Stimulation of proliferation in the colorectal mucosa by gastrin precursors is blocked by desferrioxamine

Audrey Ferrand,* Shamilah Lachal,* Gianni Bramante, Suzana Kovac, Arthur Shulkes, and Graham S. Baldwin

University of Melbourne Department of Surgery, Austin Health, Melbourne, Victoria, Australia

Submitted 9 February 2010; accepted in final form 12 April 2010

Ferrand A, Lachal S, Bramante G, Kovac S, Shulkes A, Baldwin GS. Stimulation of proliferation in the colorectal mucosa by gastrin precursors is blocked by desferrioxamine. Am J Physiol Gastrointest Liver Physiol 299: G220–G227, 2010. First published April 15, 2010; doi:10.1152/ajpgi.00046.2010.—Precursors of the peptide hormone gastrin stimulate proliferation in the colorectal mucosa and promote the development of colorectal carcinoma. Gastrins bind two ferric ions selectively and with high affinity, and the biological activity of glycine-extended gastrin (Ggly) in vitro is dependent on the presence of ferric ions. The aim of the present study was to determine whether or not iron is required for biological activity of progastrin and Ggly in vivo. Rats that had undergone a colostomy were infused with Ggly, and proliferation was measured in the defunctioned rectal mucosa. Proliferation was also measured in the colonic mucosa of hGAS and MTI-Ggly mice, which, by definition, overexpress progastrin and Ggly, respectively. The requirement for iron was assessed by thrice-weekly injection of the chelating agent desferrioxamine (DFO). The proliferation index in the defunctioned rectal mucosa was significantly increased in the Ggly-infused rats, and the increase was significantly reduced after treatment with DFO. Treatment with DFO significantly reduced the crypt height and proliferation index in the colonic mucosa of hGAS and MTI-Ggly mice but had no effect on the same variables in wild-type mice. These observations are consistent with the hypothesis that the biological activity of progastrin and Ggly in vivo is dependent on the presence of ferric ions and further suggest that chelating agents may block the stimulatory effects of gastrin precursors in the development of colorectal carcinoma.

Gastrins have also been implicated in the development of colorectal carcinoma (CRC). Increased concentrations of amidated gastrins (9) and their precursor progastrin (26) are present in the serum of patients with colorectal cancer compared with control subjects. In animal models, mice overexpressing progastrin developed more aberrant crypt foci in response to the chemical carcinogen azoxymethane than wild-type controls (10, 30), and formation of aberrant crypt foci in response to azoxymethane was also increased in rats infused with exogenous Ggly (2). In addition, increased concentrations of Ggly in gastric mucosa and in serum have been reported in animals and humans with the iron overload disease hemochromatosis (32), and patients with hemochromatosis have an increased risk of CRC (1, 25), as do apparently normal subjects with elevated concentrations of gastrin (34).

Gastrins bind two ferric ions with high affinity, via a region of five consecutive glutamate residues (4, 18). Nuclear magnetic resonance spectroscopy and fluorescence quenching experiments with Ggly showed ferric ions bound to glutamates 7, 8 and 9 (18). The substitution Glu7Ala reduced ferric ion binding and completely abolished Ggly-induced proliferation and migration of CRC cells (18). Further evidence that the binding of ferric ions was essential for the biological activity of Ggly was provided by the observation that both iron chelation with desferrioxamine (DFO) (18) and iron competition by trivalent bismuth ions (19) inhibited Ggly activity. In contrast, neither the Glu7Ala substitution nor iron chelation or iron competition had any significant effect on the biological activity of Gamide (20). Moreover, iron chelation did not affect the binding of Gamide to the cholecystokinin-2 receptor (CCK2R) or CCK2R-mediated cellular function (20).

The obvious conclusion from these studies is that nonamidated gastrins play an important role in the proliferation of normal and neoplastic colon cells and therefore in the pathogenesis of colorectal cancer. Given that human trials of the effect of blocking gastrin via immunotherapy have been rather disappointing (31), there is a need to examine other strategies by which the effects of this growth factor could be inhibited. In the present study, the iron dependence of the biological activity of nonamidated gastrins in the colorectal mucosa in vivo has been investigated. The animal models used were rats, which were infused with Ggly after their rectal mucosa had been rendered hypoplastic by colostomy, and MTI/Ggly and hGAS mice, which overexpress Ggly and progastrin, respectively.

