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Cell kinetics of repair after allyl alcohol-induced liver necrosis in mice

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Summary. The cellular kinetics of repair and scarring which occurs after induction of periportal necrosis in mice by allyl alcohol were examined by histology and immunohistochemistry. Thirty-six six-week-old female C57Bl/6J mice were injected intraperitoneally with two doses of allyl alcohol on day 0 and tissue sections were taken at various times and stained by haematoxylin and eosin or immunostained for proliferating cell nuclear antigen (PCNA), bile duct/oval cell marker A-6, and DNA fragments (apoptosis). Within 6 hours, periportal necrosis was seen extending to produce large zones of confluent, pan-acinar irregular necrosis, predominantly in the right and medial lobes with sparing of the left and caudate lobes. Restoration of liver mass was accomplished mainly by proliferation of mature hepatocytes in the surviving lobes of the liver (hyperplasia). In the right and medial lobes where necrosis was limited to the periportal zone, there was some, but much less, proliferation of small, oval periportal cells. The large necrotic zones in the right and median lobes shrank and were replaced by granulomatous inflammation. This cellular contribution of liver regeneration in the mouse was different from that previously reported in the rat and provides a means of inducing only a small proliferation of oval cells.

Keywords: allyl alcohol, liver injury, hyperplasia, stem cell

The role of different cell types in carcinogenesis or in restoration of chemically induced liver injury has been studied by following the phenotype of cells repopulating the areas of liver cell loss (Sell *et al.* 1987; Sirica 1992). Following centrolobular (zone III) necrosis of the liver by chemicals such as carbon tetrachloride, mitosis of adjacent hepatocytes was seen and interpreted to mean that the 'mature' liver cells in zone II proliferated and repopulated the necrotic central areas (Engelhardt *et al.* 1984). However, within the last 15 years it has been shown using more sensitive assays for DNA synthesis that, after zone III (Engelhardt *et al.* 1984; Tournier *et al.* 1988) or diffuse injury (Kuhlmann & Wurster 1980; Lemire *et al.* 1991; Dabeva & Shafritz 1993), proliferation of small periportal cells also occurred. These findings supported earlier conclusions regarding the presence of putative liver 'stem' cells that proliferate during the early response to chemical hepatocarcinogens (Sell & Leffert 1982). To test the hypothesis that intraportal 'stem' cells may respond to liver injury, the cellular restitutive response to periportal (zone I) necrosis induced by allyl alcohol was tested in rats (Yavorkovsky *et al.* 1995). It was found that the periportal necrotic zones were reconstituted by small 'null' cells that proliferated and acquired markers of liver cell development as the necrotic zone was resolved. We reasoned that the allyl alcohol model might provide a means of increasing the numbers of liver

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stem cells so that they could be isolated, cultured and used for transplantation studies. In order to take advantage of the model systems in mice, the possibility of applying this approach to mice was tested.

Materials and methods

Experimental animals/protocol

Thirty-six 6-week-old female C57Bl/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). They were housed 5 to a plastic cage, maintained at 21°C with a 12 hour on, 12 off light cycle. Allyl alcohol (AA, Aldrich, Milwaukee, WI) was injected intraperitoneally (i.p.) into each mouse in two doses: 0.67 mmol/kg on day −7 and 1.1 mmol/kg on day 0. Preliminary experiments demonstrated that a single dose of 0.67 mmol/kg did not consistently produce liver damage, whereas a single dose of 0.9 mmol/kg or above was lethal. Three mice were sacrificed under ether anaesthesia at 6, 12, 24 and 48 hours, 3, 5, 7 and 14 days, and 12 weeks after the second injection. The body and liver weights were measured, the liver examined grossly for pathologic changes, and tissue from each lobe of the liver was taken and fixed in 10% neutral formalin.

Histology and immunohistochemistry

After 12–16 hours fixation, the liver tissue blocks were processed and embedded in low melting point paraffin for histology (haematoxylin and eosin) and immunolabelling. For proliferating cell nuclear antigen (PCNA), the formalin fixed tissues were pretreated by boiling in 10 mmol citric acid, pH 6.0, in a microwave oven for 10 minutes (Gown *et al.* 1993). Endogenous peroxidase was blocked by incubation of the slides with 2.5% H_2O_2 in methanol for 10 minutes. For PCNA the primary antibody was mouse anti-human PCNA diluted 1:80 (PC10) (DAKO, Carpinteria, CA), while A-6 (generously supplied by Natalya Engelhardt, Moscow), a rat monoclonal antibody to mouse 'oval' cells (Engelhardt *et al.* 1990), was used at a 1:10 dilution. After overnight incubation at 4°C and washing, the slides were exposed to prediluted biotin-conjugated secondary antibodies for 60 minutes at 37°C and then to ExtrAvidin-conjugated peroxidase (Sigma Diagnostics, St Louis, MO). The peroxidase reaction was developed by incubating in 0.005% H_2O_2 and 0.2% 3,3′-diaminobenzidine (DAB). DNA fragments of apoptotic bodies were detected by enzymatic labelling with digoxigenin-linked nucleotide catalysed by terminal deoxynucleotide transferase (TUNEL method) and then reacted with peroxidase conjugated anti-digoxigenin antibody (ApopTag Plus Peroxidase Kit) (Oncor, Gaithersburg, MD). Peroxidase labelled DNA fragments are stained by development of the DAB substrate. Controls for immunostaining included sections treated with dilutions of normal serum or non-antibody hybridoma culture fluid replacing the 'primary' antibody.

