INSULIN MODULATES INTESTINAL RESPONSE OF SUCKLING MICE TO THE *ESCHERICHIA COLI* HEAT-STABLE ENTEROTOXIN

Ahmad M. Al-Majali,[1] Elikplimi K. Asem,[2] Carlton Lamar,[2]
J. Paul Robinson,[2] James Freeman,[1] and A. Mahdi Saeed[1]

Departments of Veterinary Pathobiology[1] and
Basic Medical Sciences[2]
School of Veterinary Medicine
Purdue University
West Lafayette, Indiana 47907

1. SUMMARY

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 two day old Swiss Webster suckling mice were used. Five, 10, 25, and 50μg of insulin were given orally to half the mice in each group respectively. The rest of the mice in each group were given normal saline as intra-litter controls. After 7 days, the suckling mouse assay for STa was performed on three mice from each insulin-treated and control groups. 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 in diarrhea in newborn animals and human infants.

2. 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) produced by many ETEC 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 (Sears and Kaper, 1996). The other classes of *E. coli* toxins are low molecular weight, heat-stable toxins (ST). There are two types of ST, STa and STb. STb has mostly been observed in ETEC infecting pigs and calves, but has not been well characterized (Dreyfus et al., 1985; Epstein et al., 1986). STa, on the other hand, has been purified to homogeneity (Staples et al., 1980; Saeed and Greenberg, 1985). 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 toxin size were observed in ETEC from some host species, but heterogeneity in the size of STa is not reflected in a difference in their mechanism of action, or potencies (Rao et al., 1981; Saeed and Greenberg, 1985). 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 (Guandalini et al., 1982; Rao et al., 1981; Wada et al., 1994). Guanylate cyclase has been identified as the receptor for STa by cloning and expression analysis of the STa receptor gene from rat, human, and pig intestinal DNA libraries (de Sauvage et al., 1991; Schulz et al., 1990; Wada et al., 1994). It was reported that guanylate cyclase C, which is part of the extracellular domain of the membranous guanylate cyclase, is an N-linked glycoprotein receptor that accounts for multiple heat-stable enterotoxin-binding proteins in the intestine (Vaandrager et al., 1993).

Growth factors such as insulin and epidermal growth factor (EGF) are commonly found in milk of lactating women and other mammals (Steeb et al., 1995). A major site for the synthesis of these circulating growth factors is the liver, but insulin-like growth factor-1 (IGF-1) is also produced locally in other tissues (Simmons et al., 1995; Steeb et al., 1995). The biological effect of growth factors is mediated by their binding to specific receptors on the plasma membrane. The number of these receptors on the membranes can be regulated by the concentration of growth factors in the environment (Ziegler et al., 1995). One major site for the action of growth factors is the intestinal brush border membrane. Many studies have attempted to explain normal developmental patterns for different intestinal disaccharidase, alkaline phosphatase and peptidase. Some reports suggest the involvement of some growth factors in regulating intestinal brush border membrane disaccharidase (Menard et al., 1981; Steeb et al., 1995). The differentiation of the absorptive surface of the intestinal epithelial cells is characterized by increases in sucrase and lactase activities (Menard et al., 1981; Schmidt et al., 1988).

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

3. MATERIAL AND METHODS

3.1. STa Purification

STa was isolated and purified to homogeneity by growing ETEC strains in a chemically defined medium, desalting, and concentrating 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 resulted in high 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 (Saeed and Greenberg, 1985).

3.2. Insulin Feeding of Suckling Mice

Four litters (8–10 mice in each group) of two 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) respectively. The rest of suckling mice in each litter received normal saline and were kept as intra-litter controls. All mice were given insulin or saline orally each day for 7 days using a 0.5-ml syringe and 50-µm diameter polyethylene tube. We assumed that all mice had a similar chance to suckle their mothers during the seven-day experiment.

3.3. Suckling Mouse Assay

Mice were separated from their mothers immediately before use and randomly divided into groups of three. These mice were starved for two hours 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 hours, 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. The minimum amount of STa that induced a gut to remaining body weight ratio of 0.087 or higher was considered one mouse unit (mu) (Giannella, 1976).

3.4. Electron Microscopy

One half centimeter of small intestine from each mouse of the different groups was fixed in 3% glutaraldehyde, and then postfixed 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.

3.5. Isolation of Suckling Mouse Enterocytes

Enterocyte suspensions were prepared as described previously (Al-Majali et al., 1998). 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 were determined and cell viability was assessed by exclusion of 0.2% trypan blue by cells. Dry smears were fixed in 100% methanol for both immunofluorescence and Giemsa staining. The remaining cells were used for flow cytometric analysis.

