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Microflora of Intestinal and Respiratory Tract in AIDS Process

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Abstract

Bacterial DNA isolated from the intestinal tract/rectum as well as bacteria and yeasts from the respiratory tract of HIV patients were positive in hybridization assay for HIV-1 specific sequences. Further, HIV-like sequences homologous for more than 85% with the corresponding HIV-1 sequences in the selected DNA samples were detected by PCR using primers specific for the HIV-1 genes *gag*, *pol* and *env* and then by sequence analysis of PCR products. Bacteria and yeasts isolated from the above mentioned cohorts of patients were analyzed for detection of expressed HIV-1 antigens. By monoclonal antibodies (MAbs) against HIV-1 proteins p55+p17 (Abcam, UK) proteins with the molecular weight of 55 kDa and 35 kDa were detected in the whole cohort of the patients tested. By using MAbs against p24 was detected 55 kDa protein only in Cambodian and Kenyan samples. Using monoclonal antibodies against the HIV-1 specific protein gp41, relevant protein was found in samples of both cohorts of patients. The protein of about 80-85 kDa was detected by MAbs against gp120 only in protein extracts obtained from yeasts *Candida* sp. of Cambodian and Kenyan HIV positive children.

Keywords: AIDS patients; HIV positive children; Bacteria; Yeasts; New hypothesis

Introduction

Despite unquestionable success in diagnostics and therapy of AIDS, there are many unanswered questions. Yet without finding answers to these questions, no significant progress can be expected in the treatment of AIDS. It is predominantly the situation in Africa and Asia that is challenging to fight this disease in a more integrated way and to reject all taboos and dogmas. In 1981 was first described AIDS (Acquired Immunodeficiency Syndrome) caused by human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) as a new disease associated with increasing number of young homosexual men death from opportunistic infections and rare malignancies [1,2]. There are some specialists as Duesberg, Rasnick, Crowe and others formed a strong opposition to the HIV=AIDS dogma [3]. These opponents presented a different dogma, i.e. that HIV does not exist, or if so, it is an innocuous virus unable to induce AIDS. Similarly as the proponents of the HIV=AIDS dogma, they presented many arguments for their statement.

The presented overview clearly indicates that it is necessary to consider other factors, not only HIV, which may be involved in this disease. There is increasing evidence suggesting that the gastrointestinal tract (GIT) is important anatomical reservoir of HIV infection and is the critical target in HIV infection and $CD4^+$ T cell loss [4].

Thus the pathogenesis of HIV infection is presumably centered on these mucosal viral "target" cells [5]. Mattapallil et al. [6] showed that the major focus of destruction of memory $CD4^+$ T cells by the Simian Immunodeficiency Virus (SIV) was in mucosal and peripheral tissues, where most T cells expressing CD4 and CCR5 with a "memory" phenotype reside [7]. Antiretroviral therapy prior to acute viral replication preserves $CD4^+$ T cells in the periphery but not in the rectal mucosa during acute simian immunodeficiency virus infection [8]. The HIV was detected in the bowel epithelium from patients with gastrointestinal manifestations, as well as in bowel crypt cells and the lamina propria [9,10]. Since these cells are in close vicinity to intestinal bacteria, the idea that bacteria may be also involved in AIDS pathogenesis has been raised.

Material and Methods

Patients

Intestinal/Rectal bacteria isolated from swabs were amplified by overnight cultivation in LB medium or on McConkey agar. The swabs were taken from HIV-positive patients from the USA (Veteran Hospital, San Diego), HIV-positive patients from Slovakia (Dept. of Infectious and Geographic Medicine, Derer's Hospital, Bratislava) and from 8 healthy individuals. All selected HIV-positive patients (No. of patient/category) 23/A3, 33/A3, 44/A2, 78/A3, 30/C3, P1/C3, P15/C3 were men who had sex with men and were treated with antiretroviral therapy (ART). Bacteria and yeasts isolated from the respiratory tract of HIV positive patients were also tested. The nose, pharyngeal and rectal swabs were collected by experts from Veteran Hospital and St. Elizabeth University so, unfortunately, we have any information about conditions how swabs were collected as well as disease stage of the patients (CD4 cell count or viral load). The aim of multiple overnight incubations was the pure microbial cultures suitable for taxonomy identification in certified laboratory. The tested samples were plated on LB medium and bacteria from gastrointestinal tract were reinoculated on MacConkey medium. We reaped this procedure many times depending on the purity of the original culture, until we did not obtain pure cultures with characteristic morphology. For isolation we picked

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the prevalent colonies with similar morphology. For contamining human cells to enter the samples we tried to avoid work under sterile condition. The possible presence of contaminating human genetic or cellular material we ruled out using multiple overnight incubations of isolated bacteria in LB medium, where human cells are not able to survive and it leads to its gradual elimination.

