Effects of Heparin on Polymerase Chain Reaction for Blood White Cells

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Polymerase chain reaction (PCR) has been used with increasing frequency to diagnose infectious and genetic diseases. In this study, the effects of heparin on PCR were investigated, because heparinized blood may sometimes be used in PCR studies. HLA-DQA1 gene amplification was used as a model. PCR was clearly interfered with when heparinized blood was used as a source of template DNA, and the degree of interference was affected by the following three factors; (1) type of *Taq* DNA polymerase; (2) leukocyte count in blood; and

(3) concentration of heparin contained. When additional tests were conducted with additions of definite heparin concentrations to a PCR reaction mixture, specimens with large amounts of DNA tended to exhibit less interference by heparin. The addition of ≥ 0.1 to 0.0016 U of heparin per reaction mixture (50 μ L) suppressed DNA amplification in a dose-dependent fashion. We therefore concluded that much care should be taken when heparinized blood is used as a PCR material. *J. Clin. Lab. Anal.* 13:133–140, 1999.

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Polymerase chain reaction (PCR) is now a basic experimental procedure for diagnosing various types of genetic disorders. White cells in peripheral blood are commonly used for PCR, since they can be obtained easily and routinely from patients with little difficulty. To extract DNA from white cells, citrated blood is recommended rather than ethylene diamine tetraacetic acid (EDTA) and heparin (1,2). In the medical laboratory, acid-citrate-dextrose (ACD), EDTA or heparin is usually used for the purpose of blood anticoagulation, and heparinized blood is a good candidate for material for PCR testing. However, it has not yet been determined whether heparin can be used for this purpose. Its utility in this respect was examined in this study.

MATERIALS AND METHODS

Routine Blood Samples Submitted to the Laboratory for HLA-DQA1 Gene Analysis

Ten blood samples were anticoagulated with heparin sodium (heparin, 16U/mL blood) (Novo nordisc A/S; Bagsvaerd, Denmark) and dipotassium ethylenediamine tetraacetic acid (K2-EDTA, 1.5 mg/mL blood) (Wako Pure Chemical, Osaka, Japan), and subjected to genetic analysis.

Blood Specimens

Venous blood anticoagulated with K2-EDTA was obtained from healthy donors (aged 30 to 40 years). Total white cells

were first counted using an automatic blood cell counter (NE-8000, Sysmex Corp. Kobe, Japan).

Sample Preparation

Ten mL of EDTA treated blood was centrifuged at 200g for 2 min, and the supernatant was centrifuged at 1,000g for 5 min. The sediment, composed of leukocytes, was resuspended in a small amount of plasma. Leukocyte-rich plasma and leukocyte-free plasma were added to adjust white cell concentration. In this fashion, blood samples containing leukocytes at 2,700 and 12,000/ μ L were prepared (Groups A and E). In the same manner, venous blood was collected in tubes containing different concentrations of heparin. The effects of heparin were also tested with a cell suspension with constant heparin concentrations of 16 (Groups B and F), 48 (Groups C and G), and 96 U/mL blood (Groups D and H).

DNA Extraction

Whole blood (0.5 mL) was treated with 200 μ L of a lysing solution consisting of 80 μ L of TNE buffer (10 mM Tris-

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HCl, pH 8.0, 1 mM Na₂-EDTA, and 100 mM NaCl), 100 μ L of 10% sodium dodecyl sulfate (pH 8.0), and 20 μ L of 10 mg/mL proteinase K solution (Wako). The mixture was incubated for 3 hr at 55°C prior to extraction with TE-phenol (phenol saturated with TE buffer consisting of 10 mM Tris-HCl and 1 mM Na₂-EDTA, pH 8.0). A second extraction was performed with a mixture of TE-phenol:chloroform:isoamyl alcohol (25:24:1, by volume), and a third extraction with a mixture of chloroform: isoamyl alcohol (24:1, by volume), retaining the upper aqueous phase at each step. Sodium acetate was added to the final aqueous solution to make a final concentration of 0.3 M. DNA was precipitated with two volumes of chilled absolute ethanol at -20°C overnight, followed by centrifugation at 12,000g for 10 min. After washing with chilled 70% ethanol, the pellets were dried, resuspended in 1 mL of TE buffer at 55°C for 4 h, and stored at 4°C.

