# Phenotypic Heterogeneity within Clonogenic Ductal Cell Populations Isolated from Normal Adult Rat Liver (43664)

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*Abstract.* **Oval cells represent a heterogeneous population composed of ductal cells, transitional cells with characteristics of both hepatocytes and bile ductal cells, and bipotential stem cells capable of differentiation along a biliary or hepatocytic lineage. In an attempt to define markers that would distinguish between individual cell types within the oval cell population, a number of investigators have utilized hybridoma technology to produce cell type-specific monoclonal antibodies. Several of these have proved to be of value in delineating lineage relationships during fetal development and carcinogenesis in the adult liver. Most recently, monoclonal antibodies specific for OC2 and OC3, two oval cell antigens identified in our laboratory, have been used in combination with magnetic beads or a fluorescence-activated cell sorter to isolate antigenically defined subpopulations from adult and fetal rat liver. Using OC2-positive fetal liver cells as an immunogen, we have produced a monoclonal antibody identifying a bile ductal antigen, designated BD1, that is differentially expressed by oval cells and normal ductal cells. This antigen shows a heterogeneous pattern of reactivity that defines three distinct cell populations in regenerating rat liver: a BD1-negative, [$^3$H]thymidine-labeled cell population thought to contain hepatic stem cells; a BD1-positive, thymidine-negative population of terminally differentiated ductal cells; and a BD1-positive, [$^3$H]thymidine-positive population of mature ductal cells. Analysis of BD1 expression *in vitro* on continuous lines of bile duct epithelial cells (BDEC) demonstrated that BD1 was rapidly increased in late $G_1$ and lost during $G_2$/M. High passage cultures of BDEC and primary cultures of oval cells expressed low or undetectable levels of BD1 and high passage BDEC failed to express BD1 when arrested in late $G_1$. Taken together, these results suggested that oval cells and high passage BDEC might share a subtle defect in cell cycle regulation marked by an inability to upregulate the expression of BD1.** [P.S.E.B.M. 1993, Vol 204]

**Hepatic Stem Cells: To Be or Not to Be?** It has been argued that in rapidly renewing tissues, such as skin, intestinal mucosa, or bone marrow, stem cells are one of the most likely targets of carcinogenic agents simply because their immortality assures a continued presence during the long latency period between carcinogen exposure and the development of carcinomas (1). This reasoning is consistent with the concept of Pierce and Speers (2) that carcinomas arise by the malignant conversion of stem cells which retain an infrequently exercised capacity for differentiation. Although there is good evidence for stem cell progenitors in cancers that arise in the breast, colon, skin, and bone marrow (3–6), the involvement of stem cells in liver cancer and even the existence of hepatic stem cells have remained areas of controversy.

The strongest argument against hepatic stem cells has been the immense proliferative capacity of mature hepatocytes and bile ductal cells, a capacity more than sufficient to produce regeneration following partial hepatectomy without the need to invoke stem cell involvement (7, 8). Moreover, phenotypic analysis in several murine models of hepatocarcinogenesis has implicated the fully differentiated hepatocyte as the primary progenitor of carcinoma. Indeed, lineage studies with markers like $\gamma$-glutamyl transpeptidase and glutathione-*S*-transferase-*p* (9) suggest that the majority of, if not all, primary hepatocellular carcinoma are derived from fully differentiated hepatocytes (10), a widely held view that casts oval cells, small basophilic ductal cells

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that rapidly expand after treatment with a large variety of hepatocarcinogens, as unimportant bystanders in liver carcinogenesis (11–13).

Renewed interest in oval cells has been generated by evidence from a number of more recent investigations indicating that the elusive hepatic stem cells postulated by Wilson and Leduc (14) or their immediate offspring are contained within the population of morphologically defined oval cells. The idea that oval cells have stem cell properties was founded in observations made in livers from rats treated with azodyes showing a step-wise morphologic progression leading from oval cells to small basophilic hepatocytes (15, 16). Recent findings have given credence to the role of oval cells as understudies that step in to play the leading role in regeneration and carcinogenesis when the proliferative capacity of hepatocytes has been compromised. This has been shown *in vivo* by transfer of [$^3$H]thymidine from oval cells to basophilic hepatocytes, by the presence of transitional cells with antigenic and enzymatic characteristics of both oval cells and hepatocytes, and by the ability of primary or continuous lines of rat liver epithelial cells or oval cells to express hepatocyte markers *in vitro* or to form hepatic foci or hepatocellular carcinoma *in vivo* (11–13, 17, 18).

