Detection of protein homologues with HIV-1 antigens in bacteria of positive patients – phase II

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Abstract

OBJECTIVES: Human immunodeficiency virus type 1 is widely accepted as the cause of AIDS (Acquires Immunodeficiency Syndrome) but it is necessary to consider other factors, not only HIV, which may be involved in AIDS process. It is apparent that a viral reservoir persists in virtually all infected individuals receiving HAART. Reservoirs were detected in macrophages and other cells of the blood system, in which even very effective HAART was not able to eliminate the virus. Over the last period of time AIDS research has been focused on the gut and other mucosal tissue as the major site of HIV infection and CD4+ T cells loss. Intestinal bacteria and cells associated with GIT are in close vicinity and so has been raised the idea that bacteria may be involved in AIDS pathogenesis.

MATERIAL/METHODS: Bacteria and yeast isolated from a cohort of 67 Cambodian and Kenyan HIV positive children and from a cohort of 62 Slovak and American AIDS patients were analyzed for detection of expression of HIV-1 antigens p17, p24, p55, gp41 and gp120 (Abcam, UK).

RESULTS: By monoclonal antibodies against HIV-1 proteins p17 and p55 was detected protein with molecular weight of 45–55 kDa. In samples of Cambodian and Kenyan HIV positive children was found 35 kDa protein using MAb against HIV-specific protein p17. By using MAbs against p24 was found protein of 55–60 kDa in Cambodian and Kenyan samples but, surprisingly, no proteins were detected in bacterial extracts of American and Slovak patients by this MAbs. Using monoclonal antibodies against HIV-1 specific protein gp41 was positive signal identified in 30–35% of samples from both cohorts of patients from Kenya and Cambodia and in 75% of samples from American and Slovak patients. The protein of about 75–85 kDa was detected by MAbs against gp120 only in protein extracts obtained from yeasts Candida sp. of Cambodian and Kenyan HIV positive children.

CONCLUSION: The molecular weight of 55 kDa protein was detected by MAbs anti HIV p24, p17+p55. Its molecular weight is comparable to gag-encoded Pr55Gag precursor. Surprisingly, such proteins were not found in bacterial extract from samples of American and Slovak patients by using the MAbs against HIV-specific protein p24. The protein of about 75–85 kDa was detected only in Candida species.
protein extracts of Cambodian and Kenyan HIV-positive children by the MAb against gp120. In Slovak and American samples, protein reacting with MABs anti gp120 was not found. These results suggest that there are specific differences between Slovak and American HIV positive patients bacterial proteins on one side and Cambodian and Kenyan on the other. These differences may suggest a diverse bacterial evolution in various geographical areas.

INTRODUCTION

In 1981 was first described AIDS (Acquired immunodeficiency Syndrome) caused by Human immunodeficiency virus type I (HIV-1) and Human immunodeficiency virus type II (HIV-2). The assays for identifying recent HIV infections at the population level are based on the alteration of serological parameters, as well as the newest method based on an increase of HIV genetic diversity with the progress of infection (Smolén-Dzirba & Wasik 2011). Plasma HIV RNA is dramatically reduced in HIV/AIDS patients treated with highly active antiretroviral therapy (HAART), but residual viral replication is detected after suppression of plasma viremia (Chun et al. 2000; Cusini et al. 2004). It has also been expressly proven that various forms of HIV reservoirs persist in practically all patients receiving HAART (Finzi et al. 1997; Siliciano et al. 2003).

HIV persists in peripheral blood mononuclear cells despite sustained, undetectable plasma viremia resulting from long-term antiretroviral therapy (Veazey et al. 1998; Brenchley et al. 2004). Over half of the CD4+ T cells in the gut mucosa are lost within the first few weeks after HIV-1 infection and remain consistently low, compared to peripheral blood sources, despite long term antiretroviral therapy; furthermore, of the few CD4+ T cells that persist in the gut, a significant increase in immune activation is observed (Mehandru et al. 2006).

Microbial flora is able to overcome intestinal barrier and gain the systemic circulation in case of advanced HIV infection, when the homeostatic balance between gastrointestinal indigenous bacteria and gut immunity fails. Antiretroviral therapy is not able fully controlled the microbial translocation and there is an inefficient CD4+ reconstitution (Merlini et al. 2011).

