Regional Peptide Uptake Study in the Rat Intestinal Mucosa: Glatiramer Acetate as a Model Drug

Susan Haupt,[1] Efrat Gil,[1] Regin Tirosh,[1] Ety Klinger,[2] Alexander Gad,[2] and Abraham Rubinstein[1,3]

Received February 19, 2002

Purpose. To identify regions of the rat intestine that are able to internalize from the lumen oligopeptides, using the model drug glatiramer acetate (GA).

Methods. GA was introduced into rat intestinal sacs and the integrity of GA during uptake was monitored using antibody detection. Sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of intestinal homogenates that had been exposed to GA were performed to identify GA presence. An enzyme-linked immunosorbent assay (ELISA) protocol was adapted for GA quantification. Immunohistochemistry was undertaken to examine the rat colonic wall for GA uptake, and confocal microscopy was used to differentiate adsorbed and internalized peptide in cultured colorectal adenocarcinoma cells.

Results. The colon and the ileum, respectively, were identified to be the intestinal regions in which GA was maximally preserved during uptake from the lumen. GA was identified to cross the colonic wall from the epithelium to the serosa. Internalization of GA into cultured colonic epithelial cells was demonstrated.

Conclusions. The rat colonic wall was identified to be less proteolytically active toward GA compared to the wall of the more proximal regions of the small intestine. GA has the capacity to penetrate from the lumen into the colonic wall. The maintenance of GA integrity within the wall of the colon offers the potential for local biological activity of the drug.

KEY WORDS: colon; copolymer 1; ELISA; glatiramer acetate; oral drug delivery; peptide drug.

INTRODUCTION

Oral peptide and protein drug administration for delivery into the systemic circulation is seriously jeopardized by proteolysis affected by the abundant proteases in the intestinal tract and by first pass metabolism in the liver (1). Local treatment of the mucosa is a relatively understudied alternative application of oral peptide and protein administration which requires drug stability to the target site: the mucosa. Local application to the intestinal wall may be for the purpose of treating damaged and diseased mucosa and for the induction of an immune response at the level of the mucosa which may translate to a systemic influence. The potential of locally treating the mucosa may be examined using a model drug.

Glatiramer acetate (GA, the active component of the marketed drug Copaxone, also referred to as copolymer 1) is the acetate salt form of a polypeptide series of molecular weight 4.7–11 kDa which is randomly synthesized from L-alanine, L-glutamate, l-lysine, and L-tyrosine. When delivered subcutaneously to patients with relapsing-remitting multiple sclerosis (MS), the attack frequency is reduced by 30% and disease activity decreases as detected by MRI imaging (2). The potential of the more attractive oral route for GA administration was demonstrated by the inhibited induction in mice and rats of experimental allergic encephalomyelitis (EAE, the animal model of MS) following gastric intubation of GA. The efficacy of this mode of administration suggests that the drug is able to activate the immune system within the gastrointestinal (GI) tract and effect a systemic response (3).

To gain an understanding of the mechanism of drug action within the GI tract, it is important to identify the level of drug stability and quantify local drug concentrations in a given intestinal region. The aims of this study were to examine GA stability during uptake into the mucosa of the rat intestine, develop an assay for quantifying local drug concentrations in mucosal tissues, and define an intestinal region(s) with the greatest potential for GA structural preservation which may be targeted for local therapy.

MATERIALS AND METHODS

Materials

Glatiramer acetate (Copaxone) and affinity purified rabbit polyclonal antisera directed toward GA were received from Teva Pharmaceutical Industries (Netanya, Israel). The SW480 colorectal adenocarcinoma cell line was supplied by the Amercan Type Culture Collection (ATCC, Manassas, Virginia). All other materials were purchased from Sigma Chemical Co. (St. Louis, Missouri), unless otherwise stated in the text.

Animals, Maintenance, and Euthanasia

Male Sabra rats (200–300 g), fed with standard laboratory chow and tap water and kept under constant environmental conditions (22°C, 12 h light/dark cycles), were obtained from the Animal Farm of Hadassah Medical Center and The Hebrew University (Jerusalem, Israel). All animal studies were conducted in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication No. 85-23, revised 1985). The Mutual Committee of Hadassah University Hospital and the Faculty of Medicine for Animal Welfare approved the study. Euthanasia of the anesthetized rats was carried out by chest wall puncturing.