## Oval Cell Beginnings and Endings

In most rapidly renewing tissues, stem cells usually have long cycle times relative to their immediate progeny, the so-called transit cells. It is estimated that after the initial stem cell division which leads to the production of new stem cells, transit cells, or both, there are usually between two and four amplifying divisions that produce progeny committed to subsequent differentiation into functionally mature cells (12). If oval cell expansion is viewed as a manifestation of a similar process, it follows that only a small percentage of oval cells will represent true stem cells. Most will be transit cells at various stages of differentiation along hepatocytic or biliary lineages. Alternatively, it could be argued that oval cells derive in part or in entirety from immature cells resident in existing intralobular ducts. The main difference between these two views will be in the differentiation characteristics of the resulting oval cell population. If oval cells are exclusively stem cell derived, then the resulting hepatocytes will develop by a differentiation process akin to bipotential hepatoblasts in fetal liver. On the other hand, if oval cells are produced exclusively from existing ductal cells, a process of metaplasia or transdifferentiation would have to be invoked to account for the transformation to hepatocytes.

Although there is convincing evidence from several studies for the differentiation of $\alpha$-fetoprotein (AFP)-positive oval cells into hepatocytes (19–21), the hepatocytic conversion of mature ductal cells appears to be a relatively rare occurrence observed only under extreme conditions. Regardless of their origin, it appears that the capacity of oval cells for complete differentiation along a biliary lineage has been largely compromised because well-differentiated ducts of oval cell origin are rarely seen. This apparent restriction in the bipotentiality of oval cells is reminiscent of what occurs after destruction of the exocrine pancreas by copper chelators or ethionine, a situation under which pancreatic ductal cells differentiate almost exclusively along a hepatocytic lineage (22). One could speculate that the infrequency of complete differentiation along a ductal lineage is a consequence of oval cell migration within the space of Disse, a course of movement that removes them from the morphogenic factors supplied by the portal mesenchyme and places them in direct contact with hepatocytes (12, 23). Alternatively, the restricted differentiation potential of oval cells may be a direct effect of the mutagenic or cytotoxic effects of the inducing agent.

## Antigenic Anonymity of Hepatic Stem Cells

Assuming hepatic stem cells have properties analogous to those in other epithelial tissues such as skin and intestinal mucosa, they will be present in relatively low numbers, a fact consistent with the rarity of AFP-positive ductal cells in adult liver (24). Moreover, under conditions that favor expansion, true stem cells are likely to be hidden among a crowd of transit cells with heterogeneous phenotypes, a description that applies accurately to oval cells. In an attempt to identify markers that could be used to trace the origin and fate of oval cells with stem cell-like properties during liver carcinogenesis, hybridoma technology has been exploited by several groups as a means to produce cell type-specific antibodies (12, 18). This is an approach that has been used with great success in identifying differentiation-related antigens on hematopoietic cells (25). Over the past 10 years, a number of monoclonal antibodies (mAb) specific for antigens expressed by oval cells, bile ductal cells, and hepatocytes have been described (12, 18). Several of these have been used extensively for delineating lineage relationships during fetal development and carcinogenesis in the adult liver. Most recently, mAb directed against surface antigens on hepatocytes, bile ductal cells, and oval cells have been used successfully in combination with magnetic beads, panning, or fluorescence-activated cell sorting to enrich for antigenically defined subpopulations in fetal (26, 32) and normal adult (27) rat livers.

A common element in these separation strategies has been the use of mAb reactive with the oval cell/bile duct surface antigens such as OC2 and OC3, two oval cell antigens defined by mAb produced in our laboratory (17, 28). In fetal liver, OC2 and OC3 appear initially at E10-E12, primarily on cells of hematopoietic

origin but also transiently on a subpopulation of cells positive for the hepatocyte/bile duct marker, HBd1. We have proposed that the co-expression of OC2 and HBd1 is a characteristic of endodermal cells committed to further differentiation along a liver lineage (12). AFP and albumin have also been suggested as signals for hepatic commitment that appear coincidentally with the initiation of interactions between endodermal cells and mesenchyme in the septum transversum (29, 30). At present, the temporal and phenotypic relationships between cells doubly positive for the two mAb-defined antigens and those expressing AFP and albumin are unclear.

In the adult liver, OC2 is expressed by ductal cells in intralobular bile ducts and in the canals of Hering and is present at high levels on oval cells induced after treatment with hepatocarcinogens. More importantly, the presence of OC2 on a subset of preneoplastic foci, nodules, and primary hepatocellular carcinomas (17, 31) suggests that OC2 is expressed by oval cells capable of hepatocytic differentiation. Taken together, these results delineate OC2 as an antigenic trait held in common by bipotential hepatoblasts, ductal cells, oval cells, and perhaps hepatic stem cells.