3.6. Immunofluorescence Assay

Intestinal epithelial cells isolated from suckling mouse intestines were washed 3 times in 10mM 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 10min. Slides were incubated with STa, rabbit anti-STa antibody and anti-rabbit-IgG-FITC conjugated antibody as was described by Saeed et al. (1987). As negative controls, similar samples were incubated with STa and the anti-rabbit-IgG-FITC-conjugated antibody without the anti-STa antibody.

3.7. Flow Cytometry Analysis

Enterocytes were prepared for flow cytometry following the procedure reported by Al-Majali et al. (1998). Flow cytometric analysis was preformed using an Epics ELITE flow cytometer (Coulter Electronics, Hileah, FL.). Flow cytometer was set to read 5000 cells from each enterocyte preparation. Fluorochrome excitation was done using 15mW of 488nm argon laser light. FITC-conjugated beads were run and the mean fluorescent intensity was set at a fixed value.

3.8. Statistics

Statistical analysis of the data was preformed by using the two-tailed student *t*-test for unpaired samples. Differences were analyzed for significance at $p < 0.05$.

4. RESULTS

4.1. Effect of Insulin on the Response of Suckling Mouse to the Heat-Stable Enterotoxin

Results of the suckling mouse assay indicated that the gut weight to remaining body weight ratios in all of the insulin-fed suckling mouse groups were directly related to the amount of insulin that was given (Fig. 1).

4.2. 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 (Fig. 2). The increases in cell number and viability were directly related to the amount of insulin that was fed in each group (Fig. 2). Electron microscopy revealed a significant increase in the number of lipid endocytes (fat particles) in the intestinal mucosa of insulin-fed suckling mice (Fig. 3). This increase in lipid endocytes was proportional to the amount of insulin that was fed in each group
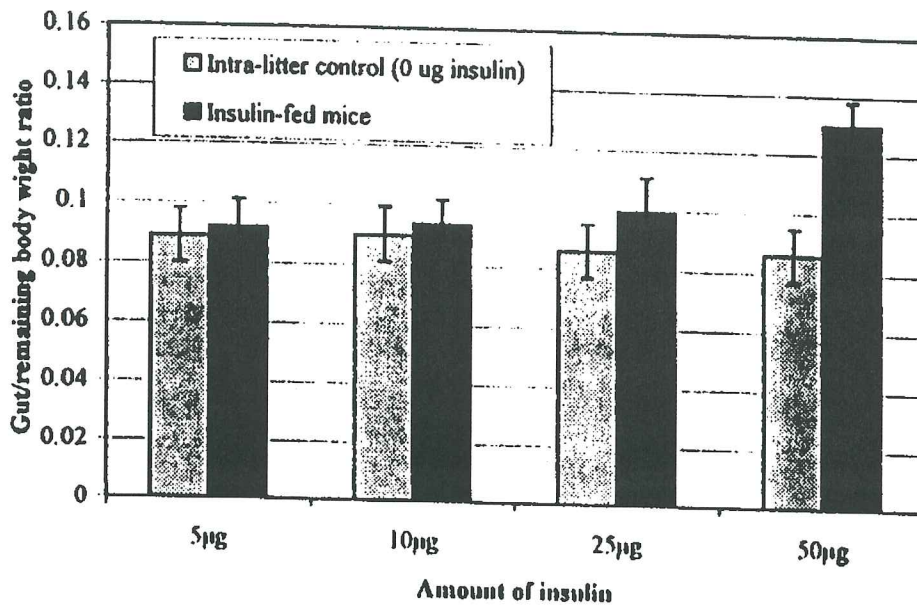
Figure 1. Ratio of gut weight to remaining body weight of control mice and suckling mice that were fed different doses of insulin (5, 10, 25, 50μg) and challenged with 1μg of STa. Each litter of suckling mice included mice that were fed the stated dose of insulin (■) for 7 days and mice (intra-litter control) that were fed saline without insulin (□). Each point represents the mean of three readings ± SEM.

