes were increased (46 and 27%, respectively) compared with those affected children with no histological inflammation (37 and 15%), although this did not achieve statistical significance ($p = 0.08$). IEL CD8$^+$ numbers exhibited a statistically significant positive correlation with histological scores of acute ($r = 0.323$, $p < 0.027$) and chronic ($r = 0.344$, $p < 0.018$) inflammation in the upper gastrointestinal biopsies, and also with overall inflammation assessed by histology ($r = 0.294$, $p < 0.045$).

Colonic Pathology—Histopathology. In the colon, 39 of 45 (87%) affected children showed evidence of acute

and/or chronic inflammation. Acute colitis was observed in 23 (51%) and chronic inflammation in 37 of 45 (82%) affected children, with evidence of both in 49%. Colonic lymph follicles were present in biopsies from all affected children compared with two of nine noninflamed controls. Within the lamina propria, an eosinophil infiltrate was reported in 24 of 45 (53%) affected children and 1 of 9 (11%) noninflamed controls (OR = 8.73; 1–403, $p < 0.03$). In affected children there is a statistically significant positive correlation between histological scores of chronic inflammation and the extent of eosinophil infiltration ($r = 0.37$, $p < 0.006$) and the increase in IEL ($r = 0.3$, $p < 0.04$). Of the 31 children in the inflammatory control group, 19 (61%) had acute inflammation, 28 (90%) had chronic inflammation, and 19 (61%) had both. Compared with affected children, there was no statistically significant difference in the prevalence of acute and chronic inflammation ($p > 0.5$). An excess of either IELs or eosinophils was observed in one (3%) and two (6%) children, respectively. Three (10%) children with Crohn's disease had granulomas and two (6%) had mucosal ulceration.

Flow Cytometry

Epithelium.   CD3$^+$ cells within the epithelium of transverse colon biopsies were greater in affected children compared with noninflamed controls ($23 \pm 3\%$ vs. 5 $\pm 1\%$, $p < 0.003$). Statistically significant increases were seen in the affected children for both CD4$^+$ ($7 \pm 1\%$ vs. $3 \pm 1\%$, $p < 0.0001$) and CD8$^+$ ($16 \pm 2\%$ vs. $4 \pm 1\%$, $p < 0.0001$) subsets. CD3$^+$, CD4$^+$, and CD8$^+$ are also statistically significantly increased in affected children compared with inflamed controls ($13 \pm 3\%$, $p < 0.03$; $2 \pm 1\%$, $p < 0.0002$; and $5 \pm 1\%$, $p < 0.01$, respectively), and for CD4$^+$ in affected children compared with IBD controls ($7 \pm 1\%$ vs. $4 \pm 1\%$, $p < 0.03$). (Fig. 4B). IEL CD4$^+$ and CD8$^+$ populations showed a statistically significant positive correlation with chronic inflammation in upper gastrointestinal biopsies ($r = 0.418$, $p < 0.008$ and $r = 0.312$, $p < 0.037$, respectively).

Lamina Propria.   Lamina propria CD3$^+$ cells were statistically significantly greater in affected children than in with noninflamed controls ($37 \pm 3\%$ vs. $15 \pm 4\%$, $p < 0.001$). Both CD4$^+$ ($19 \pm 2\%$ vs. $6 \pm 3\%$, $p < 0.006$) and CD8$^+$ ($16 \pm 2\%$ vs. $7 \pm 1\%$, $p < 0.001$) subsets were statistically significantly greater in affected children than in noninflamed controls. However, only CD8$^+$ cells were statistically significantly greater in affected children than in inflamed controls ($16 \pm 2\%$ vs. $8 \pm 1\%$; $p < 0.001$) (Fig. 4A). CD19$^+$ B cells were present in the lamina propria in greater numbers in affected children than in noninflamed ($14 \pm 2\%$ vs. $3 \pm 1\%$; $p < 0.001$) and IBD ($6 \pm 1\%$, $p < 0.004$) but not with the inflamed ($7 \pm 2\%$,