Necrotic area and proliferating and apoptotic cell measurements

The necrotic zones in each of the left, medial and right lobes of 3 mice were measured by drawing the cut surface of the liver on magnified millimetre graph paper and calculating the area involved. PC10 positive cells and apoptotic cells were counted in 30 10-mm^2 ocular grids (fields) per lobe (approximately 4000 cells) at ×400 magnification using a micrometer eyepiece (Reichert-Jung/Leica, Deerfield, IL).

A rough estimation of the stages of cell cycle for cells labelled for PCNA was determined by the following criteria:

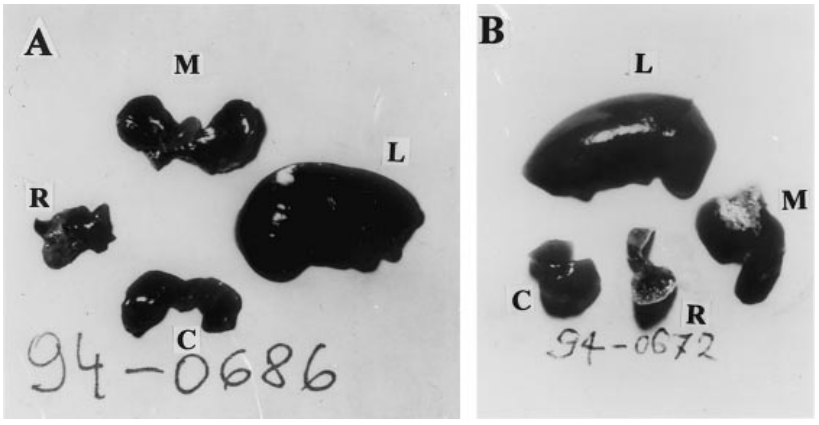

Figure 1. Gross photographs of representative livers of mice A, 3 weeks and B, 4 weeks after injection of allyl alcohol. There is enlargement of the left (L), and caudate (C) lobes with partial atrophy of the medial (M) lobes and complete atrophy of the right (R) lobes.

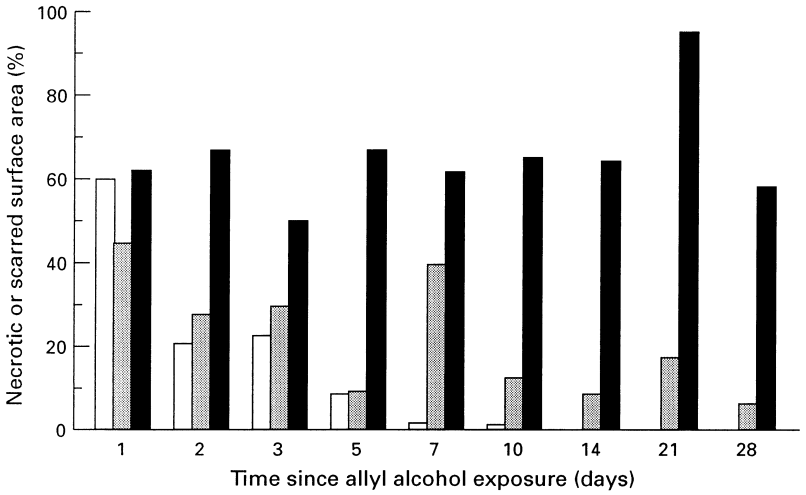

Figure 2. Graph comparing microscopic progression of necrotic zones in the ■, right; □, left and ▨, medial lobes of the livers of allyl alcohol treated mice. Although the area of liver necrosis was evaluated as being essentially the same at 24 hours, that in the right lobe progressed to geographic necrosis with massive scarring, whereas that in the left lobe resolved with hyperplasia and little or no residual evidence of injury.

G_1: patchy to light brown nuclear staining, no cytoplasmic staining

S-G_2: dark brown nuclear staining with variable cytoplasmic staining

M: diffuse cytoplasmic staining, fragmented nuclear staining

The number of labelled cells was counted per high-power field. Because of the large variation among the lobes and within different areas in the lobes, overall estimates of the number of proliferating cells are reported.

