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Effects of Fixation and Varying Target Length on the Sensitivity of Polymerase Chain Reaction for Detection of Human T-Cell Leukemia Virus Type I Proviral DNA in Formalin-Fixed Tissue Sections

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In this study, the fixation condition most suitable for maintaining the sensitivity of the polymerase chain reaction (PCR) was investigated by using the α-tubulin gene sequence, and the PCR procedure most effective for detecting human T-cell leukemia virus type I (HTLV-I) proviral sequences in fixed, embedded tissues of adult T-cell leukemia patients was explored. First, the sensitivity of the PCR targeting a 286-bp α-tubulin sequence was studied in tissue sections fixed in several fixatives for various periods at 25 or 4°C. For histological examination, fixation with 10% buffered formalin at a lower temperature for a shorter period was found to be preferable to retain the sensitivity. And the HTLV-I sequence was detected in only 7 of 18 specimens (38.9%) when the 374-bp sequence of the *gag* region was targeted, but the rate increased to 77.8% (14 of 18 specimens) when the length of the target sequence was reduced to 120 bp within the same region. Therefore, the one-round PCR targeting a shorter sequence is preferable for application of PCR to archival fixed tissue specimens, the fixation condition of which may not be ideal for DNA preservation.

The polymerase chain reaction (PCR) procedure has the potential advantage of retrospective analysis of archival fixed tissues (7, 14), but a number of factors, including fixation conditions and some potent inhibitors, etc., have been shown to influence PCR results when paraffin-embedded fixed tissues are used as the source of DNA (2, 6, 8, 10, 12, 15).

We previously demonstrated that the sensitivity of PCR is seriously affected by both the fixation time and the length of the target sequence (10) in a study with DNA extracted from fixed MT-2 cells, a line of human lymphoid cells harboring human T-cell leukemia virus type I (HTLV-I) (9). However, PCR using DNA extracted from bulk tissue or a number of tissue sections cannot localize a precise lesion. Therefore, direct application of a tissue fragment from a single section to PCR (6, 14, 15) gives a great advantage since a stained neighboring section tells the lesion.

In this study, we attempted to determine the optimal fixation conditions of tissue specimens for PCR using the sequence of the α-tubulin gene, a ubiquitous gene in the human genome. The ubiquity of the gene makes it possible to compare the results induced by various fixation conditions. In addition, we explored the most effective PCR procedure for the detection of HTLV-I proviral sequences in adult T-cell leukemia (ATL) patients using single paraffin-embedded sections from various organs fixed in several pathology laboratories.

MATERIALS AND METHODS

Sources of clinical materials. A total of 18 paraffin blocks of the spleen, lymph nodes, skin, and mandibular gland were obtained from 12 autopsy cases with clinical and serological confirmations of a diagnosis of ATL (Table 1). These tissue blocks were kindly supplied by S. Akizuki, Department of Pathology, Medical College of Oita; H. Kumamoto, Department of Oral Pathology, Tohoku University School of Dentistry; T. Masuda and K. Goto, Department of Pathology, Tohoku University School of Medicine; T. Ohto, Division of Pathology, Sendai National Hospital; and M. Yoshida, Department of Cellular and Molecular Biology, The Institute of Medical Science, The University of Tokyo. These tissue specimens were fixed in unbuffered or phosphate-buffered 10% formalin and embedded in paraffin at the above-mentioned institutions. Information on the temperature and period of fixation and the volume ratio of the tissue samples to fixatives were not available.

Tissue preparation. To determine the effect of tissue preparation on the sensitivity of detection of an α-tubulin sequence in normal spleen tissue, tissue fragments, each measuring approximately 7 by 9 by 3 mm, were fixed in 15 ml of one of the following fixatives at room temperature (25°C) or 4°C for various periods of time: Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid, pH 2.6), 10% formalin (10% dilution in water of a commercial formalin solution containing 37% formaldehyde and 10% methanol, pH 3.5), 10% neutralized buffered formalin (10% dilution of a commercial formalin solution in phosphate buffer consisting of 26 mM sodium phosphate monobasic and 46 mM sodium phosphate dibasic, pH 7.4), and Bouin's fixative (71% saturated picric acid, 8.8% formaldehyde, 2.4% methanol, 5% glacial acetic acid, pH 1.2).

