Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Chronic Psychological Stress in Rats Induces Intestinal Sensitization to Luminal Antigens

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There is increasing evidence that stress plays a role in the pathophysiology of chronic intestinal disorders, but the mechanisms remain unclear. Previous studies in rats have revealed that stress decreases gut barrier function and allows excessive uptake of luminal material. Here, we investigated whether chronic psychological stress acts to induce sensitization of intestinal tissues to oral antigens. Rats were subjected to 1 hour per day of water avoidance stress or sham stress daily for 10 days, and horseradish peroxidase (HRP) was delivered by gavage on day 5. Studies to determine sensitization were conducted on day 20. All stressed rats developed HRP-specific IgE antibodies, antigen-induced intestinal secretion, and increased numbers of inflammatory cells in gut mucosa. Luminal HRP was absorbed more readily by enterocytes of stressed animals. In addition, stressed rats had increased expression of interleukin-4 and decreased expression of interferon- γ in gut mucosa, a cytokine profile that is typical of allergic conditions. Treatment of stressed rats with an antagonist to corticotropin-releasing hormone (previously shown to inhibit stress-enhanced gut permeability) eliminated the manifestations of intestinal hypersensitivity. Our results indicate that the presence of oral antigen during chronic psychological stress alters the immune response (to sensitization rather than oral tolerance) and causes subsequent antigen-induced gut pathophysiology. (*Am J Pathol* 2006, 168:104–114; DOI: 10.2353/ajpath.2006.050575)

conditions such as inflammatory bowel disease, irritable bowel syndrome (IBS), and food allergies. In inflammatory bowel disease and perhaps IBS, there is evidence that intestinal tissues may become sensitized to a luminal antigen and that subsequent encounter with the antigen initiates an inflammatory response that is involved in the pathophysiology of disease.^{5–9} Although this theory remains controversial, it is clear that sensitization of intestinal tissues is a feature of food allergy. There is little information on the relationship between stress and intestinal anaphylaxis, although several reports indicate that psychological stress triggers allergic reactions in other organ systems.^{10–12} With respect to detrimental reactions to oral antigens, immunogenic material must penetrate the intestinal epithelial barrier to contact and activate immune cells in the lamina propria.¹³ In a sensitized host, immediate hypersensitivity reactions are initiated by antigen cross-linking of specific IgE antibodies bound to the surface of mucosal mast cells located in close proximity beneath the gut epithelium. Released mediators then act on nearby cells to induce both rapid (within minutes) changes in physiology and delayed (within hours to days) effects.^{13,14}

In allergic conditions in general, it is not clear how an individual develops sensitivity to a particular antigen. Genetic factors play a role, but persons can develop a hypersensitivity reaction with no family history of atopy.¹⁵ Normally, antigens encountered in the gut lumen induce active suppression of immune/inflammatory responses, known as oral tolerance, rather than a detrimental reaction.¹⁶ Oral tolerance is not completely understood, but likely involves antigen processing by mucosal cells. Both epithelial and dendritic cells are identified as important cells in this process.^{16–19} Therefore, the initial events after first encounter with an oral antigen appear to be critical to the outcome of oral tolerance versus sensitization.

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A number of publications^{1–4} in recent years indicate that stress plays a role in gastrointestinal pathophysiology in

The gastrointestinal tract, which constitutes one of the largest mucosal sites of exposure of an organism to the outside environment, is lined by a single cell layer of epithelial cells joined together by intercellular tight junctions. This epithelial barrier prevents invasion of microbes and also restricts uptake of macromolecular antigens and other noxious substances that may be present in ingested material. The majority of antigenic proteins are broken down by proteolytic enzymes into nonantigenic fragments before absorption. Some intact antigens are taken up by endocytosis into enterocytes, but are degraded by lysosomal enzymes after fusion of endosomes with lysosomes.²⁰ Therefore, normally very little antigenic material emerges in the lamina propria. However, under certain conditions, a defect in barrier function allows excessive uptake of intact antigens. Increased permeability has been documented in inflammatory bowel disease^{21–23} and at least in a subset of patients with IBS.²⁴ However, at the present time, there is no conclusive information regarding the significance of quantitative or qualitative changes in transepithelial antigen transport in a naive host in terms of its contribution to the development of intestinal hypersensitivity.

