CHAPTER 6

Composition of the cellular infiltrate in patients with simple and complex appendicitis

Gorter RR, Wassenaar E, de Boer OJ, Bakx R, Roelofs JJTH, Bunders MJ, van Heurn LWE, Heij HA.

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Abstract

Background

It is now well established that there are two types of appendicitis: simple (nonperforating) and complex (perforating). This study evaluates differences in the composition of the immune cellular infiltrate in children with simple and complex appendicitis.

Methods

47 consecutive children undergoing appendectomy for acute appendicitis between January 2011 and December 2012 were included. Intraoperative criteria were used to identify patients with either simple or complex appendicitis and were confirmed histopathologically. Immune histochemical techniques were used to identify immune cell markers in the appendiceal specimens. Digital imaging analysis was performed using Image J.

Results

In the specimens of patients with complex appendicitis significantly more myeloperoxidase (MPO) positive cells (neutrophils) (8.7% vs. 1.2% $p<0.001$), were detected compared to patients with a simple appendicitis. In contrast, fewer CD8+ T cells (0.4% vs. 1.3% $p=0.016$), CD20+ cells (2.9% vs. 9.0% $p=0.027$) and CD21+ cells (0.2% vs. 0.6% $p=0.028$) were present in tissue from patients with complex compared to simple appendicitis.

Conclusions

The increase in pro-inflammatory innate cells and decrease of adaptive cells in patients with complex appendicitis suggests potential aggravating processes in complex appendicitis. Further research into the underlying mechanisms may identify novel biomarkers to be able to differentiate simple and complex appendicitis.

Introduction

Recent studies indicate that acute appendicitis is not an irreversibly progressive disease, but that two distinct types of appendicitis can be identified: simple (uncomplicated or nonperforating appendicitis) and complex (complicated or perforating) appendicitis. Simple appendicitis can be treated with antibiotics only, while the second requires an appendectomy in most cases.[1,2] Initial non-operative treatment has been investigated for acute simple appendicitis both in the adult and paediatric population with good results.[3-8] Approximately, 60-85% of the adult population and 62-81% of the paediatric population an appendectomy was avoided at one-year follow-up after treatment with antibiotics only.[3-8] Selection of patients in these studies was based upon clinical and radiological variables and did not include biochemical markers. Accurate identification of patients with simple or complex appendicitis will prevent unnecessary surgical interventions in patients with simple appendicitis, while making sure patients with complex appendicitis receive the surgical treatment that they require.

To identify biomarkers that help to distinguish simple and complex appendicitis it is essential to better understand the individual underlying pathogenesis of both types. These are predisposing factors for appendicitis in general, such as appendicular obstruction, infection, diet and ischemia.[9] All the previously abovementioned factors eventually lead to invasion of the appendiceal wall by intraluminal bacteria and activate innate immune cells such as macrophages, while danger signals due to ischaemia induced cell death can further augment immune responses.[9-11] For example increase of extra-cellular adenosine triphosphate (ATP) due cell death in ischaemic tissue is a strong inducer for innate cells such as macrophages and dendritic cells (DCs) which together with Toll like receptors (TLRs) triggering results in interleukin (IL)-1b production.[12,13] T cells activated by DC are subsequently recruited to the site of inflammation and can further contribute to inflammation or by means of T regulatory cells decrease inflammation. However, it is unknown why in one person this inflammation is impeded while in others leads to complex appendicitis.

Studies focussing on the composition of the cellular infiltrate in patients with appendicitis are scarce.[14,15] It has been noted that in all patients with appendicitis there is an influx of neutrophils in the lamina propria.[14] Only one study compared the composition of the cellular infiltrate in patients with perforated appendicitis and nonperforated appendicitis and detected increased numbers of cluster of differentiation (CD)-8+ cells (T-Lymphocytes) in the specimens of the appendix of patients with perforated appendicitis.[15] Subsequent studies mainly focussed on the systemic immune response and investigated the cytokine profiles in blood samples showing elevated levels of IL-6, IL-17 and interferon-α (INF- α) in patients with complex appendicitis compared to simple appendicitis.[16,17]

The aim of this study is to evaluate differences in the composition of the infiltrate of mononuclear immune cells in the appendix between patients with simple and complex appendicitis. The results will help identify critical cell types distinguishing between these to phenotypes and can be used to design further studies to identify new biomarkers.