In an attempt to discover additional markers that would further define the subset of oval cells committed to a hepatocytic lineage, we have undertaken the production of monoclonal antibodies against OC2-positive fetal liver cells isolated using OC2-specific monoclonal antibodies in combination with magnetic beads. In this articles, we review the production and characterization of an antigen, designated BD1, defined by an mAb produced by immunization with OC2-positive cells isolated from fetal rat liver (32). Unlike previously defined ductal antigens, BD1 is the first to our knowledge to show differential reactivity with oval cells and normal ductal cells. Moreover, BD1 displays a heterogeneous pattern of reactivity with normal intralobular ducts that defines three distinct populations with differing capacities for proliferation and/or differentiation.

## Materials and Methods

Methods for isolation of bile duct epithelial cells from rat liver and establishment of long-term cultures have been described (33). Essentially identical methods were used for the isolation and culturing of oval cells from livers of Fischer 344 rats maintained on a choline-deficient diet containing 0.1% ethionine (CDE) for 4 to 7 weeks. In addition to CDE, oval cells were also induced by performing a partial hepatectomy on rats treated with 2-acetylaminofluorene (AAF/PH) using a modified resistant hepatocyte protocol that omits the initiation step with diethylnitrosamine (17).

Protocols for collection, freezing, fixation, and storage of liver tissue; isolation of fetal liver cells; immunoblot analysis of detergent extracts; and hybridoma construction and screening have been described (17, 28, 32). Bile duct ligation (BDL) was performed as described by Steiner *et al.* (34). Two-thirds partial hepatectomy (PH) was performed as described by Higgins and Anderson (35).

Immunocytochemical analysis was carried out on frozen sections of adult, CDE, AAF/PH, regenerating, or BDL livers singly or doubly stained with mAb as described previously (32). Cell cycle analysis of cultured bile duct epithelia was performed on cultures stained by indirect immunofluorescence for BD1 and counterstained with propidium iodide (32). Cultures were blocked in $G_1$/S by treatment with $2 \times 10^{-7}$ *M* methotrexate (MTX) for 17 hr or in M phase by sequential treatment with MTX and 4 $\mu$g/ml nacodozole. Cells were released from MTX by treatment with $10^{-5}$ *M* thymidine. DNA content and average intensity of BD1 fluorescence were measured by quantitative microfluorimetry using an Axiovert inverted microscope equipped with epifluorescence illumination and a Sit-66 video camera. Image analysis was carried out using Biovision software (Perceptics, Knoxville, TN). Frequency distributions of DNA content and BD1 fluorescence intensity were plotted using Statview II software (Abacus Concepts Inc., Berkeley, CA).

Labeling with [$^3$H]thymidine was accomplished by a single intraperitoneal injection of [*methyl*-$^3$H]thymidine (80.9 Ci/mole, 1 $\mu$Ci/g body wt; Amersham, Arlington Heights, IL). Livers harvested at 4 hr or 1–41 days after injection were frozen at −70°C as described previously (17). Autoradiography was carried out on frozen sections fixed in 2% paraformaldehyde and 0.1% glutaraldehyde and covered with Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1/1 with distilled water. Slides were developed after 3–5 weeks in Kodak D-19 developer.

## Results and Discussion

**Identification of BD1, an Antigen Differentially Expressed by Normal Ductal Cells and Oval Cells.** Although OC2-positive fetal liver cells were detectable as early as embryonic day E10–E12, from a practical standpoint, the number of OC2-positive hepatic cells recovered from 10- to 12-day fetal livers was too small to be useful for immunization. It was further reasoned that oval cells/hepatic stem cells in the adult liver were likely to hold more in common with ductal cells or hepatoblasts appearing at later stages of liver development when associations between the newly formed ducts and the cords of fetal liver were being formed.

Initial screens of hybridomas produced from mice immunized with OC2-positive cells from 17-day fetal rat livers identified several monoclonal antibodies displaying reactivity with both normal bile ducts and oval cells but only one that distinguished between these two cell types. Further analysis showed that the antigen

recognized by the latter mAb, designated BD1, was expressed heterogeneously by adult bile ductal cells but was absent on oval cells induced by CDE or AAF/PH protocols. BD1 was also detected at high levels on newly formed HBd1-positive ducts at gestation day 16–17, but only on the side in direct contact with the portal mesenchyme (12, 32). By gestation day E20–E21, when ducts had become integrated into the portal mesenchyme, this asymmetry was no longer apparent and most OV6-positive ducts displayed a uniform expression of BD1. In newborn rat, a few OV6-positive ducts partially or completely negative for BD1 were observed. This heterogeneity in BD1 expression increased gradually, reaching adult levels by 2 weeks after birth when approximately 30% of the ducts were weakly positive or negative for BD1 and 70% displayed moderate to high levels of expression (32).

