Mechanisms of innate immune activation by gluten peptide p31-43 in mice

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Submitted 9 December 2015; accepted in final form 2 May 2016

Mechanisms of innate immune activation by gluten peptide p31-43 in mice. Am J Physiol Gastrointest Liver Physiol 311: G40–G49, 2016. First published May 5, 2016; doi:10.1152/ajpgi.00435.2015.—Celiac disease (CD) is an immune-mediated enteropathy triggered by gluten in genetically susceptible individuals. Innate immunity contributes to the pathogenesis of CD, but the mechanisms remain poorly understood. Although previous in vitro work suggests that gliadin peptide p31-43 acts as an innate immune trigger, the underlying pathways are unclear and have not been explored in vivo. Here we show that intraluminal delivery of p31-43 induces morphological changes in the small intestinal mucosa of normal mice consistent with those seen in CD, including increased cell death and expression of inflammatory mediators. The effects of p31-43 were dependent on MyD88 and type I IFNs, but not Toll-like receptor 4 (TLR4), and were enhanced by coadministration of the TLR3 agonist polyinosinic:polycytidylic acid. Together, these results indicate that gliadin peptide p31-43 activates the innate immune pathways in vivo, such as IFN-dependent inflammation, relevant to CD. Our findings also suggest a common mechanism for the potential interaction between dietary gluten and viral infections in the pathogenesis of CD.

celiac disease; innate immunity; p31-43; polyinosinic:polycytidylic acid; small intestine

CELIAC DISEASE (CD) is a multifactorial disorder triggered by the ingestion of gluten in susceptible individuals who carry the HLA-DQ2 and/or HLA-DQ8 predisposing alleles. Both innate and adaptive immune mechanisms are involved in the pathogenesis of CD. Whereas the adaptive immune response has been well studied, less is known about innate mechanisms and their triggers (1). Some gluten-derived peptides could initiate this process, but there is not enough in vivo experimental evidence to confirm this hypothesis. The study of whether and how innate immune mechanisms are induced by gluten peptides is relevant to CD pathophysiology.

Several nonimmunogenic gluten peptides that stimulate innate immune responses (termed toxic peptides) but not the adaptive immune response have been proposed. Studies have shown that a mix of gluten peptides or pepsin-trypsin-digested gliadin (FT-gliadin) can activate dendritic cells (26) and peripheral blood mononuclear cells (16) in vitro. However, the peptides responsible have not been identified. The most studied toxic peptide is the derived α-gliadin p31-43 (LGQQQPPFP-QQQPY) that is part of the longer peptide p31-55 (LGQQQPPFPQQQPYQFPQFPQSQQPY) and is resistant to digestive enzymes in the gut (19). Increased IL-15 production and enterocyte apoptosis were reported in duodenal biopsies of patients with CD incubated with p31-43 (18). p31-43 was also shown to interact with epidermal growth factor receptor (EGFR) (5) and with the IL-15/IL-15R complex (6, 24) to affect proliferative activity in intestinal biopsies, influence human fibroblasts (23), and induce oxidative stress and endosome maturation in enterocytes (17). In murine tissues, p31-43 induced proinflammatory cytokines by macrophages (34). Altogether, these studies suggest a role for p31-43 in the stimulation of innate immune mechanisms in CD. However, the underlying pathways and in vivo relevance remain unclear.

Type I IFNs are thought to play a role in CD pathogenesis, as there is increased expression of IFN-α in duodenal mucosa from patients with CD (22) and blockade of IFN-α inhibits gliadin-induced IFN-γ expression in ex vivo experiments (28). Furthermore, epidemiological studies suggest that enteric viral infections such as rotavirus might trigger inflammatory or functional gastrointestinal disease (20, 35). The aim of this study was to determine whether p31-43 elicits innate immune activation in murine small intestine in vivo and to investigate potential underlying pathways. We also analyzed the effect of combined intraluminal administration of p31-43 and polyinosinic:polycytidylic acid (poly I:C), which mimics a viral infection, a proposed trigger of CD.

