Jpn. J. Cancer Res. **86**, 885–889, September 1995

Double Determinant Immuno-Polymerase Chain Reaction: A Sensitive Method for Detecting Circulating Antigens in Human Sera

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A sensitive method for the detection of antigens in sera, termed double determinant immuno-polymerase chain reaction (double determinant immuno-PCR) was developed, using two monoclonal antibodies (MoAbs), in which the antigens are sandwiched, and a specific DNA molecule is used as a marker. Instead of the antigen itself, the first MoAb to bind the circulating antigens was immobilized. After the biotinylated second MoAb was bound to the antigen, free streptavidin was used to attach a biotinylated DNA to the biotinylated second MoAb. The biotinylated DNA complexed with antigen-antibody-streptavidin was amplified by PCR, and the PCR products were analyzed by Southern blot hybridization after agarose gel electrophoresis. Compared with the conventional enzyme linked immunosorbent assay (ELISA) using soluble intercellular adhesion molecule-1 (sICAM-1) in the supernatant of cultured Panc-1 cells as an antigen, our double determinant immuno-PCR was 10^3 times more sensitive in terms of the detection limit. Not only in culture medium, but also in sera from gastric cancer patients of high sICAM-1 titer, an approximately 10^3-fold enhancement in detection sensitivity was obtained compared with ELISA. In addition, this system can detect the antigen in sera at a level below the detection limit of traditional ELISA methods with high sensitivity. Thus, double determinant immuno-PCR has the significant advantage that it can be readily applied to any antigen-antibody system for which two MoAbs are available.

Key words: ELISA — PCR — Immuno-PCR — Double determinant immuno-PCR — sICAM-1

A very sensitive antigen detection system, termed immuno-PCR, has recently been developed by Sano *et al.*[1] They combined the system of polymerase chain reaction (PCR)[2,3] with enzyme linked immunosorbent assay (ELISA) by using a specially designed chimeric protein with bispecific binding affinity for DNA and antibodies as a linker molecule.[4] The exponential amplification of the DNA attached to the immune complex made it possible to dramatically improve the sensitivity (approximately $\times 10^5$) in the system to detect pure bovine serum albumin with a monoclonal antibody (MoAb). Instead of using the recombinant chimeric protein, Ruzicka *et al.*[5] used commercially available avidin to link the biotinylated antibody to the biotinylated DNA to be amplified. They could detect as little as 10 fg/ml of mouse antibody to apolipoprotein E using this system. A similar approach to detecting the recombinant human proto-oncogene ETS-1 immobilized in ultra thin-wall PCR tubes has also been reported,[6] with a huge enhancement ($\times 10^5$) in detection sensitivity compared to that of conventional ELISA. Since these methods are extremely sensitive in detecting immobilized pure antigens with specific antibodies, they may be of practical use in detecting circulating autoantibodies. However, for the purpose of detecting rare circulating antigens such as tumor-associated antigens, it would be impossible to apply these techniques as described, because a double determinant immunoassay is usually required to detect these antigens. We have therefore developed a double determinant immuno-PCR system using MoAbs. In preliminary experiments using a microtiter well, we failed to obtain enhanced detection sensitivity, mainly because of a high background signal on the first MoAb immobilization. In general, the background signal on the first immobilization in ELISA tends to be higher in a microtiter well than in a pin plate. Here we report a double determinant immuno-PCR system using a pin plate to detect soluble intercellular adhesion molecule-1 (sICAM-1). A 10^3-fold higher sensitivity was obtained with immuno-PCR, compared to that of the control ELISA.

MATERIALS AND METHODS

Cell line A pancreatic cancer cell line, Panc-1,[7] was maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C in Dulbecco's modified Eagle's medium (D-MEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Cansera International Inc., Canada).

Monoclonal antibodies MoAbs CL207 (IgG_1) and HA58 (IgG_1) were prepared by Maio *et al.*[8] and Hirata *et al.*,[9] respectively. MoAb HA58 was biotinylated by mixing 1 mg/ml of the MoAb in phosphate-buffered saline (PBS) with 0.1 mg of N-hydroxy-succinimidobiotin (Sigma, St. Louis, MO) in 0.1 ml of N,N-dimethylformamide (Sigma). After having been incubated for 4 h

at room temperature, the mixture was dialyzed with PBS overnight at 4°C.[10]

Antigens We used serial dilutions of supernatant from cultured Panc-1 cells as the standard antigen. Twenty-four serum samples were collected from gastric carcinoma patients admitted to our university hospital. Aliquots of serum samples were diluted at 1/200 in PBS containing 1% bovine serum albumin (1% BSA-PBS), and were subjected to ELISA and immuno-PCR.