**Expression of BD1 after PH and BDL.** Positive expression of BD1 was consistent with the maturation of ductal cells in both fetal and adult liver, but the accumulation of BD1-negative cells in the postnatal period was less easily explained. It seemed unlikely that these were exclusively cycling cells since the reported labeling index (LI) for ductal cells was only 0.5–3% in adult rat liver. The alternative possibility that the BD1-negative cells were immature ductal cells was suggested by the absence of BD1 on oval cells, but the percentage of negative cells was clearly too high to be entirely of stem cell origin. Also at odds with this concept was the low level of BD1-negative cells observed in late gestation and neonatal rat liver (60–70% less than in adult liver), a time period when the levels of immature oval cell progenitors would be expected to be much higher. As discussed below, this apparent discrepancy may be related to changes in the regulation of BD1 during the neonatal period.

Analysis of BD1 expression during liver regeneration and after bile duct ligation has provided additional insights into the origin of negative cells and the regulation of BD1. As shown in Figure 1, the percentage of BD1-negative bile ducts increased dramatically by 72 hr after PH, a time point by which most of the ductal cells have completed their first round of division. This suggested that selective expansion of the BD1-negative cell population had occurred. Analysis of BD1 expression by [$^3$H]thymidine-labeled cells after PH showed, however, that both BD1-positive and -negative cells were labeled with thymidine at 48 hr after PH. During the next 24 hr, the percentage of [$^3$H]thymidine-labeled, BD1-positive cells decreased rapidly, a change that was mirrored by an almost identical increase in [$^3$H]thymidine-labeled, BD1-negative cells (Fig. 2). From these results, it was apparent that both BD1-negative and -positive cells were capable of proliferation after PH. The reciprocal changes in BD1-negative and -positive cells further suggested a precursor product relationship,

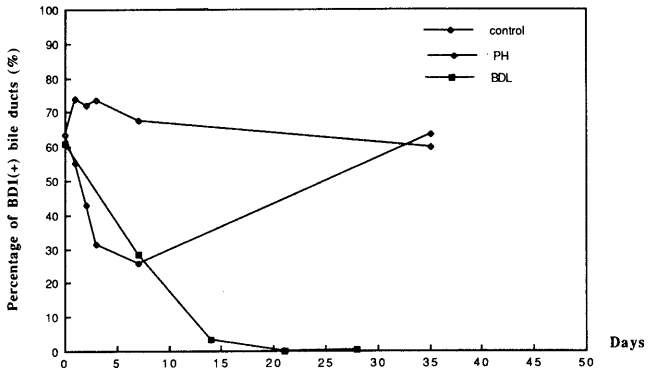


**Figure 1.** Expression of BD1 on bile ducts after partial hepatectomy or bile duct ligation. Frozen sections prepared from right lateral liver lobes of male Fischer 344 rats at various times after PH or BDL were stained by a double-label immunofluorescence technique with mAb specific for BD1 and OV6 (32). The numbers of OV6-positive ducts stained positive or negative for BD1 were determined by microscopic examination. A duct was defined as negative if less than one third of the cells showed positive fluorescence. Each time point represents the percentages of positive ducts observed in four sections/rat liver from two different rats. Partial hepatectomy or bile duct ligation was performed at Time 0.

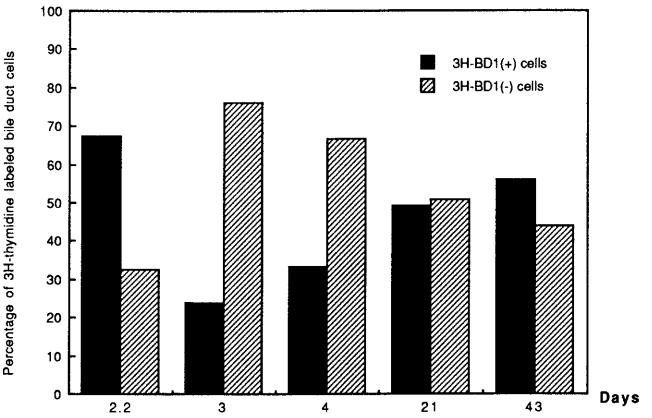


**Figure 2.** BD1 expression on ductal cells labeled with [$^3$H]thymidine after partial hepatectomy. Rats were given a single intraperitoneal injection of [$^3$H]thymidine at 48 hr after PH. At various times after injection, autoradiography and immunofluorescence analysis for BD1 were performed on pairs of serial frozen sections prepared from the right lateral lobes. The percentage of BD1-positive and -negative ductal cells labeled with [$^3$H]thymidine was calculated from four sections at each time point.

