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Vet. Res. 29 (1998) 527–536

Original article

Effect of dietary insulin on the response of suckling mice enterocytes to *Escherichia coli* heat-stable enterotoxin

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(Received 13 October 1997; accepted 24 July 1998)

Abstract – Effect of insulin on the response of suckling mice to the enterotoxigenic *Escherichia coli* heat-stable enterotoxin (STa) was studied. Four groups (8–10 in each group) of 2-day-old Swiss Webster suckling mice were used. For this study, 5, 10, 25 and 50 µg of insulin was given orally to half the mice in each group for 7 days. The rest of the mice in each group were given normal saline as intra-litter controls. After 7 days, a suckling mouse assay in which 1 µg of STa was given to all mice in insulin-treated and control groups was performed. Enterocyte suspensions were prepared from mice in all groups. Intestinal tissue samples were taken for electron microscopy. Interaction of STa with its putative receptor on the enterocytes was evaluated using indirect immunofluorescence and flow cytometry. The suckling mouse assay revealed a significant increase in the gut weight to body weight ratio in all mice in the insulin-treated groups compared to control mice ($P < 0.05$). Flow cytometry and indirect immunofluorescence analyses suggested that insulin had an up-regulatory effect on the STa-receptor level. Similarly, insulin was found to increase intestinal brush border membrane differentiation as indicated by the increase in the inward movement of milk particles through the intestinal mucosa. Insulin seems to modify the structure-function of the brush border membrane including the response of suckling mice to STa. This study may provide further insights into the mechanism of STa/receptor interaction, which is a major cause of diarrhea in newborn animals and human infants. © Inra/Elsevier, Paris.

insulin / enterocytes / *Escherichia coli* / heat-stable enterotoxin / receptor

Résumé – Effet de l'administration orale d'insuline sur la réponse des entérocytes de souriceaux à la toxine thermostable d'*Escherichia coli*. L'étude a porté sur l'influence de l'insuline sur la réponse à la toxine thermostable (STa) de l'*Escherichia coli*, chez les souriceaux. Quatre groupes de

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souriceaux Swiss Weber, âgés de 2 j (8 à 10 sujets par groupe) ont été utilisés. Dans chaque groupe, la moitié des souriceaux a reçu une dose de 5, 10, 25 ou 50 µg d'insuline par voie orale pendant 7 j. Le reste des souriceaux dans chaque groupe a servi de témoin (sans insuline). Tous les souriceaux ont reçu 1 µg de STa par voie orale. Sept jours plus tard, le test de détection de la toxine thermostable a été realisé sur trois souriceaux traités à l'insuline et trois sujets témoins. Une suspension d'entérocytes a été réalisée pour chaque groupe de souriceaux. Des échantillons d'intestins on été prélevé pour étude au microscope électronique. L'action de la STa sur ses récepteurs entérocytaires a été étudiée par immunofluorescence indirecte et cytométrie de flux. Une augmentation significative du rapport entre le poids de l'intestin et le poids du corps, a été montrée dans tous les groupes ayant reçu de l'insuline ($p < 0,05$). Les résultats de la cytométrie de flux et de l'immunofluorescence indirecte suggèrent que l'insuline provoque une augmentation du niveau des récepteurs de la STa. L'augmentation de l'endocytose des globules de lait indique que l'insuline favorise la différentiation des membranes des bordures en brosse. Ces changements structuraux et fonctionnels des membranes des bordures en brosse semblent liés à la modification par l'insuline du rapport entre la STa et le niveau des récepteurs. Ce changement donne une idée du mode d'interaction STa/récepteurs, phénomène à l'origine des diarrhées des animaux nouveau-nés et des enfants. © Inra/Elsevier, Paris.

