Antimicrobial Agents and Chemotherapy, Nov. 1998, p. 2950–2955 Vol. 42, No. 11
0066-4804/98/$04.00+0
Copyright © 1998, American Society for Microbiology. All Rights Reserved.

Effects of *Salmonella typhimurium* Infection and Ofloxacin Treatment on Glucose and Glutamine Metabolism in Caco-2/TC-7 Cells

LETA POSHO,[1] LAURENCE DELBOS-BOCAGE,[1] DELPHINE GUEYLARD,[1] ROBERT FARINOTTI,[1,2]* AND CLAUDE CARBON[1]

Centre Hospitalier Universitaire Bichat-Claude Bernard, Institut National de la Santé et de la Recherche Médicale, Unité 13,[1] and Département de Pharmacie Clinique, Faculté de Pharmacie, Université de Paris XI,[2] Paris, France

Received 29 December 1997/Returned for modification 12 April 1998/Accepted 5 August 1998

Downloaded from http://aac.asm.org/ on November 19, 2019 by guest

The effects of both *Salmonella typhimurium* infection and 5 mM ofloxacin treatment on 2 mM glutamine and 5 mM glucose metabolism in the enterocyte-like Caco-2/TC-7 cell line were studied. These cells utilized glutamine (212.07 ± 16.75 [mean ± standard deviation] nmol per h per 10^6 viable cells) and, to a lesser extent, glucose (139.63 ± 11.52 nmol per h per 10^6 viable cells). Metabolism of these substrates in Caco-2/TC-7 cells resembled that in rat, pig, or human enterocytes. Infection by *S. typhimurium* C53-enhanced glucose and glutamine substrate utilization by 32 and 22%, respectively and enhanced glucose and glutamine substrate oxidation by eight- and twofold, respectively. These increases in glucose and glutamine metabolism (especially glucose metabolism) were due in part to the metabolism of intracellular bacteria and/or to the activation of cellular metabolism. Substrate metabolism (especially glucose metabolism) in C53-infected cells was partially reduced by treatment with ofloxacin. It was concluded that cellular fuel metabolism is stimulated at the earliest stage of infection (3 to 4 h) and that treatment with 5 mM ofloxacin does not completely restore substrate metabolism to the levels observed in uninfected cells, possibly because this treatment does not eradicate intracellular *S. typhimurium* completely.

Salmonella species are facultative intracellular parasites capable of penetrating (invading), surviving within, and often multiplying within eukaryotic cells of various types, including phagocytic and epithelial cells. Most infections due to *Salmonella* share a common route, i.e., oral ingestion followed by penetration of the intestinal epithelium (11, 12, 18).

Infections caused by intracellular bacteria constitute a challenge for current antimicrobial therapies because the concentration of the antibiotic administered needs to be at a sufficiently high level at the site of infection. Many antibiotics which are active in vitro are often inactive against internalized bacteria due to poor penetration into the cells, inactivation by lysosomal enzymes, or impairment of intracellular conditions. In addition, the internalized bacteria must be in a metabolic state which renders them sensitive to the drug under study (5, 35). There has been considerable interest over the last decade in the development and clinical use of fluoroquinolones (17, 19) that demonstrate favorable intracellular pharmacokinetics for the treatment of intracellular infections.

The intestinal epithelium plays an important role in the absorption of intact drugs immediately after oral administration and in the first step of *Salmonella* infection (13). As in vitro model of intestinal epithelial cells that is based on the human colon carcinoma cell line Caco-2 (16, 20) has been developed for the study of interactions between intestinal mucosa and bacteria or antimicrobial drugs. These cells differentiate spontaneously under standard culture conditions into monolayers of polarized cells possessing microvilli and many enterocyte-like properties (41).

* Corresponding author. Mailing address: Centre Hospitalier Universitaire, Bichat-Claude Bernard (Pharmacie), 46 rue Henri-Huchard, 75877 Paris Cedex 18, France. Phone: 33 142635825. Fax: 33 140258005. E-mail: robert.farinotti@bch.ap-hop-paris.fr.

Intestinal mucosal enterocytes, which are mainly involved in the absorption of nutrients arising from intestinal digestion, have also been shown to use some of these nutrients for their own metabolism. Indeed, as reported for rats (39), pigs (32), and humans (3), glutamine and, to a lesser extent, glucose represent the major oxidative substrates of small-intestine cells. In addition, glycolysis and glutaminolysis provide metabolic intermediates for biosynthetic pathways: glycolysis provides pentose phosphate for DNA and RNA synthesis and glycerol phosphate for phospholipid synthesis, and glutaminolysis provides glutamine, glutamate, ammonia, and aspartate for the production of purines and pyrimidines for DNA, RNA and, therefore, protein synthesis. Infection of the intestinal mucosa may damage the epithelium; consequently, the requirement for metabolic intermediates for repair processes will be increased (2, 9, 21, 30, 31).

The objective of the present study was to examine the effects of both enteropathogen (*Salmonella typhimurium*) infection and fluoroquinolone (ofloxacin) treatment on the metabolism of the substrates glucose and glutamine in the enterocyte-like Caco-2/TC-7 cell line. These two substrates play an important role in intestinal cell metabolism, as they are the main sources of energy for these cells and are involved in nucleotide and protein synthesis and in cell repair processes. In addition, glutamine stimulates intestinal sodium and chloride absorption in bacterial or viral diarrhea (30, 34). The Caco-2/TC-7 clone was chosen because it demonstrates more enterocyte-like metabolic features than the parental Caco-2 cell line (6). We infected these cells with *S. typhimurium*, which is a gram-negative, facultative intracellular bacterium that is able to invade, survive within, and multiply within Caco-2 cells (12, 13). The systemic infection induced by this bacterium is lethal in certain strains of mice and has many of the hallmarks of the human disease induced by *S. typhi*, including anorexia, dissemination

through the reticuloendothelial system, splenomegaly, and diarrhea (16).