with positive cells giving rise to negative cells as ductal cells progress through BD1 permissive and nonpermissive stages of the cell cycle. Interestingly, the percentage of [$^3$H]thymidine-labeled, BD1-positive cells never reached zero (Fig. 2), the expected result if all of the thymidine-labeled cells are cycling cells. This probably reflects a lack of synchrony in the proliferative response, which results in a partial equilibrium where the increase in negative cells is offset by the recovery of BD1 by cells that are no longer cycling.

Of further significance was the fact that the recovery of presurgical BD1 levels was not achieved for 5 to 6 weeks after PH (Fig. 1). This was in sharp contrast to

the rapid decrease in BD1 expression, which reached a minimum within 3–7 days after PH (Fig. 1). Since liver regeneration is essentially complete by 3 weeks, it seems likely that other factors in addition to cell cycle must be involved in the regulation of BD1 expression. A rapid decrease in expression was also observed after BDL, thus eliminating the possibility that this loss was peculiar to liver regeneration (Fig. 1). However, unlike PH, in which 20–30% of the ducts remained positive during regeneration, the percentage of BD1-positive ducts dropped essentially to zero after BDL and no appreciable recovery in expression was observed.

Taken together, these observations suggest that ductal cells in the adult rat liver are a heterogeneous mix of at least three distinct cell populations distinguished by differences in their expression of BD1 in resting and regenerating liver. The first is composed of BD1-positive cells that remain so during liver regeneration. These cells are thought to represent terminally differentiated ductal cells that are unable to proliferate after PH. The exact size of this population is not clear at present because the minimum percentage of ducts that remain positive during liver regeneration is the result of a transient steady state in a dynamic cell population that includes BD1-positive cells that will eventually divide and become negative, BD1-negative daughter cells that have recovered BD1, and terminally differentiated BD1-positive ductal cells that no longer proliferate. The percentage of BD1-positive cells that do not incorporate thymidine after continuous labeling during the proliferative phase of liver regeneration would provide a closer estimate of the size of this persistently BD1-positive cell population. In this regard, the complete loss of BD1-positive cells after BDL suggests that the number of cells in this category may be negligibly small. This conclusion is based on the assumption that the constant cycling and/or cytotoxicity produced by BDL precludes any significant recovery of BD1-positive cells, leaving the nonproliferating ductal cells as the only positive cells remaining in the first few days or weeks after BDL. It should be considered, however, that the increase in the size of the ductal cell population after BDL is several times greater than the compensatory increase observed after PH (11). As a consequence, the apparent absence of a persistently positive cell population may be largely a dilution effect. Alternatively, the nondividing BD1-positive ductal cells may be lost as a consequence of the increased apoptosis that occurs at later time points after BDL (11).

A second population defined by BD1 is composed of the BD1-negative ductal cells that incorporate [$^3$H] thymidine after PH. Whether daughter cells produced following division of these negative cells retain their BD1-negative status or enter a BD1-permissive state is presently unknown. Also included in this population are immature ductal cells such as those in the canals of Hering that have not progressed to a BD1-permissive stage of differentiation. The third and largest population is represented by the BD1-positive, [$^3$H]thymidine-labeled cells that lose BD1 during liver regeneration. BD1-positive cells are considered to be the most mature ductal cell population. After division, these cells are thought to reaccumulate BD1.

Viewed as a whole, these three populations of ductal cells compose a cell system that has the basic characteristics of a two-compartment cell renewal system: a proliferative compartment composed of BD1-negative cells and a quiescent or functional compartment represented by the thymidine-labeled, BD1-positive and the persistently BD1-positive ductal cells. Whether the cells in these three cell populations are oriented relative to each other in the manner consistent with the streaming liver model of Arber *et al.* (36) remains to be determined.