MATERIALS AND METHODS

Mice. Eight-week-old male C57BL/6J mice were purchased from the Animal Care Facility of the Facultad de Ciencias Exactas y Naturales of the Universidad de Buenos Aires. Eight-week-old male MyD88 KO [B6.129P2(SJL)-Myd88tm1.1Defr/J] mice were purchased from the Jackson Laboratory. IFNαR knockout (KO) mice (IFNαR−/−, IFNαRΔ−−) on C57BL/6 background were kindly provided by M. Albert (Institute Pasteur, Paris, France). Eight-week-old male C3H-HeJ mice were kindly provided by Dr. Martin Rumbo from Instituto de Estudios Inmunológicos y Fisiopatológicos (IIFP-CONICET, Buenos Aires, Argentina). Mice were housed in a specific pathogen-free condition and fed ad libitum with balanced food and autoclaved water. They were maintained on a 12-h:12-h light/dark cycle and acclimatized to the surrounding conditions for 1 wk before the experimental procedures. All the studies were performed in accordance with international protocols for laboratory animal care (Canadian Council on Animal Care). Experiments were conducted with approval from the Institutional Animal Care and Use Committee of the Facultad de Ciencias Exactas, Universidad Nacional de La Plata.
Intraluminal administration of peptides and poly I:C. p31-43 peptide (LGQQQFPFFQPY, Biomatik), nonrelated peptide (NRP) (LD-PLIRGLLARPACALQV, Think Peptides), poly I:C (Sigma Aldrich), tridecapeptide (LGQQQPFPPQQPY, Biomatik), nonrelated peptide (NRP) (LD-PLIRGLLARPACALQV, Think Peptides), poly I:C, and 10 mg/kg xylazine. Once asleep, 100 μl of 100 μg/ml peptide solution in PBS, 30 μg/g poly I:C solution, or a combination of p31-43 and poly I:C were injected into the small intestinal lumen, 2 cm below the pylorus, to avoid degradation by pancreatic enzymes. Control mice received PBS. After surgery, fluid replacement was administered, and mice were monitored until recovery. C57BL/6 mice were killed 2–72 h posttreatment, whereas C5H-HeJ, IFNαR KO, and MyD88 KO mice were killed 12 h posttreatment.

To compare the effects of p31-43, poly I:C, and p31-43 + poly I:C in C57BL/6 mice, histological evaluation was performed at 72 h posttreatment. This time point was chosen based on the previous finding indicating significant differences between treatments at this time.

Histological evaluation. Sections of proximal small intestine of treated mice were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation using a Nikon Eclipse Ti fluorescence microscope with X-Cites Series 120 Q light source. Images were taken with Nikon Digital Sight DS Ri1 camera using Nis-Elements software, and measurements were performed using Image J software.

Two sections of the proximal small intestine were scored for inflammation in a blinded fashion, with at least 30 villi-to-crypt (V/C) ratios assessed in each mouse. Intraepithelial lymphocytes (IELs) per 30 enterocytes in 10 randomly chosen villus tips were counted according to previously described methods and expressed as IELs/100 enterocytes (7). Histological scores were obtained following the Park-Chiu criteria (27): 0, normal mucosa; 1, subepithelial space layer injury; 7, transmucosal infarction; 8, transmural infarction. villi, dilated capillaries; 5, disintegration of lamina propria; 6, crypt formation; 9, villus shortening and widening. At 12 h post p31-43 administration, we observed shortening and widening of villi, changes in small intestinal mucosa. At 12 h post p31-43 administration, we observed shortening and widening of villi, changes in small intestinal mucosa.

RESULTS

Intraluminal p31-43 peptide induces pathological changes in the murine small intestine. We used a previously developed technique to deliver molecules of interest intraluminally (3) and tested the capacity of p31-43 to induce morphological changes in small intestinal mucosa. At 12 h post p31-43 administration, we observed shortening and widening of villi, increased cell infiltration in the lamina propria, and edema. Administration of PBS or NRP did not cause intestinal damage (Fig. 1A). At this time point, we also observed reduction in V/C ratios, increased IEL counts, and higher histological scores in mice treated with p31-43 compared with PBS and NRP (Fig. 1B). At 72 h, mice treated with p31-43 exhibited persistent edema and cellular infiltration in the lamina propria (Fig. 1A), reduced V/C ratios, increased number of IELs, and a higher histological score compared with PBS- and NRP-treated mice (Fig. 1B). Although the surgical procedure itself altered intestinal histology transiently (3), PBS- and NRP-treated (controls) experienced faster recovery than p31-43-treated mice (Fig. 1B). We also evaluated the proliferative activity in