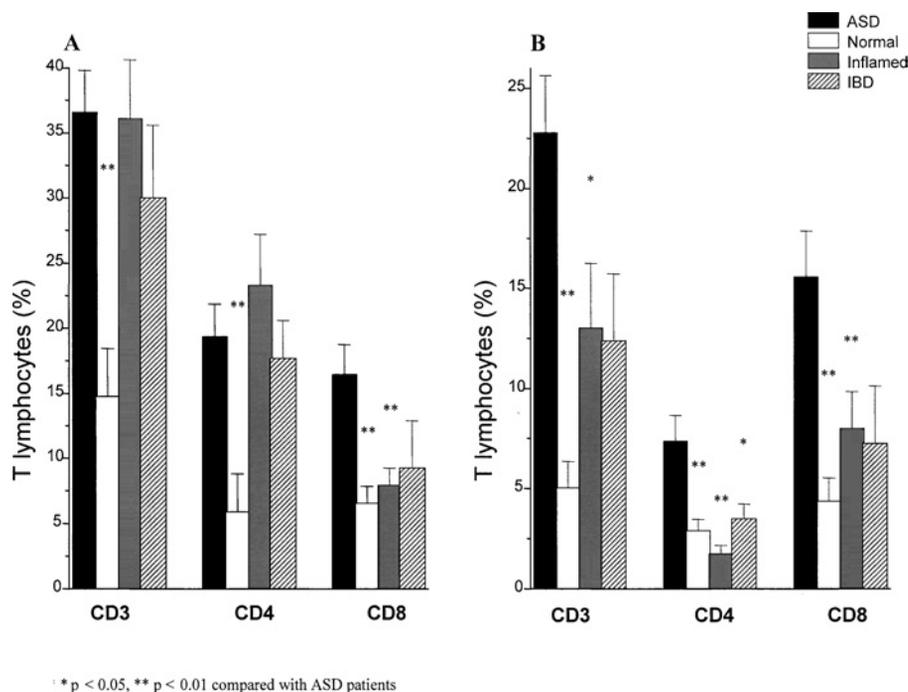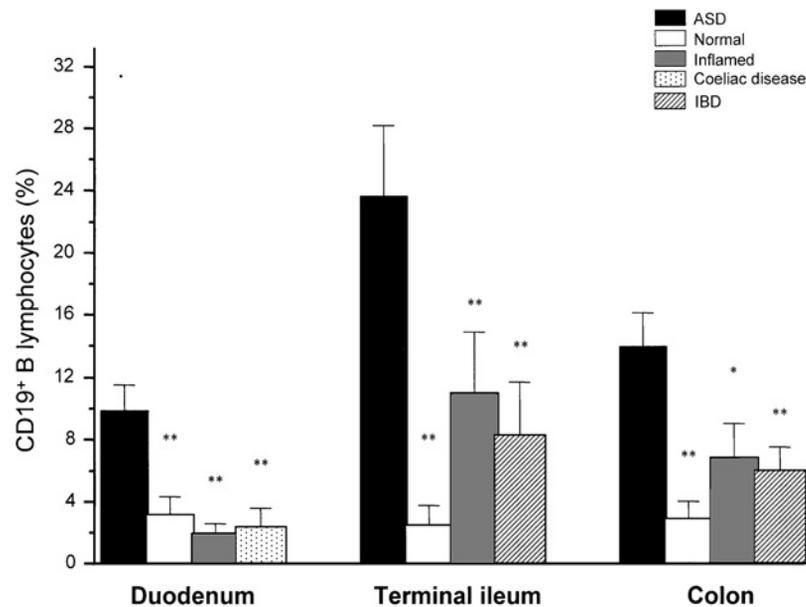*p < 0.05, **p < 0.01 compared with ASD patients

Fig. 4. Increased CD3$^+$, CD4$^+$, and CD8$^+$ T-cell populations within the lamina propria (A) and epithelial (B) compartments of colonic biopsy specimens obtained from affected children (blue) compared with noninflamed (white), inflamed (yellow), and IBD patients (red), *p < 0.05 and **p < 0.01.

* p < 0.05, ** p < 0.01 compared with ASD patients

Fig. 5. Increased CD19$^+$ expressing B-lymphocyte populations in duodenal, terminal ileal, and colonic biopsy specimens from affected children compared with controls, * $p < 0.05$ and ** $p < 0.01$. Comparisons were made between affected children (blue), noninflamed (white), inflamed children (yellow). For duodenal biopsies the red solid bar represents celiac disease controls, whereas for ileal and colonic biopsies the red bars represent IBD controls as described in the text.

