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Detection of measles virus genome in bone-marrow aspirates from adults

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We investigated the presence of the measles virus genome in order to identify asymptomatic infections in the adult population. Bone-marrow aspirates were obtained from 179 patients, 20–96 years of age, for the diagnosis of malignant diseases (29 with malignant lymphoma, 28 with acute leukaemia, 21 with myelodysplastic syndrome, five with multiple myeloma and 96 with other diseases). The measles virus genome was detected in 17 (9·5%) of 179 individuals by RT–PCR and 28 (15·6%) through hybridization. The genomes detected in bone marrow were all in the same cluster, D5, the strain circulating during the study period, and no evidence of persistent infection was obtained. We conclude that asymptomatic infections of measles virus are common in adults and the presence of the measles virus genome would not be related to the pathogenesis of illness.

After measles infection, immunity against measles virus was thought to be life-long. The measles vaccination was also considered to induce life-long immunity. It has been recognized, however, that the measles virus can infect previously immune individuals, producing a wide range of illnesses: typical measles, mild modified measles and asymptomatic infection. Recently, the number of cases of measles among previously immunized individuals has increased, probably caused by a waning of vaccine-induced immunity. Several reports have indicated that measles virus continues to circulate in highly vaccinated populations (Markowitz *et al.*, 1989). We examined the presence of the measles virus genome in peripheral blood mononuclear cells (PBMC) of healthy children. The virus genome was detected in 40 of 159 samples from healthy individuals who had been immunized more than 2 months before and seven of 26 after natural infection. Thus, we identified asymptomatic infections by RT–PCR with an unexpectedly high incidence in infants and children in Japan, where annual measles outbreaks are observed (Sonoda & Nakayama, 2001).

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In Japan, where measles virus has been circulating, we speculated that asymptomatic infection occurs frequently and life-long immunity is therefore sustained by boosts through asymptomatic re-infection. A further attenuated measles vaccine has been in use since 1978 and individuals over 30 years of age have had natural infections. In adult populations, neutralizing antibodies were maintained at high levels even in those over 80 or 90 years of age. There are no available data on subclinical infection in adult populations. Initially, we examined PBMC obtained from 30 healthy adults but found no measles virus genome. In this report, we tested for the presence of the measles virus genome from bone-marrow aspirates obtained for the diagnosis of malignant illnesses.

The study involved 179 individuals (101 males and 78 females), aged from 20 to 96 years. Details are shown in Table 1. They were diagnosed as follows: 29 with malignant lymphoma, 28 with acute leukaemia, 21 with myelodysplastic syndrome, five with multiple myeloma, 13 with other malignant tumours (lung, stomach, colon, breast and brain cancer) and 83 with other illnesses (pernicious anaemia, idiopathic thrombocytopenic purpura, iron-deficient anaemia, disseminated intravascular coagulation, polycytemia and hypereosinophilic syndrome). Bone-marrow aspirates were obtained to make a diagnosis and the remaining portion was used for the detection of measles virus genome. Informed consent was obtained after explaining the purpose of the study. The study was conducted from December 1994 to May 1997.

Total RNA was extracted using guanidinium thiocyanate/phenol/chloroform (Chomczynski & Sacchi, 1987). Measles RT–PCR was performed in the haemagglutinin (H) region as reported by Nakayama *et al.* (1995). Briefly, 3 μl RNA was reverse-transcribed with AMV reverse transcriptase (Gibco Life Sciences) using the primer MF1 (5′ GCTTCCCTCTGGCCGAACAATATCG 3′), located at genome positions 7209–7233 in the F coding region. The first PCR was performed with primers MH2+ (5′ GGGCTCCGGTGTTCCATATG 3′; positions 8067–8086) and MH6− (5′ CTTGAATCTCGGTATCCACTCCAAT 3′; 8677–8701) and the nested PCR with

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Table 1. Subjects included in the study

Basic disease	No. of patients	Male/female	Age range (years)
Malignant lymphoma	29	15/14	30–79
Acute leukaemia	28	19/9	20–78
Myelodysplastic syndrome (MDS)	21	10/11	25–89
Multiple myeloma	5	4/1	55–87
Other malignant diseases*	13	11/2	28–77
Other diseases†	83	42/41	21–96
Totals	179	101/78	20–96

* Including lung, stomach, colon, breast and brain cancers.
† Pernicious anaemia, idiopathic thrombocytopenic purpura, iron-deficient anaemia, disseminated intravascular coaglopathy, polycytemia and hypereosinophilic syndrome.

