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Detection of BK Virus and JC Virus in Urine and Brain Tissue by the Polymerase Chain Reaction

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DNAs of the human polyomaviruses BK virus (BKV) and JC virus (JCV) were amplified by the polymerase chain reaction (PCR) by using a single pair of 20-base oligonucleotide primers that were complementary to the same regions of both viruses. The sequences flanked by the primers were unique for each virus and could be differentiated by hybridization with 40-base, ^{32}P-labeled oligonucleotide probes or by cleavage with *Bam*HI. The DNA fragments resulting from amplification of BKV and JCV were 176 and 173 nucleotide pairs, respectively. The sensitivities of PCR for amplification of cloned BKV and JCV DNAs were 10 and 100 copies, respectively. Hybridization with the oligonucleotide probes was specific for each virus. A total of 57 urine samples from three groups of subjects were processed by DNA extraction or boiling and were tested by PCR. Urine samples collected from immunosuppressed patients (n = 11) and previously documented to be positive for BKV, JCV, or both were positive by PCR. Ten percent of urine samples from healthy adults (n = 30) that were previously negative for BKV and JCV were positive for one or both viruses by PCR. Urine samples (n = 16) from four seronegative bone marrow transplant recipients were uniformly negative for BKV. JCV was detected in deparaffinized brain tissue from a patient with progressive multifocal leukoencephalopathy. Specific diagnosis of virus in clinical specimens could be made within 1 day of receipt of the specimens. The PCR method is attractive for use in diagnosing polyomavirus infections because of its sensitivity, specificity, and rapid turnaround time.

The human polyomaviruses BK virus (BKV) and JC virus (JCV) are ubiquitous and infect a high proportion of the population (8, 30, 36). Primary infection occurs during childhood (30, 36) and is usually inapparent but may be accompanied by mild respiratory illness (20, 27). During primary infection, there is viremia and the virus is transported to the kidney, where it persists indefinitely. Immunocompromising conditions result in reactivation of virus and viruria. Polyomavirus viruria has been documented most extensively in recipients of renal allografts and bone marrow transplants and in pregnant women (5, 10, 12, 17, 24, 29). JCV is neurotropic and produces progressive multifocal leukoencephalopathy, a fatal neurological disease, in individuals with immunological deficits (28, 31, 40). BKV is associated with ureteral stenosis in renal allograft recipients (11, 17) and hemorrhagic cystitis in recipients of bone marrow transplants (1, 4). BKV genomes have been frequently recovered from tumors of the brain and pancreatic islet cells (13, 14).

The methods used for the detection of BKV and JCV viruria include isolation in cell culture (16, 33), electron microscopy (16, 26), cytological examination (39) and immunofluorescent staining (25) of exfoliated urinary cells, enzyme-linked immunosorbent assay for antigen (6), and DNA hybridization with labeled nucleic acid probes (2, 3, 19, 22). JCV has been detected in brain tissue by similar techniques (7, 18, 21, 32). The polymerase chain reaction (PCR), through repetitive thermal cycles and use of oligonucleotide primer-directed DNA synthesis, amplifies target DNA sequences by a factor of 10^5 or greater (34). We report the development of a PCR technique for BKV and JCV and its application for the detection of these viruses in urine and brain tissue.

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MATERIALS AND METHODS

Diagnosis by PCR. (i) Selection of sequences for primers and probes. BKV and JCV genomes have extensive (75%) nucleotide sequence homology. The nucleotide sequences of early regions of BKV (isolates Dun and MM) (35, 41) and JCV (isolate Mad1) (15) were examined, and the oligonucleotide sequences for primers and probes were selected (Fig. 1). Each of the two 20-base oligomer primers (PEP-1 and PEP-2) was complementary to a region of DNA where BKV and JCV have identical nucleotide sequences, so that this single set of primers was capable of amplifying both BKV and JCV DNAs. A 40-nucleotide sequence was selected from each virus from the region flanked by the primers (BEP-1 for BKV and JEP-1 for JCV) for use as a virus-specific probe to identify viral DNA in the PCR products. BEP-1 and JEP-1 lacked homology at 12 of the 40 nucleotides. The lengths of the BKV and JCV genomes targeted for amplification were 176 and 173 nucleotide pairs (np), respectively.

