

Immunopathogenesis of human gastrointestinal infection by *Anisakis simplex*

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Background: *Anisakis simplex* is a parasite of fish, and in the case of human infestation, it should be considered as a possible cause of gastrointestinal disease, especially in countries where raw or undercooked fish is a frequent food. Clinical features of anisakiasis may simulate acute abdominal pain, such as that found in patients with gastric ulcers, appendicitis, and Crohn's disease. Furthermore, many cases of anisakiasis are diagnosed as eosinophilic gastroenteritis, which is a broad term for a specific disease.

Objective: The purpose of this study was to investigate the immunopathogenesis of human gastrointestinal infestation by *A simplex*.

Methods: Thirteen intestinal biopsy specimens from patients with anisakiasis were analyzed for the presence of messenger (m)RNA for different cytokines and inflammatory mediators by RT-PCR. Specific IgE, eosinophil cationic protein, eosinophil protein X, and tryptase levels were measured in each patient's serum. Also, cell cultures were set up with lymphocytes from some patients and stimulated in vitro with *Anisakis* and *Ascaris* antigens.

Results: We performed immunologic phenotyping in 13 patients. All patients underwent biopsy after emergency surgery caused by episodes of acute abdominal pain. In all cases inflammatory infiltrate composed of eosinophils and lymphocytes was found in the intestinal wall. We demonstrated that after infestation, a T_{H2}-type immune response occurred. Also, major basic protein, nitric oxide, and eotaxin were found in the tissue, and eosinophil cationic protein and eosinophil protein X levels were elevated in sera.

Conclusion: These data and in vitro lymphocyte cultures indicate that a T_{H2} mechanism plays an important role in the inflammatory infiltrate produced by the anchorage of para-

sites in the gastrointestinal wall. (J Allergy Clin Immunol 1999;104:637-43.)

Key words: Anisakiasis, *Anisakis simplex* allergy, T_{H2} response, cytokines, eosinophilic gastroenteritis, eosinophils

Intestinal infestation with parasites is a frequent cause of eosinophilic gastroenteritis (EG),¹ but *Anisakis simplex* is rarely considered as a cause of this disorder in spite of its being a relatively common parasite in fish.

Human anisakiasis (herring worm disease) is caused by parasitic infection of the gastrointestinal tract caused by ingestion of raw or undercooked fish parasitized with the third larvae stage of the nematode *A simplex* (superfamily Ascaridae, family Anisakidae, genus *Anisakis*).² Besides cephalopods, hake, sardines, tuna, cod, mackerel, and other fish that are intermediate hosts (secondary hosts) for this parasite, marine mammals are the definitive hosts.³ Another larval nematode is pseudoterranova (cod-worm or seaworm disease). This nematode, in contrast with *A simplex*, does not usually penetrate into the stomach or the gastrointestinal wall.⁴ The penetration of *A simplex* into the wall of the stomach and the intestine produces a marked inflammatory (eosinophilic) response.

Clinical features of infestation with *A simplex* are often similar to those of acute appendicitis, gastric ulcer, intestinal occlusion, and even Crohn's disease. For this reason, *A simplex* is rarely considered as a cause of the disorder. Granuloma and abscess may appear with eosinophilia, fever, and abdominal pain. Endoscopic confirmation and identification of the worm are definitive for diagnosis.^{5,6} In cases of intestinal invasion by the parasite, patients frequently undergo laparotomy for appendicitis, and inflammatory eosinophilic reactions are described in the resected small intestine. Sometimes the parasite is found in the intestinal wall.^{7,8}

EG and anisakiasis infestation both are marked by an eosinophilic infiltrate of the gastrointestinal wall.⁹ Anisakiasis is differentiated from EG in some rare cases because the parasite is visualized either by endoscopic analysis or in serial studies of the histologic sections. In regard to the differential diagnosis, some points should be considered, such as dietary habits of the patients as to fish consumption and the onset of symptoms in relation with

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TABLE I. Characteristics of patients with intestinal anisakiasis

