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High frequency of antibodies to *Mycoplasma penetrans* in HIV-infected patients

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Mycoplasma penetrans, a novel mycoplasma isolated from HIV-1-infected patients with AIDS, has pathogenic properties associated with in-vivo virulence. Enzyme-linked immunosorbent assay and western blotting detected a more than 100 times higher frequency of antibodies to the mycoplasma in serum from HIV-1-infected patients with AIDS (40%) than from HIV-negative controls (0·3%). Serum from 20% of HIV-1-infected, symptom-free individuals also had *M penetrans* specific antibodies. The antibodies' major immunoreactivity was directed against P35 and P38, the two main lipid-associated membrane protein antigens of the organism. Patients attending sexually transmitted disease clinics had a low frequency of antibody (0·9%). None of 178 HIV-negative patients with different non-AIDS diseases, many associated with immune dysfunction and/or low white cell counts, tested positive for the antibodies. *M penetrans*, apparently not a commensal and not a simple opportunist, is uniquely associated with HIV-1 infection and AIDS.

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Introduction

HIV-1 is probably a necessary but not sufficient cause of AIDS.[1,2] In the search for other infectious agent(s) that might have a role in AIDS pathogenesis, attention has

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focused on several species of mycoplasma.[2] Mycoplasmas are a heterogeneous group of the smallest organisms capable of self-replication, and can cause systemic debilitating diseases and profoundly alter immune function.[3,4] *Mycoplasma fermentans*, which systemically infects AIDS patients[5,6] and previously healthy individuals who have fulminant illness unrelated to HIV,[7,8] enhances the cytocidal effects of HIV-1 in human CD4 lymphocytes.[9,10] Thus mycoplasma is a reasonable candidate as a cofactor that might increase the development of AIDS in HIV-1 infected patients.[1,2]

We have isolated a previously unknown mycoplasma on 12 occasions from urines of 6 HIV-positive patients with AIDS.[11] Biochemical, serological, and DNA analyses identify it as a new species of Mollicutes.[11,12] The mycoplasma has a tip-like structure and displays adhesion, haemadsorption, and cytadsorption, all pathological properties associated with in-vivo virulence. Using its tip, the mycoplasma penetrates mammalian cells, hence *M penetrans*.[12] We report here the frequency of *M penetrans* antibodies in serum from subjects with or without HIV.

Subjects and methods

Serum samples

We tested: (1) 234 HIV-positive patients with clinical AIDS; (2) 118 HIV-positive patients without AIDS (symptom-free blood donors); (3) 85 stored serum samples from homosexual patients dying of "gay-related infectious disease" (GRID), an early term for immunodeficiency or infectious diseases in homosexuals, all 85 were confirmed anti-HIV positive by Abbott kit; (4) 336 patients attending sexually transmitted disease (STD) clinics in San Bernardino, California (159 patients), Brooklyn, New York (79), and Milwaukee, Wisconsin (98); (5) 180 patients with various diseases, often associated with immunological or autoimmune disorders (dialysis [44], systemic lupus erythematosus [30], rheumatoid arthritis [5], multiple sclerosis [20], lymphoma/leukaemia [32], other cancer [40], and paroxysmal nocturnal haemoglobinuria [9]), most with cancer or lymphoma or leukaemia were receiving chemotherapy and had low white cell counts; and (6) 384 HIV-negative healthy blood donors.

Mycoplasma isolates

M penetrans was isolated from urine of AIDS patients.[11] In addition *M pirum* was provided by Dr J. G. Tully, National Institute of Allergy and Infectious Diseases, and *M salivarium* from the American Type Culture Collection.

Mycoplasma membrane proteins

The procedure[13-15] for preparation of mycoplasma lipid-associated membrane proteins (LAMP) was modified. Log-phase mycoplasmas were pelleted, washed twice in phosphate-buffered saline (PBS) pH 7·2 (Biofluids) at 4°C, and stored at −70°C. Pellets were briefly sonicated at 4°C in buffer A (50 mmol/l tris-HCl pH 8·0, 150 mmol/l NaCl, 1 mmol/l edetic acid), adjusted to 1 mg/ml protein in buffer A, solubilised by adding Triton X-114 (TX-114, Boehringer, diluted to 10% with sterile deionised H_2O) to a final concentration of 2% TX-114 at 4°C for 2·5 h. Lysate was cleared by centrifugation at 5°C for 20 min at 20 840 *g*. Supernatant was subjected to three cycles of phase-fractionation. The final TX-114 phase was resuspended in 4°C buffer A to original volume, and centrifuged at 5°C for 20 min at 20 840 *g*. Supernatant was saved, designated as TX-114 extract, and used as antigens for enzyme-linked immunosorbent assay (ELISA) and western blots.