insuline / entérocytes / *Escherichia coli* / toxine thermostable / récepteur

1. INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* (ETEC) produce peptide toxins, which alter intestinal water balance and lead to acute diarrheal illnesses in humans and animals. A large (85–90 kDa), heat-labile toxin (LT) has been well described and shares many structural and functional homologies with cholera toxin, including the ability to ADP-ribosylate and irreversibly activate intestinal adenylate cyclase [3, 16]. The other classes of *E. coli* toxins are low molecular weight, heat-stable enterotoxins (ST). There are two types of heat-stable enterotoxin, STa and STb. STb has mostly been observed in pigs and calves, but has not been well characterized [4, 5]. STa, on the other hand, has been purified to homogeneity [16, 18, 20]. STa-producing *E. coli* is a major cause of diarrhea in newborn animals and human infants. STa is composed of 18 amino acids and has a molecular weight of 2 kDa. Slight variations in size were observed in some host species, but heterogeneity of size of STa is not reflected in a difference in their mechanism of action or potencies [14, 16, 17]. STa has been observed to dramatically alter guanosine 3',5'-cyclic monophosphate (cGMP) metabolism via the activation of intestinal guanylate cyclase followed by a blockade of inward ion transport and subsequent secretion of water into the intestinal lumen [5, 14, 22]. The sequence of events which ends in stimulation of intestinal fluid secretion and diarrhea is initiated by STa binding to a specific receptor located on the brush border membrane of the intestinal epithelial cells of the host [17, 22]. The events end with an increase in the level of intracellular cGMP, and an accumulation of fluids and electrolytes intraluminally, which leads to diarrhea [22]. Several reports suggest that the STa-receptor has a polymeric structure of a glycoprotein with a molecular size of 140 kDa and a topological organization that is similar to the atrial and brain peptide receptors [23]. The STa-receptor is part of the extracellular motif of the brush border-associated guanylyl cyclase [22, 23].

It has been reported that some growth factors such as insulin and epidermal growth factor (EGF) are commonly found in milk of lactating women and other mammals [9, 21]. A major site for the synthesis of these circulating growth factors is the liver, but insulin-like growth factor-I (IGF-I) is also produced locally in other tissues [19, 21]. The biological effect of growth factors is mediated by their binding to specific recep-

tors on the plasma membrane. The number of these receptors on the membranes can be regulated by the concentration of growth factors in the environment [24]. One major site for the action of growth factors is the intestinal brush border membrane.

This study was initiated to explore the effect of insulin on the intestinal response of suckling mice to STa. The effect of insulin on the intestinal brush border membrane differentiation was studied using electron microscopy. Flow cytometry and indirect immunofluorescence were utilized to study the effect of insulin on the STa/receptor interaction.

2. MATERIALS AND METHODS

2.1. STa purification

STa was isolated and purified to homogeneity by growing an ETEC strain in a chemically defined medium, desalted and concentrated by batch adsorption chromatography on Amberlite XAD-2 resin, reversed phase silica and preparative reverse-phase high performance liquid chromatography (RV-HPLC). This rapid purification scheme gave high recovery yields of pure STa, which exhibited biochemical homology to STa purified by different procedures. No contamination was detected in the HPLC-purified STa. This procedure has been described in detail, and homogeneity of the purified STa was established as described by Saeed and Greenberg [16].

2.2. Insulin feeding of suckling mice

Four litters (8–10 mice in each group) of 2-day-old Swiss Webster suckling mice were used. Half the mice in each litter received 5,10, 25 and 50 μg of insulin (isophane insulin suspension, USP, Eli Lilly & Company, Indianapolis, IN, USA). The rest of the suckling mice in each litter received normal saline and were kept as intra-litter controls. All treatment groups were given the different oral insulin doses daily for 7 days using a 0.5-mL syringe and a 50-μm diameter polyethylene tube. We assumed that all mice had a similar chance to suckle their mothers during the 7-day experiment.

