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Internalization property of intestinal bacteria in colon cancer and HIV/AIDS patients

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Abstract

OBJECTIVES: Bacteria from the intestinal tract of Slovak and American HIV/AIDS patients and Slovak colon cancer patients were tested for the capacity to be internalized by cells of the HL-60 cell line as well as by normal human lymphocytes. They were anticipated to possess a specific characteristic, i.e. a vigorous ability to be internalized by HL-60 cells and human lymphocytes. This assumption was confirmed by gentamicin protection assay.

RESULTS: Internalization of bacteria from HIV/AIDS patients frequently resulted in partial (patients SKM1, SKM22) or complete lysis (patients SKK1-1, SKM12) of HL-60 cells. In comparison with intramucosal bacteria isolated from patients with colorectal cancer (TSG, 883, 660, 838, 536, MZRa), their capacity to internalize HL-60 cells was found to be 15–20 times higher (USP15/7, USP1/4, USP3/3, SK725/5). Partial lysis (patients USP15/7, USP3/3 and SKM22) and complete lysis (patients USP1/4, SKK1-1/1, SKM1/6, SKM12/5) were detected also after internalization of bacteria by normal human lymphocytes. Compared to the amount of intracellular bacteria isolated from patients with HIV/AIDS, the ability of bacteria from patients with colorectal cancer to internalize normal human lymphocytes was significantly lower (10–15 times), yet still higher than that of bacteria isolated from healthy people.

CONCLUSIONS: Our results present the ability of bacteria of colon cancer patients and HIV/AIDS patients to internalize HL-60 cells and normal human lymphocytes. The findings underline the potentially important function of bacteria in the induction of colorectal cancer and immunodeficiency. The particularly high detection ability of bacteria from HIV/AIDS patients to internalize normal human cells emphasizes their potentially important role in the process of AIDS.

INTRODUCTION

The incidence of colorectal cancer (CRC) has been increasing in recent years and the mortality rates are very high. Patients with inflammatory bowel disease (IBD) have a higher risk of 10–15% to develop CRC (Tomasello *et al.* 2014). There is increasing evidence that mucosa-associated flora plays an important role in the etiology of colon cancer and IBD (Martin *et al.* 2004; Tomasello *et*

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al. 2014). Gut inflammation may induce changes in nutrient microenvironments via production of oxygen and nitrogen radicals that allow outgrowth of facultative anaerobes, as e.g. members of the *Enterobacteriaceae* family. Intraepithelial *Escherichia coli* were found in biopsy specimens from malignant and macroscopically normal tissue of patients with colorectal adenoma and carcinoma (Swidsinski 1998). Wang *et al.* (2012) showed an enrichment of bacteria belonging to the genus *Enterococcus, Escherichia, Shigella, Klebsiella, Streptococcus* and *Peptostreptococcus* in the luminal compartment of CRC patients compared to controls. In IBD, bacteria belonging to the *Clostridia* genus, *Fusobacterium*, *Mycobacterium*, adherent-invasive *E.coli* (AIEC), *Proteus mirabilis*, *Klebsiella pneumoniae* and *Proteobacteria* such as *Helicobacter spp* were overrepresented (Grivennikov 2013; Zhu *et al.* 2013). A number of culture-based and molecular-based studies support the theory that *Escherichia coli* (*E. coli*) is a microbiological factor implicated in Crohn´s disease (Martinez-Medina *et al.* 2014).

In Crohn's disease, independent studies reported increased numbers of *E. coli* adjacent to the ileal (Martinez-Medina *et al.* 2009) or colonic mucosa (Swidsinski 2002; Swidsinski *et al.* 2005). The adherence of bacteria to enterocytes is mediated by intimin, an outer membrane protein encoded by the *eae* gene (Nataro & Kaper 1998). The *eae* gene is carried by enteropathogenic *E.coli* (EPEC). However, there is not enough data addressing the question whether colonization of the colonic mucosa by intracellular *E.coli* is primary or secondary to the pathology of CRC (Magdy *et al.* 2015) and IBD. The presence of mucosa-associated adhesive *E.coli* in colon cancer and Crohn´s disease raises the possibility that colon cancer might be the result of inflammation induced by bacteria in a way analogous to the process in stomach cancer and *Helicobacter pylori* (Martin 2004).