MATERIALS AND METHODS

Peptides. Ggly (86% purity; Auspex, Melbourne, Australia) was prepared at 0.53 mg/ml in PBS containing 0.1% bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) and filtered through a 0.4-μm Millipore filter (Millipore, North Ryde,
Australia). DFO (Sigma, St. Louis, MO) was prepared at 50 mg/ml in PBS for use as a ferric ion chelating agent in the treatment of mice overexpressing progastrin and Ggly and stored in aliquots at −20°C.

Animals. Male Sprague-Dawley rats weighing between 250–300 g were used for the colostomy model (2). Twelve-week-old male and female transgenic FVB/N mice overexpressing progastrin (hGAS mice, Ref. 36) or Ggly (MTI/Ggly mice, Ref. 14) were utilized as models of high-circulating nonamidated gastrins. All colonies were genotyped to confirm the presence of the transgene, and FVB/N mice were used as wild-type controls. All animals were maintained in standard laboratory conditions on a 12-h:12-h light/dark cycle with free access to water and pellets of sterilized standard rat chow or mouse feed (Ridley AgriProducts, Pakenham, Australia) as appropriate. All experiments were conducted at the Austin Health Department of Surgery (Melbourne, Australia) with ethics approval from Austin Health Animal Ethics Committee.

Rat surgery. Rats were anesthetized with inhaled Enflurane (Abbott, North Ryde, Australia) throughout the operation. Incision was made with a scalpel through the midline skin, then through subcutaneous tissue and fat, and finally through the muscular layer. A small 4-mm incision was made in the left iliac fossa or the hypochondrium for the formation of a stoma. After mesenteric vessels had been divided, the rectosigmoid colon was divided just proximal to the lymph node between the rectum and sigmoid colon. The proximal end of the rectum was closed with two hemoclips (Weck Closure Systems, Research Triangle Park, NC) and allowed to fall back into the pelvis. The distal end of the descending colon was brought out through the previously made incision for the stoma and sutured once in each quadrant with 5.0 prolene (Johnson and Johnson, Sydney, Australia) to form the stoma.

Alzet minipumps (reservoir volume of 2 ml, pumping rate of 2.5 μl/h for 28 days; Alza, Mountain View, CA) were filled with peptides and primed in 0.9% saline overnight at 37°C. The pump was inserted flow moderator first into the peritoneal cavity at the same time that the stoma was created, and the muscle layer was then sewn with 4.0 prolene and skin and subcutaneous tissue and skin oversewn with 4.0 Surgilene (Johnson and Johnson). Rats were caged in pairs for 28 days, and DFO (Novartis, North Ryde, Australia) was injected intraperitoneally three times a week (400 mg/kg, 250 mg/ml in 0.9% saline) (22). There were four treatment groups, with sizes as indicated in parentheses: group 1, 0.1% bovine serum albumin in PBS as control (n = 10); group 2, Ggly, 0.53 mg/ml in 0.1% bovine serum albumin in PBS (n = 9); group 3, DFO, 400 mg/kg three times a week (n = 8); group 4, DFO 400 mg/kg three times a week and Ggly 0.53 mg/ml in 0.1% bovine serum albumin in PBS (n = 9).

Measurement of proliferation indices in the rat model. On the day of culling all rats were injected with vincristine (100 mg/kg, Oncotain; Mayne Pharma, Mulgrave, Australia) to arrest proliferating cells in metaphase and were euthanized 3 h later. All culling was done between 1200 and 1300 h to avoid diurnal variation in mucosal proliferation. Rats were anesthetized with inhaled Enflurane and bled from the inferior vena cava with a 20-gauge intravenous cannula. Cecum, descending colon, rectum, and liver were collected, fixed in 10% formalin overnight, and embedded in paraffin (Department of Anatomical Pathology, Austin Health, Victoria, Australia). Sections were taken 30 μm apart to avoid the same crypt being examined more than once and stained with hematoxylin and eosin according to standard techniques. Only crypts with intact surface epithelium with no fixing artefact visible, with the base of the crypt in close proximity to the muscularis mucosa, and the full length of the crypt from lumen to basal surface sectioned and visible, were examined. The nuclei arrested in metaphase were counted in 20 crypts for each specimen using an Olympus BH-2 light microscope. Crypt height in 20 crypts per specimen was measured in pixels using Image Proplus software (MediaCybernetics, Silver Spring, MD).