HIV infection is associated with dramatic damage to the gastrointestinal (GI) tract, including substantial disruption of gut microbiota composition with presence of microbes at higher pathogenic potential compared to less aggressive indigenous organisms, massive loss of gut-residing CD4+ T-cells, and down-regulation of GI tract genes expression (Veazey & Lackner 2005; Brenchley et al. 2006; Gori et al. 2007; Douek et al. 2009). HIV-1 was also detected in bowel crypt cells and the lamina propria in HIV-positive patients (Nelson et al. 1988).

These recent findings support the idea that the mucosal and intestine immune system is the major site of viral replication, persistence and CD4+ T-cells loss in HIV-1 infected persons (Guadalupe et al. 2006; Danekar 2007; Ling et al. 2007; Lackner et al. 2009; Ling et al. 2010).

Gut associated lymphatic tissue (GALT) cells are in close contact with intestinal microflora, so it is possible, that bowel bacteria are involved in the pathogenesis of the disease. Consequences of the anatomo-functional gastrointestinal barrier breach occurs the progressive failure of mucosal immunity and leakage into the systemic circulation of bacterial by-products, such as lipopolysaccharide (LPS) and bacterial DNA fragments, which contribute to systemic immune activation (Brenchley et al. 2006; Jiang et al. 2009; Estes et al. 2010; Ferri et al. 2010). Highly active antiretroviral therapy (HAART) only partially amends gastrointestinal tract anatomo-functional damage (Brenchley et al. 2006; Marchetti et al. 2010) and intestinal microbiota, further hampering intestinal homeostasis (Hooper et al. 2010) and sustaining microbial translocation (Brenchley et al. 2006; Paiardini et al. 2008). Thus, although circulating microbial products have been shown to decrease during HAART, they remain elevated, in turn affecting immune restoration (Brenchley et al. 2006; Jiang et al. 2009; Marchetti et al. 2010). Augustin et al. determined, that HIV-infected children on HAART who received any antibiotic were significantly more colonized by clotrimoxazole-resistant E. coli (<0.01) than those not receiving any antibiotic prior to colonization (Augustin et al. 2008).

In untreated HIV/AIDS, the highest degree of microbial translocation has been shown in patients with severe immune depression (Brenchley et al. 2006; Jiang et al. 2009). Similarly, following HAART initiation, patients with blunted long-term CD4+ recovery show persistently elevated circulating LPS and bacterial DNA independently of HIV viremia reduction (Brenchley et al. 2006; Marchetti et al. 2008; Jiang et al. 2009).

These findings, confirming that the gut and other mucosal tissue, rather than blood, is the major site of HIV infection and CD4+ T cell loss, suggest the possibility that bacteria bearing HIV-like sequences (Zajac et al. 2007; Zajac et al. 2011) might play very important role in AIDS process and serve as a viral reservoir as well. Study of the expression of these HIV-like sequences may confirm this presumption.

MATERIAL AND METHODS

Bacteria and yeasts isolated from a cohort of 67 Cambodian and Kenyan HIV positive children of the respiratory tract (nose, pharyngeal swabs) and from a cohort of 62 Slovak and American AIDS patients (intestinal tract) were used for preparation of protein extracts. The prepared extracts were analyzed for detection of expression of HIV-1 antigens by Western blotting.
HIV-1 antigens p17, p24, p55, gp41 and gp120 (Abcam, UK) were detected. Isolated proteins after overnight at 45 mA electrophoresis were transferred from acrylamide gel to nitrocellulose (NC Hybon membrane). The membrane was incubated in TBS-T buffer and blocked using 5% milk for 1 hour at room temperature. The blocking buffer was removed and the membrane was washed with TBS-T buffer. Appropriately diluted monoclonal mouse antibodies p17, p24, p55, gp41 and gp120 (Abcam, UK) in TBS-T buffer with 5% milk was added to the membrane and incubated overnight at 4 °C on a shaker with a rocking motion. The membrane was washed with TBS-T buffer and incubated for 1 hour at room temperature in appropriately diluted goat anti-mouse antibody SC-2005 (Santa Cruz Biotech) in buffer containing 5% milk. Consequently the membrane was washed with TBS-T buffer and for visualization ECL solutions were used.