The fixed tissues were dehydrated in graded ethanol and embedded in paraffin. With caution to avoid cross-contamination, 3-μm-thick serial sections were made from the paraffin blocks. The microtome and cutting blade were carefully cleaned with xylene when blocks were changed. As controls, 3-μm-thick frozen sections were prepared from some un-

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TABLE 1. Detection of HTLV-I-specific sequences in fixed and embedded tissues from ATL patients

Specimen no.	Case no.	Organ	Detection by:		
			One-round PCR targeting:		Nested PCR
			374 bp	120 bp	
1	1	Spleen	+	+	+
2		Lymph node	+	+	+
3	2	Spleen	−	+	+
4		Lymph node	−	+	+
5	3	Spleen	−	+	+
6		Skin	−	−	+
7	4	Spleen	+	+	+
8		Lymph node	+	+	+
9	5	Spleen	+	+	+
10		Lymph node	+	+	+
11		Mandibular gland	−	+	+
12	6	Spleen	−	+	+
13	7	Spleen	−	−	−
14	8	Spleen	−	−	−
15	9	Lymph node	−	−	−
16	10	Spleen	−	+	+
17	11	Spleen	−	+	+
18	12	Spleen	+	+	+

fixed tissues embedded in a Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, Ind.) and chilled in liquid nitrogen.

Sample preparation for the assessment of PCR sensitivity. Direct detection of the DNA sequences in tissue sections was done by the method of Wright and Manos (15). For detection of the α-tubulin sequence in the normal spleen tissue, after removal of paraffin or the O.C.T. compound with xylene or water from tissue sections mounted on glass slides, approximately 10 mm^2 of each tissue fragment was scraped off with a disposable razor blade. To avoid fixation variability, sections were taken from the surface of the tissue block. In the case of ATL, an abundance of $CD4^+CD45RO^+$ cells, the main target cells of HTLV-I infection (11), was immunohistochemically confirmed in neighboring sections with the OPD4 monoclonal antibody (Dako Japan Co., Ltd., Kyoto, Japan) (16). The tissue samples were completely digested in 100 μl of the buffer (50 mM Tris hydrochloride, pH 8.5, 1 mM EDTA) containing 200 μg of proteinase K (E. Merck, Darmstadt, Germany) per ml and 0.5% of Tween 20 at 42°C overnight. After complete digestion, proteinase K was inactivated at 100°C for 8 min, and 10 μl of the aliquot or 10 μl of the dilution was subjected to PCR.

Enumeration of nuclei in the tissue fragments subjected to PCR. The sections adjacent to those for PCR were stained with hematoxylin-eosin and photographed. Nuclear profiles in these areas were enumerated with a model SE/30 computer (Macintosh, Capertino, Calif.) equipped with a GT-400 scanner (Epson, Tokyo, Japan). In the case of ATL, the number of cells positive for the OPD4 antibody was counted in the neighboring sections at a magnification of ×100.

Extraction of DNA from tissue sections. DNA was extracted from frozen or paraffin-embedded tissue sections by following the method of Goelz et al. (5). After removal of embedding media, tissue fragments from 10 consecutive sections were pooled in a buffer solution composed of 500 mM Tris hydrochloride (pH 9.0), 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate, and 500 μg of proteinase K per ml. The samples were digested at 48°C for 24 h and phenolized three times, after which DNA was finally precipitated by ethanol.

Assessment of PCR sensitivity. The protocol for PCR and hybridization was essentially the same as described previously (10) except that *Taq* polymerase and the buffer (10 mM Tris hydrochloride [pH 8.8], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

To examine the sensitivity of PCR, serial 10- or 2-fold dilutions of the digestion of either fixed or unfixed tissues were subjected to PCR. First, the final dilutions with a specific band detected by ethidium bromide staining and/or hybridization were determined. In parallel, the number of nuclear profiles contained in a tissue fragment subjected to PCR was enumerated as described above, and the number of nuclear profiles contained in the final dilution was calculated. PCR was performed in a duplicate manner, and the sensitivity of PCR was expressed as the ratio of the mean number of nuclear profiles contained in the final dilution of the unfixed tissue to that of the fixed tissue.

Primers and probes. The primers and the probe were synthesized with a DNA synthesizer (model 391 PCR-MATE EP; Applied Biosystems, Foster City, Calif.). For amplification of the α-tubulin sequence, 5′-GACAGAATTC CAGACCAACC-3′ and 5′-GCACCAATCCACAAACTG GA-3′, corresponding to nucleotide positions (n.p.) 756 to 775 and 1041 to 1022, respectively, were used (3). For amplification of the HTLV-I sequences, two sets of primers were designed according to the sequence of the *gag* region reported by Seiki et al. (13). 5′-ATTAAGCAAGAAGTCTC CCAA-3′, corresponding to n.p. 1258 to 1278, and 5′-AAGG CGTGGTAAGGCTCCTCC-3′, corresponding to n.p. 1631 to 1611, were designed as the outer primers for the longer target product (374 bp). Primers targeting the 120-bp product (n.p. 1301 to 1420) were the same as in the previous paper (10) and were used as the inner primers, which were nested within the outer primers. The probe sequence was 5′-ATCA CCAATGCTTGCTTTGA-5′ for the α-tubulin sequence (n.p. 871 to 890), and the same sequence (10) as in the previous paper was used for HTLV-I (n.p. 1359 to 1378).