Studies of GA Uptake into Rat Intestinal Sacs

GA uptake by the rat gastrointestinal (GI) tract was monitored *in vitro*. Briefly, intestinal segments were excised from the duodenum, jejunum, ileum, and colon of four anesthetized rats and rinsed with PBS containing protease inhibitors (PBS-PI; Complete Cocktail tablets, Boehringer Mannheim, Mannheim, Germany). Sacs (2-cm long) filled with GA (10 mg/ml PBS-PI) and secured by surgical suture were sub-

[1] The Hebrew University of Jerusalem, Faculty of Medicine, School of Pharmacy, P.O. Box 12065, Jerusalem 91120, Israel.

[2] Teva Pharmaceutical Industries, Innovative R&D Division, P.O. Box 8077, Kiryat Nordau, Netanya 42080, Israel.

[3] To whom correspondence should be addressed. (e-mail: avri@cc.huji.ac.il)

ABBREVIATIONS: EAE, experimental allergic encephalomyelitis; ECL, enhanced chemiluminescence; GA, glatiramer acetate; GI, gastrointestinal; HRP, horse radish peroxidase; IgG, immunoglobulin G; MBP, myelin basic protein; MS, multiple sclerosis; PI, protease inhibitors; TMB, 3,3′,5,5′-tetramethylbenzidine.

0724-8741/02/0600-0832/0 © 2002 Plenum Publishing Corporation

merged in PBS-PI and agitated for designated time intervals at 37°C. At the completion of the incubation period, the sac contents were removed and the sacs rinsed thoroughly in PBS-PI. Samples were snap-frozen in liquid nitrogen prior to homogenization. Control tissue from each intestinal region that had not been exposed to GA was sampled. Snap-frozen tissues were homogenized in PI-containing ice-cold phosphate buffer (0.2 M) using a polytron homogenizer (Kinematica, Lucerne, Switerland). Protein content was quantified using the Bradford method.

Homogenized samples (100 μg protein equivalent) were subjected to SDS-PAGE (18% acrylamide) in reducing sample buffer and Western transferred. Immunoblotting was undertaken using affinity purified rabbit anti-GA antibodies (anti-GA antibodies), which were detected using a horse radish peroxidase (HRP)-antirabbit immunoglobulin (IgG) antibody. Antibody staining was detected using the enhanced chemiluminescence (ECL) detection method.

GA levels in the tissue homogenates were quantified using an enzyme-linked immunosorbent assay (ELISA) modified by the introduction of a sample denaturing step to impede proteolysis and to enhance protein unfolding. Briefly, homogenized samples (100 μg protein equivalent) were boiled for 2 min in reducing sample buffer (in the absence of bromophenol blue) prior to application to the ELISA plate. Antigen detection was undertaken using the anti-GA antibodies and an HRP-antirabbit IgG antibody. Antibody staining was detected using 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard and Perry, Gaithersburg, Maryland).

Assessment of [^{125}I]GA Uptake into Rat Intestinal Everted Sacs

To compare [^{125}I]GA regional uptake in the rat intestine, GA (100 μg) was iodinated using iodine-125 (1 mCi; IMS30, Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England) and chloramine T (1 μmol) (4) for 30 s at room temperature, stopped with sodium disulfide (added to a final concentration of 0.5 M), then potassium iodide was added (200 μg). Labeled GA (120 μl) was separated from free ^{125}I on a 10-ml Sephadex G-10 column (Pharmacia, Uppsala, Sweden). The iodination efficiency in the active fractions was determined by precipitating fraction aliquots (5 μl) with trichloracetic acid (final concentration of 10%) in the presence of BSA (0.5% final concentration) on ice for 10 min (conditions that precipitate long peptide fragments). Centrifugation (12,000 rpm, 3 min; Micro 12-24, Hettich, Tuttlingen, Germany) separated supernatants, and pellets were subsequently monitored for radioactivity. Labeling efficiency of 96% was achieved as determined by:

$$\text{Labeling efficiency (\%)} = (\text{pellet cpm/total cpm}) \times 100$$

Everted sacs (2-cm long) from the duodenum, jejunum, ileum, and colon segments were prepared (5) and filled with PBS-PI and secured with a 3/0 silk suture at each end. Each everted sac was incubated in 3 ml of PBS-PI alone (controls) or containing [^{125}I]GA (70,000–120,000 cpm per sac) and nonlabeled GA (1 mg/ml). The sacs were incubated at 37°C in a shaking bath (130 oscillations per min) for 30 min. The sacs were then removed, rinsed, and cut open. The serosal fluids were collected and the empty sacs weighed. The radioactivity of the tissues and the fluids were monitored in a γ-counter. Measurements were normalized to a sac wet weight of 350 mg (a value close to the weight of all the individual sacs) and expressed as a fraction of the total radioactivity (%) introduced at the beginning of the incubation (where the total radioactivity was in excess). Two rats were used in each study, and 2–3 sacs were prepared from each intestinal segment.