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 at the intestinal absorptive surface. Electron microscopic studies did not reveal any significant 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.
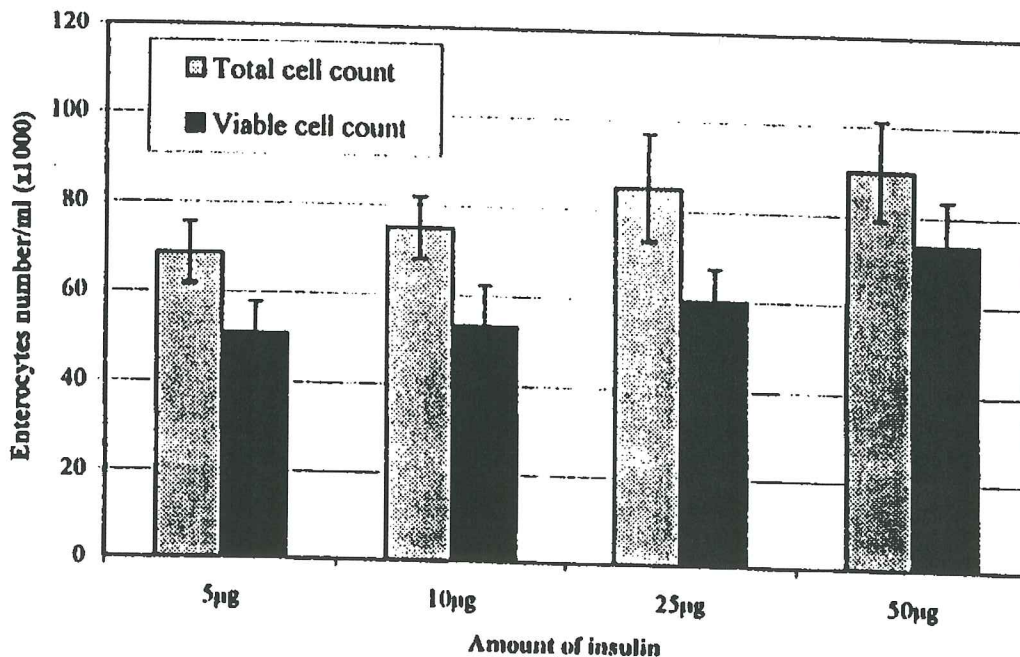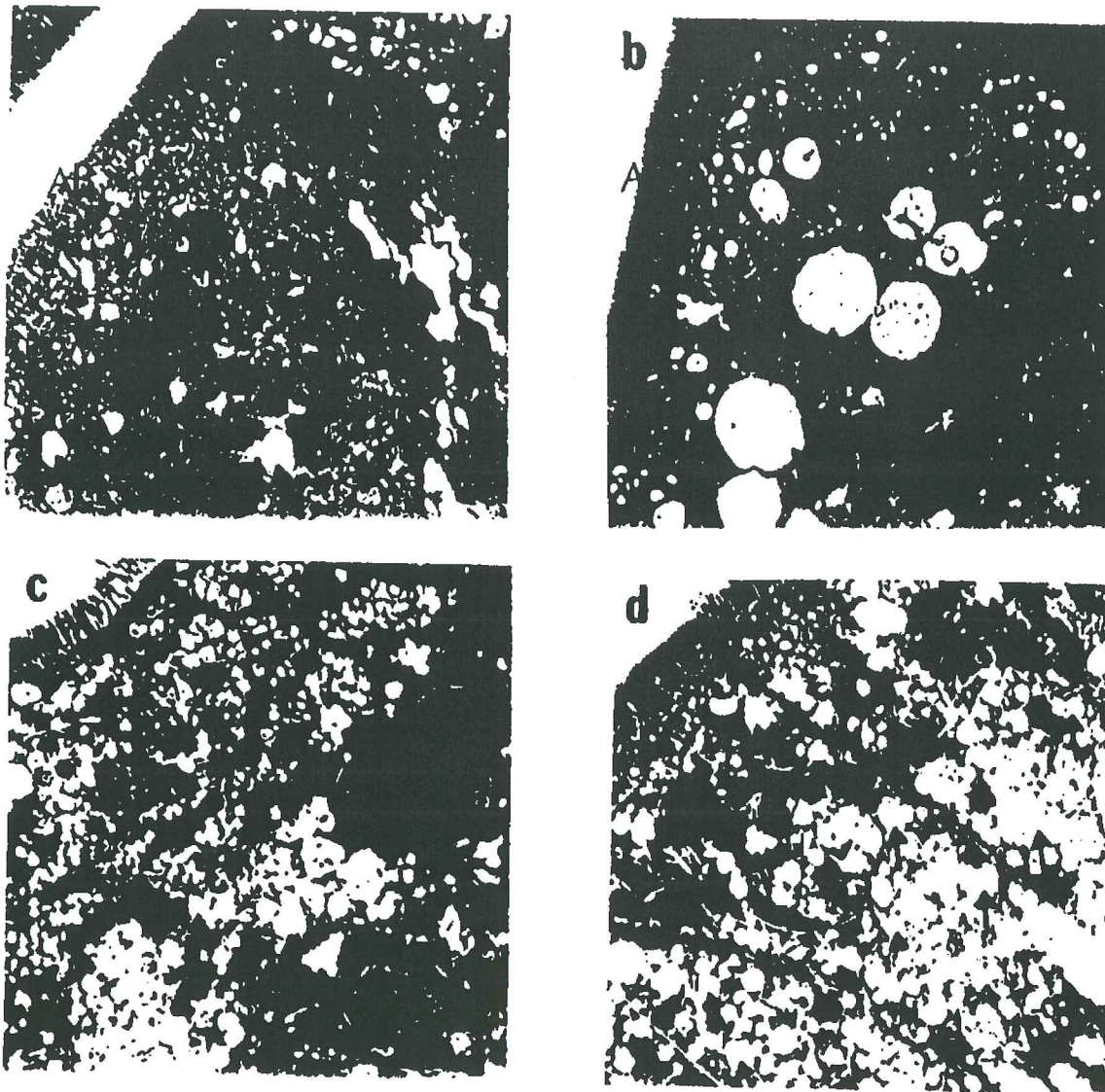