Patient No.	Sex	Age (y)	Total IgE (kU/L)	Specific IgE (kUA/L) to <i>A simplex</i>	Specific IgE (kUA/L) to <i>Ascaris lumbricoides</i>
1	M	59	96.1	15.1	3.3
2	M	46	23.7	7.91	0.35
3	F	42	45.7	13.9	0.51
4	F	47	15.1	0.97	0.35
5	M	82	ND	ND	ND
6	F	54	52.2	16.3	0.59
7	F	60	0.35	0.35	0.35
8	F	65	65	32.9	0.35
9	M	31	202	34.8	0.35
10	F	37	24.6	1.60	0.35
11	M	32	114	12.9	0.38
12	M	46	119	5.72	0.35
13	M	31	138	12.2	0.35
Mean	—	48 ± 15	70.1 ± 58	12.88 ± 11.3	0.65 ± 0.88*

ND, Not determined.

*Differences between IgE specific to *A simplex* and IgE specific to *Ascaris lumbricoides* are statistically significant ($P < .005$).**Abbreviations used**

ECP: Eosinophil cationic protein
 EG: Eosinophilic gastroenteritis
 EPX: Eosinophil protein X
 iNOS: Inducible nitric oxide synthase
 MBP: Major basic protein
 PBL: Peripheral blood lymphocyte

eating undercooked fish. Gomez et al¹⁰ demonstrated the presence of IgE antibodies against *A simplex* in 80% of patients with anisakiasis; by contrast, these antibodies were found in only 10% of control subjects. There was a good correlation between skin prick test responses with *Anisakis* extract and in vitro IgE responses.

After the first description of human infestation, numerous cases have been described in Japan, France, the UK, the US, and Spain, but immunopathologic studies are notably scarce.

We performed immunologic phenotyping in 13 patients, 9 of whom were diagnosed as having EG of unknown etiology (patients 1 to 7, 9, and 10; Table I). After allergologic review and immunologic analysis, patients given a diagnosis of EG were shown to have acute intestinal anisakiasis caused by parasitic infection of the gastrointestinal tract. Another 3 patients (patients 8, 12, and 13) were directly diagnosed as having gastric anisakiasis, and 1 patient was given a diagnosis of parasitic granuloma (patient 10). All patients underwent biopsy after emergency surgery caused by episodes of acute abdominal pain. In all cases inflammatory infiltrate in the mucosa and submucosa composed of eosinophils and lymphocytes was found.

Two types of response were analyzed in our study: serum IgE antibody and cellular immune response. We have determined specific IgE (CAP and immunoblotting) against *A simplex* and studied the possible immunologic mechanism implicated in this pathology by means of several approaches: (1) analysis of the T_{H1}/T_{H2} response and chemokines in the cellular infiltrate of intestinal biopsy specimens; (2) analysis of the release of effector molecules associated with eosinophil activation; and (3) analysis of the PBMC response after in vitro stimulation with *Anisakis* and *Ascaris* antigens, studying the proliferation and the transcription and release of cytokines into the supernatants.

The results presented in this article indicate that after penetration of the parasite into the mucosa, an important T_{H2} -dependent activation cascade was induced, with the subsequent gastrointestinal eosinophilic infiltration responsible for the pathologic features described in these patients.

METHODS**Patient selection**

This study conformed to the guidelines specified by the Spanish National Institute of Health and was approved by the Ethical Committee of the Fundación Jiménez Díaz.

Thirteen patients comprised the patient group. Patients 1 to 7, 9, and 11 were primarily diagnosed as having EG; patient 10 had a parasitic granuloma, and patients 8, 12, and 13 were diagnosed as having gastric anisakiasis. Clinical features of patients 1 to 4 and patient 11 have been described elsewhere.¹⁰ After allergologic and immunologic review, all of the patients were finally given a diagnosis of anisakiasis.

