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Variations in Inflammation and Nerve Fiber Loss Reflect Different Subsets of Achalasia Patients

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Background. **Achalasia is a debilitating motility disorder with an unknown etiology. Past research has demonstrated a spectrum of histological findings. The objective of this study was to further characterize the histopathology of achalasia, with attention to subsets of findings that may exist, possibly reflecting different pathogeneses.**

Materials and methods. **Lower esophageal muscle was obtained during surgery for achalasia (n = 12) or cancer (n = 9). Immunohistochemistry was performed to identify various inflammatory cells and nerve fibers, and grading was done by an expert pathologist. Clinical data were taken from medical records.**

Results. **There were two subsets of achalasia specimens with different histological findings. Group A (7/12; 58%) had an inflammatory infiltrate in the myenteric plexus consisting primarily of T-lymphocytes. Group B (5/12; 42%) had no such infiltrate and had less myenteric plexus macrophages *versus* Group A (P = 0.03). The loss of nerve fibers was most evident in the muscularis propria in achalasia as compared to controls (P = 0.01), and this loss was more striking in Group B *versus* A (P = 0.04). The mean duration of symptoms was 16.6 (A) *versus* 6.4 years (B) (P = NS).**

Conclusions. **Two subsets of achalasia patients exist with different histological findings. Group A had T-cell-rich inflammation present with associated macrophages. Group B, with no inflammation, had greater loss of nerve fibers in the muscularis propria *versus* Group A, and therefore, may represent more aggressive disease with shorter duration of symptoms. These results suggest that various pathogeneses of achalasia may exist that share a common pathway of aganglionosis.**

Key Words: **achalasia; dysmotility; megaesophagus; inflammation; neuron; nerve; etiology; pathogenesis; enteric; aganglionosis.**

INTRODUCTION

Achalasia is the most common primary esophageal motility disorder with approximately 2000 new diagnoses per year in the United States [1]. It is characterized manometrically by an inability to relax the lower esophageal sphincter (LES) and the absence of coordinated peristalsis. Although various disease mechanisms have been proposed, the etiology and pathogenesis of achalasia remains to be elucidated. As such, treatment options for patients afflicted with this condition are palliative and aimed at reducing LES pressure to facilitate esophageal emptying.

The efferent innervation of normal human LES consists of both extrinsic and intrinsic components. The extrinsic system is composed of cholinergic, preganglionic nerve fibers arising from the dorsal motor nucleus in the brainstem. These neurons synapse with two distinct sets of postganglionic (intrinsic) neurons in the myenteric plexus (MY) of the lower esophagus. These postganglionic fibers are either cholinergic, and result in contraction of the LES upon stimulation, or nitrergic, and inhibit LES smooth muscle, thus causing relaxation. Although extrinsic nerve derangement has been associated with achalasia in several earlier studies [2–4], it is now well understood that the primary pathophysiology involves aganglionosis in the MY [5] and loss of inhibitory nitrergic neurons, thus resulting in unopposed contractile stimuli [6, 7].

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The majority of achalasia research in the past decade have focused on unveiling the etiology and pathogenic sequence of these findings. Goldblum and colleagues were among the first to demonstrate the presence of an inflammatory infiltrate in achalasia [8], lending support to an infectious or autoimmune mechanism. Ruiz-de-Leon *et al.* established associations with various HLA class II alleles in achalasia, including DQA1*0103, DQB1*0603, and DQA1*0103-DQB1*0603 heterodimer. Interestingly, all women and two-thirds of men with the latter two alleles had antimyenteric autoantibodies present in their serum [9]. Bruley and coworkers demonstrated that achalasic serum induced neurochemical phenotype changes in isolated normal human fundus muscle such that nitrergic neurons were decreased in number with a corresponding increase in cholinergic nerves [10]. Additionally, serological studies have shown a greater prevalence of measles [11] and varicella zoster virus [12] in achalasia. Castagliuolo *et al.* found that lymphocytes isolated from achalasic LES had a higher proliferation index in response to herpes simplex virus as compared to controls [13]. These results led Park and Vaezi to hypothesize that achalasia may be caused by a viral insult that results in the generation of antimyenteric autoantibodies in genetically susceptible individuals [14].

