ABSTRACT

AIM: It has been suggested that appendicitis protects against ulcerative colitis. We hypothesize that early poor hygiene protects against ulcerative colitis (UC) and predisposes to appendicitis. Our aim was to elucidate the immunological characteristics of rectal mucosa in two populations protected against UC development: appendectomised subjects and inhabitants of developing countries.

METHODS: this was an age-matched prospective case-control study. Each consecutive individual case appendectomised (group A) was compared to another control from a developing country (group B) and to a control from the general population (group C). Four biopsies from rectal mucosa were taken from all subjects, two for histological and two for histochemical study; specific antibodies were used for T lymphocytes CD3+, CD4+, CD8+ and B lymphocytes CD20+ populations.

RESULTS: Mucosa samples of 45 non-smoker healthy subjects were studied, of which 15 were from group A, 15 from group B and 15 from group C. In appendectomised subjects, the proportion of CD8+ cells was higher than in the control group (p < 0.001), but similar to that in B group. The proportion of CD3+ and CD20+ cells was significatively lower than in Ecuadorians, but similar to the control group. In Ecuadorians, the proportion of CD3+ and CD8+ cells was significatively higher than in the control group (p < 0.001), and were similar to that of CD20+. There were no significative differences in the proportion of CD4+.

CONCLUSION: Appendectomy and deficient environmental hygiene are associated with an increase of CD8+ T lymphocytes in the rectal mucosa. Moreover, deficient environmental hygiene is associated with an increase of CD3+ and CD8+ lymphocytes. The CD8+ increase is the only common significant alteration in the mucosa of both groups protected against the development of ulcerative colitis, suggesting that the factors causing changes in lamina propria lymphocytes of both groups are different.

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Immunological changes at rectal mucosa in appendectomised subjects and inhabitants of developing countries

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INMUNOLOGÍA DE LA MUCOSA RECTAL: ALTERACIONES EN INDIVIDUOS APENDECTOMIZADOS Y EN LOS PROCEDENTES DE PAÍSES CON HIGIENE DEFICIENTE

OBJETIVO: Se ha sugerido que la apendicitis protege contra la colitis ulcerosa (CU). Nuestra hipótesis de trabajo es que una higiene deficiente protege contra la CU y predispone a la apendicitis. Nos proponemos estudiar las características inmunológicas de la mucosa rectal en dos poblaciones con baja prevalencia de CU: los individuos apendectomizados y los habitantes del tercer mundo.

MÉTODOS: Se trata de un estudio de casos y controles prospectivo en el que se enfrentan tres grupos de estudio de igual edad y sexo. Cada individuo apendectomizado (grupo A) se comparó con un ecuatoriano (grupo B) y otro sano representante de la población general del Área Sanitaria 2 de Madrid (Grupo C). Se tomaron cuatro biopsias rectales de cada uno de los sujetos. Dos se procesaron para estudio histológico convencional y dos para estudio histoquímico, en el que se usaron anticuerpos específicos para linfocitos T CD3+, CD4+, CD8+ y CD20+ linfocitos B.

RESULTADOS: En total se estudiaron 45 mucosas rectales de otros tantos sujetos no fumadores, 15 de cada grupo. En el grupo A la proporción de CD8+ fue mayor que en el grupo C (p < 0,001), pero similar a la de B grupo. En Ecuadorianos, la proporción de CD3+ y CD8+ fue significativamente menor en el grupo A que en ecuatorianos, pero similar al grupo C. En ecuatorianos se encontró la proporción de CD3+ y CD8+ fue mayor que en el grupo C (p < 0,001), pero similar al grupo B. La proporción de CD3+ y CD20+ fue significativamente menor en el grupo A que en ecuatorianos, pero similar al grupo C. En ecuatorianos se encontró la proporción de CD3+ y CD8+ fue mayor que en el grupo C (p < 0,001), pero similar al grupo B. La proporción de CD3+ y CD20+ fue significativamente menor en el grupo A que en ecuatorianos, pero similar al grupo C. En ecuatorianos se encontró la proporción de CD3+ y CD8+ fue mayor que en el grupo C (p < 0,001), pero similar al grupo B. La proporción de CD3+ y CD20+ fue significativamente menor en el grupo A.
INTRODUCTION

Several investigators have found a significant negative association between appendectomy and ulcerative colitis (UC)\(^1\) although, Anderson et al\(^6\), found that only appendectomy for a real inflammatory condition (appendicitis or lymphadenitis) was associated with a low risk of subsequent UC, specifically in patients who undergo surgery before the age of 20 years.

Despite the fact that short time after the appendectomy, changes have been demonstrated in the immunity of the mucosa of the colon\(^6\), it remains unclear why these changes have been demonstrated in the immunity of UC patients who undergo surgery before the age of 20 years.

The «Hygiene Hypothesis» proposes that early and frequent exposure to bacterial and other antigens, common in developing nations, leads to normal Th1 response and protects from UC. Additionally, it suggests that decrease ratios in the incidence of appendectomy decrease in industrialized countries, whereas they continue to rise in developing countries. Environmental factors might explain the divergent incidences of appendectomy and UC rates in recent years. The «Hygiene Hypothesis» proposes that early and frequent exposure to bacterial and other antigens, common in developing nations, leads to normal Th1 response and protects from UC. Additionally, it suggests that decrease ratios in the incidence of appendectomy observed in industrialized nations were caused by hygiene improvements\(^10\)–\(^12\).