Evaluation of GA Degradation by Peptidases of the Lumen and Wall of the Colon

To examine the contribution of the colonic lumenal contents and the epithelial surface to the proteolytic degradation of GA, the following experiment was undertaken. Intestinal sacs from the rat colon were prepared either with rinsing (as described above using PBS-PI) or without removing the lumenal contents, and exposed to GA both in the absence and presence of PI for 15 min.

Visualization of GA Uptake into the Rat Colonic Wall

The uptake of GA into the colonic wall was established by mounting rinsed colonic tissue (sampled from anesthetized rats as described above) into diffusion chambers (Easy Mount, Physiologic Instruments, San Diego, California) with the epithelia oriented toward the donor compartment. The donor compartment was filled with GA (10 mg/ml PBS). The tissues were exposed to GA for designated times (15 and 60 min) prior to removal, rinsing (3 times in PBS), and fixation (4% formaldehyde, 1-h RT prior to immersion in a 2.1 M sucrose solution until tissue submersion). Maintenance of transepithelial resistance across the mounted tissue indicated that leakage did not occur and that GA was transferred through the intact wall. Fixed tissues were snap-frozen (liquid nitrogen), mounted on a microtome stub with OCT embedding media (Sakura, Torrance, California; –28°C), cryostat sectioned (10 micron, Leica CM 3000, Limburg, Germany; –20°C), and transferred to cationically charged microscope slides (Superfrost, Electron Microscopy Sciences, Fort Washington, Pennsylvania). Sections were allowed to air dry and stored at –20°C prior to staining. Sections were then processed for immunohistochemical staining.

Sections were blocked in normal goat serum (10%), 1-h room temperature (RT). The primary antibody (anti-GA antibodies) was then added for 1-h RT. Sections were rinsed 3 times in PBS. The secondary antibody FITC-goat antirabbit IgG was added for 1 h, and the tissues were subsequently rinsed 3 times with PBS. Coverslips were mounted onto slides in Molwiol containing DABCO antiquenching agent. Staining was monitored using an inverted microscope (Nikon Eclipse TE300, Belmont, California).

Studies of GA Internalization into Immortalized Human Epithelial Cells

The ability of the human carcinogenic colonic epithelial cells (line SW480) to internalize GA in culture was examined. SW480 cells grown in 24-well trays on coverslips (2×10^4/ 200 μl) were exposed to GA for defined time intervals (15, 30, 60, and 120 min) at 37°C. At the completion of the designated exposure time, the cells were rinsed in PBS thoroughly, snap-frozen in methanol (–20°C), and, following rehydration in PBS, processed for immunohistochemistry (as described

above). GA internalization into the cells was examined using confocal microscopy (Zeiss, Gottingen, Germany).

RESULTS

Western Immunoblot Analysis of GA Integrity in Regions of the Rat Intestine

GA uptake into intestinal sacs prepared from the rat duodenum, jejunum, ileum, and colon (rinsed to clear lumenal content) was identified *in vitro* via Western immunoblot analysis (Fig. 1 is a representative result from the 4 rats). GA uptake in the presence of protease inhibitors, was highest in the colon and ileum. Lower levels were detected in the jejunum and duodenum, respectively.

Importantly, antibody detection requires a minimum recognition sequence (of around six amino acids; thus, fragments of potentially biologically relevant size (i.e., those able to activate the T-cell receptor in the context of MCH-II [6]) would be identified using this approach. Smaller fragments, the product of enzymatic or chemical hydrolysis, would not be identified using this approach.

ELISA Quantification of GA Levels in the Rat Intestinal Sacs

Quantification of GA levels in the intestinal sacs was performed using an ELISA modified to maintain the integrity of GA (Fig. 2). Consistent with the qualitative observations of GA in the tissues as viewed via Western blot analysis, GA levels were maximal after 15-min incubation in the colon and ileum with lower levels detected in the jejunum and duodenum, respectively. The relative levels of GA bound by the colon, ileum, jejunum, and duodenum were calculated to be 22.02 ± 4.15, 12.08 ± 2.68, 3.82 ± 0.67, and 0 ± 0.56 ng/100 μg protein, respectively (determined by subtracting from the levels of GA determined in the presence of GA: 28 ± 3.15, 17.88 ± 1.90, 7.82 ± 0.42, and 5.95 ± 0.22 ng/100 μg protein, respectively; the background levels determined in the absence of GA: 5.98 ± 0.98, 5.8 ± 0.78, 4.00 ± 0.25, and 6.4 ± 0.34 ng/100 μg protein, respectively).