**Cell Cycle-Dependent Expression of BD1 *In Vitro*.** To provide more insight into the nature of the cell cycle-dependent changes in BD1 expression, detailed analysis during each phase of the cell cycle was performed on established cultures of bile ductal epithelial cells (BDEC) shown previously to display antigenic and enzymatic phenotypes identical to their counterparts *in situ* (33, Table I). In accordance with results from the analysis of frozen sections of intact liver, the expression of BD1 on cultured BDEC was heterogeneous. Moreover, the percentage of BD1-positive cells and the levels of expression increased with cell density, a finding consistent with the decreased proliferation observed at higher cell densities. In contrast, other characteristic bile duct markers such as OV6, OC2, CK19, and $\gamma$-glutamyl transpeptidase were expressed uniformly regardless of cell density. Heterogeneous expression of BD1 was also observed *in vivo* on well-differentiated ducts formed by BDEC transplanted into the pancreas.

When BDEC were blocked in late $G_1$ by treatment for 17 to 20 hr with MTX, a dramatic increase in BD1 levels was observed (Fig. 3). Subsequent release from $G_1$/S block by replacement of MTX with thymidine resulted in a rapid decrease in BD1 expression, a result consistent with the rapid doubling of DNA content signifying entrance into $G_2$/M (Fig. 3). When BD1-positive BDEC cultures blocked with MTX were released into medium containing *nocodozole*, an agent that prevents progression into the M phase, BD1 levels rapidly decreased, suggesting that BD1 may be degraded during the M phase of the cell cycle. Consistent with this conclusion was the constancy in BD1 levels for up to 10 hr after release from MTX, a time span that should place most of the BDEC in M phase (33). Significantly, BD1 was the only bile ductal marker examined that showed a cell cycle-dependent pattern of expression. Cell cycle status thus appears to be an important determinant of BD1 expression both *in vivo*

**Table I.** Phenotypic Comparison of Established Rat Liver BDEC Lines with Normal Bile Ducts, Hepatocytes, and Chemically Induced Oval Cells

| | Marker[a] | BDEC line[b] | Bile ducts[c] | Oval cells[d] | Hepatocytes[e] |
|---|---|---|---|---|---|
| Bile ducts only | BD1 | (− to +++) Heterogeneous | (− to +++) Heterogeneous | (−) | (−) |
| Bile ducts/oval cells | OC2 | (+ to +++)[c] | (+++) | (+++) | (−) |
| | OC3 | (+ to +++) | (+++) | (+++) | (−) |
| | OV1 | (+) | (−/+) Heterogeneous | (+) | (−) |
| | OV6 | (++ to +++) | (+++) | (+++) | (−) |
| | BDS7 | (++ to +++) | (+++) | (+++) | (−) |
| | CK19 | (++ to +++) | (+++) | (+++) | (−) |
| | CK7 | (++ to +++) | (+++) | (+++) | (−) |
| | GGT | (+ to +++) | (+++) | (+++) | (−) |
| | Connexin 43 | (+++) | (+++) | ND[f] | (−) |
| Hepatocyte/bile ducts | DPPIV | (+ to +++) | (++) | ND | (+++) |
| | HBD1 | (− to +) Rare positive cells | (+) | (−) | (+++) |
| | Desmoplakin I | (+++) | (+++) | (+) | (+++) |
| Hepatocytes only | H.1 | (−) | (−) | (−) | (+++) |
| | H.2 | (−) | (−) | (−) | (+++) |
| | H.5 | (−) | (−) | (−) | (+++) |
| | Cell CAM 105 | Early passage: a few (+); later passage: mostly (+) | (−) | ND | (+++) |
| | Connexin 32 | (− to +) <10% | (−) | ND | (+++) |
| | Albumin | (−) | (−) | (− to +) subpopulation of cells | (+++) |
| Fetal rat liver | CK14 | Mostly (−); <1% weakly positive | (−) | ND | (−) |
| Intermediate filaments | Vimentin | Mostly (−); 2–5% positive | (−) | (−) | (−) |
| | Desmin | (−) | (−) | (−) | (−) |

[a] The production and characterization of antibodies is described in Refs. 17, 18, 28, and 32.
[b] The phenotype of all BDEC lines (BDEC 1, 2, 3, and 4) established in the present investigation is essentially identical, regardless of passage number (p1–p17).
[c] Relative reactivity was assessed subjectively on a scale from + for the weakest level of positive fluorescence to +++ for the strongest. Plus (+) was assigned when the fluorescence intensity was significantly above background levels displayed in sections stained with an unreactive mAb. Two plus signs (++) indicated an intermediate intensity significantly less than +++, the highest level observed with the mAb being tested. If a heterogenous pattern of reactivity was observed, a range of intensities has been given. For mAb against intercellular junction (connexin 43, connexin 32, and desmoplakin I), intensity was measured by the frequency of membrane plaques.
[d] *In situ* phenotype of hepatic oval cells induced by feeding rats a choline-deficient diet containing 0.05% ethionine for 6 weeks.
[e] *In situ* phenotype of normal rat liver.
[f] ND, not determined.