We have previously reported that psychological stress in rats causes disruption of the epithelial barrier.^{25–28} Acute restraint or water avoidance stress (WAS) for 1 hour transiently increases epithelial permeability of both the transcellular and paracellular pathways.²⁶ This effect is mimicked by peripheral (intraperitoneal) administration of the stress hormone, corticotropin-releasing hormone (CRH), and the effects of both CRH and stress are abolished by pretreatment of rats with a general CRH receptor antagonist also administered intraperitoneally.²⁷ On the other hand, chronic psychological stress results in longer lasting effects: rats exposed to WAS 1 hour per day for 5 to 10 days develop dramatically enhanced macromolecular permeability such that at least 1 week is required for the permeability to normalize.^{28,29} Other aspects of abnormal host defense, including adherence of commensal bacterial to epithelial cells and their internalization, have been documented.²⁹

This study was designed to test the hypothesis that chronic psychological stress can be a factor involved in inducing sensitization of intestinal tissues to oral antigens. We found that the presence of an antigen load in the intestinal tract during the course of chronic stress increased antigen uptake and resulted in sensitization such that subsequent encounter with antigen stimulated mast cell activation associating with intestinal pathophysiology. Treatment of rats with the CRH antagonist prevented both enhanced transepithelial antigen transport and manifestations of gut hypersensitivity.

Materials and Methods

Animal Treatment

Stress Protocol

All procedures were approved by the Animal Care Committee at McMaster University. Male Wistar Kyoto

rats (mean body weight 300 g; Charles River Breeding Laboratories, St. Constant, Quebec, Canada), a stress-sensitive strain,²⁵ were housed in cages equipped with filter hoods, maintained on a 12:12-hour dark/light cycle, and provided with food and water *ad libitum*. The rats were exposed to chronic WAS, a psychological stressor that results in a mucosal barrier defect as described.²⁹ Briefly, WAS rats were placed on a platform surrounded with water in a container 1 hour per day for 10 consecutive days; sham stress control rats (Con/Ag group) were placed on a similar platform in a container without water. Rats were weighed at the beginning and end of the experiments to confirm the effectiveness of the stress protocol since we previously identified that exposure of rats to chronic WAS inhibits normal weight gain.^{28,29} We did not measure corticosterone because preliminary experiments indicated no elevation of this stress hormone at the time of study (10 days after the last stress session).

Sensitization Protocol

For sensitization, horseradish peroxidase (HRP) (type II; Sigma Chemical Co., St. Louis, MO) was used as the protein antigen. In contrast to earlier studies^{30–32} in which rats were sensitized systemically with a subcutaneous injection of 1 mg of protein [in alum with 50 ng of intraperitoneal pertussis toxin (Sigma) as adjuvants to stimulate IgE production], here we administered HRP by gavage (adjuvants remained the same) immediately after the 1-hour WAS period on day 5 during the course of the 10-day stress protocol. HRP (2 mg in 1 ml of alum) was delivered via an intragastrically positioned cannula. At day 15 after antigen administration (ie, 20 days after beginning the stress procedure), rats were rapidly killed by decapitation. Truncal blood was obtained for serum IgE determinations. Intestinal segments were removed from the jejunum (beginning 5 cm distal to the ligament of Treitz) and pieces were immediately fixed for morphological studies, frozen for mRNA measurements, or prepared for functional studies, as described below.

Additional Rat Groups

Naïve rats, which were not exposed to stress/sham or antigen, were included as a baseline control group (Con group). In addition, to confirm the role of enhanced gut permeability in the sensitization of rats to oral antigen, a group of WAS rats was treated with the receptor subtype nonspecific CRH antagonist, α -helical CRH^{9–41} (50 μ g/kg injected intraperitoneally 30 minutes before each stress session, Sigma), since we had previously inhibited stress-induced mucosal barrier defect by blockade of peripheral CRH receptors.²⁷ These rats also received HRP by gavage on day 5. The remaining procedures were identical to those described above (α hCRH/WAS/Ag group). One more group of rats were treated only with WAS without exposure to luminal antigen (WAS group).

Functional Studies

Transepithelial antigen transport and electrophysiological responses to antigen challenge were determined by studying intestinal tissues in Ussing chambers.^{25–32} From a 10-cm piece of jejunum (10 to 20 cm distal to the ligament of Treitz), the external muscle with adherent mesenteric plexus was carefully removed. For each rat, four adjacent sheets of stripped tissue were mounted in Ussing chambers (WPI Instruments, Narco Scientific, Mississauga, Ontario, Canada), exposing 0.6 cm² of surface area to Krebs buffer containing 115 mmol/L NaCl, 8 mmol/L KCl, 1.25 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 2.0 mmol/L KHPO₄, 25 mmol/L NaCO₃ (pH 7.35 ± 0.02). The serosal buffer included 10 mmol/L of glucose as an energy source osmotically balanced with 10 mmol/L of mannitol in the luminal/mucosal buffer. A circulating water bath maintained the buffers at 37°C. The electrical current crossing the tissue (a measure of net ion transport) was determined in the voltage-clamp mode (zero volts potential difference) and expressed as short-circuit current (I_{sc}, in μ A/cm²). At intervals, the circuit was opened to measure the potential difference, and the tissue conductance (G = conductance, in mS/cm²) was calculated according to Ohm's law.