MATERIALS AND METHODS

Chemicals. Ofloxacin, bovine serum albumin (fraction V, fatty acid free), HEPES, methylbenzethonium hydroxide, Tris buffer, D-glucose, L-glutamine, and Triton X-100 were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Perchloric acid and potassium hydroxide were obtained from E. Merck AG (Darmstadt, Germany), and EDTA and all inorganic products were obtained from Prolabo (Paris, France). All enzymes and coenzymes used for enzymatic assays were purchased from Boehringer (Meylan, France), and D-[U-^{14}C]glucose and L-[U-^{14}C]glutamine were purchased from Amersham Life Science (Les Ulis, France). The radiochemical purity of the isotopes used was greater than 98%. The scintillation cocktail for radioactivity counting was purchased from EG&G (Evry, France).

Cell culture. All tissue culture reagents were obtained from Life Technologies (Cergy Pontoise, France). The Caco-2/TC-7 clone (passage 33), derived from the human colon carcinoma cell line Caco-2 (6) established by J. Fogh (14), was kindly provided by A. Zweibaum and M. Rousset (Institut National de la Santé et de la Recherche Médicale U178, Villejuif, France). The cell line between passages 37 and 45 was used in this work. The culture medium was Dulbecco's modified Eagle medium (DMEM) supplemented with 15% heat-inactivated (56°C, 30 min) fetal calf serum and 1% nonessential amino acids. The D-glucose concentration in the culture medium was 4.5 g/liter, and that of L-glutamine was 580 mg/ml. Caco-2/TC-7 cells were routinely cultured in 25-cm^2 plastic tissue culture flasks (Corning, Polylabo, Paris, France) at 37°C in a humidified atmosphere of 10% CO_2 in air. The medium was changed 48 h after seeding and on a daily basis thereafter until the cells reached 90% confluence (5 to 6 days after seeding). Confluent cell monolayers were detached by treatment with trypsin (0.25%)-EDTA (0.1%) in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) at pH 7.4 and 37°C for 5 min. Cells were seeded at a density of 1.4×10^4 cells/cm^2 in either 25- or 75-cm^2 plastic tissue culture flasks (Corning) and grown under the conditions just described, except that penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively) and amphotericin B (Fungizone) (1 μg/ml) were added to the culture medium. Cells were used after 15 to 17 days of culturing.

Bacterial strains and growth conditions. *S. typhimurium* strains (virulent C53 and its avirulent mutant, C53::Tn*5*-6) were kindly provided by M. Y. Popoff (Institut Pasteur, Paris, France). The C53::Tn*5*-6 strain was obtained from parent strain C53 by Tn*phoA* mutagenesis. The mutant strain is able to adhere to the brush border membrane of host cells but cannot invade cells. These two strains demonstrated similar growth rates in tryptic soy broth (Diagnostics Pasteur, Marnes La Coquette, France) and equal susceptibilities to gentamicin and ofloxacin (MICs, 1 and 0.125 mg/liter, respectively).

Bacteria were grown in tryptic soy broth, and overnight bacterial cultures (12.5×10^8 bacteria per ml) were stored in glycerol (20%) as 1-ml aliquots at −80°C. Some of the frozen stock was rapidly thawed and cultured for 18 h at 37°C for each experiment. One-milliliter aliquots of these cultures were then inoculated into 9-ml volumes of tryptic soy broth and incubated at 37°C until the mid-logarithmic growth phase (6×10^8 to 7×10^8 bacteria/ml) (22) had been attained. The bacteria were then pelleted, washed three times in sterile PBS, and used at the appropriate dilution in fresh culture medium (DMEM supplemented with 1% nonessential amino acids and 15% FCS) to infect Caco-2/TC-7 cells. The inoculum density was controlled by plating 0.1-ml volumes of serial dilutions on tryptic soy agar and counting CFU after 24 h of incubation at 37°C. Each assay was conducted in duplicate.

Infection of Caco-2/TC-7 cells and invasion assay. Flasks (75 cm^2) of Caco-2/TC-7 cell monolayers were washed three times with 10 ml of fresh culture medium prior to inoculation with 10 ml of the bacterial suspension adjusted to obtain a multiplicity of infection of 100 bacteria per cell. The avirulent C53::Tn*5*-6 strain was used as a negative infection control. Penetration was allowed to proceed for 1 h at 37°C in a humidified atmosphere of 10% CO_2. Infected cells were washed three times with 10 ml of fresh culture medium containing gentamicin (100 μg/ml), and then 10 ml of medium containing gentamicin (50 μg/ml) was added to the flasks; the flasks were incubated for 1 h at 37°C in a 10% CO_2 incubator. This treatment rapidly killed extracellular bacteria adhering to the Caco-2/TC-7 cell brush border but not bacteria located within the cells. The cell monolayers were washed three times with 10 ml of sterile PBS, and the cells were harvested with a sterile solution containing 0.25% trypsin and 1% EDTA in Ca^{2+}- and Mg^{2+}-free PBS. The cell pellet, obtained after centrifugation (150 × *g*, 5 min), was resuspended in sterile Krebs-Henseleit bicarbonate buffer (incubation buffer, pH 7.4, 37°C) (33) containing 10 mM HEPES, 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM Na_2SO_4, 1.3 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% fatty-acid-free albumin or in sterile DMEM. The cell density of the suspension was assessed by counting the cells in an aliquot by use of a Malassez hemocytometer. A 0.2-ml volume of this suspension was then added to 0.6 ml of 1% Triton X-100 in PBS. This mixture was incubated for 5 min; during this incubation, cells were lysed and intracellular bacteria were released (10). Appropriate dilutions were plated to determine the number of viable intracellular bacteria. Each assay was conducted in triplicate for three successive passages of Caco-2/TC-7 cells.