DNA Quantitation

Extracted DNA was quantified by measurement of optical density at 260 nm (OD₂₆₀) using a spectrophotometer (U2000; Hitachi, Tokyo). To determine the purity of extracted DNA, the optical density at 280 nm (OD₂₈₀) was measured, and the OD₂₆₀/OD₂₈₀ ratio was evaluated.

Polymerase Chain Reaction

To determine the feasibility of amplification, the HLA-DQA1 locus was used and studied with GH26 and GH27 (3) primers.

A) Routine blood samples were prepared as follows: The PCR reaction mixture (50 μ L) included 100 ng of DNA extracted from the whole blood anticoagulated with heparin or K₂-EDTA, 200 μ M of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Takara, Kyoto), 1.25 units of

rTaq DNA polymerase (Takara, Takara *Taq*), and 5 μ L of appropriate 10 $\times$ reaction buffer, and 10 pmol of each HLA-DQA1 primer.

B) Amplification of a HLA-DQA1 gene from 7 μ L of extracted DNA samples (100, 50, 33, and 16 ng of DNA) from the whole blood mixed with heparin or K₂-EDTA was performed.

C) Amplification of a HLA-DQA1 gene from 100 or 10 ng of extracted DNA from EDTA-blood was performed in the presence of a 5-fold dilution series of quantities of heparin (5 to 0.00032 U per PCR reaction mixture, 50 μ L).

The *Taq* DNA polymerases used in the above experiments (B and C) were Takara *Taq* (Lot No. 100), *ExTaq* DNA polymerase (Takara) (*ExTaq*) (Lot No. A1201), *AmpliTaq* DNA polymerase (Perkin Elmer Cetus Instruments, Norwalk; Lot No. G1362) (*PE Taq*), *Taq* DNA polymerase (Amersham Pharmacia Biotech, Ltd, Little Chalfont, England; Lot No. 6110798011) (*Ph Taq*), *rTaq* DNA polymerase (Wako; Lot No. 61108A) (*Wako Taq*), and *rTaq* DNA polymerase (Qiagen K.K., Tokyo, Lot No. AU97012) (*QIA Taq*).

Samples were heated at 94°C for 5 min in the first round of denaturation and were then subjected to 32 cycles of PCR consisting of 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C. Cycling was performed in an automated DNA thermal cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus Instruments). After the last cycle, the samples were incubated for an additional 5 min at 72°C.

Amplified DNAs that should have resulted in a 242-bp product were checked on a 3% (w/v) NuSieve GTG agarose gel (Takara) in a minigel apparatus (Mupid, Cosmo Bio, Tokyo) and subjected to electrophoresis. DNA was visualized by ethidium bromide staining and photographed under ultraviolet light. To type the HLA-DQA1 gene, PCR products were digested with three restriction endonucleases from representative HLA typing sets, *Hae* III, *Dde* I, and *Rsa* I (4). Restriction

Fig. 1. Amplification of the HLA-DQA1 gene. 1 and 14, Φ X174/*Hae*III; 3, 5, 7, 9, 11, and 13, results of amplification with 100 ng of template DNA from leukocyte-poor blood specimens in presence of 16 U/mL blood of heparin; 2, 4, 6, 8, 10, and 12, with 100 ng of template

DNA from leukocyte-poor blood specimens in presence of 1.5 mg/mL blood of K₂-EDTA as control group; 2 and 3, with *ExTaq*; 4 and 5, with Takara *Taq*; 6 and 7, with *PE Taq*; 8 and 9, with *Ph Taq*; 10 and 11, with Wako *Taq*; 12 and 13, with *QIA Taq*.

tion fragments were detected by staining with ethidium bromide as described above.

Statistics

Student's *t*-test was used to determine the significance of differences between groups.