RESULTS

Comparison of PCR sensitivities with extracted DNA and frozen tissues. In parallel to the detection of the α-tubulin sequence by PCR in serial dilutions of 1 μg of DNA extracted from a normal spleen, a frozen tissue section of the same spleen was digested, serially diluted, and subjected to PCR targeting the same 286-bp sequence. The number of nuclei in the final dilution of the extracted DNA with a positive signal was calculated on the basis of the fact that 1 μg of DNA was equal to 1.7×10^5 diploid cells. The number of nuclei in the final dilution of the frozen tissue section with a positive signal was deduced from the nuclear profiles in an adjacent section. The ratio of nuclear numbers in the final dilution of extracted DNA and the frozen section was approximately 1:1.1. Thus, the purification process does not seem to improve the sensitivity of PCR.

Effects of fixatives and fixation time. When a pair of primers targeting 286 bp of the α-tubulin sequence was used, the sensitivity of the PCR definitely decreased as a function of fixation time regardless of which fixative was used. Among four fixatives, Carnoy's fixative gave the best result. Even after 10-day fixation, the sensitivity was reduced by less than 1/10. In contrast, when tissue was fixed in Bouin's fixative, the sensitivity rapidly declined and no signals were detected

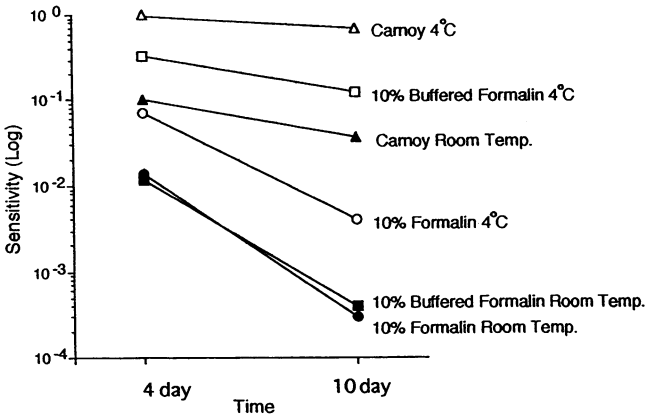

FIG. 1. Effect of fixation at 4°C on the sensitivity of PCR. Normal spleen was fixed in three kinds of fixatives at 4°C for 4 or 10 days. The sensitivity of PCR was calculated as described in Materials and Methods, and the values at 4°C were plotted (open symbols). For comparison, the values at 25°C were also plotted (closed symbols).

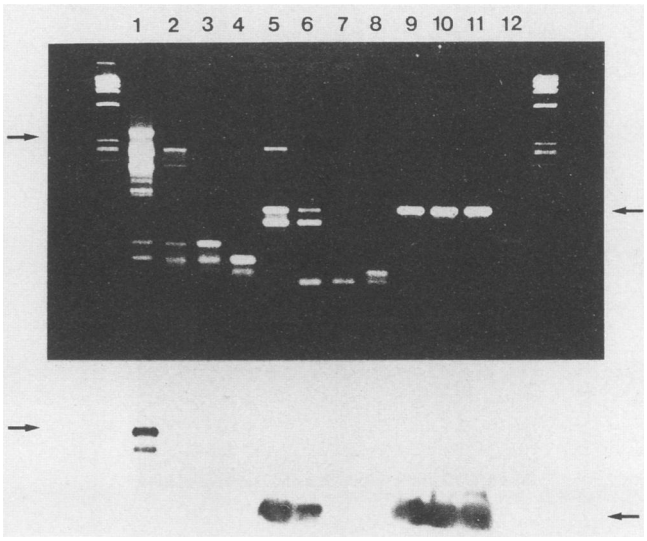

FIG. 2. Representative electrophoresis and hybridization of PCR. At the top is ethidium bromide staining of a 4% agarose gel, and at the bottom is a hybridization with a digoxigenin-labeled probe. The longer product with 374 bp (lanes 1 to 4) was amplified only in specimen 1 (lane 1). The shorter product with 120 bp (lanes 5 to 8) was amplified in specimens 1 and 3 (lanes 5 and 6). Nested PCR (lanes 9 to 12) amplified the shorter product in specimens 1, 3, and 6 (lanes 9, 10, and 11). However, no HTLV-I-specific sequences were amplified in specimen 13 (lanes 4, 8, and 12). The numbers of the specimens are referred to in Table 1. Lanes 1, 5, and 9, specimen 1; lanes 2, 6, and 10, specimen 3; lanes 3, 7, and 11, specimen 6; lanes 4, 8, and 12, specimen 13. Lanes on both sides contain PhiX DNAs digested by *Hae*III as size markers. The arrows on the left and the right sides indicate the 374- and the 120-bp products, respectively.

