Detection of HIV-1 sequences in intestinal bacteria of HIV/AIDS patients

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Abstract

OBJECTIVES: Bacterial DNA isolated from the intestinal tract of 11 American and 30 Slovak HIV/AIDS patients were analyzed by colony and dot blot hybridization assay for HIV-1 specific sequences. Secondly, PCR using primers specific for the HIV-1 *gag*, *pol* and *env* genes for detection of HIV-1 sequences in these DNA were performed.
RESULTS: Intestinal bacteria DNA of HIV/AIDS patients hybridized in colony and dot blot hybridization assay for HIV-1 specific sequences. PCR products synthesized on specific primers of HIV-1 *gag* (115 bp), *env* (142 bp), *pol-env* (1484) genes were found to be for more than 90% homologous to the corresponding sequence in HIV-1.
CONCLUSIONS: Intestinal bacteria of HIV/AIDS patients are bearing sequences for more than 90% identical with those of HIV-1.

INTRODUCTION

Plasma HIV RNA is dramatically reduced in HIV/AIDS patients treated with highly active anti-retroviral therapy (HAART), but residual viral replication is detected after the suppression of plasma viremia [1,2]. It has been also expressly proven that various forms of HIV reservoir persist in practically all patients receiving HAART [3,4]. These reservoirs were detected in macrophages and the other cell of the blood system, in which very effective HAART is not able to eliminate the virus. Recent studies have indicated that range of viral reservoirs in the human body is very probably much broader.

Over the last period of time AIDS research has been focused on the gut, and other mucosal tissue, with the major site of HIV infection and CD4+ T cell loss [5]. Matapallil et al. [6] and Li et al. [7] showed that simian immunodeficiency virus (SIV) rapidly and selectively infected and destroyed the memory CD4+ T cells. The major focus of destruction of these cells and the "functioning" CD4+ CCR5+ T cells is in mucosal cells, where most T cells expressing CD4 and CCR5 with a "memory" phenotype reside. The loss of CD4+ T cells in the intestine occurred simultaneously with productive infection of large numbers of mononuclear cells at this site [8]. These recent findings support the hypothesis that the mucosal immune system in general and the intestine immune system in particular, is the major site of viral replication, persistence and CD4+ T cell loss in HIV-infected

subjects [5,9]. The pathogenesis of HIV infection is centered on these mucosal viral "target" cells. HIV-1 was also detected in bowel crypt cells and the lamina propria [10]. Since these cells are in close proximity to intestinal bacteria, it is possible that bacteria also may be involved in the pathogenesis.

In order to study the pathogenesis of HIV infection, we investigated whether the bowel bacteria might be the possible vector of HIV transmission. The idea grew of our extensive laboratory and field experience with an other retrovirus, the bovine leukemia virus [11,12]. This experience, coupled with Temin's hypothesis suggesting that retroviruses generally originated from mobile genetic elements [13,14], and with Montagnier's and Lo's contention that mycoplasma could be an important "co-factor" in the progression of AIDS, stimulated our interest in investigating bacteria [15,16].

To our knowledge, there are no literature data concerning detection of retroviral sequences in bacteria. Here we present the second experimental evidence to show that bacteria isolated from the colon of AIDS patients carry HIV sequences [17].

MATERIAL AND METHODS

Patients

Intestinal bacteria were isolated from patients' swabs by overnight cultivation in LB medium or on McConkey agar of 11 HIV-positive patients from the USA (Veteran Hospital, San Diego) and 30 HIV-positive patients from Slovakia (Dept. of Infectious and Geographic Medicine, Derer's Hospital, Bratislava) and from 10 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 having sex with men treated with antiretroviral therapy (ART).

DNA isolation and PCR amplification

Bacterial chromosomal DNA was prepared from amplified bacteria by standard protocol [18]. Extra chromosomal bacterial DNA was purified by an alkaline lysis procedure [19]. Polymerase chain reaction specific for HIV sequences was carried out using these primers:

In PCR bacterial DNA isolated from the intestinal tract of 10 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.

Southern hybridization

For colony blotting, bacterial suspension was diluted to the concentration of 10^{-9} and 10^{-10} on LB plates and 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). As probes all three aforementioned PCR products were used. The combined PCR probe was prepared as mixture one or the mixture of two or all three of these PCR products. 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 chromosomal, plasmid and phage DNA [20].

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 150bp were in some cases cloned into pCR TA cloning vector (Invitrogene). Amplified fragments longer than 150bp were directly sequenced on the ABI 373 DNA Sequencer and ABI PRISM 310 Genetic Analyzer (Applied Biosystem).

