Infection of Mouse Liver by Human Adenovirus Type 5

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SUMMARY

CBA mice, inoculated intravenously with large doses of adenovirus type 5, showed raised levels of serum aspartate aminotransferase (SAAT; EC 2.6.1.1) and died within a few days from histologically demonstrable hepatic necrosis. After inoculation of 1 LD₅₀, virus was rapidly taken up by the tissues where infectivity then declined greatly. Organ titres then increased about 100-fold by 48 h p.i. but, in the liver, which showed intranuclear inclusion bodies, and by electron microscopy, scattered intranuclear and intracytoplasmic adenovirions, the increase was 10000- to 100000-fold. P antigen was detected by single radial diffusion in liver extracts, and by immunofluorescence in 80% of liver cells at 36 h p.i. Hexon, penton base and fibre antigens appeared later and in fewer cells. The maximum amount of hexon, of demonstrable type 5 specificity, was shown by radioimmunoassay to be equivalent to up to 5 x 10¹¹ whole adenovirions/g liver. It is concluded that human adenovirus type 5 undergoes an abortive but lytic infection in most liver cells but that replication may proceed to completion in a few.

INTRODUCTION

Infections of cultured cells by human adenoviruses (Ad) are permissive, abortive or 'semi-permissive' depending on the type of virus and cell used (Philipson & Lindberg, 1974). In monkey cells the infection may be abortive or persistent (Baum, 1977), and abortive infections of rodent cells are associated with transformation. Thus transformed hamster cells arise from an abortive, though mainly lytic infection by Ad 12 (Strohl et al. 1970), or when the usually permissive infection by Ad 5 is rendered abortive by the use of temperature-sensitive (ts) mutants at the restrictive temperature (Williams, 1973) or of fragmented, but not intact, virus DNA (Graham et al. 1975). Similarly, transformed rat embryo cells grow out from the abortively-infected survivors of a 'semi-permissive' infection by group C adenoviruses (Gallimore, 1974). The transformed phenotype is associated with the expression of a small integrated part of the virus genome (Sharp et al. 1974).

Infections of cultured mouse cells by human adenoviruses have not been widely studied

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but, as in hamster cells, Ad 12 infection of 3T3 or L cells is abortive yet lethal, with synthesis of early T antigen but not of virus-specific DNA or capsid proteins. Even when these latter are synthesized, as in certain mouse–human hybrid cells, 'no increase of infectious virus above background' was detected (Biron & Raska, 1976). These in vitro results may be relevant to the oncogenicity of Ad 12 in whole mice (Rabson et al. 1964; Allison et al. 1967).

A further in vivo model with features of an abortive infection was recently provided when Postlethwaite (1973) showed that adult CBA mice, inoculated with large amounts of Ad 5, died within a few days from hepatic necrosis. This model, already used to examine the immunogenicity and protective capacity of capsid antigens (Mautner & Willcox, 1974), has been thoroughly characterized here with a view to further work. It is concluded that the infection is abortive in most liver cells but may proceed to completion in a few.

**METHODS**

*Viruses.* Human adenovirus types 2 and 5 were grown in KB cells, extracted and purified by the method of Russell et al. (1967), and stored at \(-70^\circ C\) after addition of glycerol to a concentration of 50% (v/v). A representative preparation of Ad 5 had a titre of \(1.2 \times 10^{12}\) p.f.u./ml and a particle: p.f.u. ratio of 10. Mice were inoculated intravenously with 0.2 ml of virus diluted in 5% (v/v) glycerol in physiological saline.

*Cells, media and plaque assays.* HeLa and KB cells were grown in Eagle's minimal essential medium (E) with 10% (v/v) tryptose phosphate broth (T), 10% (v/v) calf serum (C), 0.06% (w/v) sodium bicarbonate (0.1% for KB cells) and 100 units of penicillin and 100 μg of streptomycin/ml (ETC/80.10.10).

HeLa cells were used for plaque and neutralization assays with extra MgCl₂ as in Williams (1970) modification of the method of Kjellén (1961). The titrations of organ extracts were normalized by reference to a simultaneously titrated standard, the titres of which varied over a threefold range with 95% confidence limits of \(\pm 58\%\). Since high concentrations of liver extract interfered with plaque production, low dilutions were not used.