(ii) Amplification. Target sequences were amplified in a total reaction volume of 100 μl containing 10 μl of sample DNA, 200 μM of each of the four deoxynucleotide triphosphates, 0.5 μM of each high-pressure liquid chromatography-purified oligonucleotide primer (PEP-1 and PEP-2), 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 1× reaction buffer (50 mM KCl, 10 mM Tris hydrochloride [pH 8.3], 1.5 mM $MgCl_2$, and 0.01% [wt/vol] gelatin). For the testing of tissue sections, the 100-μl reaction mixture was added to the tissue fragments, after they were deparaffinized and desiccated as described below. Reaction mixtures were overlaid with mineral oil and subjected to thermal cycling. Amplification of target DNA was accomplished by using a thermal cycle of 1.5 min at 94°C for denaturing the DNA, 1.5 min at 55°C for annealing of primers, and 2 min at 72°C for sequence extension. A 10-min denaturing step (94°C) was included in the first cycle when

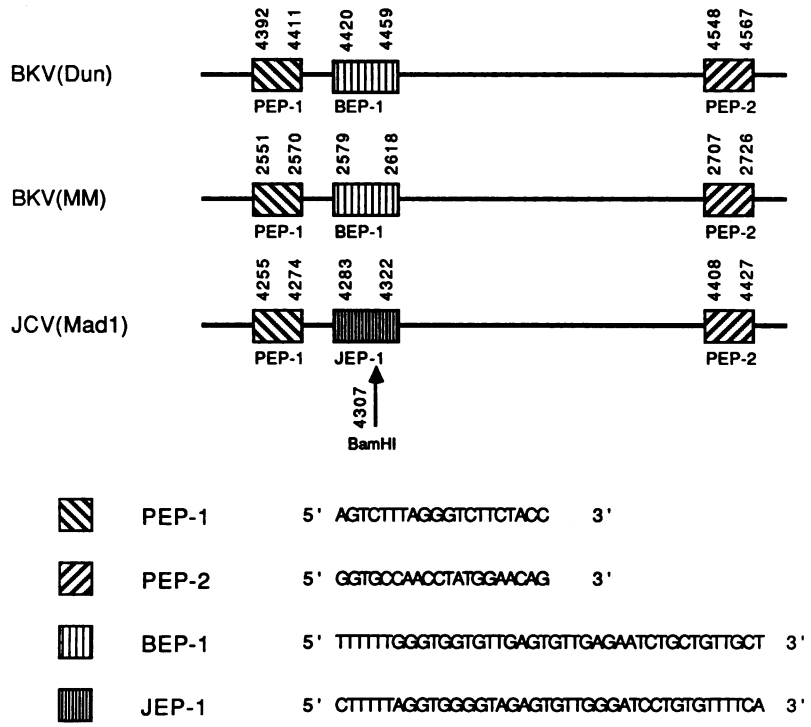

FIG. 1. Synthetic oligonucleotide primers (PEP-1 and PEP-2) used for amplification of BKV and JCV by PCR. Also shown are probes specific for BKV (BEP-1) and JCV (JEP-1). The DNA sequences targeted for amplification are in the early regions of the Dun and MM strains of BKV (35, 41) and the Mad1 strain of JCV (15) that code for large and small T antigens. Numbers are in nucleotides.

the tissue specimen was tested. Each sample was subjected to 40 cycles of amplification in a DNA thermal cycler (Perkin-Elmer).

(iii) Detection and identification of viral sequences in reaction products. BKV and JCV DNA sequences amplified by PCR were identified by three methods: visualization of bands of the appropriate size on agarose gels after electrophoresis, slot blot hybridization, and Southern hybridization. In the agarose gel method, 10 μl of the PCR product was electrophoresed on 4% gels (3% NuSieve–1% SeaKem; FMC Bioproducts, Rockland, Maine), and the gels were stained with ethidium bromide and examined for bands of the appropriate size. Digestion of the PCR products with *Bam*HI prior to electrophoresis was used to discriminate between BKV and JCV sequences. The JCV sequence targeted for amplification contained a *Bam*HI site, whereas the BKV sequence was not cleaved (Fig. 1 and 2). The JCV DNA was cleaved into fragments of 120 and 53 np by *Bam*HI.

