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MOLECULAR BIOLOGY APPROACH TO THE SEARCH FOR NOVEL HIV PROTEASES AND ANTIRETROVIRAL DRUGS.

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ABSTRACT

Currently, there is no definitive chemotherapy for HIV/AIDS treatment. In addition, the high degree of variability of amino acid sequence of HIV membrane envelope glycoprotein (gp120) constitutes an impediment in the preparation of an effective vaccine against the virus. This difficulty in treatment exacerbates the spread of this virus, whose mode of transmission is though the blood and or body fluids; thus suggesting that viral transmission may be possible through HIV infected malarial patients.
Surprisingly, the virus has not been found to be transmitted along side malaria thus exonerating mosquito as vectors of the virus. The presence of HIV proteases and or antiretroviral molecules in the mosquito gastrointestinal system (GIT) is then deduced.
Sequencing the entire mosquito genome, thus identifying its gene maps, can easily identify these proteases and or antiretroviral genes. The application of recombinant DNA and polymerase chain reaction (PCR) techniques to the mapped genes would elaborate their protein products. The vulnerability of the HIV to the protein products could then be developed into potential drugs, which could be tested in the animal models of HIV infection before subjection to clinical trials. Optimistically, the magic HIV therapeutics may be hidden in such insects and may require the application of molecular biology techniques to unravel.

KEYWORDS: Antiretroviral drugs, malaria, proteases, restriction enzymes, polymerase.

INTRODUCTION

Various therapeutic agents for the treatment of HIV/AIDS have appeared in pharmacies since the discovery of the virus about two decades ago. Drug toxicities, cost and other requirements for their administration place a limitation on their universal acceptance (Anonymous 2001). These setbacks present a challenge to all manner of researchers . Despite the massive investments put into these search for definitive HIV treatment, no single therapeutic agent has fulfilled the objectives in the treatment strategy (Miller, 1989). Nevertheless, combination therapy with some of these drugs though effective, have not lost their toxicities. Despite the successes recorded in the effective control of these scourge by vaccines, definitive therapy still remain only conjectures. As the search for definitive HIV/AIDS therapy continues, it becomes imperative to approach it from the viral survival in nonpermisive vectors.

Blood Transmission and Insect Immunity HIV transmission is mostly reported through blood and other body fluids. In the tropics where malaria ranks as one of the most serious and common infections, it could be inferred that the incidence of HIV/AIDS would be parallel with malaria. This is sequel to the implication of mosquito as a vector for blood parasites, like *plasmodium falciparium* and some viruses (Muller et al 1993, Muller *et al* 1995) which probably include HIV. Surprisingly, there is no evidence implicating the mosquito as a vector for HIV (Corbett *et al* 2002) despite the exposure of a large number of people living with HIV/AIDS to mosquitoes in the tropics. This is supported by the fact that a greater percentage of the tropical population is spared the scourge despite the widespread malaria endemicity.

The significance of the nontransmittance of HIV by mosquito in contrast to its vector capacity for *P. falciparium* may suggest the non-viability of the virus in the host. This may be supported by the loss of viability by some parasites in the mosquitoes (Bowman et al 1986). This has been transmitted by mosquitoes other than Anopheles gambiae (Shahabuddin et al 1995). Genetic studies have subsequently established a linkage between certain loci in the mosquito genome and susceptibility of plasmodium species (Shahabuddin et al 1995).

This phenomenon of insect immunity has been enunciated by the response shown for example to bacteria proteins by some insects (Harris and Watkin 1965). These insects were associated with the secretion of some antibacterial immune proteins called cecropin and attacins, which are

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thought to form channels in the membrane of this bacterial, thus leading to their lysis. The non-transmittance of HIV by a gambiae may be similar to the phenomenon of insect immunity resulting in viral lysis.

DEDUCTION

There is a possibility that the anti-HIV immune protein in A. gambiae may neither be specifically cecropin nor attacins, nor even the frog skin compound maiganin, which independently kill malarial parasites within oocysts in the mosqu o midgut if injected into the mosquito during infections (Miller, 1989). Other genes responsible for the expression of mosquito HIV immune proteins might be implicated in the virus lysis. Taking the biology of HIV into consideration, it is likely that these lytic proteins may function as either proteases or antiretroviruses. The identification and cloning of the genes encoding the mosquito proteases/retroviruses in its gut is therefore important for elucidation of the mosquito–virus interaction and may proceed thus.