Template DNA As a template DNA, we used a 253-base-pair (bp) fragment corresponding to nucleotides from 4917 to 5169 of leucocyte common antigen related molecule (LAR), which is a member of the membrane associated protein tyrosine phosphatase family.[11] The DNA was biotinylated by a reverse transcription polymerase chain reaction (RT-PCR) using biotinylated primers. Nucleotide sequences of the primers were 5′-TCCTGGCCTTCCTACGACGG-3′ for the sense direction and 5′-CACGTGGCAGCCTCCAGCAG-3′ for the antisense direction.

ELISA The ELISA system for measurement of circulating ICAM-1 was developed in our laboratory.[9] The first MoAb (CL207 5 μg/ml in PBS) was immobilized at 4°C overnight. The plate was blocked with 3% BSA-PBS at 37°C for 2 h, then Panc-1 supernatant or 1/200 diluted serum samples were added and incubation was continued at 4°C overnight. The plate was washed with PBS containing 0.05% Tween 20 (Tween-PBS) for 5 min twice and with PBS for 5 min once, then biotinylated second MoAb (HA58 1 μg/ml in 1% BSA-PBS) was added and incubation was continued at 37°C for 1 h. Avidin-conjugated peroxidase (Vector, Burlingame, CA) diluted 1/1000 in 0.05 *M* PBS with 0.05 *M* NaCl at pH 8.0, was added and incubation was continued at room temperature for 30 min. Then 1 mg/ml *ortho*-phenylenediamine in 0.1 *M* citric acid/0.2 *M* $Na_2HPO_4 \cdot 12H_2O$ with 1 μg/ml H_2O_2 was added and color was allowed to develop for 5 min, at which time the reaction was stopped by adding 2 *N* sulfonic acid. Absorbance (A) was measured at 492 nm (A492) in a micro ELISA autoreader EAR400 (SLT-Lab Instruments, Austria).

Immuno-PCR Procedures up to addition of the biotinylated second antibody were identical to those in ELISA except for the use of 1 mg/ml salmon testis DNA with 3% BSA-PBS as the blocking agent instead of 3% BSA-PBS alone. The plate was washed to remove unbound biotinylated second antibody, then free streptavidin (Sigma) at 1 μg/ml in 1% BSA-PBS was added and incubation was continued at room temperature for 30 min. The plate was washed with Tween-PBS for 5 min four times, and with PBS for 5 min three times, then biotinylated DNA molecules were added at the concentration of 10^5 copy/ml in 1% BSA-PBS and the plate was incubated at room temperature for 30 min. The pins were washed with Tween-PBS for 5 min five times and with distilled water for 5 min four times. They were detached from the plate and put into 50 μl of PCR mixture in a 500 μl Eppendorf tube. Each tube was subjected to PCR using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). PCR was carried out under the following conditions: 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.8 m*M* deoxyribonucleotide (0.2 m*M* each), 2 μM of each primer, and 1.25 units of AmpliTaq DNA polymerase. The temperature profile used was: initial denaturation (94°C, 2 min), and 35 cycles of denaturation (94°C, 1 min), annealing and extension (60°C, 1 min). Ten μl of the resulting PCR products were run in a 3% (w/v) agarose gel and transferred to nylon membrane, Hybond N^+ (Amersham, Arlington Heights, IL), in 0.4 *N* NaOH/1 *M* NaCl. The blots were hybridized with ^{32}P-labeled LAR cDNA probe.[12]