RESULTS

HLA Typing for EDTA Blood and Heparinized Blood

EDTA blood and heparinized blood were collected from single donors ($n = 10$), and capability of HLA typing was examined for the two different sources of blood using Takara *Taq*. The typing was successfully performed for all of EDTA blood samples, while amplification failed for 2 of 10 heparinized blood samples. When hematological data were checked thereafter, the 2 specimens with low amplification were found to have low leukocyte counts less than $3,000/\mu\text{L}$, although specimens with a leukocyte count between 2,800 to $9,500/\mu\text{L}$ were selected as materials. The results suggested two possibilities for heparinized blood: (1) heparin affected the yield of DNA; or (2) heparin interfered with amplification of DNA in PCR.

Relationships Between Concentration of Heparin in Blood and Amount of Extracted DNA

In order to examine the effects of heparin on yield of DNA (Assumption 1), DNA extraction was attempted for specimens with definite leukocyte counts but different concentrations of heparin. The amounts of DNA extracted from whole blood in the presence of 0, 16, 48, and 96 U/mL blood of heparin were 7.12 ± 0.725 , 7.55 ± 0.388 , 7.48 ± 1.00 , and 7.80 ± 0.538 pg/leukocyte, respectively. No significant difference ($P > 0.05$) was observed in amount of DNA extracted from blood between samples with and without heparin. The OD260/OD280 ratio was between 1.80 and 1.93 and purity was equivalent among those extracted DNAs. These findings indicated that heparin did not affect on the yield of DNA when a sufficient number of leukocytes was used.

Gene Amplification With the DNA Extracted From Whole Blood Mixed With Heparin

In order to test Assumption 2, the relationship between leukocyte count and the effect of anticoagulant (heparin) on DNA amplification was studied. Results of PCR amplification of HLA-DQA1 genes were as follows. PCR+: the amount of PCR product was sufficient for digestion with restriction enzymes for HLA typing, and PCR+W: PCR product was adequate for digestion with restriction enzymes but a slightly less intense band was observed electrophoretically. To type HLA-DQA1 genes, PCR products were therefore digested with restriction endonucleases and electrophoresed.

Inter-reagent differences among *Taq* DNA polymerases when 100 ng of extracted DNA was used for PCR as its template

The above experiments were conducted using Takara *Taq* DNA polymerase. In the next trials, differences in results of amplification with polymerases were studied using Takara

TABLE 1. Results of PCR Amplification With DNA Prepared From Whole Blood Anticoagulated With Heparin^a

<i>Taq</i> DNA polymerase	Group	Amount of DNA extracted from heparinized blood used as template			
		100 ng	50 ng	33 ng	16 ng
Ex <i>Taq</i>	B	+w	+	+	+
	C	—	+w	+w	+
	D	—	—	—	+w
	F	+	+	+	+
	G	+	+	+	+
	H	+w	+	+	+
Takara <i>Taq</i>	B	—	—	+w	+
	C	—	—	—	—
	D	—	—	—	—
	F	+	+	+	+
	G	+w	+	+	+
	H	—	+w	+	+
PE <i>Taq</i>	B	—	—	+w	+w
	C	—	—	—	—
	D	—	—	—	—
	F	+	+	+	+
	G	+w	+	+	+
	H	—	—	+w	+w
Ph <i>Taq</i>	B	—	—	+	+
	C	—	—	—	—
	D	—	—	—	—
	F	+	+	+	+
	G	+	+	+	+
	H	—	+w	+	+
Wako <i>Taq</i>	B	—	+	+	+
	C	—	—	+w	+w
	D	—	—	—	—
	F	+	+	+	+
	G	+w	+	+	+
	H	—	+	+w	+w

^aGroup B, DNA materials from leukocyte-poor blood with 16 U/mL blood of heparin; Group C, DNA materials from leukocyte-poor blood with 48 U/mL blood of heparin; Group D, DNA materials from leukocyte-poor blood with 96 U/mL blood of heparin; Group F, DNA materials from leukocyte-rich blood with 16 U/mL blood of heparin; Group G, DNA materials from leukocyte-rich blood with 48 U/mL blood of heparin; Group H, DNA materials from leukocyte-rich blood with 96 U/mL blood of heparin; + shows PCR+, amount of PCR product was sufficient for digestion with restriction enzymes for HLA typing; +w shows PCR+, amount of PCR product was adequate for digestion with restriction enzymes for HLA typing, but a slightly less intense band was observed electrophoretically (partial inhibition); —, PCR amplification was suppressed.