Results

Gross findings

At 6, 12 and 24 hours the liver of each mouse was mottled with alternating whitish grey and dark-red zones in each lobe. After 24 hours large grey-white zones of necrosis were present representing approximately 50% of the total liver mass. In general, the left and caudate lobes recovered, so that by 10–17 days they had an essentially normal colour and consistency and were enlarged 30–40%, whereas the right and medial lobes retained large, shrunken, whitish zones. After 21 days, the left lobes and caudate lobes were enlarged up to 60% more than normal, the medial lobe had variable scarring or enlargement, and the right lobes were massively scarred and up to 100% atrophied (Figure 1). The percentage of the liver lobes that appeared necrotic or scarred at different times as estimated grossly is presented graphically in Figure 2. Throughout the experiment, the liver mass, measured as a ratio between body weight and liver weight, remained constant (data not shown).

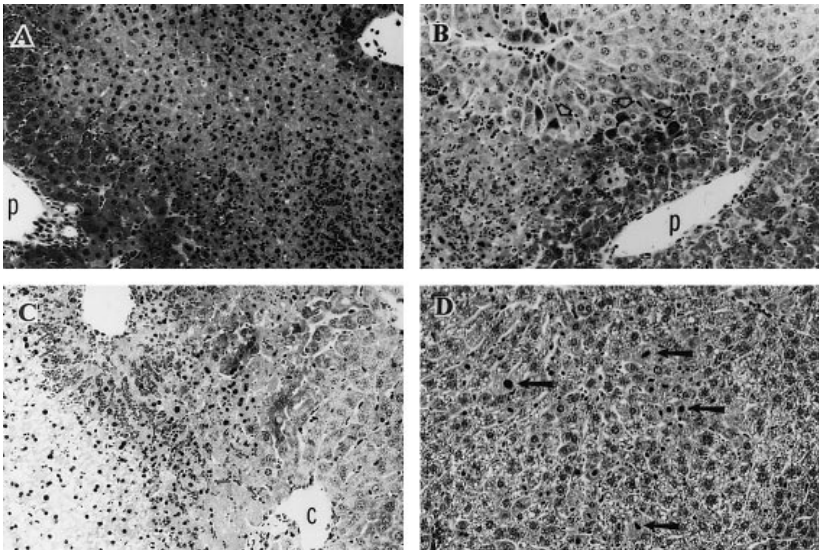

Figure 3. Photomicrographs of haematoxylin and eosin stained tissue sections of livers from allyl alcohol treated mice from 6 to 48 hours. A, Areas of incipient necrosis show loss of nuclear staining and increased staining of cytoplasm. P, Portal vein, (6 hours) ×200. B, Apoptotic bodies located at margin of necrotic tissue, short open arrows. P, Portal vein. (12 hours) ×200: C, Central zones next to necrotic zone. C, Central vein. (24 hours) ×200. D, Mitotic figures in non-necrotic liver, closed arrows (48 hours). ×200.

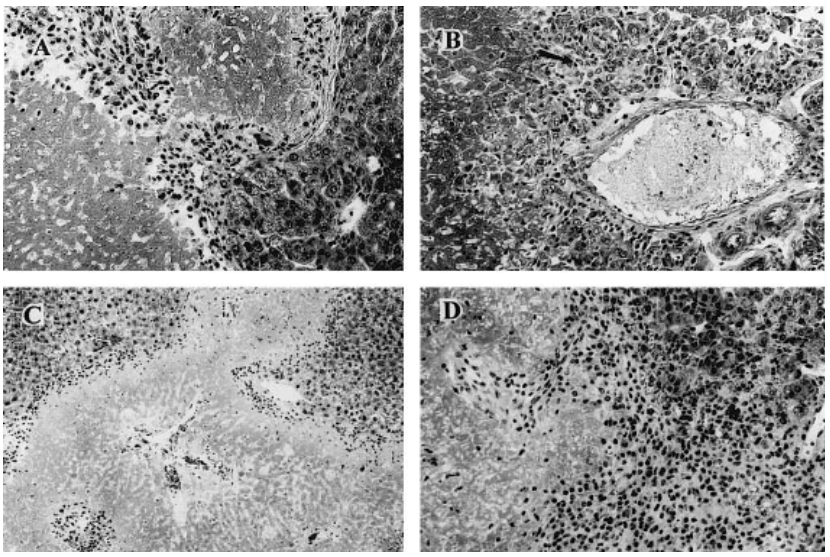

Figure 4. Photomicrographs of haematoxylin and eosin stained tissue sections of livers from allyl alcohol treated mice from 3 days to 7 days. A, Margin of necrotic area showing layer of round cell infiltrate (Day 3). ×200. B, Portal zone next to area of necrosis showing some oval cells (arrow) and round cells (Day 3). ×200. C, Area of geographic necrosis (5 days). ×100. D, Margin of necrotic areas showing increasing chronic inflammation. (7 days). ×200.