RESULTS

Intestinal bacteria from the original cultures made of bacteria harvested from AIDS patients were diluted to the concentration 10^9–10^{12} and plated at concentra-
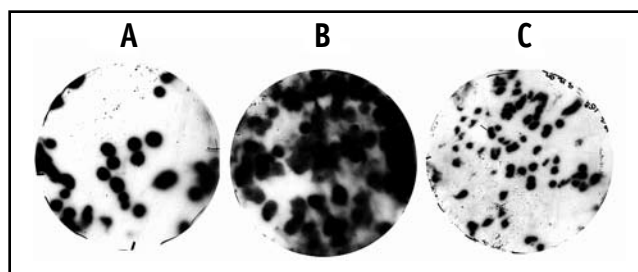
Gene	Primer name	Primer sequences	Product size (bp)	Position
gag	38for	ATAATCCACCTATCCCAGTAGGAGAAT	115	1047
	39rev	TTTGGTCCTTGTCTTATGTCCAGAATG		1162
pol env	Pfor	CATTTGGAAAGGACCAGCAAAACTACT	1484	4430
	Erev	TCATATGCTTTAGCATCTGATGCACAA		5914
env	68for	AGCAGCAGGAAGCACTATGG	142	7281
	69rev	CCAGACTGTGAGTTGCAACAG		7423

Figure 1. Colony hybridization. Bacteria isolated from patients P1 and P15 were diluted to : A) P15, dilution 10^{-10}; B) P15, dilution 10^{-9}; C) P1, dilution 10^{-10}, transferred to Hybond membrane and hybridized with probe of PCR fragments determined by primers 68;69 synthesized on the template pBH10.
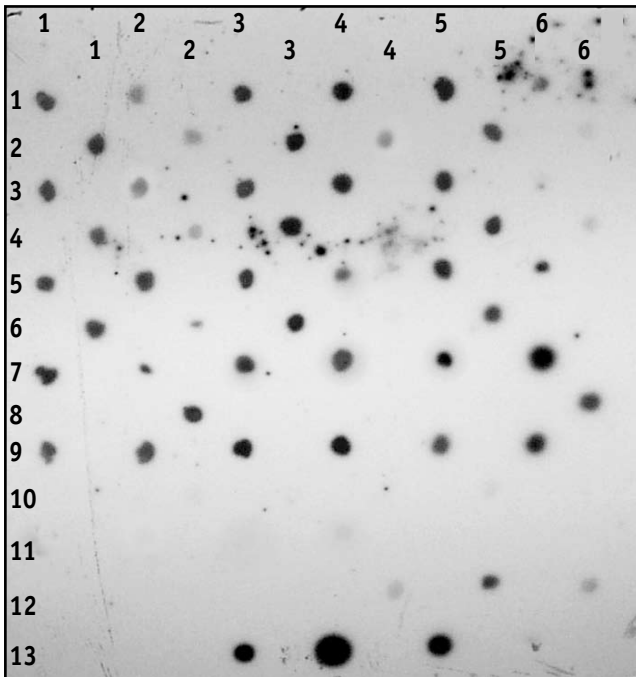
Figure 2. Dot blot hybridization. DNA of lymphocyte/bacterial DNA (0.2µg) of HIV positive patients are in lines 1-9 and of healthy subjects are on lines 10-12. The selected patients 23, 33, 44, 78, 30, P1 and P15 are localized as follows: line 2 – 1,2; line 1 – 5,6; line 4 – 3,4; line 6 – 1,2; line 7 – 1,2; line 7 –3,4 and line 7 – 5,6 ;. The PCR products 38;39, mixture 38;39 and 68;69, 68;69 used as probes diluted 1:100 on line 13 in position 3,4,5.Probe: LMP agarose purified the PCR products 38;39 and 68;69 synthesized on the template of patient 30's lymphocyte DNA.

Figure 3. The longest PCR products of 1484 bp determined by primers P10;E1 (HIV-1 env gene), synthesized on the template of 11 bacterial chromosomal DNA of AIDS patients are shown. Lines 1 - 11: M11; P1; P3; P6; P15; P9; M1; M2; M15; M12; M22; line12 - PCR without DNA; line 13 - negative control; line 15 - pBH10; line 16 – marker λDNA×HindIII; line 17 - marker øX174×HaeIII.

