Flow Cytometric Analysis of DNA Content of Mouse Liver Cells Following *in vivo* Infection by Human Adenovirus Type 5

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**SUMMARY**

Human adenovirus type 5 caused acute hepatocellular damage when injected intravenously into C57B1/6 mice. Early protein (P) and late virus protein (V) antigen staining by immunofluorescence was located principally, if not exclusively, in hepatocytes. Autoradiography following incorporation of tritiated thymidine into Ad5-infected liver cells *in vivo* indicated that DNA synthesis was initiated in hepatocytes. Flow cytometry showed that cells with a 4n DNA content prior to infection were highly susceptible to infection, with 75% being lost from the liver cell population by 5 days post-infection. This observation was correlated with the observed pathology during the course of the infection.

Human adenoviruses induce cellular DNA replication in cultured non-permissive hamster (Strohl, 1969) and semi-permissive mouse (Younghusband *et al.*, 1979) cells arrested in the G1 stage of the cell cycle by serum starvation. Flow cytometry (FC) analysis shows that increased numbers of cells are found in the S and G2 phases of the cell cycle by 24 to 48 h after infection of cycling rat and mouse embryo fibroblasts by adenovirus type 5 (Ad5) (J. D. Murray, I. W. Taylor, A. W. Braithwaite & A. J. D. Bellett, unpublished results). The applicability of this type of analysis of changes in cell DNA content after virus infection of cultured cells has also been demonstrated by Lehman and his co-workers using rodent cells infected with simian virus 40 (SV40) (for review, see Lehman *et al.*, 1979).

Postlethwaite and co-workers (Postlethwaite, 1973; Duncan *et al.*, 1978) have described the course of infection *in vivo* in mouse liver following intravenous injections of large doses of Ad5 (LD$_{50} = 10^4$ p.f.u. of purified Ad5). By 36 h post-infection, up to 80% of liver cells were positive when stained by indirect immunofluorescence for Ad5 early proteins (P antigens). Late virus proteins (V antigens) were detectable by 48 h, although fewer cells were positive. Histological observations showed hepatocellular damage by 24 h with progressive hepatocellular death occurring during the third and fourth days post-infection. Increased mitotic activity was observed in surviving hepatocytes by day 4. Increased amounts of infective virus were recovered from livers 24 to 48 h post-infection, with a subsequent decrease after this time. They concluded that human Ad5 undergoes an abortive but lytic infection in most liver cells, but that some liver cells were probably permissive.

Postlethwaite's (1973) model of infecting mouse liver by intravenous injections of Ad5 is a suitable system in which to study the cellular consequences of lytic virus infections *in vivo*. In this study FC analysis has been used to investigate (i) whether DNA synthesis is induced in liver cells infected by Ad5, and (ii) which liver cells are susceptible to infection. Infections were done with both Ad5 and the temperature-sensitive mutant ts125, as they have been observed to have different effects on the DNA content of rat and mouse cells infected *in vitro* (J. D. Murray *et al.*, unpublished results).

Ad5 and ts125 were grown in KB cells and titrated in human embryo kidney cells (Younghusband *et al.*, 1979). Six-week-old male C57B1/6 mice, bred at the John Curtin School of Medical Research, were used for all experiments. An intravenous injection of
Fig. 1. (a) Section of formal saline-fixed mouse liver 5 days after intravenous inoculation with $1.5 \times 10^8$ IU of wild-type Ad5 illustrating degenerating hepatocytes (closed arrow) in association with inflammation and mitotic figure in surviving hepatocyte (open arrow). Haematoxylin and eosin stained. (b) Acetone-fixed 6 μm frozen section of Ad5-infected mouse liver stained 2 days post-infection for the presence of Ad5 early proteins (P antigens) by the indirect immunofluorescent method (Bellett & Younghusband, 1979). Positive cells appear predominantly in the matrix of liver rather than near the bile duct (bd) and blood vessel (bv) walls. (c) Acetone-fixed 6 μm frozen section of Ad5-infected mouse liver treated for autoradiography. Sections were dipped in Ilford K2 Nuclear Research Emulsion, dried, exposed for 3 days at 8 °C and developed in Kodak D19 developer (diluted 1:1 with distilled water) for 1.5 min. The distribution of cells with autoradiographic grains is the same as that seen with P and V antiserum staining. Bar marker represents 10 μm in all photographs.
between $1.5 \times 10^8$ and $3.1 \times 10^8$ infectious units (IU) of crude preparations of either wtAd5 (wild-type) or ts125 diluted in 0.16 ml phosphate-buffered saline (PBS) resulted in the death of approx. 50% of the mice by day 4 post-infection. In the experiments described $1.5 \times 10^8$ IU of virus was injected. Control mice were injected with 0.16 ml crude lysate from uninfected KB cells diluted 1:2 with PBS. At 2, 5 or 9 days post-infection two mice were killed from each group, the livers removed, and pieces of liver were processed as follows: placed in medium (Autopow + 10% foetal calf serum) for FC analysis; placed in 10% formal saline for histology or snap frozen in liquid nitrogen for immunofluorescence staining. In one experiment 20 μCi of tritiated thymidine/mouse was injected intravenously 20 h post-infection. Two mice/group were then killed and their livers sampled as described on days 2 and 4 post-infection.