ELISA

In the ELISA for antibodies to LAMP antigens, TX-114 extract, diluted in 20 mmol/l $NaHCO_3$ pH 9·6 and 0·15 mol/l NaCl to about 2 μg/ml protein, was coated on Nunc-Immuno F96 MaxiSorp plates at 37°C for 4 h with 100 μl in each well, overcoated with 0·1% bovine serum albumin (BSA) plus 0·02% sodium azide at 25°C for 2 h, and washed twice with solution A (PBS pH 7·2 plus 0·05% Nonidet P-40 [NP-40]). 100 μl human serum or plasma, diluted 1/250 in 10% normal goat serum, 2% BSA, and 0·3% NP-40 in PBS was added to each well and incubated at 5°C overnight, then at 37°C for 90 min. Excess fluid was aspirated to stop the reaction and plates were washed six times with solution A. Each well received 100 μl of 1/1000 biotin-labelled antibody of goat anti-human IgG-γ (Kirkegaard & Perry [KP]) in diluent I (10% normal goat serum, 2% BSA, and 0·1% NP-40 in PBS), was incubated at 37°C for 90 min, washed as described above, incubated with 1/20 000 peroxidase-labelled streptavidin (KP) in diluent I at 37°C for 90 min, washed as described above, developed with 100 μl of 2, 2'-azino-di-[3-ethyl-benzthiazoline-sulphonate] peroxidase substrate solution at 37°C for 20 min, and reaction stopped by adding 100 μl 1% sodium dodecyl sulphate (SDS).

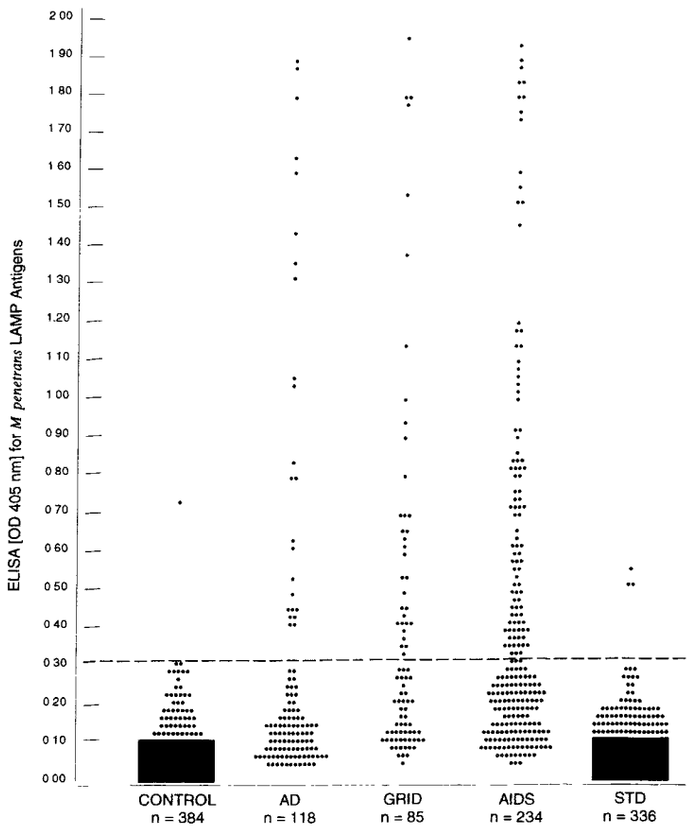

Fig 1—*M penetrans* antibodies in serum.

Black blocks (OD ≤0·10) represent 330 dots (86%) and 255 dots (76%) of samples from HIV-negative blood donors and STD patients, respectively. AD = symptom-free HIV-positive blood donors.