Total and specific IgE antibody measurement

Total serum IgE, specific IgE to *A simplex*, *Ascaris lumbricoides*, and fish were measured by using the Pharmacia CAP System (Uppsala, Sweden). Immunoblot was performed as described previously.¹¹

Determination of mediators in sera

Quantitative measurement of eosinophil cationic protein (ECP) and tryptase in human serum was made by fluoroimmunoassay (Unicap Pharmacia). Quantitative determination of eosinophil protein X (EPX) in human serum was determined by Pharmacia EPX RIA (Uppsala, Sweden).

RT-PCR

Messenger (m)RNA was extracted¹² from 13 paraffin-embedded intestinal biopsy specimens, and mRNA (0.5 μ g) was converted to complementary (c)DNA by the reverse-transcriptase enzyme reaction (AMV transcriptase-reverse; Promega, Madison, Wis) for a total volume of 20 μ L.

PCR was performed in a final volume of 50 μ L containing 2 to 4 μ L of reverse transcriptase reaction product. β -Actin, IL-2, IL-4, IL-5, IFN- γ , IL-8, and human inducible nitric oxide synthase (iNOS; Clontech Laboratories, Palo Alto, Calif) were amplified according to the manufacturer's instructions. Primers used for the amplification of major basic protein (MBP) were derived from the published cDNA sequence for MBP¹²: 5'-TGCCCCATGCAACT-TACAGG-3' as sense and 5'-AGATGAAGAGCAGAACTGCC-3' as antisense. Primers used for eotaxin amplification were derived from the published sequence for eotaxin¹³: 5'-CCCAACCACCT-GCTGCTTTAACCTG-3' as sense and 5'-TGGCTTTGGAGTTG-GAGATTTTGG-3' as antisense. Thirty cycles of PCR were per-