Incubation conditions and assay of metabolites. Incubations were carried out with 25-ml polycarbonate Erlenmeyer flasks (Nagle Company, Rochester, N.Y.) containing 1 ml of uninfected or infected cell suspension (6×10^6 to 10×10^6 cells) in a final volume of 2 ml of incubation buffer, in the presence or absence of 5 mM D-[U-^{14}C]glucose or 2 mM L-[U-^{14}C]glutamine, and with or without 5 mM ofloxacin. The flasks were sealed and incubated in a shaking water bath (37°C, 100 oscillations/min for 1 h). Neither the incubation buffer nor flasks were gassed with oxygen (19:1 [vol/vol] O_2/CO_2 mixture), unlike in previous studies (3, 8, 32), since the capacity of *S. typhimurium* for entry into and growth in host cells is greater under low-oxygen conditions (15). Incubations were stopped by adding 0.25 ml of ice-cold perchloric acid (final concentration, 4%). The glucose and glutamine which remained and the lactate, pyruvate, glutamate, and ammonia which had been produced were assessed in the neutralized, non-protein-containing supernatant of the incubation medium by specific enzymatic methods (4) and with a UVIKON 810 recording spectrophotometer (KONTRON, Les Ulis, France). Carbon dioxide production was determined by measuring $^{14}CO_2$ release during incubation with ^{14}C-labelled substrates (1.7 to 2.4 MBq/mmol). $^{14}CO_2$ was trapped with methylbenzethonium hydroxide (90 min of shaking at 100 oscillations/min, room temperature) after the incubations were stopped with perchloric acid, and counts were determined with a liquid scintillation counter (LKB-Rackbeta 1218; Wallac, Turku, Finland). Blank CO_2 production was obtained from incubation flasks containing ^{14}C-labelled substrates but no added cell suspension. Glucose utilization and glutamine utilization were calculated from the net quantities of each substrate that disappeared from the incubation medium. Metabolite production was calculated from the net quantities of metabolites generated by both substrates.

Substrate utilization and oxidation by the two strains of *S. typhimurium* were assessed as described above, bacteria being taken at the mid-logarithmic growth phase (6×10^8 to 7×10^8 bacteria/ml).

Enzyme activity assays. Enzyme activity assays were performed with uninfected cells or with cells infected as described above and grown in 25-cm^2 flasks. The extracellular medium was removed after gentamicin incubation and replaced with fresh sterile incubation medium. The cells were then incubated from time zero for an additional 6 h, washed three times with PBS incubation (5 mM ofloxacin was added to certain flasks after a 2-h period), and collected after trypsinization and centrifugation in sterile PBS. The final volume of the cell suspension and the cell density were subsequently determined. An aliquot (0.2 ml) of the cell suspension was lysed with 1% Triton X-100 so that the number of viable intracellular bacteria could be determined. The remaining suspension was centrifuged, and the pellet was stored at −80°C. Enzyme activities in homogenates of pellets were measured later. Sucrase-isomaltase (EC 3.3.1.48) activity was determined as described by Messer and Dahlqvist (25), and dipeptidylpeptidase IV (EC 3.4.14.2) activity was determined as described by Nagatsu et al. (29). These brush-border-associated hydrolases were used as markers of cell differentiation. The protein content of uninfected or infected Caco-2/TC-7 cells was determined by the method of Lowry et al. (23).

Data presentation and statistical analysis. Substrate utilization and metabolite generation were expressed as nanomoles per hour per 10^6 viable cells, viability being determined at the onset of incubation. Enzyme activities were expressed as milliunits per milligram of protein. The efficiency of infection was expressed as the percentage of surviving bacteria relative to the inoculum. Statistical differences between experimental groups were determined by variance analysis. Data were expressed as the mean ± standard deviation. *P* values equal to or less than 0.05 were considered statistically significant. *n* values are numbers of separate experiments.

RESULTS

Caco-2/TC-7 cell protein content and viability. The protein contents in uninfected and C53-infected Caco-2/TC-7 cells (n = 20) were 0.57 ± 0.25 and 0.59 ± 0.20 mg/10^6 cells, respectively. The viabilities of uninfected cells, as tested by trypan blue exclusion, were 95.9% ± 0.3% (n = 20) (time zero), 90.7% ± 0.8% (n = 10) after 2 h in incubation buffer, and 84.1% ± 0.4% (n = 6) after 4 h in incubation buffer. Corresponding values for infected cells were 92.1% ± 1.3% (n = 20) at the end of gentamicin incubation, 88.6% ± 3.2% (n = 10) after 2 h in incubation buffer, and 74.4% ± 4.6% (n = 6) after 4 h in incubation buffer. Infection significantly decreased the viability of isolated cells ($P < 0.001$). Incubating isolated cells for 2 h with 5 mM ofloxacin did not modify viability of any experimental group.

Entry of *S. typhimurium* into Caco-2/TC-7 cells and intracellular growth. The capacity of *S. typhimurium* to enter and grow within Caco-2/TC-7 cells is illustrated in Fig. 1. The efficiency of invasion for the virulent strain was 15% ± 10% of the inoculum at the end of gentamicin incubation and correspond-

Downloaded from http://aac.asm.org/ on November 19, 2019 by guest

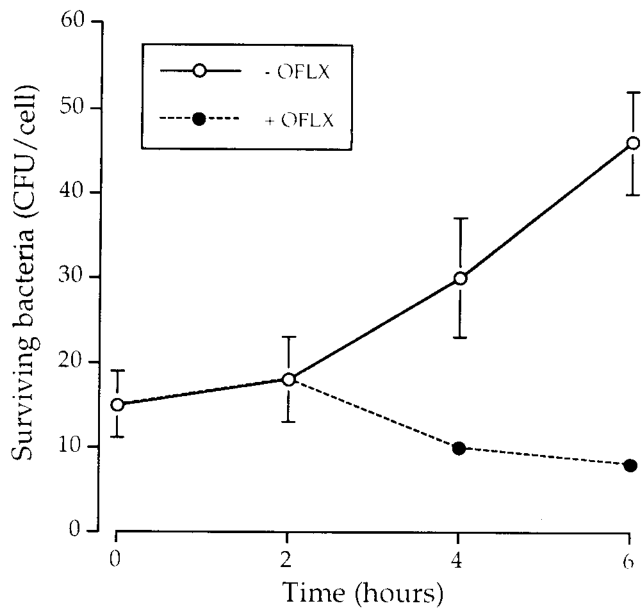

FIG. 1. Penetration into and growth in Caco-2/TC-7 cells of *S. typhimurium*. Cell monolayers were infected with strain C53 (1 h). After gentamicin treatment (1 h), cells were incubated for an additional 6 h. At various times, viable intracellular bacteria were counted and expressed as the percentage of intracellular bacteria per inoculum for six experiments. Simultaneously, the effect of 5 mM ofloxacin (OFLX) was assessed by adding this drug to the incubation medium after 2 h of incubation ($n = 2$).