DNA isolation and PCR amplification

Bacterial chromosomal DNA was prepared from amplified bacteria by standard protocol [11]. Extrachromosomal bacterial DNA was purified by an alkaline lysis procedure [12]. Polymerase chain reaction specific for HIV sequences was carried out as described previously [13]. These primers were using:

gag gene: 38for ATAATCCACCTATCCCAGTAGGAGAAT

39rev TTTGGTCCTTGTCTTATGTCCAGAATG

G3for TTGGACATAAGACAAGGGCCAAAA

G4rev GTCGTTGCCAAAGAGTGATTTGAG

gag/pol genes: 38for ATAATCCACCTATCCCAGTAGGAGAAT

P3rev AAGGTACTATTT TTAGATGGCATA

env gene: 68for AGCAGCAGGAAGCACTATGG

69rev CCAGACTGTGAGTTGCAACAG

In PCR bacterial DNA isolated from the intestinal tract of 8 healthy subjects was used as control. To avoid false positive results, in every set of reactions one PCR reaction was performed without any DNA. Plasmid pBH10 (GENEBANK accession number M15654) was used as a reference source of HIV DNA and lymphocyte DNA of AIDS patient 30 served as a template for PCR products used as probes in DNA hybridization.

For analysis was used mostly the plasmid DNA for two reasons - it was possible to isolate them cleaner and the second reason is better work in the PCR. Positive PCR results we have achieved with the use of bacterial chromosomal DNA as well. Whereas these forms cannot be separated in such a way that one of them could be excluded we don't know exactly determine in which form HIV sequences are presented in bacteria. We assume that there are presented in the extra chromosomal form but we cannot exactly excluded that they are well integrated into the bacterial chromosome.

DNA sequencing

The sequencing reaction was performed using fluorescent dyes of ABI Prism Big Dye Terminator sequencing kit (Applied Biosystems) and afterwards extension products were purified by Auto-Seq G-50 columns (Amersham Biosciences). PCR products smaller than 130 bp were in some cases cloned into pCR TA cloning vector (Invitrogene). Amplified fragments longer than 130 bp were directly sequenced on the ABI 373 DNA Sequencer and ABI PRISM 310 Genetic Analyzer (Applied Biosystem).

Southern hybridization

Intestinal bacteria from the original cultures made of bacteria harvested from AIDS patients were diluted to the concentration 10^9-10^{12} and plated on media so as to yield single colonies. LB plates containing from 30 to 50 grown colonies were blotted to the Hybond N+ membrane lyses, washed and prehybridized. ^{32}P-labeled probes were obtained by Ready-To-Go DNA Labeling kit (-dCTP) (Amersham Bioscience). The combined PCR probe was prepared as mixture of two of these PCR products determined by primers 38; 39 and 68; 69. Hybridization was performed for 16 hours in standard hybridization buffer at 42°C or in Rapid-hyb buffer (Amersham Bioscience) at 60°C. Subsequently, membranes were washed at the final temperature of 60°C or 65°C. This technique was also used for dot-blotting of purified 0.2-0.3 µg chromosomal and plasmid DNA [14].

Western blotting

Bacteria and yeasts isolated from the respiratory tract (nose, pharyngeal swabs) of HIV positive children and from the intestinal tract of AIDS patients were used for preparation of protein extracts after overnight incubation in LB medium at 37°C. As a negative bacterial control were used bacteria isolated from healthy person. After electrophoresis at 45 mA, the extracted proteins were overnight transferred from acrylamide gel to nitrocellulose membrane (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 p24, p55+p17, gp41 and gp120 (Abcam, UK) in TBS-T buffer with 5% milk were 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. Subsequently the membrane was washed with TBS-T buffer and for visualization ECL solutions were used.