The optical density (OD) of each well at 405 nm corrected with a reference wavelength at 650 nm was measured with a microtitre plate reader with automatic subtraction of OD of the blank. Absorbance readings greater than the mean reading of 383 HIV-negative normal subjects plus four SDs were considered to be positive for *M penetrans* antibodies. Each sample was tested at least twice. All positive samples were repeated three times and were also tested in plates coated with BSA without mycoplasma antigens to assess non-specific binding. In this study, antisera from rabbits immunised with *M penetrans*, *M pirum*, and *M salivarium* were also used to evaluate the antigenicity and immunoreactivity of the mycoplasmal surface antigens coated on the ELISA plates.

Western blotting

Proteins (about 90 μg) from *M penetrans* TX-114 extract were separated by SDS-polyacrylamide gel electrophoresis[16] and electroblotted on a BA-85 nitrocellulose membrane (Schleicher & Schuell).[17] The membrane was blocked with 5% fetal bovine serum and 1% BSA in PBS pH 7·2 and cut into 4 mm strips. Each strip was incubated for 5 min with 1/250 human serum 25°C for 15 h with

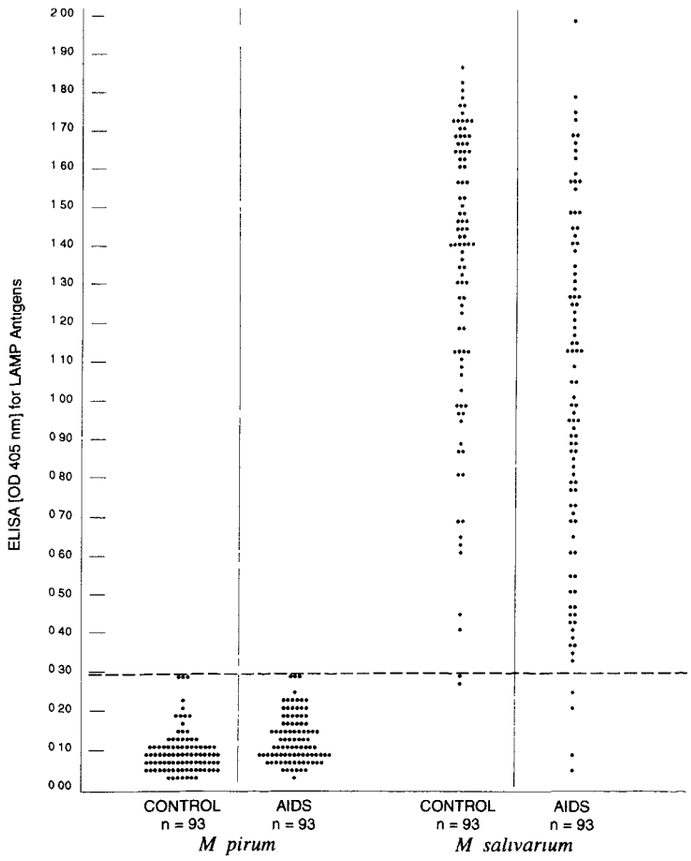

Fig 2—Antibodies to *M pirum* and *M salivarium* in serum.

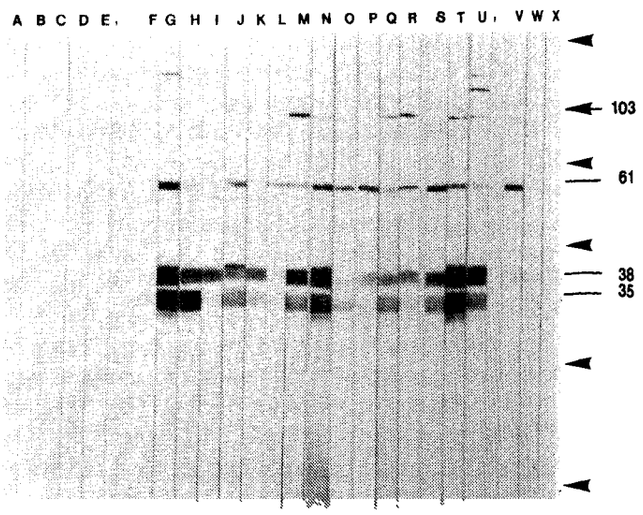

Fig 3—Western blots of *M penetrans* LAMP antigens.