Two competing hypotheses were examined: (A) the inverse association between appendectomy and UC is conferred by a mutual determinant such as poor hygiene conditions during childhood, and (B) the appendix itself has biologic effects that promote the development of UC.

We hypothesize that early poor hygiene protects against UC and predisposes to appendicitis. Lymphoid tissue of rectal mucosa, always involved in UC patients, should have changes and these should persist during the whole life.

Therefore, our aim was to determine whether same immunological changes exist in lamina propria lymphocytes (LPLs) of rectal mucosa from appendectomised subjects, and inhabitants from developing countries.

METHODS

Study design

This was an age- and sex-matched prospective case-control study. Each consecutive appendectomised case was compared to another control from a developing country (Ecuador) and to a control from the general population.

Assessment of exposure

A history of appendectomy before the age of twenty years, born and living in Ecuador and less than two years of residence in Spain were assessed retrospectively during a face to face interview. All the subject had agreed to participate in the study. The presence of digestive disease was excluded by means of a total colonoscopy and a comprehensive medical interview, always performed by the same investigator (J.M.J.). All patients presenting diarrhea had a stool culture performed.

Sources of cases

This study included 45 non-smoker cases and controls selected from subjects in whom total colonoscopy was normal and performed for the study of blood in stools suggestive of haemorrhoidal origin and/or altered bowel habit suggestive of irritable bowel syndrome. None of them was receiving medication when the biopsies were taken. All patients underwent the same colon cleaning by means of 15 mg polietilenglicol (Solo- tion Evacuatno Bolus). The samples were obtained over proctoscope by a forceps rigid (Richard Wolf) of 6mm aperture.

The Ethics Committee of La Princesa Hospital approved the protocol and each participating subject signed an informed consent form.

Patients and controls

- Group A. Fifteen healthy Spanish patients inhabitants in Madrid (health area 2) had appendectomy before the age of 20 years and before 1984.
- Group B. Fifteen healthy patients with hygiene deficient environment during childhood and adolescence in their country (Ecuador) and had obtained residence permit in Madrid (health area 2) after 2002.
- Group C. Fifteen healthy Spanish patients inhabitants in Madrid (health area 2) had not undergone appendectomy. All cases and controls were matched for age (± 5 years).

Histology and immunohistochemistry

Four biopsies from rectal mucosa were taken from all subjects. Two biopsies were fixed in 10% formalin overnight, embedded in paraffin and processed for histological study by a pathologist (C.G.). The other two biopsies were obtained for the histochemical study. CD3, CD4, CD8 or CD20 immunostaining were performed by a peroxidase-based immunohistochemical approach (Envision + HPR method). Briefly, 3-µm thick paraffin embedded tissue sections were deparaffined in xylene, rehydrated through graded alcohols and washed in PBS. Prior endogenous peroxidase blocking, the antigen was retrieved by heating the slides in citrate buffer 1 mM pH 8 for two minutes in a pressure cooker. Monoclonal antibodies (clon SP7 for CD3, clon 4B12 for CD4, clon 4B11 for CD8 and clon L26 for CD20) (Master Diagnostica S.L. Granada, Spain) were used as prediluted primary antibody and Envision\(^TM\) + Peroxidase Anti-mouse Polymer labelled with horseradish peroxidase (DAKO Co Carpinteria, CA 93013, USA), as the secondary one. The reaction was developed with diaminobenzidine (DAB) substrate-chromogen solution (DAKO Co). Finally, the sections were counterstained with Harris haematoxylin and mounted with Eukitt medium.

The number of lymphocytes was then quantified with a CAS 200 image analysis microscope (Becton-Dickinson, NJ, USA). 10 fields (40x) were counted and the mean of lymphocytes of mucosa (intramucosal and lamina propria lymphocytes) stained area per area unit was calculated. CD3+ and CD4+ counts were semiquantitative. Samples both from controls and from UC patients were counted by a blind observer.

Statistical analysis

The number of cells and standard deviation were calculated and compared by using Mann-Whitney U test. A p value < 0.05 was considered statistically significant.

RESULTS

Mucosa samples of 45 healthy subjects were prospectively studied, of which 15 had undergone appendectomy at a mean of 22 years prior to the time of their visit (1.1 male/female,
59 (± 12) years, group A), 15 from hygiene deficient environmental countries (0.7 male/female, 52 (± 13) years, group B) and 15 healthy controls from Madrid urban area (0.6 male/female, 57 (± 13) years, group C). The presence of haemorrhoids was considered to be the origin of blood in the stools in 30 patients, while in the remaining 15 patients no endoscopic or histological alteration was found and the diagnosis of irritable bowel syndrome was established.

