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Sensitivity and Specificity of *in Situ* Bacterial Chain Reaction (BCR) in Detecting Sparse Human Tumor Cells in Peripheral Blood

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We report here the development of bacterial chain reaction (BCR), a system using micro organisms as nanodevices to amplify and visualize signals from molecular bioprobes such as antibodies, binding proteins, lectins, and oligonucleotides. Unlike conventional enzyme-linked amplification systems in which the amount of enzyme is a constant parameter, in the BCR an enzyme (penicillinase) is used to trigger a proliferative chain reaction producing an exponential increase in enzyme. The detection limits and specificity of BCR were determined using a model system designed to detect and enumerate MCF-7 (a human breast adenocarcinoma cell line) cells disseminated at extremely low frequency (e.g., one tumor cell per million normal cells) among monocluclear cells (MNCs) of human peripheral blood. Results of testing 83 specimens of peripheral blood from presumably healthy donors showed 97.6% specificity. The system was capable of detecting tumor cells at a frequency of 2×10^{-7}. © 1996 Academic Press, Inc.

The presence and number of tumor cells disseminated in blood or bone marrow of cancer patients has been shown to have significant clinical implications. For example, presence of occult (clinically undetectable) tumor cells in bone marrow of breast cancer patients at the time of diagnosis or initial treatment may serve to identify patients at high risk for disease recurrence and progression (1). Currently only two techniques, immunocytochemistry and reverse transcriptase polymerase chain reaction (RT-PCR), are sensitive enough to detect occult tumor cells. However, both these techniques are labor-intensive and, therefore, not suitable for routine applications in clinical or research laboratories. We describe here a new amplification methodology, termed bacterial chain reaction (BCR), and its application to a model system for detecting *in situ* tumor cells present at extremely low frequency (e.g., one tumor cell per million normal cells) among monocluclear cells (MNCs) of peripheral blood. The BCR uses micro organisms as nanodevices to amplify and visualize signals from molecular bioprobes such as antibodies, binding proteins, lectins, and oligonucleotides. In contrast to other enzyme-linked amplification systems in which the amount of enzyme is a **constant** parameter, in the BCR an enzyme (penicillinase) is used to trigger a proliferative chain reaction producing an **exponential** increase in enzyme. In the present study we have used the model system to measure detection limits and specificity of *in situ* BCR.

Principle of BCR. The initial procedural steps for *in situ* BCR are similar to those used in immunocytochemistry. For example, cells to be tested are first fixed on a microscope slide, and then treated sequentially with monoclonal antibody (mAb) to cytokeratin (CK) and penicillinase-labeled secondary antibody (e.g., a polyclonal antibody against mouse IgG). After each treatment, the slide is rinsed to remove unbound antibody. As a result of this procedure, only tumor cells are coated with significant amounts of penicillinase. At this stage, the slide is

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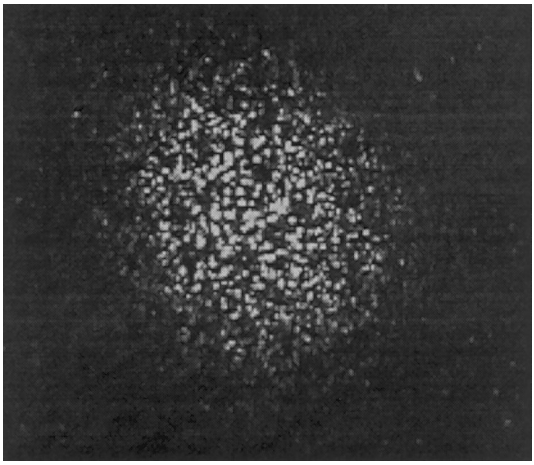

FIG. 1. Satellite colony (actual size ca. 4 mm diameter). Scale 8.75×.

covered with ''BCR indicator agar'' containing nutrients, bacteria and a concentration of penicillin high enough to prevent bacterial growth. Under these conditions, a BCR is triggered because of the following interrelated events:

(1) Bacteria can grow only near a penicillinase-labeled tumor cell where penicillin concentration has been lowered as a result of penicillinase activity.