The role of microbiota in the pathogenesis of HIV infection has become the subject of intensive research in recent years. Despite effective viral suppression with antiretroviral therapy, individuals with HIV continue to have excessive non-AIDS morbidity and mortality, which appears to be driven in part by microbial translocation and resultant immune activation (Klatt *et al.* 2013; Tenorio *et al.* 2014). Much work has been invested into elucidating the mechanisms by which intestinal microbiota augment or disrupt intestinal barrier function, immune response to antigen, and systemic immune activation. The presence of microbial products in peripheral blood of HIV-infected subjects was also linked to immune activation and increased morbidity and mortality (Sandler *et al.* 2011). Defects in the mucosal barrier have been associated with decreased gastrointestinal epithelial barrier integrity and accelerated disease (Favre *et al.* 2010).

Mechanisms leading to immunologic failure despite effective viral suppression remain elusive, though there appears to be a link between poor CD4 recovery and bacterial translocation (Merlini *et al.* 2011). Dillon *et al.* (2014) found a trend toward lower CD4 count with increased abundance of *Bacteroides*. In the proximal gut, low CD4 count was associated with colonization by environmental *Burkholderia* and *Bradyrhizobia*, most likely secondary to loss of colonization resistance, as these organisms were not seen in HIV negative or HIV infected subjects with normal CD4 (Yang *et al.* 2014).

Observations concerning especially expressive characteristics of bacteria of cancer patients to internalize human cells prompted us to analyze bacteria isolated from HIV/AIDS patients for such a capacity. We started with the presumption that cancer and AIDS have many common features and this may be reflected by the nature of intestinal bacteria. One of the very actual AIDS problems is that in individuals treated with highly active anti-retroviral therapy (HAART) plasma HIV RNA is reduced below the level of detection, but there is strong evidence of residual viral replication after complete suppression of plasma viremia (Chun *et al.* 2000; Cusini *et al.* 2004). It has been reported that various forms of viral reservoir persist in virtually all infected individuals receiving HAART (Finzi *et al.* 1997; Siliciano *et al.* 2003; Veazey & Lackner 2005). HIV-1 was also detected in bowel crypt cells and the lamina propria in AIDS patients (Nelson *et al.* 1988; Wang *et al.* 2015). Since these cells are in close proximity to intestinal bacteria, they may be involved in the process of pathogenesis (Veazey & Lackner 2005). Vujkovic-Cvijin hypothesized that persistence of even a subset of the "disease-associated microbial community" could continue to sustain pathologic chronic immune activation in this population despite suppression of viral replication (Vujkovic-Cvijin *et al.* 2013).

Our assumption is based on extensive laboratory experience with internalized bacteria isolated from biopsies of patients with colorectal problems (Mego *et al.* 2005; Mego *et al.* 2006). To study this phenomenon of bacteria isolated from the GIT of HIV/AIDS patients, we chose cells of the promyelocytic cell line HL-60 and normal human lymphocytes as host cells. This approach may give an answer to the question whether bowel bacteria play some role in the pathogenesis of HIV infection and consequently in the AIDS process generally.

MATERIAL AND METHODS

Patients

Intestinal bacteria of HIV-positive patients (USP1, USP3, USP15) from the USA (Veteran Hospital, San Diego) and HIV-positive patients (SKM1, SKM12, SKM22, SKK1-1, SK725) from Slovakia (Department of Infectious Diseases and Geographic Medicine, Derer's Hospital, Bratislava) were isolated from patients´ rectal swabs by overnight cultivation in LB medium or on McConkey agar. Bacteria of cancer patients (TSG, 883, 660, 838, 536, MZRa) were collected in the National Cancer Hospital, Bratislava. Swabs of healthy individu-

als were used as controls. The majority of HIV-positive patients were men who had sex with men and were treated with antiretroviral therapy (ART). Informed consent from all subjects was required prior to testing.