METHODS

Outline of procedure for identification of HIV lytic proteins HIV lytic proteins can be identified from the A. gambiae by creating its gene libra., A gene library or gene bank being a collection of cloned DNA fragments representing the entire genetic material of an organism (Xrefer, 2002). This facilitates screening and isolation of any particular gene. Gene libraries are created by fractionating the genomic DNA into fragments using restriction enzymes and /or physical methods. These fragments are cloned and the host cells containing the recombinant fragments are centrifuged and frozen. Alternatively, the phage vectors can be identified using specific gene probes with the southern blotting technique or, through their protein products, using Western-blotting (Xrefer. 2002). The gene libraries of A gambiae can therefore be created as follows:

Isolation of Total Nucleic Acids

The DNA of mosquito can be obtained from about 200 female mosquitoes killed by freezing them in liquid nitrogen in accordance with the modified description of Muller et al (1993). Biochemistry hotmail internet 2002. The pooled mosquitoes may be ground in 10 ml of 50mM of 1mg/ml proteinase k, and then incubated at 50oC for 1hr

Insoluble material may be removed by centrifugation, leaving the required genetic material. DNA (and RNA) along with the proteins. Phenol is then added to insoluble solution that may be obtained. When mixed, this creates an emulsion of all the tube contents. Centrifugation of this will lead to the formation of two layers, the genetic material being in the upper phase, the phenol in the lower and the insoluble protein on the interface between the two layers. The upper layer contains RNA and DNA, which can be separated by "buoyant density centrifugation". This process involves putting the mixture in a centrifuge tube with caesium chloride at high concentration. Once a gradient is established by centrifugation at 40,000RPM for 40 hr, the concentration in the center of the tube will enable the DNA to concentrate there by equilibrium since it has a slightly lower density than DNA.

The denser RNA moves towards the sides of the tube or the bottom depending on the tube orientation. Addition of a large concentration of ethanol and centrifugation can then precipitate the separated DNA. The remaining ethanol is then removed by drying and the pellet redissolved in a buffer

Creation of Gene Libraries

Restriction Digestion of Mosquito Genomic DNA Restriction endonucleases or Restriction enzymes are then used in cleaving the DNA. The enzymes that originate from different forms of bacteria have the ability to cut DNA into defined fragments that have been created may be of an unknown length and the total number of fragments may be unknown. To resolve the resultant number and size of fragments the technique known as "agarose gel electrophoresis" is employed. Because of these differences in sizes of the fragments, they separate out as different bands on the gel in accordance with the voltage set up across the gel. The positions of these bands are genetic material which fluoresce under ultra-violet light since the gels were ab initio mixed with ethidium bromide, which is a fluorescent material. These band positions can be measured, recorded and used to determine the size of each fragment.

Cloning of the DNA

The cohesive ends of the digested DNA fragments could join a vector (Bacteriophage/plasmid) DNA that has also been digested with the same enzyme or enzymes that produce the samesticky ends. In this process, the recombinant DNA is legated into a suitable vector and can then be introduced into a system that will amplify it. such as a bacterium. Under normal conditions bacteria will only take up this free plasmids when it is made "competent" through its treatment with a solution of calcium chloride and then "heat shocking" it. In this process of "transformation", the plasmid and bacterial

solutions are mixed and some of the bacteria will take up the plasmids. Less than one percent usually does so, whereas each bacterium should take up a maximum of one plasmid and should only contain one recombinant molecule.

By careful choice of a plasmid containing a gene that codes for resistance to an antibiotic such as the Amp gene (which gives resistance to ampicillin), it can be determined which bacteria have taken up a plasmid. The bacteria are plated out at low concentration of an agar jelly containing the said antibiotic, and if one of them had resistance before the treatment, only the ones containing the plasmid and resistance gene will contain a single type of DNA molecule. This process is known, as DNA cloning as each cell in a colony is identical to the rest. Inside the host cell, the recombinant DNA undergoes replication. Thus, a bacterial host will give rise to a colony of cells containing the cloned target gene.