2.3. Suckling mouse assay

Mice were separated from their mothers immediately before use and randomly divided into groups of three. These mice were starved for 2 h before inoculation. Each mouse was inoculated orally with a diarrheagenic dose of HPLC-purified STa (0.1 mL PBS containing 1 μg STa) with one drop of 2 % Evans blue. After 3 h, mice were killed by cervical dislocation, the abdomen was opened, and the entire intestine (excluding the stomach) was removed with forceps. The intestines from each group were pooled and weighed. The ratio of the intestinal weight to the remaining carcass weight was calculated. Animals with no dye in the intestine or with dye within the peritoneal cavity were excluded from the calculations. One mouse unit (MU) of STa was considered as the minimum amount of STa that induced a gut to remaining body weight ratio of 0.087 or higher in a 2-day-old suckling mouse [6]. Each 1 μg STa dose contained 200 MU to ensure a diarrheagenic effect on suckling mice older than 2 days.

2.4. Electron microscopy

One half centimeter of small intestine from each mouse of the different groups was fixed in 3 % glutaraldehyde, and then post-fixed in 1 % osmium tetraoxide. Tissues were dehydrated, infiltrated in different concentrations of epon and incubated under high vacuum. Thin sections were prepared and stained with uranyl acetate and lead acetate and viewed in a JEOL, JEM-100, CX electron microscope.

2.5. Isolation of suckling mouse enterocytes

Several published methods for preparing enterocytes were utilized, but the following procedure gave the best results for the suckling mouse. Mice were killed by cervical dislocation; the entire intestine from each group was collected and placed in tissue culture medium (Medium 199). The intestine was moved to a petri dish containing dithiothreitol-EDTA solution (1.5 mM EDTA, 0.5 mM dithiothreitol (DTT), and 1 000 I.U. of penicillin/streptomycin dissolved in phosphate buffer solution PBS (pH 7.2)). The intestine was chopped into very small pieces using a sterile blade and left in the DTT solu-

tion for 45 min. The mixture of the intestine and the DTT solution was filtered through a cotton filter to remove particulate material. Enterocyte suspension was filtered through a nylon-mesh filter (50 μm), centrifuged at 1 000 *g* for 5 min and then washed three times with PBS to remove any traces of the DTT solution. The population of cells harvested was monitored by periodic wet mount examination through the whole procedure to assess the quantity and quality of the isolated enterocytes. Cell counts and cell viability were determined by exclusion of 0.2 % trypan blue-stained enterocytes. Dry smears were fixed in 100 % methanol for both indirect immunofluorescence and Giemsa staining. The remaining cells were used for flow cytometric analysis.

2.6. Indirect immunofluorescence assay

Intestinal epithelial cells isolated from suckling mouse intestine were washed three times in 10 mM PBS (pH 7.2) to remove any traces of the DTT solution. Smears of enterocytes were made on glass slides, air-dried, and then fixed in absolute methanol for 10 min. Slides were incubated with STa, rabbit anti-STa antibody and anti-rabbit-IgG-FITC conjugated antibody as described by Saeed et al. [17]. As negative controls, similar samples were incubated only with STa and anti-rabbit-IgG-FITC-conjugated antibody. Non-specific fluorescence was estimated using isotope controls (enterocytes incubated with irrelevant specific FITC-conjugated antibodies).

2.7. Flow cytometry analysis

Enterocytes were prepared for staining by three additional washings in PBS, pH 7.2, containing 0.5 % BSA. In a volume of 100 μL, 10^5 enterocytes, in PBS–BSA, were incubated with 50 μL of HPLC purified STa (10 μg/mL of 10 mM PBS) for 45 min at 37 °C. After washing three times in PBS–BSA, enterocytes were resuspended in 100 μL of PBS–BSA. Fifty microliters of STa-specific antiserum produced in rabbits were diluted 1:10 in PBS and added to the enterocyte suspensions and incubated for 30 min at 4 °C. Cells were then washed three times with PBS–BSA and then resuspended in 100 μL PBS–BSA. Fifty microliters of goat anti-rabbit serum, FITC-conjugated (KPL, Gaithersburg, MD, USA) diluted 1:100 in PBS were added to the enterocyte suspension and incubated for 30 min on ice. Cells were then washed three times with PBS–BSA, resuspended in 1.0 mL of PBS, and kept on ice until flow cytometric analysis was performed. As negative controls, similar samples were incubated with STa and anti-rabbit-IgG-FITC-conjugated antibody and used to determine the threshold of specific staining. Isotope controls were used to control the non-specific fluorescence. Flow cytometric analysis was preformed using Epics ELITE flow cytometer (Coulter Electronics, Hileah, FL, USA). Flow cytometer was set to read 5 000 cells from each enterocytes preparation. FITC-stained cells were excited by using 15 mW of 488 nm argon laser light. FITC-conjugated beads were run and the mean fluorescent intensity was set at a fixed value.

