We suggest that, when breastfed children who are over 12 months old show reluctance to take supplementary foods, they should be weaned completely to enhance their food intake. We also suggest that, in Ghana, breastfed children who eat well may be weaned at about 18 months without adverse effects on their health. However, in view of the many advantages of long-term breastfeeding, as well as the disadvantages, more work will have to be done to substantiate our observation.

We thank the Chief and mothers of the children of Ashaley-Botwe, Dr A. Asiedu, and Dr D. A. Armah of the Princess Marie Louise Hospital for their cooperation and valuable assistance. We are also grateful to the Japan International Cooperation Agency and the Ghana Government for sponsoring this study.

Correspondence should be addressed to L. A. B., Noguchi Memorial Institute for Medical Research, University of Ghana, PO Box 25, Legon, Accra, Ghana.

REFERENCES

IDENTIFICATION OF HIV-INFECTED SERONEGATIVE INDIVIDUALS BY A DIRECT DIAGNOSTIC TEST BASED ON HYBRIDISATION TO AMPLIFIED VIRAL DNA

MICHEL LOCHE BERNARD MACH
Department of Microbiology, University of Geneva Medical School, Geneva, Switzerland

Summary There is a need for direct detection of the virus in people infected with human immunodeficiency virus (HIV), independently of a serological response. In this study, after enzymic amplification of a specific segment of the HIV genome, a simple slot-blot hybridisation procedure allowed unequivocal identification of HIV DNA in all seropositive subjects tested. More importantly, the hybridisation test allowed the detection of HIV DNA in several seronegative subjects from very high risk groups. This new direct approach towards the diagnosis of HIV infection, which can easily be carried out on a large scale, is therefore capable of identifying HIV-infected individuals before the development of antibodies.

Introduction Routine diagnostic procedures used to identify individuals infected with human immunodeficiency virus (HIV) rely on the presence of antibodies against viral epitopes in serum. Approaches based on the identification of viral antigens by specific polyclonal or monoclonal antibodies suffer from a lack of sensitivity. Yet there is a need, in several clinically important situations, for direct detection of the virus independently of circulating antiviral antibodies in infected individuals. There is growing evidence that the period during which a person infected with HIV does not produce antiviral antibodies can be as long as several months and even over a year. It follows that seronegative blood donors who are nevertheless HIV infected might not be exceptional, and the need for an earlier diagnostic procedure has been emphasised. Another important group is children born to HIV-seropositive mothers; serological diagnosis is not possible for them for several months.

HIV is an RNA virus from the group of retroviruses. The viral genome is copied into complementary DNA by reverse transcriptase and eventually integrated as DNA into the chromosomal DNA of infected cells, primarily T lymphocytes, macrophages, and microglial cells in the brain. Attempts have been made to identify HIV DNA in lymphocyte DNA by hybridisation with the Southern blot procedure. This approach has not been successful, presumably because the number of HIV-infected lymphocytes is generally very small and the HIV DNA sequences are too rare to be detected.

We have therefore explored the possibility that extensive enzymic amplification of a specific segment of viral DNA, from total DNA prepared from the blood of infected subjects, could allow the detection of viral DNA by hybridisation. Two oligonucleotides corresponding respectively to the plus and minus strands of the 5' portion of the gag gene of HIV, 213 base-pairs apart, were synthesised according to published DNA sequences, at the positions in the HIV sequence indicated below. This portion of the HIV sequence is strictly conserved among different viral isolates. The enzymic amplification procedure was first applied to small quantities of cloned DNA prepared from a plasmid clone containing the entire HIV viral genome (kindly provided by S. Wain-Hobson). Samples from the amplification reaction were spotted on membrane filters and hybridisation was carried out with a phosphorus-32-labelled oligonucleotide (19 mer) corresponding to the relevant region of the HIV genome. We have shown that under these conditions, hybridisation is strictly sequence specific.

Amplification of HIV DNA has been described previously for cell lines infected in culture by HIV. We have tested the amplification procedure for direct detection of HIV DNA sequences in blood samples. Only a small subset of T lymphocytes is infected and HIV DNA is therefore present in much smaller amounts than the cellular genes previously analysed with that procedure.