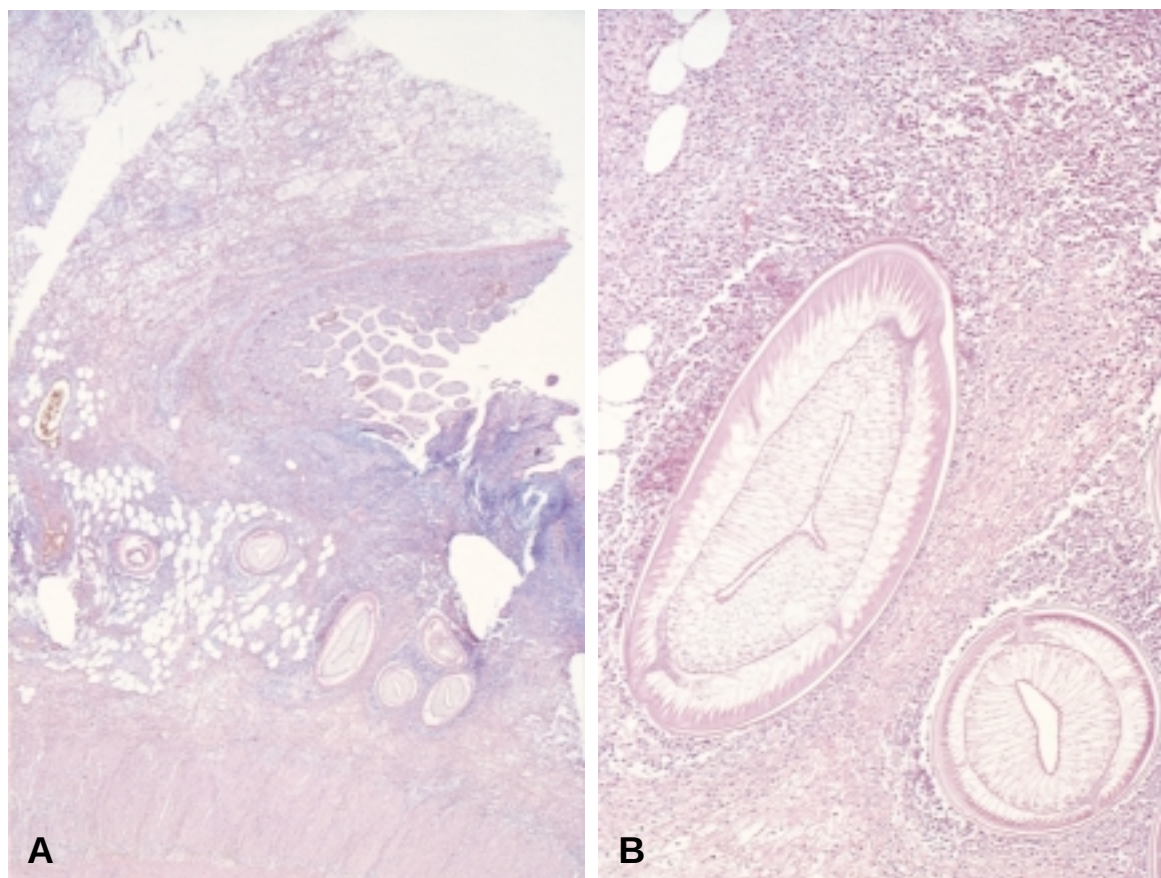


FIG 1. A, Histologic section showing larva in the submucosal layer of intestine, with cellular infiltrate composed of eosinophils and lymphocytes, of a patient with anisakiasis (patient 13). B, Microscopic view of the parasite in the submucosa.

formed by using DNA thermal cycle (Gene Amp 2400, Perkin Elmer). Each PCR cycle consisted of 1 minute of denaturation at 95°C, 1 minute of annealing at 60°C, and 2 minutes of extension at 72°C. After the final cycle, the temperature was held at 72°C for 7 minutes to allow annealing of the amplified products. A 15- μ L aliquot from each PCR underwent electrophoresis in a 2% agarose gel containing 0.5% ethidium bromide. The gel was then photographed under UV transillumination and submitted to a Southern blot hybridization, as previously described.¹²

PCR products were not obtained when reverse transcriptase was omitted, indicating that there was no DNA contamination. The positive control was a known positive cDNA provided by the manufacturer; in the case of MBP and eotaxin we used eosinophil-infiltrated lung as the positive control.

Cell cultures

After informed consent was obtained, human peripheral blood lymphocytes (PBLs) from some patients and healthy control subjects were isolated by density sedimentation gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). Cells were adjusted to 1×10^6 cells/mL in RPMI-1640 culture medium (Flow Laboratories, Scotland, UK) supplemented with 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 1% sodium pyruvate. Different concentrations of *Anisakis* and *Ascaris* extracts were added (0, 5, 25, and 100 μ g/mL). A final volume of 200 μ L/well was dispensed in a 96-well, flat-bottom plate (Costar, Cambridge, Mass), and cells were cultured at 37°C in a 5% CO₂ atmosphere. After 4

days of culture, tritiated thymidine (New England Nuclear, Boston, Mass) was added, and its incorporation was measured after 18 hours in a β -scintillation counter (Beckman LS 2800, Palo Alto, Calif).

Cells from the same patients were cultured for 48 hours in the same conditions as described above, centrifuged at 800g for 5 minutes, and washed. mRNA was extracted, and the supernatants were stored for cytokine determination.

Quantitative determination of human IL-5 and IFN- γ levels

For the quantitative determination of human IL-5 in cell culture supernatant, we used the Quantikine Human IL-5 Immunoassay (R&D systems Europe, Oxon, UK). For determination of IFN- γ , we used the Interferon- γ kit (Genzyme, Cambridge, Mass).

Statistical analysis

Statistical significance of differences in cytokine profile was made by using Fisher's exact test. A type I error of less than .05 was considered statistically significant. All statistical analyses were performed with the GraphPAD InStat 1990 Software (V 1.14).

RESULTS

Characteristics of patients

Table I summarizes the total and specific IgE against *Anisakis* and *Ascaris* antigens found in these patients.