and *in vitro*, with its loss occurring during $G_2$/M. This is consistent with *in vivo* results suggesting that the loss of BD1 is associated with cycling ductal cells or ductal cells in a replication competent status incompatible with high levels of expression. Recovery of BD1, in contrast, appears to be dependent on a maturation event associated with entrance into a state of BD1-permissive quiescence. The slow kinetics of BD1 re-expression after PH suggest a delay of variable duration after cell division may occur before ductal cells become permissive for BD1.

Although [$^3$H]thymidine labeling studies clearly show that both BD1-negative and -positive cells proliferate after PH, there is currently no evidence to rule out the possibility that BD1-negative ductal cells undergo selective expansion in carcinogen-treated rats. Preferential ductal cell proliferation due to differences in resistance to the mitoinhibitory effects of carcinogens would in essence be the ductal cell equivalent of the resistant hepatocyte model. In support of this possibility, there are a number of phenotypic characteristics exhibited by hyperplastic ductal cells that are not detected on their normal counterparts (11). One way to test this possibility experimentally would be to examine changes in the LI of BD1-negative and -positive cells during the induction of oval cells by AAF/PH. If there

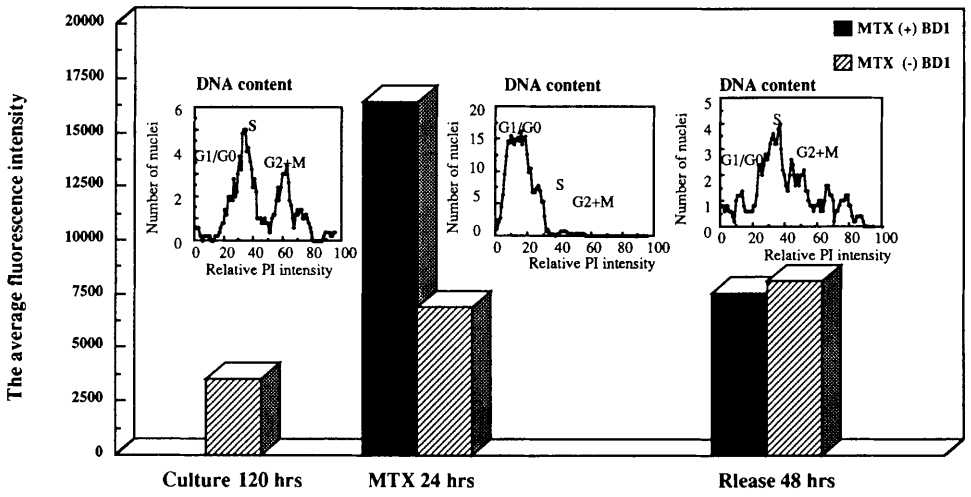


**Figure 3.** Microfluorimetric analysis of BD1 expression on BDEC cultures treated with MTX. BDEC ($5 \times 10^{-4}$) at Passage 9 were maintained in chamber slides until 40–60% confluent (48 hr). Cultures were stained for BD1 by indirect immunofluorescence before and after a 24-hr treatment with MTX and at 48 hr after release in thymidine-containing medium. Cultures were counterstained with propidium iodide. Average fluorescence intensity for BD1 was determined as described in Material and Methods for five randomly selected fields. DNA content was estimated from the total fluorescence for nuclei within five separate fields. Relative nuclear intensities below 30 were operationally defined as $G_1/G_0$.

is preferential expansion of BD1-negative ductal cells, only the negative cells should incorporate [$^3$H]thymidine and the overall LI should be much lower than after PH without AAF, when both BD1-positive and -negative ductal cells can proliferate. Studies of this nature are currently in progress.

Why oval cells do not express BD1 is not clear at present. Cytotoxic effects do not seem to be involved since expression is maintained on mature bile ducts in AAF/PH-, CDE-, and galactosamine-treated animals. Rapid proliferation and an impaired ability to differentiate into mature ductal cells are the most likely causes for the inability of oval cells to express BD1, but other factors may also be operative. This is suggested by the fact that the few oval cell ducts still present 20 months after withdrawal of animals from CDE diet are negative for BD1. In addition, preliminary studies on the expression of BD1 in primary oval cell cultures established from rats maintained on CDE diet indicate that BD1 is not expressed at detectable levels even after 15–25 days in culture. In contrast, primary cultures of BDEC established and maintained under identical conditions displayed high levels of BD1. Decreased levels of BD1 were also exhibited by BDEC maintained for more than 30 passages *in vitro*. High passage cultures also failed to induce BD1 expression when blocked in $G_1$ after treatment with MTX for 17 to 20 hr (Fig. 4). This change in BD1 expression does not appear to be related to differentiation competence or neoplastic conversion because BDEC at high passage are not tumorigenic and form well-differentiated ducts when transplanted into the pancreas. These results are intriguing because they suggest that oval cells and high passage BDEC may both possess a subtle defect in cell cycle regulation manifested by an inability to upregulate the expression of BD1.

