CD45RO Expression on Circulating CD19⁺ B Cells in Crohn's Disease Correlates With Intestinal Permeability

BRUCE R. YACYSHYN* and JON B. MEDDINGS*
*Department of Medicine, Division of Gastroenterology, University of Alberta, Edmonton, Alberta; and *Gastrointestinal Research Group, University of Calgary, Calgary, Alberta, Canada

Background/Aims: Increased intestinal permeability is observed in Crohn’s disease and a subset of first-degree relatives. An alteration in isoform expression of the common leukocyte antigen (CD45) is also found in a significant fraction of patients. Because this alteration may be a measure of antigen exposure, the hypothesis of the study was that this alteration would be observed in both patients and relatives of patients with Crohn’s disease and that this would correlate with increased intestinal permeability. Methods: Lactulose and mannitol permeability were defined in healthy controls, patients with Crohn’s disease, and their first-degree relatives. Simultaneously, peripheral blood was assayed using flow cytometry for CD45RO expression on CD19⁺ B cells. Results: A subset of relatives had significantly increased permeability, as did the majority of patients with Crohn’s disease. A small fraction of peripheral B cells from controls expressed the CD45 isoform (<6%). This fraction was significantly increased for patients with Crohn’s disease and their relatives. Relatives with no clinical evidence of Crohn’s disease were only found to have increased CD45RO expression if they had increased permeability. Conclusions: Individuals at risk for developing Crohn’s disease include a subset with increased intestinal permeability. These people have an associated phenotypic alteration of circulating B cells that is not observed in controls or relatives with normal intestinal permeability.

Crohn’s disease is an immunoregulatory disorder of the intestine often associated with systemic manifestations. Although the etiology of this disease is poorly understood, an increase in intestinal permeability has been suggested as a key initiating event. Increased permeability may be secondary to external factors that include viral infections or luminaly ingested toxins such as nonsteroidal anti-inflammatory drugs or ethanol. However, consideration has recently been given to a primary disturbance of intestinal permeability in the genesis of Crohn’s disease. This suggestion was originally advanced by Hollander et al.¹ and recently confirmed by us.² The evidence for this proposal relies on the observation that a subset of patients at greatest risk for the development of Crohn’s disease, first-degree relatives of afflicted individuals, have increased intestinal permeability in the apparent absence of the disease itself. To date, this work has not been replicated in other laboratories, although similar findings have been recently reported in abstract form.³

In support of the immunologic basis of this disease, a wide variety of abnormalities have been reported, but it is often unclear whether they represent primary events or are secondary to the inflammatory process itself. We have recently identified a population of CD19⁺ B cells expressing a CD45 isoform in the peripheral blood from patients with Crohn’s disease but not from the blood of patients with either ulcerative colitis or celiac sprue.⁴ CD45, the leukocyte common antigen, is the most prevalent antigen on the surface of B and T lymphocytes and plays a key role in intracellular signaling through the tyrosine phosphatase activity of its cytoplasmic domain. The isoforms of this antigen change during T-cell differentiation with the transition from expression of high-molecular-weight CD45RA (p220) isoform on naive cells to the low-molecular-weight CD45RO (p180) isoform on memory T cells.⁵ A similar transition of CD45 isoforms occurs on B cells.⁶-¹⁰ Pre-B cells exclusively express CD45RA at low density with an increase in CD45RA density as differentiation proceeds towards mature B-cell function (reviewed in Pilarski and Jensen¹¹). A transition from CD45RA to CD45RO expression seems to occur on B cells after antigenic stimulation in vivo; this has been confirmed using in vitro techniques.⁹,¹⁰ We have identified the presence of CD45RA on CD19⁺ B cells in peripheral blood of patients with Crohn’s disease.⁴ Furthermore, the percentage of CD19⁺ B cells expressing the CD45RO isoform seemed to correlate with the Crohn’s disease activity index.

Abbreviations used in this paper: FITC, fluorescein isothiocyanate.
© 1995 by the American Gastroenterological Association
0016-5085/95/$3.00
finding this isoform on the B cells of relatives of patients with Crohn's disease without evidence of disease would implicate this immunologic alteration either in disease pathogenesis or as a marker of early disease. Therefore, the purpose of this study was twofold. First, we wished to confirm our earlier findings that a subset of relatives have increased intestinal permeability using patients from a different geographical location. Second, we wished to ascertain whether the CD45RO isoform could also be found on B cells in a subset of relatives. Finally, and perhaps most importantly, if both markers could be identified in a subset of relatives, would they identify the same subset?