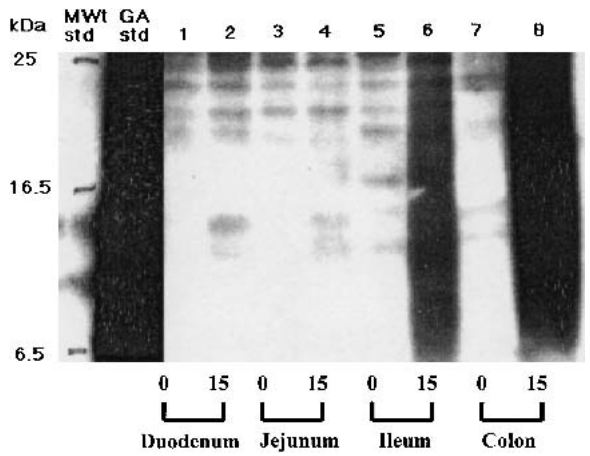

Fig. 1. GA uptake into rat intestinal segments (0, 15 min after incubation) as monitored by Western blot analysis.

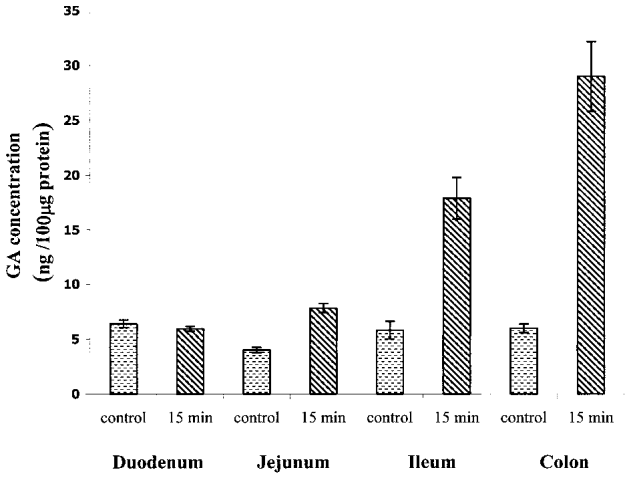

Fig. 2. Quantification of GA uptake into rat intestinal segments as determined by ELISA (where "control" refers to tissues not exposed to GA and "15 min" refers to the incubation time in the presence of GA). Results are the mean of three studies ± SD.

Monitoring of [^{125}I]GA Uptake into Rat Intestinal Everted Sacs

Low detectable GA levels in the duodenum and the jejunum segments may have resulted from either of the following possibilities: GA did not get taken-up by these intestinal tissues; GA was taken-up intact and transported through the serosa, leaving no detectable GA within the tissue; GA was degraded at the epithelial surface and was not taken-up into the tissue; or GA was degraded at the epithelial surface and the GA fragments were taken up by the mucosa. To address these questions, [^{125}I]GA was incubated within everted sacs and the accumulation of the radioactive label was monitored within the intestinal sacs and the serosal fluids.

The levels of ^{125}I monitored in the serosal fluids and intestinal walls of the everted sacs prepared from rat duodenum, jejunum, ileum, and colon, after incubation with [^{125}I]GA in PBS for 30 min, are displayed in Fig. 3. ^{125}I accumulated most abundantly in the wall and the serosal fluids of the ileum with lower levels detected in the jejunum, the duodenum, and the colon, respectively. The discrepancies between the levels of GA detected using anti-GA antibodies and the levels of ^{125}I monitored likely reflect the ability of the antibody to bind peptide fragments (≥6 amino acids) whereas radioactive monitoring detects ^{125}I-label without discrimination on the basis of peptide size. By comparing the data concerning GA uptake as detected using antibodies with the levels of ^{125}I uptake, it may be suggested that the low levels of intact GA (or large peptidic GA fragments) detected in the duodenum and the jejunum resulted from proteolysis (presumably of brush border origin) of the GA. The high levels of ^{125}I-label in these wall segments suggest that GA had been degraded during uptake into the wall and serosal fluid.