The marked increase in the heterogeneity of BD1 expression during the first 2 weeks after birth may also mark a change in the cell cycle regulation of BD1. In both fetal and neonatal liver, the LI is considerably higher than in the adult, but in spite of this, the levels of BD1-negative cells are much lower. This inconsistency suggests that differences exist between neonatal and adult ductal cells in the rate of BD1 degradation during $G_2/M$ or in the rate of recovery after cell division. What these differences may be is pure speculation at present, but one reasonable possibility would be a maturation-related change in a protease expressed during $G_2/M$ that has cleavage sites within BD1.

**Relationship of BD1 to Known Ductal Cell Antigens.** At present, relatively little is known about the structural characteristics of BD1. In BDEC cultures stained by indirect immunofluorescence, BD1 is distributed as a dense meshwork of cytoplasmic filaments. Immunoblots of detergent extracts from BDEC contain two peptides of 165 kDA and 176 kDA that show reactivity with BD1-specific mAb. In addition to these peptides, a minor peptide of approximately 220 kDA was detected in immunoblots of microsomal extracts from the human hepatoma line HTB52 and the rat hepatoma line H5D 16.12. The apparent size of BD1 differs significantly from cytokeratins commonly found in ductal cells. A close relationship between any presently known bile duct components, such as GGT, BDS7, or OV6, also seems unlikely because all are coexpressed at high levels by oval cells (11, 12). Additional insight into the identity of BD1 should be forthcoming

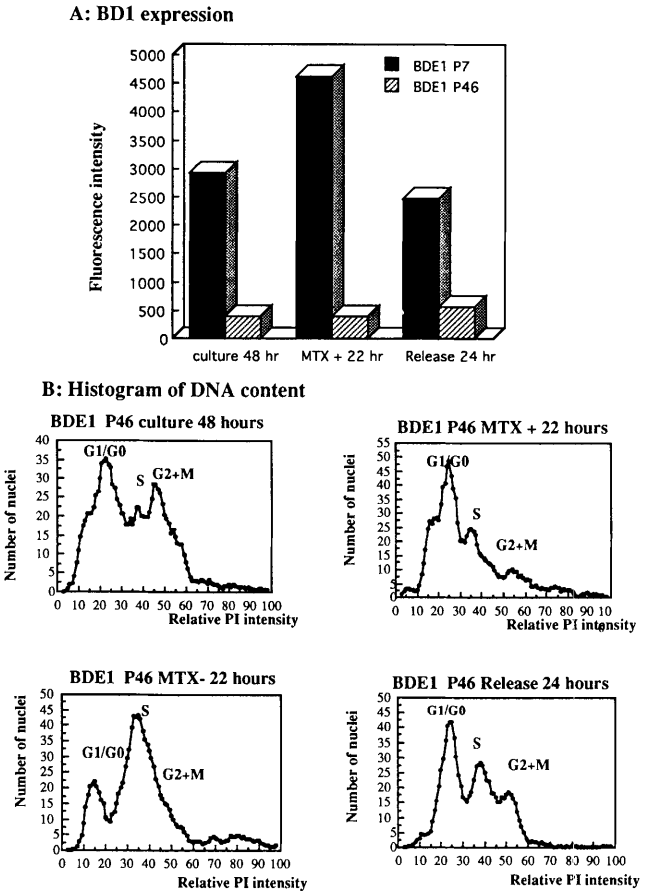


**Figure 4.** Effect of MTX on BD1 expression by low and high passage BDEC. BDEC were stained for BD1 by indirect immunofluorescence and counterstained with propidium iodide before and after treatment for 22 hr with MTX and at 24 hr after release in thymidine. Fluorescence intensities for BD1 and nuclear staining were determined as described in Figure 3. (A) Relative fluorescence intensities for BD1 labeling; (B) histograms of propidium iodide fluorescence intensities.

when sufficient quantities of purified BD1 become available for microsequence analysis.

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