

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

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## 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 significantly lower than in Ecuadorians, but similar to the control group. In Ecuadorians, the proportion of CD3+ and CD8+ cells was significantly higher than in the control group ( $p < 0.001$ ), and were similar to that of CD20+. There were no significant 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.

## 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 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 la proporción de CD3+ y CD8+ fue mayor que en el grupo C ( $p < 0,001$ ), pero similares las proporciones de CD20+. No se encontraron diferencias en la proporción de CD4+.

**CONCLUSIONES:** Apendectomía y un medio ambiente con higiene deficiente se asocian con un incremento de linfocitos CD8+. En los individuos con higiene deficiente se asocia, además, un incremento de CD3+. El incremento de CD8+ es

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**la única alteración que comparten las mucosas de los dos grupos que están protegidos contra la CU. Creemos que los factores que causan estos cambios en las poblaciones de linfocitos son diferentes en cada grupo.**

## INTRODUCTION

Several investigators have found a significant negative association between appendectomy and ulcerative colitis (UC)<sup>1-5</sup> although, Anderson et al<sup>6</sup>, 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<sup>7,8</sup> it remains unclear why these changes in the mucosa persist so long, and whether the protective factor against the subsequent development of UC could be attributed to the inflammatory condition responsible for the appendectomy and its etiopathogenic factors rather than the extirpation of a small intestinal fragment.

It is well known that UC is more prevalent in industrialized countries and is almost absent in developing countries<sup>9</sup>. On the contrary, the rates 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 also suggests that decrease ratios in the incidence of appendicitis observed in industrialized nations were caused by hygiene improvements<sup>10-12</sup>.

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 after 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 diarrhoea 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 mean of 15 mg polietilenglicol (Solution Evacuante Bohm). The samples were obtained over proctoscope by a forceps rigid (Richart Wolf) of 8/mm 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 undergone appendectomy before the age of 20 years and before 1984.
- Group B. Fifteen healthy patients with hygiene deficient environmental 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 ( $\pm 5$  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

M 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<sup>TM</sup> + 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 (40 $\times$ ) were counted and the mean of lymphocytes of mucosa (intraepithelial and lamina propria lymphocytes) stained area per area unit was calculated. CD20+ 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 a mean of 22 years prior to the time of their visit (1.1 male/female,

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)

	Groups					
	A	B	C	A vs. C	B vs. C	A vs. B
CD4+ (mean $\pm$ SD)	1.13 $\pm$ 1	1.43 $\pm$ 1	0.93 $\pm$ 1	NS	NS	NS
CD8+ (mean $\pm$ SD)	18.7 $\pm$ 12	16.4 $\pm$ 7	6.31 $\pm$ 3	< .0001	< .0001	NS
CD3+ (mean $\pm$ SD)	12.7 $\pm$ 9	28.3 $\pm$ 15	13.3 $\pm$ 10	NS	< .001	< .001
CD20+ (mean $\pm$ SD)	1.0 $\pm$ 1	2.08 $\pm$ 1	1.42 $\pm$ 2	NS	NS	0.016

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

59 ( $\pm$  12) years, group A), 15 from hygiene deficient environmental countries (0.7 male/female, 52 ( $\pm$  13) years, group B) and 15 healthy controls from Madrid urban area (0.6 male/female, 57 ( $\pm$  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 glandules, 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<sup>13</sup>. 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 found from normal rectal mucosa in long term appendectomised subjects or inhabitants of countries with deficient hygiene, reason for which we cannot contrast our data.

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+ can evolve to Th1 cytotoxics 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<sup>14</sup>.

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 dysregulation and protect against UC<sup>15,16</sup>. 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<sup>17-20</sup>. 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<sup>21</sup>.

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.

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