Comparison of Peptidase Activity in the Lumen and Wall of the Colon

The proteolytic activity of the lumenal contents and the mucosa toward GA were assessed (Fig. 4). GA was identified using antibody staining in the colonic segments (free of lumenal contents) even in the absence of PI, suggesting that

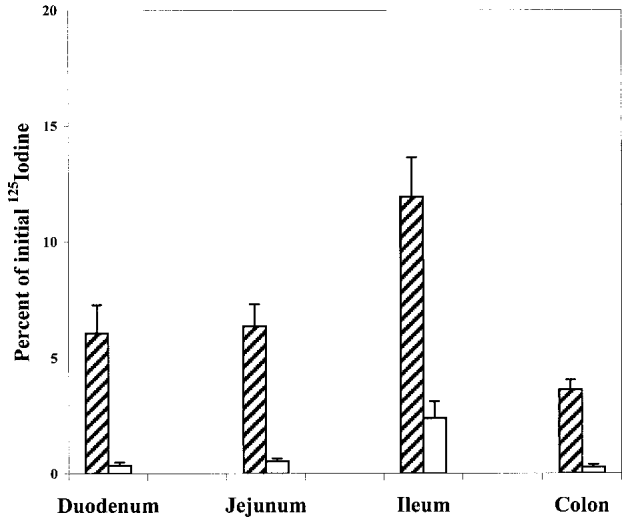

Fig. 3. Uptake of ^{125}I-label by everted sacs of equivalent wet weight (350 mg) prepared from the duodenum, jejunum, ileum, and colon of the rat (cross-hatched columns) and the corresponding serosal fluid (empty columns) as measured after 30 min incubation with [^{125}I]GA. The values are expressed as a percentage of the total ^{125}I radioactivity introduced at the commencement of incubation. Shown are the mean values of four studies ± SD.

colonic epithelial proteolytic activity was either weak or absent. In contrast, when GA was incubated in intestinal sacs containing lumenal contents (in the presence of PI), little GA was detected in the intestinal tissue. These findings either suggest that the GA is complexed by the lumenal contents and is consequently not available for epithelial uptake, which is unlikely due to the abundant excess of GA, or more probably, that lumenal proteases either produced by the colonic microflora or accumulated in this region are able actively to degrade the GA much faster than epithelial proteases.

Immunohistochemical Detection of GA Uptake into the Rat Colonic Wall

The studies described above of tissue homogenates are unable to distinguish between GA adherance to the mucosal surface and GA uptake into the epithelia. Immunohistochemical analyses of intestinal sacs incubated with GA were undertaken to resolve this question. GA uptake into the wall of the rat colon, from the epithelia to the serosa, occurred in a time-dependent manner (Fig. 5). Examination of GA uptake into *in vitro* cultured SW480 cells further indicated that GA could be internalized rather than merely adhering to the surface of the cells (Fig. 6).

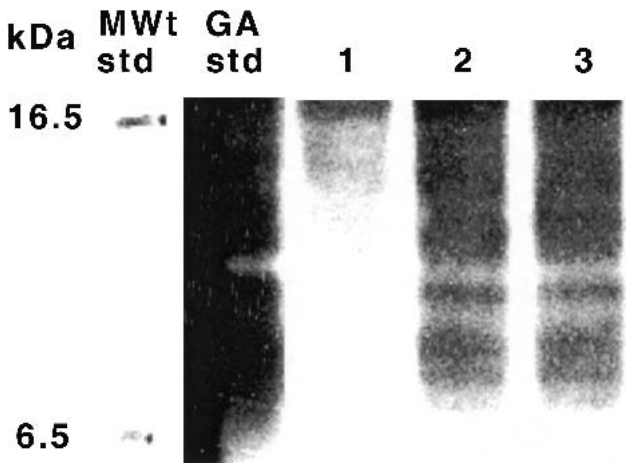

Fig. 4. GA stability in the wall segments of the rat colon following 15 min of incubation as determined by Western blot analysis. Lane 1: plus colon contents, plus PI; lane 2: no colon contents, no PI; lane 3: no colon contents, plus PI.

DISCUSSION

The colon and the ileum, respectively, were identified in this study as the intestinal regions where the structure of the peptide drug GA was best preserved during local uptake onto the intestinal wall. The colon lacks villi and is therefore morphologically distinct from the more proximal regions of the small intestine which expose a membrane brush border rich in proteolytic enzymes (7). An important feature of the distal ileum is the presence of Peyer's patches covered by specialized cells bearing sparse irregular microvilli or microfolds (microfold cells; M-cells) which are able to sample large molecules for immune priming (8).