Results

PCR and DNA sequencing

Bacterial DNA of positive isolates was then amplified by the PCR using three sets of primers. PCR products of 635 bp limited by primers G3,G4 from *gag* gene HIV-1 were synthesized on the template of bacterial DNA isolated from Slovak and American AIDS patients. Lines 1-9: Patients P1, P3, P6, P9, P13, P15, Mok2, Mok11, Mok12 shown in figure 1.

PCR products we obtained using commercial primers 68.69 and 38.39 in all tested patients. PCR product of 1465 bp determined on HIV-1 *env* gene was found in more than 50% of analyzed patients [13]. In some cases, we did not obtain PCR products due to the following reasons: poor quality of bacterial DNA because some bacteria are very difficult to lyse and then isolate DNA, DNA sequences could be present

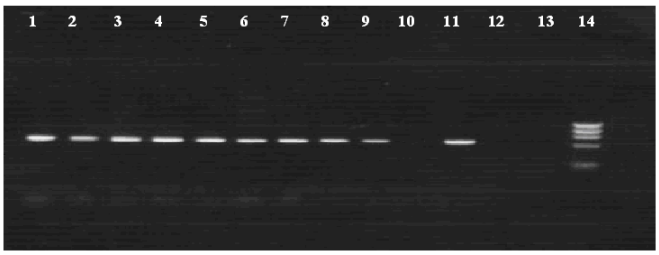

Figure 1: PCR products of 635 bp synthesized on the template of bacterial DNA isolated from Slovak and American AIDS patients. Lines 1-9: Patients P1, P3, P6, P9, P13, P15, Mok2, Mok11, Mok12; line 10: PCR without template DNA; line 11: PCR product synthesized on template pBH10; line 12: DNA of HIV-negative patient; line 13: DNA of negative bacteria control; line 14: marker ø X174xHaeIII. Primers G3, G4 from *gag* gene HIV-1 were used.

in hard to cultivated species like mycoplasmas, the other reason could be no homology with HIV-1 sequences. Globally, we have prepared more than 500 PCR products from tested patient's samples using before mentioned primers.

The PCR products synthesized on the template of bacterial DNA using the above mentioned primers from *gag, pol* and *env* genes were sequenced on the ABI 373 DNA Sequencer. Sequences of patient's intestinal/rectal bacteria revealed at least 85% homology in selected samples with three crucial HIV-1 genes *gag, pol, env*. Sequences of 420 bp PCR product limited by primers 38; P3 of *gag-pol* HIV-1 genes, synthesized on template of bacterial DNA P15-17 isolated from AIDS patient P15 were for 98% identical with HIV-1 isolate HXB2 (Figure 2).

We sequenced the majority of PCR products - 280 PCR products - and sequencing file is given as an example. Other gag sequences were correlated with the HIV-1 gag sequences to 85-95%. PCR products are obtained using primers from all supporting HIV-1 genes - *gag, pol* and *env*. These results could confirm that the bacteria may be present the entire HIV-1 genome.

Southern hybridization

Based on the given idea, bacteria isolated from the gastrointestinal tract and blood of 51 American and Slovak AIDS patients and bacteria and yeasts isolated from the respiratory tract of Cambodian and Kenyan HIV positive children were tested for the presence of HIV-1 like sequences by dot blot hybridization [13-18]. The majority of intestinal microbes were characterized as *Escherichia coli*, *Proteus mirabilis, Citrobacter freundii, Staphylococcus* sp., *Klebsiella pneumoniae* and *Enterobacter aerogenes* using classical microbiological diagnostic tests. Positive hybridization for 80-90% was achieved in the cohort of American and Slovak AIDS patients by using PCR product probes synthesized using before mentioned primers from all main parts of the HIV-1 genome (Figure 3).

These PCR products were prepared on templates of: a) plasmid pBH10 bearing HIV-1 provirus; b) DNA isolated from lymphocytes of an AIDS patient. On the other hand, positive hybridization was detected in bacteria and yeasts of 41, i.e. 31%, of Cambodian and Kenyan HIV positive children [17,18]. The majority of these respiratory tract microbes were characterized as *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans,* and in some cases *E. coli*, *Streptococcus pyogenes*, *Proteus mirabilis* and *Candida tropicalis*.