Lanes A–E = serum from 5 HIV-negative health blood donors. F–U = serum or plasmas from 16 HIV-positive individuals with positive ELISA. F–K = patients from HIV-positive symptom-free blood donors. L–R = AIDS patients. S–U = GRID patients. V–X = AIDS patients with negative ELISA to *M penetrans* antigens. Arrows = prestained protein size-markers with apparent molecular weight from top to bottom of 205, 103, 67, 42, 28, and 18 kDa, respectively.

shaking. The strips were washed six times with solution A, incubated at 25°C with 1/1000 biotin-labelled antibody of goat anti-human IgG-γ, incubated at 25°C with 1/10 000 peroxidase-labelled streptavidin in diluent I for 2 h, and developed at 37°C for 20 min with the 4-chloro-1-naphthol peroxidase substrate system (KP).

Results

97 (41·5%) HIV-positive patients with clinical AIDS had antibody titres to *M penetrans* (fig 1). In addition, 34 (40·0%) homosexual patients dying of GRID had serological evidence of *M penetrans* infection. 24 (20·3%) HIV-positive symptom-free blood donors also tested positive for antibodies to *M penetrans*. In contrast, only 1 (0·3%) HIV-negative blood donor had a positive titre.

We tested 336 serum samples from STD patients in three cities to assess whether *M penetrans* represents a previously unrecognised sexually transmitted infection not directly associated with AIDS or HIV infection. Only 3 of 159 STD patients in southern California, 0 of 79 in Brooklyn, and 0 of 98 patients in Milwaukee tested positive. 5 of the 336 serum samples were HIV-positive. However, these 5 were negative for *M penetrans* antibodies. Only 2 lymphoma patients from the 180 patients with various diseases had positive titres to the mycoplasma. These 2 samples were anti-HIV positive by ELISA. None of the other 178 samples tested positive for antibodies to either *M penetrans* or HIV.

Overall, 157 (35·4%) serum samples from 444 HIV-positive subjects were positive for antibodies to *M penetrans* LAMP compared with 4 (0·4%) from 893 HIV-negative subjects. The relative odds of HIV-positive individuals being exposed to or infected with *M penetrans* compared with HIV-negative subjects was 122 to 1 (odds ratio = $157 \times [893-4]/[444-157] \times 4 = 121{\cdot}6$).

Parallel ELISA with *M pirum* and *M salivarium* TX-114 phase-fractionated LAMP antigens validated the antibody assay to LAMP antigens. Like *M penetrans*, *M pirum* infects AIDS patients, uses glucose and arginine, and attaches via a tip.[18,19] *M salivarium* is a commensal almost universally found in the oral cavity and rarely in the urogenital tract.[20] Serum from 93 HIV-positive AIDS patients and 93 HIV-negative blood donors randomly selected from the previous sample pools were tested. Samples positive for *M pirum* specific antibodies were uncommon in both populations (fig 2). Samples positive for *M salivarium* antibodies were highly frequent in both groups (fig 2). A mean antibody titre to the commensal *M salivarium* was lower in serum from AIDS patients than in that from controls (mean absorbance 0·98 *vs* 1·31).

We analysed the immunoreactivity of antibodies in serum from HIV-positive patients to fractionated LAMP antigens with western blot. 35 kDa (P35) and 38 kDa (P38) molecular weight proteins were the most prominent antigens detected by serum from patients with AIDS (strips F-U) (fig 3). These antigens corresponded to the two major LAMPs of *M penetrans*. Serum from HIV-negative blood donors did not react with P35 and P38 (strips A–E). Some serum samples from HIV-1-infected AIDS patients that tested negative by ELISA (strips V–X) showed low positive reactions to P35 and P38. Additional 61 and 103 kDa proteins (P61 and P103) were also recognised by many samples from HIV-1-infected patients (fig 3). P103 seems to react specifically with serum from HIV-positive subjects. P61, although producing a more intense reaction with serum from HIV-positive subjects, did occasionally have a low reaction with some of the control serum samples from HIV-negative subjects.