Intestinal Responses to Antigen Challenge

In several earlier studies,^{30–32} we documented that antigen challenge to sensitized intestine stimulates epithelial ion secretion (indicated by a rise in I_{sc}) and results in increased tissue G. These changes were associated with mast cell activation. Electrophysiological parameters, I_{sc} and G, of the intestinal epithelium were recorded at baseline and 30 minutes after HRP antigen challenge, ie, 5×10^{-5} mol/L HRP added to the luminal buffer. (For comparison, some tissues were challenged with the non-specific protein ovalbumin.) Mast cells were examined by microscopy for signs of activation/degranulation (see section below).

Transepithelial Antigen Transport

We previously showed in sensitized rodents that epithelial permeability is specifically enhanced for the antigen to which the animal has been sensitized;³¹ this phenomenon is due to interleukin (IL)-4-up-regulated expression of CD23 (low-affinity IgE receptor) on epithelial cells, binding IgE antigen, and transporting the complex via endosomes across the cell (transcellular pathway).^{33,34} Antigen delivered to the lamina propria then activates mast cells, and released mediators act on epithelial receptors to further increase permeability by loosening intercellular tight junctions (paracellular pathway). Here, we measured HRP flux across the tissue as an indicator of overall transepithelial antigen transport. After HRP was added to the luminal buffer, the serosal buffer was sampled at 30-minute intervals for 90 minutes. The concentration of intact HRP in the samples was determined by assaying enzyme activity using a modified Worthington method.²⁶ Briefly, 150 μ l of

sample were added to 800 μ l of phosphate buffer containing 0.003% H₂O₂ and 80 μ g/ml o-dianisidine (Sigma Chemical Co.). Enzyme activity was determined from the rate of increase in OD at 460 nm. Fluxes were calculated according to standard formulae and were expressed as pmol/cm²/hour.

Morphological Studies

Pathways of Antigen Transport

To determine uptake of intact antigen into epithelial cell endosomes, jejunal tissues were obtained from all groups of rats for transmission electron microscopy (EM) at 90 minutes after HRP was added to the luminal buffer. Tissues were fixed in 2% glutaraldehyde for 2 hours at room temperature, then incubated for 30 minutes in 5 mg of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 10 ml of 0.05 mol/L Tris buffer and 0.01% H₂O₂ (pH 7.6), and subsequently processed for EM. Tissues were cut to obtain longitudinal sections of epithelium. Ultrathin sections were placed onto copper grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope. EM photographs of jejunal epithelial cells were taken at magnification of $\times 5000$. The total area of HRP-containing endosomes was measured within a fixed size window (300 μ m²) in epithelial cells using image analysis and expressed as μ m²/window. Twelve photographs (one window per photograph with each window covering one to two cells) for each rat, 72 for each experimental group, were analyzed by the same observer who was unaware of the treatment group. The presence or absence of HRP in the paracellular pathway was noted.

Inflammatory Cells

Our previous studies documented that sensitization increases the numbers of certain inflammatory cells in the gut mucosa.^{14,35} Here, we determined the numbers of inflammatory cells by light microscopy and the activation state of mast cells by EM before and after antigen challenge of tissues in the Ussing chambers. For light microscopy, jejunal segments were fixed in 4% phosphate-buffered saline (PBS) and formaldehyde and stained with hematoxylin and eosin. Eosinophils and mononuclear cells were counted by one investigator (P.C.Y.) using a light microscope (magnification, $\times 400$) in 10 random fields for each rat (60 fields/rat group). Mast cells were counted in sections fixed in Carnoy's fixative and stained with 0.5% toluidine blue. Cell numbers were expressed per mm² mucosa. For EM, tissue segments were fixed in 2% glutaraldehyde and processed routinely. Twenty sections containing mast cells were randomly selected from each rat, and granules were categorized as intact or degranulated by density analysis using a computerized imaging system. All sections were coded to avoid observer bias.

