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Tumor Cell Detection Method Using Complement-Mediated Cytolytic Reaction and Imaging Sensor System

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ABSTRACT

A novel tumor-detection system consisting of complement-mediated cytolytic reaction and an image processing system was developed for the simple and rapid determination of tumor cells.

The present system consists of a CCD image sensor, image memory board, personal computer, and microscope.

When monoclonal antibody 3C4, which is specific to the guinea pig hepatoma L-10, was added to cell suspension, only L-10 cytolysis occurred. Cytolysis caused a decrease in brightness of the cells observed by phase-contrast microscopy. The phase contrast image of the cells before cytolysis was converted to a digitalized signal and stored in computer memory. After cytolysis, a brightness threshold above that of lysed cells was subtracted from the digitalized signal and compared to the signal stored before reaction.

L-10 cells in mixed cell suspension were determined specifically by the system. Measurement time was only 2 sec and overall time, including reaction time, was approximately 30 min. Since this method does not require a cell washing process, automation of the whole system is possible.

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Index Entries: Hepatocarcinoma cell; monoclonal antibody; complement; cytolytic reaction; imaging sensor system; phase-contrast microscope.

INTRODUCTION

Analysis at the cell or tissue level is developing as one of the most important topics for future clinical diagnosis. This is especially true in the case of cancer detection, where a prognosis is required at an early stage and, consequently, highly sensitive and rapid detection methods for specific cells are essential. In addition, the substances currently used for specific cancer detection, such as AFP (alphafetoprotein) or CEA (carcinoembryonic antigen) can only be applied in the case of progressive cancer, making the developing of alternative techiques that can be used in early diagnosis of great importance.

Cell diagnosis is performed mainly by the visual inspection of trained experts or using an automated cell analyzer (*1–4*). In this study, a novel method is described that has enabled us to detect specific cells in a suspension or in a tissue slice, rapidly and quantitatively.

Progress in the technology for data processing by computer has enabled various image analysis systems to be developed (*5,6*), several being commercially available (*7*). They are, however, very expensive, since they are designed for multi-functional application, with the potential for performing several image analyzing techniques, many of which are of no application for routine clinical analysis. Sixteen-bit personal computers have now become relatively inexpensive, making their use as a laboratory tool a familiar sight. Consequently, we were able to construct a "homemade" image analysis system using a readily available 16-bit computer and compatible image memory board. The system possesses only basic image analyzing functions, but they are sufficient for normal image analysis methods.

The solid state CCD (Charge Coupled Device) image sensor (*8*) has become widely used. It possesses several advantages over a conventional vidicon. It

1. is compact,
2. is highly sensitive,
3. has no after-image,
4. is distortionless,
5. has low power consumption, and
6. has a long operational life.

The CCD image sensor, therefore, was used for the transducer of our biosensor, converting optical information to a prossable electrical signal.

In this study, the CCD image sensor and homemade image analysis system was applied to the automatic detection of the cytotoxic reaction of

hepatocarcinoma with monoclonal antibody, based on complement activation, highlighting the wider application of the "bioimage sensor" for specifically determining cells, such as tumor cells.

MATERIALS AND METHODS

Cell and Antibody Preparation

Line-10 hepatocarcinoma cells (*10,11*) and L2C leukemia cells (*12,13*) of strain-2 guinea pigs were used. The cells were frozen and stored in liquid nitrogen until use.

Monoclonal antibody 3C4, which is specific to Line-10 cells (*14*), was prepared by hybridoma methods (*15*). Antibody was produced by ascite methods (*15*).

Cytotoxic Reaction

The specific detection of tumor cells in this study was based on the complement-mediated cytotoxic reaction with monoclonal antibody (*9*).

In this study, lysis was followed using a phase-contrast microscope. In the case of normal cells, the difference between intracellular and extracellular compartments produces a phase-lag of transmitted light. Normal cells, therefore, look bright under the phase-contrast microscope, whereas damaged cells that have lost membrane integrity appear darker.

Applying the decrease in brightness of lysed cells, specific, and nonspecific cells are distinguished.

