NHE3 modulates the severity of colitis in IL-10-deficient mice

C. B. Larmonier,1* D. Laubitz,1,3* R. D. Thurston,1 A. L. Bucknam,2 F. M. Hill,1 M. Midura-Kiela,1 R. Ramalingam,1 P. R. Kiela,1,3* and F. K. Ghishan1*

1Department of Pediatrics, Steele Children’s Research Center, 2Department of Immunobiology, University of Arizona Health Sciences Center, Tucson, Arizona; and 3Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Submitted 24 February 2011; accepted in final form 11 March 2011


© 2011 the American Physiological Society http://www.ajpgi.org

DIARRHEA. Observed commonly in inflammatory bowel disease (IBD), is a direct result of perturbations in colonic absorptive and secretory processes (4, 21). Electroneutral Na+/H+ exchange (NHE), mediated primarily by NHE3, plays a critical role in intestinal Na+ and water absorption, regulation of intracellular pH, and acidity of the apical microenvironment, and its inhibition is believed to significantly contribute to IBD-associated diarrhea. NHE3 is a target of proinflammatory cytokines such as TNFα and IFNγ, major proinflammatory cytokines associated with intestinal inflammation (1, 21), as well as of the enteropathogenic bacteria (13). In vivo, NHE3 activity and transport function are severely impaired in different models of spontaneous colitis, such as IL-2 knockout (KO) and IL-10 KO mice, with negligible changes in NHE3 gene expression (2, 27). In a model of systemic T cell activation driven by anti-CD3 injection, TNFα was shown to simultaneously inhibit NHE3-mediated Na+ transport and promote myosin light chain kinase-mediated barrier dysfunction (5). NHE3 expression has been shown to be significantly downregulated (>87% decrease) in the mucosa of patients with active IBD (30). Other recent studies with biopsy specimens obtained from control and ulcerative colitis patients demonstrated a significant impairment of colonic NHE3 activity without changes in mRNA or protein expression (11, 34).

Chronic NHE3 inhibition in intestinal inflammation may have consequences beyond alteration of ion transport. There is a significant upregulation of IFNα and IFNγ-inducible genes in the jejunal mucosa of NHE3-deficient mice (18, 32), with the infiltrating CD8+ and asialoGM1+ NK cells as the primary sources of the cytokine (16). Moreover, NHE3-deficient mice spontaneously develop colitis restricted to distal colonic mucosa (18). NHE3−/− mice housed in a conventional facility exhibited phenotypic features such as mild diarrhea, occasional rectal prolapse, and reduced body weight. Furthermore, NHE3−/− mice display alterations in epithelial gene and protein expression patterns that predispose them to a high susceptibility to dextran sulfate sodium (DSS), with accelerated mortality resulting from intestinal injury and bleeding, hypovolemic shock, and sepsis (16). Our studies suggest that NHE3 participates in mucosal responses to epithelial damage, acting as a modifier gene determining the extent of the gut inflammatory responses in the face of intestinal injury.

The aim of this study was to investigate the contribution of NHE3 to the development of colitis, immune activation, and mucosal homeostasis in IL-10-deficient mice, an established Crohn’s disease (CD) model of unrestrained activation of a T-helper (Th)-1/Th-17-mediated immune response (8, 33). Severe IBD has been described to be more prominent in patients with IL-10 receptor deficiency. We created double-KO mice lacking both IL-10 and NHE3, which represents a conjunction of several key factors implicated in the pathogenesis of IBD: genetic predisposition (22), loss of immune tolerance to colonic commensal bacteria (24, 28), and defective intestinal NHE with the ensuing consequences (16, 18). Examination of proximal and distal colon established important histological alterations, such as a dramatically increased neutrophil and mononuclear cell infiltration in NHE3×IL-10 double-KO mice compared with single-KO littermates. Leukocyte infiltration was accompanied by elevated expression of macrophage inflammatory protein-2 (MIP-2) and keratinocyte chemoattractant (KC) neutrophil chemokines and the IFNγ-inducible T cell chemokines CXCL10 and CXCL11. Colonic IFNγ and IL-17

* C. B. Larmonier and D. Laubitz contributed equally to this work. P. R. Kiela and F. K. Ghishan share senior authorship.