(2) Growing bacteria (in contrast to dormant or slowly growing bacteria) are induced by penicillin to synthesize endogenous penicillinase, and concomitantly become more resistant to penicillin.

(3) Bacteria with endogenous penicillinase further reduce the antibiotic concentration near the tumor cell thus promoting additional bacterial growth. The end point of this proliferative chain reaction is a **visible cluster of bacterial microcolonies (termed satellite colony)** surrounding the tumor cell. Satellite colonies can be enumerated by naked eye to obtain quantitative data (Figure 1).

Theoretical considerations. The sensitivity of the assay hinges on achieving a rate of penicillin hydrolysis high enough to lower the penicillin concentration from $\boldsymbol{C_i}$, the concentration initially present in the BCR indicator agar, to $\boldsymbol{C_g}$, a critical concentration that triggers bacterial growth near individual target cells. Under the assay conditions, enzyme activity is probably limited by diffusion of penicillin towards the analyte because $\boldsymbol{C_i}$ (0.035 μM) is 1400-fold lower than the enzyme K_m (48 μM). Consistent with this premise, we have observed that increasing the diffusion rate of penicillin in the agar (by increasing agar concentration) increases assay sensitivity.

MATERIALS AND METHODS

Chemicals. Penicillinase (β-lactamase I, E.C. 3.5.2.6) and penicillin V were obtained from Sigma Chemical Co. (St. Louis, MO). Bacterial culture medium (Difco Antibiotic No. 1) was prepared according to the manufacturer's formulation except that glucose was omitted.

Antibodies. Two IgG murine mAbs were used for most of the experiments. A mAb directed against human CK polypeptide 18 (clone CY90, IgG1 isotype, Sigma Chemical Co., St. Louis, MO), and a mAb to penicillinase of *Bacillus cereus* 569/H (2). The latter mAb belongs to a family of functional anti-enzyme antibodies, i.e., antibodies enhancing enzymatic activity and protecting enzymes from heat denaturation (3). This mAb was used as an enzyme-antibody soluble complex that was prepared in advance and stored at 0-4°C in the presence of 0.05% sodium azide.

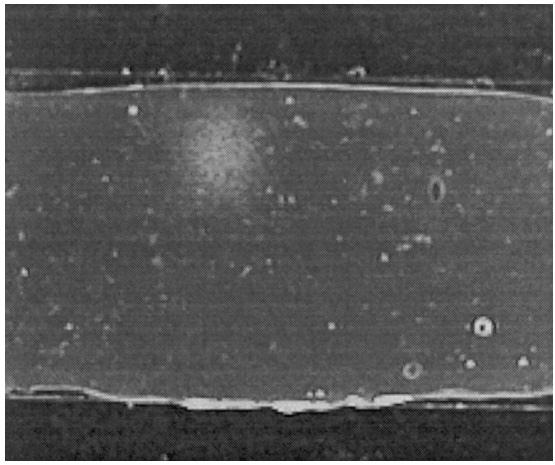

FIG. 2. A bright area near top-center of a glass slide indicates a satellite colony (actual size ca. 5 mm diameter) present after a BCR assay using MNCs mixed with a small number of MCF-7 tumor cells. The BCR indicator agar is visible at the edges of the slide. Scale 1.7×.

Under these conditions, the enzymatic activity of the complex remained constant for more than six months. A purified polyclonal IgG from goat antiserum to mouse IgG (Sigma Chemical Co., St. Louis, MO) was used to bridge the anti-CK mAb to the penicillinase-anti-penicillinase mAb soluble complex.