Stored Line-10 cells (2×10^7 cells $\cdot$ mL^{-1}, 2 mL) were thawed at 38°C and suspended in 5 mL of RPMI1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). After centrifugation (900 rpm, 8 min) and decantation, the cells were washed twice by the same procedure and reconstituted in 3 ml of RMPI1640 medium (Cell concentration : 6×10^6 cells $\cdot$ mL^{-1}). 100 μL of cell suspension, 100 μL of 3C4 antibody solution, and 50 μL of rabbit serum (complement source) were mixed in a 1.5 mL microfuge tube (Eppendorf, Hamburg, FRG). After 30 min incubation at 37°C, the sample was transferred into a hemacytometer (Improved Neubauer type, EKDS Co., Tokyo Japan) and analyzed using the imaging sensor system.

Instrumentation and Computer Hardware

A schematic diagram of the image sensor system is shown in Fig. 1. A phase-contrast transmitted light microscope (Vanox Model AHB-LB, Olympus, Tokyo, Japan) was fitted with a dark-field condenser to obtain contrasting cell images. Video images of cell suspensions contained in the counting chamber of the hemacytometer were obtained using a CCD video camera (Model TI-25A, NEC, Tokyo, Japan), mounted vertically onto the microscope with a standard C-mount adapter. Video images of cells were displayed on a 12-inch black-and-white monitor (Model TMP-712B, NEC),

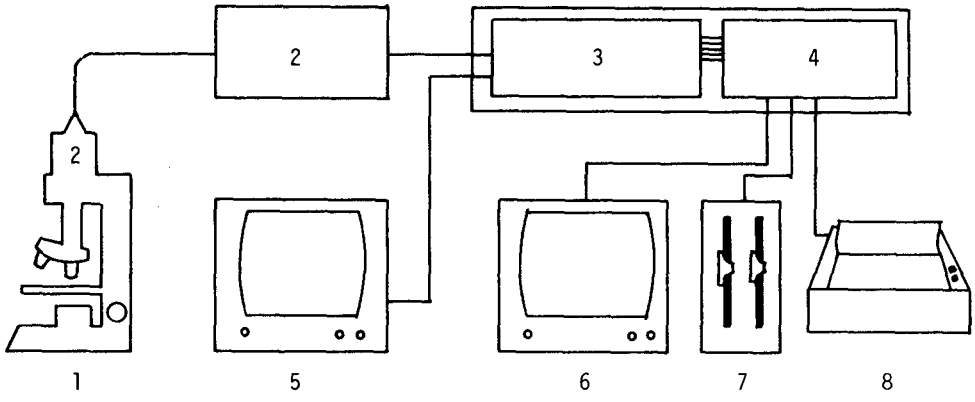

Fig. 1. Schematic diagram of the imaging sensor system—1—Microscope, 2—CCD video camera and controller, 3—Image memory board, 4—Personal computer, 5—Video monitor, 6—Color monitor, 7—Floppy Disk Drive, 8—Printer.

and the image was focused by adjusting the focal length between the microscope objective lens and the hemacytometer. The video display was fed into an image memory board (Model FDM 98-1, Photoron, Tokyo, Japan), and connected to the expanded bus of a 16-bit personal computer (Model PC-9801E, NEC, Tokyo, Japan). FDM 98-1 has a 6-bit A/D converter, an 8-bit D/A converter, and a 64 KB RAM.

Computer Software

The image-digitizing program had the main function of scanning a defined frame area (256 by 256 picture elements [pixels], scan time 1/60 second) of the image, binarization of the frozen image, and recording the cell image counts (number of white level pixels) for different 10-frame scans obtained by manually moving the field of view. To obtain one data set (scanning, freezing, binarization, and counting of the cell image area) requires approx 2 sec.

RESULTS

Definition of Threshold Level

As mentioned above, "high" level pixels were counted after the image area was binarized to eliminate both the injured cell image and the background noise level. The definition of threshold level is thus very important. An example of the brightness distribution change caused by the cytotoxic reaction is shown in Figure 2. Figure 2(a) shows the brightness distribution before cytotoxic reaction, and Fig. 2(b) shows the brightness distribution of the same sample after cytotoxic reaction. The most suitable

