Monoclonal Antibody to 5-Bromo- and 5-Iododeoxyuridine: A New Reagent for Detection of DNA Replication

Abstract. *Monoclonal antibodies specific for 5-bromodeoxyuridine have been produced and applied in detecting low levels of DNA replication on a cell-by-cell basis in vitro. The immunoglobulin-producing hybridomas were derived from spleen cells of mice immunized with a conjugate of iodouridine and ovalbumin. The cells were fused with the plasmacytoma line SP2/0Ag14. The antibodies produced are highly specific for bromodeoxyuridine and iododeoxyuridine and do not cross-react with thymidine. DNA synthesis in cultured cells exposed to bromodeoxyuridine for as short a time as 6 minutes can be detected easily and rapidly by an immunofluorescent staining method and quantitated by flow cytometry.*

The detection of replicating cells is usually accomplished by demonstrating [^{3}H]thymidine incorporation into DNA by autoradiography or scintillation counting (*1*). Autoradiography is time-consuming, and scintillation counting, although rapid, is a "batch" method and cannot provide quantitative information on the frequency of replicating cells within a population. An immunological method based on heteroclonal rabbit antibodies specific for 5-bromodeoxyuridine (BrdUrd) was developed by Gratzner *et al.* (*2*) and adapted to flow cytometry (*3*). This antibody technique, however, was variable in specificity, depending on the rabbit immunized; moreover, the antibody was difficult to separate from immunoglobulin species that cross-react with thymidine. These difficulties could be obviated by production of a chemically defined antibody in unlimited amounts to serve as a consistent reagent for the rapid quantitation of DNA replication. The hybridoma technique of Kohler and Milstein (*4*) was used to produce monoclonal antibodies highly specific for BrdUrd and 5-iododeoxyuridine (IdUrd) and applicable to detecting incorporation of these molecules into replicating DNA.

BALB/c mice were immunized by peritoneal injection of 200 μg of a conjugate of iodouridine and ovalbumin emulsified in Freund's complete adjuvant (*5*). The initial injection was followed every 2 weeks for about 1 month by injections prepared in incomplete adjuvant. An intravenous injection of the iodouridine-ovalbumin conjugate in phosphate-buffered saline (PBS) was then given through the tail vein, and 3 days later spleen cells from the mouse were dispersed and fused with the plasmacytoma cell line SP2/0Ag14 (*6*) by the method of Gefter *et al.* (*7*). Hybrid clones were selected by growth in hypoxanthine-aminopterin-thymidine medium. Portions of culture medium from each well that contained a clone were screened with an enzyme-linked immunosorbent assay (ELISA) to detect antibody that was specifically bound to a conjugate of iodouridine and bovine serum albumin (BSA). The second antibody used in the assay was sheep $F(AB)_2$ against mouse immunoglobulins coupled to β-galactosidase, and the substrate was *p*-nitrophenyl-β-D-galactopyranoside (*8*). The supernatants from 24 of 170 clones showed iodouridine-BSA binding. Six of these positive clones were screened by ELISA and a hapten inhibition test with IdUrd and thymidine. Binding of the antibody was blocked by IdUrd in four of the clones tested. One clone, B44, secreted an antibody that was inhibited in its binding to iodouridine-BSA by IdUrd but not by thymidine. This clone was selected for further cloning in agarose and by limiting dilution.

The ability of B44 supernatants to specifically bind to BrdUrd in cellular DNA was tested on plasmacytoma cells. The cells were incubated for 2 hours in 10 μ*M* BrdUrd plus 1 μ*M* fluorodeoxyuridine (FdUrd) in Dulbecco's minimal essential medium (GIBCO), fixed in a mixture of methanol and acetic acid (3:1), spread on microscope slides, air-dried, and denatured in 0.07*N* NaOH for 2 minutes (*2*). The slides were then incubated for 60 minutes with various dilutions of a dialyzed 50 percent $(NH_4)_2SO_4$ precipitate (20 mg/ml) of the B44 supernatant culture medium from the hybridoma cells. The slides were washed twice for 5 minutes, incubated with fluoresceinated goat antiserum to mouse immunoglobulin (Cappel), diluted 1:40 in PBS containing 5 percent goat serum for 30 minutes, and again washed in PBS. The cells were then observed by epifluorescence optics. Cells that had been incubated in BrdUrd medium and stained with antibody diluted 10^5-fold displayed bright fluorescence. In contrast, control cells grown in medium without BrdUrd, as well as nonreplicating regions of nuclei, were almost invisible under fluorescence excitation. Cells engaged in localized synthesis of DNA showed fluorescence only at the