The comparatively low protease activity at the apical surface of the colonic epithelium and the extended residence through this intestinal region have led the colon to be recognized as a potential portal for peptide and protein drugs into the circulatory systems (reviewed in Refs. 9 and 10). Membrane extracts of the wall of the colon have been identified to be less proteolytic than those of the small intestine to the drugs insulin and calcitonin (11,12). The capacity of the colon to take-up peptide and protein drugs has been inferred from *in vitro* studies of internalization by cultured immortalized epithelial cells (13–15) and diffusion across intestinal segments prepared as everted sacs (16) (reviewed in Ref. 4) or mounted in diffusion chambers (17). The capacity of the colon to take-up protein drugs *in vivo* has largely been assessed in terms of systemic levels circulating or biological action of the administered drugs such as insulin (where circulating insulin levels and reduced glucose levels demonstrate efficacy; reviewed in Ref. 10). Also, leuprolide, a nonapeptide, has been shown to be maximally bioavailable in anesthetized rats following administration to closed loops of the colon and the ileum, respectively, with significantly lower levels following administration to the jejunum (17). In the current study in contrast to those described, we examined *in vitro* the integrity and concentrations of the peptide drug GA during uptake into specific regions within the small and large intestine of the rat intestinal wall. Clearly, the colon and the ileum have unique properties that favor local peptide uptake and which may be exploited for local drug administration.

Identification of GA using antibody detection (an approach that appears to recognize fragments of at least a six-amino-acid length [6]) led to the detection of GA at the epithelia, across the width of the mucosa to the serosa. Peptide fragments of around 10 to 30 amino acids can bind the MHC-II of antigen-presenting cells required to stimulate a T-cell response (6). Distribution of drug fragments of a sufficient magnitude to bind MHC-II, in the proximity of the relevant cells, would suggest that stimulation of an immune response through colonic uptake is a theoretical possibility.

In contrast to the structural preservation of GA during exposure to the colonic wall, the inclusion of the contents of

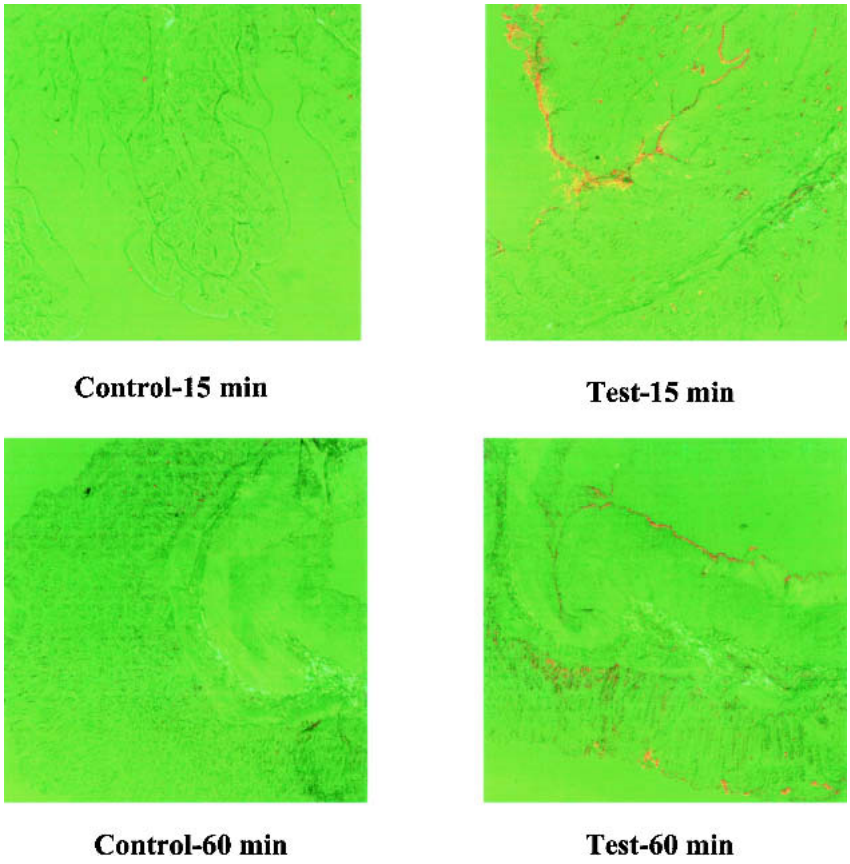

Fig. 5. Immunohistochemical detection of GA infiltration into rat colonic tissues after incubation in diffusion chambers for 15 and 60 min.