Discussion

We have developed an ELISA and an immunoblot serological assay to detect *M penetrans* specific antibodies in HIV-positive patients with or without clinical AIDS. The serum samples positive for *M penetrans* specific antibodies consistently reacted to two major LAMP of *M penetrans* (P35 and P38). The validity and specificity of the serological assays with cell surface antigens of *M penetrans* were further verified by results in our control study with LAMP similarly

prepared for *M pirum* and *M salivarium*. We found a high frequency (about 40%) of specific antibodies to *M penetrans* in HIV-positive patients, especially those currently diagnosed as manifesting AIDS or those who had previously died of AIDS. In contrast, less than 0·3% of the general population is positive for antibodies to this mycoplasma. Because the organism was identified and isolated from the urogenital tracts of HIV-infected patients, *M penetrans* is most probably transmissible through sexual contact. However, antibody to this novel mycoplasma was not common among sexually active patients without HIV-1 infection. The frequency of positive tests for *M penetrans* antibodies in patients attending STD clinics in three cities was higher (0·9%) but still low. Moreover, there was no serological evidence of *M penetrans* infection in patients with a wide variety of diseases not associated with AIDS. Interestingly, the two serum samples from lymphoma patients that tested positive for antibodies to *M penetrans* were HIV-1 antibody-positive.

The mycoplasma was uniquely associated with patients with HIV-1 infection (odds ratio 122). Although the clinical significance of *M penetrans* in AIDS is not clear, our findings showed that the organism is circulating in some communities with specific high-risk factors of AIDS or HIV-1 infection (eg, homosexuality).

A large part of our study was designed to answer whether *M penetrans* is a commensal or an opportunist. If the organism is a commensal, the antibody to the mycoplasma should be found in all groups, as occurs with antibodies to the known commensal *M salivarium*. In addition, highly immunocompromised patients with AIDS are unlikely to mount stronger immune responses and produce higher titres of antibody to a commensal than are healthy controls. Normally patients with AIDS have weaker immune response and produce lower antibody titres to a foreign agent, as shown by the lower titres we found in AIDS patients to *M salivarium*. Inconsistent with the idea of *M penetrans* being a simple opportunist, we found that about 20% of HIV-positive symptom-free donors were positive for *M penetrans* antibody. These patients showed no evidence of immunosuppression, low white cell count, or opportunistic infections. In contrast, 178 patients with other diseases, many with abnormal immune functions and low white cell counts after receiving immunosuppressive anticancer drugs, had no evidence of *M penetrans* infection. We conclude that *M penetrans* is not a commensal in man and may not be a simple opportunistic infection that commonly occurs in immunocompromised patients.

Studies from our laboratory and from others' showed that patients with AIDS had systemic infections with *M fermentans*.[1,5,6,18,21] The mycoplasma was identified in urine, blood, oral swab, and various diseased organs. However, we have had great difficulty studying antibody responses to *M fermentans* infections in these patients. We have found, like our laboratory findings of atypical antibody responses in monkeys,[22] that many patients with AIDS known to be infected with *M fermentans* failed to produce detectable antibodies to the mycoplasma. More specifically, patients with AIDS who were positive by polymerase chain reaction or even by culture for *M fermentans* often had only low or no antibody to the organism. Therefore, the frequency of antibodies to *M fermentans* among AIDS patients might be artificially low and the serological correlation between *M fermentans* infections and AIDS may appear to be less important. This complicated problem has not been completely resolved. In our study with *M fermentans* LAMP antigens, we found that 15% of HIV-1-infected patients with AIDS and 1·3% of healthy controls tested positive. Half the AIDS patients who had *M fermentans* specific antibodies also had antibodies to *M penetrans*. The other half had evidence of *M fermentans* infections only.

M penetrans has prominent pathobiological properties of virulence, and was originally isolated only from patients with AIDS.[11,12] We have now shown that the frequency of antibodies to this mycoplasma is far higher in HIV-1-infected individuals than in the general population, and was twice as high in AIDS patients compared with symptom-free HIV-1 infected individuals. Thus infection with *M penetrans* may relate to the development of clincial AIDS.