tions yielding single colonies. LB plates containing from 30 to 50 grown colonies were then tested for HIV-like sequences by colony hybridization. All colonies formed by bacteria from AIDS patients P15 and P1 hybridized positively with the HIV-1 specific probes. PCR products were synthesized on the template of plasmid pBH10 or lymphocyte DNA of AIDS patient 30 (Figure 1). The strongest hybridization signal was obtained with probes derived from PCR product 68; 69 and P; E. Contamination with human cell debris appears unlikely because of the high factor of dilution of the bacterial culture. Subsequent experiments were performed with purified subclones positive in hybridization. Bacterial colonies from healthy subjects did not react to the HIV-1 probe. Dot blot hybridization of human chromosomal and bacterial DNA (in the amount of 0.2 µg/sample) of 27 AIDS patients, using the same probes as used in the colony hybridization assay, confirmed the above results (Figure 2, lines 1–9). DNAs of healthy subjects are on lines 10, 11, 12 and PCR products used as probes diluted 1:100 on line 13. The lymphocytes/bacterial DNA of patients 23, 33, 44, 78, 30, P1, P15 are localized as follows: line 2/1,2; line 1/5,6; line 4/3,4; line 7/1,2; patients 96, 83, 62 are localized on line 6/1,2; line 8/3,4; line 4/5,6). PCR product of 1484 bp was produced from the genomic DNA of 11 different bacteria isolated from AIDS patients using the
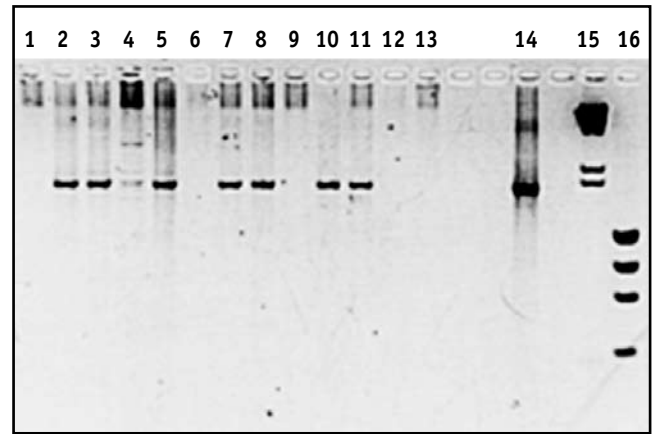
primers P1; E1 of HIV-1 *env* gene (Figure 3). No PCR products were detected in PCR using bacterial DNA of patients M11 (line 1), P9 (line 6) and M15 (line 9).

The 142 bp amplicon limited by primers 68; 69 produced using template DNA from both bacteria and lymphocytes of patients 23, 33, 44, 78, 30, P1 and P15 was sequenced (Figure 4). Amplicons from these different sources were >95% identical. The sequences were remarkably similar, with some differences occurring not between isolates, but between the isolates as a group and the reference sequence in pBH10.

DISCUSSION

Despite great progress in the diagnosis and treatment of AIDS, there are still many unanswered questions about how HIV causes the multiple pathogenic manifestations of full-blown AIDS. Based on our previous experiments with bovine leukemia virus (BLV) resulting in detection of BLV sequences in intestinal bacteria of virus infected animals, we performed this project to study the role of intestinal bacteria in AIDS and to determine if they serve as a possible vector of transmission or reservoir of the virus. In our experiments, HIV sequences in bacteria of HIV-positive patients were confirmed by colony hybridization using three probes from all major HIV-1 genes. Bacteria bearing HIV-1 sequences were mostly (40%) specified as *E. coli* (negative in serotypization), *Proteus mirabilis* (15%), *Citrobacter freundii* (10%), *Staphylococcus sp.*(6%*)* and *Enterobacter aerogenes* (3%). This fact indicates that HIV sequences are spread in a broad variety of commensal bacteria, but their host range is very limited, transmission is possible only between numbers of one species [17]. At least 70% to 100% of bacterial colonies derived from bacteria taken from all HIV-positive patients tested were found positive for any
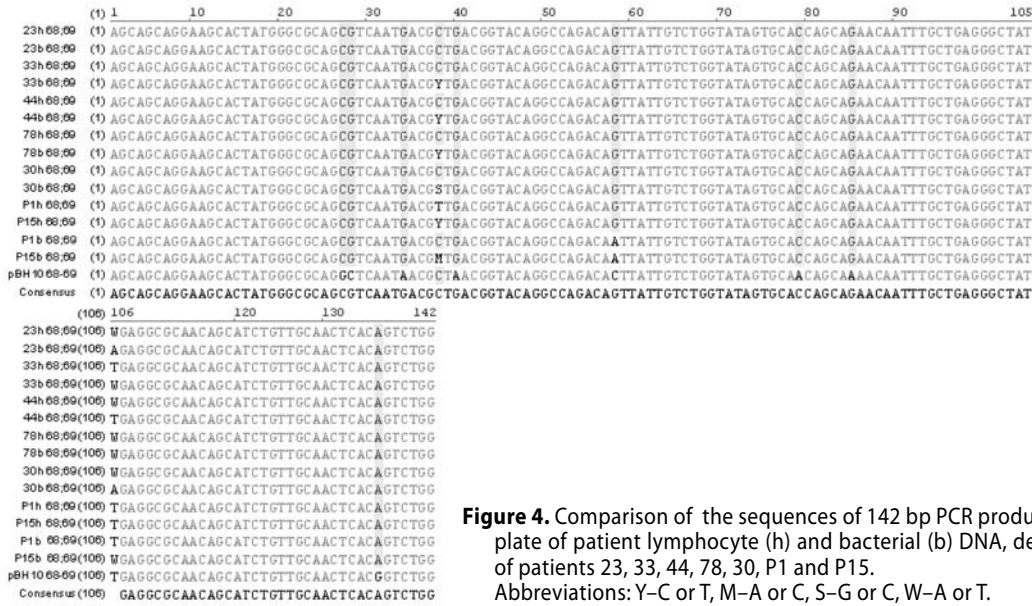
Figure 4. Comparison of the sequences of 142 bp PCR products synthesized on the template of patient lymphocyte (h) and bacterial (b) DNA, determined by primers 68;69, of patients 23, 33, 44, 78, 30, P1 and P15.
Abbreviations: Y–C or T, M–A or C, S–G or C, W–A or T.