Microscopic findings

The histopathologic changes evolved from cell injury and cell death (necrosis and apoptosis) to either regeneration or scarring are shown in Figures 3 (6–48 hours), 4 (3–7 days) and 5 (10 days–12 weeks). Early necrotic changes were seen in all liver lobes examined. Scattered periportal necrosis was seen in the left and caudate lobes, but most of these lobes did not show large areas of necrosis. Focal irregular, partly confluent, pan acinar ('geographic') necrosis was seen in the medial lobe and extensive irregular, confluent necrosis was present in the right lobes. Whereas the necrosis in the right lobes evolved to scarring and atrophy, that in the left and caudate lobes resolved and generally these lobes had an essentially normal histologic appearance after 21 days. The medial lobe showed variable necrosis and regeneration.

6–12 hours. At 6–12 hours (Figure 3A and B) approximately one-third of the liver was normal, one-third showed confluent haemorrhagic necrosis with some acellular zones and some areas containing 'ghosts' of liver cells, and one-third showed increased eosinophilia of periportal cells without necrosis. There was little or no inflammation.

24 hours. There was marked acellular pan acinar necrosis of 50 to 60% of the liver with remaining areas essentially normal (Figure 3C). The necrotic zones had a rim of haemorrhage similar to a vascular infarct.

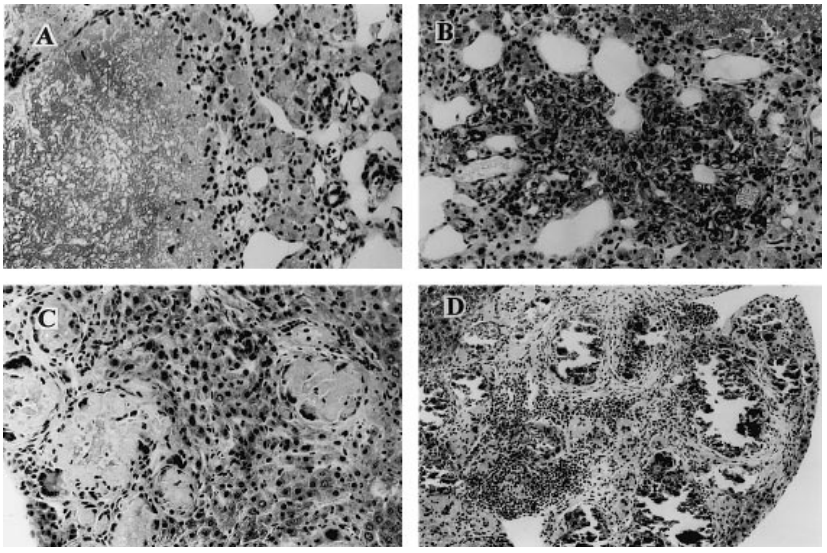

Figure 5. Photomicrographs of haematoxylin and eosin stained tissue sections of livers from allyl alcohol treated mice from 10 days to 12 weeks. A, Margin of necrotic area showing vascularization of inflammed margin (10 days). ×200. B, Area of 'oval' cells around portal zones within necrotic area (14 days). ×200. C, Granulomas forming around necrotic areas (4 weeks). ×200. D, Large granuloma in atrophic liver (12 weeks). ×10.

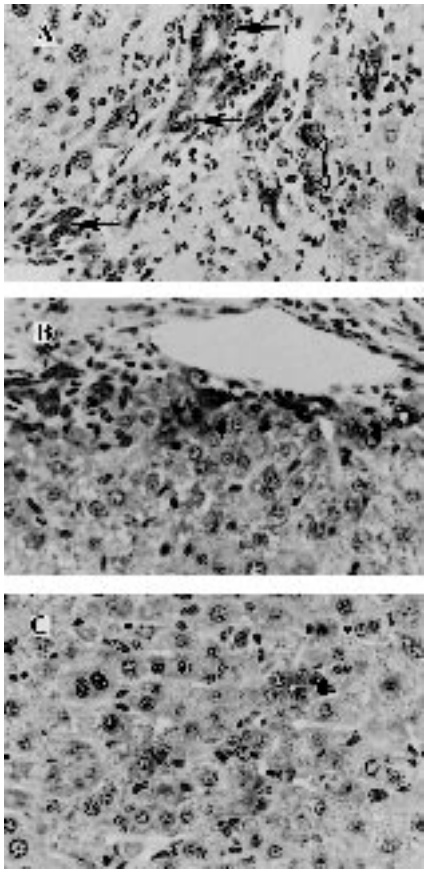

Figure 6. Immunohistological immunization of A-6 antibody staining 3 days after allyl alcohol exposure. In the portal area shown in A there is labelling of ducts (large arrows) and a few small periportal 'oval' cells (small open arrows) as well as some small hepatocytes (small closed arrows). At the portal plate, shown in B, there is also labelling of oval cells (open arrows) and hepatocytes (closed arrows). Nearby, in C, there is labelling of individual hepatocytes, as well as a cluster of hepatocytes around a canaliculus (arrow).