Taq, *Ex Taq*, *PE Taq*, *Ph Taq*, *Wako Taq*, and *QIA Taq*. Four concentrations of heparin (0, 16, 48, and 96 Units/mL blood) were studied for blood specimens with low leukocyte count (2,700/ μ l) (Group A, B, C, and D, respectively) and for blood specimens with high leukocyte count (12,000/ μ l) (Groups E, F, G, and H, respectively).

In the control groups (Groups A and E), PCR amplification of the HLA-DQA1 gene was successful with each *Taq* DNA polymerase tested.

In the leukocyte-poor blood specimens, low-concentration heparin (16 U/mL blood) yielded inter-reagent differences in DNA amplification among *Taq* DNA polymerases (Group B) (Fig. 1), but higher concentrations of heparin (48 and 96 U/mL blood) failed to yield amplification with any *Taq* reagents (Groups C and D) (Table 1).

In the high-leukocyte blood specimens, control (Group E), low and medium concentrations of heparin (16 and 48 U/mL blood; Groups F and G, respectively) yielded clearly observable bands with each *Taq* DNA polymerase except *QIA Taq*, although high-concentration heparin (96 U/mL blood; Group H) yielded negative results for all *Taq* DNA polymerases except *ExTaq* (Table 1).

Those findings indicated that differences in DNA amplification exist between reagents.

Inter-reagent differences among *Taq* DNA polymerases in dilution tests with extracted DNA as template

It was reported that heparin was extracted together with DNA by binding (5). Therefore, to determine the minimum inhibitory concentration of heparin-DNA complex in the PCR amplification mixture, dilution tests for DNAs extracted with heparin were carried out.

When three different amounts of DNA specimens extracted from EDTA blood (50, 33, 16 ng) of control groups A and E were amplified, all specimens exhibited clear bands electrophoretically for each type of *Taq* DNA polymerase, and were identified as PCR+. However, DNA extracted from heparinized blood exhibited inter-reagent differences in DNA amplification due to *Taq* DNA polymerase. DNA materials obtained from leukocyte-rich blood with heparin were more suitable than those obtained from leukocyte-poor blood with heparin. When the amounts of the extracted DNAs from heparinized blood used as templates, were reduced in stepwise fashion (50, 33, and 16 ng) in the amplification mixture, interference of DNA amplification tended to decrease.

Takara *ExTaq*, PCR amplification for 50, 33, and 16 ng of DNA materials prepared from leukocyte-poor blood with 16 and 48 U/mL blood of heparin and leukocyte-rich blood with 96 U/mL blood of heparin (Groups B, C, and H) was succeeded, but PCR amplification with 16 ng of DNA materials prepared from leukocyte-poor blood with 96 U/mL blood of heparin (Group D) was PCR+w (Fig. 2 and Table 1).

With Takara *Taq*, PCR amplification with 33 and 16 ng of extracted DNA prepared from leukocyte-poor blood with 16 U/mL blood of heparin and 50, 33, and 16 ng of extracted DNA prepared from leukocyte-rich blood with 96 U/mL blood of heparin (Groups B and H) succeeded, but those electrophoretic analyses demonstrated suppression of PCR with the DNA materials prepared from leukocyte-poor blood with 48 or 96 U/mL blood of heparin (Groups C and D) (Table 1).

With *PE Taq*, when 33 and 16 ng of extracted DNA prepared from leukocyte-poor blood with 16 U/mL blood of heparin and from leukocyte-rich blood with 96 U/mL blood of heparin (Groups B and H) were used as templates, PCR

Fig. 2. Amplification of the HLA-DQA1 gene with DNAs extracted from leukocyte-poor blood (2,700 / μ l). **a**, 1, ϕ X174/*Hae*III; 2 to 5, results of amplification from prepared DNAs in presence of 1.5 mg/mL blood of K2-EDTA as control group; 6 to 9, results of amplification from prepared DNAs in presence of 16 U/mL blood of heparin; 10 to 13, results of amplification from prepared DNAs in presence of 48 U/mL blood of heparin. **b**, 1, ϕ X174/

*Hae*III; 2 to 5, results of amplification from prepared DNAs in presence of 96 U/mL blood of heparin. **A** and **B**, 2, 6, and 10, results of amplification with 100 ng of DNA; 3, 7, and 11, results of amplification with 50 ng of DNA; 4, 8, and 12, results of amplification with 33 ng of DNA; 5, 9, and 13, results of amplification with 16 ng of DNA.

amplification was successful. However, suppression of PCR amplification was observed in DNA prepared from leukocyte-poor blood with 48 or 96 U/mL blood of heparin (Groups C and D) (Table 1).