Address for reprint requests and other correspondence: F. K. Ghishan, Dept. of Pediatrics, Steele Children’s Research Center, Univ. of Arizona Health Sciences Center, 1501 N. Campbell Ave., Tucson, AZ 85724 (e-mail: fghishan@peds.arizona.edu).
protein secretion was significantly higher in double-KO mice. We also documented a significant increase in the number of apoptotic colonic epithelial cells (CECs) compared with single-KO mice. These results strongly suggest the importance of NHE3 in the maintenance of intestinal epithelial homeostasis and in modulating the inflammatory response in IL-10-deficient mice.

**MATERIALS AND METHODS**

*Experimental animals.* SLE9a3-deficient (NHE3−/−) mice on a mixed genetic background (129/Black Swiss) (26) were obtained from Dr. Gary Shull (University of Cincinnati, Cincinnati, OH). They were backcrossed for >10 generations into the 129/SvEv background and then bred to IL-10−/− (also 129/SvEv) mice obtained from the laboratory of Ballouf Sartor (University of North Carolina at Chapel Hill, Chapel Hill, NC). The mice were maintained in the animal facility at the University of Arizona Health Sciences Center as described previously (18). All mice were maintained in identical conditions as one colony. Wild-type (WT) mouse mice are homozygous IL-10−/− or NHE3−/− (single-KO) mice, and compound-homozygous mice are IL-10−/−/NHE3−/− (double-KO) mice. Sentinel mice were routinely monitored and determined as free from common murine pathogens (mouse hepatitis virus, mouse parvovirus, minute virus, Theiler’s murine encephalomyelitis, Mycoplasma pneumoniae, Sendai, epizootic diarrhea of infant mice, mouse minute virus, and endoparasites). All animal protocols and procedures were approved by the University of Arizona Animal Care and Use Committee.

*Histology and scoring.* Proximal and distal colons from WT, NHE3−/−, IL-10−/−, and NHE3−/−IL-10−/− mice were harvested and fixed in 10% neutral buffered formalin (Fisher Scientific, Tustin, CA). Fixed tissues were embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin for light-microscopic examination. Sections were graded according to previously published criteria (17) by a veterinary pathologist blinded to the study design.

*Hematology.* Hematological profile analysis with complete differential was done by an experienced pathologist at the University Animal Care Pathology Services (University of Arizona) using the Hemavet 850 Mascot (Drew Scientific, Farmington, CT).

*Immunohistochemistry.* Sections of proximal and distal colon were prepared as described above. After deparaffinization and rehydration, antigen retrieval was performed by heating slides in citrate buffer (10 mM sodium citrate and 0.05% Tween 20, pH 6.0). Slides were washed in PBS, and residual endogenous peroxidase activity was quenched by incubation in 3% H2O2 in water for 10 min. Slides were then incubated for 1 h in normal goat serum blocking buffer (KPL, Gaithersburg, MD) or normal rabbit serum blocking buffer (Vector Laboratories, Burlingame, CA), depending on the secondary antibody.

Next, sections were incubated with a primary antibody against cleaved caspase-3 (1:50 dilution; Cell Signaling Technology, Danvers, MA), which binds to the active (cleaved) form of the molecule, as determined by 10.220.32.247 on April 19, 2017 http://ajpgi.physiology.org/ Downloaded from
Fig. 1. Histology of proximal and distal colon of wild-type (WT), IL-10−/−, NHE3−/−, and IL-10−/−/NHE3−/− mice. A: histological analysis (hematoxylin-eosin staining) of colon morphology in WT, IL-10−/−, NHE3−/−, and IL-10−/−/NHE3−/− mice. Sections were scored on a scale of 0–5, based on degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, and architectural distortion, by an unbiased pathologist according to previously described criteria (17).

B and C: penetration of colitis, expressed as percentage of mice with histological score ≥2, in proximal and distal colon.
changes. IL-10−/− mice showed signs of colonic inflammation consistent with those described by others (7). Proximal and distal colon were affected, with degenerative lesions and excessive regenerative mucosal hyperplasia, leading to a marked thickening of the intestinal wall, accompanied by marked mixed leukocytic infiltrate in the lamina propria. Colonic sections were evaluated in a blinded manner by a veterinary pathologist according to the previously published criteria (17), taking in consideration lymphocytic and neutrophilic infiltration, crypt hyperplasia, mucosal ulcerations, focal or multifocal transmural necrosis, and penetration of the colonic wall. Double-KO mice exhibited a worsening of the inflammation pattern in distal and proximal colon (Fig. 1A). We observed significantly increased penetration of the disease, with significantly more mice exceeding the threshold of inflammation arbitrarily set to 2 (Fig. 1, B and C).