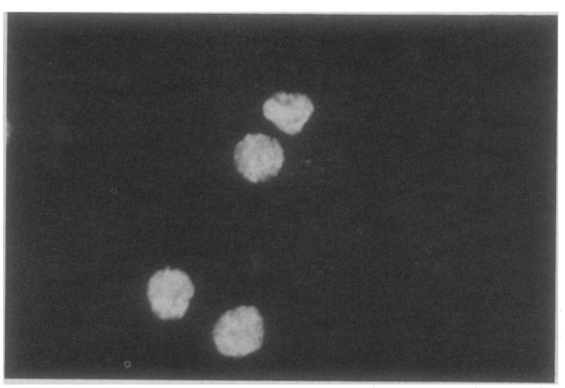

Fig. 1. Immunofluorescent staining of BrdUrd-labeled plasmacytoma cells with monoclonal antibody. The Sp2/0Ag14 cells were grown in medium containing 10 μ*M* BrdUrd and 1 μ*M* FdUrd for 2 hours, fixed in a mixture of methanol and acetic acid (3:1), and spread on slides. Primary staining was with dialyzed 50 percent $(NH_4)_2SO_4$ precipitate, which was diluted to 1:1000 in PBS. Areas of localized fluorescence are seen in some of the nuclei. Unlabeled nuclei are visible only when cells are transilluminated.

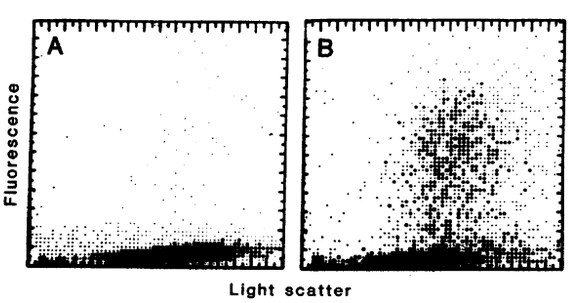

Fig. 2. Flow cytometric analysis of BrdUrd-labeled WiL2 human lymphoblast cells. The cells were exposed to 10 μ*M* BrdUrd for 6 minutes, fixed in 70 percent ethanol, and stained in suspension with concentrated (sixfold) supernatant medium from B44 cultures as in (*3*). The second antibody was fluoresceinated goat antiserum to mouse immunoglobulin (Cappel) diluted 1:80 in PBS plus 5 percent goat serum. Flow cytometric analysis was carried out with a cell sorter (EPICS IV, Coulter Electronics). The laser was tuned to 488 nm, at 65 mW. (A) Cells incubated without BrdUrd. (B) Cells incubated in 10 μ*M* BrdUrd. Ten thousand cells were analyzed.

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sites of BrdUrd incorporation (Fig. 1).

Other experiments tested the immunofluorescent staining in a more quantitative manner. Human lymphoblast cells (WiL2) were incubated briefly in medium containing BrdUrd, fixed in 70 percent ethanol, and stained in suspension. No cytoplasmic fluorescence was observed by fluorescence microscopy.

The cells were then analyzed by flow cytometry for light-scatter size and the amount of indirect immunofluorescence of the antibody to BrdUrd. Exposures as short as 6 minutes were sufficient to demonstrate the incorporation of BrdUrd (Fig. 2). The cells that were not labeled with BrdUrd exhibited about tenfold less fluorescence than the labeled cells (Fig. 2). With concentrated antibody preparations (20 mg/ml, compared to the 100 μg/ml routinely used), the fluorescence intensity was increased by only 0.8 percent, as judged by the fluorescence peak channel values of 3 and 4, respectively. In previous observations with heteroclonal rabbit antibodies to BrdUrd, careful titrations were necessary to arrive at a serum concentration that specifically stained the BrdUrd-labeled cells and did not stain the nuclei of unlabeled cells (*3*). Successively decreasing the protein concentration from 20 mg/ml to 10 μg/ml did not change the number of BrdUrd-labeled cells (the labeling index), but did cause decreases in the fluorescence intensity of individual cells. These observations demonstrate the importance of titrating to antibody excess in order to maximize the antibody binding to the BrdUrd in individual nuclei.