Tumor cell line. MCF-7, a human breast adenocarcinoma cell line (4), was obtained from the American Tissue Culture Collection. Cells were grown at 37°C under 5% CO_2 in Eagle's minimal medium supplemented with Earle's salts, nonessential amino acids, sodium pyruvate (100 μg/ml), L-glutamine (292 μg/ml), bovine insulin (10 μg/ml), and 10% heat-inactivated (56°C - 30 min) fetal bovine serum (all components of the culture medium were obtained from Sigma Chemical Co. (St. Louis, MO). Cultures were routinely tested for mycoplasma contamination. Cells were harvested by a brief treatment with trypsin, resuspended in phosphate-buffered saline (PBS), counted in a hemocytometer, and cellular viability was measured using fluorescein diacetate (5). For some experiments, the MCF-7 cells were covalently labeled with carboxyfluorescein using N-hydroxy-succinimidyl ester of 5-(and 6)-carboxyfluorescein diacetate (6), a fluorogenic substrate that accumulates intracellularly and is deacetylated by esterases yielding highly fluorescent cells (5). After labeling, the cells were fixed with formaldehyde and permeabilized with saponin to increase penetration of antibody (7).

Human blood cells. Clinical blood specimens were diluted 1:2 with PBS, and MNCs were separated using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation (8). The cells at the interface were collected, washed twice with PBS, resuspended in 0.5 ml PBS, and counted in a hemocytometer.

Bacteria. Cells of *Sarcina lutea* were grown at 37°C in Difco Antibiotic No. 1 medium. Cultures were aerated in a tube roller, and harvested before reaching stationary phase. For cryopreservation, cultures were mixed with glycerol (20% final concentration), and aliquots were stored at −20°C.

Immunoenzymatic cell labeling. Cells to be tested were smeared on several slides (using about 5×10^5 cells per slide), air-dried for 1-2 hours or overnight, fixed in methanol-acetone (1:1) for 2 min, and blocked for 60 min at 37°C with PBS containing 5% dried milk. The slides were wrapped with plastic film and stored at −20° C. For immunoenzymatic labeling, the cells were treated sequentially with: (i) mAb to CK18 (diluted 1:100); (ii) bridge goat antibody (0.2 μg/ml); and (iii) soluble immune complex of penicillinase and mAb to penicillinase containing 0.92 ng/ml of penicillinase. Each treatment included a 30-min incubation at 37°C followed by one or more rinses with PBS-1% bovine serum albumin to remove unbound reagents. Although sterile conditions were not necessary, sterile reagents and plasticware were used, and slides were handled with rubber gloves to avoid gross microbial contamination.

In situ amplification and visualization. Immediately following immunoenzymatic labeling, each slide was placed on a plastic Petri plate, and covered with 14 ml of melted (45°C) BCR indicator medium. The medium contained nutrients (Difco Antibiotic medium No. 1), 3×10^6 cells/ml of *S. lutea*, 34.5 nM penicillin V, and 0.6% Oxoid agar. After standing for about 15 min to allow for agar solidification, the slides were incubated at 37°C overnight. Next day, satellite colonies were visible by naked eye (Fig. 2), and the location of each colony was marked on the back of the slide. The BCR indicator agar was removed, and the presence of tumor cells within the marked areas was ascertained using a fluorescent microscope.

TABLE 1
Specificity of the BCR Assay System

Experiment number	No. of specimens tested	No. of negatives observed	No. of positives observed
3-111	6	6	0
3-112	7	7	0
3-115	10	10	0
3-116	8	8	0
3-117	6	4	2*
3-121	10	10	0
3-123	7	7	0
3-124	5	5	0
3-125	10	10	0
3-127	14	14	0
Total	83	81	2

* Satellite colonies were smaller than those present in positive control slides (see text). Altogether, the data indicate 97.6% specificity.

RESULTS

Results of preliminary experimental supported the premise that the BCR model system could detect discrete numbers of tumor cells dispersed among large MNC populations (ca. 5×10^5 cells). Namely, the following results were obtained: (i) no satellite colonies were observed on slides with only MNCs; (ii) satellite colonies were present on all slides with mixtures of MNCs and MCF-7 cells, and the number of colonies on individual slides corresponded roughly to the number of MCF-7 cells present in the respective sample. (iii) no satellite colonies were observed on slides with mixtures of MNCs and MCF-7 cells when either the anti-CK18 mAb or the bridge polyclonal IgG was omitted; (iv) satellite colonies of different sizes were present on some of the slides. We ascribed the size variation to clusters of MCF-7 cells because we observed that suspensions of MCF-7 cells often contain clusters of 2-10 cells. Similar observations have been reported by other investigators (9).