RESULTS

Effect of the copy number of biotinylated DNA on nonspecific amplification A double determinant ELISA for sICAM-1 using two MoAbs (CL207 and HA58) with different specificities, which was established in our laboratory, was used in this study to examine the availability of double determinant immuno-PCR. Free streptavidin was employed to attach the biotinylated DNA to the biotinylated second MoAb. A major problem we encountered while developing the immuno-PCR was that even the negative control for the first MoAb (1% BSA-PBS) generated some nonspecific amplification. This signal was not affected by the concentrations of MoAbs or streptavidin, or by the incubation time or temperature for binding of MoAbs, blocking agent, streptavidin, and biotinylated DNA. Also, PCR conditions such as number of cycles and annealing temperature and time, and the compositions of blocking agents and washing solutions did not affect the nonspecific signal. However, the copy number of biotinylated DNA had a marked influence on the intensity of false-positive signals. Fig. 1 shows the results of immuno-PCR with 1% BSA-PBS as a negative control for the first MoAb. Serial ten-fold dilutions of the biotinylated DNA were tested and the conditions except for the biotinylated DNA copy number were the same as described in "Materials and Methods." The nonspecific amplification, which was detected as a 253-bp band, started to appear at 10^5–10^6 copy/ml biotinylated DNA. As the copy number was increased, the intensity of the 253-bp band grew stronger until the PCR amplification was saturated at 10^9 copy/ml of biotinylated DNA. Thus, we employed the condition of 10^5 or 10^6 copy/ml biotinylated DNA for further analyses.

Analysis of the nonspecific amplification We then attempted to clarify which agent in our immuno-PCR

was responsible for nonspecific binding with biotinylated DNA. The biotinylated DNA (10^6 copy/ml) was added at each step and each resulting complex was subjected to PCR. As a result, no nonspecific amplification was observed until the biotinylated DNA was applied at the step

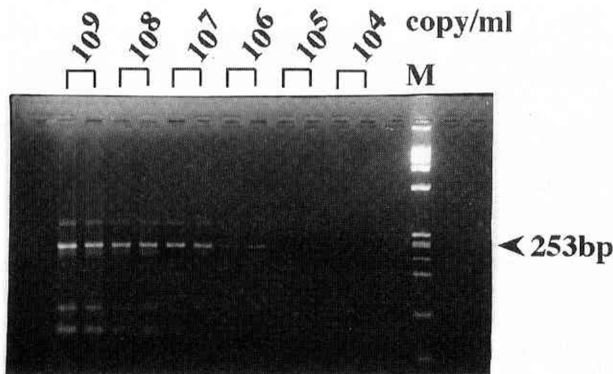

Fig. 1. Effect of biotinylated DNA copy number on nonspecific amplification of double determinant immuno-PCR. A 1% BSA-PBS solution was used as an antigen and conditions except for the biotinylated DNA copy number were the same. Serial ten-fold dilutions of the DNA from 10^9 copy/ml to 10^4 copy/ml were applied to each of two antigen-antibody-streptavidin complexes and amplified by PCR. The PCR products (253 bp) were analyzed by gel electrophoresis and staining with ethidium bromide. M is a DNA molecular weight standard marker (ϕX174-*Hae* III digest).

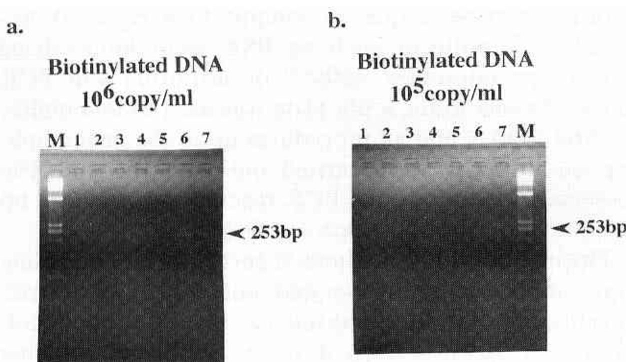

Fig. 2. Step analysis of nonspecific binding. Applied biotinylated DNA copy number; (a) 10^6 copy/ml and (b) 10^5 copy/ml. Lanes 1–6 indicate the steps in which biotinylated DNA was applied. Lane 1, application directly to the pin; lane 2, application after immobilizing the 1st MoAb; lane 3, after adding the blocking agent; lane 4, after incubating with 1% BSA-PBS; lane 5: after incubating with biotinylated 2nd MoAb; lane 6, after incubating with streptavidin (i.e. all steps were completed). Lane 7 represents the application of biotinylated DNA after the streptavidin without the step of incubating with biotinylated second MoAb. M is a DNA molecular weight standard marker (ϕX174-*Hae* III digest).

just after the addition of streptavidin. To determine whether streptavidin alone can cause nonspecific binding, the biotinylated DNA was applied after the addition of streptavidin without the biotinylated second MoAb, generating a nonspecific band with weak intensity (Fig. 2a). The same analysis was performed at 10^5 copy/ml biotinylated DNA. In this case, no nonspecific band could be seen, even after binding of streptavidin (Fig. 2b). These results indicated that streptavidin and the biotinylated second MoAb were mainly responsible for the nonspecific amplification. Thus, we determined that the copy number of biotinylated DNA affording maximum sensitivity without nonspecific amplification is 10^5 copy/ml. The other conditions employed were as described in "Materials and Methods."