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<td>TNFa</td>
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<td>CTGCTCTGATGTTAACCTGG</td>
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<td>MCP1</td>
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<td>Bcl-3</td>
<td>GATGCCTTGTTGGTGTAC</td>
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FACSCalibur flow cytometry (BD Bioscience), and data were processed using CELLQuest (BD Bioscience) software.

Confoocal microscopy. Small intestinal sections were deparaffinized and treated with Antigen Retrieval AR-10 Solution (BioGenex). After the sections were blocked with 2% goat serum, a primary antibody was added for 1 h. Anti-Ki-67 antibody (Novus Biologicals) was added at 4°C, and Alexa488 goat anti-rabbit antibody (Molecular Probes) was added at 10 μg/ml for 1 h. Anti-cleaved caspase-3 antibody conjugated to FITC (Cell Signaling) was added for 1 h at RT. Nuclei were stained with propidium iodide at 1 μg/ml for 15 min. Images were obtained and analyzed in a TCS SP5 Confocal Microscope combined with Leica LAS AF software.

TUNEL reaction. Cell death was quantified using the In Situ Cell Death Detection Kit (Roche). Paraffin-embedded small intestinal tissue sections were dewaxed, rehydrated, and treated with Proteinase K for permeabilization. TUNEL reaction mixture was then added, and samples were analyzed by confocal microscopy. Images were taken from a confocal microscope Olympus FV1000 using a ×20 NA 0.75 objective and a zoom of ×2. A 473-nm solid-state laser was used to detect apoptotic cells, whereas a 405-nm state laser was used to identify nuclei stained with DAPI. Images were analyzed with ImageJ software.

Statistical analysis. Statistical analysis was performed with GraphPad Prism software. When two groups were compared, an unpaired t-test was used. When more than two groups were compared, a one-way ANOVA test was used; P < 0.05 was considered significant. Data are displayed as means ± SE.
small intestinal crypts by counting Ki-67+ epithelial cells. At 12 h, proliferative activity was significantly higher in p31-43-than in PBS-treated mice (Fig. 2A).

**Intraluminal p31-43 increases mRNA expression of inflammatory cytokines.** We next explored the expression of proinflammatory mediators induced by p31-43. Compared with PBS-treated mice, there was a rapid and marked increase in IFN-γ mRNA 2 h after p31-43 treatment, followed by increases in CXCL10 and IFN-β mRNA, which peaked 6 h after intraluminal administration of p31-43 (Fig. 2B). The expression of mRNA for IL-15, IL-18, IL-1β, IL-6, TNF-α, and chemokines such as MCP1, CXCR3, and CXCL2 was similar in all groups (data not shown).

**Intraluminal p31-43 induces cell death in the mucosa.** Cell death by gluten-specific and -nonspecific cytotoxic mechanisms plays a role in intestinal damage in CD (31). To study whether p31-43 has cytotoxic activity, we examined TUNEL staining of small intestinal sections 12 h after treatment. This revealed a large increase in the number of TUNEL-positive cells in the lamina propria compared with PBS-treated mice (Fig. 3A). TUNEL-positive cells were also found in the epithelium of p31-43-treated but not in PBS-treated mice (white arrows, Fig. 3A). Automated counting confirmed an increase in the frequency of total TUNEL-positive cells, when both the lamina propria and epithelium of p31-43-treated mice were analyzed (Fig. 3B). The expression of anti- and proapoptotic mediators, Bcl2 and Bax, respectively, was evaluated by qPCR analysis of whole small intestinal mucosa. At 12 h, we found increased Bax/Bcl2 ratio in p31-43-treated mice compared with PBS-treated controls (Fig. 3C), suggesting that p31-43 has a proapoptotic effect in the small intestine in vivo. Mice treated with p31-43 had increased numbers of TUNEL-positive cells in epithelium compared with PBS-treated control mice (Fig. 3D). A similar trend was seen in lamina propria alone although this did not attain statistical significance. To further explore the hypothesis that p31-43 caused death of epithelial cells, we first...
used qPCR analysis of isolated epithelial cells, which showed a trend toward an increase in the Bax/Bcl2 ratio in intraepithelial cells (IEC) from p31-43-treated mice. Although this difference did not reach statistical significance (Fig. 3E), flow cytometry showed an increased number of annexin V-positive/propidium iodide-positive IECs from mice treated with p31-43 (Fig. 3F).