Materials and Methods

Study Subjects

Fifteen patients with Crohn's disease, determined by standard clinical criteria and followed up at the University of Alberta Hospital, were asked to participate in this study. Patients had a mean age of 43.9 years (range, 18–68 years), and all had normal renal function as indicated by serum creatinine levels. In addition, 13 of their first-degree relatives agreed to participate in the study. Ten control volunteers of similar age were also recruited. Controls were not matched; however, controls were chosen to represent closely the study population in age, sex, and ethnicity. All relatives and controls were entirely free of gastrointestinal symptoms, had no history of renal disease or diabetes, and had not taken nonsteroidal anti-inflammatory medications or alcohol for at least 2 weeks before the study. The same drug and alcohol exclusion criteria were also applied to the patient population. Protocols were approved by the Ethics Committee of the Faculty of Medicine, University of Alberta.

Intestinal Permeability Study Protocol

After an overnight fast, all participants drank a 250-mL solution containing 5 g lactulose, 2 g mannitol, and 5 g glucose made isosmotic with NaCl. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest grade available. Urine was collected for 5 hours into preweighed containers to which 7.5 mL of 10% thymol had been added as a preservative. During the urine collection, the subjects were only allowed to drink water, black coffee, or tea. Urine was assayed immediately for sugar concentrations by high performance liquid chromatography as described previously. On completion of the urine collection, 10 mL of peripheral blood was immediately obtained from each participant. Peripheral blood mononuclear cells were purified by centrifugation over Ficoll Paque (Pharmacia, Dorval, PQ) followed by two washes.

Antibodies

The CD45 common determinant marker (HLE–fluorescein isothiocyanate [FITC]) and the control antibodies immunoglobulin (Ig) G1–FITC, IgG1–PE, IgG2a–FITC, and IgG2a–phycoerythrin were purchased from Becton Dickinson (Mountain View, CA). β-FITC, β-RD1 (CD19), β–FITC, or β-RD1 (CD20) were purchased from Coulter (Hialeah, FL). Biotinylated goat anti-mouse Ig and Tandem Avidin were purchased from Southern Biotechnology (Birmingham, AL), and UCHL1 (CD45RO) was a generous gift from Dr. P. Beverley. Monoclonal antibodies to CD45RA were CD45RA–FITC and were purchased from Gen Track (Wayne, PA). The specificity of CD45 antibodies used was confirmed by their appropriate molecular weights, and all were tested for their reactivity with a panel of CD45 transfected.

Three-Color Immunofluorescence

Cell surface antigens present on isolated mononuclear cells were evaluated by three-color immunofluorescence using a four-stage combined direct and indirect staining procedure. In the four stages, the cells were (1) stained with an uncoupled antibody; (2) stained with goat anti-mouse biotin (Jackson, Mississauga, Ontario, Canada); (3) blocked with mouse Ig (Jackson; 1 µg/mL); and (4) stained with Streptavidin–Tandem, together with the two remaining antibodies directly conjugated to either FITC or phycoerythrin. One million mononuclear cells were resuspended in 50 µL of uncoupled monoclonal antibody that was diluted in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.02% sodium azide. The cells were incubated for 30 minutes at 4°C, spun down, washed twice in buffer solution, incubated for 10 minutes at room temperature, spun down, and resuspended in 20 µL of Streptavidin–Tandem. The other two monoclonal antibodies coupled to FITC and phycoerythrin were added directly, and 25 µL of buffer was added. This was incubated for 30 minutes at 4°C. Cells were washed three times and fixed with 1% formalin for flow cytometric analysis. Analysis of samples was performed on a FACScan (Becton Dickinson). Dead cells and red cells were excluded by gating on forward-angle light scatter and side scatter. All samples included staining with isotype-matched control antibodies and unstained cells. List mode files were collected of 20,000 cells from each sample, and measurements of all three fluorochromes as well as forward and side scatter were recorded. Flow cytometry histograms of this technique are available elsewhere.