2.8. Statistics

Statistical analysis of the data was preformed by using the two-tailed Student's *t*-test for unpaired samples. Values of $P < 0.05$ were considered to be significant.

3. RESULTS

3.1. Effect of insulin on the response of suckling mouse to the heat-stable enterotoxin

Results of the suckling mouse assay indicated a significant increase in the gut weight to remaining body weight ratios in all of the insulin-fed suckling mouse groups ($P < 0.05$). This increase was directly related to the amount of insulin that was given ($P < 0.05$) (*figure 1*).

3.2. Effect of insulin on intestinal development and differentiation

Cell number and viability were relatively higher in insulin-fed suckling mice groups than mice of the control groups (*figure 2*). The increases in cell number and viability were directly related to the amount of insulin that was fed to each group (*figure 2*). Electron microscopy revealed a significant increase in the number of lipid endocytes (fat particles) in the intestinal mucosa of

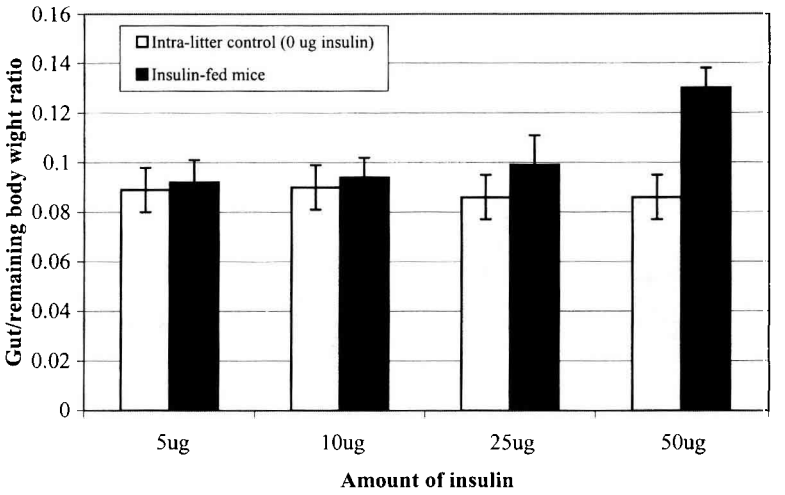


Figure 1. Ratio of gut weight to remaining body weight of control mice and suckling mice that were fed different doses of insulin (5, 10, 25, 50 μg) and challenged with 1 μg of STa. Each litter of suckling mice included a group of mice that were fed the stated dose of insulin (■) for 7 days and a group of mice (intra-litter control) that were fed saline without insulin (□). Each point represents the mean of three readings ± SEM.