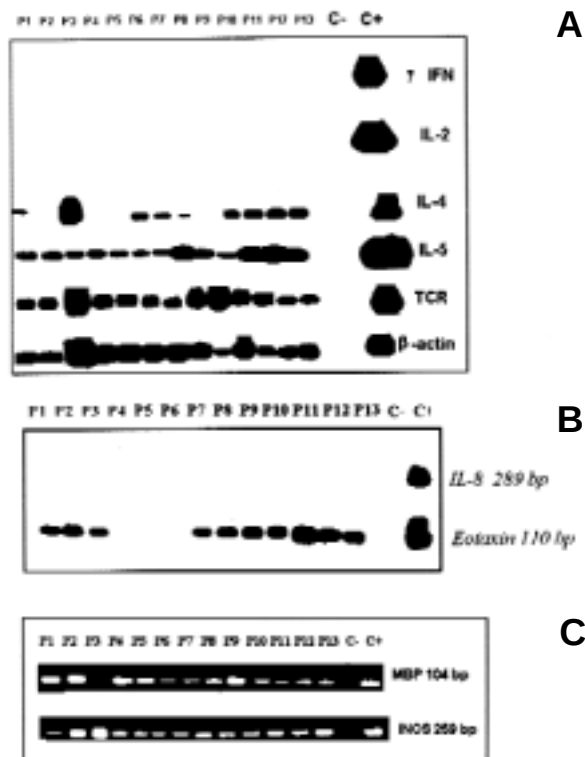


FIG 2. mRNA expression of cytokines, chemokines, iNOS, and MBP in intestinal biopsy specimens. A, Cytokine profile of mRNA for T_{H1} (IL-2 and IFN- γ) and T_{H2} (IL-4 and IL-5) cytokines in intestinal biopsy specimens from patients with EG caused by *A simplex* infestation. The presence of T cells was confirmed by T-cell receptor amplification. β -Actin was used to normalize RNA from tissues. B, Chemokines studied in tissue sections from patients with anisakiasis. IL-8 and eotaxin gene expression was studied by RT-PCR followed by Southern blot. C, Tissues positive for iNOS and MBP in the intestinal biopsy specimens of patients with EG detected by RT-PCR.

Total IgE levels ranged from 0.35 to 202 kU/L, with a mean of 70.1 kU/L (normal range, <100 kU/L). Levels of specific anti-*A simplex* IgE were 12.88 ± 11.3 kUA/L (CAP class 3), and levels of anti-*Ascaris* IgE were 0.65 ± 0.88 kUA/L. The differences between specific anti-*Anisakis* and anti-*Ascaris* IgE levels were significant ($P < .005$), indicating response specificity to *A simplex* compared with *Ascaris* species. Specific IgE levels to fish were not detected in the sera of these patients.

With regard to biopsy specimens, in all cases inflammatory infiltrate composed mainly of eosinophils and lymphocytes was found in the mucosa and submucosa. In some cases mucosal disruption may signal the larval entrance in the intestinal wall, and the larva was clearly identifiable (Fig 1).

Cytokine profile (T_{H1}/T_{H2}) and chemokines in tissue sections from intestinal biopsy specimens

To analyze which cytokine profile is involved in these patients, RT-PCR for different T_{H1} and T_{H2} cytokines was performed by using a collection of paraffin-embedded

A biopsy specimens from the gastrointestinal tracts of the patients presented in Table I.

The results presented in Fig 2, A, show a polarized T_{H2} response in the studied patients. First of all, neither IL-2 nor IFN- γ was detected in any of the patients studied. However, 9 of 13 patients had mRNA for IL-4, and all 13 patients had transcripts coding for IL-5 and T-cell receptor. PCR products were not obtained when reverse transcriptase was omitted, indicating that there was no DNA contamination.