Patients and Methods Blood samples were taken from healthy seronegative controls and from HIV-seropositive individuals, including patients with AIDS, patients with AIDS-related complex, and seropositive subjects with no AIDS symptoms. In addition, samples were obtained from

well-studied seronegative sexual partners of seropositive individuals. Peripheral blood lymphocytes were prepared from 2-3 ml blood by centrifugation through a 'Ficoll' gradient. DNA was prepared by incubation in 500 µg/ml proteinase K, 0.5% sodium dodecylsulphate, 10 mmol/l "tris"-HCl pH 7.5, 30 mmol/l sodium chloride, 20 mmol/l ethylenediamine tetra-acetate (EDTA) at 50°C for 2 h, followed by three extractions with phenol/chloroform (50/50), ethanol precipitation, and resuspension in 10 mmol/l "tris"-HCl pH 7.5 and 1 mmol/l EDTA.

The oligo primers and the oligo probe indicated below were synthesised by the β-cyanoethyl amide procedure by means of a Pharmacia 'Gene Assembler' instrument. They all correspond to the 5' portion of the highly conserved gag gene of HIV1.13

Primer I (5'): GAAGGAGAGAGATGGGTGCG (Position 325-344)
Primer II (3'): GGGATGGTTGTAGCTGTCCC (Position 518-537)
Probe: GGGAGCTAGAACGATTCGC (Position 451-469)

Amplification of a specific segment of DNA was carried out by the principle of Mullis et al.,7,19 with slight modifications: 1 pg of each DNA sample was mixed with 1.5 mmol/l each of dATP, dTTP, dCTP, and dGTP, 7 mmol/l "tris"-HCl pH 8.8, 16 mmol/l ammonium sulphate, 10 mmol/l magnesium chloride, 10 mmol/l β-mercaptoethanol, 0.02% gelatin, 7 µmol/l EDTA, 1 µmol/l primer I, and 1 µmol/l primer II in a volume of 100 µl. After incubation at 98°C for 7 min (annealing of primers). Then 2 units of Taq polymerase (New England Biolabs) was added and the tubes were transferred to 70°C for 3 min (DNA synthesis). This cycle was repeated 25 times, with all subsequent denaturation steps limited to 1 min at 92°C and without further addition of enzyme. In early experiments, Klenow DNA polymerase (Boehringer) was used, with the addition of 1 unit of enzyme after each denaturation step.12

Samples of 5 µl were taken before incubation and after different cycles of the amplification procedure, adjusted to 0.4 mol/l sodium hydroxide, 25 mmol/l EDTA for 5 min, neutralised with 10 µl 2 mol/l ammonium acetate and applied to a nitrocellulose membrane (pore size 0.45 µm) with a slot-blotter apparatus (Schleicher and Schüll). Hybridisation and washing were done essentially as described previously for HLA typing by oligonucleotide hybridisation.18 The membranes were treated at 80°C for 1 h then prehybridised in 4 × SSC (SSC = 150 mmol/l sodium chloride, 15 mmol/l sodium citrate, pH 7), 5 × Denhardt (Denhardt = 0.02% ficoll), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 5% sodium dodecyl sulphate, 20 mmol/l sodium phosphate, 100 µg/ml sheared and denatured herring sperm DNA and 1.5 × 106 cpm of phosphorus-32-labelled oligo probe. The membranes were then washed twice in 3 × SSC, 8 × Denhardt, 5% sodium dodecylsulphate at 50°C for 15 min and autoradiographed for 2-6 h with Cawso intensifier screens. Labelling of the oligo probe with polynucleotide kinase (Boehringer) was done as described previously.15

Results

Fig IA shows that the amplification signal with the HIV probe became detectable after hybridisation and increased with the number of amplification cycles. 2 pg DNA from plasmid clone pB71-LAV (provided by S. Wain-Hobson) was subjected to successive cycles of primer extension and denaturation in the presence of the two primers and Klenow DNA polymerase. Samples were collected every five cycles, spotted, and hybridised.