*Mice.* Six- to ten-week-old male CBA/Ca mice were used. They appeared to be free from a latent murine adenovirus infection, as shown by sensitive radioimmunoassays for group-reactive anti-hexon antibody and by attempted isolation of virus in cultured mouse embryo cells.

*Collection of mouse organs and preparation of extracts.* After collection of tail vein blood, mice were killed by cervical dislocation and organ samples were either stored at \(-70 ^\circ C\), fixed for histological and electron microscopic examination, or snap frozen for immunofluorescence studies. Portions were extracted in ETC/95.3.2., centrifuged at 430 g for 15 min, and the supernatants used for infectivity assays. Saline liver extracts [1:2 (w/v) in PBS/A; Dulbecco & Vogt (1954)], cleared by centrifuging at 1100 g for 15 min, were used for antigenic analysis.

*Immunological reagents and techniques*

*Virus antigens and antisera.* Ad 5 'soluble' antigen and purified preparations of Ad 2 and Ad 5 hexon were obtained by the methods of Pereira et al. (1968). Purified hexon was labelled with \(^{125}\)I by the chloramine-T method (Hunter & Greenwood, 1962), at a level of 0.2 to 1 μCi/μg of protein.

Antisera to whole virus (in Freund's complete adjuvant) and to purified Ad 5 hexon (as alum precipitates; Mautner & Wilcox, 1974), were raised in rabbits and mice respectively, with booster doses of antigen in saline. Rabbit antisera to Ad 5 P antigen and to purified hexon and fibre antigens, and guinea pig antiserum to penton-base antigen, were kindly
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provided by Dr W. C. Russell, specificity of the latter serum having been achieved by passage through hexon- and fibre-containing immunoabsorbent columns (Haase & Pereira, 1972). Rabbit anti-mouse immunoglobulin (Ig) was kindly provided by Professor J. H. Humphrey.

Mouse sera were tested for antibodies to Ad 5 by double gel diffusion.

Detection of antigens in liver extracts. P antigen was detected by the reversed radial immunodiffusion technique of Pereira et al. (1972), with gels containing a 1 in 100 dilution of a rabbit anti-P serum and 2 μl of extract being placed in each well. Results are expressed as the area (mm²) of the zone of opacity, which is proportional to the antigen concentration. A standard liver extract was included in each assay.

Hexon was detected by the saturation method for inhibition of radio-immunoprecipitation (Willcox & Mautner, 1976). Liver extract (30 μl) was pre-incubated with 5 μl of a limiting dilution of a mouse anti-hexon antiserum for 30 min at 37 °C. ¹²⁵I-Labelled Ad 5 hexon (2 ng) was then added to each tube and incubation was continued for a further 30 min at 37 °C. Enough rabbit anti-mouse Ig antiserum was then added to precipitate all the mouse Ig present, and the mixtures were incubated for a further 30 min at 37 °C. The precipitate and supernatant fluids were separated by centrifugation and counted in a Packard Autogamma scintillation counter to give the percentage of radioactivity precipitated. Non-immune (SPF) mouse serum gave 2 to 5% precipitation and this value was not subtracted from any of the results reported.

Immunofluorescence. Acetone-fixed sections were examined by the indirect immunofluorescence technique using immune and pre-immune sera absorbed with mouse liver homogenate (Nairn, 1962), and fluorescein isothiocyanate-conjugated goat anti-rabbit (Behring) and swine anti-guinea pig (Nordic) globulins. They were counter-stained with 0.1% (w/v) Evans Blue (Gurr).

Assay of serum aspartate aminotransferase (SAAT). The method of Dickie et al. (1970) was used, with a Technicon A II autoanalyser.

Histological studies. Organ samples were fixed in 10% (w/v) neutral buffered formalin and paraffin sections (4 μm) were stained by haematoxylin and eosin, phloxine–tartrazine, Feulgen, methyl green–pyronin and periodic acid–Schiff (PAS; with and without diastase pre-treatment).