Figure 2. Number and viability of enterocytes obtained from control mice and mice fed 5, 10, 25, and 50μg of insulin. Each point represents the mean of three readings ± SEM.

Figure 3. Transmission electron micrographs of small intestine epithelium (5000X) of suckling mice that were fed different 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.

4.3. Effect of Insulin on STa Interaction with Its Receptor

4.3.1. 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 in feeding suckling mice fed insulin for 7 days, there was increased intensity of fluorescence staining on the brush border membrane of enterocytes (Fig. 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 1).

4.3.2. Flow Cytometry. Flow cytometric histograms revealed a significant increase in fluorescence intensity among enterocytes from insulin-treated mice when compared with that of control mice. The staining results of freshly isolated enterocytes
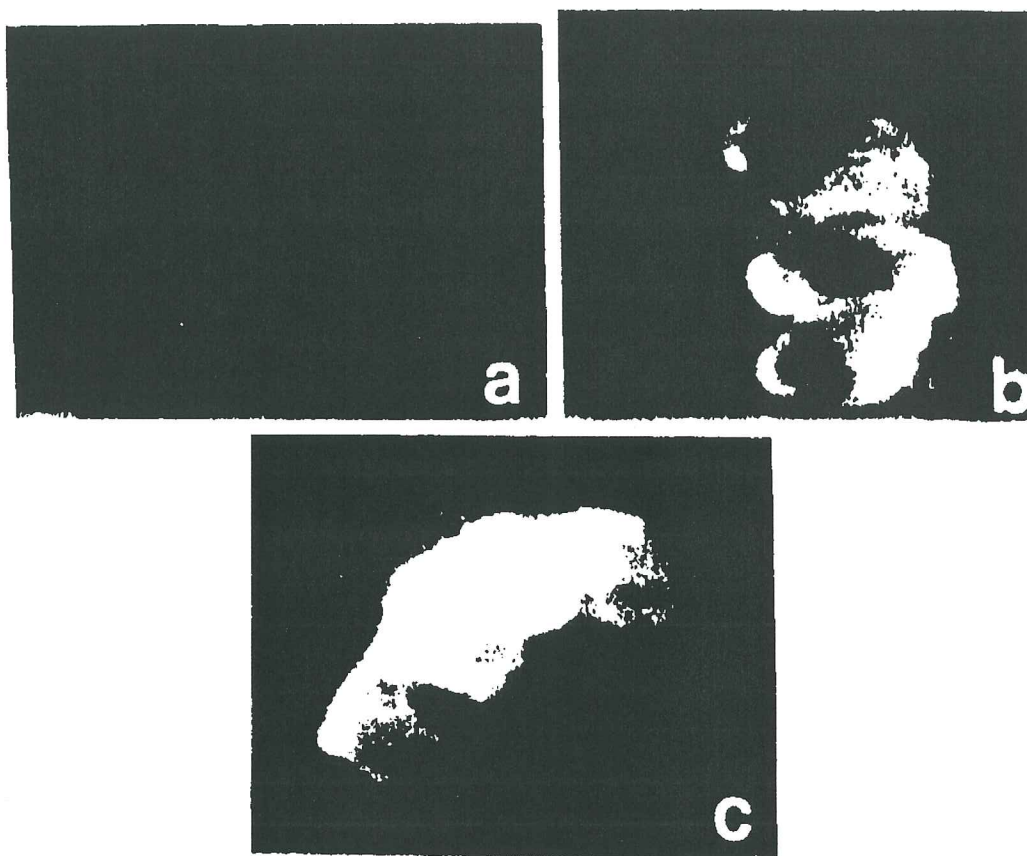
Figure 4. Fluorescence micrographs (1000X) of enterocytes obtained from the different insulin-fed groups. A: Negative control, no anti-STa antibody was added; B and C, Enterocytes obtained from mice that were fed 10 and 50μg of insulin, respectively. All cells were incubated with STa, rabbit anti-STa antibody, and anti-rabbit-IgG-FITC conjugated antibody. Arrowheads indicate the location of the brush border membrane of the enterocytes.