Mucosal changes induced by p31-43 are MyD88 and type I IFN dependent but not Toll-like receptor 4 dependent. To investigate possible signaling pathways that might mediate the effects of p31-43, we used MyD88 KO, IFNαR KO, and Toll-like receptor 4 (TLR4)-deficient (C3H-HeJ) mice. No histological changes were observed in MyD88 KO mice 12 h after administration of p31-43 (Fig. 4A). There were no differences in V/C ratio, IEL counts, global histological scores (Fig. 4B), or in cell death analysis (Fig. 4C) between p31-43- and PBS-treated mice. However, TLR4-deficient C3H-HeJ mice had decreased V/C ratios, increased IELs counts, and increased global histological scores after administration of p31-43 (Fig. 4D). The effects of p31-43 were absent in IFNαR KO (Fig. 4E).

p31-43 and poly I:C cause mucosal damage via independent mechanisms. Intraluminal administration of poly I:C, a synthetic analog of dsRNA that mimics the innate response to viral infection acting via TLR3 receptor, induces mucosal damage (3). We therefore investigated the effect of intraluminal administration of p31-43 and poly I:C on mucosal damage. On the basis of previous work that determined an optimal time point for the induction of intestinal damage and inflammation with poly I:C and reduced effect of surgery at 72 h (3), we used this time point to evaluate the combined effect of p31-43 and poly I:C. As expected, p31-43-treated mice had reduced V/C ratios compared with control mice at 72 h (Figs. 1C and 5A), but poly I:C alone or the combination of p31-43 + poly I:C had a more pronounced decrease in V/C ratios (Fig. 5A).

p31-43 and poly I:C induce distinct patterns of inflammatory mediators. The analysis of mRNA at different time points after treatment showed distinct patterns of expression for the proinflammatory cytokines IFN-α, IFN-β, and CXCL10 in p31-43-, poly I:C-, or p31-43 + poly I:C-treated mice. Increased expression of IFN-β in the mucosa was found 2 h after treatment with poly I:C, whereas this increase was only noted 4 h after p31-43 + poly I:C treatment. Induction of IFN-β was modest and delayed until 6 h after treatment with p31-43 alone. TNF-α expression was increased by poly I:C or p31-43 + poly I:C but not by p31-43 treatment. Consistent with previous results, p31-43 induced IFN-γ expression, which was not observed in p31-43 + poly I:C-treated or poly I:C-treated mice. Poly I:C is a strong inducer of CXCL10 (8), which was also upregulated by p31-43 + poly I:C treatment, whereas CXCL10 induction by p31-43 was weaker and delayed. A synergistic effect of p31-43 + poly I:C was only observed for CXCL2 and MCP1 (Fig. 5B). Altogether, these results suggest that mucosal dam-
age caused by p31-43 and poly I:C may employ different pathways, which can interact in a complex fashion.

Poly I:C enhances cell death induced by p31-43. Treatment with p31-43 led to increased cell death in the intestinal mucosa as assessed by TUNEL staining (Fig. 3), and this was further increased in mice given p31-43 + poly I:C together. However, poly I:C alone had no effect on cell death (Fig. 6A). Confirming our previous findings, p31-43 alone also induced a proapoptotic pattern of Bax/Bcl2 ratio 12 h after treatment, but this was not seen in mice receiving poly I:C alone or in combination with p31-43 (Fig. 6B). On the other hand, treatment with p31-43 + poly I:C induced a marked increase in the number of cleaved-caspase 3-positive cells in lamina propria compared with mice treated with PBS, poly I:C, or p31-43 alone (Fig. 6C). As caspase 3 is central to both the intrinsic and extrinsic pathways of apoptosis, these results suggest that p31-43 + poly I:C is a stronger stimulus for cell death than poly I:C or p31-43 alone and that the pathways involved may be different.