**Hematologic parameters in WT, single-KO, and double-KO mice.** To further characterize the contribution of NHE3 deficiency in IL-10 KO mice, basic hematologic parameters were evaluated. Although the neutrophil count was elevated in IL-10−/− and double-KO mice compared with WT or NHE3−/− mice, the overall white blood cell count showed only a tendency for increase, without reaching statistical significance (Fig. 2A). A small, but significant increase in the numbers of erythrocytes was observed in IL-10−/− mice but was normalized in double-KO mice. No significant differences in monocyte, basophil, or eosinophil counts were observed between groups (Fig. 2B). Hematologic parameters of the double-KO mice did not demonstrate any significant difference from homozygous single-KO mice.

**Increased neutrophil and mononuclear cell recruitment and related chemokine expression in the colon of IL-10−/−NHE3−/− mice.** Immunohistochemical staining of neutrophils was performed using the Ly-6B.2 antibody, which specifically recognizes a 40-kDa antigen expressed by polymorphonuclear cells but is absent in resident tissue macrophages. A significant increase in neutrophil infiltration in IL-10−/−NHE3−/− compared with WT, IL-10−/−, or NHE3−/− mice was observed in proximal (Fig. 3A) and distal (Fig. 4A) colon. This observation was further confirmed by real-time RT-PCR analysis of the mucosal expression of MMP8, a neutrophil-specific collagenase. In proximal colon, MMP8 mRNA expression was increased 10-, 17-, and 89-fold in IL-10−/−, NHE3−/−, and double-KO mice, respectively (Fig. 3B). In distal colon, the differences in MMP8 expression were also highly pronounced: 8-, 2.5-, and 137-fold in IL-10−/−, NHE3−/−, and double-KO mice, respectively (Fig. 4B). Consistent with this observation, expression of MIP-2 (CXCL2), a potent murine neutrophil chemokine and a functional IL-8 homolog, was also significantly elevated in double-KO mice. In proximal colon, MIP-2 transcript increased 5-, 3, and 71-fold in IL-10−/−, NHE3−/−, and double-KO mice, respectively (Fig. 3C), while in distal colon the respective differences were 32-, 16-, and 84-fold, respectively (Fig. 4C). We also assessed the expression of CXCL1 (KC), which, similar to IL-8, has been shown to positively correlate with disease activity in IBD patients (14). In agreement with increased neutrophil infiltration and MIP-2 expression data, KC mRNA expression was significantly increased in proximal and distal colon of IL-10−/−NHE3−/− mice compared with WT or single-KO littermates (Fig. 5).

To investigate the mechanism responsible for the histologically apparent increase in mononuclear cell infiltration in both colonic segments in double-KO mice, we analyzed the mRNA expression of major IFNγ-inducible T cell chemoattractants: CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (IP9). Mucosal CXCL9 expression was not significantly increased in any of the mouse genotypes or in either colonic segment (Figs. 6A and 7A). In proximal colon, CXCL10 and CXCL11 were significantly increased in IL-10−/− mice, but not in NHE3−/− littermates, and there was no significant effect of the double-KO status in this colonic segment (Fig. 6). However, distal colonic expression of CXCL10 was enhanced in IL-10−/−NHE3−/− mice (16- and 28-fold over WT in IL-10−/− and IL-10−/−NHE3−/− mice, respectively; Fig. 7B), whereas CXCL11 mRNA in IL-10−/− and IL-10−/−NHE3−/− mice was increased 25- and 84-fold, respectively (Fig. 7C).