Protein analysis of patient's bacteria and yeasts

The expression of HIV-1 antigens in bacteria and yeasts of all cohorts of HIV positive patients was analyzed by using specific monoclonal antibodies against HIV-1 antigens p24, p55+p17, gp41 and gp120 (Abcam, UK). The 55 kDa protein was detected in 30-35% protein extracts from bacteria and yeasts of the respiratory tract (nose, pharyngeal swabs) of Cambodian (Km) and Kenyan (Ke) HIV positive children 14 Ke, 3 Km, 32 Ke, 21 Ke, 17 Ke, 23 Km, 28 Km using MAbs against HIV1 p24 (Figure 4).

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gi|1906382|gb|K03455.1|HIVHXB2CG  Human immunodeficiency virus type 1 (HXB2), complete genome;
           HIV1/HTLV-III/LAV reference genome
          Length = 9719

 Score =  761 bits (384), Expect = 0.0
 Identities = 408/416 (98%)
 Strand = Plus / Plus


Query: 5     ttgttgttccaaaatgggaacccagattgtaagattattttaaaagcattgggaccagcg 64
              |||||| ||||||||| |||||||||||||||| ||||||||||||||||||||||||
Sbjct:1750  ttgttggtccaaaatgcgaacccagattgtaagactattttaaaagcattgggaccagcg 1809


Query:65    gtaacattagaagaaatgatgacagcatgtcagggagtaggaggacccggccataaggca 124
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Sbjct:1810 gctacactagaagaaatgatgacagcatgccagggagtaggaggacccggccataaggca 1869


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Query: 365   tacaagggaaggccagggaattttcttcagagcagaccagagccaacagccccacc 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2110 tacaagggaaggccagggaattttcttcagagcagaccagagccaacagccccacc 2165
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Figure 2: The part of sequences (420 bp) of PCR product limited by primers 38; P3 of gag-pol HIV-1 gene synthesized on template of bacterial DNA P15-17 isolated from AIDS patient P15. More than 85% identities with HIV-1 isolate HXB2.

By monoclonal antibodies against HIV-1 p55+p17 diluted 1:1000 proteins of about 55 kDa and 35 kDa were detected in extracts from gastrointestinal tract bacteria of American and Slovak HIV positive patients P14, P3, P15, 725, 723, 79, K1-1 and 116 (Figure 5).

In some samples (P15, 725, 723, K1-1) also 35 kDa protein was found. Western blotting of proteins isolated from intestinal bacteria of Slovak and American AIDS patients P14, Mok11, P15, Mok1, 79, K1-1 and 116 using monoclonal antibodies against HIV-1 gp41 revealed a protein of about 40 kDa (Figure 6).

In samples P14, Mok11, P15, P8, Mok1, K1-1 was also detected protein about 80 kDa. Using the monoclonal antibodies against HIV-1 gp120 diluted 1:1000 respiratory tract microflora was tested. Analysis of samples 23 Ke, 28 Km, 22 Km, 3´Km, 21´Ke, 14´Ke are shown in figure 7.

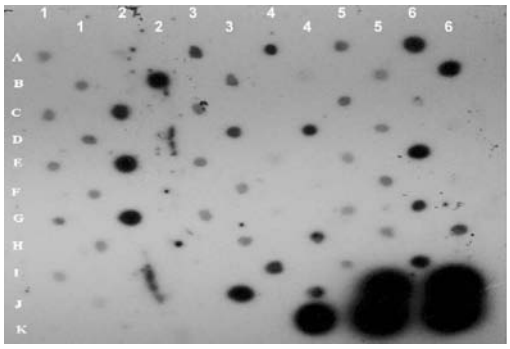

Figure 3: Dot blot hybridization. DNA of lymphocyte/bacterial DNA of HIV positive patients are in lines A-J. Mixture of 38;39 and 68;69 LMP agarose purified PCR products, synthesized on the template of AIDS patient 30's lymphocyte DNA were used as probes. Probes diluted 1:50 are on line K in position 5, 6. Lymphocyte DNA of patient 30 is on line K in position 4.

