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Research Article

A Th2 Cytokine Profile in Appendicular Lavage Fluid Suggests Allergy as a Possible Etiology for Acute Appendicitis

Nuno Carvalho,[1,2] André Barros,[3] Hélder O. Coelho,[4] Catarina F. Moita,[3] Ana Neves-Costa,[3] Dora Pedroso,[3] Filipe C. Borges,[1] Luís F. Moita,[3,5] and Paulo M. Costa[1,2]

[1]*Serviço Cirurgia Geral, Hospital Garcia de Orta, Almada, Portugal*
[2]*Faculdade de Medicina, Universidade de Lisboa, Portugal*
[3]*Innate Immunity and Inflammation Laboratory, Instituto Gulbenkian de Ciência, Oeiras, Portugal*
[4]*Serviço Anatomia Patológica, Hospital Garcia de Orta, Almada, Portugal*
[5]*Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, Portugal*

Correspondence should be addressed to Luís F. Moita; lmoita@igc.gulbenkian.pt

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Acute appendicitis is the most frequent surgical abdominal emergency, but its etiology remains poorly understood. Histological examination of the appendix, following its removal due to acute appendicitis, consistently shows features in common with bronchial asthma, suggesting an allergic reaction as a candidate etiologic factor. Here, we propose the concept of appendicular lavage and use it to study the levels of the Th2 cytokines IL-4, IL-5, and IL-9 in patients with a clinical diagnosis of acute appendicitis. The study group included 20 patients with a histological diagnosis of phlegmonous appendicitis, 13 patients with gangrenous appendicitis, and a control group of 8 patients with a clinical diagnosis of appendicitis but with normal histology. Cytokine levels were higher in acute appendicitis. The difference was more pronounced when comparing phlegmonous appendicitis with nonpathological appendicitis ($p = 0.01$) for IL-4 (48.3 vs. 21.3 pg/mL), IL-5 (29.2 vs. 8.0 pg/mL), and IL-9 (34.1 vs. 16.6 pg/mL). This Th2 cytokine profile is compatible with the hypothesis of allergy as an etiologic factor for acute appendicitis and may have important implications for the diagnosis, prevention, and treatment of this condition.

1. Introduction

Acute appendicitis (AA) is a frequent disease whose etiology cannot be explained by any single factor. Luminal obstruction is the trigger event that culminates in inflammation of the appendix. Fecaliths are found in one-third of specimens. In the other cases, obstruction is thought to be caused by hypertrophy of mural lymphoid follicles in response to diverse causes [1]. The peak incidence of appendicitis coincides with the age when the immune response is most vigorous, and the lymphoid follicles are at their maximum development [2].

Aravindan has reported histological features in AA that are similar to those of bronchial asthma, a paradigm for an allergic reaction. Based on these findings, it was proposed that AA may be triggered by a type I hypersensitivity reaction and, therefore, could be caused by an allergic reaction [3]. Cytokines from Th2 lymphocytes are responsible for the histological features of asthma [4]. Th2 effector cells secrete mainly interleukin-4 (IL-4), IL-5, IL-9, and IL-13, which are known to be involved in allergic responses [5].

Bronchoalveolar lavage (BAL) is a useful tool for investigating inflammatory cells and mediator profiles, like cytokines, in various bronchopulmonary diseases [6]. High levels of IL-4 and IL-5 are found in BAL in asthma [7]. Similar to BAL, we have developed the concept of appendicular lavage (AL), where saline is instilled and collected in the appendicular lumen of appendectomy surgical specimens. The aim of this study was to test the hypothesis that AA might be the consequence of an allergic reaction by evaluating the

levels of Th2 cytokines in AL fluid of patients submitted to appendectomy due to a clinical diagnosis of AA.

2. Materials and Methods

2.1. Study Population. The study group, evaluated between April 2016 and June 2017, consisted of patients with the clinical diagnosis of AA, admitted to the emergency department of Hospital Garcia de Orta, when one of the authors (NC) was on call to perform the appendicular lavage. The only exclusion criterion was the absence of the author (NC). The histological diagnosis of AA discriminates acute phlegmonous appendicitis (APA) and acute gangrenous appendicitis (AGA). The control group consisted of patients admitted with the clinical diagnosis of AA, submitted to appendectomy, but with normal histology (nonpathological appendix (NPA)). No type of allergy test was performed. Laparoscopic appendectomy was performed in 33 patients and open surgery in 8 patients, including 3 conversions from laparoscopy. There were 9 localized and 3 generalized peritonitis.