**DISCUSSION**

In this study, we found that intraluminal administration of p31-43 reduced V/C ratio, increased IEL infiltration, and led to higher histological scores in wild-type (C57BL/6) mice. p31-43 caused an inflammatory response in the small intestine, characterized by elevation of IFN-γ expression followed by elevations in IFN-β and CXCL10. p31-43 also induced cell death in epithelial cells. Treatment with p31-43 in mice lacking TLR4 induced similar morphological changes than in wild-type but not in mice lacking the MyD88 molecule. The results indicate a direct proinflammatory effect of p31-43 in vivo that requires the central adaptor of the TLR pathway, MyD88, but is independent of TLR4. Finally, we demonstrated that the mucosal damage induced by p31-43 is type I IFN dependent.

There is controversy on the potential induction of the innate immune response by gliadin peptides. Critics are based on the lack of specific receptor identification and reports on in vivo effects. p31-43 has been shown to trigger inflammation using cell lines and duodenal biopsies, whereas instillation of p31-49 into the duodenum of treated patients with CD led to reduced V/C ratios and increased IEL counts within 4 h after administration (4, 33). Others have shown that chemokines IP-10 (CXCL10) and MCP-5, which recruit monocytes and T cells, were increased in vitro by p31-43 (34), as well as cell proliferation and proapoptotic activity (4, 6, 12). In this study, we provide evidence for in vivo innate immune stimulation and apoptosis by p31-43. We found that intraluminal p31-43 stimulated a broad spectrum of proinflammatory genes such as IFNγ, CXCL10, and IFNβ, increased the number of Ki-67-positive cells in crypts of C57BL/6 mice, and increased cellular death in lamina propria and in epithelial cells. A high number of TUNEL-positive cells was found in p31-43-treated mice, which was associated with a proapoptotic profile (high Bax/
Bcl2 ratio). Finally, cell death evaluated by qPCR (Bax/Bcl2 ratio), fluorescence microscopy (TUNEL reaction), and flow cytometry (annexin V/propidium iodide) indicated that p31-43 may induce enterocyte death in vivo.

Some previous studies demonstrated that PT-gliadin induced proinflammatory genes in a MyD88-dependent manner but a TLR2- and TLR4-independent manner (34), whereas others showed that gliadin-derived peptides increased inflammatory mediators through TLR4/MyD88/TRIF/NFkB and NLRP3 inflammasome pathways (25). Although these findings suggest that innate response via TLR signaling and inflammasome can be elicited by gliadin peptides, p31-43 was not specifically evaluated. Type I IFNs play a critical role in our experimental model, as p31-43 induced the expression of type I IFNs in vivo, and its effects on intestinal pathology were absent in IFNαR KO mice. Type I IFNs have been suggested as early mediators of CD pathogenesis, and MxA, a downstream element of the type I IFN pathway, has been reported to be increased in duodenal biopsies of untreated patients with CD (13). Although it is not known what factors might drive the induction of type I IFNs in patients at risk of CD, viral infection is an obvious potential candidate (15, 31, 32). A role for type I IFNs might also overlap with the proposed involvement of IL-15 in CD (15), as, although these mediators activate different downstream pathways, IL-15 upregulation during experimental virus infection depends on IFNαR signaling (11).

Our data suggest that p31-43 and viral infection could act in synergy to induce the innate immune responses such as IL-15 production thought to be critical for the initiation of tissue pathology in CD. To test whether pathways induced by p31-43 and other stimuli synergize to worsen the innate immune response, we employed a poly I:C model (3). We observed distinct proinflammatory patterns in mice with p31-43, poly I:C, or p31-43/poly I:C treatment. Poly I:C alone increased IFN-β, TNF-α, and CXCL10. p31-43 alone induced IFN-β and CXCL10 at lower levels, and it was the only stimulus that rapidly increased IFN-γ. The combination of p31-43 and poly I:C increased IFN-β, TNF-α, and CXCL10 and was the only stimulii that increased CXCL2 and MCP-1. CXCL10, CXCL2,
and MCP-1 are relevant for the recruitment of T cells, polymorphonuclear cells, and monocytes.