Polymorphonuclear leucocytes were scattered in the margins between the 'normal' and necrotic tissues.

48 hours. There were large areas of 'geographic' necrosis alternating with nearly normal areas. There were now round cells at the margins of necrotic zones. The right and medial lobes were almost completely necrotic, whereas the left and caudate lobes were partially or almost completely normal. Numerous mitotic figures (up to 3 per high-power field, Figure 3D) were present in non-necrotic zones. In surviving portal zones near or within the margins of the necrotic zones there was a slight increase in small 'oval' cells.

3–5 days. The findings described on day 2 were now more marked. In the right lobe the pan acinar, confluent necrotic areas were surrounded by more round cells and macrophages with some margins having a marked giant-cell reaction (Figure 4A and C). There were more periportal 'oval' and round cells as well as small ducts, in portal zones adjacent to or just within the necrotic zones (Figure 4B). There were still up to three mitotic figures in hepatocytes per high-power field in non-necrotic areas.

7 days. Mitotic figures were rarely seen. The organizing inflammation around the necrotic zones had the appearance of granulation tissue with increasing numbers of small capillaries and many chronic inflammatory cells (Figure 4D). There was a cuff of round cells and 'oval' cells in some portal zones.

10–21 days. The left and caudate lobes showed little or no evidence of residual necrosis. Organization continued around the zones of acellular necrosis in the right and medial lobes with increasing vascularization and giant-cell reaction. By 21 days many foamy macrophages surrounded the necrotic zones and these took on the appearance of a foreign-body reaction. Clusters of granulomas were seen containing Langhans' giant-cells, and occasional small granulomas were seen in otherwise normal liver. There was neovascularization (granulation tissue, Figure 5A) and oval cell proliferation around portal areas that survived within the margins of the necrotic zones, in a few instances giving rise to 'islands' of small oval cells (Figure 5B).

5 weeks and later. Residual foreign body granulomas of various sizes were seen in otherwise normal liver (Figure 5C and D). With some exceptions, the right lobes were almost completely atrophic or showed extensive areas of scarring. The medial lobes were scarred as well as apparently normal areas.

Immunohistology

A-6. Labelling with A-6 in ductular cells as well as small periportal 'oval' cells and transitional hepatocytes in periportal or perinecrotic areas of the liver is shown in Figure 6. A-6 was also found in some larger individual hepatocytes as well as in clusters of small hepatocytes that appeared to be forming ducts.

Apoptotic cells and bodies. These were identified by *in situ* labelling as single cells with halos and differentiated from the karyorrhexis of necrosis which resulted in weak staining. Apoptotic cells were seen in the periportal areas as early as 6 hours and in areas at the margins of the necrotic and non-necrotic areas at 24 hours (Figure 7). The mean numbers of apoptotic cells in the right and left lobes of the liver at different times after AA injection are presented in Figure 8.

PCNA. PCNA labelling at 4 days is shown in Figures 9

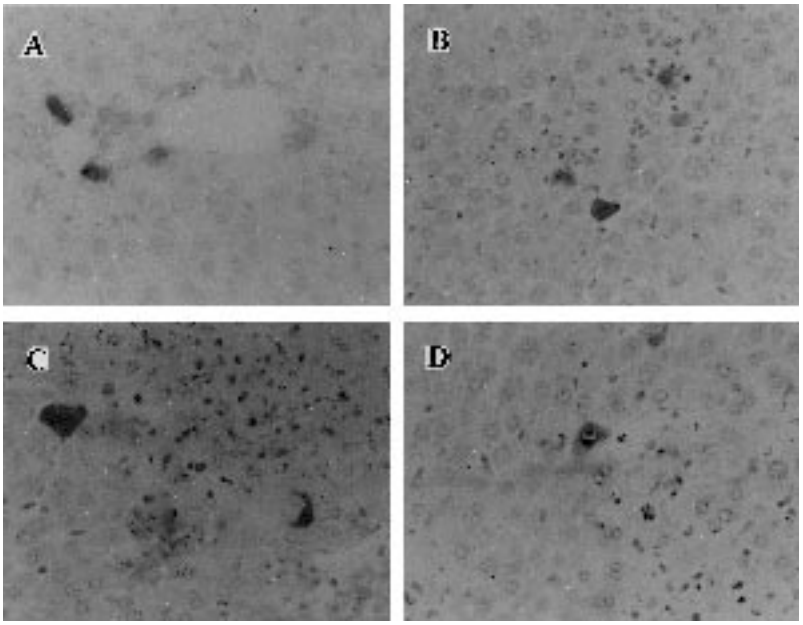

Figure 7. Terminal transferase labelled cells. A, 6 hours showing very early periportal labelled cells. ×200. B–D, 24 hours showing apoptic cells adjacent to areas of necrosis. ×200.