Electron microscopy. The fixative and intermediate washing buffer solution were formulated to have an osmotic pressure equal to that of fresh mouse blood (= 360 mosM). The samples of liver tissue were fixed in 1.8% (w/v) glutaraldehyde in 0.045 M-phosphate buffer pH 7.2 containing 2.5 mM-MgCl₂ at 0 to 4 °C for 3 to 3.5 h. After washing for 1 to 3 nights in several changes of 0.1 M-phosphate buffer pH 7.2 containing 0.156 M-sucrose and 2.5 mM-MgCl₂ at 4 °C they were post-fixed in 1% (w/v) OsO₄ in 0.1 M-phosphate buffer, pH 7.2, containing 2.5 mM-MgCl₂ at 20 °C for 1 h. They were dehydrated with a graded series of ethanol solutions and embedded in Araldite. Thin sections were cut with a tungsten-coated glass knife (Roberts, 1975) and stained with uranyl acetate and lead citrate (Reynolds, 1963).

RESULTS

Preliminary experiments

When CBA/Ca or Fl (CBA/C₅₇Bl/6) mice were inoculated intravenously with 2 × 10⁶ p.f.u. of purified Ad 5, 9 out of 10 sickened and died of a profound hepatic necrosis within 5 days, but all of 10 mice survived one third this dose (Postlethwaite, 1973). This abrupt mortality threshold was reflected in the extent of histologically demonstrable liver necrosis.
Fig. 1. Serum aspartate aminotransferase levels. Mean values from two experiments with range indicated by bars. Inoculation of diluent alone had no effect.

and in the dose–response relationship revealed by assays for SAAT in CBA mice. Thus, 2 days after inoculation of approx. 0, 3, 10, 30 or 90 × 10⁷ p.f.u. of virus into groups of four mice, enzyme levels in serum pools were 66, 95, 160, 1120 and 3200 units/l respectively. Mice which survived made a rapid and complete recovery with no apparent sequelae up to 6 months later. No adverse effects were seen in mice inoculated with u.v.-irradiated virus equivalent to three lethal doses nor when this amount of live virus was treated with a 1 in 250 dilution of a rabbit anti-Ad 5 antiserum for 1 h prior to inoculation. Normal rabbit serum did not protect even at a 1 in 5 dilution and the same dilution of the anti-Ad 5 serum did not protect against two lethal doses of Ad 2 virus.

Although small amounts of infectious virus were detected in organ extracts and virus antigen was seen in hepatic parenchymal cells by immunofluorescence microscopy, no virus particles could be found by electron microscopy in these early experiments, and the question of complete or abortive growth was thus undecided.

**Detailed analysis of the acute disease over its natural time course**

To facilitate the demonstration of virus growth, should this indeed occur, a 50% lethal dose of virus (10⁶ p.f.u.) was used (in two identical experiments) in order to infect as many cells as possible, whilst permitting sufficient survivors for groups of three mice to be studied at intervals up to 14 days during the development and resolution of the disease. The zero time group was bled and then killed for organ sampling 2 and 6 min after infection respectively.

**Development of clinical disease, levels of SAAT and gross and microscopic pathology**

After onset of disease at 48 h p.i., mice were profoundly sick from days 3 to 7 with sluggish movement, hunched posture and yellow urine. All deaths occurred between the fourth and seventh days. Survivors were clearly recovering by the eighth day and had regained their normal appearance and activities by day 14. Levels of SAAT increased about 100-fold between 24 and 36 h p.i. and returned to near normal by the tenth day (Fig. 1). At autopsy, naked-eye changes were confined to liver, spleen, adrenals and thymus. From 2 to 6
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Days p.i. the liver became progressively pale, friable and enlarged and showed scattered petechiae. Patchy recovery was seen at 8 days. The spleen almost doubled in size by day 8, the mean wet weights on days 0, 2, 4, 8 and 10 being 58, 87, 76, 80 and 106 mg respectively. Adrenals were red and enlarged from days 3 to 6 and the thymus was noticeably small during the period 6 to 10 days p.i. The liver, adrenals and thymus appeared grossly normal again by days 10, 10 and 14 respectively, but the spleen was still enlarged at day 14.