**Elevated IFNγ, IL-17, and IL-12/23 p40 protein secretion in the colon of IL-10−/−NHE3−/− mice.** IFNγ, IL-17, and IL-12/23 p40 were chosen as key cytokines involved in the Th-1/Th-17 T cell differentiation pathways implicated in the pathogenesis of Crohn’s disease and IL-10-deficiency colitis. Cytokine secretion was evaluated by ELISA in colonic explant culture supernatants. Consistent with previously published data (18), colonic expression of IL-17, IFNγ, and IL-12/23 p40 subunit was not elevated in NHE3−/− mice (Fig. 8). Production of all three cytokines in the colon of 10-wk-old IL-10−/− mice was increased, although IL-17 did not reach the level of statistical significance (Fig. 8A). In IL-10−/−NHE3−/− mice, colonic secretion of IL-17, IFNγ, and IL-12/23 p40 was sig-
nificantly higher than in WT mice. Moreover, secretion of IL-17 and IFN$\gamma$ by the colonic explants was significantly higher in double-KO than IL-10$^{-/-}$/NHE3$^{-/-}$ mice (Fig. 8). Real-time RT-PCR analysis of the cytokine mRNA level in colonic mucosa followed the same pattern (data not shown).

Increased epithelial cell apoptosis in IL-10$^{-/-}$/NHE3$^{-/-}$ mice. In our initial description of spontaneous colitis in NHE3$^{-/-}$ mice, microarray analysis of colonic gene expression identified a significant number of genes associated with regulation of programmed cell death (18). Although NHE3$^{-/-}$ mice were found to be highly susceptible to DSS-induced epithelial injury (18), which is typically associated with increased epithelial cell apoptosis (31), NHE3 status in mice did not influence the numbers of cleaved caspase-3-positive cells after short-term exposure to DSS (18). However, a chronically increased level of proinflammatory cytokines, such as IFN$\gamma$, is...
believed to significantly contribute to the pathology of IBD, in part by interfering with antiapoptotic signals in the epithelium as well as by modulating the integrity of the epithelial barrier (9). Therefore, we investigated whether loss of NHE3 could contribute to the worsening of the symptoms observed in double-KO mice through increased epithelial cell apoptosis. Proximal and distal colonic sections were stained for cleaved caspase-3, and the numbers of cleaved caspase-3-positive cells were evaluated (Figs. 9 and 10). The number of cleaved caspase-3-positive cells was significantly increased in proximal (Fig. 9) and distal (Fig. 10) colon of the IL-10−/− NHE3−/− mice. The differences were more dramatic in proximal colon (typically more affected in IL-10 deficiency), where the number of apoptotic epithelial cells was increased ∼20-fold in double-KO mice compared with IL-10−/− mice (Fig. 9). Interestingly, in proximal colon, the majority of the cleaved caspase-3-positive cells were positive for NHE3, as determined by immunohistochemistry.
Caspase-3 staining was observed in surface epithelial cells, where expression of NHE3 is normally the highest.

**DISCUSSION**

Under physiological conditions, the intestinal epithelium serves as a strict barrier between luminal content and the intestinal mucosa. As a very dynamic tissue, the epithelial barrier requires the maintenance of a balance between intestinal cell proliferation, differentiation, and apoptosis. At the interface between the intestinal microflora and the gastrointestinal-associated lymphoid tissue, the intestinal epithelium not only serves as a physical barrier, but it also plays an essential role in shaping the mucosal immune system, actively participating in the sensing of commensal and pathogenic bacteria, alongside its critical role in transport and nutrient absorption. Decreased expression of NHE3 or its key accessory proteins or greatly depressed NHE3 activity without changes in its expression has been reported in IBD (11, 30, 34), likely contributing to inflammation-associated diarrhea. However, loss of NHE3 function in Slc9a3 KO mice also leads to spontaneous distal colitis and extreme susceptibility to DSS-mediated mucosal injury (16, 18). Since NHE3 deficiency could not be studied in chronic settings using chemically induced models of IBD, we investigated its contribution to the development of colitis in IL-10⁻/⁻ mice, an accepted model of chronic Crohn’s-like disease. To this end, we crossed NHE3 mice into the 129/SvEv genetic background for >10 generations with no adverse effects on their phenotype. We also evaluated the penetration and severity of colitis in 10-wk-old single-KO (NHE3⁻/⁻ or IL-10⁻/⁻) and double-KO (IL-10⁻/⁻ NHE3⁻/⁻) mice.