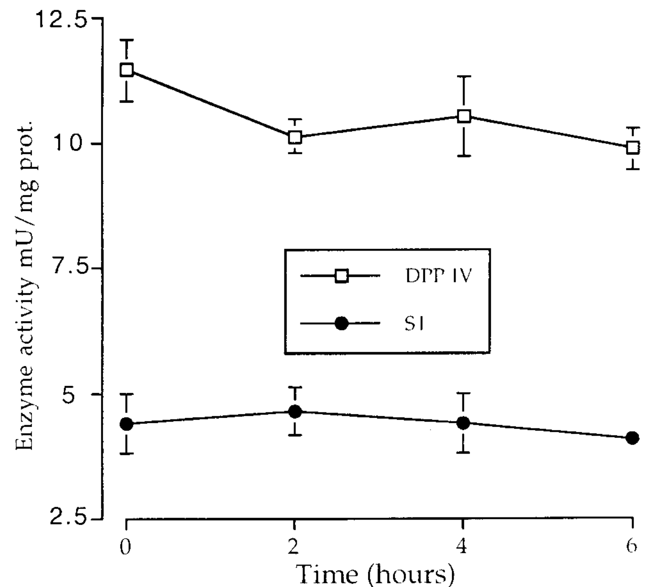

FIG. 2. Dipeptidylpeptidase IV (DPP IV) and sucrase-isomaltase (SI) activities in Caco-2/TC-7 cells. Cell monolayers were infected with strain C53 (1 h). After gentamicin treatment (1 h), cells were incubated for an additional 6 h. Enzyme activities were assayed as described in Materials and Methods ($n = 4$). prot., protein.

ed to approximately 15 bacteria per cell. The efficiency of invasion for the avirulent strain was very weak (0.02%), however. Two hours elapsed before internalized virulent bacteria began to multiply, and the number of intracellular bacteria was approximately 45 bacteria per cell after 6 h of incubation. The number of intracellular bacteria decreased after 4 h of incubation when ofloxacin (5 mM final concentration) was added to the extracellular medium, but total eradication was not observed, since invading bacteria still represented 10% of the inoculum at the end of this experiment. Mean maximal activities for sucrase-isomaltase and dipeptidylpeptidase IV ($n = 4$) were 4.40 ± 0.32 and 10.56 ± 0.86 mU per mg of protein, respectively. These activities were not significantly modified by infection or ofloxacin treatment (Fig. 2).

Glucose utilization and glycolysis. Glucose disappearance and lactate and pyruvate production were measured in uninfected (control) and Caco-2/TC-7-infected cells incubated with 5 mM D-[U-^{14}C]glucose. There was no difference in glucose utilization between control cells (139.63 ± 11.52 nmol per h per 10^6 viable cells) and cells infected with the avirulent strain (Fig. 3). Treatment with ofloxacin (5 mM final concentration) did not affect glucose utilization in either of these two groups. Glucose utilization by Caco-2/TC-7 cells infected with the virulent strain of *S. typhimurium* increased by 32% ($P < 0.0001$), and that of infected cells treated with ofloxacin increased by 22.6% ($P < 0.001$), both compared to the values for control cells. Glucose was mainly metabolized into lactate plus pyruvate, which accounted for 72 to 84% of the glucose utilized. The fraction of glucose completely converted to CO_2 (Fig. 4) by uninfected Caco-2/TC-7 cells amounted to 2% of the glucose utilized. This fraction increased almost sevenfold following infection of these cells with strain C53 and accounted for 13.6% of glucose utilization. CO_2 generation fell significantly, by 32% ($P < 0.0001$), when C53-infected cells were treated with ofloxacin.

Glutamine utilization and metabolism. Uninfected cells utilized 212.07 ± 16.75 nmol of 2 mM glutamine substrate per h per 10^6 viable cells. Glutamine utilization was not modified by ofloxacin treatment. Infection with strain C53 significantly increased the capacity of the cells to utilize glutamine, by 22% ($P < 0.0001$), compared to the values for uninfected cells or cells infected

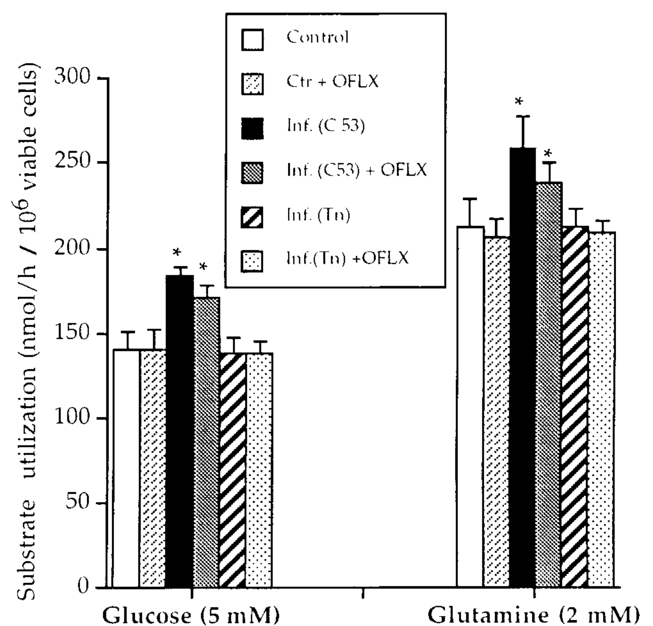

FIG. 3. Substrate utilization. Isolated uninfected Caco-2/TC-7 cells ($n = 8$) or Caco-2/TC-7 cells infected with strain C53 ($n = 5$) or strain C53::Tn*5*-6 ($n = 5$) were incubated for 1 h in the presence of 5 mM D-[U-^{14}C]glucose or 2 mM L-[U-^{14}C]glutamine and with or without ofloxacin (OFLX). Substrate utilization corresponds to the net amounts of substrates that disappeared from the incubation medium. Asterisks indicate values statistically different from those for uninfected cells ($P < 0.0001$). Ctr, control cells; Inf., infected; Tn, C53::Tn*5*-6.