Histological examination of liver, lung and spleen for up to 7 days after inoculation of virus-free diluent (containing glycerol and CsCl) revealed no abnormality. Sections of infected liver (Fig. 2) showed centrilobular congestion which progressed to diffuse hepatocellular damage by 24 h with prominent cytoplasmic eosinophilia and focal necrosis. The subsequent formation of acidophilic apoptotic bodies (Kerr et al. 1972) was accompanied by a sparse, mainly lymphocytoid, inflammatory cell infiltrate and Kupffer cell hyperplasia. Progressive hepatocellular necrosis with focal haemorrhage occurred during the third and fourth days. Surviving hepatocytes showed increased mitotic activity from day 4 onwards and reparative features, with increased numbers of macrophages, were prominent by the eighth day. The liver appeared normal after 14 days.

Intranuclear inclusions, first seen at 15 h p.i. as tiny eosinophilic dots, became large and irregular by 36 h. They were phloxine-positive but negative for DNA and RNA when stained by Feulgen and methyl green–pyronin techniques respectively. PAS-positive,
Table I. Mean tissue concentrations of infective virus at 0 and 6 h post-infection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0 h</th>
<th>6 h</th>
<th>% of blood concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>125</td>
<td>109</td>
<td>27.1</td>
</tr>
<tr>
<td>Lung</td>
<td>180</td>
<td>38</td>
<td>3.7</td>
</tr>
<tr>
<td>Liver</td>
<td>500</td>
<td>16</td>
<td>10.4</td>
</tr>
<tr>
<td>Adrenal</td>
<td>63</td>
<td>2.6</td>
<td>3.7</td>
</tr>
<tr>
<td>Heart</td>
<td>160</td>
<td>1.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>57</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Blood</td>
<td>4800</td>
<td>0.8</td>
<td>100.0</td>
</tr>
<tr>
<td>Thymus</td>
<td>18</td>
<td>0.4</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* Mean values for two experiments. Tissues showed the same ranking in each experiment.

diastase-labile glycogen was absent from most of the hepatocytes with nuclear inclusions, especially at 36 and 48 h, but persisted in inclusion-free cells.

Occasional intranuclear inclusions were identified in the bronchial epithelium, especially at 36 h, but not in the adrenals which were congested. The spleen showed follicular hyperplasia and marked lymphocytolysis, most marked at 72 h.

Recovery of virus from organ extracts

Infectivity assays gave highly consistent results, in the two experiments, for the distribution of virus between tissues (Table I and Fig. 3). Virus in the blood was quickly taken up into other organs where titres then declined rapidly. By 6 h, when the total virus recovered was only 2.3% of that obtained at zero time and about 0.16% of that inoculated, residual infectivity was largely concentrated in spleen, lung and liver, only the thymus showing a lower concentration than the greatly depleted blood. Virus concentrations continued to decline and, for all tissues, were lowest ($P < 0.05$) between 24 and 36 h p.i.

Between 24 and 48 h p.i. virus concentrations increased, much more so for the liver (10000- to 100000-fold) than for other tissues (about 10- to 100-fold; Fig. 3). For all tissues except spleen, virus concentrations were maximum at 48 h and, for liver and lung, were significantly ($P < 0.05$) greater than at 36 or 72 h. Virus in the blood may also have increased by 48 h, but the relative titres from 15 h to 4 days were not significantly different from those observed over a similar period when virus was mixed with mouse serum in vitro and incubated at 37 °C. The spleen appeared to be exceptional since the titres at 4 days were not significantly different from those at 2 days. From peak tissue concentrations, virus then declined to low or undetectable levels by 4 to 6 days p.i. The virus recovered from a 48 h liver extract was antigenically similar to the inoculum virus since the 50% neutralization titre of a potent Ad 5 antiserum lay between 1/200000 and 1/400000 for both.

Demonstration of virus antigens in liver cells by immunofluorescence

Antigen-containing cells (Fig. 4 and 5) were randomly distributed. P antigen was first seen 15 h p.i., was present in nearly 80% of cells by 36 to 48 h and then declined. Cells with capsid antigens were fewer, were not seen until 36 h p.i. and reached peak levels at 48 to 72 h p.i. Fibre antigen was detected in a higher proportion of cells than other capsid antigens. No antigens were detected later than 6 days p.i.
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Fig. 3. Infectivity in mouse organs from one experiment. ●, Values for separate extracts and ○, for pooled extracts from three mice respectively. ▲, Absence of plaques at lowest dilution tested.