and 10. After PC10 labelling, G1 and S phase cells were counted in 30 randomly selected fields in each zone of the liver at different times after AA injection (Figure 11). Because of the variation in labelling in the different lobes of the liver it was not possible to present representative counts, so the data are presented in the graph as indices (1 to 4+). Proliferation of 'mature' hepatocytes was greater in periportal zones and next to necrotic zones early after injury; then increased in non-necrotic areas after 24 hours, peaking at 72–120 hours and then declining. From 48 to 120 hours the number of cells in each of the four phases of the cell cycle was very high and each hepatocyte appeared to divide at least once. Labelling of smaller hepatocytes (? oval cells) in perinecrotic areas, particularly in periportal zones, began at 2 days, peaked at 5 days and then continued at a low rate in areas adjacent to necrotic areas. In periportal areas, ductular cells, small A-6 positive 'oval' cells and hepatocytes, as well as stromal cells, were strongly positive for S and G2 phases (Figure 10D). After 7 days, proliferating 'mature' hepatocytes in non-necrotic areas were no longer seen, whereas proliferation of small peri-necrotic hepatocytes continued in lobes with extensive necrosis.

Discussion

The purpose of this study was to evaluate a mouse model in which hepatocytic progenitor cells (stem cells) could be activated and induced to proliferate. Such a model would be useful in studies of cellular lineages in

Figure 8. Graph of number of apoptotic cells per 400 cells in ■, right and ○, left lobes of the liver at different times after allyl alcohol exposure. Throughout the course of the experiment the number of apoptotic cells was much higher in the right lobe than in the left lobe.

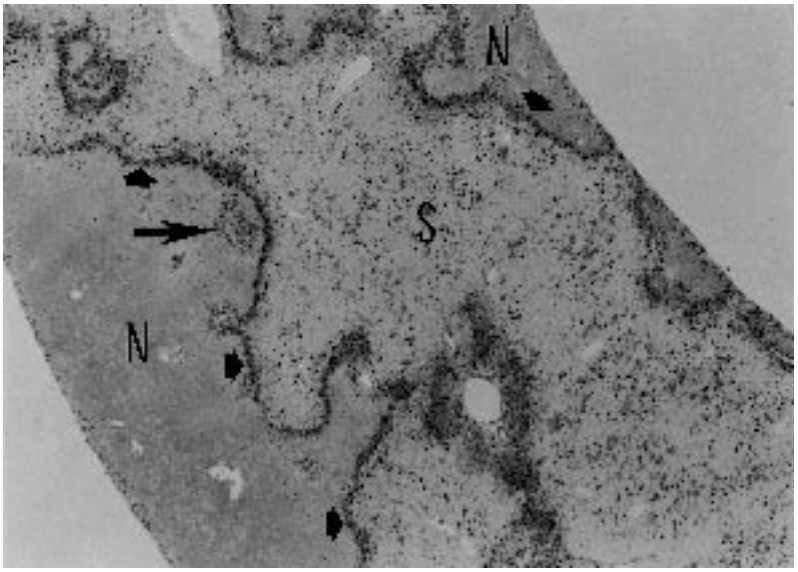

Figure 9. PCNA labelling 4 days after allyl alcohol injection. ×100. High percentages of cells are labelled at the junction of necrotic (N) and non-necrotic areas (small arrows), as well as in periportal areas near or just within the necrotic margin (large arrow). There is a smaller proportion of labelling of mature hepatocytes in the surviving area (S) between the necrotic areas.

the liver, and as a valuable source for isolation, cultivation and further experimental utilization of mouse liver stem cells. Several similar models already exist in rats, using either fetal liver or carcinogenic or non-carcinogenic injury to activate putative liver stem cells and cultivate them *in vitro* (Sirica 1992; Sell & Leffert 1982; Grisham 1983; Sirica *et al.* 1990; Germain *et al.* 1988; Reid 1990). In addition, experimental models have been reported in mice, in which small, oval hepatocytes, that may function as progenitor cells in the liver, appear after treatment with carcinogens (Engelhart *et al.* 1990; Factor *et al.* 1994; He *et al.* 1994) or in lines of transgenic mice (Dunsford *et al.* 1990; Schirmacher *et al.* 1991; Bennoun *et al.* 1993; Orian *et al.* 1990), in which proliferation of 'oval' cells is associated with liver cell transformation and liver tumour growth. If liver tumours do originate from stem cells (Sell & Pierce 1994), then proliferating immature liver cells induced by carcinogens or in transgenic mice may have already been transformed and phenotypically changed. Therefore, a model of short-term, non-carcinogenic chemical injury was sought to activate proliferation of putative mouse liver stem cells.