Cell lines and cell culture

The following cell models were used in our experiments: the HL-60 cell line established from human promyelocytic cells and HIV-negative normal human lymphocytes. The cells were grown in MEM medium, 5% CO_2, supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics.

Gentamicin protection assay (GPA)

Gentamicin protection assay (GPA) is a simple test based on the resistance of intracellular bacteria to gentamicin used for detection of intracellular bacteria as described by Swidsinski *et al.* (1998). HL-60 cells were seeded in a density 5×10^6 cell/ flask dish and incubated at 37 °C until a confluent monolayer was formed and then washed with PBS. Each monolayer was infected with approximately 5×10^7 bacteria in culture medium without antibiotics. After 3 hours of incubation at 37 °C, the monolayer was washed with PBS. To determine bacterial internalization, HL-60 cells were treated with culture medium containing gentamicin (100ul/ml) to kill all extracellular bacteria. After 1-hour incubation, the cells were washed with PBS. Cells in monolayer were lysed by addition of water containing 1% (vol/vol) Triton X-100 for 5 minutes to release internalized bacteria. The cell lysate was plated onto MacConkey or LB agar plates. It progressed well during infection of normal human lymphocytes by bacteria from cancer and HIV/AIDS patients with respect to their properties. The number of colonies was counted and the bacteria were identified using a commercial identification system VITEK (Bio-Mérieux, Marcy L'Etoile, France).

All isolated bacteria were assessed for antibiotic sensitivity by controlled disk diffusion with disk containing ampicilin, tetracycline, gentamicin, ofloxacin, chloramphenicol, trimethoprim, compound sulfonamides, colistin, cefuroxim or cefalotin. Bacterial samples were stored in Luria-Bertani (LB) broth with glycerol (15% vol/vol) media at –80 °C until further analysis.

RESULTS

Intestinal bacteria isolated from the patients tested were first typed and the most frequent isolates were found to be *Escherichia coli* (70%), *Proteus mirabilis* (10%), followed by *Citrobacter freundii* (5%), *Staphylococcus sp.* (7%), *Enterococcus aerogenes* (4%), and *Enterobacter cloacae* (4%). The bacteria were diluted to the concentration 10^{-7}–10^{-10} and plated at concentrations yielding single colonies for their subcloning. Because of bacterial heterogeneity, further analysis was performed on bacterial subclones. The extrachromosomal replicons were commonly presented and their coding capacity was detected in some cases (patients USP1 and USP15) to be around 50 kbp (data not shown). The gentamicin protection assay (GPA) was used to valuate the ability of intestinal bacteria to enter into cells of the HL-60 cell line and human lymphocytes. As shown in Table 1, bacterial subclones USP15/7, USP1/4 and USP3/3 derived from American AIDS patients, as well as bacteria of subclone SK725/5 of the Slovak patient SK725 were very efficiently internalized into HL-60 cells without lysis.

Bacterial subclones SKM1/6, SKM22/5 of Slovak AIDS patients SKM1 and SKM22 accumulated inside HL-60 cells to avoid their partial (30–50%) lysis. Complete lysis of host HL-60 cells after 3 hours of incubation was performed by bacterial subclones SKM12/5 and SKK1-1/1. Bacterial subclones TSG+/2, TSG/1, 883S/3, 660/3, 838/4, 536/1, 883/1 and MZRa/1 of cancer patients TSG, 883, 660, 838, 536 and MZRa with adenomas and carcinomas were positive in internalization. The level of their internalization into HL-60 cells was 15 to 20 times lower in comparison to bacteria of HIV/AIDS patients, but much higher than in normal subjects.

Tab. 1. Results of gentamicin protection assay (GPA) in HL-60 cells.