The acute disease was similar for mice infected with either the wtAd5 or the ts125 mutant. Clinical signs were first noted on day 2 post-infection and characterized by immobility, hunched posture and ruffled fur. Fifty percent of the infected mice died, with a median survival time of 3.5 days. Survivors continued to show fluctuating clinical signs throughout the study. Control mice injected with KB cell lysate did not show clinical signs or die.

Livers taken from mice infected 2 days previously with either strain of Ad5 were grossly normal. However, microscopic examination showed foci of degenerating hepatocytes. Their nuclei had become fragmented or disappeared such that some cells appeared as anuclear, globular, eosinophilic structures. No inclusion bodies were seen but a mixed polymorphonuclear and mononuclear infiltration occurred within the necrotic areas. On days 5 and 9 post-infection, the livers were enlarged, pale and friable. Microscopically, the day 5 livers showed hepatocellular damage, and inflammation was diffuse and associated with focal haemorrhages. Blood vessels and bile ducts showed no pathological changes. The mitotically active cells observed in day 5 post-infection livers (Fig. 1 a) are probably non-infected cells which are dividing in response to the liver damage resulting from the virus infection. However, even though the infection is largely lytic (Duncan et al., 1978), it is not known if some of the virus-infected cells could have survived and could still be capable of proliferating. Livers from control mice injected with the KB cell lysate were normal by gross and microscopic examination.

The presence of early (P antigen) and late (V antigen) virus proteins was determined at 2 and 4 days post-infection. Frozen liver samples were cut into 6 μm sections, acetone fixed on to gelatin subbed slides and stained by the indirect immunofluorescent method. Both P and V antigen-positive cells were present at 2 and 4 days post-infection, although there were less V antigen-positive cells at both times than P antigen-positive cells. There were fewer P antigen-positive cells at 4 days than there were at 2 days post-infection. The antigen-positive cells were scattered randomly throughout the matrix of the liver and generally were not located near bile ducts or blood vessels (Fig. 1 b). This suggests that the principal cell type making early and late virus proteins were hepatocytes. In mice injected with tritiated thymidine the distribution of cells showing autoradiographic grains at 2 and 4 days post-infection was similar to the pattern seen with immunofluorescent staining for virus antigens (Fig. 1 c), with cells in the liver matrix accumulating autoradiographic grains rather than the cells lining the ducts and vessels. There were no P or V antigen-positive cells in sections cut from control animals injected with uninfected KB cell lysates. From 1.2 to 4.8% of cells in liver sections from control animals showed tritiated thymidine incorporation on day 2 post-infection, while in virus-infected samples from 30 to 47% of the cells showed autoradiographic grains. There was no noticeable difference between wtAd5 and ts125 in the distribution or frequency of P or V antigen-positive cells or cells showing tritiated thymidine incorporation.

FC analysis of a suspension of single nuclei from the liver samples was carried out as
Fig. 2. DNA content distribution histograms of liver cells. The percentage of cells in cell cycle phases was calculated by a computer programme which excludes cells with greater than 4n (G2 + M) DNA contents. The initial peak in all histograms represents chicken red blood cells added as an internal marker. The G1 peak is arbitrarily centred on channel 50 and CV is the coefficient of variation of the G1 peak. (a) Control liver cells 9 days post-injection of KB cell lysate. G1 = 70.5%; S = 6.7%; G2 + M = 22.8%; CV = 2.4%. (b) Liver cells 2 days post-infection with ts125. Note increased frequency of cells in S phase, decreased frequency of cells in G2 + M, and the appearance of a peak of cells with 8n DNA content (channel 200). G1 = 70.5%; S = 12%; G2 + M = 17.5%; CV = 2.6%. (c) Liver cells 5 days post-infection with wtAd5. The G2 + M peak is greatly reduced and 8n peak, seen at 2 days post-infection, is absent. G1 = 84.3%; S = 9.9%; G2 + M = 5.8%; CV = 3.3%.