Measurement of iron status. Blood from the rats was collected from the tail vein at 0, 1, and 4 wk into lithium heparin tubes (Terumo, Tokyo, Japan). The whole blood was centrifuged at 10,000 g for 10 min, and blood cells were counted and serum markers of iron status (serum hemoglobin, ferritin, iron, transferrin saturation, and total iron binding capacity) measured (Network Pathology, Austin Health). The dry weight of iron in liver was also measured (Analytical Research Laboratories, Melbourne, Australia).

Measurement of proliferation indices in the mouse model. Twelve-week-old hGAS, MTI/Ggly, and FVB/N mice were treated with DFO (400 mg/kg in PBS) or PBS by intraperitoneal injection three times weekly for 4 wk. Mice were weighed before euthanasia and anesthetized by Fortthane (isoflurane) inhalation before blood was collected by cardiac puncture. Colonos were harvested, fixed, and paraffin embedded according to standard techniques. Sections of colon tissue were dewaxed and stained for PCNA with a monoclonal anti-mouse primary antibody (Dakopatts, Copenhagen, Denmark) and a goat anti-mouse IgG-horseradish peroxidase secondary antibody (Dako-patts) with diaminobenzidine as substrate as described previously (15). The labeling index was calculated as the number of immunopositive cells multiplied by 100 and divided by the total number of cells per colonic crypt. The heights of 20 colonic crypts per specimen were measured in pixels using Image Proplus software (MediaCybernetics).

Radioimmunoassay. Serum concentrations of progastrin and Ggly were measured by radioimmunoassay as described previously with antibodies 1137 and 7270, respectively (9).

Statistical analysis. All statistics were analyzed by t-tests with Bonferroni’s correction with the program SigmaStat (Jandel Scientific, San Rafael, CA). The results are expressed as means ± SE, and the statistical significance is indicated by ***P < 0.001, **P < 0.01, and *P < 0.05.

RESULTS

Circulating gastrin concentrations. To determine the effectiveness of the osmotic pumps used for infusion of Ggly in the rat model, serum Ggly concentrations were measured by radioimmunoassay. Using antibody 7270, which detects the COOH terminus of Ggly, the plasma Ggly concentration significantly increased from 63 ± 7 fmol/ml at day 0 to 525 ± 124 fmol/ml at day 7, and a similar increase was observed in the Ggly/DFO group.

The serum concentrations of progastrin or Ggly were also measured in control FVB/N mice and in hGAS and MTI/Ggly mice by radioimmunoassay (Table 1). The concentration of progastrin in the circulation of hGAS mice was over 450-fold greater than in wild-type FVB/N mice (P < 0.001). A modest, but statistically significant (P < 0.01), threefold increase in Ggly was noted for MTI/Ggly mice compared with wild-type controls. Treatment with DFO did not significantly affect the expression of progastrin or Ggly, with similar concentrations measured in hGAS and MTI/Ggly mice treated with DFO compared with PBS-treated mice.

DFO inhibits Ggly-stimulated proliferation in rat colorectal mucosa. Proliferation in the colorectal mucosa of rectally defunctioned rats was assessed by measurement of crypt height and by counting metaphase-arrested nuclei. There was no significant difference in crypt height between the treatment groups in the cecum, descending colon (data not shown), and defunctioned rectum (Fig. 1A). In agreement with our previous results (2), Ggly significantly stimulated proliferation in the colon (184%, data not shown) and defunctioned rectum (281%, Fig. 1B) but not in the cecum (data not shown). Administration of DFO partially reversed the Ggly-stimulated proliferation in the defunctioned rectum (Fig. 1B) but not in the colon. Administration of DFO itself was able to stimulate prolifera-
No significant differences were observed between the treatment groups in serum hemoglobin concentrations (Fig. 2A), serum iron (data not shown), total iron binding capacity (Fig. 2B), transferrin saturation (Fig. 2C), or serum ferritin (data not shown). Similarly no significant difference was observed between the treatment groups in liver iron content (Fig. 2D), or in leukocyte or platelet counts (data not shown). The same regime of DFO treatment has previously been reported to have no effect on iron status in mice (27, 28).