2.2. Ethics Considerations. This study is part of a research project approved by the Ethics Committee of Garcia de Orta Hospital (reference 05/2015). Each enrolled subject gave written informed consent. The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The authors declare no conflict of interest.

2.3. Appendicular Lavage. After removal of the appendicular specimen, a gauge was inserted in the proximal luminal aspect of the appendix and 3 mL of saline 0.9% was instilled and collected for AL. Saline was reinstilled and collected 3 times. The appendicular lavage process was standardized and performed exclusively by one of the authors (NC). The appendicular fluid samples were collected to a Sarstedt Monovette tube and centrifuged. 1 mL of the supernatant was extracted and stored at -20°C. The ELISA protocol was used for IL-4, IL-5, IL-9, and IL-6 determinations (human IL-4, IL-5, IL-9, and IL-6 MAX, BioLegend, San Diego, CA 92121, USA) according to the manufacturer's protocol. The cytokine levels, IL-4, IL-5, IL-9, and IL-6, were expressed in pg/mL.

2.4. Pathologic Analysis. After AL procedures, the appendices were preserved in 10% formalin for histopathological examination. A minimum of 24 hours was allowed for adequate tissue fixation. Appendicular sections were sampled from the tip, base, and intermediate length for fixation and paraffin processing. Two sections of 5-micron thickness were cut from each paraffin block and stained by hematoxylin and eosin [8]. The criteria for AA were polymorphous nuclear neutrophil infiltration at the muscularis propria [9]. APA was defined by the presence of neutrophil infiltrate in the muscular propria, and AGA was defined by the presence of necrosis of the wall of the appendix in a background of transmural inflammation [10]. The presence of neutrophils in the mucosa was considered a variant of normal with no clinical relevance, when no other inflammatory cells were detected in abnormal numbers. The specimens were classified as negative for appendicitis when no neutrophil infiltrate was shown in the muscular propria (NPA) [10]. All the histopathological analyses were performed by one of the authors masked to the results of cytokine measurements (CH).

Table 1: Characterization of the population according to histological categories.

Variable *N* (%)	APA 20 (49)	AGA 13 (32)	NPA 8 (19)	*p* value
Age (y)	36.2 ± 16.9	20.0 ± 15.1	34.2 ± 12.9	0.898
Gender (M/F)	14/6	4/9	6/2	0.054
BMI	25.3 ± 6.0	26.6 ± 3.1	23.7 ± 3.7	0.211
Allergy	3	2	2	NA
WBC	14.3 ± 4.4	14.4 ± 3.8	12.3 ± 2.9	0.161
CRP	3.7 ± 5.6	9.4 ± 6.2	4.6 ± 6.3	0.001
LOS	2.8 ± 1.8	5.4 ± 3.9	3.3 ± 3.1	0.002

Results presented as number (valid percentage) or mean ± standard deviation. APA = acute phlegmonous appendicitis; AGA = acute gangrenous appendicitis; NPA = nonpathological appendix; M = male; F = female; BMI = body mass index; NA = not applicable; WBC = white blood count; CRP = C-reactive protein; LOS = length of stay.

2.5. Statistical Analysis. Data are presented as descriptive statistics, mean and standard deviation. For continuous variables, considering the distribution of number of cases among the categories, a nonparametric approach was followed to assess statistical differences among the considered groups: Kruskal-Wallis tests were used, with *a posteriori* pairwise Wilcoxon tests; p values were then corrected for multiple comparisons using the Holm correction. For categorical variables, such as gender, a strategy based on Fisher's exact test overall and pairwise, using the aforementioned correction, was used. Statistical analysis was performed on R (https://cran.r-project.org), using the stats package for hypothesis testing and ggplot2 for the plots.

3. Results

We analyzed 33 patients with a histological diagnosis of AA, 20 patients with APA, 13 patients with AGA, and 8 patients, the control group, with normal histology. History of allergy was present in 7 patients (4 to antibiotics, 1 to Metibasol®, and 2 with allergic rhinitis); no differences between groups were identified. None took medication. No differences in age, gender, and BMI among groups were found ($p = 0.898$, $p = 0.054$, and $p = 0.211$, respectively) (Table 1). A significant difference was found for C-reactive protein levels and length of stay ($p = 0.001$ and $p = 0.002$, respectively) (Table 1).