Ph *Taq* yielded findings similar to those for PE *Taq* except in Group H. When 50, 33, and 16 ng of DNA prepared from leukocyte-rich blood with 96 U/mL blood of heparin (Group H) were used as templates, PCR amplification was successful (Table 1).

With Wako *Taq*, when 50, 33, and 16 ng of DNA materials prepared from leukocyte-poor blood with 16 U/mL blood of heparin, and 33 and 16 ng of DNA materials prepared from leukocyte-poor blood with 48 U/mL blood of heparin (Group C) and leukocyte-rich blood with 96 U/mL blood of heparin (Groups B and H) were used as templates, PCR amplifications were successful, but suppression of PCR amplification was observed when DNA prepared from leukocyte-poor blood with 96 U/mL blood of heparin was used (Group D) (Table 1).

With QIA *Taq*, PCR amplification was suppressed for DNA prepared from heparinized blood.

Figure 3 shows the relationship between calculated heparin amount and DNA as template with the assumption that added heparin was yielded completely together with the extracted DNA.

Figure 4 shows the calculated amount of maximally-available heparin in the PCR mixture. The quantities obtained were as follows: 0.106 U (100 ng of DNA)/PCR reaction mixture (50 μ L) for Ex*Taq*, 0.055 U (100 ng of DNA) for Takara *Taq*, 0.055 U (100 ng of DNA) for PE *Taq*, 0.055 U (100 ng of DNA) for Ph *Taq*, 0.055 U (100 ng of DNA) for Wako *Taq*, and less than 0.017 U/PCR reaction mixture (50 μ L) for QIA *Taq*.

Fig. 3. Relationship between calculated amount of heparin and extracted DNA as template with the assumption of complete yield of heparin. B, C, and D; Groups B, C, and D, with DNA from blood poor in leukocytes in the presence of 16, 48, and 96 U/mL of heparin, respectively, used as template; F, G, and H, Groups F, G, and H, with DNA from blood rich in leukocytes in the presence of 16, 48, and 96 U/mL of heparin, respectively, used as template.

Effects of Direct Addition of Heparin in the PCR Mixture

Figure 5 shows the inhibitory effects of added heparin on DNA amplification. The addition of heparin at more than 0.0016 to 0.1 U per 50 μ L of reaction mixture suppressed the appearance of a visible band on electrophoresis in a dose-dependent fashion.

The maximal tolerated amount of heparin for PCR for the various *Taq* DNA polymerases for 100 and 10 ng of template DNAs were as follows: 0.1 and 0.05 U for Ex*Taq*, 0.025 and 0.0125 U for Takara *Taq*, 0.1 and 0.025 U for PE *Taq*, 0.025

Fig. 4. Maximal tolerated amount of heparin in PCR mixture (50 μ L) derived by calculation for various *Taq* DNA polymerases. PCR+, Amount of PCR product was sufficient for digestion with restriction enzymes for

HLA typing; PCR+w, Amount of PCR product was adequate for digestion with restriction enzymes for HLA typing, but a slightly less intense band was observed electrophoretically.

Fig. 5. Maximally permissible amount of heparin added directly to PCR mixture for various *Taq* DNA polymerases. PCR+, Amount of PCR product was sufficient for digestion with restriction enzymes for HLA typing; PCR+w, Amount of PCR product was adequate for digestion with restriction enzymes for HLA typing, but a slightly less intense band was observed electrophoretically.

and 0.0125 U for *Ph Taq*, 0.05 and 0.025 U for *Wako Taq*, and 0.0016 and 0.0016 U for *QIA Taq* for 100 and 10 ng of template DNA, respectively (Figs. 5 and 6).