Patient/bacterial clone	Number of bacterial colonies
HIV positive patients	
USP15/7	2264
USP1/4	1340
USP3/3	1680
SK725/5	1140
SKM1/6	481/partial lysis of HL-60 cells
SKM22/5	27/partial lysis of HL-60 cells
SKM12/5	complete lysis of HL-60 cells
SKK1-1/1	complete lysis of HL-60 cells
Colon cancer patients	
TSG+/2	67
TSG/1	71
883 S/3	104
660/3	84
838/4	98
536/1	76
883/1	72
MZRa/1	15
negative controls (healthy persons)	<5

The number of colonies represents the number of intracellular bacteria in HL-60 cells after infection. Number of cells used for assay: HL-60 5×10^6; number of bacteria 1×10^8. Bacteria SK725/5 were classified as *Enterobacter cloacae*. All other clones tested were characterized as *E. coli*.

Normal human lymphocytes were partially lysed after infection by bacterial subclones USP15/7, USP3/3 and SKM22/5 of HIV/AIDS patients USP15, USP3 and SKM22 (Table 2). Complete lysis occurred after infection by subclones USP1/4, SKK1-1/1, SKM1/6 and SKM12/5 of patients USP1, SKK1-1, SKM1 and SKM12. And again, normal human lymphocytes were permissive for infection by subclone SK725/5 but they were not lysed. After three-week incubation of infected lymphocytes, no intracellular bacteria could be detected by GPA. Bacterial subclones TSG+2, TSG/1, 883S/1, 660/3, 838/4, 536/1, 883/1 and MZRa/3 were internalized into human lymphocytes up to 15–20 times more weakly than bacteria from HIV/AIDS patients. Human lymphocytes were found greatly susceptible to bacteria of AIDS patients in GPA. There were however difficulties in evaluating the results since some analyzed cells underwent degradation during the assay.

DISCUSSION

In the presented work we performed an untraditional approach to study the role of intestinal bacteria in the process of AIDS and colorectal cancer. The idea developed from our laboratory experience with bacteria isolated from patients with colorectal problems (Mego *et al.* 2005). We confirmed that the special characteristic of these bacteria was their ability to internalize into colon epithelial cells.

Tab. 2. Results of gentamicin protection assay (GPA) in human lymphocytes.

Patient/bacterial clone	Number of bacterial colonies
HIV positive patients	
USP15/7	1121/partial lysis of human lymphocytes
USP1/4	complete lysis of human lymphocytes
USP3/3	320/partial lysis of human lymphocytes
SKM22/5	423/partial lysis of human lymphocytes
SKK1-1/1	complete lysis of human lymphocytes
SKM1/6	complete lysis of human lymphocytes
SKM12/5	complete lysis of human lymphocytes
SK725/5	1140
Colon cancer patients	
TSG+2	27
TSG/1	14
883 S/3	20
660/3	12
838/4	13
536/1	25
883/1	19
MZRa/3	11
negative controls (healthy persons)	<5

Number of colonies represents the number of intracellular bacteria in normal human lymphocytes after infection. Number of cells used for assay: normal human lymphocytes 1.5×10^7; number of bacteria 1×10^8.

Bacteria isolated from the intestinal tract of cancer patients were mostly identified as *E.coli*. Swidsinski reported that only 3% of colon mucosa biopsies from asymptomatic controls tested positive for bacteria. In contrast, biopsies from 92% of patients with colonic adenomas or carcinomas held bacteria, with *E. coli* being the predominant bacterium in 70% of patients (Swidsinski *et al.* 1998). Because of bacterial heterogeneity, further analysis was performed on bacterial subclones. GPA was used to valuate the ability of intestinal bacteria to enter into cells of the HL-60 cell line and human lymphocytes. On average, we found 74 colonies representing the number of intracellular bacteria in HL-60 cells after infection and an average of 18 colonies representing the number of intracellular bacteria in normal human lymphocytes after infection. Bacterial subclones used in GPA are indicative of the intracellular mechanism of colonization, similar to that described for some enteropathogenic *E.coli* strains. The possible role of mucosal adherent *E. coli* in colon cancer pathogenesis is currently speculative, but there is growing interest in the potential role of inflammation and perhaps particularly NFκB activation in colon cancer (Francis *et al.* 1991; Rhodes & Campbell 2002). Bacterial adhesion has the potential to induce epithelial cell changes that could promote cancer development (Greten *et al.* 2004).