Materials & Methods

Study population

All children aged 0-17 years who underwent an appendectomy for suspected acute appendicitis in the Academic Medical Centre Amsterdam between January 2011 and December 2012 were included. Patients with a noninflamed appendix, a parasitic infection, those who underwent an appendectomy as a routine procedure (for instance in case of malrotation) or as an interval appendectomy after initial non-operative treatment of appendicitis were excluded. The medical charts from the included patients were reviewed and the specimens from the original histopathological examination of the appendix were collected and additional staining procedures, as described below, were performed. Patients without histopathological specimens, missing data or those unsuitable for staining were also excluded. Study approval was obtained from the medical ethics review board prior to the start of this study.

Patients were allocated into either the simple or complex appendicitis group according to the following definitions for simple and complex appendicitis.[18,19]
Simple appendicitis is diagnosed on the basis of (1) intraoperative findings: inflamed appendix without signs of gangrene, perforation, purulent fluid, contained phlegmon or intra-abdominal abscess *and* (2) histopathological examination confirming the diagnosis of appendicitis without necrosis or perforation. Complex appendicitis is diagnosed on the basis of (1) intraoperative findings: signs of a gangrenous appendix with or without perforation, intra-abdominal abscess, appendicular contained phlegmon or purulent free fluid and (2) histopathology confirming the diagnosis based on extensive necrotic tissue in the muscular layer of the appendix or signs of perforation. In case of discrepancies between clinical and pathological findings, discussion was held by the pathologist (JR) and one member of the surgical team (RG). In case of disagreement, a third reviewer was approached.

Medical chart review

A standardised data extraction form was used in order to review the medical charts. (Appendix 1)

Staining and scanning

The original specimens of appendices were handled using a standardised protocol developed by the department of pathology, Academic Medical Centre of Amsterdam. The haematoxylin and eosin-stained sections and tissue blocks were retrieved from the archive and re-evaluated to identify the most severely affected appendiceal segment based on microscopic examination by three of the authors (EW, RG, JR), which was then selected for additional staining. Based upon the literature, we decided to use the immune histochemical stains with antibodies specific for immune cells from the innate and adaptive immune response and these are listed in Table 1.

In short, after deparaffinization and rehydration, endogenous peroxidase was blocked in methanol and H_2O_2. Next, heat induced antigen retrieval was performed in Tris-EDTA pH 9.0 for 20 minutes at 98C. Then the sections were incubated with appropriate dilutions of the primary antibodies for 1 hour at room temperature. After washing, sections were incubated with horseradish peroxidase (HRP) conjugated anti mouse or anti rabbit polymers (Bright vision). After detecting HRP activity with 3,3'-Diaminobenzidine (DAB)+, sections were counterstained with haematoxylin and mounted.

All histological slides were screened for eligibility prior to scanning. One patient was excluded because of contamination with an unknown substance of his slide due to which scanning was not possible. Slides were digitised using an Olympus dotSlide scanner (Tokyo, Japan) at 10x, and the areas of interest were saved as jpg.

Table 1. Overview of immunohistochemical stains used in the present study. Targeted cells are displayed in the right column.