Table 1. 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

	Fluorescence intensity	
Doses of insulin (μg)[a]	Using indirect immunofluorescence assay	Using flow cytometry (%) of fluorescence intensity[b]
0[c]	+	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.
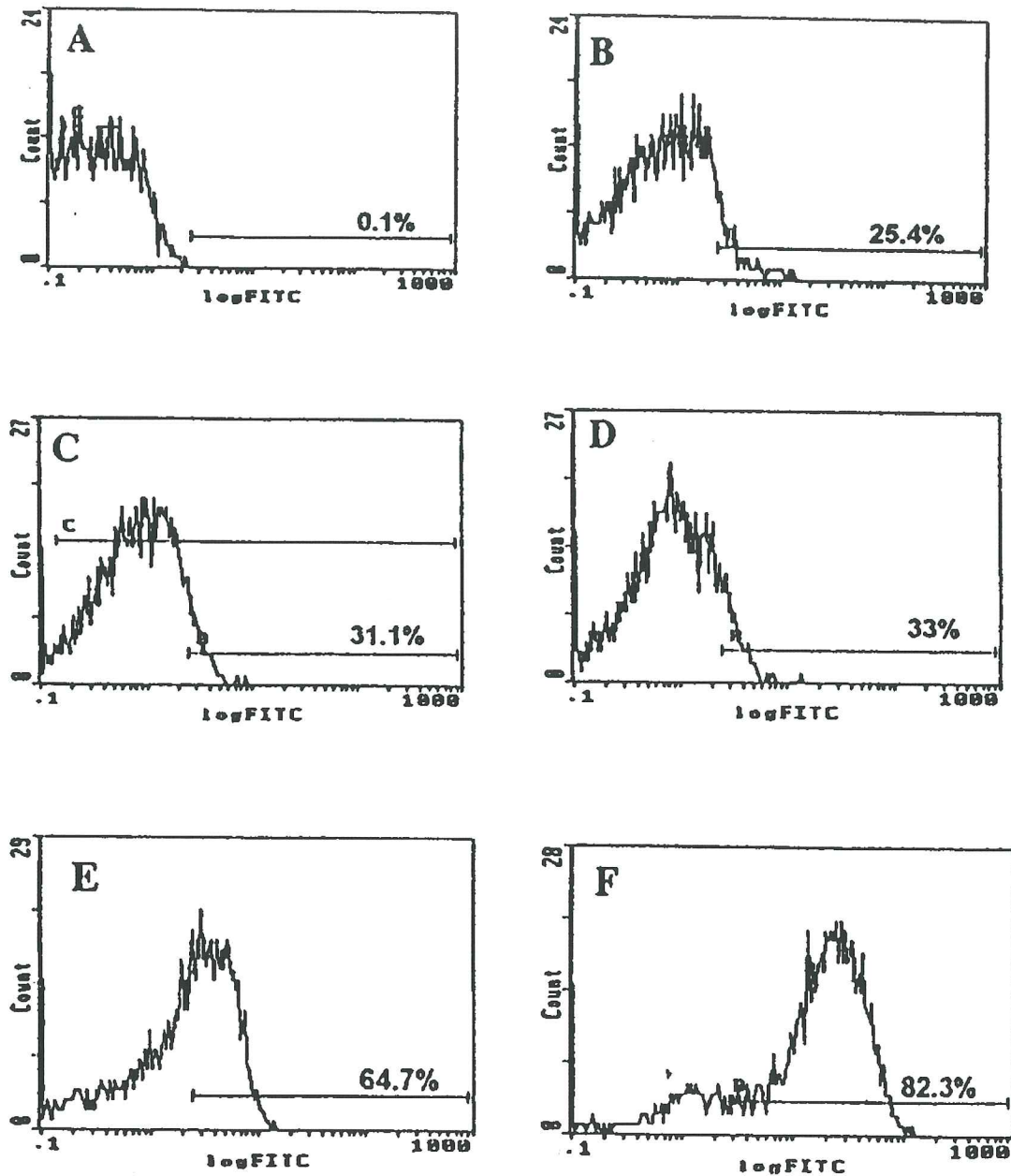
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Figure 5. Representative flow cytometric histograms for enterocytes obtained from the intestine of sucklin mice. A: negative control, no STa added; B: Control, no insulin was given; C, D, E, and F: Treated mice fe 5, 10, 25, and 50 ug of insulin, respectively, for 7 days. All cells were incubated with STa, rabbit anti-STa ant body, and anti-rabbit-IgG-FITC conjugated antibody.