**DFO reduces crypt height in the colonic mucosa of mice overexpressing nonamidated gastrins.** To determine the effect of DFO on progastrin- and Ggly-induced changes in the colonic epithelium, colonic crypt height was measured in 12-wk-old hGAS and MTI/Ggly mice and in FVB/N wild-type mice (Fig. 3). Control PBS-treated hGAS mice had significantly increased crypt height (125%, P < 0.001) compared with PBS-treated FVB/N mice, in agreement with the data of Koh and coworkers (36). Following treatment with DFO, colonic crypt height in hGAS mice was significantly (P < 0.05) reduced. Although Koh and coworkers have reported previously that Ggly overexpression resulted in an increase in colonic crypt height (14), the crypt height in MTI/Ggly mice from our colony was not significantly different from wild-type controls. However, treatment with DFO significantly (P < 0.05) reduced crypt height in MTI/Ggly mice, and no such difference was detected in wild-type FVB/N mice after treatment with DFO (Fig. 3G).

**DFO inhibits proliferation in the colorectal mucosa of mice overexpressing nonamidated gastrins.** Similar trends were observed upon analysis of proliferating cells within colonic crypts by immunohistochemical staining for PCNA (Fig. 4). The number of proliferating cells in hGAS mice (194%) was significantly greater than in FVB/N wild-type mice (P < 0.05). The number of proliferating cells was significantly reduced following DFO treatment of either hGAS mice (by 52%, P < 0.05) or MTI/Ggly mice (by 48%, P < 0.05) (Fig. 4).

**DISCUSSION**

Progastrin and its derivatives bind two ferric ions selectively and with high affinity (3, 4). The significance of the association between gastrins and iron was first demonstrated by preventing the interaction between ferric ions and Gamide or Ggly. Mutation of the binding region, or competition with chelators or bismuth ions, abolished Ggly-induced proliferation and migration in vitro (18, 19), but the presence of ferric ions was not

**Table 1. Circulating gastrin concentrations in mice overexpressing nonamidated gastrins**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Ggly, pM</th>
<th>Progastrin, pM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+PBS</td>
<td>+DFO</td>
</tr>
<tr>
<td>MTI/Ggly</td>
<td>This work</td>
<td>38 ± 10‡</td>
<td>41 ± 10‡</td>
</tr>
<tr>
<td>(14)</td>
<td></td>
<td>85 ± 28‡</td>
<td></td>
</tr>
<tr>
<td>hGAS</td>
<td>This work</td>
<td>10 ± 2</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>(36)</td>
<td></td>
<td>&lt;30</td>
<td></td>
</tr>
<tr>
<td>FVB/N</td>
<td>This work</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(14, 36)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Circulating gastrin concentrations in mice overexpressing nonamidated gastrins were taken from the indicated references or measured by radioimmunoassay as described in MATERIALS AND METHODS. Statistical comparisons of gastrin concentrations in overexpressing mice and the corresponding wild-type strain were made by t-test with Bonferroni’s correction. The concentrations in desferrioxamine (DFO)-treated animals were not significantly different from PBS-treated animals. *P < 0.05; †P < 0.01; ‡significance not reported. MTI-Ggly, mice that overexpress glycine-extended gastrin; hGAS, mice that overexpress progastrin.

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Desferrioxamine (DFO) inhibits glycine-extended gastrin (Ggly)-stimulated proliferation in rat colorectal mucosa. Rats that had been subjected to a colostomy to induce hypoplasia of the defunctioned rectal mucosa were infused with Ggly (0.53 mg/ml in 0.1% bovine serum albumin in PBS) or with 0.1% bovine serum albumin in PBS alone via Alzet miniosmotic pumps. DFO (250 mg/ml in 0.9% saline) or 0.9% saline was injected intraperitoneally 3 times a week at a dose of 400 mg/kg. After 4 wk animals were injected with vincristine and euthanized 3 h later. Sections from the colorectal mucosa were prepared as described in MATERIALS AND METHODS and examined microscopically to determine the crypt height (A) and the metaphase index (mean number of metaphase-arrested nuclei per crypt) (B) in defunctioned rectum. Data are means ± SE, where N = 8–10. The significance of differences from the control (⁎P < 0.05; ⁎⁎⁎P < 0.001) or between the DFO-treated and corresponding untreated samples (##P < 0.01) was determined by t-test with Bonferroni’s correction.
essential for the activity of Gamide (20). The aim of the present study was to assess the role of iron in the biological activity of gastrin precursors in vivo.