$p < 0.09$) control groups (Fig. 5). As in the duodenum, CD14$^+$ monocytes were marginally raised in affected children compared with noninflamed controls (9% vs. 4%, $p = 0.04$). In addition, CD56$^+$CD3$^-$ NK cells were increased in affected children compared with noninflamed controls (5% vs. 2%, $p = 0.02$). In keeping with previous immunohistochemical observations (16) increased $\gamma\delta$ TCR expression was seen in CD3$^+$ cells within the lamina propria of affected children compared with noninflamed controls (10% vs. 3%, $p = 0.03$).

Extent of Pathological Changes and Correlation Between Sites

Overall, inflammation was present in the upper and/or lower GI tract in 48 of the 52 (92%) affected children. Mucosal lymphoid follicles were observed histologically in all affected children, with reactive follicular hyperplasia seen in 75%. There was a strong positive correlation between CD3$^+$ cells at all intestinal sites (duodenum, ileum, and colon) in both the lamina propria ($p < 0.00005$) and IEL ($p < 0.0004$) of affected children, with a consistent pan-enteric increase in T cells. Similarly, the numbers of CD19$^+$ lamina propria B cells correlated between all intestinal sites in af-

fected children ($p < 0.013$). The degree of chronic inflammation in upper gastrointestinal biopsies, assessed histologically, correlated significantly with intraepithelial CD4$^+$ and CD8$^+$ cells in the colon ($r = 0.418$, $p < 0.008$, and $r = 0.312$, $p < 0.037$, respectively) and intraepithelial CD8$^+$ cells in the ileum ($r = 0.344$, $p < 0.018$). In addition, both ileal and colonic intraepithelial CD3$^+$ cells correlated with the extent of acute inflammation in colonic biopsies ($r = 0.33$, $p < 0.03$ and $r = 0.35$, $p < 0.025$, respectively). In the colon, the degree of chronic inflammation and the increase in IEL also exhibited a significant positive correlation ($r = 0.3$, $p < 0.04$).

Including all sites, an eosinophil infiltrate was evident in 35 of 52 (67%) affected children, including 2 of 4 affected children with no histological inflammation, compared with 2 of 25 (8%) noninflamed controls (OR= 23.68; 4.73–220.46, $p < 0.00001$). Chronic inflammation in both upper and lower GI biopsies showed a statistically significant positive correlation with the degree of eosinophil infiltration ($r = 0.34$, $p < 0.01$, and $r = 0.37$, $p < 0.006$, respectively). The degree of mucosal eosinophil infiltration exhibited a positive correlation with absolute numbers of eosinophils in the blood ($r = 0.3$, $p < 0.04$).

Including all sites, increased IEL were seen in 19 of 52 (37%) affected children compared with none of the noninflamed controls ($p < 0.001$). In addition, epithelial damage, as assessed histologically, was seen in 17 (33%) affected children compared with none of the noninflamed controls ($p < 0.004$). Finally, colonic CD3$^+$ cells showed a statistically significant positive correlation with absolute peripheral blood lymphocyte counts ($r = 0.39$, $p < 0.01$) and absolute numbers of neutrophils in the blood ($r = 0.36$, $p < 0.027$).

Influence of Sex

The majority of affected children were boys, whereas the noninflamed controls were relatively evenly distributed between the sexes. To rule out a systematic bias due to a gender-related difference in mucosal lymphocyte populations in normal mucosa, these were compared in the noninflamed controls. There are no statistically significant differences according to sex in any of the mucosal lymphocyte populations in either the epithelial or lamina propria compartments at any site ($p > 0.05$).

Dietary History and Pathological Changes

Some children with autism are on exclusion diets, particularly gluten-free and casein-free (GF/CF) which, from parental reports, may lead to improvements in behavioral and gastrointestinal symptoms in some children (22). Exclusion diets may influence the immune/inflammatory response in the intestinal mucosa. To examine this possibility, the dietary status of the affected children was recorded at the time of endoscopy. Dietary modification was reported in 37 (71%), which, in our experience, is associated with a high level of compliance. Of the 15 affected children on a normal diet, 13 (87%) had an excessive mucosal eosinophil infiltrate and 14 (93%) showed histological inflammation, 10 (67%) in upper GI biopsies, and 13 (87%) in ileocolonic biopsies (9 (60%) in both). Of 19 affected children reported to be on a strict GF/CF diet, 9 (42%) had an eosinophil infiltrate, and inflammation was seen in 17 (89%). For those affected children not on a GF/CF diet there was a statistically significant increased prevalence of a mucosal eosinophil infiltrate (OR= 7.22; 1.07–78.54, $p < 0.05$). Dietary status did not influence the presence or extent of mucosal inflammation ($p > 0.05$). No statistically significant effect of diet was seen on B- and T-cell populations measured by flow cytometry ($p > 0.05$). Radioallergosorbent test (RAST) for dietary allergens was available on 14 affected children. Only one patient showed a positive score (2) for wheat, while no reaction was observed against milk allergen. Antiendomyseal antibody and routine stool and sero-

logical pathogen screenings were negative, irrespective of dietary history.