Analysis of Lactulose and Mannitol

Urine samples (10 mL) were deionized by adding 1 g of a 1:1.5 (wt/wt) mixture of Amberlite IR–120 and IRA–400 resin (BDH Chemicals, Toronto, Ontario, Canada). Sucrose was added as an internal standard, and the supernatant was filtered through a 40-µm Millipore filter (Millipore, Bedford, MA). Samples were separated on a Hamilton RCX–10 anion exchange column (The Hamilton Co., Reno, NV) in an HP 1090 high-performance liquid chromatograph (Hewlett Packard, Toronto, Ontario, Canada) at room temperature using 30 mmol/L NaOH as the isocratic mobile phase. Peak identification was accomplished with the use of authentic standards and
Table 1. Patient Description

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease location</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>7 (47)</td>
</tr>
<tr>
<td>Large intestine</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Both</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Previous therapy</td>
<td></td>
</tr>
<tr>
<td>Mesalamine products</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Steroids</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Both</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Neither</td>
<td>2 (13)</td>
</tr>
</tbody>
</table>

NOTE. Disease location is shown as well as prior therapy of disease in the patient group.

detected using pulsed amperometric electrochemical detection on a gold electrode. Samples were diluted as necessary after addition of the internal standard. Quantitation was performed using known standards at multiple concentrations with linear interpolation between concentrations. All samples were diluted so that concentrations of interest fell within the range of standards. Data were expressed as the fractional excretion of lactulose or mannitol, and the lactulose/mannitol ratio was calculated directly from these numbers.

Statistics

The data acquired (percentage of CD45RO CD19+ B cell expression and intestinal permeability lactulose/mannitol ratio) were compared using the Statistical Analysis System (SAS Inc., Cary, NC) and analysis of variance.

Results

Study Group

Table 1 shows the location of Crohn’s disease found in our patients by radiological or endoscopic methods. A variety of previous treatments had been used, and these are also outlined in Table 1.

Intestinal Permeability

Figure 1 shows the lactulose/mannitol ratio found in all study groups. A group of historical controls (n = 40) is included, and these represent an ongoing control group studied in our laboratory as internal controls. Because numerous permeability measurements are performed in this laboratory, these serve as ongoing quality controls. None of the patients were taking nonsteroidal anti-inflammatory drugs or ethanol for 2 weeks before testing. The upper limit of normal (defined as the mean of this group plus 2 SD) is shown by the dotted line in Figure 1. Controls used in this study (current controls) had lactulose/mannitol ratios uniformly within this normal range. In contrast, a significant proportion of patients with Crohn’s disease had increased intestinal permeability, some with very high values. Ten of the 15 patients (67%) had high permeability, whereas 5 patients’ permeability was within our normal range. Seven of 13 relatives (54%) also showed high permeability in the absence of either symptoms or signs of disease. These data confirm our earlier findings that a subset of relatives have increased permeability, albeit a higher proportion than showed previously.

Immunologic Alterations

In Figure 2, the percentage of peripheral CD19+ B cells bearing the CD45RO isoform is shown for both controls and patients with Crohn’s disease. In all 10 controls, <6% of peripheral B cells were found to be positive for this isoform. Therefore, this value was used as the upper limit of normal and is represented by the dotted line in Figure 2. As we have previously reported, a significant number of patients (67%) had increased expression of this marker on peripheral B cells. It was also apparent that this correlated with the intestinal permeability of these patients. In Figure 2, patients are shown as either having normal permeability or high permeability based on the data from Figure 1. Of the 10 patients with high permeability, 8 showed an abnormally high fraction of B cells positive for CD45RO. This situation was found in only 2 of the 5 patients with normal permeability.
Figure 2. Fraction of CD45RO⁺ B cells in patients with Crohn's disease. The fraction of peripheral B cells (CD19⁺) that had detectable CD45RO isoform is shown both for controls and patients with Crohn's disease. In all control patients, this value was <6% and was used as the upper limit of normal (dotted line). Patients with Crohn's disease were subdivided into those with normal and high intestinal permeability as defined in Figure 1. Patients with high permeability had a significantly increased fraction of B cells positive for this isoform as compared with either the control group or patients with normal permeability. A close statistical association (analysis of variance) was found between intestinal permeability as assessed by lactulose/mannitol fractional excretion and CD45RO expression on CD19⁺ B cells ($t$ test = 4.5787; degrees of freedom ($n - 2$) = 22; $P = 0.0003$).

Because this observation could be related to disease activity in these patients rather than the permeability defect per se, we were interested in examining this relationship in the relative population. These data are shown in Figure 3. The same control data are shown, but now the fraction of CD45RO⁺ peripheral B cells is shown for the relatives. Once again, we have subdivided the relatives into those with normal and high intestinal permeability based on the data from Figure 1. All relatives with normal permeability had a normal fraction of CD45RO⁺ B cells (<6%), whereas all relatives with increased permeability showed an increased fraction of CD45RO⁺ B cells. Clearly, in the absence of symptoms or signs of Crohn's disease, this finding cannot be related to the presence of clinically detectable inflammation.