Despite the supporting evidence for virally mediated autoimmunity in achalasia, there are several reports that contradict these findings. For instance, two PCR studies collectively demonstrated no evidence for herpes or measles infection in 25 achalasia patients [15, 16]. Furthermore, a recent study by Moses and colleagues[17] showed that antimyenteric autoantibodies were found with similar frequency in achalasia (51%) and gastroesophageal reflux disease (50%) patients, suggesting that their presence may be due to a nonspecific reaction to a disease process as opposed to a primary pathologic factor. Due to this wide spectrum of findings, we hypothesized that different pathogeneses may exist in achalasia. The objective of this study was to characterize the nature of the inflammatory infiltrate in achalasia as well as to assess the regional distribution of nerve fibers, with special attention to subsets of histological findings that may exist, possibly reflecting different disease mechanisms.

MATERIALS AND METHODS

Muscle Specimens and Clinical Data

Muscle specimens were obtained at the time of surgery for achalasia ($n = 12$) or for cancer or high-grade dysplasia ($n = 9$). These muscle slips were approximately 1 cm in length and taken from the region of the LES. The control tissue (cancer/high-grade dysplasia) was observed to be normal both grossly and microscopically. Tissues were immediately formalin-fixed and paraffin-embedded. Clinical data, including age, gender, prior endoscopic therapy (Botulinum toxin injection or pneumatic dilation), preoperative resting LES pressure (LESP; normal = 6–26 mmHg), preoperative and postoperative dysphagia score (1, no dysphagia to 5, dysphagia to saliva), and duration of symptoms were obtained through patients' medical charts. All specimens and data were obtained through protocols approved by the Institutional Review Board at the University of Pittsburgh.

Immunohistochemistry and Data Analysis

Immunohistochemistry was performed by preparing histological sections (4-μm-thick) and affixing them to electrostatically charged slides. After deparaffinization and rehydration in xylene and graded alcohols, slides were heated in a microwave oven for 15 min with commercial monoclonal antibodies specific for helper T-lymphocytes ($CD4^+$; Vector, Burlingame, CA), cytotoxic T-lymphocytes ($CD8^+$; Dako, Carpinteria, CA), natural killer cells (NK) ($CD56^+$; Neomarkers, Fremont, CA), dendritic cells (DC) ($CD1a^+$; Immunotech, Marseilles, France), macrophages (MAC) ($CD68^+$; Ventana, Tucson, AZ), B-cells ($CD20^+$; Ventana), and nerve fibers ($S100^+$; Dako). Fibrosis was assessed using Trichrome and routine hematoxylin and eosin staining.

An expert pathologist graded levels of immune cells as follows: 0 (0–5% of cells), 1 (6–25% of cells), 2 (26–50% of cells), 3 (51–75% of cells), and 4 (>75% of cells). Nerve fibers were graded as follows: 1 (rare), 2 (moderate), and 3 (numerous). Fibrosis was scored as follows: (no fibrosis), 1 (mild fibrosis), 2 (moderate fibrosis), and 3 (marked fibrosis). Grading was done for each specimen at the level of the muscularis propria (MP) and the MY separately. Statistical analysis was performed using the unpaired *t*-test, Mann-Whitney *t*-test, or Fisher's exact test.

RESULTS

Inflammatory Infiltrate

Seven of the 12 (58%) achalasia specimens had inflammation present (Group A). There were substantially more inflammatory cells present in the MY as compared to the MP ($P = 0.027$) (Fig. 1A-B). This infiltrate consisted primarily of T-lymphocytes (mean grade: cytotoxic T-lymphocytes = 1; helper T-lymphocytes = 0.6) (Fig. 1C-D). B-cells were found in only 2/7 (29%) of Group A specimens (mean grade = 0.3) (Fig. 1E). MAC were found in close association with this infiltrate in all seven patients, and higher levels of MAC were observed in the MY of Group A as compared to Group B (mean grade = 1.4 *versus* 0.5; $P = 0.03$) (Fig. 1F). In contrast to Group A, no inflammation was present in Group B specimens (Fig. 2). No NK or DC were found in either the MP or the MY of Group A or B. There was a trend toward more fibrosis in achalasia MY as compared to controls ($P = 0.06$) with no differences in Group A *versus* B.

Nerve Fiber Distribution

Overall, patients with achalasia had less nerve fiber staining as compared to controls, and this loss was most striking in the MP (mean grade = 0.33 *versus* 1.22; $P = 0.01$) (Fig. 3). All achalasia specimens exhibited aganglionosis, and 8/12 (67%) showed marked atrophy of the MY. There were significantly less MP