We have previously reported that administration of Ggly to rats that had undergone a colostomy significantly increased proliferation in the defunctioned rectal mucosa (2). The present study demonstrates for the first time that the proliferation induced by Ggly in the defunctioned rectal mucosa of rats can be reversed at least in part by injection of the iron chelator DFO for 4 wk (Fig. 1B). The failure to reverse the Ggly-induced increase completely may be a result of the increase in proliferation observed in the animals treated with DFO alone. Similarly treatment with DFO for 4 wk significantly reduced crypt height (Fig. 3) and proliferation index (Fig. 4) in the colon of mice overexpressing progastrin or Ggly. No such reduction was detected in wild-type FVB/N mice. The failure to corroborate the previously observed increase in crypt height (Fig. 3) and proliferation index (Fig. 4) in the colon of MTI/Ggly mice may be a reflection of a lower than expected overexpression of Ggly as measured by radioimmunoassay. Whereas progastrin expression was consistent with the value reported previously (36), radioimmunoassay results suggested that the serum Ggly concentration was less than reported by Koh and coworkers (14) (Table 1). Taken together, these observations are consistent with the hypothesis that nonamidated gastrins require ferric ion binding to exert their biological activity in vivo.

Although the simplest explanation for reversal by DFO of the increase in proliferation induced by nonamidated gastrins is that the chelator is removing an essential ferric ion from nonamidated gastrins and hence reducing their biological efficacy, alternative explanations for the inhibitory effects of DFO should also be considered. The presence of iron is essential for cellular metabolism and DNA synthesis, and iron depletion has been shown to induce cell cycle arrest and apoptosis (16). However, at the dose used in the present study, treatment with DFO did not have any detrimental effect on crypt height or proliferation index of the normal colonic mucosa in FVB/N control mice (Fig. 3) and indeed significantly stimulated proliferation in colonic and defunctioned rectal mucosa in rats (Fig. 1). Similarly, no difference was observed between control rats and rats treated with DFO in hemoglobin concentration or in iron status as assessed by serum markers (including serum iron, transferrin saturation, total iron binding capacity, or serum ferritin) or tissue markers (including dry weight of iron in livers) (Fig. 2). These observations are in agreement with previous reports that the same dose of DFO had no significant effect on iron hematological parameters in mice (27) and that growth-related changes in rats were observed only after 12 wk of DFO administration (22). The failure to detect any change in iron status indicates that the inhibitory effect of DFO described in the present work is not caused by general depletion of iron stores. Hence the data presented herein establish that nonamidated gastrins require ferric ions to exert their biological activity in both rats and mice in vivo and are consistent with the working hypothesis, on the basis of experiments with cell lines in vitro (18), that DFO inhibits proliferation by removal of the ferric ions bound to nonamidated gastrins.

Iron chelators have been frequently described as effective antiproliferative agents (37). Significantly, inhibition of proliferation by iron chelators seems to be specific to aggressively growing tumor cells because in a landmark study an antiproliferative effect of DFO (with 90% cell death) was demonstrated in two human neuroblastoma cell lines but not in various other cell lines of different origin (7). Neuroblastoma cells were more sensitive to iron deprivation than normal bone