**Discussion**

A large body of evidence exists suggesting that intestinal inflammation secondary to Crohn's disease is associated with increased intestinal permeability. Recently, we revisited this controversy and proposed a hypothesis to reconcile these disparate findings. If all relatives were ultimately to develop Crohn's disease, it would not be unreasonable to expect all to have increased permeability at some point before the onset of disease. However, because only a fraction of the relative population ever develops Crohn's disease, it seemed inappropriate to discard the permeability hypothesis merely because the entire relative group could not be shown to have increased intestinal permeability. Instead, we suggested that if this hypothesis was correct, we should be able to show a subgroup of relatives with increased intestinal permeability. The size of this subgroup would dictate whether the entire group could be shown to have a statistical increase in permeability. This was exactly what we found in a previous study, and we have reported that approximately 10% of first-degree relatives show increased permeability in the apparent absence of disease.

In this study, we report findings that confirm this earlier report using patients and relatives from a different geographical location. Of 13 relatives, 7 had increased intestinal permeability. Although the fraction of relatives with increased permeability is greater than the fraction previously reported, this is a small study in terms of patient number. The observation that a fraction of the relative population had increased intestinal permeability remains valid. Furthermore, another group has found similar results, although these have only been presented

Figure 3. Fraction of CD45RO⁺ B cells in relatives of patients with Crohn's disease. The format of this figure is identical to that of Figure 2. Relatives having normal intestinal permeability were indistinguishable from controls. However, the entire subgroup of relatives with increased intestinal permeability had increased expression of this CD45 isoform on peripheral B cells.
in abstract form to date. Thus, the hypothesis that increased permeability exists before the onset of Crohn's disease seems to be gaining support.

If increased paracellular permeability is important in the genesis of Crohn's disease, the underlying mechanism is presumed to be abnormal antigen presentation to the mucosal immune system and/or the systemic immune system. With increased antigen presentation, there is a shift in isoform expression of the leukocyte common antigen CD45 from the RA form to the RO form. This has been shown to occur on B cells using both in vitro and in vivo experiments. We have recently reported that this seems to be detectable in patients with Crohn's disease. Patients with this disease express a greater proportion of the RO isoform on circulating B cells, and this correlates with disease activity. However, because increased intestinal permeability is a hallmark of active Crohn's disease, it is unclear whether this association is with active inflammation or increased intestinal permeability.

In this study, we had a group of patients in which both the presence of disease and intestinal permeability status had been determined. Furthermore, we had a group of relatives of known permeability status in the absence of clinically detectable disease. This enabled us to address this question. A significant fraction of patients had an increased proportion of circulating B cells positive for the CD45RO isoform (10/15), confirming our earlier findings. Of these patients, 80% were found to have increased intestinal permeability; in only 20% of the patients was the CD45RO isoform found in patients having normal intestinal permeability. Furthermore, in the relative population without clinically detectable disease, we also found 5 of 13 individuals with this CD45 isoform. Of great interest to us was the observation that all of these individuals had increased intestinal permeability. Thus, this isoform is found in the identical subgroup of relatives that showed increased intestinal permeability. It would seem that the presence of this CD45 isoform is determined by the presence of increased intestinal permeability rather than the presence of intestinal inflammation itself. The 2 patients with normal permeability and increased CD45RO expression would argue against this interpretation; however, intestinal permeability was only determined on a single occasion, and we have no information regarding the intestinal permeability status of these patients before this determination. It is entirely conceivable, and in our view quite likely, that the time course of alterations in permeability and CD45 isoform expression are radically different. To answer this question, a much larger study with a very different focus would need to be performed.

We conclude that individuals at greatest risk for the development of Crohn's disease (relatives of existing patients) include a subset with increased intestinal permeability. In this group, there is an associated immunologic alteration of circulating B cells that is not observed in either control patients or relatives with normal intestinal permeability. Therefore, not only is increased permeability seen in a subgroup of relatives, but it seems to have an immunologic correlate. These data provide further support to the hypothesis that increased intestinal permeability is an early initiating event in the genesis of Crohn's disease.

References

14. Murphy MS, Eastham EJ, Nelson R, Pearson AD, Laker MF. Intes-


Received June 16, 1994. Accepted September 23, 1994.
Address requests for reprints to: Bruce Yacyshyn, M.D., 2E3.11 Walter MacKenzie Health Sciences Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2R7. Fax: (403) 492-3340.
Supported by the Medical Research Council of Canada, the Crohn’s and Colitis Foundation of Canada, and the Grawin Foundation.