Monoclonal antibody	Source	Pretreatment* and dilution	Main cellular expression
CD8	DAKO, clone C8/144B (M7103)	Boiling 1:100	Cytotoxic T cells
CD56	Thermo Scientific, clone 123C3.D5 (MS-204)	Boiling 1:400	Natural killer cells
MPO	DAKO (A0398)	None; 1:4000	Neutrophils
CD20	DAKO, clone L26 (M0755)	Boiling 1:1000	B cells
CD21	Thermo Scientific, clone 2G9 (MS-1086)	Boiling 1:50	Follicular dendritic cells and B cells
CD3	Thermo Scientific, clone SP7 (RM-9107)	Boiling and 1:500	Pan-T cells
CD4	Thermo Scientific, clone 4B12 (MS-1528)	Boiling and 1:100	T helper cells
CD68	DAKO, clone PG-M1 (M0876)	Boiling 1:200	Monocytes/ macrophages
CD138	DAKO, clone MI 15 (M7228)	Boiling 1:200	Plasma cells

* Boiling: 10 minutes in 10/1 mM Tris/EDTA with autoclave

ImageJ analysis

Image J software (version 1.49; National Institutes of Health, USA, v 1.49) was used for measuring the percentage of immune positivity for the respective immune histochemical markers on the digitised whole-slide images. Image J is a public domain, Java-based image-processing program. Digital imaging analysis using Image J has been performed in many previous studies and has been identified as a reliable method.[20,21] The color deconvolution plugin was used for segmentation of the DAB signal, using the standard settings for haemotoxylin and DAB. A threshold was established for detection of immune positive cells. A manually selected area including the layers of mucosa, submucosa and muscular layer was selected by two authors (EW, JR). They excluded the subserosa, background, lumen and mesoappendix from further analyses. Finally, the degree of positivity for a specific marker of the examined selected surface of the appendix was calculated. Figure 1 shows an example of specimens illustrating the appendices of both simple and complex appendicitis. Also the figure illustrates the process of analysing. One author performed the image analysis (EW) and was blinded for categorisation of the patient into simple or complex appendicitis. (Figure 1,2)

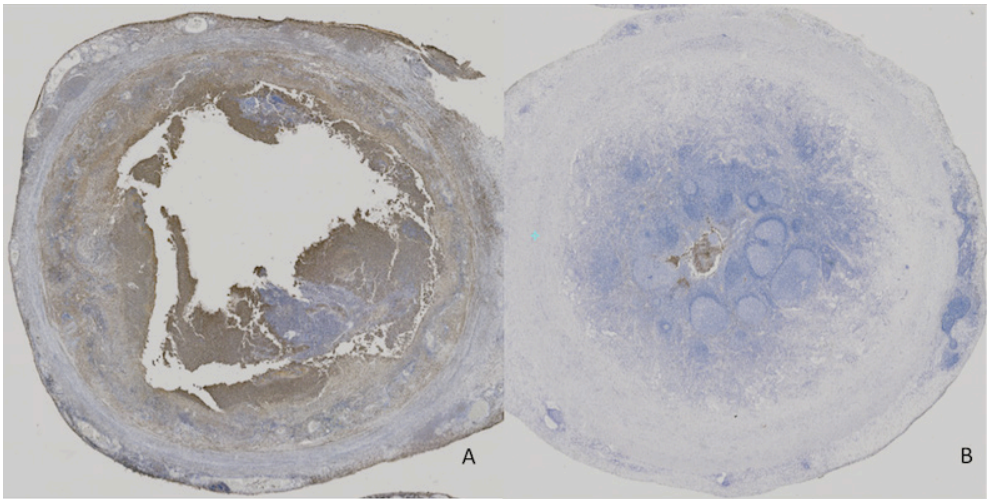

Figure 1. Appendiceal specimen with MPO staining (MPO+ cells are colored Brown). A Complex appendicitis B Simple appendicitis
The abundance of MPO+ cells in patients with complex appendicitis can be seen (figure 1A), whereas in simple appendicitis (figure 1b) only limited MPO+ cells are detected and mostly limited to mucosa.