Table 1. Oligonucleotide Sequences (5' to 3') of the Upstream and Downstream Primers Used for RT-PCR of IL-4, IFN- γ , and CD23

Primer	Upstream	Downstream
IL-4	ACCTTGCTGTCACCCCTGTTCTGC	GTGTGTGAGCGTGGACTCATTACAG
IFN- γ	ACACTCATTGAAAGCCTAGAAAGTCTG	AGAAGAGCTATGAGCTGCCTGACG
CD23	CTCAGCTCGTGTGTTGGGG	CAACGTTGCATGCCGGTCCC

Molecular Studies

Tissue mRNA Markers

Sensitization results in a skewing of cytokine production toward a T-cell helper (Th)2 cell profile, with IL-4 more than interferon (IFN)- γ .³⁶ Here, we examined mRNA expression in gut mucosa of these two cytokines as well as the expression of CD23. Rat mucosa was removed from tissue pieces by scraping with a glass slide, snap-frozen in liquid nitrogen, and stored at -70°C . Total RNA was extracted using RNeasy mini kit (Qiagen, Mississauga, Ontario, Canada) and 1 μg of RNA was reverse-transcribed by Oligo d(T)₁₆ using the Perkin-Elmer RNA polymerase chain reaction (PCR) core kit (Perkin-Elmer, Mississauga, Ontario, Canada). The resulting complementary DNA (20 μl) was then subjected to PCR by addition of the series of reagents, according to the manufacture's directions. To control for sample-to-sample variation, primers for GAPDH were used to amplify a 198-bp product. Negative controls were performed with samples lacking reverse transcriptase enzyme. PCR products were electrophoresed in 1.5% agarose gel in the presence of 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Amplified DNA bands were visualized with a UV transilluminator and digital photographs were then recorded. The intensity of the DNA bands was analyzed using the software of the same system (Kodak Digital Science 1D; Life Technologies, Inc., Rockville, MD). Primers used for PCR amplification of cDNA for IL-4, IFN- γ , CD23, and GAPDH are shown in Table 1.

Tissue Protein Markers

Using immunohistochemical techniques we determined the expression of cytokines and CD23 protein in gut mucosa as well as the presence of IgE. All procedures were performed at room temperature. Five- μm frozen sections of rat jejunum were treated with 0.3% H_2O_2 and incubated in 1% bovine serum albumin in PBS for 30 minutes. Sections were then incubated for 1 hour with anti-rat IL-4, IFN- γ , or IgE antibodies (diluted 1:100, 1:200, and 1:50, respectively). The site of the antigen-antibody reaction was revealed by sequential incubation with biotinylated goat anti-rabbit antibody (1:300), and HRP-conjugated streptavidin (Dako Diagnostics, Mississauga, Ontario, Canada) 1:300 in PBS containing 1% bovine serum albumin for 1 hour, followed by development in AEC (Sigma). Positive stained cells appeared red in color and were counted in a light microscope. Ten contiguous nonoverlapping fields ($\times 400$) were evaluated

for each rat (60 fields for each group). The results were expressed as cells/ mm^2 of mucosa. Anti-CD23 antibody was prepared according to a previous report³³ and diluted 1:50. A secondary antibody of rabbit anti-mouse conjugated with fluorescein isothiocyanate was used to treat sections for 1 hour that were observed under a confocal microscope. Positively stained epithelial cells were then counted. For these studies, spleen tissues were used as positive controls. Negative controls included sections where the primary antibodies were omitted or replaced with isotype control antibodies.

IgE Antibodies

The presence of antigen-specific IgE antibodies in the circulation is a standard method to document sensitization. HRP-specific IgE titers were measured in serum by passive cutaneous anaphylaxis using standard procedures, as described in a previous report.³⁰ Values greater than 1:16 were accepted as positive.

Statistical Methods

Data are expressed as mean $\pm$ SEM. Differences between groups were tested by analysis of variance, with post hoc analysis by Newman-Keuls test or Student's *t*-test when appropriate. *P* values less than 0.05 were accepted as significant.

Results

Reduced Weight Gain Confirmed the Effectiveness of the Stress Protocol

As in previous studies,^{28,29} chronic psychological stress inhibited weight gain. At the time of study, the weight gain of WAS rats was only 25.2 ± 3.7 g, significantly less ($P < 0.05$) than the value of 44.0 ± 3.7 g in sham stress rats and 52.5 ± 6.8 g in naïve rats ($n = 6$ in each group).