from insulin-treated and control mice are shown in Table 1 and Fig. 5. The increase i fluorescence intensity of the surface of enterocytes was directly proportional to th amount of insulin that was fed to each group of suckling mice ($p < 0.005$) (Table 1). N fluorescence was noticed on the enterocytes of the control groups where no STa wa added. A similar observations were made upon repeating the experiment.

5. DISCUSSION

Diarrheal disease caused by *Escherichia coli* that produce heat-stable enterotoxi (STa) is common in newborn animals and human infants. The reason for higher preva

lence and greater severity of the disease in younger subjects is not well elucidated. In order to formulate efficacious 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 (Rouyer-Fessard et al., 1990). IGF-I and insulin are present in breast milk (0–80 microunit/liter for insulin and 1.3–7 ng/ml for IGF-I), and free IGF-I has been found in human saliva (Costigan et al., 1988; Grosvenor et al., 1992; Koldvsky and Strbak, 1995). 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 to the E. coli STa using electron microscopy, brush border enzyme analysis, indirect immunofluorescence and flow cytometric analysis.

The suckling mouse assay is considered specific for differentiating between STa and other enterotoxins produced by enterotoxigenic E. coli cultures (Wada et al., 1994). Insulin-fed mice had a gut weight to remaining body weight ratio higher than the control group, when both groups were inoculated with similar diarrheagenic doses of STa. These findings suggest that insulin had a modulating effect on intestinal epithelium response to E. coli STa. The small intestine in mice acquires its capability to respond to insulin during the suckling period (Menard et al., 1981; Menard and Dagenais, 1993). The fact that insulin is able to increase epithelial cell proliferation in both small and large intestines supports, at least in the mouse model, the view that the developmental pattern of epithelial cell proliferation and differentiation is under some modulatory influence of insulin. Results of electron microscopic studies suggest that insulin has increased the absorptive surface as indicated by the increase in the inward movement of milk particles (Fig. 3). It was reported that IGF, epidermal growth factor (EGF), transforming growth factor α (TGF) and β have important modulatory roles in small intestinal crypt cell proliferation, particularly after intestinal injury (Arsenault and Menard, 1984; Menard and Dagenais, 1993; Potten et al., 1995). A premature rise in the circulating insulin level induces 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 brush border membrane enzyme activity, namely that was trehalase, glucoamylase, sucrase and lactase (Menard et al., 1981). Increasing the amount of insulin in the intestinal lumen causes an increase in its own receptors in that area (Heaton and Gelehrter, 1981; Menard and Dagenais, 1993). The fact that insulin up-regulates production of its own receptor (Heaton and Gelehrter, 1981) may explain the dose-dependent effect of the insulin on the STa-receptor binding. This study suggest 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 of the STa receptor density or increase in the affinity of these receptors to the STa toxin. The flow cytometric analysis demonstrated that enterocytes from both insulin-fed groups and control group contained STa receptors. However, the fluorescence intensity suggesting receptor activity was higher on the enterocytes of the insulin-fed groups compared to the control (Fig. 5). 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. The biological function of insulin within the small intestine is not completely known. It was reported that the presence of insulin and IGF-I receptors on the brush border membrane suggest an autocrine/paracrine role for these growth factors (Simmons et al., 1995; Steeb et al., 1995).

In summary, the results of this study suggest that insulin has a modulatory effec on the response of suckling mouse intestine to STa. The changes in the structural func tion of the intestinal brush border membrane due to the effect of insulin may modify the STa/receptor interaction. Knowledge of this modification may provide insights into how to intervene with the STa/receptor interaction.

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