Early in infection P antigen was seen as small cytoplasmic and perinuclear dots, which appeared to migrate to the nucleus where they coalesced to form large granules. From 48 h onwards some nuclei showed rings of fluorescence similar to the late P antigen described in cultured cells by Hayashi & Russell (1968). By 4 days p.i. most antigen-containing nuclei showed uniform and complete fluorescence. In contrast, fluorescence due to capsid antigens never completely filled the nuclei. Hexon was first observed as small nuclear dots which increased in size and number during infection. Fibre and penton base antigens both appeared initially as flecks which increased and enlarged to give the appearance of wrinkled silver paper or of fine intranuclear cobwebs respectively. The complete filling of nuclei by P antigen but not by capsid antigens is contrary to the situation reported in the permissive infection of human embryo kidney cell cultures by Hayashi & Russell (1968). Cells containing P antigen and particularly capsid antigens were more markedly autofluorescent than uninfected cells, especially at 3 to 4 days p.i.

Detection of P antigen in liver extracts by radial immunodiffusion

P antigen was first detected at 24 h p.i., reached maximum levels around 48 h and became undetectable between 4 and 6 days p.i. (Table 2).
Detection of hexon antigen by radioimmunoassay

Hexon in the liver extracts was detected by its ability to block a limiting dilution of specific anti-hexon antibody and so prevent it from binding $^{125}\text{I}$-labelled hexon added subsequently. Without any blocking agent, the antibody formed soluble complexes with the labelled antigen, which were then precipitated by subsequent incubation with anti-immunoglobulin antiserum. The percentage of the labelled hexon thus precipitated was determined and was a measure of the amount of antibody present. Blocking of the antibody by pre-incubation with unlabelled hexon reduced the percentage precipitation of labelled antigen.

Time of appearance

Hexon first became clearly detectable at 36 h p.i. and persisted in substantial amounts until the fourth day (Table 2). The modest amount of blocking from 0 to 15 h may have reflected inoculum-derived hexon in these early samples. There was no blocking by liver...
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Fig. 5. Development of P and capsid antigens in infected liver: ○—○, P antigen; ●—●, fibre antigen; □—□, penton base antigen; ▲—▲, hexon antigen. The lines join the mean values from two experiments. At each point, counts were made from about 1000 cells from a single liver section except at 36 and 48 h when, in one experiment, counts were made from each of 5 to 7 different sections (shown for P antigen as closed circles).

Table 2. Detection of P and hexon antigens in infected liver extracts

<table>
<thead>
<tr>
<th>Liver extracts (h or days p.i.)</th>
<th>P antigen*</th>
<th>Hexon antigen† (Expt. 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>Control uninjected 0 h</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 h</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15 h</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 h</td>
<td>0.5 (0.0–0.9)‡</td>
<td>0.3 (0.0–1.0)</td>
</tr>
<tr>
<td>36 h</td>
<td>1.6</td>
<td>2.7 (2.0–3.5)</td>
</tr>
<tr>
<td>48 h</td>
<td>3.0</td>
<td>3.0 (2.5–3.5)</td>
</tr>
<tr>
<td>72 h</td>
<td>2.4</td>
<td>2.1 (2.0–2.4)</td>
</tr>
<tr>
<td>4 days</td>
<td>1.5</td>
<td>0.7 (0.0–1.2)</td>
</tr>
<tr>
<td>6 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No extract</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as mean area (mm²) of radial immunodiffusion zone versus anti-P antiserum.
† Expressed as percentage of labelled hexon precipitated by known antibody in presence of liver extracts.
‡ Where a range is given, results are the mean values from three mice; otherwise they are pools from three mice.

extracts from uninjected mice. As shown in separate assays, anti-hexon antibodies were present from 8 to 14 days p.i. These presumably explain the increased precipitation of labelled hexon and, coupled with the demonstration by gel-diffusion of serum antibody from the sixth day, may account for the apparent absence of hexon in liver extracts after the fourth day.
Fig. 6. Quantification of hexon in 48h liver extract. The curve (○—○) represents the plot of percentage of labelled hexon precipitated by anti-hexon serum (ordinate) after pre-incubation of the serum with known concentrations of unlabelled pure hexon (abscissa). Intercepts on this curve indicate the percentage precipitation obtained with different dilutions of the 48 h liver extract (E). In this experiment volumes of extract were one third those used in Table 2, so a 1/3 dilution in Fig. 6 is equivalent to a 1/1 dilution in Table 2. Total volumes were the same throughout.