Specific Antigen Challenge of Intestinal Tissues Evoked a Rapid Ion Secretory Response and Increase in Conductance Only in WAS/Ag Rats

There were no significant differences in short-circuit current (Isc) and conductance (G) values at baseline in jejunal tissues from WAS/Ag rats compared with those from rats exposed to Con/Ag (both groups having re-

Figure 1. Electrophysiological responses of jejunal tissues to antigen challenge. Ussing chambers were used for the study. Jejunal tissues were obtained from naive control rats (Con), stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, WAS-only (WAS) rats, and WAS rats pretreated with the CRH antagonist, α -hCRH (α hCRH/WAS/Ag). All rats except Con group received a bolus of intragastric HRP at day 5 of the stress/sham protocol; 15 days later tissues were studied in Ussing chambers. **A:** Secretory response. Isc was recorded at baseline and 30 minutes after adding HRP antigen to the mucosal (apical) buffer. **B:** Conductance. G was recorded at baseline and 30 minutes after adding HRP antigen to the apical buffer. Values indicate mean $\pm$ SEM; $n = 6$ rats in each group (four tissues per rat averaged to obtain each rat value).

ceived oral HRP on day 5) or naive control rats (Figure 1). Tissues from stressed rats responded to HRP antigen added to the luminal buffer with a rapid (beginning after ~ 2 minutes) elevation in Isc, consistent with Isc changes previously reported in systemically sensitized rats and shown to be due to secretion of chloride ions.³² The values for Isc at 30 minutes after HRP antigen challenge were significantly greater ($P < 0.01$) in tissues from stressed rats than values in tissues from sham stressed rats or naive controls (Figure 1A). Jejunal tissues from the latter two groups did not respond at all to HRP challenge. Similarly, values for G were significantly greater 30 minutes after HRP antigen challenge in tissues from stressed rats compared with the two other groups (Figure 1B). There was no response to challenge with ovalbumin. These results indicate that intestinal tissues became sensitized to oral HRP antigen only in stressed rats.

Figure 2. Effect of sensitization and Ag challenge on mucosal to serosal flux of HRP. Flux of HRP was determined across the jejunal epithelium that was obtained from naive control rats (Con), stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, WAS-only (WAS) rats, and WAS rats pretreated with the CRH antagonist, α -hCRH (α hCRH/WAS/Ag). HRP was measured in serosal samples after addition of 10^{-5} mol/L HRP to the luminal buffer, and fluxes were calculated for three flux periods: 0 to 30 minutes, 30 to 60 minutes, and 60 to 90 minutes. Values are expressed as means $\pm$ SE, pmol/cm²/hour; $n = 4$ rats/group; * $P < 0.05$, compared with Con rats; # $P < 0.05$ compared with values in WAS-only rats in each period.

Transepithelial Antigen Transport Was Enhanced Only in Stressed Rats

The overall rate of transepithelial antigen transport, as indicated by HRP flux across jejunal tissues in Ussing chambers, was significantly greater in WAS/Ag rats compared with WAS rats or naive control rats (Figure 2). EM photomicrographs of mucosal tissues from WAS/Ag rats demonstrated HRP penetrating and between epithelial cells (Figure 3C, arrowheads). Figure 3 also shows many large HRP-containing endosomes (arrows) within enterocytes. Such endosomes were observed in all regions of the cell and some HRP was also identified in the lamina propria (open arrow). However, in photomicrographs of tissues from Con/Ag rats (Figure 3A) and α hCRH/WAS/Ag rats (Figure 3B), there was no HRP visible between cells and only a few HRP-containing endosomes of small size were identified in the apical region of enterocytes. Image analysis revealed that the total area of epithelial HRP endosomes in fixed-size windows was increased significantly ($P < 0.01$) in WAS/Ag rats compared with both naive rats and Con/Ag animals (Figure 3D). These findings are in keeping with the concept that chronically stressed rats developed sensitization to orally delivered HRP and subsequently demonstrated accelerated protein transport across the epithelial barrier.

Inflammatory Cell Numbers Were Increased Only in Gut Mucosa from Stressed Rats

The numbers of inflammatory cells, including mast cells, eosinophils, and mononuclear cells, were significantly greater in intestinal tissues from the six WAS/Ag rats that had been given HRP by gavage 15 days earlier compared with those from the similarly treated six Con/Ag rats or the six naive control rats (Figure 4). In addition, after

Figure 3. Pathways of transepithelial transport of HRP. Jejunal tissues were obtained from naive control rats (Con), stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, WAS-only (WAS) rats, and WAS rats pretreated with the CRH antagonist, α -hCRH (α hCRH/WAS/Ag). All rats except naive controls received a bolus of intragastric HRP at day 5 of the stress/sham protocol; 15 days later the tissues were obtained and processed for Ussing chamber study and electron microscopy. **A–C:** Electron photomicrographs show endosomes containing HRP (arrows) within enterocytes. **A:** Representative section from a Con/Ag rat. **B:** Representative section from a WAS/Ag/ α -hCRH-treated rat. **C:** A representative section from a WAS/Ag rat shows HRP endosomes (arrows) in the epithelial cells, HRP products in the paracellular spaces (arrowheads), and HRP endosome in the lamina propria (open arrow). **D:** Image analysis of the total area of HRP-containing endosomes within a fixed sized window ($300 \mu\text{m}^2$) in enterocytes. Values indicate mean $\pm$ SEM; $n = 6$ rats in each group (12 views per rat section averaged to obtain each rat value).