Histological, immunohistochemical, and gene expression analyses of proximal and distal colon established important architectural features consistent with exacerbated colitis in IL-10⁻/⁻ NHE3⁻/⁻ mice compared with WT or single-KO mice. Increased lamina propria infiltration with granulocytes and mononuclear cells was accompanied by elevated expression of key neutrophil and T cell chemokines (MIP-2, CXCL1, caspase-3 staining was observed in surface epithelial cells, where expression of NHE3 is normally the highest.

**FIG. 5.** Real-time PCR analysis of CXCL1 (keratinocyte chemoattractant (KC)) mRNA expression in proximal (A) and distal (B) colon of WT, IL-10⁻/⁻, NHE3⁻/⁻, and IL-10⁻/⁻ NHE3⁻/⁻ mice. Values were normalized and calculated as described for MMP8 in Fig. 3B legend. Statistical significance was determined as described in Fig. 3 legend.

**FIG. 6.** Real-time RT-PCR analysis of CXCL9 (A), CXCL10 (B), and CXCL11 (C) mRNA expression in proximal colon of WT, IL-10⁻/⁻, NHE3⁻/⁻, and IL-10⁻/⁻ NHE3⁻/⁻ mice. Values were normalized and calculated as described for MMP8 in Fig. 3B legend. Statistical significance was determined as described in Fig. 3 legend.
and it is believed that regulation of intestinal cell survival and related signaling pathways may represent a therapeutic approach for the treatment of IBD. It is very likely that NHE3 deficiency leads to lowering of the threshold for inflammation-induced apoptosis, and several hypotheses could be put for-

**Fig. 7.** Real-time RT-PCR analysis of CXCL9 (A), CXCL10 (B), and CXCL11 (C) mRNA expression in distal colon of WT, IL-10−/−, NHE3−/−, and IL-10−/− NHE3−/− mice. Values were normalized and calculated as described for MMP8 in Fig. 3B legend. Statistical significance was determined as described in Fig. 3 legend.

**Fig. 8.** Secretion of IL-17 (A), IFNγ (B), and IL-12/23 p40 (C) by colonic explants obtained from WT, IL-10−/−, NHE3−/−, and IL-10−/− NHE3−/− mice. Colonic tissues were cultured for 18 h, and concentration of the cytokines accumulated in the medium was measured by ELISA. Values are means ± SE. Duplicate measurements were performed with tissues obtained from individual animals. Statistical significance was determined as described in Fig. 3 legend.

CXCL10, and CXCL11). Accordingly, colonic secretion of IFNγ, IL-17, and IL-12/23 p40 was increased in the colon of IL-10−/− NHE3−/− mice above the levels observed for single-KO littermates. We also established that the colonic epithelium of the double-KO mice exhibited increased hallmarks of apoptosis, including a significant increase in the number of cleaved caspase-3-positive epithelial cells.

Tightly regulated epithelial cell apoptosis plays a key role in the maintenance of epithelial integrity and restitution. In patients with IBD, increased apoptosis has been found in the acute inflammatory sites throughout the entire crypt-villus axis,
ward that could explain increased epithelial cell apoptosis in mice lacking both NHE3 and IL-10.

Changes in ionic fluxes in the NHE3-deficient colonocytes may contribute to increased epithelial cell apoptosis. The magnitude and scope of changes in the expression of membrane transport-related genes in the colon of NHE3−/− mice seem to support this hypothesis (18). Na+ deprivation experienced by NHE3-deficient cells could lead to a decreased apoptotic threshold similar to that reported in HeLa cells, in which medium Na+ depletion leads to increased Na+ efflux, normotonic shrinkage, and increased caspase-3 cleavage (23). However, the results from an acute in vitro study are unlikely to be replicated in a model of chronic NHE3 deficiency, where numerous compensatory mechanisms are expected to be activated. Some of these include cation-sparing processes, such as downregulation of the Kcnn4 K+ channel and upregulation of the nongastric H+-K+-ATPase (Atp12a) and Na+-K+-ATPase (Atp1a1) (18). Interestingly, although this was not investigated in the gut epithelial cells, inhibition of K+ efflux in neutrophils prevented mitochondrial dysfunction, caspase-3 activation, and apoptosis (10). If applicable to the intestinal epithelial cells (IECs), this finding could explain why we do not observe increased constitutive apoptosis in the colonic epithelium in NHE3−/− mice (16) and would imply that other superimposed stimuli are necessary to elicit such a response.