after 1-day fixation. In the case of formalin, the most common fixative, the use of buffered or unbuffered diluent had little effect on the sensitivity of PCR. Loss of sensitivity took place gradually, reaching less than 10^{-3} on day 10.

Effects of fixation temperature. As a definite decrease in sensitivity was seen with longer fixation at room temperature in a previous experiment, the possibility of preservation of the sensitivity of PCR at a lower temperature was explored. The tissues were fixed in Carnoy's solution and 10% formalin, buffered or unbuffered, at 4°C for 4 or 10 days. The sensitivity substantially improved more than 10-fold in fixation with both Carnoy's solution and formalin (Fig. 1). In particular, the decrement of the sensitivity was negligible even after 10-day fixation with Carnoy's fixative. Of particular practical importance was a more than 100-fold increase in sensitivity after 10% buffered formalin fixation.

Detection of the HTLV-I sequence. Since the conditions of tissue fixation vary and are not optimized in most archival tissue samples, we attempted to improve the sensitivity by modification of the PCR protocol in the detection of HTLV-I sequences in the ATL tissue samples processed in different laboratories.

First, tissue specimens were subjected to PCR using outer primers with an expected product length of 374 bp. As shown in Fig. 2 and Table 1, the HTLV-I-specific sequence was detected by ethidium bromide staining and/or hybridization in only 7 of 18 specimens (38.9%). Next, PCR was carried out using inner primers with an expected length of 120 bp. Positive signals were then detected in 14 of 18 specimens (77.8%) (Fig. 2 and Table 1). The HTLV-I-specific sequence was clearly detected in 7 of 11 specimens which were negative by the former PCR using outer primers.

Using these two pairs of inner and outer primers, the so-called nested PCR or two-step PCR was carried out with four specimens formerly found to be negative by the PCR targeting 120-bp sequences. The HTLV-I sequence was then detected in one of these specimens. The nested PCR amplified the HTLV-I sequence in 15 of 18 specimens (83.3%), but 3 specimens remained negative (16.7%) (Fig. 2 and Table 1).

Study of PCR using extracted DNA from bulk tissue. Since no HTLV-I sequences were detected in three cases, DNA was extracted from 10 consecutive sections from those cases and the nested PCR was performed. When 1 μg of DNA was subjected to PCR, the specific band of the HTLV-I sequence was detected in one of three cases but not in the others (Fig. 3). When 10-fold serial dilutions were subjected to PCR, no specific bands were detected in any of those dilutions (data not shown).

Then, by using a section adjacent to one for PCR, the total number of nuclear profiles and the number of OPD4-positive cells in the fragment subjected to PCR were estimated. The number of OPD4-positive cells was 5,835, and the total number of cells was 41,420, a ratio of approximately 1:7. Although the ratio varies among these 10 consecutive sections, the number of OPD4-positive cells in 1 μg of extracted DNA was roughly estimated to be 1.4×10^4 (one-seventh of 10^5 cells). Therefore, 10,000 to 20,000 infected cells were required to obtain HTLV-I-specific signals in this specimen (specimen 13). The number of nuclei of OPD4-positive cells in the fragment subjected to PCR was definitely smaller than the required number.

In the remaining two cases, even the α-tubulin-specific sequence could not be amplified from 1 μg of extracted DNA (data not shown), indicating that the sensitivity of PCR had decreased at least 10^5-fold in these specimens.