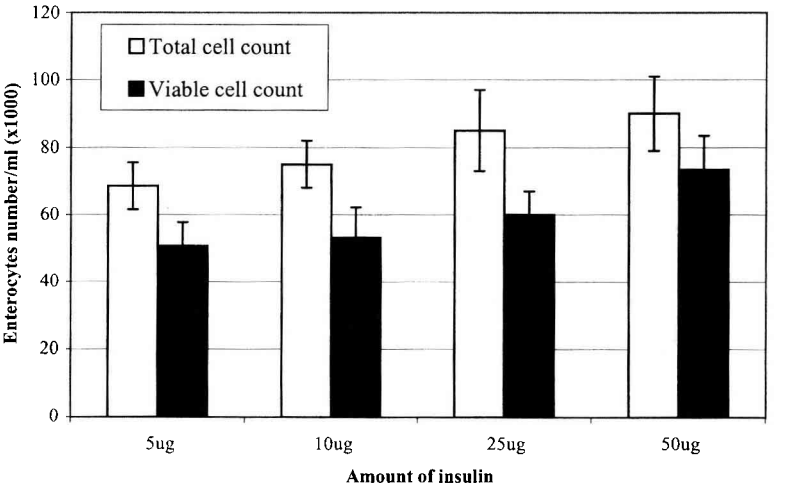


Figure 2. Number and viability of enterocytes obtained from groups fed 5, 10, 25 and 50 μg of insulin. Each point represents the mean of three readings ± SEM.

insulin-fed suckling mice (*figure 3*). This increase in lipid endocytes was proportional to the amount of insulin that was fed to each group of suckling mice. The lipid endocytes were mostly concentrated in the apical part of the epithelial cell mucosa. This increase in lipid particles was due to an increase in the uptake of milk particles by the intestinal absorptive surface. Electron microscopic studies did not reveal any significant

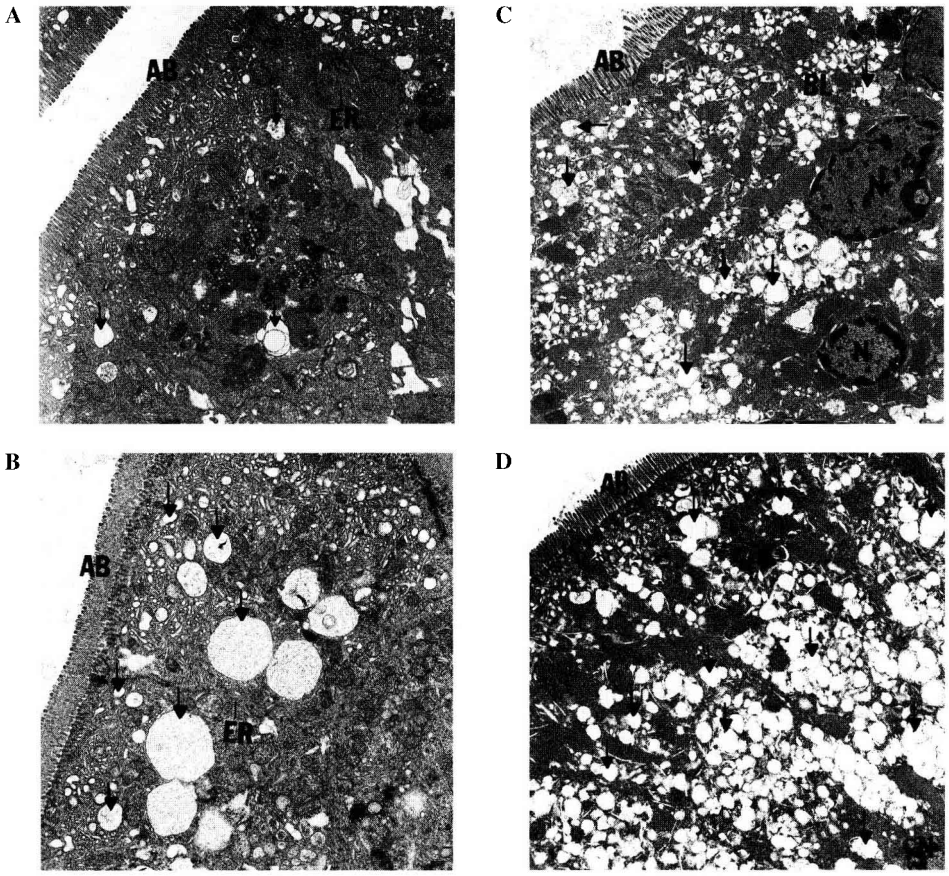


Figure 3. Transmission electron micrographs of small intestine epithelium (× 5 000) of different groups of suckling mice that were fed increasing amounts of insulin. A, Epithelium of control group; B, C, D, epithelium of mice fed 5, 10, 25 µg of insulin, respectively. **Arrows,** fat droplets (lipid endocytes); **AB,** absorptive surface, **N,** nucleus, **BL,** basolateral membrane, **ER,** endoplasmic reticulum.

changes in the morphology of the intestinal mucosa nor the ultrastructural characteristics of the absorptive surface in samples obtained from mice fed different doses of insulin.