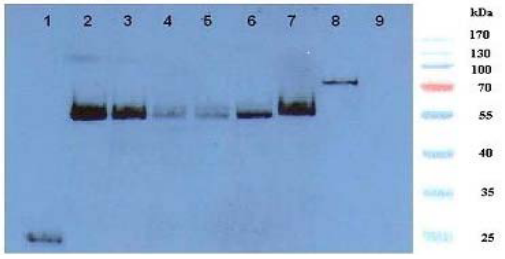

Figure 4: Western blotting of proteins isolated from bacteria and yeasts of the respiratory tract of Cambodian (Km) and Kenyan (Ke) HIV positive children. Used monoclonal antibodies against HIV-1 p24 (1:1000). Line 1: protein p24 diluted 1:1000; line 2: 3´Km; line 3: 32 Ke; line 4: 21´Ke; line 5: 17Ke; line 6: 23´Km; line 7: 28 Km; line 8: serum of AIDS patient diluted 1: 500; line 9: negative bacterial control.

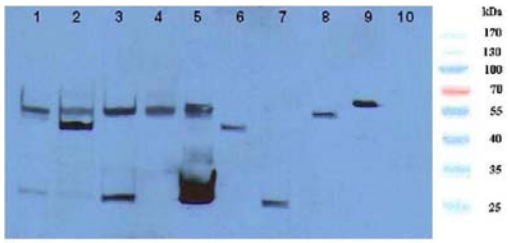

Figure 5: Western blotting of proteins isolated from bacteria of the gastrointestinal tract of American and Slovak HIV positive patients. Used monoclonal antibodies against HIV-1 p55+p17 (1:1000). Line 1: P14; line 2: P3; line 3: P15; line 4: 725; line 5: 723; line 6: 79; line 7: K1-1; line 8: p55 protein diluted 1:1000; line 9: serum of AIDS patient diluted 1:500; line 10: protein extract of healthy person bacteria.

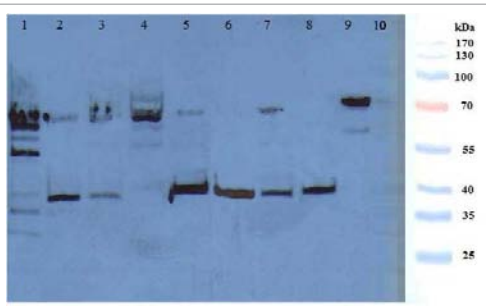

Figure 6: Western blotting of proteins isolated from intestinal bacteria of Slovak and American AIDS patients. Used monoclonal antibodies against HIV-1 gp 41 (1:750). Line 1: P14; line 2: Mok11; line 3: P15; line 4: P8; line 5: Mok1; line 6: 79; line 7: K1-1; line 8: 116; line 9: serum of AIDS patient diluted 1:500; line 10: protein extract of healthy person bacteria.

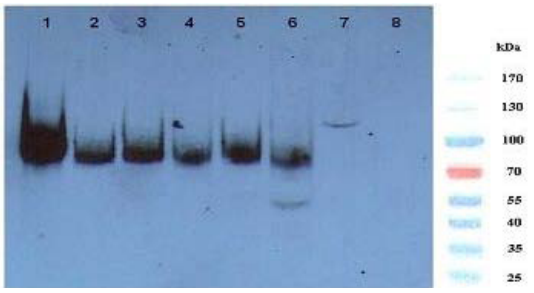

Figure 7: Western blotting of proteins isolated from bacteria and yeasts of the respiratory tract of Cambodian (Km) and Kenyan (Ke) HIV positive children. Used monoclonal antibodies against HIV-1 gp120 (1:1000). Line 1: 23 Ke; line 2: 28 Km; line 3: 22 Km; line 4: 3´Km; line 5: 21´Ke; line 6: 14´Ke; line 7: serum of AIDS patient diluted 1:1000; line 8: protein extract of healthy person bacteria.

In majority of tested samples we can observe overlap between the positive samples in PCR and Western blott analysis. If positive sample in PCR is negative in Western blotting we can predict that HIV-like sequences are contained in bacteria but these are not expressed.

Discussion

In intestinal and respiratory tract microflora of analysed HIV positive persons, HIV-1 like genetic information was confirmed by colony hybridization using probes from all major HIV-1 genes. Sequencing of a large amount of PCR products synthesized on the template of bacterial and yeast DNA confirmed HIV-1 like sequences and revealed their homology for more than 85% with various HIV-1 isolates.