Comparison of sensitivity between ELISA and immuno-PCR using Panc-1 supernatant In our conventional double determinant ELISA system of sICAM-1, the reliable cut-off value was 44 ELISA unit/ml (EU). One EU corresponds to 29 pg/ml of purified ICAM-1 (Bender MedSystems, Austria). We prepared serial logarithmic dilutions of the supernatant from Panc-1 cells, with 1% BSA-PBS, from 440 EU to 0.0044 EU to use for ELISA and immuno-PCR. The resulting PCR products were Southern blot-hybridized with a specific probe for LAR cDNA, to detect weakly positive signals. Positive signals were obtained from 440 EU to 0.044 EU, and a negative control for the first MoAb (1% BSA-PBS) did not generate any signal (Fig. 3). This showed that our immuno-PCR could detect ICAM-1 molecules at a level as low as 0.044 EU, and was therefore approximately 10^3 times more sensitive than ELISA.

Comparison of sensitivity between ELISA and immuno-PCR using serum samples For the purpose of the clinical

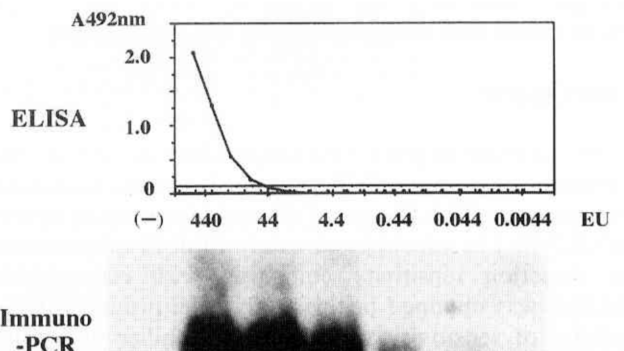

Fig. 3. sICAM-1 detection in Panc-1 supernatant. Serial ten-fold dilutions of Panc-1 supernatant from 440 EU to 0.0044 EU were used as antigens; 1 EU corresponds to 29 pg/ml of purified ICAM-1 antigen. (−) indicates negative control antigen, 1% BSA-PBS.

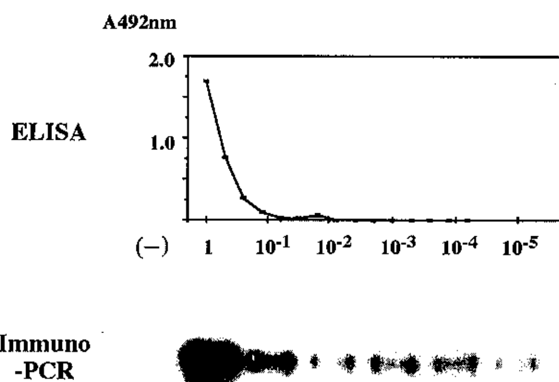

Fig. 4. Comparison of detection limit between ELISA and immuno-PCR using patient's serum as an antigen. Serial ten-fold dilutions of 1/200 diluted serum (which is indicated as 1) were used as antigens. (−) indicates negative control antigen, 1% BSA-PBS.