challenge with HRP antigen in Ussing chambers, mast cells appeared activated (granules empty or reduced in density) only in tissues from rats that had been stressed at the time of first HRP exposure (Figure 5C), whereas mast cells appeared unactivated in tissues from Con/Ag rats (Figure 5A) and α hCRH/WAS/Ag rats (Figure 5B). The percentage of activated mast cells was significantly increased ($P < 0.01$) in gut mucosa of stressed rats compared with those in the control groups (Figure 5D).

Cytokine Profile Was Altered in Stressed Rats

IL-4 was selected to represent Th2 type cytokines and IFN- γ to represent Th1 cytokines. Densitometry showed that there was greater expression of IL-4 mRNA and less expression of IFN- γ mRNA in intestinal mucosa of stressed rats, the difference being significant ($P < 0.01$), when compared with control groups (Figure 6, A and B). Tissue sections showed many more IL-4-positive stained

Figure 4. Inflammatory cells in gut mucosa. Numbers of mast cells (MC), eosinophils (Eo), and mononuclear cells (Mono) were counted in coded sections. Jejunal tissues were obtained from naïve control rats (Con), stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, WAS-only (WAS) rats, and WAS rats pretreated with the CRH antagonist, α -hCRH (α hCRH/WAS/Ag). Tissues were fixed and stained as indicated in Materials and Methods. The results are expressed as number of cells per mm². Bars indicate means $\pm$ SEM; values were obtained from 25 fields per rat (150 per group). * $P < 0.05$, compared with naïve controls. # $P < 0.05$, compared with the rats of WAS group.

cells and less IFN- γ -stained cells in the mucosa of WAS/Ag rats compared with those in Con/Ag rats and naïve control rats (Figure 7, A and B). The expression of epithelial CD23 (both mRNA and protein) was also enhanced only in WAS/Ag rats compared with the control groups (Figures 6C and 7C). Quantified cell counts of IL-4, IFN- γ , and CD23 are shown in Figure 7D.

Specific Anti-HRP IgE Was Detected in Serum from Stressed Rats

The mean titer for anti-HRP IgE in serum was 1:64 (range, 1:16 to 1:256) in serum from rats in the WAS/Ag group sensitized to oral HRP. In contrast, no serum sample from rats in the other groups had an IgE titer greater than 1:2 (the lowest dilution tested).

CRH Antagonism Prevented Stress-Induced Sensitization

Rats treated by intraperitoneal injection of the CRH antagonist before each WAS session did not demonstrate any indications of sensitization to oral HRP. In these rats, there were no functional changes in *I*_{sc} or conductance in response to challenge with luminal antigen (Figure 1, A and B). In addition, the value for the flux of HRP across the tissues was comparable to those of both control groups and significantly less ($P < 0.01$) than in untreated WAS rats (Figure 2A). EM photomicrographs of HRP-challenged tissues showed only occasional endosomes containing HRP in enterocytes and no HRP in paracellular spaces between adjacent epithelial cells (Figure 3B); this finding was confirmed by image analysis (Figure 3D).

There were no increases in numbers of inflammatory cells in the mucosa of WAS rats treated with the CRH antagonist (Figure 2B) and no signs of mast cell activa-

tion after HRP antigen challenge (Figure 4, B and D). In addition, there were no changes in the expression (mRNA or protein) of Th1 or Th2 cytokines in CRH antagonist-treated WAS rats (Figure 5, A and B). In contrast to the elevated expression of CD23 on enterocytes in untreated stressed rats, the expression of this low-affinity IgE receptor was similar to that in control animals. Finally, serum from CRH antagonist-treated WAS rats did not contain HRP-specific IgE despite the fact that these rats received oral HRP during the stress protocol. No serum sample from rats in this group demonstrated a positive passive cutaneous anaphylaxis reaction.

Discussion

To our knowledge, this is the first study showing that the concurrence of chronic stress with a large antigen load in the intestinal tract results in the sensitization of intestinal tissues. Sensitization was demonstrated by both systemic and local responses. These included production of antigen-specific IgE antibodies; antigen-induced intestinal functional changes including stimulated ion secretion and enhanced permeability; recruitment of mast cells (which were activated after antigen challenge), eosinophils, and mononuclear cells to the gut mucosa; a shift in cytokine profiles toward a Th2 profile; and enhanced expression of IgE receptors on intestinal epithelial cells. Antagonism of peripheral CRH receptors, which has been shown to be effective in inhibiting stress-induced antigen uptake, prevented all of the changes associated with sensitization.