In the slot blot hybridization method, 10 μl of PCR product was diluted in 190 μl of 0.5 M NaOH–25 mM EDTA, heated to 100°C for 2 min, and then chilled rapidly on ice and added to prewetted (with 20× SSPE, which is 3.6 M NaCl, 200 mM NaH_2PO_4 [pH 7.4], and 20 mM EDTA [pH 7.4]) nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) in a slot blot apparatus (Bethesda Research Laboratories-Life Technologies, Gaithersburg, Md.). Each slot was then washed with 2 volumes of 20× SSPE. The membrane was removed from the apparatus, exposed to UV light for 2 min (9), and air dried. Duplicate filters were prepared in this manner. The filter was incubated for 1 h at 42°C in a prehybridization solution containing 3× SSPE, 5× Denhardt solution (10× Denhardt solution is 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll), 0.5% sodium dodecyl sulfate, and 0.25 mg of tRNA per ml. Five nanograms of oligonucleotide probe per milliliter, either BEP-1 or JEP-1 (Fig. 1), that was end labeled with [^{32}P]ATP and T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, Ohio) to a specific activity of 2×10^8 to 5×10^8 cpm/μg was added and incubated for 1 h at 42°C. The filter was then washed for 5 min at room temperature in 3× SSPE, three times for 5 min in 1× SSPE containing 0.1% sodium dodecyl sulfate, and once for 10 min at 70°C in 5× SSPE with 0.1% sodium dodecyl sulfate. Autoradiograms were prepared by exposure of filters to X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens for 1 h.

Specific virus sequences were also identified by Southern transfer hybridization from agarose gels (38). Nylon filters were hybridized with ^{32}P-labeled BEP-1 and washed as described above for the slot blot hybridization procedure. Autoradiograms were prepared after 1 to 3 days of exposure at −70°C. The filters were then washed at 95°C to remove the BKV probe, and the hybridization procedure was repeated with the JCV probe JEP-1. Of the 40 nucleotides in JEP-1, 29 were complementary to the 53-np *Bam*HI cleavage fragment and 11 were complementary to the 120-np fragment.

Specimens. To evaluate the amplification of BKV and JCV genomes in clinical specimens, 57 urine specimens stored from previous studies were tested by PCR. These were (i) 11 urine specimens from renal allograft and bone marrow transplant recipients which were positive by in situ filter hybridization by using mixed BKV and JCV probes, (ii) 30 urine specimens from healthy adults which had previously tested negative with BKV and JCV probes, and (iii) 16 urine specimens from 4 BKV-seronegative bone marrow transplant recipients. These urine specimens were not tested previously, but they were obtained from BKV-seronegative

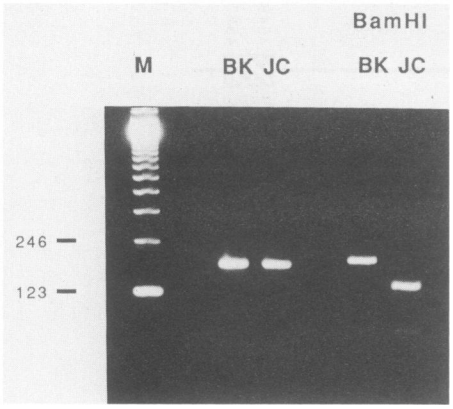

FIG. 2. Detection of PCR-amplified BKV and JCV DNA in agarose gels. Lane M contains size markers (123-base-pair ladder; Bethesda Research Laboratories-Life Technologies). Amplified BKV and JCV resulted in sequences of 176 and 173 np, respectively. The amplified JCV sequences were cleaved by *Bam*HI to produce fragments of 120 and 53 bases. The BKV sequence was not cleaved by *Bam*HI. Numbers to the left of the gel are in nucleotide pairs.