DNA Synthesis

The RNA extracted from the mosquito can be reverse transcribed for amplification into large amounts of single-stranded DNA. In a typical reaction reported by Miller et al (1995)4, total RNA of 3 mosquitoes (prepared from a pool of 20 mosquitoes) was reverse transcribed in a volume of 40l and precipitated by adding 40l of 5M ammonium acetate and 80l ethanol. The pellets were washed with 80% ethanol, air dried, and dissolved in 100l 1mM EDTA, 10mM Tris-HCL, ph 8.0.

PCR Reactions

Polymerase chain reaction (PCR) was then performed; PCR being a technique, which is used to replicate a fragment of DNA to produce many copies of a particular DNA sequence. This technique therefore, enables the amplification of minute traces of genetic material. It starts with the separation of the two strands of the DNA by heating and short sequences of a single strand (primers) are added, together with a supply of free nucleotides and DNA ploymerase obtained form a bacterium that can withstand extreme heat. In a series of heating and cooling cycles, the DNA sequence doubles with each cycle and is thus rapidly amplified. Typical reaction (Miller et al 1995) were performed on a lab-line programmable Thermal Blok machine, in a volume of 100 1 of 10mm Tris-HCl, PH 8.3, 50mm KCl, 2mMMgcl2 200M each deoxynucleotide. 50p moles of Ty-act and Ty-s primers, 1 unit of Tth-polymerase, and 1% of first strand cDNA template, overlaid with 801 mineral oil. PCR was performed in three steps of 2 minutes at 94°C, 1 minute at 65°C, and 2minutes at 74°C (25 cycles).

The amplified sequences can be cloned in the plasmid vector and expressed in E. coli as explained next.

Expression of Recombinant DNA in E.coli

On expression, recombinant proteins could be produced in the E.coli strain M15 carrying the lac repressor overproducing plasmid. Induction could be performed in LB medium by adding IPTG (isopropylthiogalactoside) to a final concentration of 0,2mM at an OD600 of 0.6, and further agitation for 4hr at 37°C. The recombinant proteases are known to have a high affinity for nickel ions (Hochuli et at 1988) and this allows the rapid purification in a single-step procedure, by nickel-chelate affinity chromatography (Stuber et at 1990). In brief, 1 litre of an IPTC-induced culture of E.coli M15 plasmid cells transformed with desired protein constructs was centrifuged, the bacteria dissolved in 100ml 6M guanidine hydrochloride, 100mM Na_2HP0_4, Ph 8 and stirred for 3hours at room temperature. The suspension was centrifuged at 10,000g and the supernatant was directly loaded on a 5-ml nickel column. After washing with 30ml 6M guanidine hydrochloride, 100mM Na2HP04, was equilibrated with 20ml 8M urea, 100mM Na_2HP0_4, 10mM Tris-HCl, PH 8. The recombinant proteins were eluted with a Ph step gradient (until ph 4) in 8M urea, 100mM Na2HP04, 10mM Tris-HCl.

Fractions containing the purified proteins were pooled and dialyzed against PBS. The precipitate formed during PBS-dialysis was removed by centrifugation and the soluble protein was stored in aliquots at-20°C.

Antibody Production

After purification on nickel-chelate chromatography followed by dialysis against PBS. recombinant the recombinant DNA proteins could be used to immunize Balb/c mice for example Typically, the immunization could be intraperitoneally three times with 50g of purified protein in complete (first immunization) and incomplete Freund's adjuvant. Three weeks after the last immunization, 1ml of blood could be collected by heart puncture. The antibody titre of the obtained sera could then be tested on recombinant DNA proteins spotted on nitrocellulose.

Immunoblotting

In the Southern blotting technique, the separated DNA fragments in agarose gel electrophoresis are denatured to single strands. These are

transferred, or "blotted", onto a nitrocellulose filter where they are immobilized in their relative positions. Specific gene probes labeled with a radioisotope are then added. The probe has a base sequence complementary to the target sequence, which it attaches to it by base pairing. The probes whose length may reach up to about 100 nucleotides can be constructed in the laboratory. These hybridize with any complementary fragments on the filter, which are subsequently revealed by autoradiography. Thus they can be used to identify particular DNA fragments and in conjunction with restriction mapping to map certain sequences.