The levels of cytokines in AL according to the histologic groups are presented in Table 2. In all the studied subjects, cytokines IL-4, IL-5, and IL-9 were detected in AL fluid. For IL-4, there were significant differences among the histological groups ($p = 0.034$). The difference between APA and NPA groups was significant ($p = 0.017$). No significant differences for the AGA group with the remaining groups were found (AGA vs. APA, $p = 0.421$; AGA vs. NPA, $p = 0.421$) (Figure 1). For IL-5, the differences were subtle ($p = 0.056$).

Table 2: Cytokine levels (pg/mL) according to the histological categories.

	IL-4 Mean ± SD	IL-5 Mean ± SD	IL-9 Mean ± SD
Phlegmonous	158.1 ± 228	76.3 ± 106.5	101.9 ± 141.7
Gangrenous	109.2 ± 154.7	49.2 ± 81.6	72.5 ± 110.3
Nonpathological	23.5 ± 6.8	9.9 ± 4.0	17.9 ± 7.0

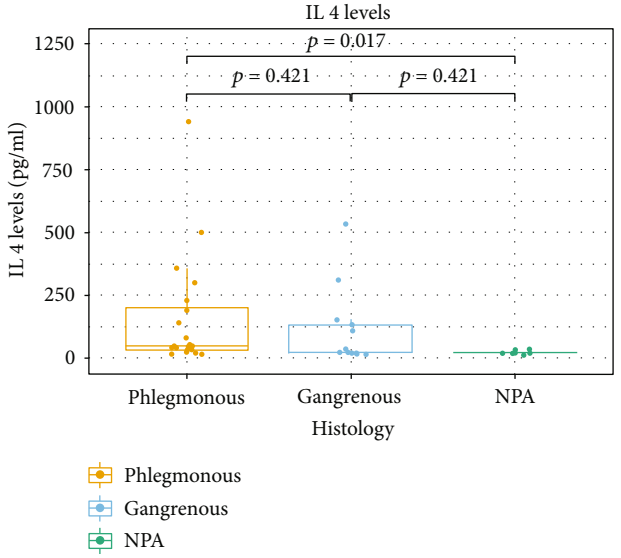

Figure 1: Box plots of IL-4 levels according to the different histological categories.

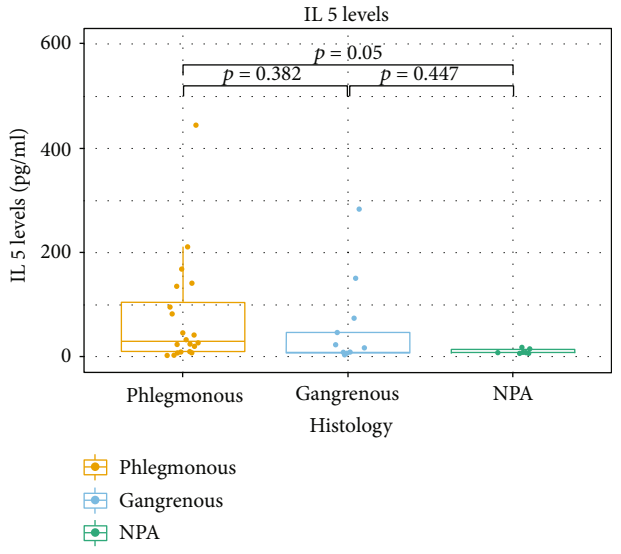

Figure 2: Box plots of IL-5 levels according to the different histological categories.

Differences among groups showed a tendency for different levels of IL-5 between APA and NPA ($p = 0.05$) (Figure 2). As for IL-9, there was no clear evidence of differences for the AGA group (AGA vs. APA, $p = 0.587$; AGA vs. NPA, $p = 0.587$). IL-9 was the cytokine whose levels revealed less