DISCUSSION

PCR was clearly interfered with when heparinized blood was used as a source of template DNA, and the degree of interference differed depending on the *Taq* DNA polymerase used. Reduction or dilution of the DNA used in PCR sometimes eliminated interference. When DNA was extracted from whole blood that contained the same concentration of heparin, suppression of amplification was greater for samples of DNA from blood poor in leukocytes than for that rich in leukocytes.

When tests of addition of heparin at definite concentrations were undertaken, specimens with large amounts of DNA exhibited less interference by heparin. The addition of ≥ 0.1 to 0.0016 U of heparin per reaction mixture (50 μ L) suppressed DNA amplification in a dose-dependent fashion. Interference studies were also carried out with HLA-DQA1 gene amplification as a model and considerable differences were found among *Taq* DNA polymerases in PCR amplification. The most dramatic inhibitory effect of heparin was observed when *QIA Taq* was used, followed by *Ph Taq*, *Takara Taq*, *Wako Taq*, *PE Taq*, and *Ex Taq* in order. The permissible level of heparin with *Ex Taq* was largest when 100 ng of template DNA was used. These findings suggest that the inhibition caused by heparin depends on the amount of template DNA

Fig. 6. Amplification of the HLA-DQA1 gene. 1 and 14, Φ X174/*Hae*III; 2 and 3, results of amplification for 100 and 10 ng of template DNA, respectively, with *Ex Taq*; 4 and 5, results of amplification for 100 and 10 ng of template DNA, respectively, with *Takara Taq*; 6 and 7, results of amplification for 100 and 10 ng of template DNA, respectively, with *PE Taq*; 8 and 9, results of amplification for 100 and 10 ng of template DNA, respectively, with *Ph Taq*; 10 and 11, results of amplification for 100 and 10 ng of template DNA, respectively, with *Wako Taq*; 12 and 13, results of amplification for 100 and 10 ng of template DNA, respectively, with *QIA Taq*. **a**, Heparin added at 0.1 U/50 μ L of PCR mixture; **b**, Heparin added at 0.05 U/50 μ L of PCR mixture; **c**, Heparin added at 0.025 U/50 μ L of PCR mixture; **d**, Heparin added at 0.008 U/50 μ L of PCR mixture; **e**, Heparin added at 0.0016 U/50 μ L of PCR mixture.

Fig. 6. (continued).

and on the type of polymerase used. The data indicate that heparin probably adsorbs to DNA during isolation of DNA from white cells and subsequently interferes with PCR.

Not only we but also Holodnity et al. found inhibition of gene amplification by heparin (6). Heparin, a strongly anionic physiological substance, anticoagulates blood by conjugating with thrombin and antithrombin III (7,8). Thus, the interference of heparin with PCR might have resulted from similar conjugation between heparin and *Taq* DNA polymerase peptide chain.

Suppression of gene amplification by heparin was dose-dependent, and the degree of inhibition by heparin in PCR varied considerably among *Taq* DNA polymerases, but the degree of interference was clearly influenced by leukocyte count in sample blood and the concentration of heparin added.

Assuming that the heparin added is yielded completely in the extraction process and that the dose of remaining heparin in the extracted DNA is calculated, the maximum per-

missible amount of heparin in PCR is 0.106 U/50 μ L PCR mixture (*Ex Taq*). The calculated maximal tolerated amount of heparin in the PCR mixture was nearly the same as that of heparin added directly to the PCR mixture. These findings indicate that heparin is extracted together with DNA after the liberation of DNA from cells and thereby interferes with PCR, as Beutler previously speculated (5). Therefore, almost all heparin added to blood appears to be collected together with DNA.

Routine specimens have sufficient numbers of white cells and amounts of DNA yielded from them will be sufficient for PCR. However, some specimens have insufficient sample volume or leukocyte count, and excess heparin will be concentrated in them, e.g., to 2 $\times$ or 3 $\times$ or more, and extracted DNA will be unsuitable for PCR due to relatively high concentration of heparin. Therefore, when PCR amplification yielded negative results, whether the DNA sample applied included interfering heparin should be determined.

In conclusion, our findings show that most heparin was extracted together with DNAs obtained from white cells, and that much care should be taken in PCR assays if white cell count in sample blood is insufficient given the amount of heparin present.

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