The presence of antigen-specific IgE antibodies in the circulation is a commonly accepted criterion for sensitization.³⁷ In our previous studies of food allergy,^{30–35} rodents were sensitized by systemic injection of antigen because oral antigen rarely resulted in positive responses. However, in this study, all of the rats exposed to both WAS and antigen developed HRP-specific IgE titers greater than 1:16 with some values as high as 1:256. In contrast, sham stress rats that received the bolus of antigen but were not stressed had either no positive passive cutaneous anaphylaxis reactions or reactions only at the lowest dilution (1:2). Naïve control rats had no positive passive cutaneous anaphylaxis reactions.

A more sensitive indicator of sensitization of intestinal tissues is the response to antigen challenge directly to the gut mucosa. Previous studies have shown that antigen added to the small intestine results in ion secretion, as indicated by a rise in *I*_{sc} in tissues studied in Ussing chambers.^{13,30–32} This response occurs very rapidly, within minutes after addition of antigen to the luminal surface, despite the fact that the intact antigen must cross the epithelium to reach and activate mast cells located in the lamina propria. Mast cell mediators such as histamine, serotonin, prostaglandins, and proteases mediate the ion secretory response.^{13,32} Evidence has been provided that transepithelial transport of antigen in sensitized rodents is both faster and of greater magnitude because of its protected transcytosis within enterocyte endosomes.³¹ Subsequent to mast cell activation, a sec-

Figure 5. Mast cell activation. Jejunal tissues were obtained from naive control rats (Con), stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, WAS-only (WAS) rats, and WAS rats pretreated with the CRH antagonist, α -hCRH (α hCRH/WAS/Ag). All rats except naive controls received a bolus of intragastric HRP at day 5 of the stress/sham protocol; 15 days later tissues were obtained and mounted in Ussing chambers. Ninety minutes after HRP antigen challenge, tissues were removed and processed for electron microscopy. **A–C:** Electron photomicrographs show mucosal mast cells in the lamina propria. **A** and **B:** Representative sections from a Con/Ag rat (**A**) and rat treated with α -hCRH (**B**) show normal mast cells with electron dense granules. **C:** Representative section from a WAS/Ag rat shows a mast cell with depleted granules. **D:** Percentage of mast cells degranulated after HRP antigen challenge as measured by empty granules or reduced density of granules. Values indicate mean $\pm$ SEM; $n = 6$ rats in each group (20 random views per rat section averaged to obtain each rat value). * $P < 0.05$, compared with Con group; ** $P < 0.05$, compared with WAS-only group.

ond phase of enhanced transepithelial transport of antigen occurs because of loosening of intercellular tight junctions allowing additional intact antigen to move through the paracellular spaces.

Here, we confirmed by HRP flux that the antigen was transported across the epithelium, both more rapidly and in larger quantity, in sensitized stressed rats than in naïve controls or in rats exposed to sham stress. The increased uptake fits the pattern seen in sensitized rats.³¹ Although both stress and antigen challenge increase gut perme-

ability, it is likely that the change was due to sensitization rather than to stress alone because the effects of chronic stress repair within 1 week²⁸ and our studies were conducted 10 days after the last stress session. Therefore, we surmise that the contribution of stress-induced permeability at that time is minimal (by the fact that the conductance of the WAS group did not change significantly and HRP flux was much less than the WAS/Ag group). The pathways of transepithelial antigen transport in WAS/Ag rats visualized 90 minutes after adding HRP to

Figure 6. Message and protein expression of INF- γ , IL-4, and CD23. Jejunal tissues were obtained from naive control rats, stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, and WAS/Ag rats pretreated with the CRH antagonist, α -hCRH. All rats except naive controls received a bolus of intragastric HRP at day 5 of the stress/sham protocol; 15 days later tissues were obtained. **A:** mRNA was purified from jejunal mucosa. Reverse transcriptase-PCR was used to determine mRNA (primers as in Table 1); the ratio of intensity of mRNA bands was normalized to GAPDH. Values indicate mean $\pm$ SEM; $n = 6$ rats in each group. **B:** Numbers of positively stained cells (per mm²) in the lamina propria (for INF- γ and IL-4) or epithelium (for CD23). Values indicate mean $\pm$ SEM; $n = 6$ rats in each group (10 random fields per rat averaged to obtain each rat value).

the luminal buffer included both the endosomal transcellular route and the paracellular route, as would be expected in sensitized rats, since both phases are involved at that time point. In contrast, little endosomal transport and no paracellular transport were evident in the two control groups.