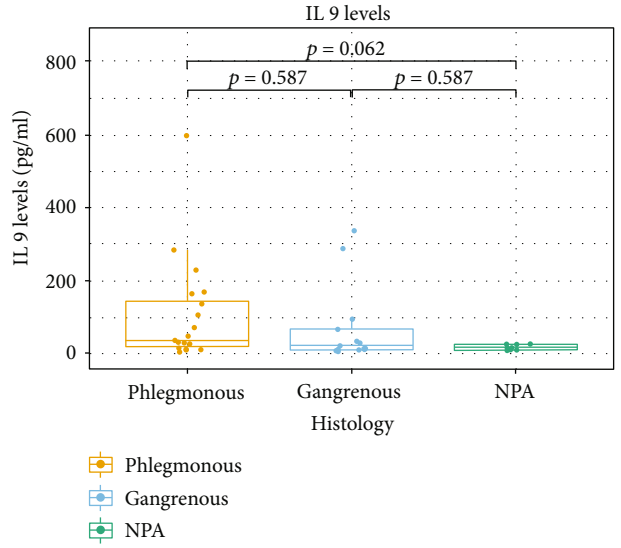

Figure 3: Box plots of IL-9 levels according to the different histological categories.

differences among groups ($p = 0.083$). However, while not statistically significant, there was a tendency indicating that differences exist between APA and NPA groups for this cytokine ($p = 0.062$) (Figure 3). No differences were found between groups for IL-4 and IL-5 in the peripheral blood.

Th2 cytokines can be elevated in the course of an acute-phase response together with other inflammatory mediators [11]. To test whether Th2 cytokines were specifically associated with a histological diagnosis of AA, we have also measured the levels of IL-6 in the AL fluid, a prototypical general (nonallergic) acute-phase cytokine [12]. No differences were found among the groups (Supplementary Figure 1) in the study ($p = 0.627$).

To test an independent indicator of the contribution of an allergic reaction to the pathophysiology of AA, we have quantified IgE levels in the histological samples of the different groups (Figure 4). While differences among groups did not reach statistical significance ($p = 0.29$), both the median (49 for APA, 42 for AGA, and 26 for NPA) and mean (68.16 for APA, 48.85 for AGA, and 23.50 for NPA) were substantially higher for APA and AGA when compared to NPA. These results, while not reaching statistical significance due to high variation in the APA and AGA, strongly suggest that IgE levels are substantially higher in histologically documented cases of AA.

4. Discussion

The most common theory for the etiology of AA is intraluminal obstruction, which is not supported by histological findings in surgical specimens. In most cases, no obstruction can be found [13]. In one study, only in 0.4% of 1969 appendectomy specimens was there evidence of luminal obstruction by vegetable fibers [14].

Based on histologic findings, specifically eosinophilic infiltration, mastocyte degranulation, and muscular edema, an allergic reaction was proposed as a possible etiology of

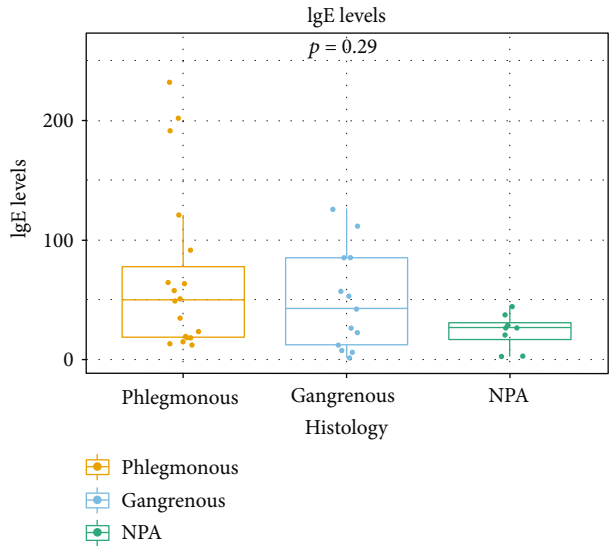

Figure 4: Box plots of IgE levels according to the different histological categories.

AA [3]. The concept is attractive: the appendix is a lymphoid organ; therefore, an immune response to a local antigen could be a factor in the pathogenesis of AA [15]. The gastrointestinal system is one of the main entrances into the body for allergens during all life stages [9]. Atopy may be a risk factor for appendicitis [9]. By analogy with asthma, the contraction of the muscular wall of the appendix in response to antigenic stimulation may result in luminal obstruction culminating in AA. This response may occur in any segment of the intestine, but the appendix is more vulnerable because of its small lumen size and limited capacity to accumulate fluids [3]. The identification of the offending allergen(s) is not always straightforward [16].