To measure the size of the product of the amplification reaction, samples were amplified and at cycle 20, 1 µCi P32-dCTP was added to each tube. After ethanol precipitation, the samples were subjected to agarose gel (2%) electrophoresis and autoradiography. Fig IB confirms that the fragment is the expected size, with either HIV plasmid DNA (lane 3) or DNA from a seropositive subject (lane 2).

In fig IC, the results of a quantification experiment are reported. A fixed amount of HIV plasmid DNA (4 × 106 molecules) was mixed with 1 µg control human DNA and was serially diluted with human DNA. 1 µg of each dilution was used for amplification with Taq polymerase and one tenth of each reaction mixture was tested by hybridisation. The lowest amount easily detectable in that sample corresponds to less than 4 molecules of HIV DNA. This sensitivity allows the detection of 1 HIV DNA molecule in about 100 000 lymphocytes.
Fig 2—Identification of HIV-specific DNA by hybridisation to amplified DNA samples.
Lanes 1–3 = three seropositive subjects.

Fig 3—Hybridisation to amplified DNA (25 amplification cycles) of control individuals, HIV-seropositive subjects, and HIV-seronegative partners.
Controls = seronegative, not at risk; seronegative partners = samples from sexual partners of seropositive individuals.

When samples from the amplification reactions for the DNA of HIV-seropositive individuals were spotted and hybridised with the HIV DNA oligonucleotide probe, an unequivocal hybridisation signal was observed after 10–25 cycles of amplification, depending on the individual (fig 2). The number of amplification cycles required to produce detectable HIV DNA was not related to whether the subject had AIDS or was a symptom-free seropositive. These differences might reflect the relative abundance of HIV-infected T lymphocytes in the blood samples of the different subjects. DNA from uninfected control subjects, handled in an identical manner, was consistently negative (fig 3).

Discussion

From these results, we conclude that DNA hybridisation of amplified DNA can be used as a direct test of HIV infection, independently of an antibody response against the virus. This HIV DNA diagnostic test can be carried out on a large scale. Its main advantage is that it does not rely on an antibody response and, as shown here, can identify HIV-infected individuals in an earlier phase of the infectious process. Used on a large scale, this test could form an additional safeguard in the identification of all infected individuals, particularly in the analysis of blood donors, since specific cases of HIV infection due to transfusion with seronegative blood have already been reported.6 Our test will be useful for the confirmation of serological diagnosis, in particular when false-positive assays might be expected,20 in all suspected cases of AIDS in seronegative patients, and for early diagnosis in high-risk groups, such as sexual partners of seropositive individuals. An area of immediate practical importance is the identification of HIV DNA in children born to HIV-seropositive mothers. This identification is currently only possible by long and complicated in-situ hybridisation procedures.21 We have identified HIV DNA in blood samples from 20 out of 50 children under 18 months old, born in Switzerland to HIV-seropositive mothers (unpublished). Since a positive diagnosis of HIV
infection might determine the therapeutic strategy in these children, systematic use of the DNA hybridisation test in such cases would be valuable.

Epidemiological studies of high-risk groups with this diagnostic assay will yield important information about the extent of the latent period between HIV infection and the development of a serological response. The increasing awareness of this latent period,3,4 together with the observation that mental and behavioural abnormalities might constitute early manifestations of HIV infection,22 before the onset of other clinical symptoms, suggest a widespread use of the direct HIV diagnostic test.

Since this work was completed, Ou and colleagues24 have reported that HIV DNA can be identified in blood from seropositive subjects after DNA hybridisation. Their identification relied on hybridisation to an HIV probe in liquid, followed by digestion with a restriction enzyme, gel electrophoresis, and autoradiography.

This work was supported by the Armagnac Fund.

Correspondence should be addressed to B. M., Department of Microbiology, University of Geneva Medical School, 9 Avenue de Champel, CH 1211 Geneva 4, Switzerland.

REFERENCES