The results reported here indicate that monoclonal antibodies specific for BrdUrd can provide a sensitive method for detecting DNA replication in single cells in a manner analogous to the use of [^{3}H]thymidine. When the technique is used to measure cell proliferation kinetics, significant increases in statistical reliability and speed have been achieved (*9*). The fluorescence intensity per cell due to monoclonal antibody to BrdUrd was directly related to the amount of BrdUrd incorporated (*3*) and provides a rapid method for quantifying the rate of cellular DNA synthesis. The analytical applications of this monoclonal antibody detection method may be useful not only for measuring cellular DNA replication and DNA synthesis rate in vitro but for investigating DNA repair phenomena and DNA replication in situ.

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Placental Mononuclear Phagocytes as a Source of Interleukin-1

Abstract. *Mouse and human placental tissue contains a large number of mononuclear phagocytes. These cells, isolated from placenta, were shown to produce the multifaceted immune factor interleukin-1. Activity in the supernatants of 48-hour mononuclear phagocyte cultures was associated with a 12,000- to 18,000-dalton protein, consistent with known interleukin-1 characteristics. Stimulation of phagocytosis with latex beads increased the production and release of interleukin-1 from these placental cells, which may be a useful source of this protein.*

Activated mononuclear phagocytes and macrophages are known to produce a number of immunologically important factors (*1*). Interleukin-1 (IL-1), a 12,000- to 18,000-dalton protein, is produced by macrophages and is responsible for a number of T and B lymphocyte actions (*2*). Recently, the effects of IL-1 were equated with the responses noted for other macrophage factors of similar molecular weight, such as leukocyte endogenous mediator (LEM). It was reported that LEM modulates the central nervous system, producing fever; the bone marrow, causing the release of neutrophils and stimulating granulopoiesis; and the liver, causing the production of acute-phase proteins (*3*). It was also shown that IL-1 is similar to endogenous pyrogen (EP), in that IL-1 binds to and elutes from an affinity column for EP (*4*). Further study of IL-1, LEM, and EP requires a source of cells that are activated or can be activated to produce sufficient quantities of mononuclear phagocyte culture supernatants.

Wood (*5*) showed that human placenta contains approximately 65 percent mononuclear phagocytes. He reported that most cells released from tissue rich in chorionic villi had receptors for the third component of complement (C3) and for Fc, and that they morphologically resembled mononuclear phagocytes and phagocytized opsonized erythrocytes. Wood (*6*) subsequently demonstrated that large amounts of immunoglobulin G (IgG) are bound within the placenta to Fc receptors on mononuclear phagocytes, suggesting a mechanism for the removal of incompatible antibodies.

Since, in pregnancy, many events mimic the effects of IL-1 or LEM (*7*), we questioned whether the mononuclear phagocytes in placenta could actively produce IL-1 or be stimulated to produce this factor. We initially examined placentas taken from C57B1/6 mice 2 days prior to term and subsequently human placentas collected after normal delivery. Whole mouse placentas and tissue rich in chorionic villi from human placentas were separately minced and enzymatically digested with trypsin and collagenase in calcium-free Hanks balanced salt solution for 30 minutes (*5*). The cell suspension was washed twice with medium (*8*) and cultured at 1.0×10^6 cells per milliliter on tissue-culture plates for 2 hours (*8*). The plates were then washed with medium to remove nonadherent cells and the percentage of adherent cells was estimated by counting the cells collected from the washing. Mononuclear phagocyte characteristics were determined by counting the number of adherent cells that phagocytized latex beads (*9*) and stained positively for nonspecific esterase (*10*). The adherent cell population (in human cultures a placenta can provide greater than 1×10^{11} adherent cells) was cultured for 48 hours, and the supernatants were collected, dialyzed (40 times), and filtered. The supernatant fluids were analyzed for IL-1 and interleukin-2 (IL-2) activity. The IL-1 activity was estimated by the thymocyte proliferation assay reported by Farrar *et al.* (*11*). Because this assay also detects IL-2, the samples were also examined for IL-2 activity by a conventional T cell growth assay (*12*) to ensure that the

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