RESULTS

Microbes of Slovak and American HIV/AIDS patients were most often identified as *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter aerogenes*. Microbes from respiratory tract of Cambodian and Kenyan HIV/AIDS patients were most often identified as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Candida albicans*, *Candida tropicalis*, *Enterobacter aerogenes*, but also as *Escherichia coli* and *Proteus mirabilis*. Taxonomic classification of analyzed microbes was performed in commercial diagnostic laboratory using standard microbiological tests.

Protein of 35 kDa was also detected in bacteria and yeasts of the respiratory tract of Cambodian (Km) and Kenyan (Ke) HIV positive children: 3’Km, 10 Km, 14’Ke, 17’Ke, 21 Ke, 28 Km, 14´Km, using MAbs against HIV1 p17 (Figure 1).

In our research was detected 55–60 kDa protein using MAbs against HIV1 p24 approximately in 30 Cambodian (Km) and 35% Kenyan (Ke) bacterial extracts of HIV positive patients: 14’Ke, 17´Ke, 21 Ke, 32´Ke, 3’Km, 14’Km, Muta 104-0 (Figure 2).

45–55 kDa protein was also detected in bacteria isolated from intestinal tract Slovak and American HIV positive patients: 132/3, 18315, Tu Sevcikova 3, Tu S6, Bact 28/38 II, 883 Ca+/2, 18363, serum of AIDS patient diluted 1:500, 21 Ke using MAbs against HIV-1 p55+p17 (Figure 3).

Protein of 41 kDa was also detected in bacteria isolated from intestinal tract Slovak and American HIV positive patients: Mok1, P8, 79, P55 + IPTG, K1-1, 116x, serum of AIDS patient diluted 1:500 using MAbs against HIV-1 gp41 (Figure 4).

Using monoclonal antibodies against HIV-1 gp120 (1:750), proteins of 75–85 kDa were detected in protein extracts from bacteria and yeasts of the respiratory tract (nose, pharyngeal swabs) of Cambodian (Km) and

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Fig. 1. Western blotting of proteins isolated from bacteria and yeasts of the respiratory tract (nose, pharyngeal swabs) of Cambodian (Km) and Kenyan (Ke) HIV positive children. Detection was performed using monoclonal antibodies against HIV-1 p17 diluted 1:750. Line 1: 3’Km, line 2: 10 Km, line 3: 14’Ke, line 4: 17’Ke, line 5: 21 Ke, line 6: 28 Km, line 7: 14´Km, line 8: negative bacterial control, line 9: serum of AIDS patient diluted 1:500, line 10: protein p17 diluted 1:750.

Fig. 2. Western blotting of proteins isolated from bacteria and yeasts of the respiratory tract of Cambodian (Km) and Kenyan (Ke) HIV positive children. Used monoclonal antibodies against HIV-1 p24 (1:1000). Line 1: 14’Ke, line 2: 17’Ke, line 3: 21 Ke, line 4: 32’Ke, line 5: 3’Km, line 6: 14’Km, line 7: Muta 104-0, line 8: negative bacterial control, line 9: serum of AIDS patient diluted 1:500, line 10: protein p24 diluted 1:1000.

Kenyan (Ke) HIV positive children: 14 Ke, 21 Ke, 3’Km, 22 Km, 28 Km, 25’Km, serum of AIDS patient diluted 1:500 (Figure 5).

**DISCUSSION**

Recent studies suggest that the main fight against the HIV diseases is performed in gut-associated lymphatic tissue closed to the gastrointestinal tract (Li et al. 2005; Guadalupe et al. 2006; Mach et al. 2007). Severely immune depressed HIV-infected patients fail to efficiently control translocation of microbial macromolecules following HAART initiation and maintaining heightened microbial translocation on virologically-suppressive HAART display a circulating microbiota that is polymicrobial at the genotype level and that is not substantially modified by therapy (Merlini et al. 2011). It is questionable if bacteria play some role in process of dramatic loss of CD4+ T cells, predominantly from the mucosal surfaces. Less efficient control over microbial translocation in HIV patients’ cohort might be consistent with the dramatic structural damage of the intestinal barrier described in untreated advanced HIV/SIV infection (Brenchley et al. 2006; Estes et al. 2008), with ever-increasing content of microbial by-products infiltrating intestinal lamina propria and gaining the circulation. The gastrointestinal tract damage and occurring microbial translocation might be hardly repaired by the late institution of therapy, in turn favouring the continuous passage into the systemic circulation of a highly polymicrobial intestinal flora. Increased levels of circulating microbial by-products and markers of immune response to microbial translocation have been associated with increased HIV disease progression and mortality even in the context of continuous antiviral therapy and independently of CD4+ count and HIV-viremia (Marchetti et al. 2010; Sandler et al. 2011). Interestingly, more than 90% of HIV-infected patients harbour a bacterial population enriched with Enterobacteriales, whereas less than 60% display the probiotic Lactobacillales, with the same proportion being maintained on virologically-suppressive HAART (Brenchley et al. 2006).