Peripheral Blood Mononuclear Cell (PBMC) Populations

Data on absolute PBMC values at the time of ileocolonoscopy were available on 43 affected children. Data are shown in Fig. 6. For lymphocytes, 17 (40%) had values below the 5th centile of the age-standardized reference range. Of the remaining affected children, no value exceeded the 50th centile of this reference range. For lymphocyte subsets, a similar pattern was observed: CD3$^+$ cells were below the 5th centile in 13 of 36 (36%) affected children and only 3 were above the 50th centile: for both CD4$^+$ and CD8$^+$ cells, 13 of 36 affected children had counts below the 5th centile and only 2 reached the 50th centile. For CD19$^+$ B lymphocytes, 7 of 36 (19%) affected children had counts below the 5th centile, and only one exceeded the 50th centile (Fig. 6B). Although, the majority of counts for NK cells (26 of 36; 72%) were below the 50th centile, there was a wider distribution compared with other subsets.

A similar pattern was observed for peripheral blood monocyte counts, with only one value higher than the 50th centile and the remainder below the 25th centile. Eosinophil numbers were low with only 3 above the 50th centile (Fig. 6C) and 8 (20%) below the 5th centile. Absolute basophil, red blood cell, and neutrophil counts were evenly distributed within the normal ranges. In contrast, absolute platelet counts were raised; 15 (35%) were above the 95th centile, with only 3 patients with values below the 50th centile.