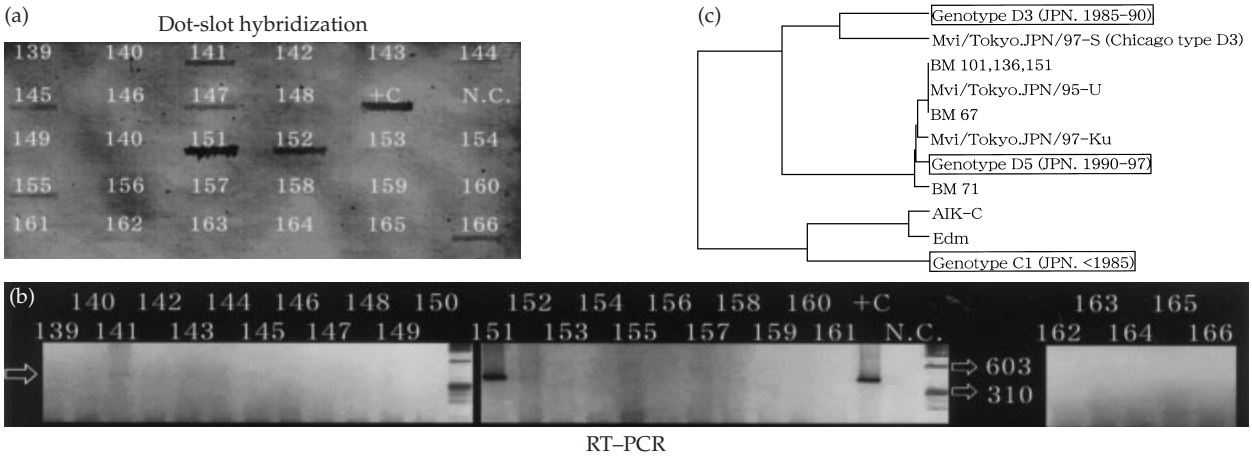

Fig. 1. (a)–(b) Results of dot-slot hybridization (a) and RT–PCR (b) of samples 139–166. In (b), the arrow on the left shows the size of the measles H gene PCR product and the arrows next to the DNA marker indicate the positions of markers of 603 and 310 bp. +C; Positive control; N. C.; negative control. (c) Sequence analysis of PCR products detected from bone-marrow samples. PCR was done in part of the haemagglutinin (H) region (positions 8106–8672) and the nucleotide alignment was determined by direct sequencing. Phylogenetic analysis was performed by the neighbour-joining method. Consensus sequences of genotypes C1, D3 and D5 were used. BM 67, 71, 101, 136 and 151 were PCR-positive samples from bone-marrow aspirates.

primers MH3+ (5′ CAGTCAGTAATGATCTCAGCAACTG 3′; 8106–8130) and MH4− (5′ ATCCTTCAATGGTGCCCACTCGGGA 3′; 8458–8482). Our RT–PCR was sufficiently sensitive to detect measles virus genome at 10^0 to 10^{-1} $TCID_{50}$. Negative controls for each step, genomic RNA extraction, cDNA synthesis, first PCR and nested PCR, were interspersed and RNA extracts from uninfected B95a cells were processed as a negative control in parallel with the test samples in the experiment. For the positive control, RNA extract from the Edmonston strain was used in each experiment.

For a more sensitive detection of the nested PCR products, a hybridization method was employed using a photobiotin labelling and detection kit (Wako Junyaku), as recommended by the instruction manual. Briefly, 20 µl of nested PCR products was mixed with an equal volume of denaturing solution (0·8 M NaOH, 20 mM EDTA), heated at 100 °C for 10 min and then placed on ice. To neutralize the pH, 40 µl 2 M ammonium acetate was added. Samples were subjected to dot-slot aspirating hybridization (Bio-Rad) and fixed on a membrane. PCR products of the H gene of the Edmonston strain were labelled with photobiotin. The dot-slot membrane was prehybridized overnight at 42 °C with prehybridization solution and incubated with the measles virus H probe at 42 °C for 20 h. After several washing steps and blocking, the membrane was processed for enzymatic detection using streptavidin, alkaline phosphatase and BCIP/NBT. For each experiment, positive and negative controls were interspersed. PCR products were excised from the gel and sequenced by dye terminator methods with an ABI 373A sequencer (Applied Biosystems). Nucleotide alignment and dendrogram analysis were performed with GENETYX Mac version 9.0.

Table 2. Detection of measles virus genome from bone-marrow materials obtained from patients with different diseases

Values are numbers positive/numbers tested.

Basic disease	RT–PCR positive	Hybridization positive
Malignant lymphoma	3/29	5/29
Acute leukaemia	7/28	7/28
Myelodysplastic syndrome (MDS)	1/21	1/21
Multiple myeloma	0/5	0/5
Other malignant diseases	2/13	3/13
Other diseases	4/83	12/83
Total	17/179	28/179