Downloaded from http://aac.asm.org/ on November 19, 2019 by guest

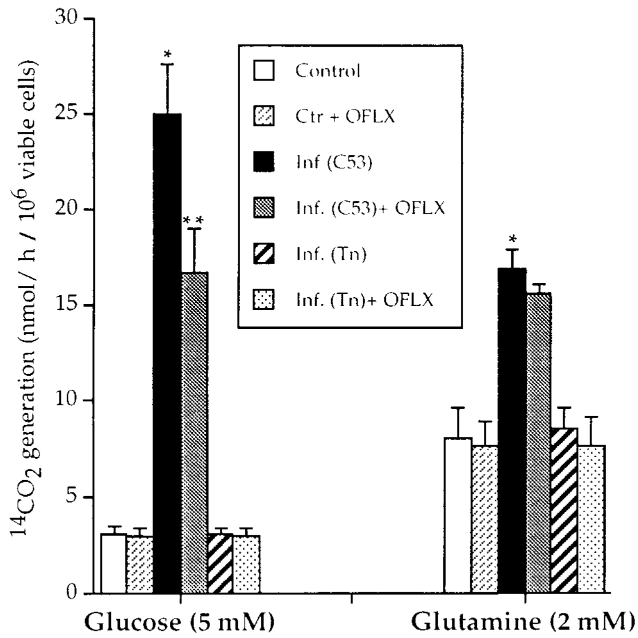

FIG. 4. Substrate oxidation. Isolated uninfected Caco-2/TC-7 cells ($n = 8$) or Caco-2/TC-7 cells infected ($n = 5$) were incubated for 1 h in the presence of 5 mM D-[U-^{14}C]glucose or 2 mM L-[U-^{14}C]glutamine and with or without ofloxacin (OFLX). Substrate oxidation was determined as described in Materials and Methods. Asterisks indicate values statistically different from those for uninfected cells ($P < 0.0001$); double asterisks indicate a value statistically different from that for C53-infected cells ($P < 0.0001$). See the legend to Fig. 3 for definitions of abbreviations.

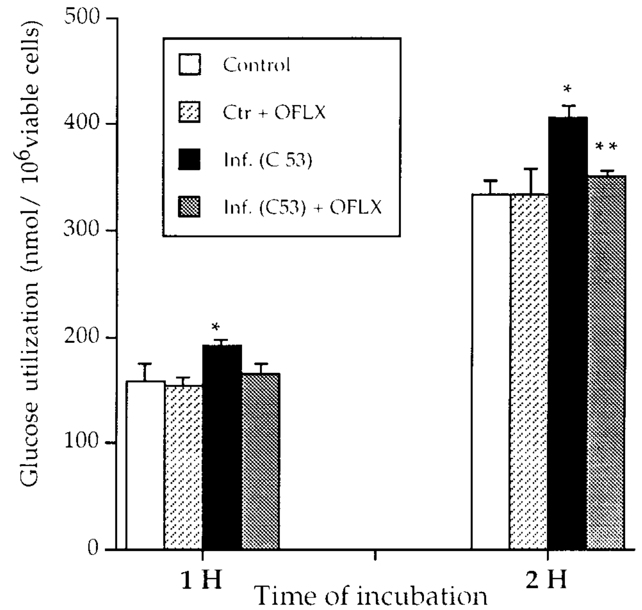

FIG. 5. Time-dependent effect of ofloxacin (OFLX) on glucose utilization. Isolated uninfected or C53-infected Caco-2/TC-7 cells were incubated for 1 or 2 h at 37°C in the presence of 5 mM D-[U^{14}C]glucose and with or without 5 mM ofloxacin ($n = 3$). Asterisks are as defined in the legend to Fig. 4. See the legend to Fig. 3 for definitions of abbreviations.

with the avirulent strain (Fig. 3), both before and after ofloxacin treatment. A net production of ammonia (Table 1) and a net production of glutamate, which accounted for approximately 40% of the glutamine utilized, were observed for all experimental groups. Generated CO_2 (Fig. 4) accounted for 4 to 7% of the glutamine utilized.

Time-dependent effect of ofloxacin on glucose utilization in C53-infected cells. We decided that the time-dependent effect of ofloxacin treatment on glucose utilization should be examined, since the major consequence of infection was to cause an increase in glucose utilization by Caco-2/TC-7 cells. Glucose utilization by control or C53-infected Caco-2/TC-7 cells was twice as high after 2 h of incubation as after 1 h of incubation (Fig. 5), while glucose utilization by C53-infected Caco-2/TC-7 cells incubated with ofloxacin fell by 9.4 and 17% after 1 and 2 h of incubation, respectively.

Glucose and glutamine metabolism in *S. typhimurium*. The utilization and oxidation of glucose and glutamine by the two bacterial strains were assessed. Both strains behaved similarly (Table 2).

DISCUSSION

These experiments were designed to study the effects of infection by *S. typhimurium* at an early stage and of subsequent ofloxacin treatment on glucose and glutamine metabolism in isolated, metabolically active, enterocyte-like Caco-2/TC-7 cells. Isolated columnar absorptive cells have frequently been used (3, 38) for the study of the metabolic properties of enterocytes. The present results provided information on the capacities of uninfected and infected Caco-2/TC-7 cells for glucose and glutamine metabolism both before and after treatment with ofloxacin, although exposure of all surfaces of isolated enterocyte-like cells to nutrients at the same concentration may be considered a nonphysiological situation.