previously tested HIV fragments. All colonies analyzed from patients P1 and P15 hybridized positively very probably as a consequence of life style (homosexuality; drugs) and antiretroviral therapy. In this way several multiresistant bacterial clones, mostly bearing HIV sequences, are amplified in the intestinal tract of the patient. This presumption was indirectly confirmed by isolation of the prevalent uniform presence of one form of cca 50kb plasmid from the mixture of patient P15'colonic bacteria (data not presented).

In dot blot hybridization, the lymphocyte/bacterial DNA of HIV/AIDS patients were in most cases positive (Figure 2). Of 27 patient samples 23 lymphocyte DNA were positive (85%) and 20 bacterial (74%). Yet positivity of all the 41 patients tested was 92% and 84%. The results were partially affected by failure to isolate pure DNA from some patient's bacteria. The presented results are in compliance with the probes used, the intensity of the hybridization signal was in correlation with the origin the probes used, the signal of human samples was more intensive with the applied human probes and vice versa for bacterial probes (data not shown). This findings suggests some differences in sequences between two sources of material. The hybridization signal of samples from patients 96, 83, 62 were not intensive either in lymphocyte or bacterial DNA. These patients were classified as A1 stage of their CDC clinical category with CD4+ around 400/µl. The strongest signal was obtained with probes derived from PCR products 68; 69 and P; E synthesized on the pBH10 template or human chromosomal DNA of patient 30. These results are in correlation with the common usage of 68; 69 primers in AIDS diagnostics. The hybridization signal was not detected in samples of control subjects with the exception of samples from one subject (line 12/5,6).

Subsequently, the DNA of positive isolates was amplified by the PCR reaction using all three sets of primers.

The sequences – in the range of 112 to 412bp – synthesized on DNA templates of intestinal bacteria of patients revealed at least 90% homology with three crucial HIV-1 genes (*gag, pol, env*). The sequences were remarkably similar, with some differences occurring not between isolates but between the isolates as a group and the reference sequence in pBH10. These minor differences in the isolates strongly suggest that the DNA has a common recent ancestry in HIV-1. Important is the finding of rather marked differences between bacterial sequences and sequences of pBH10 in all fragments tested. On the basis of these results, the possibility of contamination of our samples by pBH10 plasmid DNA is practically excluded.

There is increasing evidence that the mucosa-associated bacteria may play important roles in the pathogenesis of inflammatory bowel disease, ulcerative colitis, Crohn's disease and potentially even colon cancer [21,22]. Invasive strains of *E. coli* that undergo lyses upon entry into mammalian cells can act as a stable DNA delivery system to their hosts [21]. They are the system "hit and run away" and mainly their extrachromosomal content remains in the host cell even when the bacterial carriers are not detectable. The horizontal gene transfer from bacteria to yeast, to plant and mammalian cells has been reported by other investigators [23,24,25,26]. According to our preliminary results, we may conclude that the ability of invasive bacteria containing HIV sequences in the form of "virus-like particles" to enter into HL-60 cells or human lymphocytes represents an ideal system for the primae impressionis of horizontal transfer of genes between eukaryotic and prokaryotic cells. By this way "virus-like particles" or other particles are introduced as on a tray into cells of the lymphoproliferative system and consequently their genetic informations may interact or be integrated into the host DNA [27].

Our primary detection of HIV sequences in a form as virus or "virus-like particles" in bacteria of HIV/AIDS

patients may be explained hypothetically by the following: /1/ some specific intestinal bacteria attend as a natural host of viruses or "virus-like particles" containing HIV sequences. They serve as genuine vectors for transmission of HIV genetic information; /2/ intestinal bacteria were infected by viruses or "virus-like particles" previously produced by human lymphoproliferative cells or by liberation of these particles from cells after their destruction.

Neither of these two recources is experimentally confirmed at present. However, confirmation one of them may have signficant importance for further AIDS reseach and the development of therapy of this disease.

ACKNOWLEDGEMENT

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