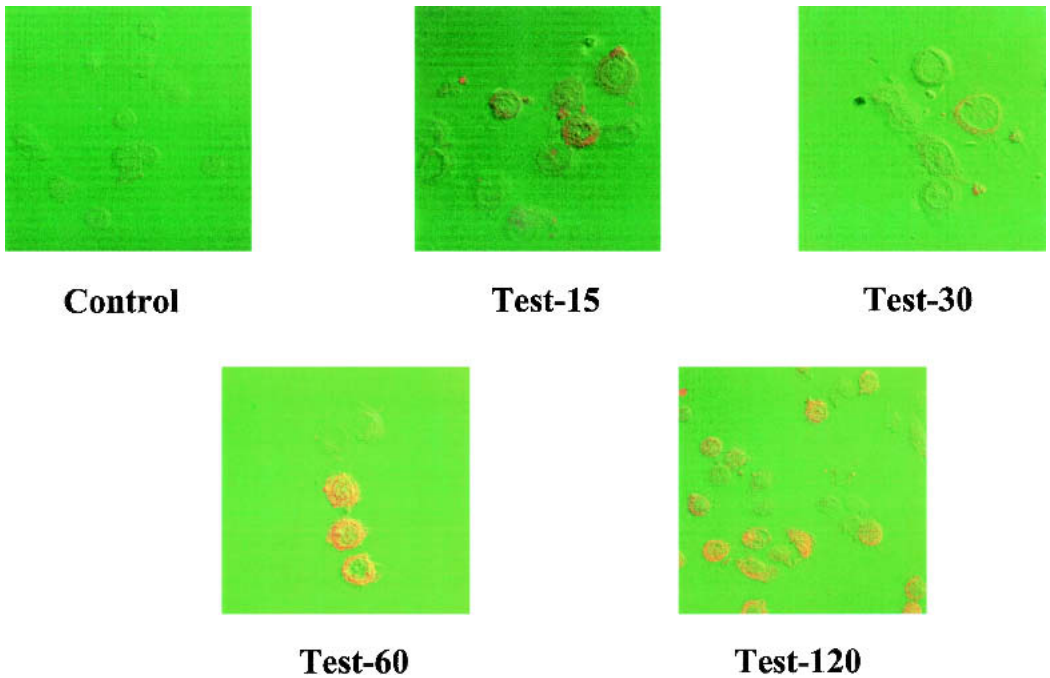

Fig. 6. GA internalization into SW480 colon adenomacarcinoma cells after 0, 15, 30, 60, and 120 min of incubation as monitored by confocal microscopy.

the colonic lumen resulted in comparatively rapid degradation of GA. Similarly, insulin and calcitonin were found to degrade rapidly in the presence of the lumenal contents of the colon as compared to degradation induced by the membrane homogenates (11,12). These findings indicate that while the colonic wall may be a relevant drug delivery site for local therapy, the harsh surrounds pose a major challenge to approaching this target site.

Peptide preservation during oral delivery to effect local therapy is likely to be enhanced by incorporation into a protective delivery system. A successful example is the oral delivery of the anti-inflammatory cytokine interleukin-10 (IL-10), produced by genetically engineered *Lactoccus lactis* to treat ulcerative colitis in a mouse model. Interestingly, although the cyotokine was apparently released from the bacteria along the length of the intestine, only in the region of the apparently less proteolytic colon was it detected (18). This study demonstrated that proteins, presented in a relevant delivery system to the colon, are able to stimulate a biological response. Thus, incorporation of GA into a pertinent delivery platform may promote intact delivery to the target site.

While these studies indicate the potential relevance of the colon and possibly the ileum as target sites for drug delivery to evoke local therapy, the pertinence of these regions to the oral delivery of GA in the Teitelbaum study (3) is remote. In light of the observed degradation of GA in the upper small intestinal tract in this current study, it is unlikely that rats orally fed GA (3) were able to take up large quantities of bioactive drug fragments from the region distal to and including the duodenum. One possibility may be that only minute amounts of GA are required to stimulate the therapeutic immune response in the small intestine of the EAE-induced rats. Another possible scenario is that GA administered in PBS using a feeding needle stimulated an immune response through penetration into the lymphatics at a site proximal to the small intestine. Nasally administered myelin basic protein (MBP) has been shown to stimulate immune suppression although it was shown to effect suppression of disease onset only when administered prior to disease induction or up to the first day of appearance of clinical signs (19).