In protein analysis of patient's bacterial extracts, a protein of the molecular weight of about 55 kDa was detected by MAbs anti HIV-1 p55+p17. By MAbs against HIV p24, a protein of 55 kDa was found in Cambodian and Kenyan samples, yet this protein was not found in bacterial extracts of American and Slovak AIDS patients. According to our results, these *gag*-coded proteins seem to exist in bacteria as 55 kDa protein, whose molecular weight is comparable to the *gag*-encoded Pr55Gag precursor. The relevant protein was detected by MAbs against gp41 in samples from all cohorts of patients. By MAbs against gp120 the protein of about 85 kDa was detected only in *Candida* species

protein extracts. These results suggest that there are specific differences between bacterial HIV-like proteins from various geographical areas, due to differences in their evolution.

Generally we do not know enough about our intestinal bacteria, this second kingdom in our body, which represents 10^{14} of living organisms. The role of microbial flora and its influence on our body is still underestimated.

In light of our results, bacteria and yeasts of the human gastrointestinal and respiratory tract are carrying HIV sequences in the form of virus-like genetic information or in another extra chromosomal form. This may be resistant or multiresistant bacteria, especially in patients from USA, Slovak republic and the other industrialized counties, where is resistance to antibiotics widespread. But to confirm this claim, antibiograms of analyzed bacteria are needed. The resistance to ATBs is largely coded by plasmids in which also genes for adhesion to intestinal cells may be localized [19-21]. After adhesion, bacteria equipped in this manner are able to penetrate via colonic epithelial cells into the lymphatic system and consequently into the bloodstream and to internalize human cells with an affinity to various organs [22]. In same specific cases such as gut immunity fail and violation of the homeostatic balance between gastrointestinal bacteria, the microorganisms are able to overcome the intestinal barrier and pass into the systemic circulation. This situation may be associated with inefficient reconstruction of $CD4^+$ T cells [23].

The explanation of the presence of HIV sequences in patient's commensal microflora is as follows: 1) intestinal bacteria are infected by HIV released from degraded human cells, in particular by macrophages and lymphocytes; 2) intestinal bacteria of AIDS patients are a natural host of HIV sequences in some specific extra chromosomal forms. The form and presence of HIV-like sequences are questionable, as mentioned before it is very likely that bacteria not bearing the complete viral form (the LTR sequences were not detected). Analyzed HIV sequences are not existentially important for bacteria so some changes and mutations in these sequences are tolerated by the bacteria. We assume that there are presented in the extra chromosomal form but we cannot exactly excluded that they are well integrated into the bacterial chromosome. It is an open question and more research is needed.

Based on our results we can agree with the statement, that HIV has hypothetically been an integral part of the human population. Many epidemics in Europe, Asia, North America, North Africa were associated with new patterns of communication and transportation between separately populated areas and claimed many lives. The result was the reduction of pathogenic intestinal bacteria - some with HIV genetic information. Due to application of antibiotics, pharmaceuticals, drugs and lifestyle changes became pathogenic bacteria mostly multiresistant - including those containing HIV sequences. Subsequently they may overcome the intestinal barriers, penetrate into organisms and begin to attack continuously the immune system [24]. The $CD4^+$ T cell count was decreasing and when it was below 200/µl, the immune system became dysfunctional and the syndrome of immunodeficiency could start [25].

In the presented hypothesis, we can find the new opinion and new answers to the questions in HIV research as: origin of HIV, large scale HIV positivity in Africa, connection of AIDS with TBC in Africa, presence of HIV reservoirs after antiretroviral therapy, rarity of detecting complete viral particles in the material from AIDS patients but detection of HIV sequences and HIV proteins therein, and high AZT toxicity [24].

The confirmation of our hypothesis may open new perspectives in diagnostics and AIDS therapy, aimed at recovering the host-microorganism relationships and based on elimination of bacteria and yeasts bearing HIV genetic information, an approach that should not be very burdensome for patients.

Our results are quite preliminary and they can be considered only as hypotheses generating. Also that our study is a pilot project and still more research is warranted. In the future it should be very useful to rule out human cell contamination using PCR to a human housekeeping gene and to find missing link between the DNA and protein experiments we would like to analyze presence of HIV RNA in patient´s bacteria through RT-PCR.

Acknowledgement

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