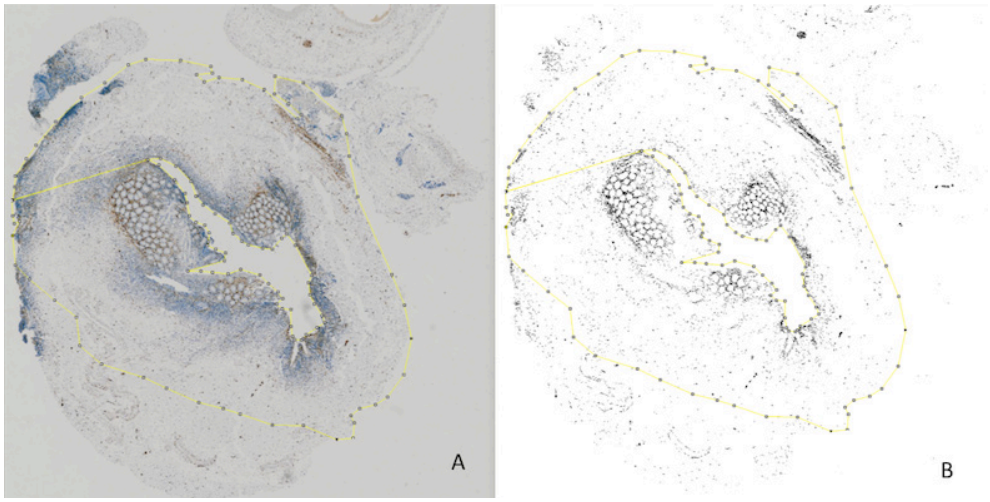

Figure 2. Appendiceal specimens A MPO + cells are marked with DAB (brown color) B. Same image with RGB colors demounted. The yellow line represents the borders of the area of interest (mucosa, submucosa and muscular layer). Figure 1b was used to calculate the percentage of positivity.

Primary outcome parameter

The primary outcome parameter was the percentage of immune positivity for the respective immune histochemical marker compared to the total surface area.

Statistics

Based on the distribution of the data, non-parametric Mann-Whitney U test or parametric Student's t-test was used in case of continuous outcome variables and chi-square or Fisher's Exact Test in case of binary outcome variables as appropriate. Missing data were left out of the analysis. A P value < 0.05 was considered statistically significant. Statistical Package for Social Sciences (SPSS) (version 22.0; IBM) was used for all analysis.

Results

General characteristics

In total 71 patients were treated for acute appendicitis in this time period. Excluded were 23 patients for the following reasons: Missing data (N=16), interval appendectomy (N=2), non-inflamed appendix (N=3), conservative treatment (N=2). None of the patients suffered from a parasitic infection. The remaining 48 patients with appendicitis were identified of whom one had to be excluded from further analysis due to contamination of his specimen. Analysis was therefore performed on 47 patients; 23 patients were classified as simple appendicitis and 24 patients as complex appendicitis. The general characteristics of both groups are shown in Table

2. Patients with complex appendicitis had a longer duration of complaints (1 [0-6] days versus 2 [0-13] days; p=0.038), higher body temperature (37.2 [36.4 39.1] degree Celsius versus 38.0 [36.4-39.5] degree Celsius; p=0.022) and level of C-reactive protein (CRP) (9 [0-93] mg/L versus 110 [1-400] mg/L; p=<0.001) than patients with simple appendicitis. No difference was noted regarding age between the two groups. None of the patients were neutropenic, suffered from human immunodeficiency virus (HIV) or were on immunosuppressive medication that might affect the cellular infiltrate at the time of appendectomy.

Table 2. General characteristics of patients with simple and complex appendicitis.

	Simple appendicitis (n=23)	**Complex appendicitis** (n=24)	P-value
General			
Age (years)*	11 (1 – 16)	9 (1 – 16)	NS
Sex - male	14 (61)	13 (54)	NS
Medical history			
Duration abdominal pain (days)*	1 (0-6)	2 (0-13)	0.038
Physical examination			
Temperature (degree Celsius)*	37.2 (36.4-39.1)	38.0 (36.4-39.5)	0.022
Heart rate (beats/minute)*	89 (56-127)	132 (89-156)	< 0.001
Biochemical testing			
C-reactive protein (mg/l)*	9 (0-93)	110 (1-400)	< 0.001
CRP group			
0-50 mg/l	19 (83)	6 (25)	
50-200 mg/l	2 (9)	3 (12)	< 0.001
>200 mg/l	1 (4)	12 (50)	
Missing Data	1 (4)	3 (13)	
White blood cell count (10^9/l)*	13.6 (2.2-25.2)	16.0 (6.2-22.2)	NS
Type of surgery			
Laparoscopic	18 (78)	16 (67)	
Open	5 (22)	6 (25)	NS
Conversion	0(0)	2 (8)	