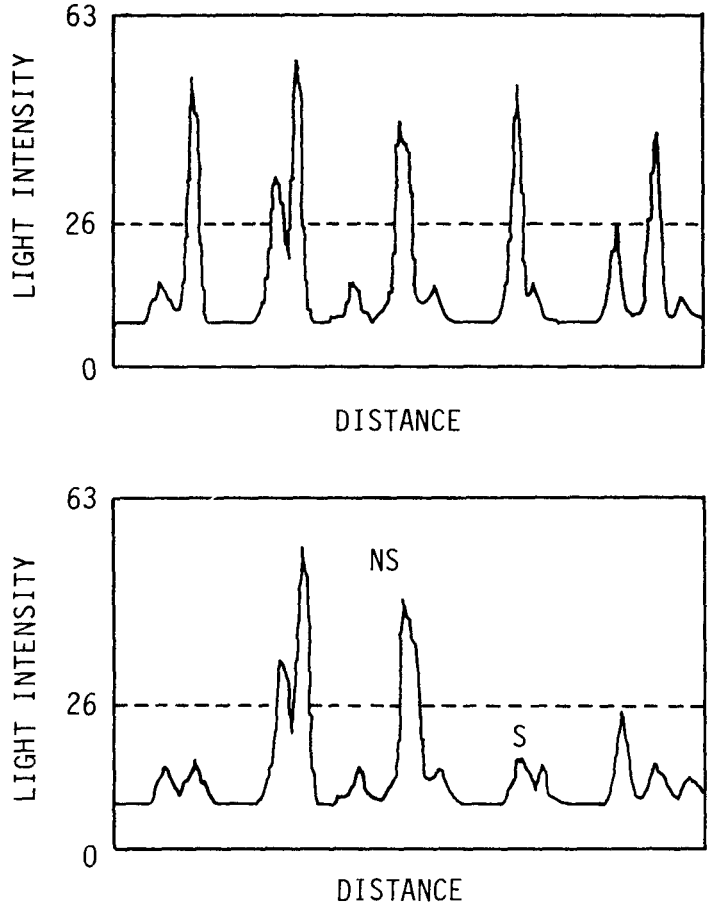

Fig. 2. Definition of threshold level, (a) Brightness distribution before cytotoxic reaction, (b) Brightness distribution after cytotoxic reaction (37°C, 60 min.), S:L-10 cell (specific), NS:L2C cell (nonspecific).

brightness threshold level, which leaves the normal cell image and eliminates those that are damaged, was determined from these brightness distribution diagrams. In this system, the most suitable brightness threshold level was 26.

Relationship Between Cell Number and Image Counts

In order to count cells automatically, the relationship between cell number and image counts was investigated. Normal (non-injured) Line-10 cells were diluted to various concentrations with PBS. Samples were transferred to the hemacytometer and cell image area was counted using the image sensor system (brightness threshold level; 26). This result is shown in Fig. 3. A good linear relationship was obtained between Line-10 cell number and cell image counts in the range of $6\times10^5-1\times10^7$ cells $\cdot$ mL^{-1}

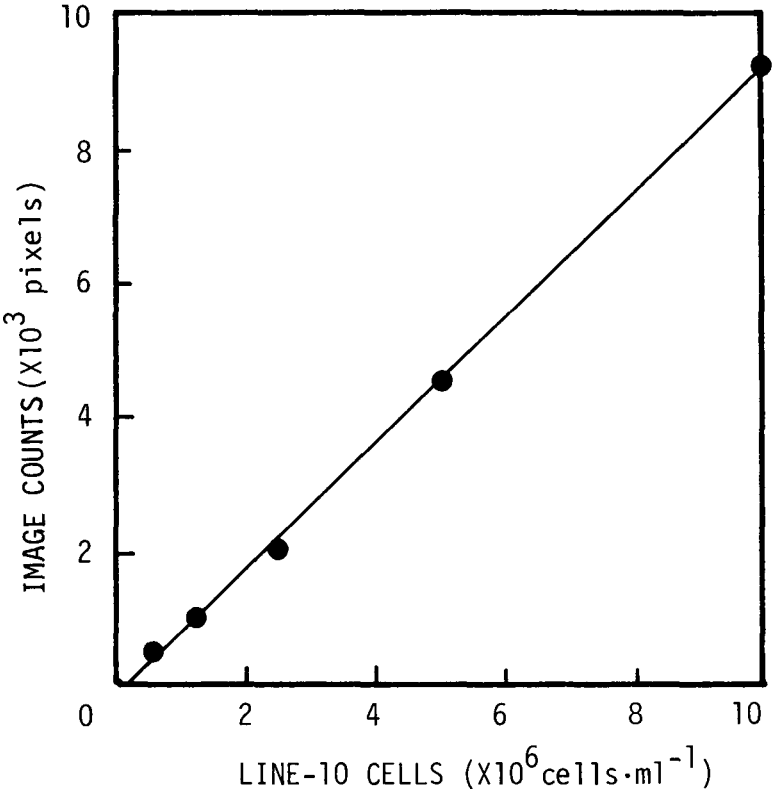

Fig. 3. Effect of L-10 cell concentration on image counts, Threshold level: 26.