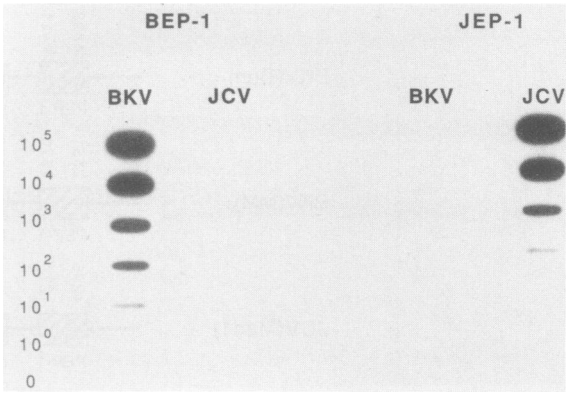

FIG. 3. Sensitivity of PCR for the detection of cloned BKV and JCV DNAs and the specificity of detection with ^{32}P-labeled oligonucleotide BEP-1 and JEP-1 probes by slot blot hybridization. The numbers on the left represent the number of genome copies contained in 10 μl (1/10) of the reaction mixture prior to amplification. Viral DNA was diluted in human placental DNA (0.1 μg/μl). The autoradiographic exposure time was 1 h.

patients who were not shown to excrete BKV in a previous prospective study (5). Urine specimens (10 to 50 ml) were centrifuged (1,500 × *g*, 15 min), and the sediments were stored at −70°C. Either DNA extracted from urinary cell pellets or boiled urinary cell pellets were tested by PCR. DNA was extracted (23) from urine sediments and was suspended in 50 μl of 10 mM Tris hydrochloride and 1 mM EDTA (pH 8.0). Alternatively, the urine pellet was suspended in an equal volume of PCR buffer and boiled for 10 min, and 10 μl was added to the PCR mixture. Parallel studies in which urine samples were tested by the two methods showed that both methods were adequate for making viral DNA in the sample available for amplification.

Paraffin-embedded brain tissue from a needle biopsy of a patient with acquired immunedeficiency syndrome with progressive multifocal leukoencephalopathy (diagnosed on the basis of histopathology and JCV antigen detection) was processed as described by Shibata et al. (37). Briefly, a single 5- to 10-μm section was placed in a microcentrifuge tube and deparaffinized with xylene. Following centrifugation, the xylene was decanted and the pellet was washed twice with 95% ethanol. The tissue pellet was desiccated and tested by PCR.

RESULTS

To standardize PCR, known quantities of molecularly cloned BKV and JCV DNAs (purified from vectors by restriction endonuclease digestion and gel electrophoresis [2]) were used as templates and amplified by using a single pair of 20-mer oligonucleotide primers (PEP-1 and PEP-2). Agarose gel electrophoresis of the PCR products revealed bands of the appropriate size, as predicted by sequence data for BKV (176 np) and JCV (173 np) (Fig. 2). Furthermore, treatment of the PCR products with *Bam*HI resulted in cleavage of the JCV DNA to produce the predicted 120- and 53-np fragments. As expected, BKV DNA was not cleaved by *Bam*HI. Further confirmation of amplification of BKV and JCV sequences was obtained by hybridization of the reaction products with the ^{32}P-labeled, virus-specific oligonucleotide probes BEP-1 and JEP-1 (Fig. 3 and 4). The probes hybridized only to the fragments of the appropriate size. The 10 μl of PCR products tested by slot blot hybridization represented amplification and detection of 10 and 100 copies of BKV and JCV DNAs, respectively, in the reaction mixture (Fig. 3). Negative hybridization results were obtained when the same DNA standards were tested prior to amplification (data not shown). Cross-hybridization between BKV and JCV DNAs was not observed.

Eleven urine specimens previously found (by in situ filter hybridization with a mixed probe) to contain BKV, JCV, or both were amplified by PCR; and the reaction products were analyzed by agarose gel, slot blot, and Southern hybridization. Of the 11 specimens, 7 were positive for BKV, 1 was positive for JCV, and 3 were positive for both viruses (Table 1). BKV DNA (176 np) was seen in all lanes except lane 5 in Fig. 4A. The cleavage of JCV sequences was seen in lanes 1, 3, 5, and 10 of Fig. 4A. The larger JCV fragments (120 np) were visible on the agarose gel, but the smaller JCV fragments (53 np) were somewhat obscured by nonspecific primer bands. On the autoradiograms, the JEP-1 probe hybridized with the 53-base sequence. Specimens in lanes 1, 3, and 10 of Fig. 4B) were positive for both viruses. The signal observed in lane 5 (Fig. 4B) at the 173-np position was seen on the JCV autoradiogram but not on the BKV autoradiogram. It represented undigested JCV DNA. Nonspecific bands were present on the ethidium bromide-stained gel, but they did not hybridize with either probe. The PCR products of the urine specimens were also tested by slot blot hybridization; the results were identical to those obtained by Southern hybridization.