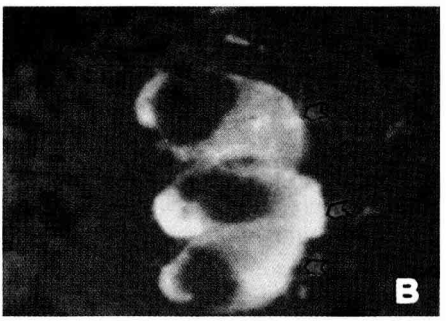
3.3. Effect of insulin on STa interaction with its receptor

3.3.1. Indirect immunofluorescence assay

Indirect immunofluorescence study of STa-susceptible mice revealed the localization of intensely stained areas mostly at the brush border membrane region of the enterocytes. It was found that feeding suckling mice with insulin for 7 days increased the fluorescence intensity observed on the brush border membrane of enterocytes (*figure 4*). This increase in fluorescence intensity was directly proportional to the amount of insulin that was given to each group of the suckling mice (*table I*).

3.3.2. Flow cytometry

Flow cytometric histograms revealed a significant increase in fluorescence inten-



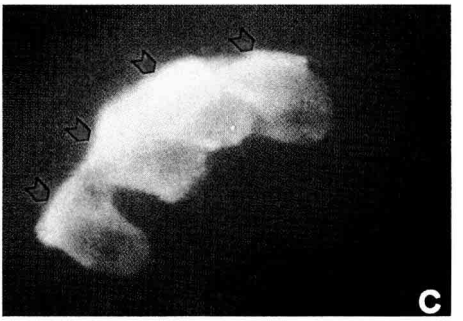


Figure 4. Fluorescent micrographs (× 1 000) of enterocytes obtained from the different insulin-fed groups. A, Negative control, no anti-STa antibody was added; B and C, enterocytes obtained from mice that were fed 10 and 50 µg of insulin, respectively. All cells were incubated with STa, rabbit anti-STa antibody and anti-rabbit-IgG-FITC conjugated antibody. Arrowheads indicate the location of the brush border membrane of the enterocytes.

Table I. Intensities of fluorescence on enterocytes observed with indirect immunofluoresence and flow cytometric analysis of samples obtained from mice that were fed increased doses of insulin. Enterocytes were reacted with STa, rabbit anti-STa antibody and anti-rabbit-IgG-FITC-conjugated antibody.

Doses of insulin (µg)[a]	Fluorescence intensity using indirect immunofluorescence assay	Fluorescence intensity using flow cytometry (%) of fluorescence intensity[b]
0 [c]	+ [d]	25.4
5	++	31.1
10	+++	33
25	++++	64.7
50	++++	82.3

[a] Mice groups that were fed different doses of insulin for 7 days.
[b] Statistical analysis indicated significant difference ($P < 0.05$) between the values of fluorescent intensities for control and treated groups.
[c] Enterocytes from the intra-litter control mice that were fed saline without insulin.
[d] Level of fluorescence: +: insignificant fluorescence; ++: slight fluorescence; +++: clear fluorescence; ++++: bright yellow fluorescence.

sity using enterocytes from insulin-treated mice when compared with that of control mice. The staining results of freshly isolated enterocytes from insulin-treated and control mice are shown in *table I*. The increase in fluorescence intensity of the surface of enterocytes was directly proportional to the amount of insulin that was fed to each group of suckling mice ($P < 0.005$) (*table I*). No fluorescence was observed on the enterocytes of the control groups where no STa was added. A similar trend was found upon repeating the experiment.