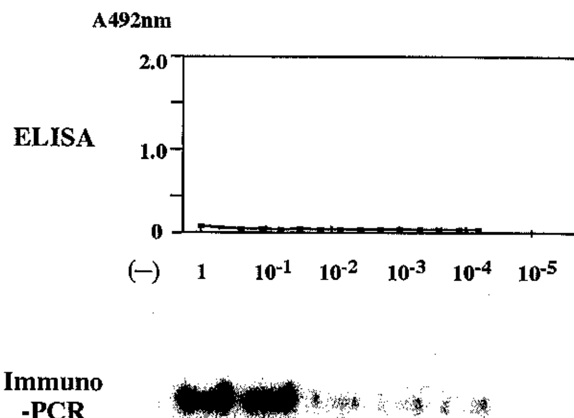

Fig. 5. Detection of sICAM-1 by immuno-PCR in serum below the detection limit in ELISA. Serial ten-fold dilutions of 1/200 diluted serum below the detection limit were used as antigens. (−) indicates negative control antigen, 1% BSA-PBS.

application of this system, the sera from gastric cancer patients with a high titer of sICAM-1 (over 300 EU in 1/200 diluted sera in conventional ELISA) were then tested. Serial ten-fold dilutions from the 1/200 diluted sera were used for ELISA and immuno-PCR. A representative case is shown in Fig. 4. Whereas the detection limit was at about 1/10 dilution for ELISA, it was at $1/10^5$ dilution for immuno-PCR, with the elimination of nonspecific amplification. Furthermore, as shown in Fig. 5, a patient's serum in which sICAM-1 was below the detection limit for ELISA was also investigated by immuno-PCR. Interestingly, from 1/200 dilution to $1/200 \times 10^4$ dilution clear signals were observed without nonspecific amplification. These results suggest that double determinant immuno-PCR can detect circulating antigens with very high sensitivity, which may enable us to detect rare antigens thus far not encountered.

DISCUSSION

We have developed a new and sensitive system, double determinant immuno-PCR, for the detection of circulating antigens. With the use of the antigen-antibody system of sICAM-1 in sera, approximately 10^3-fold enhancement in detection sensitivity compared with conventional ELISA was obtained in this study. The principal determinant of sensitivity was the copy number of applied biotinylated DNA. Nonspecific binding of the second MoAb and streptavidin was a major limiting factor in applying more biotinylated DNA. The concentrations of the second MoAb and streptavidin had little influence on nonspecific binding at a given copy number of biotinylated DNA (data not shown).

Targets of nonspecific binding of biotinylated second MoAb and streptavidin remain to be determined. The immobilized first MoAb and/or blocking agent could be targets. Some component of serum may interact with the MoAb or streptavidin. The affinity of streptavidin or proteins may vary depending on the physicochemical characteristics of the antibodies and antigens used in the assay system.

In all other techniques of immuno-PCR reported previously,[4, 8, 9] antigens such as BSA were immobilized directly on microtiter wells[4, 8] or ultra-thin-wall PCR tubes.[9] In this study, a pin plate was used to immobilize the first MoAb, and all procedures up to the final amplification by PCR were carried out with the pin. The presence of the pin in the PCR reaction mixture had no effect on amplification (data not shown).

The immuno-PCR methods reported so far, including ours, are semiquantitative and not suitable for strict quantitative analysis. Some quantitative PCR methodologies have recently been developed,[13–15] and applying those methods to immuno-PCR may be useful to increase the quantitative reliability.

In this study, we used sICAM-1 as a model antigen system to examine the sensitivity of double determinant immuno-PCR. However, sICAM-1 may not be the best target if this system is to be applied to diagnostics, because the expression level of sICAM-1 in sera from patients with various diseases is high enough to be detected by the conventional ELISA system,[9, 16] and sICAM-1 is expressed not only in the sera of patients with malignant diseases, but also in the sera of patients

with benign inflammatory diseases.[17, 18] A better target for our method might be certain tumor-related antigens for which detection by conventional ELISA has a high specificity but a low sensitivity. For example, circulating antigens which are barely detectable using conventional methods, such as the ErbB-2 product,[19, 20] would be good candidates as target molecules of our method. Our present data indicate that the double determinant immuno-PCR system is highly sensitive and might be useful as a clinical diagnostic tool for early stage malignancy, or to monitor tumor burden in the circulation of patients before and after surgical resection.

ACKNOWLEDGMENTS

We are very grateful to Ms. E. Takeda for her technical assistance and to Dr. P. Baginsky (University of California, San Francisco) for his editorial assistance. This work was supported by a Grant-in-Aid for Cancer Research (K.I.) and a Grant for Scientific Research Expenses for the Health and Welfare Program (2nd Term Comprehensive 10-Year Strategy for Cancer Control) (K.I., F.I.) from the Ministry of Health and Welfare, Japan.

(Received March 9, 1995/Accepted May 29, 1995)

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