A number of investigations are obvious extensions of the current findings. Defining the actual site of immune stimulation in the Teitelbaum (3) study remains an issue. Assessing whether the Waldeyer's ring (the rat adenoid/tonsil equivalent) is the site where GA encounters the immune system or whether the immune system can be stimulated by extremely low levels of GA in the small and large intestine remains to be addressed. In addition, this study has stimulated the question whether the colon and the ileum may be appropriate sites to target with GA for the induction of Bystander suppression. An appropriate *in vivo* examination of these questions would be to administer GA site- and dose-specifically to the nasal-pharyngeal region and specific locations within the small and large intestine and evaluate the impact of the drug on the rate of disease onset. In light of any clear suppression of onset, the system should then be more specifically dissected for cytokine stimulation in the appropriate location to ascertain the nature of the immune suppression of the autoimmune disease EAE.

REFERENCES

1. J. F. Woodley. Enzymatic barriers for GI peptide and protein delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **11**:61–95 (1994).
2. B. G. Arnason. Immunologic therapy of multiple sclerosis. *Annu. Rev. Med.* **50**:291–302 (1999).
3. D. Teitelbaum, R. Arnon, and M. Sela. Immunomodulation of experimental autoimmune encephalomyelitis by oral administration of copolymer 1. *Proc. Natl. Acad. Sci. U S A* **96**:3842–3847 (1999).
4. D. M. Weir. *Labelling Proteins and Polypeptides*, 2nd edition, Blackwell Scientific, Abingdon, Oxon, United Kingdom 1973.
5. L. Barthe, J. Woodley, and G. Houin. Gastrointestinal absorption of drugs: Methods and studies. *Fundam. Clin. Pharmacol.* **13**:154–168 (1999).
6. A. A. Abbas, A. H. Lichtman, and J. S. Pober. *Cellular and Molecular Immunology*. W.B. Saunders Co., Philadelphia, London, Toronto, 1994.
7. R. L. Bacon and N. R. Niles. *Medical Histology*, Springer-Verlag, New York, Heidelber, Berlin, 1983.
8. P. Onori, A. Franchitto, R. Sferra, A. Vetuschi, and E. Gaudio. Peyer's patches epithelium in the rat: A morphological, immunohistochemical, and morphometrical study. *Dig. Dis. Sci.* **46**:1095–1104 (2001).
9. A. Rubinstein, B. Tirosh, M. Baluom, T. Nassar, A. David, I. Gliko-Kabir, R. Radai, and M. Friedman. The rationale for peptide drugs delivery to the colon and the potential of polymeric carriers as effective tools. *J. Control. Release* **46**:59–73 (1997).
10. W. Wang. Oral protein drug delivery. *J. Drug Target.* **4**:195–232 (1996).
11. K. Ikesue, P. Kopeckova, and J. Kopecek. Degradation of proteins by Guinea pig intestinal enzymes. *Int. J. Pharm.* **95**:171–179 (1993).
12. R. H. Lu, P. Kopeckova, and J. Kopecek. Degradation and aggregation of human calcitonin in vitro. *Pharm. Res.* **16**:359–367 (1999).
13. Y. Sai, M. Kajita, I. Tamai, J. Wakama, T. Wakamiya, and A. Tsuji. Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells. *Am. J. Physiol.* **275**:G514–G520 (1998).
14. Y. Sai, M. Kajita, I. Tamai, J. Wakama, T. Wakamiya, and A. Tsuji. Adsorptive-mediated transcytosis of a synthetic basic peptide, 001-C8 in Caco-2 cells. *Pharm. Res.* **15**:1305–1309 (1998).
15. M. Heyman, A. M. Crain-Denoyelle, S. K. Nath, and J. F. Desjeux. Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2. *J. Cell. Physiol.* **143**:391–395 (1990).
16. J. A. Atchison, W. E. Grizzle, and D. J. Pillion. Colonic absorption of insulin: an in vitro and in vivo evaluation. *J. Pharmacol. Exp. Ther.* **248**:567–572 (1989).
17. Y. Zheng, Y. Qiu, M. F. Lu, D. Hoffman, and T. L. Reiland. Permeability and absorption of leuprolide from various intestinal regions in rabbits and rats. *Int. J. Pharm.* **185**:83–92 (1999).
18. L. Steidler, W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**:1352–1355 (2000).
19. L. Y. Xu, J. S. Yang, Y. M. Huang, M. Levi, H. Link, and B. G. Xiao. Combined nasal administration of encephalitogenic myelin basic protein peptide 68-86 and IL-10 suppressed incipient experimental allergic encephalomyelitis in Lewis rats. *Clin. Immunol.* **96**:205–211 (2000).