Data are represented as number of patients (percentage), unless stated otherwise
* Data are represented as median (range). Missing data N=4

Cellular infiltrate

Results from the cellular analysis are shown in Table 3. There were more myeloperoxidase (MPO)+ cells in patients with complex appendicitis compared to patients with a simple appendicitis (8.7% versus 1.2%, p<0.001), suggesting an increased infiltration of myeloid cells, in particular neutrophils. Furthermore we detected increased numbers of CD8+ T cells in the infiltrate in patients with simple appendicitis compared to tissue from patients with

complex appendicitis (1.3% versus 0.4%, p=0.016). The total number of CD3+ and CD4+ cells did not differ between the two groups. Interestingly, the number of B cells as determined by CD20+ (2.9% versus 9.0%, p=0.027) and CD21+ (0.2% versus 0.6%, p=0.028) was reduced in patients with complex appendicitis compared to simple appendicitis. We did not observe a difference in natural killer (NK) cells based on CD56 expression as well as in macrophages based on CD68.

Table 3. Measurements of positivity of immunohistochemical marker in patients with simple and complex appendicitis.

Marker	**Simple appendicitis** (n=23)	**Complex appendicitis** (n=24)	P-value
MPO - neutrophils	1.2% (0.0-33.8)	8.7% (0.1-39.0)	0.000
CD68 - monocytes/macrophages	1.7% (0.0-6.1)	2.2% (0.1-11.3)	0.882
CD20 - B cells	9.0% (0.3-18.8)	2.9% (0.4-17.1)	0.027
CD21 - B cells	0.6% (0.0-6.9)	0.2% (0.0-4.2)	0.028
CD138 - plasma cells	0.9% (0.0-7.5)	0.4% (0.0-4.1)	0.125
CD3 - pan T cells	2.5% (0.0-20.1)	1.9% (0.2-7.5)	0.328
CD4 - T helper cells	0.1% (0.0-7.4)	0.04% (0.0-9.6)	0.176
CD8 - T cells	1.3% (0.3-6.6)	0.3% (0.0-3.8)	0.016
CD56 - natural killer cells	1.6% (0.1-4.5)	1.4% (0.0-2.8)	0.259

Data are presented as median (range)

Discussion

This study shows that the cellular infiltrate in children with complex appendicitis contains significantly more MPO+ cells (neutrophils) and significantly fewer CD20+/CD21+ cells (B-lymphocytes) and CD8+ cells (T-lymphocytes) in comparison to children with simple appendicitis. Regarding clinical variables, patients with complex disease have a longer duration of symptoms, higher level of CRP and higher body temperature, which might reflect a more systematic immune response.

It is generally known that neutrophils invade the appendix during appendicitis.[14] We have shown that the level of neutrophil infiltration (MPO expression) is different between simple and complex appendicitis. The number of neutrophils is higher in complex appendicitis and CD20+/CD21+/CD8+ cells (B-and T-lymphocytes) are decreased, suggesting that pathways triggering innate immune responses and reducing potential regulating responses by adaptive immune cells may play an important role in the pathogenesis of complex appendicitis. Although MPO is expressed both in neutrophils and macrophages, the increase in MPO+

cells in our study reflects an invasion by neutrophils, as there was no significant difference in the CD68+ cells (monocytes and macrophages) between patients with simple and complex appendicitis. In the past neutrophils were considered as short-lived effector cells of the innate immune system. Nowadays their importance in the activation and regulation of the innate and adaptive immunity has been recognised.[22] Neutrophils are the effector cells in the Th17 mediated immune response.[22] It has been demonstrated that Th17 cells derived cytokines (such as IL-17 and IL-8) lead to recruitment, activation and prolonged survival of neutrophils.[23] The increased serum levels of Th17 polarizing cytokine IL-6 and IL-1b and elevated levels of IL-17 in patients with complex appendicitis detected by Ruber et al support the hypothesis that a Th17 immune response might be activated in patients with complex appendicitis.[16,17]