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SHORT REPORTS

Nonsense mutation of glucokinase gene in late-onset non-insulin-dependent diabetes mellitus

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A nonsense mutation at codon 186 in exon 5 of the gene for glucokinase, an enzyme important for glucose-induced insulin secretion, was identified in a Japanese patient with late-onset non-insulin-dependent diabetes mellitus (NIDDM). All affected members of her family were heterozygous for the mutation and had late-onset NIDDM or impaired glucose tolerance, whereas unaffected members showed normal glucose tolerance. The early insulin response to oral glucose was impaired in affected relatives, but was normal in those unaffected. These findings suggest that the glucokinase mutation raises the set-point of pancreatic beta cells for glucose-induced insulin secretion, leading to abnormal glucose tolerance in some patients with late-onset NIDDM.

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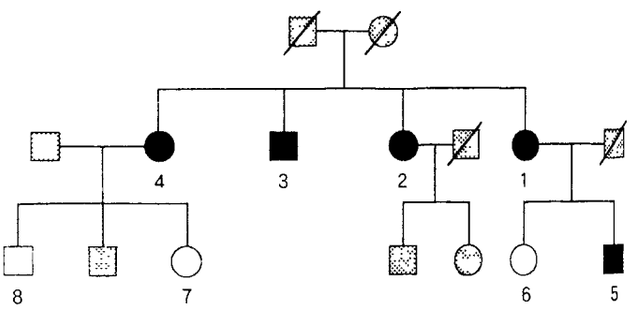
Non-insulin-dependent diabetes mellitus (NIDDM) is caused by insulin resistance, impaired insulin secretion, or both, and its development is affected by genetic factors.[1] Glucokinase is a key enzyme for glucose metabolism in pancreatic beta cells; glucose metabolism is thought to be a prerequisite for glucose-induced insulin secretion.[2] Close linkage of NIDDM to the glucokinase gene has been reported in patients with maturity-onset diabetes of the young (MODY),[3,4] and a nonsense mutation of exon 7 was found in one such pedigree.[5] We have analysed the glucokinase gene in Japanese patients with late-onset NIDDM and a family history of NIDDM in close relatives.

The exon-intron organisation of the human glucokinase gene was determined and the exon-containing regions were amplified with the polymerase chain reaction. Mutation in the glucokinase gene was sought by temperature-gradient gel electrophoresis of the amplification products and was confirmed by direct sequencing with a DNA sequencer.*

*Full details of the methods and a figure summarising the sequencing data are available from *The Lancet*.

A nonsense mutation of exon 5 of the glucokinase gene was found in a 59-year-old woman (the 23rd subject screened). She had had no glucosuria during pregnancy and her children's birthweights were 3150 g and 3300 g. She was diagnosed as having diabetes (fasting blood glucose 8·1 mmol/l) at age 41 when glucosuria was found at an annual check-up. Diet therapy lowered the fasting blood glucose concentration to 6·3 mmol/l. The 75 g oral glucose tolerance test (OGTT) and sequencing of the glucokinase gene were done for all available members of her family (see figure and table). Subject 2 had rheumatoid arthritis but had had no glucosuria on many examinations, including during pregnancy. Subject 3 had first had glucosuria at age 37. Subject 4 was found to have impaired glucose tolerance at age 37. None of these individuals had symptoms of diabetes. All the family members with abnormal glucose tolerance in the OGTT (by WHO criteria) were affected by the glucokinase mutation; those with normal glucose tolerance did not have the mutation. None of the affected individuals had MODY.[6]



Pedigree of proband's family.

Solid symbols represent family members affected by the mutation and open symbols those found not to have it. The dotted symbols represent subjects unavailable for testing (those crossed through were dead).

All the affected members were heterozygous for the glucokinase mutation; a substitution of T for C in codon 186 of exon 5 changes CGA (arginine) to TGA, an amber termination codon.* The mutation is likely to delete 60% of aminoacid residues of glucokinase derived from the affected allele. Mutation of aspartate 205 to alanine reduces rat liver glucokinase activity 500-fold.[7] Thus, truncation of human glucokinase at residue 186 is likely to abolish the enzyme's activity. Direct sequencing revealed no mutation in other regions, including the unaffected allele. However, the nucleotides at codons 107 and 446 differ from the reported sequence[8] in all Japanese subjects analysed so far, including this family.

The mean plasma insulin concentration 30 min after a 75 g glucose load was significantly lower for the affected family members than for those not affected by the mutation (31 [SD 5] *vs* 52 [5] mU/l, $p < 0{\cdot}002$). In relation to the blood glucose concentration, there was pronounced