**Quantification**

The 48 h liver extract from experiment 2 was chosen for more precise quantification. Blockage of precipitation by different dilutions of extract was interpreted in terms of hexon content by reference to the dose–response curve for known concentrations of unlabelled purified hexon (Fig. 6). The concentration of hexon appeared to be 25 μg/ml of extract which is equivalent to approx. $5 \times 10^{11}$ adenovirus particles/g liver. In a similar experiment, hexon was present at a concentration of about 1 μg/ml at 48 h, although it was not carefully titrated on this occasion.

**Serological typing of the detected hexon**

The blocking by the liver extracts shown in Table 2 and Fig. 6 appeared to be specific since it was not seen at certain times after inoculation nor with extracts from control mice. However, some non-specific effect on the antibody, for example by products of liver damage, could not be excluded. To investigate specificity therefore, the cross-reactive population of an anti-Ad 5 hexon antiserum was pre-blocked with excess unlabelled type 2 hexon (Willcox & Mautner, 1976) and the residual type-specific anti-Ad 5 hexon antibody was then used to detect hexon in the liver extracts. As a control, similarly prepared type-specific anti-Ad 2 hexon antibody was tested in parallel.

These antisera each gave 36% binding of the homologous labelled hexon and about 10% of the heterologous i.e. they were at least 80% type-specific (Willcox & Mautner, 1976). The infected liver extracts all clearly blocked the homologous (anti-Ad 5) type-specific antibody efficiently but had no effect on the heterologous (anti-Ad 2, Table 3). As previously found, uninfected liver extracts did not block at all. Moreover, the possibility that inhibition of hexon binding was spurious and due to increased amounts of mouse immunoglobulin in liver extracts was excluded by showing that inhibition remained the same in spite of increasing the amount of anti-Ig serum by 50 or 100%.
Table 3. Specificity of blocking by the hexon in liver extracts

<table>
<thead>
<tr>
<th>Type-specific antibody*</th>
<th>Inhibitor (liver extract pools)</th>
<th>% precipitation of labelled hexon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>72 h (Expt. 1)</td>
<td>21.6</td>
</tr>
<tr>
<td>Anti-Ad 5 hexon</td>
<td>48 h (Expt. 2)</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>72 h (Expt. 2)</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>Non-injected</td>
<td>38.6</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>72 h (Expt. 1)</td>
<td>36.1</td>
</tr>
<tr>
<td>Anti-Ad 2 hexon</td>
<td>48 h (Expt. 2)</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>72 h (Expt. 2)</td>
<td>35.7</td>
</tr>
<tr>
<td></td>
<td>Non-injected</td>
<td>43.0</td>
</tr>
</tbody>
</table>

* Type-specific anti-Ad 5 (or Ad 2) hexon antibody was prepared by pre-incubation of anti-Ad 5 (or Ad 2) hexon antiserum with excess (100 µg/ml) of unlabelled Ad 2 (or Ad 5) hexon. Each was assayed against 125I-labelled homologous hexon.

Fig. 7. Part of hepatocyte nucleus 24 h p.i. showing two types of abnormal inclusion, A and B, as described in Results.

Electron microscope studies

Liver samples from mice inoculated with virus-free diluent and sacrificed up to 4 days p.i., and the zero time sample of infected liver, showed no consistent abnormalities. There was an orderly arrangement of uniform hepatocytes with Kupffer cells and sinusoids. The hepatocytes contained much glycogen, many mitochondria and occasional lipid droplets, as well as endoplasmic reticulum, microbodies and lysosomes.

At 24 h p.i. a few nuclei showed two abnormal features approx. 0.5 to 2 µm diam., namely, uniformly grey patches less granular than the surrounding nucleoplasm (Fig. 7, A) and other patches (Fig. 7, B) consisting of electron-dense dots (approx. 20 nm diam.) and apparent clusters (approx. 100 to 140 nm diam.) of dots. By this time adjacent hepatocytes, in contrast to their usual uniformity, were beginning to show morphological differences.