In our study, CD20+/CD21+ cells and CD8+ T cells (B- and T-lymphocytes) were decreased in patients with complex appendicitis compared to patients with simple appendicitis. Recently the regulatory effects of B cells have been described and the higher number of B cells in simple appendicitis may have protective effects against ongoing inflammation and progression to complex appendicitis.[24,25] In contrast to the findings of our study, Kuga et al identified significantly more cytotoxic T-cells and NK cells in the appendix of patients with perforated appendicitis compared to nonperforated appendicitis.[15]

It is well known that clinical variables differ between simple and complex appendicitis. Complex appendicitis is more common in younger children, although we could not confirm this in our study.[26,27] However, there may be a relative over presentation of perforated appendicitis in our older group, as both young age and complicated appendicitis are independent indications to refer patients to our tertiary referral centre. Moreover due to the retrospective nature of this study, we were not able to include 16 patients in this study due to missing data, no histopathological specimens available or unsuitable for staining. This might have to led selection and/or information bias. The factor time is an inconsistent risk factor for the occurrence of complicated appendicitis. Regarding the factor of time, patients with complex appendicitis had a marginally significant longer duration of complaints: two days versus one day. In the literature, inconsistent results regarding the duration of complaints have been reported.[26,28,29] Laboratory results also differ between patients with simple and complex appendicitis. A recent systematic review found higher levels of inflammatory markers (both CRP and leucocytes) in children with complex appendicitis, which is confirmed by findings in our study.[30] This may reflect a more systematic inflammatory response in these patients.

Taken together, these results present a different immune response towards myeloid cell responses inducing invasion of neutrophils, to the detriment of adaptive CD8+ or B cell responses in patients with complex appendicitis. Whether or not this is the result of longer

duration of the inflammatory process cannot be concluded from this study, only that our study indicate a distinctive immune phenotype in samples of simple and complex appendicitis. The immune pathways underlying our observations can be further explored in functional studies to understand the pathogenesis of complex and simple appendicitis. Furthermore, unraveling the underlying immune response might lead to the identification of markers for complex appendicitis that can be used as a diagnostic tool to discriminate simple from complex appendicitis prior to treatment.

Conclusions

In conclusion, this study shows that there is an increased invasion of neutrophils in the appendix in patients with complex appendicitis whilst in the appendix of patients with simple appendicitis there is an increase of CD20+/CD21+/CD8+ cells (B- and T-lymphocytes). Clinically, patients with complex appendicitis in this study have longer duration of symptoms, higher body temperature and higher level of CRP. These findings suggest that simple and complex appendicitis are characterised by a unique mononuclear cellular infiltrate in the appendix. These findings may be exploited in the search for novel biomarkers to discriminate simple and complex appendicitis before treatment.

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Appendix 1: Data collected from patients chart

Medical history	Days of abdominal pain (from time of onset until presentation)
Physical examination	Body temperature (^{O}C)
	Heart rate (beats per minute)
	Diffuse abdominal guarding
Biochemical testing	C-reactive protein (CRP; mg/l)
	White blood cell count (WBC; x10^9/l)
Imaging studies	Ultrasound performed, if so characteristics on imaging studies
	CT performed, if so characteristics on imaging studies
	MRI performed, if so characteristics on imaging studies
Intraoperative variables	Type of inflammation
	Free fluid
	Perforation
	Spill
	Need for antibiotics
Postoperative	Complications
Histopathological examination	Diameter of appendix
	Type of inflammation
	Signs of perforation