Bacteria isolated from the intestinal tract of HIV/AIDS patients in our cohort were mostly specified as *E. coli* (negative in serotypization), *Proteus mirabilis*, *Citrobacter freundii, Staphylococcus sp.* and *Enterobacter aerogenes.* All these bacteria, except *Staphylococcus,* belong to the order *Enterobacteriales*. *Enterobacteriales* and members of the *Enterobacteriaceae* family (Phylum *Proteobacteria*) strongly correlate with gut CD4 and CD8 depletion and activation (Ellis *et al.* 2011; Vulkovic-Cvijin *et al.* 2013).

Nevertheless, we suppose that these elements occassionally carry also components of adherence and penetration into a wide range of receptive cells. This assumption was confirmed by GPA. On applying this test, we found that bacteria of all patients tested entered into HL-60 cells or normal human lymphocytes with a 10–15 times greater efficiency than did bacteria of patients with colorectal problems.

What is the fate of internalized bacteria and their hosts? We detected that there were two possibilities: a) bacteria continually disappeared (lysed) inside infected cells and after 12–16 days no bacteria were detectable in host cells by GPA; b) the host HL-60 cells were completely lysed by infected bacteria of patients SKM12 and SKK1-1, partially SKM1 and SKM22; or normal human lymphocytes were completely lysed by infected bacteria

of patients SKM1, SKM12 and SKK1-1, partially USP15 and SKM22. This result, confirming lyses of lymphoproliferative cells by internalized bacteria isolated from AIDS patients, represents a completely new phenomenon, undetected so far in any system studying the proces of intenalization of bacteria in human cells. This finding may become important in the development and management of HIV infection.

As mentioned above, mucosa-associated and intramuscular bacteria may play important roles in the pathogenesis of diseases, as in inflammatory bowel disease, ulcerative colitis, Crohn´s disease and potentially even colon cancer. In view of the immunomodulatory effect reported for probiotics in nonimmunocompetent and HIV/AIDS patients, many scientists studied the effects of probiotics on HIV/AIDS patients undergoing antiretroviral therapy (Gautam *et al.* 2014; d´Ettore *et al.* 2015; Falasca *et al.* 2015). The mechanisms by which probiotics modulate the immune system, are not entirely understood (Gori *et al.* 2011), but studies have shown that probiotics may counteract the inflammatory process by stabilizing the gut microbial environment and the intestinal barrier, lowering systemic inflammation and stimulating natural killer (NK) cell activity. It is well known that Lactobacillus casei *Shirota* (LcS), a commercial probiotic strain, increases the number of bacterial species in the gut that are considered beneficial, improves the balance between beneficial and potentially harmful intestinal bacteria and enhances NK cell activity (Reale *et al.* 2012; Dong *et al.* 2013).

Invasive strains of *E. coli* that undergo lysis upon entry into mammalian cells can act as stable DNA delivery systems to their host. They may function by the system "hit and run" and their extrachromosomal contents remain in the host cell even when the bacterial carriers are not detectable. The horizontal gene transfer from bacteria to yeast, plant and mammalian cells was reported by several investigators (Zambryski 1992; Grillot-Courvalin *et al.* 1998; Walters 2001). Our results strongly indicate that the property of invasive bacteria isolated from HIV/AIDS patients to enter human lymphocytes or HL-60 cells may represent an ideal system for the primae impressionis of horizontal transfer of genetic information. In our previous work, we published original findings about HIV-like sequences in bacteria of HIV AIDS patients (Zajac *et al.* 2007). The expression of these sequences was detected by monoclonal antibodies to HIV-1 antigens (Zajac *et al.* 2011). When bacteria containing HIV-like sequences penetrate human cells, in particular lymphocytes, they can infect or lyse them and induce the process of immunodeficiency.

Our hypothesis has experimentally not yet been established. Yet confirmation of this hypothetical possibility may be of significant importance for further AIDS research as well as for the management of HIV infection.

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