Allyl alcohol is known to produce periportal necrosis in mice and rats. Periportal injury is mediated by metabolism of allyl alcohol to acrolein, glutathione depletion and lipid peroxidation (Atzori *et al.* 1989; Reis & Tarlow

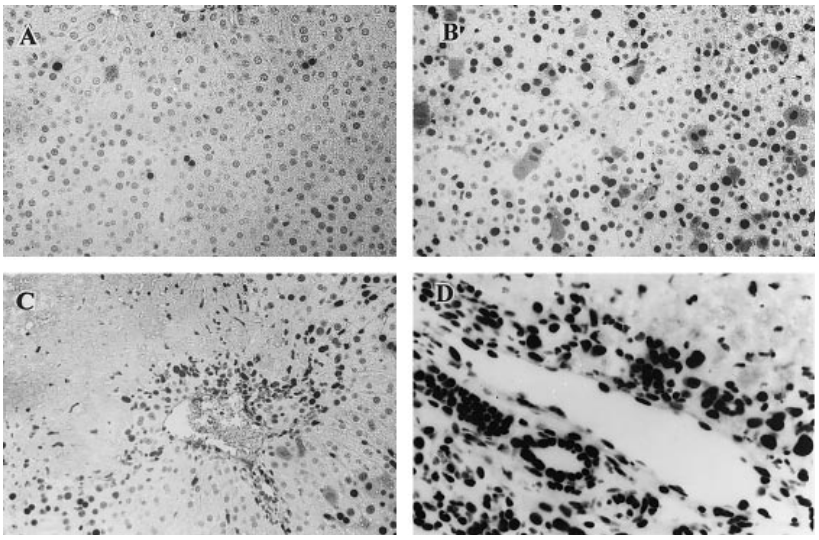

Figure 10. PCNA labelling. A, An increase in nuclear label is first seen at 12 hours. ×200. B, At 2 days there is labelling of both hepatocytes in non-necrotic zones and cells of different size in periportal areas located next to necrotic areas. ×200. C, At 3 days nuclear and cytoplasmic labelling is prominent in non-necrotic zones. ×200, and D, in periportal next to necrotic zones there is labelling of ductal cells, hepatocytes and small cells of different appearance, including 'oval' cells. ×400.

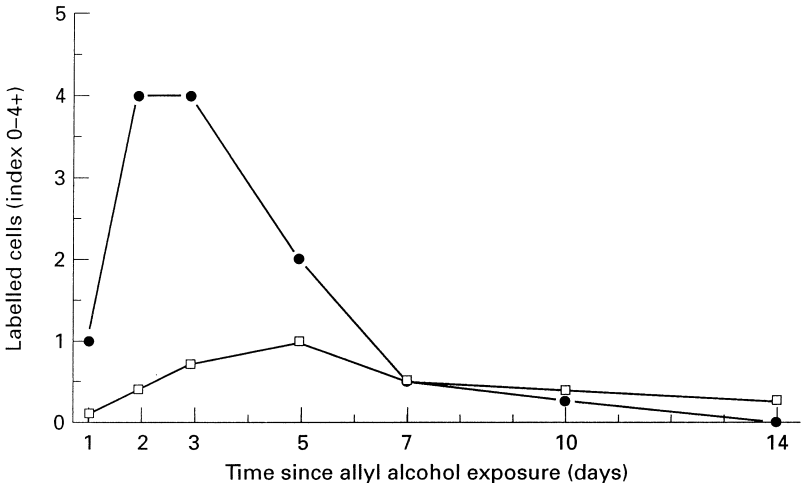

Figure 11. Graph of PCNA labelled ●, large hepatocytes compared to □, small periportal cells after allyl alcohol exposure. Because of variation among lobes and within lobules, numbers are recorded as 0 to 4+. Most of the liver lost to necrosis is replaced by proliferation of large hepatocytes in non-necrotic areas. There is proliferation of small periportal cells adjacent to necrotic areas.

1967; Reid 1972; Jaeschke *et al.* 1987; Penttila 1988). Although the mechanism and initial site of injury, namely the periportal area (zone I), appear to be similar, if not identical, in rats and mice, we have identified several important differences (Yavorkovsky *et al.* 1995).