There was a significant increase in the T_{H2} response compared with the T_{H1} response ($P < .005$) when examining the cytokine profile in the intestinal specimens from the patients with anisakiasis. Because of the observation of an important eosinophilic infiltration in all these patients, we wanted to study some of the chemokines responsible for attracting eosinophils to the inflammatory focus. By using cDNA obtained from these patients, the expression of eotaxin was detected in 77% (10 of 13) of the specimens (Fig 2, B). By contrast, IL-8 mRNA transcripts were not detected in any of the specimens studied (Fig 2, B). We also studied the mRNA from 3 normal paraffinized intestinal tissues obtained from people with unrelated intestinal pathology. We were unable to detect mRNA for IL-4, IL-5, IL-2, IFN- γ , or MBP. Only eotaxin was detected in 1 of 3 specimens (data not shown).

Analysis of eosinophil-related activation molecules: iNOS, MBP, EPX, and ECP

After stimulation, eosinophils, which represent the main cell in the infiltrate, produce ECP, EPX, MBP, and iNOS.^{13,14} To study the level of activation of eosinophils, different approaches were used. First, RT-PCR from the biopsy specimens were analyzed for the presence of iNOS and MBP, showing that all patients had detectable levels of the transcription of iNOS, and 12 of 13 had transcripts of MBP. The results shown in Fig 2, C, demonstrate that eosinophils are actively transcribing MBP and iNOS, indicating nitric oxide production as a possible inflammatory mediator.

Second, we also measured ECP and EPX in serum from these patients, and almost all of them had high levels of both proteins. The mean level of ECP was 22.4 ± 15 compared with the control level of 4.4 ± 6.9 , and differences between both are very significant ($P < .0001$). When EPX levels were measured in these patients, we found elevated levels (37.4 ± 20 μ g/L) compared with the control levels (17.7 ± 9 μ g/L, $P < .001$). The activation observed was specific for eosinophils because when we determined a typical mastocyte mediator, such as tryptase, in the same serum, the value was under the normal level (5.44 ± 2.52 μ g/L), and no significant differences were obtained compared with control sera (5.6 ± 7.5 , $P = .79$).

Cytokine profiles in cell cultures from patients given a diagnosis of anisakiasis

To confirm a T_{H2} response in this pathology, we analyzed the cytokines released by the PBLs of 3 patients