The invasive strain of *S. typhimurium* (C53) was able to enter and multiply within the Caco-2/TC-7 cells, but the avirulent

TABLE 1. Ammonia and glutamate generated from 2 mM glutamine by Caco-2/TC-7 cells[a]

Product	Amt (nmol/h/10^6 viable cells) produced under the following conditions:			
	Control	Control + ofloxacin	Infection	Infection + ofloxacin
Ammonia	218.17 ± 15.02	209.87 ± 12.14	254.23 ± 18.85[b]	240.31 ± 10.25
Glutamate	88.16 ± 20.78	85.44 ± 21.14	102.0 ± 16.36	92.06 ± 7.34

[a] Uninfected (control) and infected (with C53) Caco-2/TC-7 cells were incubated for 1 h with glutamine and without or with ofloxacin. The net generation of ammonia and glutamate was measured. Values are means ± standard deviations for five to eight experiments.

[b] The P value (in a comparison with the control) was <0.001.

TABLE 2. Glucose and glutamine metabolism in *S. typhimurium*[a]

Strain	Metabolism (nmol/h/10^6 bacteria) of:			
	Glucose (5 mM)		Glutamine (2 mM)	
	Consumption	Oxidation	Consumption	Oxidation
C53	7.49 ± 0.15	1.40 ± 0.10	0.40 ± 0.01	0.11 ± 0.09
C53::Tn5-6	7.35 ± 0.06	1.30 ± 0.01	0.36 ± 0.05	0.09 ± 0.02

[a] Bacteria in the mid-logarithmic growth phase were incubated for 1 h at 37°C in the presence of 5 mM D-glucose or 2 mM L-glutamine. Substrate consumption corresponded to the net amount of substrate that disappeared from the incubation medium, and substrate oxidation corresponded to the net amount converted into $^{14}CO_2$. Each value is the mean ± standard deviation for three determinations.

Downloaded from http://aac.asm.org/ on November 19, 2019 by guest

strain (C53::Tn*5*-6) was not. The mean value for the high efficiency of infection by strain C53 was 15% and corresponded to 15×10^6 bacteria/10^6 viable cells. The trypan blue exclusion test showed that cell viability remained elevated during a 4-h incubation period in all experimental groups. Isolated Caco-2/TC-7 cells were also found to be metabolically competent during this period, since the rates of $^{14}CO_2$ production from the two substrates remained linear in all groups.

The results of the present experiments further showed that Caco-2/TC-7 cells are able to metabolize glucose in a manner similar to that of isolated enterocytes from rats, pigs, or humans (3, 8, 38), unlike pig colonocytes (7). Indeed, the Caco-2/TC-7 cells exhibited a high capacity for glucose utilization (139.63 ± 11.52 nmol per h per 10^6 viable cells). However, the value reported here is five times higher than that recorded for Caco-2/TC-7 cells by Chantret et al. (6) and may be explained by differences in the methods used for the measurement of glucose utilization. As in enterocytes (33, 38), glycolysis is the predominant pathway for glucose metabolism in Caco-2/TC-7 cells. Lactate and pyruvate production accounted for 84% of the glucose utilized, and the possible use of glucose in other metabolic processes, such as lipid and pentose phosphate synthesis, cannot be excluded. Approximately 2% of the glucose utilized was converted into CO_2 by these cells. This percentage is at least five times lower than percentages reported for rat, pig, or human enterocytes (3, 36, 37) or pig colonocytes (7). The fact that the incubation buffer was not saturated with O_2 may provide an explanation for this difference. No modifications in glucose metabolism were observed when the Caco-2/TC-7 cells were treated with ofloxacin.

Infection of these cells by the virulent strain of *S. typhimurium* increased glucose utilization by 32%. Mészaros and coworkers (26) also observed an increase in the rate of glucose utilization by the intestinal mucosa of septic rats. This result may have been due, in part, to intracellular bacteria metabolism, as *S. typhimurium* is able to use glucose in vitro (Table 2). Infection by strain C53 seemed to modify glucose metabolism. Glycolysis dropped significantly ($P < 0.001$) and accounted for only 72% of the glucose used, whereas the fraction of glucose converted into CO_2 rose eightfold. Furthermore, intestinal infection is known to enhance RNA synthesis in intestinal cells via an increase in the concentrations of metabolic intermediates (31), including those provided by glucose metabolism. The increase in the CO_2 generated therefore might have been due to the relative increase in glucose utilization and to an increase in the capacity of C53-infected Caco-2/TC-7 cells to oxidize glucose. The results showed that the capacity of the C53-infected cells for glucose oxidation was increased compared to that of uninfected Caco-2/TC-7 cells (3.04 nmol/h per 10^6 viable cells) and seemed to indicate that the energy requirement of these cells had increased, assuming that cellular ATP production is representative of the energy requirement of these cells (24). It has been demonstrated (32) that complete oxidation of glucose into CO_2 provides approximately 58% of the total ATP required by piglet intestinal cells incubated with 2 mM glucose alone and that complete oxidation of glutamine provides almost 70% of the total ATP required by piglet enterocytes incubated with 2 mM glutamine alone.

The increase in glucose utilization observed in the C53-infected Caco-2/TC-7 cells after 1 and 2 h of incubation decreased in the presence of ofloxacin by 9.4 and 17% ($P < 0.0001$), respectively. Glucose oxidation also decreased by 33%. Such results emphasize the involvement of internalized bacteria in the upregulation of glucose metabolism and suggest that incubation of C53-infected cells with 5 mM ofloxacin did not totally eradicate the intracellular bacteria (Fig. 1). This finding has been reported for many antibiotics, including quinolones, despite high intracellular drug concentrations (27). No differences were observed in glucose metabolism between the control and C53::Tn*5*-6-infected cells, confirming that the latter strain was noninvasive.