4. DISCUSSION

Diarrheal disease caused by *Escherichia coli* that produce heat-stable enterotoxin (STa) is common in newborn animals and human infants. The high prevalence and severity of the disease among younger subjects is not well elucidated. In order to formulate efficient control and prevention methods, a clear understanding of the disease mechanism is necessary. Insulin and IGF-I have been identified throughout the small and large intestine in animals and man [15]. IGF-I and insulin are present in breast milk (0–80 microunit/liter for insulin, 1.3–7 ηg/mL for IGF-I), and free IGF-I has been found in human saliva [2, 7, 9]. In this study, we hypothesized that insulin, as a growth factor, may exert a modulating effect on the intestinal epithelium of newborn animals and may modify their response to diarrheagenic toxins. We, therefore, investigated the effect of insulin on the interaction of suckling mice enterocytes with STa using electron microscopy, indirect immunofluorescence and flow cytometric analysis.

The suckling mouse assay is considered a specific assay to differentiate between STa and other enterotoxins produced by ETEC cultures [22]. Insulin-fed mice had a gut weight to remaining body weight ratio higher than the control groups when both groups were inoculated with similar diarrheagenic doses of STa. These findings suggest the modulating effect of insulin on intestinal epithelium affected the response to *E. coli* STa.

The small intestine in mice acquires its capability to respond to insulin during the suckling period [11, 12]. Furthermore, the fact that insulin is able to prematurely increase the epithelial cell proliferation in both small and large intestine supports, at least in the mouse model, the developmental pattern of epithelial cell proliferation and differentiation consistent with the modulatory influence of insulin and other growth factors. Information from the electron micrographs suggests that insulin has increased the absorptive surface as indicated by the increase in the inward movement of milk particles (*figure 3*). It was reported that IGF, (EGF), transforming growth factors α (TGF) and β have important modulatory roles in small intestinal crypt cell proliferation, particularly after intestinal injury [1, 11, 12, 13]. A premature rise in the circulating insulin level induces a significant increase in epithelial cell labeling indices. This suggests an important role of insulin in modulating intestinal cell proliferation and modulation. Insulin was reported to increase the activity of brush border membrane enzymes, namely, trehalase, glucoamylase, sucrase and lactase [10, 11, 12].

Increasing the amount of insulin in the intestinal lumen causes an increase in its own receptors in that area [8, 11]. The fact that insulin may up-regulate its own receptor [8], may explain the dose-dependent effect of the insulin on the STa-receptor binding. This study suggests that insulin may modulate the STa-receptor number on the brush border membrane. This up-regulation of STa binding can be either through the increase in the STa-receptor density or increase in the affinity of these receptors to the STa toxin. The flow cytometric analysis demonstrated that both insulin-fed groups and control group were stained with the secondary FITC-conjugated antibody. This suggests the existence of STa-receptors on the brush border membrane of both control and

insulin-fed groups. However, the fluorescence intensity was higher on the enterocytes of the insulin-fed groups compared to the control groups (*table I*). No fluorescence was observed on the enterocytes of the negative control group where no STa was added. Our flow cytometry results suggest that due to the elevation of intestinal cell proliferation and differentiation in response to insulin, there was an increase in the STa binding. Similar results were obtained in indirect immunofluorescence studies on enterocytes prepared from insulin-fed and control groups (*figure 4*).

The use of conventional fluorescent microscopy results only in inaccurate estimates of fluorescence intensity due to the scoring protocol used (++++ for high fluorescent intensity, + for relatively low fluorescent intensity). Using flow cytometry, studying the STa/receptor interaction was possible through accurate determination of the intensity of fluorescence on enterocyte of the different suckling mice groups.

In summary, the results of this study suggest that insulin has a modulatory effect on the response of suckling mouse intestine to STa. This modulation may be due to changes in the structure-function of the intestinal brush border membrane. This modification may further provide insights into the mechanism of the STa/receptor interaction.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance provided by Carol Koons, Kathy Ragheb and Beverly Waisner. We also thank Dr Michel Levy for his help in translating the title and the abstract into French and Mr Les Booth for his computer assistance.

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