The reaction products from urine specimens from healthy adults and BKV-seronegative bone marrow transplant recipients were tested by slot blot hybridization. Of 30 specimens from healthy adults, 1 was positive for BKV, 1 was positive for JCV, and 1 was positive for both viruses (Table 1). None of the 16 specimens from the four BKV-seronegative bone marrow transplant recipients was positive for BKV (Table 1). However, two of these patients (patients B and D) each had a single specimen positive for JCV. Both patients had JCV-specific antibody in sera collected before transplantation. JCV was not detected in any of four urine specimens from the third JCV-seropositive patient (patient C).

A single paraffin section of brain tissue from the patient with progressive multifocal leukoencephalopathy was tested

TABLE 1. Detection by PCR of BKV and JCV in urine specimens from immunosuppressed and healthy subjects

Subject	Serological status		BKV and JCV in situ filter hybridization	Specimens			
				No.	No. positive by PCR for:		
	BKV	JCV			BKV	JCV	BKV and JCV
Bone marrow and renal transplant recipients (n = 11)	Unknown	Unknown	Positive	11	7	1	3
Healthy adults (n = 30)	Unknown	Unknown	Negative	30	1	1	1
Bone marrow transplant recipients (n = 4)							
Patient A	−	−	NT[a]	3	0	0	0
Patient B	−	+	NT	7	0	1	0
Patient C	−	+	NT	4	0	0	0
Patient D	−	+	NT	2	0	1	0

[a] NT, Not tested.

as described previously. The PCR products were digested with *Bam*HI and examined on agarose gels. Two fragments of 120 and 53 np were seen. The reaction products were positive for JCV and negative for BKV when tested by slot blot hybridization.

Several observations were made regarding the three methods used to identify the amplified viral sequences in the PCR products. The most rapid diagnosis was obtained by the agarose gel method. After completion of the PCR, it required 1 h for the *Bam*HI digestion and 1 h for gel electrophoresis. Ethidium bromide was added to the gel and electrophoresis buffer to eliminate a separate staining procedure. The agarose gel method was approximately 100-fold less sensitive than the slot blot hybridization method, when different quantities of reference DNA were amplified by PCR and viral DNA detection by the two methods was compared. In the limited number of specimens examined, when bands of the appropriate size were observed, they were always found to be specific, as determined by Southern hybridization.

Results with the slot blot technique were obtained within 4 to 4.5 h. The short hybridization time (1 h) and exposure time for autoradiography (1 h) made it possible to obtain results within 1 day after thermal cycling was completed. With longer periods of exposure for autoradiography, interpretation of results was more difficult because nonspecific signals occurred with negative control urine specimens and human placental DNA. The Southern technique was more specific in this respect, since signals were discriminated on the basis of size. The longer exposure times (1 to 3 days) that were required for Southern hybridization possibly reflected the loss of PCR product during transfer from agarose gels to nylon membranes.