Allergies are inflammatory diseases dependent on Th2 activation, mediated by IL-4, IL-5, and IL-9, in response to environmental allergens [7]. IL-4 acts as a growth factor for Th2 cells and promotes the production of IgE. IL-5 induces the differentiation, activation, and survival of eosinophils. IL-4 and IL-9 induce the growth of mast cells and basophils [7].

BAL has been performed for several years in lung diseases. We extend and adapt this approach to monitor the levels of cytokines in the appendix—appendicular lavage. The fluid collected from the appendicular lumen may reflect local inflammatory alterations. We used 3 washes with NaCl 0.9% at the appendicular lumen to mobilize cytokines that are adherent to the mucosa. All steps of the process were standardized, from the surgical handling of the specimens to the processing of lavage and quantitative determinations. Differences in cytokines should be accepted as reflecting local inflammatory changes. Elevated levels of IL-4 and IL-5 are present in BAL of patients with allergic asthma [6]. Higher levels are seen in symptomatic patients [17]. The results of our study show a statistical difference in the levels of IL-4 between phlegmonous and nonpathologic appendicitis. IL-4 elevation reflects a putative allergic reaction in AA. In the case of IL-5, our data suggests differences between groups, mainly between the phlegmonous and nonpathological appendix. For IL-9, there are also indications of differences between groups, mainly between the phlegmonous and nonpathological appendix.

No significant differences for the Th2 cytokine profile were found between AGA and APA or NPA. In fact, some authors claim that simple inflammatory appendicitis and necrosis represent different diseases, or different patient responses to disease, with distinct epidemiology, natural history, and microbiology and different Th17 cytokine profiles [18]. Blood inflammatory response in AA showed a positive association of Th1-mediated immunity and gangrenous appendicitis [19]. AGA etiology may be different from APA, without an allergic component, and so Th2 cytokines are not elevated in AL. Another possible explanation for IL-4 and IL-5 similar values in AGA and NPA is that the cellular destruction in AGA is so marked that Th2 cells can no longer produce IL-4 and IL-5 and the values fall down to values found in NPA. This hypothesis is compatible with the possibility that AGA represents a later phase of the disease where tissue destruction is more pronounced and where the initial cytokines have been depleted.

In asthma, the paradigm of allergic disease, the cytokine profile in BAL shows elevation of IL-4, IL-5, and IL-9 compared to control groups [6]. Our results with AL are similar to those findings in BAL in the presence of allergy. In fact, our study showed elevations of Th2 cytokines in AL fluid of patients with appendicitis, compared with the control group (nonpathologic appendicitis).

Nonsurgical therapeutic alternatives are viable options to treat AA in uncomplicated cases as recently demonstrated for the use of antibiotics [20]. Therefore, our work adds allergy not only as a possible etiology for AA but also as an alternative therapeutic target to be explored.

4.1. Strengths. Using a novel methodology, appendicular lavage, our study provides objective and original data demonstrating a correlation between Th2 cytokines and appendicular histological features that show that an allergic component is present in AA.

4.2. Limitation. Appendicular lavage should be reproduced by other researchers. The sample size is still limited. A larger dataset of patients, originating from multiple centers, is strongly recommended because it is likely to validate and extend the conclusions of the current study.

4.3. Conclusion. Differences in IL-4, IL-5, and IL-9 in AL fluid were found between the 3 study groups and especially significant between phlegmonous and negative appendicitis. Therefore, in AL fluid, we found a Th2 cytokine profile compatible with allergy. Further studies are required to assess the importance and robustness of our results regarding the allergic components in AA. The identification of an allergen will be a particularly difficult task, but our results open the possibility for novel management strategies in AA that might not always include a surgical procedure.

Data Availability

The raw data used to support the findings of this study are restricted by the ethics committee of Hospital Garcia de Orta in order to protect patient privacy. Data are available from Dr. Nuno Carvalho (nunomdcarvalho1964@gmail.com), the leading author of this study, for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors have no conflict of interest in relation to this work.

Authors' Contributions

N.D.C. designed the experiments, acquired the data, and wrote the manuscript. A.B.B., C.F.M., D.P. and A.N.C. did the analyses of the data. H.O.C. and F.C.B. acquired the data including the pathology analyses of surgical samples. L.F.M. and P.M.C. were the advisors of the project and reviewed the manuscript.

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Supplementary Materials

A single figure that displays the IL-6 levels in AA samples according to their histological classification. *(Supplementary Materials)*

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