In addition to the excessive uptake of antigen induced by stress, other factors may be involved in the sensitization of WAS/Ag rats. For instance, stress results in skewing of T-cell responses away from Th1 toward Th2 profiles.³⁶ Using the relative expression of INF- γ (a typical Th1 cytokine) versus IL-4 (a typical Th2 cytokine), we confirmed this trend using both RNA message and protein expression. In addition, by immunohistochemistry we identified greatly enhanced expression of CD23, the low-affinity IgE receptor, on the apical surface of enterocytes in WAS/antigen exposed rats. CD23 expression on B cells, particularly the b isoform, is up-regulated by IL-4.³⁸ We recently reported that the b isoform of CD23, including some unique splice variants, is expressed on intestinal epithelial cells in sensitized rodents and is up-regulated by IL-4.^{34,39} In studies involving blocking antibodies and gene-deficient mice, CD23 was identified as critical for the protected transepithelial transport of IgE-antigen

Figure 7. Immunohistochemistry of jejunal mucosa showing protein expression of INF- γ , IL-4, and CD23. Representative sections include tissues from Con/Ag rats (**A1–C1**) and WAS/Ag rats (**A2–C2**). **A:** Representative section showing cells in the lamina propria stained for INF- γ . **B:** Representative section showing cells in the lamina propria stained for IL-4. **C:** Representative section showing epithelial cells and cells in the lamina propria stained for CD23. **D:** Counts of positive stained cells in jejunal tissues that were obtained from naive control rats (Con), stressed (WAS/Ag) rats, sham-stressed (Con/Ag) rats, WAS-only (WAS) rats, and WAS rats pretreated with the CRH antagonist, α -hCRH (α -hCRH/WAS/Ag). The results are expressed as number of cells per mm². Bars indicate means $\pm$ SEM; values were obtained from 25 fields per rat (150 per group). * $P < 0.05$, compared with naive controls. # $P < 0.05$, compared with the rats of WAS group.

in endosomes. Taken together, our results suggest that the enhanced endosomal transport of antigen in WAS-sensitized rats was related to elevated CD23 expression on enterocytes induced by IL-4.

Altered host response to bacteria may have also played a role in sensitization. In our previous studies of the effects of chronic psychological stress, we docu-

mented abnormal adherence of microbes to the apical surface of enterocytes in both the small and large bowel, as well as penetration of some organisms through the epithelium.²⁹ The presence of microbes or microbial factors in the lamina propria could alter innate or adaptive immune responses. For example, certain bacterial toxins such as pertussis toxin stimulate IL-4 production.⁴⁰ In addition, lipopolysaccharide potentiates the IL-4-stimulated expression of IgE receptors on human epithelial cells *in vitro*.⁴¹ Mast cells express Toll-like receptors and thus can react to microbial products to increase the output of cytokines and other mediators.⁴² In fact, mast cells are identified as a common factor linking stress, food allergies, and IBS,⁴³ because increased numbers of mast cells are present in IBS gut mucosa and a significant proportion of individuals with IBS report adverse reactions to foods.^{44,45} Finally, probiotics have been shown to be beneficial in treating or preventing the development of food allergies,⁴⁶ although the underlying mechanisms responsible for their beneficial effects have not yet been identified.

In earlier studies examining the mechanisms involved in the stress-induced increase in epithelial permeability, we found that the pathways involve neuroendocrine factors.²⁷ In particular, the effect of stress on enhancing macromolecular transepithelial transport was mimicked by CRH injected peripherally in naïve rats and inhibited by α -hCRH, a non-(receptor subtype) specific antagonist that does not cross the blood-brain barrier. A recent report indicates that human mast cells express CRH receptors;⁴⁷ these data suggest that psychological stress may activate mast cells via liganding CRH receptors to release inflammatory mediators that may be associated the subsequent intestinal pathophysiological changes. Therefore, we used this antagonist in the current study to obtain further evidence for stress-enhanced permeability as a key factor in the sensitization of rats to oral antigen. All of the indicators of sensitization described above were within the normal range in WAS rats pretreated before each stress session with the CRH antagonist.

In summary, these studies demonstrate that chronic psychological stress in a genetically stress-susceptible host results in sensitization of intestinal tissues to a specific antigen present in excess within the intestinal tract. On subsequent antigen challenge, mast cells become activated and may be associated with the resulting intestinal pathophysiology. These studies indicate that stress-enhanced gut permeability has significant consequences that can contribute to the initiation of disorders involving replacement of oral tolerance with immunological or inflammatory responses to luminal antigens.

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