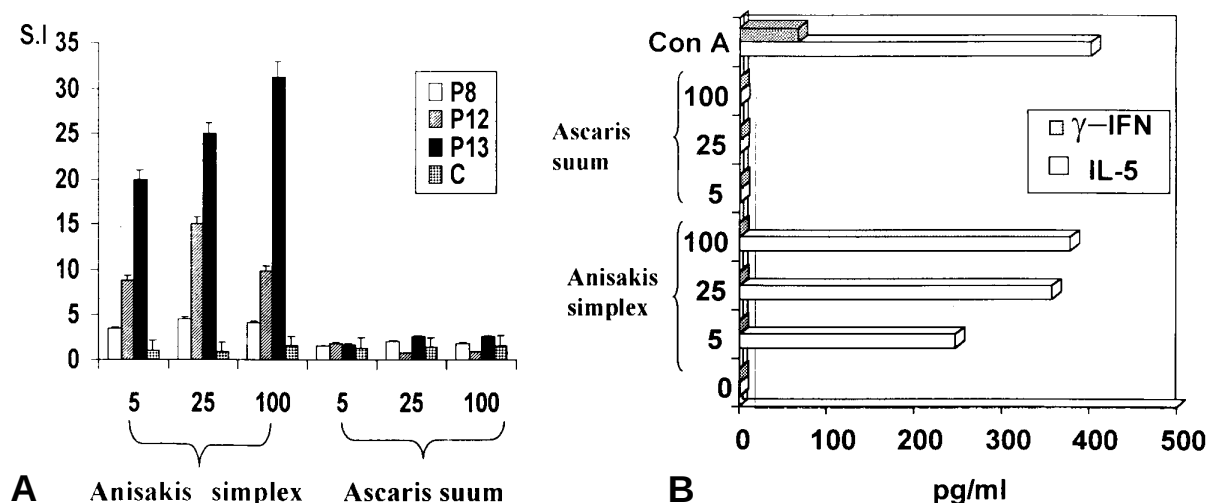


FIG 3. A, Proliferative response of lymphocytes from patients with anisakiasis and healthy control subjects. PBMCs were stimulated with different doses (5, 25, and 100 µg/mL) of *A simplex* and *Ascaris* extract. Data represent mean of stimulation indexes (SI) ± SD. The stimulation index was calculated by the ratio between the mean of disintegrations per minute from stimulated cells and the mean of disintegrations per minute from unstimulated cells from triplicate cultures. Control represents mean of stimulation index ± SD of 6 different healthy subjects. B, Cells were cultured with different doses (0, 5, 25, and 100 µg/mL) of *Anisakis* and *Ascaris* extracts. Supernatants were collected at different times (24 and 72 hours) to analyze IL-5 and IFN-γ levels. Data represents mean of 3 different patients (patients 8, 12, and 13). The values of IL-5 production are given in picograms per milliliter and correspond to 247 ± 23 pg/mL for the dose of 5 µg/mL *A simplex*, 357 ± 33.2 pg/mL for the dose of 25 µg/mL antigen, and 378 ± 36.9 pg/mL for the dose of 100 µg/mL.

(patients 8, 12, and 13), and the proliferation of these PBLs in response to the antigens from *A simplex* and *Ascaris suum*. PBLs were cultured with or without different doses of these extracts. Results showed that PBLs proliferate specifically to different antigen doses of *A simplex* (Fig 3, A). There was no response to *Ascaris* antigens. Data are represented as means of stimulation indexes ± SDs in triplicate cultures.

When PBLs from control subjects were cultured in the same conditions, no significant proliferations were found. Values represent mean stimulation indexes ± SDs of 6 different healthy subjects.

The presence of IFN-γ and IL-5 in supernatants from PBLs cultured in the same conditions was analyzed. Fig 3, B, shows that when lymphocytes from these patients were cultured with *A simplex* extract, IL-5 was detected in supernatants after 48 hours, but IFN-γ was not detected. Three different doses of the antigenic extract of parasites were used, demonstrating a dose-dependent response in the IL-5 produced. In these cells mRNA specific to IL-4 and IL-5 increased after antigen stimulation with respect to basal condition, and no T_{H1} cytokine production was detected (data not shown).

DISCUSSION

This article demonstrates that a polarized T_{H2} immune response is implicated in the response to *A simplex*, confirming and extending our preliminary results.¹¹

EG is characterized by digestive tract eosinophilic

infiltrates, which are frequently associated with peripheral blood eosinophilia and nonspecific digestive symptoms.¹⁵ Although its etiology is unknown, it can have different causes (eg, some reactions to drugs, hypereosinophilic syndrome, and infestation by parasites), and therefore differential diagnosis must be made.

We reviewed 13 patients that had been given a diagnosis of anisakiasis, and most of them had a selective increase in the level of anti-*A simplex*-specific IgE, which reacted with antigenic bands of around 39 to 42 Kd (immunoblot data not shown). Histologically, all surgical specimens had a similar appearance, with mural edema and lymphocytic and eosinophilic infiltration (Fig 1).

Human anisakiasis is a parasitic infection of the gastrointestinal tract caused by *A simplex*, and it is acquired by eating raw fish.^{16,17} Although most of the cases have been described in Japan,^{18,19} in the last few years it has increased in other countries, such as France, Spain, and the US.²⁰⁻²³ Interestingly, there is a wide geographic distribution of this nematode and a high frequency of parasitized fish, which is in contrast with the few references of human anisakiasis in the literature. In fact, pathologists have not considered it to be a frequent cause of EG.^{1,24}

In our review we found 8 of 13 patients were misclassified as having primary EG. These patients were finally diagnosed as having anisakiasis after the allergologic and immunologic review. Immunodiagnosis with parasitic antigens should be performed as an important tool for differential diagnosis of EG.