Serum Immunoglobulins

Serum immunoglobulin data were available on 43 affected children. Twelve (28%) had IgM values below the 5th centile of the paediatric reference values and only six had values above the 50th centile. Fifteen (35%) affected children had IgG values below the 5% centile (Fig. 7). Seventeen (40%) had IgA values below the 5th centile, and no value was greater than the 50% centile (Fig. 7). In addition, serum IgA showed a statistically significant negative correlation with percent of CD4 in the blood ($r = 0.38$, $p < 0.037$). For total IgE, data were available on 23 affected children, 9 (39%) of whom had values higher than the 95th centile. Anti-gliadin antibodies were assessed in 38 affected children. Consistent with the lack of evidence for coeliac disease for any of the ASD children, only three (8%) had raised anti-gliadin IgG; however, 33 (87%) were below the 50th centile. Similarly, for the anti-gliadin IgA subclass, 37 (97%) affected children had values below the 50th centile. Anti-gliadin
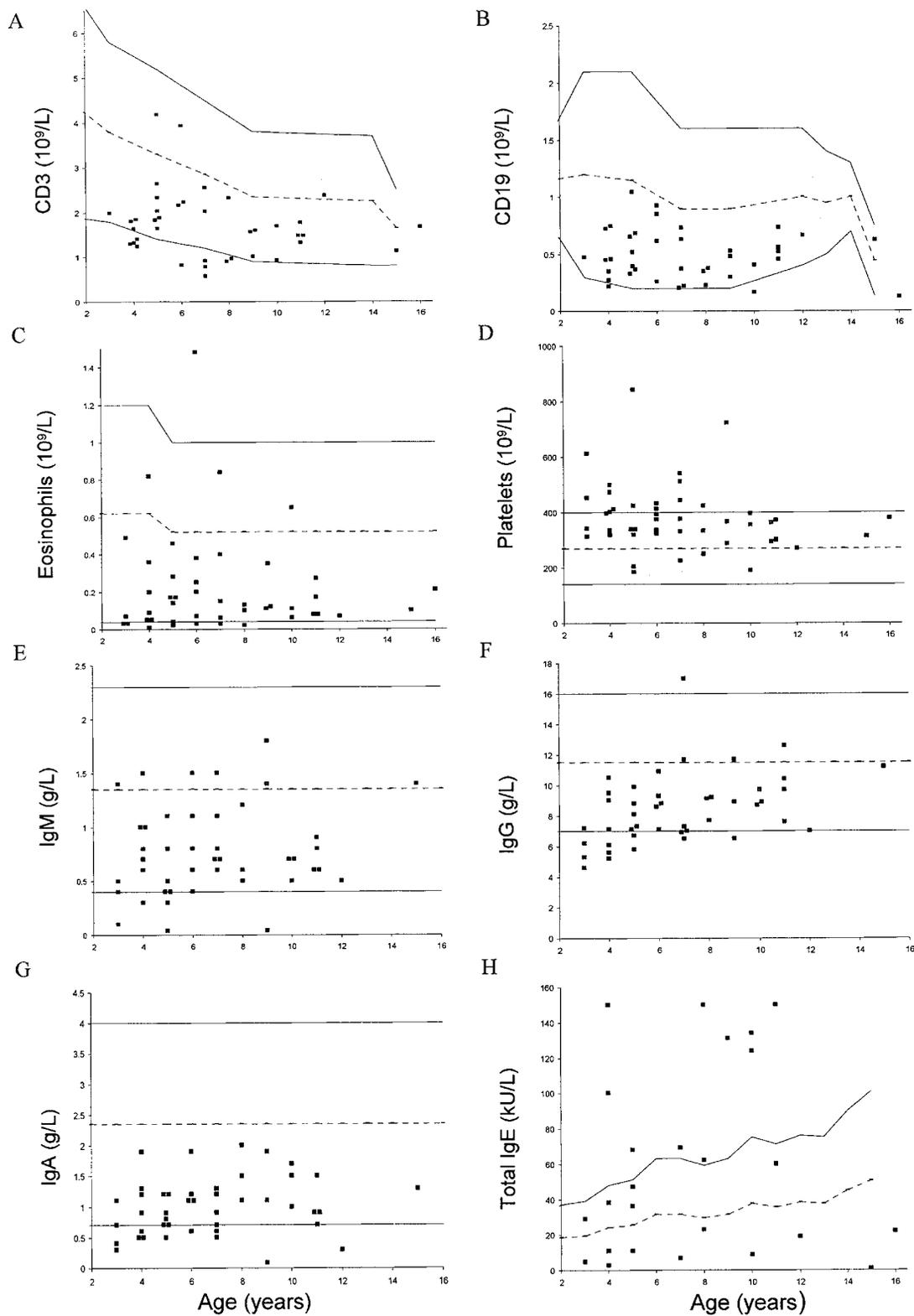
Fig. 6. Absolute peripheral blood cell counts for CD3$^+$ cells (A), CD19$^+$ cells (B), eosinophils (C), and platelets (D) from children with autistic spectrum disorder. Serum concentrations of IgM (E), IgG (F), IgA (G), and IgE (H) in affected children. All data are shown in relation to Royal Free Hospital paediatric reference values (5th, 50th (dotted line), and 95th centiles).

antibody levels were not influenced by dietary modification.

DISCUSSION

This study identifies a novel and consistent pattern of mucosal immunopathology in a cohort of children with the combination of regressive autism and gastrointestinal symptoms. The data support and extend our previous observations (7, 9, 10, 14, 15). This study also adds several important observations. It confirms that, within the same children, there is a mucosal lesion involving both small and large intestine; the lesional infiltrate is characteristic, distinguishing affected children from inflamed and histologically normal controls, and the mucosal changes are likely to be part of a continuous pathogenetic process affecting both small and large intestine. There is a prominent eosinophil component to the mucosal lesion that may vary according to dietary restriction of gluten and/or casein. It is evident that routine haematoxylin and eosin-based histopathology provides only a limited view of the lesion that tends to underestimate the discrepancy between this pathology and normal mucosa.

Clearly it would be of interest to examine children with autism who do not have GI symptoms in a similar manner. However, the ethical constraints of performing invasive investigation on asymptomatic children means that such a comparison is not feasible at this stage. A potential shortcoming of this study is that the expert developmental diagnosis was not reevaluated in our unit. This has been performed in previous studies (7, 10) and we have no reason, on the basis of these prior observations, to doubt the accuracy of the original diagnosis based on *DSM*/ICD-10. All children studied remain under review by local developmental pediatricians, and we are unaware of any case where the diagnosis has been revised or reverted.

Furthermore, there is potential in a study such as this, for differential efficiency of lymphocyte isolation from intestinal biopsies due to variable size of the specimens obtained. However, this variability should not lead to a systematic bias between the various groups and is unlikely to account for the observed differences. In addition, detailed morphometric studies of lymphocyte populations in many of the same children included in this study, and reported previous, show significant elevation in respect to lymphocyte subsets, consistent with the present study (9, 14).