Analysis by TUNEL staining, Bax/Bcl2 ratio, and cleaved caspase-3 suggests that a proapoptotic pathway is involved in the increased cell death observed in p31-43-treated mice. In contrast, poly I:C treatment did not induce a significant increase in any of these parameters, perhaps indicating that the histological damage caused by these stimuli may be driven by different pathways. As well as cell apoptosis, mechanisms such as metalloprotease- and TGF-β-induced fibrosis can all contribute to tissue pathology, and these may be induced differentially by individual triggers. Further support for complexity in the pathogenic processes could come from our finding that, in p31-43 + poly I:C-treated mice, the number of TUNEL-positive cells and of cleaved caspase-3-positive cells was increased, but there was no change in Bax/Bcl2 ratio. Because caspase-3 can be activated by both intrinsic and extrinsic apoptotic pathways, but also can be cleaved by Granzyme B (10), this may explain why in p31-43 + poly I:C-treated mice cleaved caspase-3-positive and TUNEL-positive cells were increased but not the proapoptotic ratio. Together, our results suggest that distinct or partially overlapping pathways of tissue damage may be induced by p31-43 and poly I:C.

The adaptive immune response in CD is necessary for the development of the disease; however, it is now clear that it is insufficient to cause full intestinal pathology (21). Cytotoxic
activity of IELs has been considered as a key element for enterocyte damage. Although increased number and activation of IELs are a hallmark of CD, how these cells are induced and activated is still a matter of discussion. Setty et al. (30) have recently suggested that epithelial stress and antigluten adaptive immune responses can be independently induced at early stages of the disease. In accordance with these results, previous reports from our group revealed the presence of epithelial stress in active CD (2). Altogether, the results raise the hypothesis that, by activating innate immunity, peptides such as p31-43 may lead to epithelial stress, a condition that together with the adaptive immune response would facilitate the development of enteropathy in CD. It remains to be determined whether this mechanisms could also have implications for other gluten-related disorders such as non-celiac gluten sensitivity (29). Our work shows that induction of inflammation by nonimmunogenic peptides depends on MyD88, but not TLR4, signaling. In contrast, wheat amylase-trypsin inhibitors have been identified as potent stimulators of an inflammatory reaction through activation of TLR4 signaling on monocytes, macrophages, and dendritic cells (14). Therefore, it is possible that nonimmunogenic gluten peptides and nongluten proteins in wheat induce inflammation through different pathways, facilitating the onset of CD and other intestinal inflammatory diseases.

In summary, in vivo inflammatory changes driven by p31-43 and poly I:C occur through different pathways, as judged by the kinetics of the mucosal damage and histological recovery. Although the receptor for p31-43 has not been identified yet, different cells can produce inflammatory mediators after incubation with this peptide. Because HLA-DQ2 or DQ8 molecules do not present p31-43 and the mucosal changes observed are MyD88 and type I IFN dependent, future work should determine the effect of p31-43 in other genetically modified mouse strains. Signals triggered by gliadin-derived peptides, particularly p31-43, in addition to those elicited by certain infections, may exacerbate inflammation, promoting the development of intestinal pathology in a genetically susceptible individual.
CHARACTERIZATION OF THE MUCOSAL IMMUNE RESPONSE TO GLIADIN IN COELIAC DISEASE

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Abstract

Background: Understanding the mucosal immune response to gliadin in celiac disease (CD) is critical for the development of new anti-inflammatory therapies.

Methods: We used quantitative reverse transcriptase PCR and flow cytometry to analyze mucosal immune responses to gliadin in the small intestine of DQ8 mice.

Results: Immunocompetent CD mice had a more robust mucosal immune response to gliadin, compared to naïve mice. The CD mice showed a stronger innate immune response and a more robust Th1 response, as reflected in increased levels of IFN-γ, IL-12, and IFN-α. The CD mice also had a more pronounced reduction in mucus production, as indicated by decreased MUC2 expression.

Conclusion: Our findings suggest that the mucosal immune response to gliadin in CD is characterized by a stronger innate immune response and a more robust Th1 response.