(correlation coefficient; .9996). Since a single Line-10 cell covered an image area of 20 ± 5 pixels, approx 500 cells were counted per scan when a cell concentration of 10^7 cells · mL^{-1} was used. In one set of measurement, therefore, approx 5000 cells are counted.

These results indicated that normal cells could be counted automatically by the image sensor system.

Detection of Cytotoxic Reaction Using the Imaging Sensor System

Detection of complement-mediated cytotoxic reaction was attempted by the imaging sensor system. Image areas were measured for antibody dilutions between 1:10 and $1:10^5$. Since the protein concentration of antibody stock solution was approx 1 mg · mL^{-1}, the protein concentration of the antibody solutions ranged between 10^{-1} and 10^{-5} mg · mL^{-1}.

Figure 4 shows the relationship between antibody dilution and image counts. The number of image counts was drastically decreased for antibody dilutions greater than 10^{-3} mg · mL^{-1}. This decrease indicated that a cytotoxic reaction had occurred. In the range from $10^{-4}-0$ mg · mL^{-1}, no

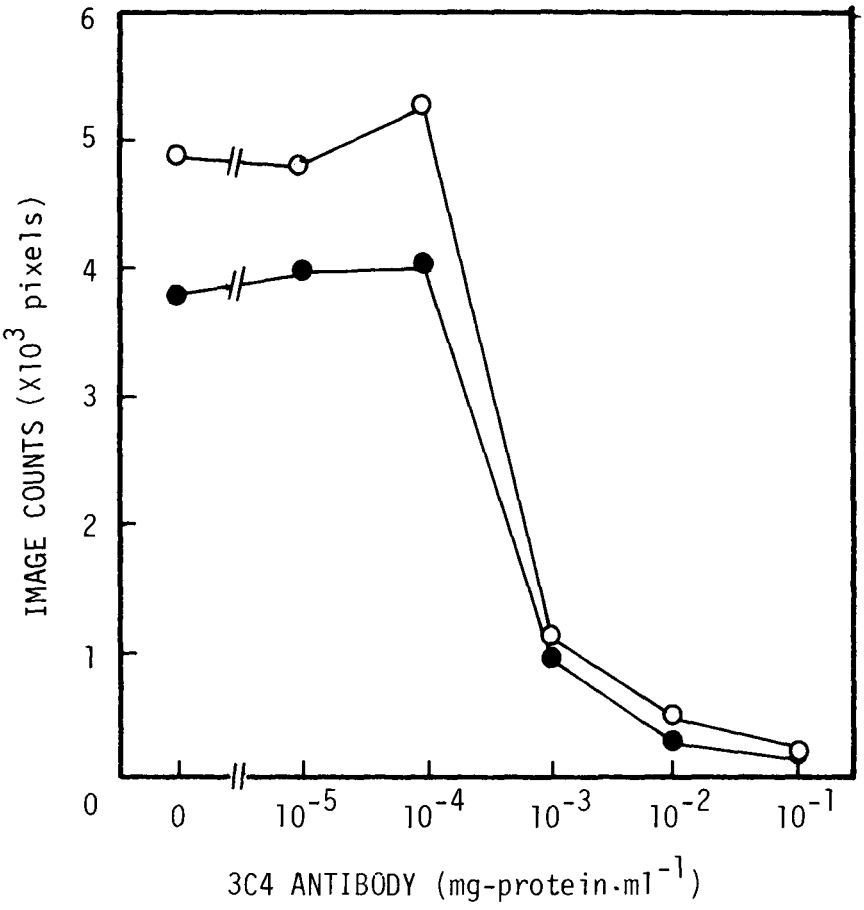

Fig. 4. Effect of antibody concentration on L-10 cytotoxic reaction, Reaction time: ○ 30 min., ● 60 min, Threshold level: 26.

decrease in image counts was observed, which is parallel to the result obtained by the Trypan blue dye exclusion test.

Quantitative Detection of Line-10 Cells in Mixed Cell Suspension

Based on the results of previous experiments, quantitative detection of Line-10 cells in mixed cell suspension was carried out. The cell concentrations of standard suspensions containing L-10 and nonspecific L2C were corrected so as to retain the same image counts throughout. The two standard suspensions were then mixed (the L-10 cell content was the volume ratio of the two suspensions), and analyzed by the imaging sensor system. Figure 5 shows the relationship between L-10 cell content (%) and image counts after treatment of the cell suspension with 3C4 antibody and complement. L-10 cells could be detected quantitatively in the range 10–100%, a linear relationship being observed between the L-10 content and image area in this same range.