Caco-2/TC-7 cells also exhibited a high capacity for glutamine utilization, which was as high as that previously found for enterocytes isolated from fed adult rats and piglets (32, 38). Net ammonia production matched glutamine utilization and suggested, as reported for piglet enterocytes (32), that ammonia production by the glutamate dehydrogenase reaction was limited. Glutamate was also generated from glutamine via the glutaminase reaction, and the fraction of glutamate recovered at the end of 1 h of incubation accounted for approximately 40% of glutamine utilization in each of the experimental groups. Four percent of the glutamine used by the Caco-2/TC-7 cells was converted into CO_2, but some glutamine may have been used by other pathways leading to purine or pyrimidine synthesis (38, 40). The oxidized fraction was smaller than that measured for rat, pig, or human enterocytes (10 to 20% of glutamine utilization), however (3, 36, 38); this finding may have been due to nonsaturation of the incubation buffer with O_2. Comparison of the results for glucose and glutamine metabolism in the present work indicates that the rate of glutamine utilization was 1.5-fold higher and that the rate of glutamine oxidation was 2.6-fold higher than the corresponding values for glucose. These observations suggest that glutamine was the better fuel for Caco-2/TC-7 cells, as reported for enterocytes. Incubation of these cells with 5 mM ofloxacin did not modify glutamine metabolism.

Infection of Caco-2/TC-7 cells with the invasive *S. typhimurium* strain enhanced glutamine utilization by 22% and CO_2 production by 109%. A similar enhancement of glutamine oxidation in enterocytes isolated from *Cryptosporidium*-infected piglets was observed by Argenzio et al. (2). This increase in glutamine metabolism may have been related to a stimulation of glutamine metabolism in the Caco-2/TC-7 host cells rather than to internalized bacteria, as ofloxacin treatment did not reduce glutamine metabolism significantly. It should also be noted that C53 infection caused a greater increase in glucose oxidation (fivefold) than in glutamine oxidation (twofold) in the Caco-2/TC-7 cells, although glutamine utilization was increased. Glucose and glutamine substrate oxidation rates were increased in the C53-infected Caco-2/TC-7 cells by 13.5 and 7.2 nmol/h per 10^6 viable cells, respectively. These findings suggest that the C53-infected Caco-2/TC-7 cells mobilized glucose rather than glutamine for energy purposes and that glutamine may have been used to promote cell repair by stimulation of proliferative events (purine, pyrimidine, polyamine, and protein synthesis) (2, 40). However, stimulation of glutamine and glucose oxidation seemed to occur only during the very early stages of infection; in fact, decreases in glucose or glutamine oxidation rates by intestinal cells from septic rates at 48 h (1) and *Cryptosporidium parvum*-infected piglets at 72 h (28), compared to the rates in the corresponding control cells, have been reported. It is therefore possible that enterocytes that have been infected longer have a lower energy requirement than control cells or that infected cells utilize another fuel (e.g., ketone bodies or lipids) for the maintenance of ATP production.

In conclusion, infection by *S. typhimurium* C53 enhanced glucose and glutamine utilization and oxidation, particularly glucose oxidation. Ofloxacin did not completely eradicate intracellular bacteria (at least under our experimental conditions with a 2-h exposure to the drug), since substrate metabolism remained elevated and did not return to the levels observed in

Downloaded from http://aac.asm.org/ on November 19, 2019 by guest

uninfected cells. It will be interesting to study the contribution of both glucose and glutamine to repair events in infected cells.

ACKNOWLEDGMENTS

The excellent assistance of P. H. Duée and his team (INRA, Jouy-en-Josas, France) with this study and the help of M. Muffat-Joly with the statistical analyses are gratefully acknowledged.