Fig. 2. DFO treatment does not significantly alter iron status in rats. The following serum markers of iron status and the dry weight of iron in liver were measured after 4 wk of thrice weekly intraperitoneal injections of DFO (400 mg/kg) as described in MATERIALS AND METHODS: serum hemoglobin (A), total iron binding capacity (TIBC) (B), transferrin (Tf) saturation (C), and liver iron (D). Data are means ± SE, where N = 8–10. No significant differences were observed from the saline-injected control.
marrow (5). Interestingly, there were marked differences in studies conducted with iron chelators in vivo compared with those performed in vitro. Treatment with DFO for 4 wk did not inhibit the xenograft growth of human neuroblastoma or cervical carcinoma cell lines (27) in nude mice (24) although growth of the cervical carcinoma cell lines was inhibited by DFO in vitro (29). The possible involvement of nonamidated gastrins in the above effects will be worthy of future investigation, as 80% of neuroblastomas have been shown to express gastrin mRNA, whereas expression of the mRNA encoding the CCK2R, which is specific for amidated gastrins, was limited to 10% of the same tumors (23).

Fig. 3. DFO reduces crypt height in the colonic mucosa of mice overexpressing nonamidated gastrins. A–F: representative sections from the colons of control FVB/N mice (A and B), hGAS mice overexpressing progastrin (C and D), or MTI/Ggly mice overexpressing Ggly (E and F), which had been treated with PBS (A, C, and E) or DFO (B, D, and F), were stained with hematoxylin and eosin. Images were taken at ×20 magnification, and the scale bars represent 50 μm. G: colonic crypts were significantly longer in hGAS mice overexpressing progastrin compared with FVB/N mice (**P < 0.001). The colonic crypts in hGAS or MTI/Ggly mice were significantly shorter following treatment with DFO (hatched bars) than following treatment with PBS (white bars) (#P < 0.05). The crypt height was measured in μm for a total of 20 crypts per section using the image analysis program ImageProPlus. Data are means ± SE, where N = 5–6. Statistical significance was determined by t-test with Bonferroni’s correction.
Similarly, studies conducted using DFO as a single agent in patients with neuroblastoma have been inconclusive. In one study, 10 children with recurrent neuroblastoma were given a 5-day course of DFO as a continuous infusion with no clear benefit (6). In a second study, only one of nine patients undergoing the same DFO treatment showed a decrease in tumor mass (11). The failure to demonstrate an effect of DFO in vivo may be a result of its short plasma half-life and high hydrophilicity, which leads to poor permeability across plasma membranes. As a result, chelators with greater activity and...
with less toxicity than DFO (38) may offer better alternatives for cancer therapy.

There has been considerable recent interest in the connection between iron and the risk of colorectal cancer in humans. However, none of the large number of studies that have addressed this issue have been randomized prospective controlled trials (17). A review of 33 mainly retrospective epidemiological case-controlled and cohort studies concluded that three quarters of the better-designed studies supported the role of iron as a risk factor for colorectal cancer (17). A recent population-based case control study confirmed that patients with hemochromatosis have an increased risk of CRC (1), and overexpression of transferrin receptor 1 (21) and of other iron import proteins (8) is a common occurrence in colon cancers. Furthermore, dietary iron has been shown to promote induction of colon tumors in mice by azoxymethane (13). One attractive hypothesis that is consistent with the above observations is that the increased risk of colorectal cancer may be at least in part due to an increased bioactivity of nonamidated gastrins.

In summary, the present study has demonstrated that iron contributes to the biological activity of nonamidated gastrins in vivo. Treatment with the iron chelator DFO significantly reduced Ggly-induced proliferation in the defunctioned rectal mucosa of rats that had undergone colostomy. Similarly, DFO treatment of mice overexpressing progastrin resulted in significantly reduced crypt height and proliferation index in the colonic mucosa. Additionally, treatment of mice overexpressing Ggly with DFO significantly reduced colonic mucosal crypt height, with a concomitant reduction in proliferation index. Together these results suggest that interference with the ability of gastrin precursors to bind iron could be a novel approach to limit the proliferative effects associated with nonamidated gastrins in the gastrointestinal tract and hence a new therapeutic option for treatment of CRC.

GRANTS

This work was supported by grant 5 R01 GM065926-08 from the National Institutes of Health (to G. Baldwin, A. Shulkes), grants 400062 (to G. Baldwin, A. Shulkes), 208926 (to G. Baldwin), and 350235 (to A. Shulkes) from the National Health and Medical Research Council of Australia, and a grant from the Austin Medical Research Foundation (to A. Ferrand).

DISCLOSURES

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

REFERENCES