Specificity. The specificity of the BCR system was determined by testing 83 consecutive specimens of human peripheral blood obtained from presumably healthy donors. The testing was done in 10 separate experiments using 6-12 fresh specimens for each experiment. The following controls were included in each experiment: (i) a positive control consisting of a mixture of MNCs and MCF-7 cells; (ii) two negative controls (both using a mixture of MNCs and MCF-7 cells) in which either the anti-CK18 mAb or the bridge polyclonal IgG was omitted; (iii) a standard negative control using MNCs from a large (300 ml) blood specimen from a presumably healthy donor (the specimen was purchased as outdated blood from a local Blood Center). To ascertain that proper conditions existed during each test, a small volume (about one μl) of diluted penicillinase was spotted on the agar near the slide. The enzyme generated a satellite colony of relatively constant diameter (ca. 7 mm) serving as quality control . As shown in Table 1, 81 of the 83 specimens tested did not produced visible satellite colonies. These data represent a 97.6% specificity. Without exception, positive control slides had satellite colonies, while no colonies were observed on any of the three negative control slides. The two putative false positive specimens had satellite colonies with bacterial microcolonies smaller than those in satellite colonies of positive controls. Under the microscope, satellite colonies in positive control slides showed bacterial microcolonies of about 80 μ^2 average area, while in slides of the false-positive specimens the area ranged from 3 to 13 μ^2.

TABLE 2
Detection Limits of the BCR Assay System

Mononucleated cells	MCF-7 cells	Ratio*	Satellite colonies
6×10^6	0	0	No
6×10^6	20	$1{:}3.0 \times 10^5$	Yes
2.5×10^7	0	0	No
2.5×10^7	20	$1{:}1.2 \times 10^6$	Yes
1×10^8	0	0	No
1×10^8	20	$1{:}5.0 \times 10^6$	Yes

* Figures indicate the ratio of MCF-7 cells to MNCs in the cell mixtures tested.

Detection limits. The detection limits of the BCR assay system was determined using different mixtures of MNCs and carboxyfluorescein-labeled MCF-7 cells. The results shown in Table 2 indicate that MCF-7 cells were still detectable at a frequency of 2×10^{-7}. Observations under a fluorescent microscope showed that each of the satellite colonies examined had clusters of fluorescent MCF-7 cells ranging from 5 to 20 cells.

DISCUSSION

The results presented above demonstrate the use of BCR for *in situ* amplification and visualization of molecular signals from immobilized tumor cells labeled with penicillinase through a mAb to CK18. Although this mAb reacts with both normal and malignant epithelial cells, it has been used extensively for immunocytochemical tumor testing because most specimens of normal peripheral blood or bone marrow do not have detectable cells reacting with Mab to CK18. In our experiments, we found only 2 (2.4%) putative false-positives out of 83 specimens of normal blood tested. Our results are similar to those obtained by other investigators, for example Pantel et al. found 2 (1.7%) false positives among 117 specimens (10).

Aside of its high sensitivity, an important characteristic of *in situ* BCR is that it allows for post-assay direct microscopic confirmation of tumor cells generating satellite colonies. This is an important property because it could serve to decrease the number of false-positive, and, at the same time, to reduce considerably the time and subjectivity inherent in prolonged microscopic screening. Additional features of the system derive from the use of penicillinase as cellular label. The catalytic activity of penicillinase ranks among the highest known enzyme activities approaching that of diffusion-limited reactions (11), and its turnover number (1×10^5 to 1×10^6 min^{-1}) is comparable to that of enzymes commonly used for immunoassays, e.g., alkaline phosphatase, β-galactosidase and horseradish peroxidase. Moreover, since penicillinase is not found in eukaryotic cells, its use precludes the need for inactivating endogenous enzyme, a process often required when other enzymes are used for immunocytochemistry.

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