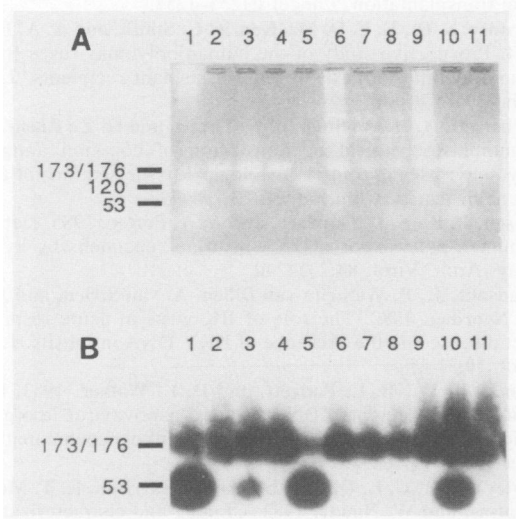

FIG. 4. Amplification of BKV and JCV in urine specimens by PCR and analysis of reaction products by gel electrophoresis (A) and Southern hybridization transfer (B). PCR products were treated with *Bam*HI prior to electrophoresis. Hybridizations with BEP-1 and JEP-1 were performed sequentially on the same filter after the probe was removed by washing it at 95°C. The photograph in panel B was prepared by superimposing the autoradiograms from the hybridizations with BEP-1 and JEP-1 (A). Autoradiographic exposure times were from 1 to 3 days.

DISCUSSION

Both BKV and JCV DNA segments were amplified by PCR by a single pair of primers which were complementary to the same regions of both viruses. The sequences flanked by the primers were sufficiently dissimilar for the two viruses, so that the viruses could be differentiated by hybridization with virus-specific oligonucleotide probes or by cleavage with a restriction endonuclease. Agarose gel electrophoresis was used as a simple and rapid method for the detection and identification of BKV and JCV sequences in reaction products after thermal cycling. Hybridizations with ^{32}P-labeled probes performed in a slot blot format were more sensitive for virus detection than examination of agarose gels. Southern hybridization was used to establish that amplified segments were of the expected size and that they hybridized specifically with oligonucleotide probes with sequences unique for each virus. The identification of PCR products by slot blot hybridization and Southern hybridization gave identical results.

PCR has several distinct advantages over existing techniques for the diagnosis of polyomavirus infections. Amplification by PCR resulted in a 100- to 1,000-fold-greater sensitivity in the detection of viral DNA. As few as 10 and 100 copies of BKV and JCV, respectively, were identified. Among healthy adults, nearly all of whom are presumed to be infected, polyomaviruses were detected in 10% of urine specimens by PCR, whereas results with in situ filter hybridization were uniformly negative. A major limitation of the PCR is that false-positive results are obtained as a result of contamination of the specimen or reaction mixture. No contamination was evident when specimens from BKV-

seronegative bone marrow transplant recipients were tested for BKV in the same manner as the specimens from healthy adults were. JCV was detected in immunosuppressed bone marrow transplant recipients, but only in JCV-seropositive individuals.

Detection and identification of BKV and JCV in the PCR products by hybridization with virus-specific oligonucleotide probes provide a level of specificity that is difficult to attain by other techniques (2, 21). In previous studies in which full-length genomic probes were hybridized with cellular DNAs from clinical samples, some cross-hybridization was seen between BKV and JCV DNAs. In the present study, there was no cross-hybridization when PCR-amplified DNA fragments were hybridized with oligonucleotide probes. Therefore, mixed infections could be diagnosed with confidence. The PCR products contained large amounts of amplified DNA. Therefore, it should be possible to identify the amplified genome with nonisotopic probes.

In addition to high sensitivity and specificity, the short turnaround time from the receipt of the specimen to the final result makes PCR attractive for diagnostic use. Results can be obtained on the day of or the day after specimen receipt. Processing of the urinary cell pellet by boiling, instead of DNA extraction, shortens the time required to perform PCR and also eliminates the risk of cross-contamination of specimens during the extraction procedure. We also found that the length of the thermal cycle can be reduced by one-half without any deleterious effect on amplification. These modifications would make PCR testing and analysis by slot blot hybridization possible within 24 h. The slot blot procedure is also particularly well-suited for the testing of many samples simultaneously, a distinct advantage for epidemiological studies.

The identification of BKV and JCV by PCR may be useful in some clinical situations, e.g., in studies of brain tissue of patients suspected of having progressive multifocal leukoencephalopathy. The excretion of human polyomaviruses in urine is correlated with some urinary tract illnesses, especially hemorrhagic cystitis in bone marrow transplant recipients; but in most instances, viruria is not associated with the disease. Therefore, the significance of virus identification in urine by PCR requires evaluation in the context of the clinical and pathological findings. A negative result would help eliminate BKV or JCV as playing any role in the disease.

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