First, in preliminary experiments the single dose that produces consistent sub-lethal necrosis in the rat (0.67 mmol/kg) was not active in the mouse. This may be because rat hepatic mitochondria, targets for lipid peroxidation, are more sensitive to allyl alcohol than are those of the mouse (Jacobs *et al.* 1987). A range of single allyl alcohol doses was tested in the mouse (data not shown), and a narrow dose range, between 0.67 and 0.8 mmol/kg, produced sublethal periportal injury; doses above 0.8 mmol/kg were usually fatal. However, the amount of sublethal injury was not consistent from mouse to mouse, and thus unreliable for studies of post-necrotic repair. We were able to produce a consistent sublethal necrosis in mice by using a two-dose regime: 0.67 mmol/kg, followed one week later by 1.1 mmol/kg. Several other chemicals (including CCl_4) show a similar effect of autoprotection after repeated doses and its suggested mechanism may be induction of division of new cells that are more resistant to the chemicals (Mehendale *et al.* 1994). However, that does not seem to play a major role in the case of allyl alcohol toxicity, since we were unable to see significant proliferation after a single 0.67 mmol/kg dose. Alternatively, the first dose may upregulate detoxifying enzymes so that the second dose, lethal when given alone, is modified to produce sublethal injury.

Secondly, once injury is induced in mice, it initially appears very similar in extent to periportal injury described in rats (Yavorkovsky *et al.* 1995). However, instead of being confined to periportal spaces and ultimately resolved, the injury in mice unexpectedly progresses to large areas of geographic necroses, with the right lobe being most affected. Species differences in the distribution of the injected toxin in the liver or differences in metabolism in different lobes may be the reason for the different pathway of injury progression in mice.

Finally, as shown in the Results, the regenerative response to allyl alcohol differs significantly from that in rats, apparently as a result of the pattern of necrosis. We were unable to compare this result with the literature data, since we could not find clearly documented reports of the evolution of cellular changes (repair process) in response to allyl alcohol induced lesions in the mouse.

Restoration of periportal cuffs of necrosis in the rat is accomplished mainly by proliferation of small 'null' cells that give rise to 'oval' cells and 'transition hepatocytes' expressing phenotypic markers of hepatocyte differentiation with eventual complete resolution of the necrotic periportal zones (Yavorkovsky *et al.* 1995). Thus, the necrotic zone is largely filled in by proliferation of putative intraportal, tissue determined liver stem cells (Sell 1994).

In the mouse the initial widespread periportal injury evolves differently in the different lobes. In the left and caudate lobes, after the initial evidence of sublethal injury, the lobes are not only completely restored but are up to 60% larger, compensating for the loss of tissue in the right and sometimes also in the medial lobes. There is essentially complete necrosis and atrophy of the right lobes and partial geographic scarring of the medial lobes. In some mice the medial lobe shows little necrosis and also hypertrophies. There is very little proliferation of oval cells. In some areas of necrosis, islands of small oval cells appear to arise from residual non-necrotic portal triads within the margins of the necrotic zone and in some animals there is an increase

in oval cells in portal zones in non-necrotic areas of the liver.

The precise reason for the lobular distribution of injury by allyl alcohol is not known. The relative limitation of allyl alcohol induced necrosis to right and medial lobes may be due to the irregular distribution of allyl alcohol by the portal system of the liver (Atzori *et al.* 1989), or because of metabolic differences among the liver lobes (Richardson *et al.* 1986). A predominance of injury in the right lobe is also observed after carbon tetrachloride or dimethylnitrosamine treatment (Lawson & Pound 1974). Similar results have been obtained for furan-induced cholangiofibrosis (Sirica *et al.* 1994). This latter result has been attributed to 'streaming' in the portal venous circulation that brings more blood to the right and caudate lobes. Although such streaming has been described for the human liver, which has right lobe predominance (Conn & Groszmann 1982), it is not clear what role this effect has in rodent livers. There may also be lobar 'differences' in furan metabolism (Elmore & Sirica 1991).

We had hoped to use the restitutive cellular response to allyl alcohol in the mouse as a way of increasing the number of putative liver stem cells and their immediate progeny for further study. From the present results, it does not appear likely that allyl alcohol induced injury will accomplish the desired result. It is possible that pretreatment with agents, such as a sucrose diet (Jaeschke *et al.* 1987), which reduces hepatic glutathione, inhibition of alcohol dehydrogenase by pyrazole, induction of aldehyde dehydrogenase by phenobarbital or administration of ferrous sulphate may alter the toxicity of allyl alcohol and change the restitutive response. However, we are now studying the effect of other hepatotoxins that produce periportal injury to develop a suitable model for a liver stem cell response in the mouse.

Acknowledgement

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