IL-10 has been shown to prevent loss of intestinal barrier integrity (20), whereas proinflammatory cytokines, such as IFNγ and TNFα, have been shown to significantly impair mucosal integrity (6, 19, 25). In the case of double-KO mice, the significant increase of proinflammatory cytokines could be the triggering factor leading to a dramatic increase in CEC apoptosis. IL-10 has also been described to modulate Fas
expression on IECs (3). Fas is a member of the TNF death-domain-containing receptor family and is expressed constitutively in epithelial cells, whereas Fas-ligand is only expressed by CD8\(^+\) cytolytic T cells under inflammatory conditions. In IL-10\(^{-/-}\) NHE3\(^{-/-}\) mice, proinflammatory cytokines could up-regulate the expression of Fas on IECs, which in the absence of IL-10 regulation, would sensitize CECs to Fas ligand-induced apoptosis. We evaluated colonic Fas expression by real-time RT-PCR in all four mouse genotypes and found it to be elevated in proximal and distal colon of double-KO mice compared with NHE3\(^{-/-}\) or IL-10\(^{-/-}\) NHE3\(^{-/-}\) mice, although the difference did not reach statistical significance (data not shown). Therefore, Fas involvement can only partially explain the changes observed in double-KO mice.

Cytotoxic T cells can also trigger CEC apoptosis degranulation of granzymes, serine proteases that, together with the pore-forming protein perforin, induce cell death. IL-10-deficient mice overexpress granzyme A in the large intestine (12). Granzyme A and perforin 1 are upregulated in the colon of NHE3\(^{-/-}\) mice (18). However, expression of neither showed an additive or synergistic trend in IL-10\(^{-/-}\) NHE3\(^{-/-}\) mice (data not shown), suggesting that CEC apoptosis in this model is not mediated via the granzyme-perforin axis.

In IBD, endoplasmic reticulum (ER) stress plays a role in the initiation and propagation of the inflammatory condition, ultimately leading to epithelial cell apoptosis (15). In addition, it has been shown that IL-10 is a potent inhibitor of the inflammation-induced ER stress response by modulating activating
transcription factor 6 nuclear recruitment to the grp78 gene promoter (29). As part of this complex process, ER stress results in the splicing of a 26-bp fragment from the mRNA encoding the transcription of transcription factor X-box-binding protein 1, which is a strong inducer of a subset of unfolded protein response target genes (15). Although the role of ER stress appears to be more significant in secretory cells than in the absorptive surface epithelium, we explored the possibility that, in double-KO mice, the combined absence of NHE3 and IL-10 could contribute to the exacerbation of ER stress and, ultimately, to IEC apoptosis. However, the analysis of X-box-binding protein 1 splicing in the colon (whole colonic extract), as well as grp78 mRNA expression, did not confirm the involvement of ER stress as the major inducer of IEC death (data not shown). Similarly, analysis of the markers of hypoxia, accumulation of hypoxia-inducible factor-α and the subsequent upregulation of intestinal trefoil factor, did not yield conclusive answers, with no significant differences between single-KO and IL-10−/−NHE3−/− mice (data not shown).

In summary, NHE3 deficiency superimposed on chronic colitis in IL-10−/− mice leads to exacerbation of colitis, likely precipitated by increased predisposition to programmed cell death in CECs. Although we were not able to determine the precise cause of CEC apoptosis in IL-10−/−NHE3−/− mice, it is plausible that a combination of multiple factors contributed by impaired electrolyte and immune homeostasis is necessary. Our results highlight the importance of NHE3 in the maintenance of intestinal barrier integrity and in modulating the inflammatory process in IL-10-deficient mice. These novel observations may be of high significance for the overall IBD pathogenesis, where NHE3 inhibition, associated with genetic predisposition (such as IL-10 or IL-10 receptor polymorphisms), may influence the extent of the epithelial barrier defect and contribute to the ultimate degree of inflammation.

GRANTS

This work is supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant 2R01 DK-041274 (to F. K. Ghishian).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