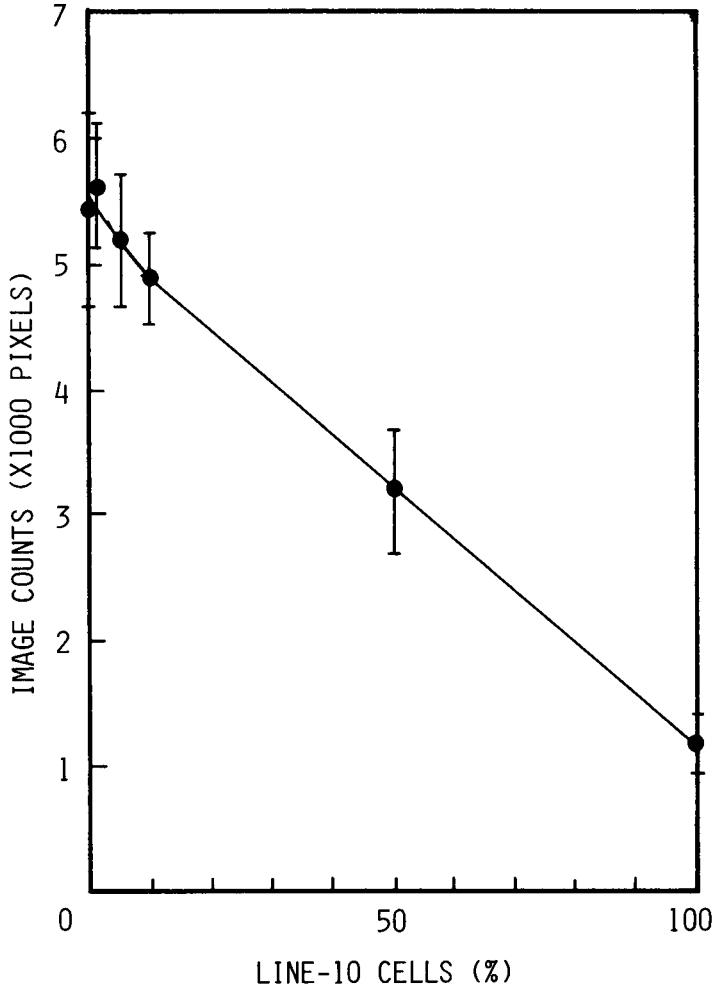

Fig. 5. Specific determination of L-10 cell content in mixed cell suspension, Reaction time: 60 min, Antibody concentration: 1×10^{-1} mg · mL^{-1}, Threshold level: 26, Nonspecific cells: L2C (guinea pig leukemia).

DISCUSSION

In this study, complement-mediated cytotoxic reaction was used for the detection of tumor cells. This method is very simple, requiring less time and fewer specialized techniques than other conventional cell staining methods or immunofluorometry.

The process does not require a cell-washing step, therefore making it possible to automate the whole system during the reaction stage.

Some researchers have reported that the cytotoxic reaction is only 70% specific for target cell recognition (*16*), yet we observed that approximately 100% of target cells were specifically damaged under the conditions used here.

During analysis of tumor cells, we chose a reaction time for cytolysis to occur of 30 or 60 min since this is the time allotted in the conventional Trypan blue dye exclusion method. This method relys on a reduction in the selective permeability of the cell membrane caused by the cytotoxic reaction, allowing stain to invade the cytosolic compartment. Our imaging sensor system, however, detects the decrease in phase contrast caused by the cytotoxic reaction, thus it may be possible that the reaction time can be shortened. It was observed using this system that the actual decrease in phase contrast had reached a minimum after 15–20 min.

These results show that this imaging sensor system can enable hepatoma cells to be determined in approximately 20 min, involving the reaction time. This method is very rapid compared with conventional methods.

The detection limit of our system for hepatoma in mixed cell suspension was 5%. This value is not sufficient for cancer diagnosis. Although the cytotoxic reaction employed in our system cannot be improved, the detection limit can be brought within practical application by enhancing the sensitivity of the detection device to a change in brightness. We have already commenced construction of an improved imaging sensor system with higher sensitivity. This system will be described elsewhere.

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