REFERENCES

1. **Ardawi, M. S. M., Y. S. Jamal, A. A. Ashy, H. Nasr, and E. A. Newsholme.** 1990. Glucose and glutamine metabolism in the small intestine of septic rats. J. Lab. Clin. Med. **155:**660–668.
2. **Argenzio, R. A., J. M. Rhoads, M. Armstrong, and G. Gomez.** 1994. Glutamine stimulates prostaglandin-sensitive Na^+-H^+ exchange in experimental porcine cryptosporidiosis. Gastroenterology **106:**1418–1428.
3. **Ashy, A. A., and M. S. M. Ardawi.** 1988. Glucose, glutamine and ketone-body metabolism in human enterocytes. Metabolism **37:**602–609.
4. **Bergmeyer, H. U.** 1974. Methods of enzymatic analysis, 3rd ed. vol. 1 to 4. Academic Press, Inc., New York, N.Y.
5. **Carlier, M. B., B. Scorneaux, A. Zenebergh, J. F. Desnottes, and P. M. Tulkens.** 1990. Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages. J. Antimicrob. Chemother. **26**(Suppl. B)**:**27–39.
6. **Chantret, I., A. Rodolosse, A. Barbat, E. Dussaulx, E. Brot-Laroche, A. Zweibaum, and M. Rousset.** 1994. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. J. Cell Sci. **107:**213–225.
7. **Darcy-Vrillon, B., M. T. Morel, C. Cherbuy, F. Bernard, L. Posho, F. Blachier, J. C. Meslin, and P. H. Duée.** 1993. Metabolic characteristics of pig colonocytes after adaptation to a high fiber diet. J. Nutr. **123:**234–243.
8. **Darcy-Vrillon, B., L. Posho, M. T. Morel, F. Bernard, F. Blachier, J. C. Meslin, and P. H. Duée.** 1994. Glucose, galactose, and glutamine metabolism in isolated pig enterocytes during development. Pediatr. Res. **36:**175–181.
9. **Darmaun, D., B. Messing, B. Just, M. Rongier, and J. F. Desjeux.** 1991. Glutamine metabolism after small intestinal resection in humans. Metabolism **40:**42–44.
10. **Finlay, B. B., B. Gumbiner, and S. Falkow.** 1988. Penetration of *Salmonella* through polarized Madin-Darby canine kidney epithelial monolayers. J. Cell Biol. **107:**221–230.
11. **Finlay, B. B., and S. Falkow.** 1989. *Salmonella* as an intracellular parasite. Mol. Microbiol. **3:**1833–18421.
12. **Finlay, B. B., and S. Falkow.** 1990. *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. **162:**1096–1106.
13. **Finlay, B. B., K. Y. Leung, I. Rosenshine, and F. Garcia-del Portillo.** 1992. *Salmonella* interactions with the epithelial cell. ASM News **58:**486–489.
14. **Fogh, J., and G. Trempe.** 1975. New human tumor cell lines, p. 115–141. *In* J. Fogh (ed.), Human tumor cells in vitro. Plenum Press, New York, N.Y.
15. **Francis, C. L., M. N. Stanbach, and S. Falkow.** 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. Mol. Microbiol. **6:**3077–3087.
16. **Gahring, L. C., F. Heffron, B. B. Finlay, and S. Falkow.** 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. Infect. Immun. **58:** 443–448.
17. **Garcia, I., A. Pascual, and E. J. Perea.** 1994. Intracellular penetration and activity of BAY Y 3118 in human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **38:**2426–2429.
18. **Garcia-del Portillo, F., and B. B. Finlay.** 1994. *Salmonella* invasion of nonphagocytic cells induces formation of macrophinosomes in the host cells. Infect. Immun. **62:**4641–4645.
19. **Graninger, W., K. Zedtwitz-Liebenstein, H. Laferl, and H. Burgmann.** 1996. Quinolones in gastrointestinal infections. Chemotherapy (Basel) **42**(Suppl. 1)**:**43–53.
20. **Griffiths, N. M., B. H. Hirst, and N. L. Simmons.** 1994. Active intestinal secretion of the fluoroquinolone antibacterials ciprofloxacin, norfloxacin and pefloxacin; a common secretory pathway? J. Pharmacol. Exp. Ther. **269:**496–502.
21. **Guarino, A., R. B. Canani, A. Casola, E. Pozio, R. Russo, E. Bruzzese, M. Fontana, and A. Rubino.** 1995. Human intestinal cryptosporidiosis: secretory diarrhea and enterotoxic activity in Caco-2 cells. J. Infect. Dis. **171:**976–983.
22. **Lee, C. A., and S. Falkow.** 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87:** 4304–4308.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
24. **Manillier, C., P. Vinay, L. Lalonde, and A. Gougoux.** 1986. ATP turnover and renal response of dog tubules to pH changes in vitro. Am. J. Physiol. **251:** F919–F932.
25. **Messer, M., and A. Dahlqvist.** 1966. A one step ultramicromethod for the assay of intestinal disaccharidase. Anal. Biochem. **14:**376–392.
26. **Mészaros, K., C. H. Lang, G. J. Bagby, and J. J. Spitzer.** 1988. *In vivo* glucose utilization by individual tissues during nonlethal hypermetabolic sepsis. FASEB J. **2:**3083–3086.
27. **Michélet, C., J. L. Avril, F. Cartier, and P. Berche.** 1994. Inhibition of intracellular growth of *Listeria monocytogenes* by antibiotics. Antimicrob. Agents Chemother. **38:**438–446.
28. **Moore, R., S. Tzipori, J. K. Griffiths, K. Johnson, L. De Montigny, and I. Lomakina.** 1995. Temporal changes in permeability and structure of piglet ileum after site-specific infection by *Cryptosporidium parvum*. Gastroenterology **108:**1030–1039.
29. **Nagatsu, T., M. Hino, H. Fuyamada, T. Hayatawa, S. Sakakibare, Y. Nakagawa, and T. Takemoto.** 1976. New chromogenic substrates for X-propyl dipeptidyl-aminopeptidase. Anal. Biochem. **74:**466–476.
30. **Nath, S. K., P. Dechelotte, D. Darmaun, M. Gotteland, M. Rongier, and J. F. Desjeux.** 1992. [^{15}N]- and [^{14}C]glutamine fluxes across rabbit ileum in experimental bacterial diarrhea. Am. J. Physiol. **262:**G312–G318.
31. **Newsholme, E. A., P. Newsholme, and R. Curi.** 1987. The role of the citric acid cycle in cells of the immune system and its importance in sepsis, trauma and burns. Biochem. Soc. Symp. **54:**145–161.
32. **Posho, L., B. Darcy-Vrillon, F. Blachier, and P. H. Duée.** 1994. The contribution of glucose and glutamine to energy metabolism in newborn pig enterocytes. J. Nutr. Biochem. **5:**284–290.
33. **Posho, L., B. Darcy-Vrillon, M. T. Morel, C. Cherbuy, F. Blachier, and P. H. Duée.** 1994. Control of glucose metabolism in newborn pig enterocytes: evidence for the role of hexokinase. Biochim. Biophys. Acta **1224:**213–220.
34. **Rhoades, J. M., E. O. Keku, J. Quinn, J. Woosely, and J. G. Lecce.** 1991. L-Glutamine stimulates jejunal sodium and chloride absorption in pig rotavirus enteritis. Gastroenterology **100:**683–691.
35. **Tulkens, P. M.** 1991. Intracellular distribution and activity of antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. **10:**100–106.
36. **Vaugelade, P., L. Posho, B. Darcy-Vrillon, F. Bernard, M. T. Morel, and P. H. Duée.** 1994. Intestinal oxygen uptake and glucose metabolism during nutrient absorption in the pig. Proc. Soc. Exp. Biol. Med. **207:**309–316.
37. **Vidal, H., B. Comte, M. Beylot, and J. P. Riou.** 1988. Inhibition of glucose oxidation by vasoactive intestinal peptide in isolated rat enterocytes. J. Biol. Chem. **263:**9206–9211.
38. **Watford, M., P. Lung, and H. A. Krebs.** 1979. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. **178:**589–596.
39. **Windmueller, H. G., and A. E. Spaeth.** 1978. Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for postabsorptive rat small intestine. J. Biol. Chem. **255:**107–112.
40. **Wu, G., C. J. Field, and E. B. Marliss.** 1991. Glutamine and glucose metabolism in rat splenocytes and mesenteric lymph node lymphocytes. Am. J. Physiol. **260:**E141–E147.
41. **Zweibaum, A.** 1991. Differentiation of human colon cancer cells. Life **218:**27–37.

Downloaded from http://aac.asm.org/ on November 19, 2019 by guest