Because infection of this parasite in human beings pro-

duces an immune response that is almost unknown, we analyzed mRNA from paraffin-embedded intestinal biopsy specimens obtained from patients with EG. Fig 2 shows the profile of cytokines present in tissues. We were able to detect T_{H2} cytokine-specific mRNA (IL-4, IL-5, or both) in 100% of the analyzed tissues, and no T_{H1} cytokines (IL-2 and IFN- γ) were detected. This cytokine pattern was confirmed in PBMCs from 3 patients with anisakiasis. First, expression of T_{H2} cytokine genes was detected after antigen challenged by RT-PCR, and second, cells cultured with *Anisakis* extract were able to proliferate, produce, and release IL-5 to the culture medium (Fig 3, B).

These positive results indicate that T_{H2} cells mediate the primary immunologic effector mechanism in the infiltrates produced by the *A simplex* invasion of the gastrointestinal mucosa. T cells release cytokines, such as IL-4, which is implicated in IgE synthesis, and IL-5, which induces selective eosinophil recruitment and activation, clearly in accordance with clinical features of the disease.

One of the main characteristics observed in the intestinal tissue of this pathology was the presence of eosinophils in the gastrointestinal wall. Therefore we investigated the role of eotaxin in these phenomena.²⁵ We found that eotaxin, one of the chemokines responsible for eosinophilic infiltration, is predominantly expressed (77% of total specimen analyzed) in these tissues, and other chemokines, such as IL-8, were not present.²⁶

Recently, the presence of eotaxin in the intestinal wall of normal mice has been described as regulating the baseline eosinophil trafficking on nonhematopoietic tissue.²⁷ For this reason, we have analyzed the presence of RNA specific for T_{H1} (IFN- γ and IL-2) and T_{H2} cytokines (IL-4 and IL-5), eotaxin, and MBP in 3 normal human intestinal biopsy specimens. Our results were negative, except for the presence of eotaxin mRNA in one of the tissues (data not shown). This result emphasizes the importance of the finding of the predominance of mRNA for T_{H2} cytokines in the histologic specimens from our patients with anisakiasis.

Eosinophils are specialized cells that carry out a wide range of functions in allergic and parasitic disease.²⁸ Some of their properties are thought to be protective because they can attack parasites. Human eosinophil MBP is one of the principal mediators of injury to parasites. Therefore we wondered whether the infiltrating eosinophils expressed mRNA for MBP. In all paraffin-embedded intestinal specimens from patients, mRNA specific for MBP was found (Fig 2, C). We previously published data on the immunolocalization of MBP in eosinophils infiltrating into the intestinal submucosa of patients with anisakiasis.¹¹

Nitric oxide is another mediator involved in parasite killing, which has been recently described in eosinophils.²⁹ Because eosinophils have iNOS and are implicated in the defense against parasites, we investigated whether iNOS activity was implicated in this pathology. The presence of iNOS in tissues was analyzed. As shown in Fig 2, C, messenger specific to iNOS was detected in all tissue, indicating a possible effector mechanism against *A simplex* in this

pathology. We confirmed eosinophil activation by testing other classical cytotoxic mediators released by eosinophils in serum, such as ECP and EPX, and both were found to be elevated in the sera of these patients.

Our studies indicate that anisakiasis is linked to a profound T_{H2} response, which is demonstrated by local detection of mRNA (eg, IL-4, IL-5, and eotaxin) with no detectable transcripts of IFN- γ , IL-2, and IL-8. Eosinophil-dependent activation cascade appears to be very active; iNOS, MBP, ECP, and EPX are implicated in parasitic attack.

In the last 2 years, an increasing number of cases of eosinophilic enteritis caused by *A simplex* have been detected. In fact, the United States Food and Drug administration recently published that parasites of fish are a risk to public health.³⁰ Our findings highlight the importance of pathologists taking into account that *A simplex* may be a common cause of EG and that it should be considered in the differential diagnosis of acute abdominal pain, especially after ingestion of fish.

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