One biopsy taken from the group B presented alterations in the architecture of the glands, thus being rejected from the study of LPL pattern. Table I shows CD3+, CD4+, CD8+, and CD20+ populations in rectal mucosa. In the appendectomised (group A) the number of cells/field of CD8+ cells was higher than in the control group (P < .001), but similar to that in B group. In Ecuadorians (group B), the proportion of CD3+ and CD8+ cells, was significantly higher the control group (P < .001). In addition, the proportion of CD20+ was significantly higher in the Ecuadorians than in appendectomised subjects. The number of cells/field of CD20+ cells was higher in group B than in those appendectomised (group A) (P < .05), and also than in higher than in group C but without reaching statistical significance. Finally, there were no statistically significant differences in the proportion of CD4+.

**DISCUSSION**

This is a pilot study, and the recruitment was stopped on finding significant differences, thus the number of cases and controls is not very large. We think that our measurements in immunohistochemically stained biopsy samples reflect the real situation of lamina propria lymphocytes from of rectal mucosa. The method of colon cleaning was identical for all three groups; therefore we are under the impression that this does not influence the results.

We found that appendectomy and deficient environmental hygiene are associated with an increase of CD8+ T lymphocytes in the rectal mucosa. Moreover, deficient environmental hygiene is associated with an increase of CD3+ and CD8+, lymphocytes. In the revised literature no studies of LPLs have been described in immunohistochemically stained biopsy samples. CD4+ lymphocytes in the rectal mucosa. Moreover, deficient environmental hygiene is associated with an increase of CD3+ and CD8+, lymphocytes. In the revised literature no studies of LPLs have been described in immunohistochemically stained biopsy samples. CD4+ lymphocytes from of rectal mucosa in long term appendectomised subjects or inhabitants of countries with deficient environmental hygiene is associated with an increase of CD8+ T lymphocytes. Moreover, deficient environmental hygiene is associated with an increase of CD3+ and CD8+, lymphocytes. In the revised literature no studies of LPLs have been described in immunohistochemically stained biopsy samples.

Our results suggest that the increase of CD8+ may play an important role as a protective factor during the whole life against UC in appendectomised subjects. The CD8+ may evolve to Th1 cytotoxic or to Th3. Some data suggest that a serious intestinal infection, as appendicitis, can induce the evolution from CD8 towards Th3, stimulating the immunological tolerance. In addition, CD8+ and CD3+, were significantly higher from the Ecuadorians group, possibly associated with the constant antigenic bacteria and helminthic exposure, which may play a protective role against gut immune disregulation and protect against UC. CD4+ T cells are increased in lymphoid tissue of rectal mucosa from UC patients, and these cells cluster in subepithelial dendritic cell-aggregates before colitis develops, suggesting that priming of CD4+ T cells, possibly by dendritic cells, is a primary event in inflammatory bowel disease. Nevertheless no differences were found between all three groups. The increase of CD8+ may play an important role in the inverse relationship between appendectomy and UC. But, CD3+ and CD20 additional changes in LPLs from Ecuadorians are more complex, and probably not just one factor is responsible. It is possible that the factors inducing the changes in LPLs of both groups have a different cause.

We can refuse the hypotheses that the inverse association between appendectomy and ulcerative colitis is conferred by a mutual determinant such as poor hygiene conditions during childhood. On the other hand, childhood hygiene has been found to be associated with Cohn’s disease but not with UC, and does not seem to play a role for the association between appendectomy and UC. Our findings support the hypotheses that the appendix itself has biologic effects that promote the development of UC. These findings show that there is not only one factor that explains the low prevalence of UC among the inhabitants of developing countries and appendectomised subjects, and does not support the hypothesis that improvements in childhood hygiene might explain both the divergent incidences of appendicitis and UC.

In summary, this study has shown that two populations with low prevalence of UC present persistent alterations in the populations of LPLs of the rectal mucosa. Further studies are needed to determine the functional implications of these morphological changes, and to evaluate if they can stimulate the immunological tolerance.

**TABLE I. Proportion of lymphocytes in lamina propria from rectal mucosa in appendectomised (group A, 15 subjects), subjects from developing countries (group B, 14 subjects), and general population (group C, 15 subjects)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>A vs. C</th>
<th>B vs. C</th>
<th>A vs. B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ (mean ± SD)</td>
<td>13.3 ± 1</td>
<td>18.4 ± 7</td>
<td>30.3 ± 1</td>
<td>0.03 ± 1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+ (mean ± SD)</td>
<td>28.3 ± 15</td>
<td>16.4 ± 7</td>
<td>13.3 ± 10</td>
<td>&lt; .0001</td>
<td>&lt; .001</td>
<td>NS</td>
</tr>
<tr>
<td>CD3+ (mean ± SD)</td>
<td>1.0 ± 1</td>
<td>2.0 ± 1</td>
<td>1.0 ± 1</td>
<td>NS</td>
<td>NS</td>
<td>0.016</td>
</tr>
<tr>
<td>CD20+ (mean ± SD)</td>
<td>1.4 ± 1</td>
<td>1.4 ± 1</td>
<td>1.4 ± 1</td>
<td>NS</td>
<td>NS</td>
<td>0.016</td>
</tr>
</tbody>
</table>

NS: statistically non-significant differences; SD: standard deviation.
REFERENCES