At all sites the mucosal infiltrate in affected children was comparable with that seen in established inflammatory conditions of the intestine. One distinguishing feature of the lesion was the intraepithelial infiltrate which, in addition to an excess of the constitutively dominant $CD3^+CD8^+$ population, showed a significant excess of the $CD3^+CD4^+$ population at all sites, that was greater than in the other inflammatory pathologies. These data, in combination with the observations that serum IgG from affected children colocalized with complement component C1q at the level of the epithelial basement membrane (14)—a finding that is not seen in normal and other inflamed controls—is consistent with the epithelium being an important focus of immunopathology. In addition, $CD19^+$ B cells were substantially increased in the lamina propria at all levels studied compared with both normal and inflamed controls. The prior observation that $\gamma\delta$ T cells were increased in the colonic mucosa was confirmed. The fact that this population is increased in the colon but not in the duodenum in affected children may indicate a local microbe-driven response, or dysbiosis in the colon rather than reflecting the presence of distinct diseases at different sites.

Within this cohort of affected children there was a relative circulating lymphopenia. However, this did not correlate with the extent of the increase in lymphocyte numbers in the gut mucosa. The data indicate that the latter phenomenon is not solely a result of infiltration of extramucosal lymphocyte populations, but most likely a local antigen-driven response, generated from within the gut mucosal lymphoid tissue. This is also consistent with the observation of reactive germinal centers in ileocolonic mucosal biopsies, particularly those within the terminal ileum that was not seen in controls. Unpublished studies of lymphocyte viability, including TUNEL staining, do not indicate an increased propensity for gut lymphocyte apoptosis in affected children compared with histologically normal and inflammatory controls. On the contrary we have detected cellular activation with the elaboration of mitogenic and proinflammatory cytokines within the mucosal immune system of affected children (23). The relative lymphopenia is associated with cutaneous anergy in the majority of these children, at a level that is statistically significantly greater than age-matched healthy controls (15). There appears to be, therefore, a paradoxical immune activation within the mucosal immune system that is associated with minor systemic immunodeficiency. Further studies of T_H1 and T_H2 cytokine production are ongoing.

Exclusion diets, particularly a GF/CF diet, have been reported to benefit children with this ASD phenotype (24, 25). In a single blind study, behavioral and developmental improvements were significantly better for autistic children on a GF/CF restricted diet compared with those on a normal diet (22). It is notable, therefore, that while a GF/CF diet did not appear to influence the inflammatory status of the mucosa it may have influenced the specific contribution of eosinophils to the overall pathological profile. These preliminary data suggest that, while dietary

allergy may not be the primary proinflammatory effector in affected children, it may contribute to the mucosal pathology in some.

Functionally, the mucosal immunopathology is variously associated with dysmotility, including constipation and oesophageal reflux. Similar findings have been made in children with cow's milk allergy (26), although the interrelationship of these features is not known at this time. Increased paracellular intestinal permeability (27), reduced brush border enzyme activity (8), defective sulphation of ingested phenolic amines such as acetominphen (28), and cognitive and behavioral responses to exclusion diets (24, 25) have been reported in affected children, by different groups. In an open-label study, the antibiotic vancomycin—given to reduce putative intestinal bacterial dysbiosis—induced striking cognitive responses in children with regressive autism, maintained only during the period of administration (29, 30). We have proposed that these features suggest an enterocolonic encephalopathy that may be analogous, in certain respects, to hepatic encephalopathy (31).

This study did not set out to examine the aetiology of the enterocolitis in affected children. While Uhlmann *et al.* have reported the presence of measles virus genomic RNA in follicular dendritic cells within ileal lymphoid follicles in affected children (32), these data do not directly support a causal relationship to the mucosal findings. Further study is clearly required, particularly as Singh *et al.* have reported atypical humoral immune response to measles virus in children with a similar autism phenotype, that correlate with abnormal serum antibody titers to myelin basic protein (33). It will be important to determine the specificity of this lymphocytic infiltrate, to determine whether or not these cells may be responding to specific bacterial or viral peptides or whether there is potential for molecular mimicry.

In summary, a pattern is emerging of mucosal immunopathology in a cohort of children with regressive autism. The features are those of panenteric lymphocytic infiltration, with particular evidence of a marked B-cell proliferative response not seen in classical IBD. Although potential links with disturbed cognition in autism have been proposed, further study is required. Study of children without obvious regression or with genetically determined autism may be required to provide a context for these findings.

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