At 48 h this cellular variation was very marked (Fig. 8), notably with regard to
Fig. 8 to 10. Features of liver tissue 48 h p.i.

Fig. 8. Characteristic 'mosaic' appearance showing that each cell looks different from its neighbours. L = Lipid droplets, R = red blood cell in sinusoid; Ap = apoptotic body.

Fig. 9. Part of hepatocyte nucleus containing large numbers of scattered adenovirus particles (arrows). Insert (magnification ×230000) shows hexagonal outline of an intranuclear adenovirus particle.

Fig. 10. Part of hepatocyte showing adenovirus particles (V) scattered in the cytoplasm. N = Part of nucleus; M = mitochondrion; E = endoplasmic reticulum.
Fig. 11. Liver tissue 4 days p.i. showing disorganization and large numbers of lipid droplets, some of which appear to be in the process of fusing (F). R = scattered red blood cells; Ap = apoptotic body; S = part of large space between cells.

Fig. 12. Liver tissue 8 days p.i. showing healthy-looking hepatocytes with areas of glycogen (G) and abundant rough endoplasmic reticulum (E). N = nucleus; R = red blood cell in sinusoid.
cytoplasmic and mitochondrial density, form and swelling of endoplasmic reticulum, and numbers of lipid droplets relative to other organelles. There was less glycogen and more lipid, and rounded areas of electron-dense degenerating protoplasm characteristic of apoptosis (Kerr et al. 1972) were seen (Fig. 8, Ap). Moreover, some of the cells with abnormal nuclei contained scattered adenovirus particles (Fig. 9) as defined by size (approx. 70 nm diam.), hexagonal outline (Fig. 9 insert) and characteristic varied appearance (Takeuchi & Hashimoto, 1976). These were present either in nuclei only or, less frequently, in both nuclei and cytoplasm (Fig. 10). No paracrystalline arrays were seen.

The general appearance of liver tissue at 3 and 4 days p.i. (Fig. 11) was of disorganization and extensive damage, but adenovirions were still seen in a few cells at 3 days p.i. Red blood cells were widespread, hepatocyte boundaries were indistinct, intercellular spaces were prominent and apoptotic bodies were still present. Glycogen had entirely disappeared but surviving cells contained enormous amounts of lipid.

The cells and architecture of the liver returned to normal during the period 6 to 14 days p.i. as revealed, even as early as the eighth day (Fig. 12), by the uniform appearance and arrangement of hepatocytes, the apparent normality of nuclei and mitochondria, the abundance of glycogen and rough endoplasmic reticulum and the reduced number of lipid droplets.

**DISCUSSION**

The random, non-clustered development of P antigen in 80% of liver cells, appearing as a single sharp peak in a non-progressive disease, is consistent with an abortive but lethal infection. A purely toxic effect seems unlikely since the inoculum virus had been purified from free fibre and penton base antigens (Pereira, 1958; Everett & Ginsberg, 1958; Levine & Ginsberg, 1967) and u.v.-irradiated virus had no effect. A role for the liver macrophage (Mims, 1964), suggested in preliminary dose-response experiments by the enhanced mortality and aminotransferase levels brought about by the prior inoculation of powdered silica (Postlethwaitc, 1973), needs further study.

Abortive adenovirus infections in cultured cells have been reviewed by Schlesinger (1969), Strohl (1973) and Philipson & Lindberg (1974). As found in some of these, the block to virus replication in the liver infection studied here may occur at the level of virus DNA synthesis, with failure of later gene transcription, from a subsequent block in translation so that capsid antigens are not produced, or from defective maturation and assembly at an even later stage of development. Certainly there is a close relationship between early (T) antigen production and cell killing in the abortive infection of cultured BHK 21 cells by Ad 12 (Strohl, 1969a), and P antiserum is now thought to react with T antigen(s) as well as with the early, 72K, DNA-binding protein (Graham et al. 1977).