The results of RT–PCR and hybridization of samples 139–166 are shown in Fig. 1(a, b). Sample 151 showed a strongly positive response and samples 141 and 152 were weakly positive by RT–PCR. Through dot-slot hybridization, measles virus genome was detected more sensitively in samples 141, 144, 145, 147, 151–153, 155, 162 and 166. All RT–PCR positives were also positive for hybridization. As for the negative control, RNA extract from uninfected B95a cells showed no positive signal in PCR or hybridization experiments. The results of detection of measles virus genome are shown in Table 2. The genome was detected in 17 of 179 individuals (9·5 %) by RT–PCR and ethidium bromide staining: three of 29 patients with malignant lymphoma, seven (25 %) of 28 patients with acute leukaemia, one of 21 patients with myelodysplastic syndrome, none of five patients with multiple myeloma, two of 13 patients with other malignant tumours and four of 83 patients with other diseases. Through dot-slot hybridization, it was detected in 28 of 179 individuals (15·6 %). We have examined the sequence of measles virus in Japan since 1984. Measles before 1985 was of genotype C1, while from 1985 to 1990 it was D3. Measles in the period 1995–1997 was D5, but D5 disappeared transiently and D3 re-emerged from late 1997 to 1999 (Nakayama *et al*., 1995; Yamaguchi, 1997; Takahashi *et al*., 2000; WHO, 2001). During the study period, circulating wild measles virus was of genotype D5. Of 17 RT–PCR-positive samples, five are shown in Fig. 1(c), including measles genotypes isolated in Japan since 1984. The measles genotype detected from bone-marrow materials in this study was D5 with a few nucleotide changes. No C1, D3 or A genotype was detected in the study.

Measles virus genome was detected on more than two occasions in four patients, two patients with acute lymphoblastic leukaemia (L2) aged 20 and 56, one with acute myeloblastic leukaemia aged 46 and one with malignant lymphoma (diffuse large cell type, stage III–IV) aged 61.

Vaccination was thought to provide life-long immunity similar to natural infection. Immunity against measles was supposedly maintained in adult populations by subclinical re-infection. Serological confirmation of subclinical re-infection was obtained by pre-exposure in household-exposed parents who developed asymptomatic secondary immune responses with a concomitant increase in specific IgG neutralizing test antibodies and haemagglutination inhibition titres (Muller *et al*., 1996; Huiss *et al*., 1997). Helfand *et al*. (1998) reported that IgM antibodies against measles virus were detected in 10 (23 %) of 44 bus-tour participants who had been exposed to measles during a 3-day trip and none developed typical measles symptoms. All cases were defined by serological evidence. Reyes *et al*. (1987) reported the isolation of measles virus from PBMC in mild cases of measles where the patient had previously been immunized. Recently, measles virus was isolated in a case of infection by household contact without any symptoms (Vardas & Kreis, 1999). The index case was identified as a secondary measles infection with positive isolation of measles virus and serological evidence. Measles virus was isolated from urine of the patient's mother without IgM antibodies or a booster of neutralizing antibodies and the genomic sequence of the isolated virus was identified as similar to that obtained from the index case.

In our previous examination in paediatric populations, the measles virus genome was detected in 36 of 78 individuals (40 of 159 samples) who were immunized more than 2 months before, and was identified as a circulating wild strain in all cases. Among 13 healthy individuals more than 2 months after natural infection, the measles virus genome was detected in six (seven out of 26 samples). There was no close relationship between the PCR positivity and the period since immunization or natural infection. PCR-positive samples were obtained from April 1993 to January 1995, in accordance with regional measles outbreaks. Thus, asymptomatic measles infections are probably very common manifestations of measles during outbreaks in highly immune populations (Sonoda & Nakayama, 2001).

Following this line of research, we examined PBMC obtained from healthy adults for the measles virus genome but failed to detect the genome. Remaining portions of bone-

marrow aspirate samples were examined after cytological examination, bacterial culture and so on. Measles virus genome was detected in 17 of 179 individuals (9·5%) by RT–PCR and ethidium bromide staining. Through dot-slot hybridization, it was detected in 28 of 179 individuals (15·6%). There was no relationship between the detection rate of measles virus genome and haematological malignant disease. In patients with malignant lymphoma and acute leukaemia, the detection rate of measles virus genome was slightly higher than in those with non-malignant haematological diseases, reflecting immunosuppression due to basic illness or chemotherapy. Subclinical infection was confirmed in adulthood and the detection of measles virus genome was not related to the illness.

Recently, it was hypothesized that some non-infectious diseases, inflammatory bowel diseases and autistic developmental disorders, might be related to the MMR vaccine. Measles virus genome has been detected in PBMC of patients with autoimmune hepatitis, inflammatory bowel illness, intractable convulsion, Crohn's disease and autism (Kawashima *et al*., 1996, 2000; Ekbom *et al*., 1994; Wakefield *et al*., 1997). However, there was no evidence of the presence of measles virus using N gene RT–PCR (Afzal *et al*., 1998) and, epidemiologically, no supportive evidence was reported (Farrington *et al*., 2001). The pathological relationship of the persistence of measles virus to the onset of diseases is still controversial. Katayama *et al*. (1995) reported the detection of measles virus genome RNA in autopsy brain material with high positivity and some were identified as current strains by sequence analysis. In this report and our previous report, we speculate that asymptomatic measles infections occur even in the adult population with unexpectedly high frequency, and this supports the preservation of measles immunity. We conclude that the presence of the measles virus genome is not directly connected to the pathogenesis of illness but might be a symptomatic infection, with no relationship between the detection of the measles virus genome and specific diseases.

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