The Western blotting (protein blotting) technique is an immunoassay for the determination of very small amounts of particular protein in tissue samples or cells. The expressed recombinant proteins obtained above is subjected to electrophoresis on SDS-Polyacrymide gel to separate constituent protein. The resultant protein bands are then "blotted" onto a polymer sheet. A radiolabelled antibody specific for the target protein is added. This binds to the protein, which can then be detected by autotadiography. A variation of this technique is used to screen bacterial colonies containing DNA clones in order to isolate those colonies expressing a particular protein. The amino acid sequence of these proteins could be determined by the Edman degradation technique, which involves removal of their terminal amino-acid resides sequentially and identified chromatogragraphically. Each step is automated and the entire process is now automated by a machine the sequenator. The gene coding for this proteins can be worked out using the genetic code.

Gene Sequencing (DNA Sequencing)

Two techniques are also of value in the elucidation of the nucleotide sequence of the gene of interest. In the Maxam-Gilbert method, each of these DNA fragments that has been cleaved with a restriction enzyme is labeled with 32P-phosphate at one end.

The fragments are subjected to four different sets of reactions, each set specifically cleaving DNA at a particular base or bases. The cleaved fragments are separated by electrophoresis according to their chain length and identified by autoradiography. The base (nucleotide) sequence is then deduced from the position of bands in each of the four lanes in the gel. The Sanger method also called the dideoxy method, involves synthesizing a new DNA strand using as template single-stranded DNA from the gene being sequenced. Synthesis of the new strand can be stopped at any of four bases by adding the corresponding dideoxy (dd) derivative of the deoxyribonucleoside phospates. For example, by adding ddGTP it terminates at an adenosine and so on. The fragments, which comprise radiolabelled nucleotides, are finally subjected to electrophoresis and autoradiography. By using fluorescent dyes as labels instead of radioisotopes, the Sanger method has been fully automated. After separation of the fragments, the products of all four reactions are detected by fluorescence spectroscopy and analyzed by computer, which gives a printout of the base sequence.

In vitro Assay for the Protease Activity

A possible in vitro assay could involve a test of proteolytic activity of the variously obtained recombinant DNA for its substrate (HIV virus) in serum. A modification of an in vitro assay procedure (Savarino et al 2001) requires infection of peripheral blood mononuclear cells with HIV-1/HIV-2 strains. The cells could then be washed three times with phosphate buffered saline, and suspended at 5 x 105/ml in fresh culture medium in the presence or absence of the suspected recombinant proteases (0-12.5M). In parallel, mock-infected cells could be incubated with 0-250M potential proteases in other to determine toxicity HIV-1P24 could be measured by ELISA in cell culture supernatants at 7 days post infection. HIV-2 replication could also be estimated using SIV p27 ELISA kits. At different intervals, post-infection, cell viability could be measured by trypan blue exclusion and by the methyl-tetrazolium (MTT) method (Savarino et al 1999).

The effects of potential antiretroviral proteins on reverse transcription and integration could then be tested using a replication-defective reporter HIV-1 construct (pRRLsin.hpGK.GFP). The effects of the drug on the viral envelope could be assessed by syncytium assays and immunoprecipitation, using antibodies to different epitopes of gp120. In this assay, H9 IIIB cells could be cultivated in the presence or absence of the recombinant proteins for 48hours, washed three times and then resuspended at 5 x 105/ml. Then, 5 x 104 recombinant proteins-treated or untreated H9 IIIB cells (100 1) and 5x105 MT-2cells (1ml) could be cultivated at 37°C for 6 hours. The culture samples could then be examined under a microscope after 6 hours of incubation.

In vivo Assay for the Protease Activity

In this assay, animal models of HIV infection could be administered with the recombinant

proteins and their blood sera monitored for HIV antibodies by ELISA.

CONCLUSION

From the foregoing experiments, a variety of methods has been highlighted for the identification of the DNA fragments of the mosquito genome, and the Gene library constructed from there. These techniques also have explained the elucidation of the HIV proteolytic genes and subsequent methods of ascertaining their proteases and antiretroviral activity. The potential HIV proteases and antiretroviral proteins so contained could be subjected to normal clinical trial protocols with the potentials of development into drugs.

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