DISCUSSION

A major reason pathologists find PCR attractive is its ability to amplify impure and fragmented DNA. This means that archival fixed and embedded tissue specimens can be used as a source of DNA. In addition, a precise lesion can be

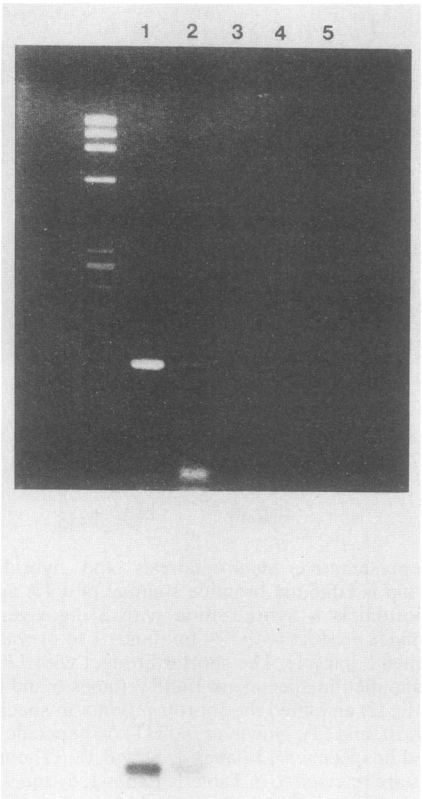

FIG. 3. Electrophoresis and hybridization of the nested PCR of DNA extracted from specimens of ATL tissues in which the specific band of HTLV-I was not amplified by the method directly using tissue fragments. One microgram of DNA extracted from consecutive sections (specimens 9, 13, 14, and 15) was subjected to nested PCR. An HTLV-I-specific band was detected in specimen 13 but not in specimens 14 and 15. Lane 1, specimen 9 as a positive control; lane 2, specimen 13; lane 3, specimen 14; lane 4, specimen 15; lane 5, DNA from normal spleen as a negative control. The lane on the left side contains PhiX DNA digested by *Hae*III as a size marker.

localized in an adjacent histological preparation. Using this method, we attempted to detect HTLV-I sequences in tissues from ATL patients. PCR failed to detect HTLV-I sequences in some specimens, although the standard PCR in our system can detect HTLV-I sequences in a 10^{-5} dilution of 1 μg of extracted DNA from unfixed MT-2 cells (10). Several papers, including our previous report (2, 6, 8, 10, 12), have suggested that the fixation process for tissue specimens is a critical factor for determining the sensitivity of PCR amplification and that a proper setting of fixation conditions should substantially increase the sensitivity of PCR. As demonstrated in extracted DNA (10), shorter fixation time is useful for maintaining the sensitivity of PCR. In addition, the present paper demonstrated that a lower temperature also helps retain the sensitivity.

Although Carnoy's fixative, an alcohol-based fixative, gave the best results in PCR, this fixative is known to be poor for histological studies (8). Thus, for general purposes, fixation with 10% buffered formalin at a lower temperature seems to be the best choice because the reduction of the sensitivity in the present study was less than 1/10 even after 10-day fixation at 4°C.

Surprisingly, the sensitivity in 10% unbuffered formalin was almost the same as that in 10% buffered formalin at room temperature. Jackson et al. reported that the DNA yield from tissues fixed with unbuffered formalin at room temperature was almost the same as that with buffered formalin (8). If the DNA yield reflects DNA preservation, our results are in agreement with their results. Since the data showed that fixation for a longer period at room temperature markedly decreased the sensitivity of PCR, the failure to detect even α-tubulin sequences in 2 specimens of 18 (11.1%) in the present study probably was at least partly due to suboptimal fixation of the tissues, although the coexistence of some inhibitors for *Taq* polymerase (4) should be taken into account.

It was also concluded that a rather short sequence, close to 100 bp, is preferable as a target sequence in tissue specimens possibly fixed and embedded under inappropriate conditions. Our results were consistent with the data obtained in an experiment using extracted DNA from fixed MT-2 cells (10). In that experiment, cells fixed for a long time were better amplified with a shorter target sequence. Nested PCR, utilized to detect human immunodeficiency virus type 1 sequences in crude lysates (1), has also been useful to circumvent the unfavorable situation induced by fixation. Coates et al. (2) also demonstrated that PCR targeting a shorter fragment and the two-step PCR are efficient procedures for detecting target DNA sequences in fixed tissues.

In 16.7% of specimens, neither the nested PCR nor the PCR targeting a rather short product could amplify HTLV-I proviral sequences with the method used in the present study. In one of these cases, however, a larger amount of purified DNA gave a positive signal. In the method used for tissue preparation in the present study, the impurity of DNA had very little influence on the sensitivity of PCR, as shown in Results. Greer et al. also reported that additional DNA purification resulted in little or no significant improvement in the amplification in this method (6). Therefore, the amount of DNA content in the tissue fragment subjected to PCR could be another factor inducing an increased chance of false-negative results with this method. With these cautions in mind, the direct application of PCR to histological sections may provide indispensable information for elucidation of the pathogenesis of disease.

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