Previous studies in our laboratory detected HIV-like sequences in gut bacteria of HIV/AIDS patients, which may confirm that bacteria could be involved in this trial (Veazey & Lackner 2005; Zajac et al. 2007; Zajac et al. 2011).

In summary, the molecular weight of 45–55 kDa protein was detected by MAbs anti HIV-1 p17 and p55. Its molecular weight is comparable to gag-encoded Pr55Gag precursor. This precursor like protein of 55–60kDa was detected by MAbs against HIV p24 in Cambodian and Kenyan samples. Surprisingly, no such proteins were found in bacterial extract of Slovak and American AIDS patients by this MAbs. Using MAbs against gp41 the protein of 41 kDa was identified in 30–35% of bacterial extracts from all cohorts of patients. The protein of about 75–85 kDa was detected only in Candida species protein extracts of Cambodian and Kenyan HIV positive children by the MAbs against gp120. In Slovak and American samples, protein reacting with MAbs anti gp120 was not found. These results suggest that there are specific differences between Slovak and American HIV positive patients bacterial proteins on one side and Cambodian and Kenyan on the other.

Detection of HIV-1 antigens in bacteria isolated from respiratory and gastrointestinal tract of HIV patients, it is possible to conclude that bacteria bearing HIV-1 specific proteins are localized not only in the intestinal tract of HIV/AIDS patients but in the other organs. Consistent with earlier observation in SIV models, Veazey reminds us that the battle against HIV-1 should focus on the intestinal mucosa with therapeutic strategies to reduce gut immune activation (Veazey & Lackner 2006). As well as systemic immune activation in SIV-infected rhesus monkeys is associated with breakdown of the intestinal epithelial lining (Estes et al. 2010;
Sandler & Douek 2012). A mechanism contributing to AIDS progression in which intestinal epithelial damage leads to translocation antigens into tissues, which contributes to systemic immune activation, increased lentiviruses replication, progressive immune deficiency and AIDS (Sandler & Douek 2012). Despite the importance of intestinal barrier damage to AIDS progression, the mechanisms responsible for AIDS enteropathy are not understood. Virgin et al. said that virome understood as a subset of the metagenome may be defined to include both viruses that infect eukaryotic cells and phages that infect other members of the microbiome. So the mammalian virome and bacterial microbiome are extremely complex and can contribute to immune status and disease in a range of settings (Virgin et al. 2009). The enteric virome contributes to the progression of SIV infection to AIDS by fostering intestinal epithelial damage and systematic immune activation via release of pathogens as well as bacterial, fungal antigens into host tissues and systematic circulation (Scott et al. 2012).

The transmission of HIV patients’ microorganisms and their role in AIDS pathogenesis is still not explained, but is intensively studied. Bacteria bearing HIV sequences may serve as a reservoir of HIV-1 antigens in the form of “virus-like HIV particles” or others extrachromosomal forms. It is likely that virus-like HIV particles does not consist of viral structures, but of cellular proteins and protein particles belonging to bacteria. Such particles could induce some inflammatory signals and probably again the actions attributed to HIV. We believe that in the process of immunodeficiency play important role combination of gastrointestinal microflora and HIV genetic information.

Thus, our research should be used to generate hypotheses to be tested in larger studies aimed at investigating the role of translocating bacteria in gastrointestinal tract. Our study will be extended to the new patients cohort. The Western blott analysis that we used, will be in next accompanied by more precise techniques such as immunoprecipitation and subsequent sequencing of isolated proteins.

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