Described previously (Taylor, 1980). The computer analysis used (Milthorpe, 1980) assigns cells to stages of the cell cycle based on DNA content. Cells in the G1 stage of the cell cycle have a diploid (2n) DNA content while cells which have completed DNA replication but not yet divided (G2 and mitotic stages) have a 4n DNA content. Cells which were replicating DNA at the time of analysis (S phase) have a DNA content intermediate between the 2n value found in G1 cells and the 4n DNA content of G2 + M (mitotic) cells. The DNA content of a G1 tetraploid cell (polyploid) would be 4n, the same value as a G2 diploid cell, while the tetraploid G2 phase cell would have an 8n DNA content. The computer analysis excludes cells with greater than a 4n DNA content from the cell cycle stage analysis. The total number of cells scored is recorded, thus the difference between the total number of cells scored and the number of cells used in the cell cycle analysis represents the number of cells with a greater than G2 DNA content. Between 5704 and 17 578 cells were analysed/sample.

The control (KB lysate-injected) samples of liver gave the same distribution of cellular DNA at 2, 4, 5 or 9 days post-injection (Fig. 2a) with a mean of 71% G1, 6% S and 23% G2 + M cells (from eight animals). Approx. 4% of cells had >G2 DNA content. The high proportion of G2 + M cells is consistent with the observation that 4- to 6-week-old mice have predominantly polyploid liver parenchymal cells, with cells being either binucleate (2n × 2) or mononucleate, i.e. 4n (Brodsky & Uryvaeva, 1977). Even though the hepatocytes are predominantly polyploid the remaining cell types, such as those of the blood vessels and bile ducts, are diploid and these, in conjunction with the diploid nuclei from binucleate hepatocytes, are represented in the G1 peak.

As no appreciable difference between the effects of wtAd5 or the ts125 mutant on cellular DNA content was observed, the results for the two virus types have been pooled. By day 2 the DNA distribution had altered in Ad5-infected samples from control values (Fig. 2b), with an increased frequency of cells in S (mean value 10.7%) and a decrease in G2 + M (mean value 15.3%) cells. The proportion of cells with a greater than G2 or 4n DNA content had also increased (9.5%) with a distinct peak of cells with an 8n DNA content being observed (data from nine animals).

On day 4 post-infection the number of cells with a 4n DNA content (G2 + M) had decreased further and the percentage of cells with > G2 DNA content had returned to control levels (G1 = 79.8%; S = 11.7%; G2 + M = 8.5%; > G2 = 4%; four animals). A further drop in the frequency of cells with G2 + M or greater DNA content was observed in samples.
taken on day 5 (Fig. 2c) and then remained constant up to day 9 post-infection (G1 = 86.2%; S = 7.9%; G2 + M = 5.8%; > G2 = 2.1%; four animals). Cells were not detected with 16n or 32n DNA contents, indicating that the cells lost from the 4n and 8n peaks (see Fig. 2b) had not moved into the next higher ploidy level.

The histological observations reported here agree with the report of Duncan et al. (1978) that the in vivo infection of mouse liver by human Ad5 results in extensive hepatocellular damage with little or no damage to the bile ducts and blood vessels. Immunofluorescent staining with Ad5-specific antiserum indicated that the infected cells are principally hepatocytes in the matrix of liver.

Tritiated thymidine autoradiography showed that thymidine was being incorporated in up to 47% of the hepatocytes in livers taken from animals 2 days post-infection. The increased DNA synthesis may represent the initiation of cellular DNA synthesis, virus DNA synthesis or DNA repair synthesis. The appearance of a number of cells with an 8n DNA content at 2 days post-infection, in conjunction with a decrease in G2 + M cells (Fig. 2b), suggests that the DNA being synthesized is largely cellular. Although the results of Duncan et al. (1978) suggest that a proportion of the DNA made in some of the infected liver cells is viral, it seems unlikely that sufficient virus DNA would be made in those liver cells permissive for virus DNA replication to approximately double the DNA content of the affected cell. The contribution of DNA repair synthesis to the overall observed increase in cells incorporating the tritiated thymidine is not known.

FC analysis revealed that up to 27% of the cells in the livers of uninfected animals have a 4n or greater DNA content. The FC analysis also indicates that as the pathology of the disease progresses the 4n to 8n DNA content cells are largely lost from the liver cell population and fail to be replaced by 9 days post-infection. Any binucleate hepatocytes (which would initially be detected as 2 x 2n cells rather than a 4n cell) stimulated to synthesize cellular DNA would move into the tetraploid cell class (see Brodsky & Uryvaeva, 1977) and thus would be included in the 4n to 8n cell group lost from the liver. The loss of these cells as observed by FC analysis correlates with the histological observations of hepatocellular damage.

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REFERENCE


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