The later appearance of capsid antigens and of complete virions in parenchymal cell nuclei, and the increase of infectivity after the apparent eclipse of inoculum virus, suggest a complete cycle of virus growth in some liver cells. This is consistent with the liver tropism of group C adenoviruses in newborn hamsters (Pereira et al. 1962; Samaille et al. 1965; Connor, 1970) and immunologically compromised humans (Aterman et al. 1973). However, the virus-inhibiting effect of low dilutions of liver extracts noted here, and the demonstration of interferon and non-specific inhibitors in Ad 1-induced hepatitis in newborn hamsters (Rudenko et al. 1972), indicate the need to assess the role of these substances in the mouse infection, since they could contribute to a spurious demonstration of eclipse of inoculum virus. The small, late increases in infectious titre noted in other organs might simply imply haematogenous spread of liver-grown virus but, in view of the common, often latent,
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association of lymphoid tissues with adenovirus infections (Pereira & Kelly, 1957; Van der Veen & Lambriex, 1973; Faucon et al. 1974), the exceptional pattern of infectivity in the spleen (Fig. 3) merits particular study.

The qualitative evidence that the blocking agent in liver extracts was hexon of the same type as the virus originally inoculated is compelling. It argues strongly against the possibility of non-specific blocking or of blocking by a reactivated mouse adenovirus. However, the estimated concentration of hexon is only approximate for technical reasons. At 48 h this amounted to a 1000- to 25000-fold excess over the detectable infectivity. This is much greater than that reported for many cell culture systems (White et al. 1969) and may reflect the production of incomplete (Sundquist et al. 1973; Wadell et al. 1973), possibly interfering particles, imbalance in synthesis of virion components, deficient assembly and maturation, or inefficient biological assay. The small proportion of apparently fully permissive cells may reflect anatomical or physiological differences, asynchrony of infection and infection-dependent changes in cell competence, host-range variants or defective interfering particles in the virus inoculum, or varying sensitivities of detection. Changes in cell susceptibility with phase of the mitotic cycle have indeed been reported (Semkow, 1974). Moreover, in the Ad 12–BHK21 system, infection leads to cell DNA synthesis (Strohl, 1969b) and subsequent survival or death are associated with culture conditions providing, respectively, for logarithmic growth or G1-phase arrest (Strohl, 1969a). The possibility of a virus variant was suggested by the high LD<sub>50</sub> and the low yield of new infectious virus; a raised body temperature could contribute to variable expression of the virus genome (Russell et al. 1972).

On balance, it seems reasonable to postulate for human Ad 5 in mouse liver a small proportion of permissive cells in a predominantly abortive infection, as indeed pertains for the uncomplemented infection of cultured green monkey kidney cells in vitro (Friedman et al. 1970; Baum et al. 1972; Baum, 1977). The absence of progressive infection may then be explained by early elimination of the few permissive cells, by inefficient release of new infectious virus, by antibody-limited spread of extracellular virus from the sixth day and, at earlier times, by the possible intervention of interfering substances. A similar restriction of hepatic infection to the cells initially infected has been reported for Ad 2 and Ad 5 in newborn hamster liver (Samaille et al. 1965). A possible role for cell-mediated immunity would be consonant with the known thymic dependence of anti-hexon and -fibre antibody formation (Mautner & Willcox, 1974), although lymphocyte infiltration of the liver was scanty and the presence of other virus-induced, possibly cell-surface, antigens was not sought. Moreover, adoptive transfer experiments suggested that cell-mediated protection was unimportant (Mautner & Willcox, 1974) and T cell-deprived and cyclophosphamide-treated mice were no more susceptible than controls (R. Postlethwaite, unpublished observations). However, while the evolution of the disease and the regeneration of liver cells are so rapid that immunological factors may have little opportunity to influence high dose infections, they may contribute to the sublethal effect of small doses.

This simple, reproducible and dose-responsive animal model, coupled with complementary study of cultured cells, is well suited for further examination of those factors of host and parasite which determine the outcome of this, as of other, adenovirus infections. If the few apparently permissive liver cells simply reflect host-range variants in the inoculum virus, then selection and propagation of these may permit detailed study of the determinants of virus virulence. Finally, whether the infections studied are permissive, abortive, transforming or persistent, they may well have general significance as well as special relevance for other virus infections of the liver which continue to pose questions for experimentalist and clinician alike.
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