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Clonal analysis of infiltrating T lymphocytes in liver tissue in viral hepatitis A

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SUMMARY

The pathogenic mechanism leading to liver tissue injury in hepatitis caused by hepatitis A virus is unclear. We have randomly established T-cell clones from liver biopsies from four patients with hepatitis A. A total of 578 clones was phenotypically analysed. During the acute phase of the disease $CD8^+$ clones dominated over $CD4^+$ clones, whereas in a biopsy taken late after onset of clinical syndromes more $CD4^+$ than $CD8^+$ clones were obtained. Interestingly, in a patient with a second exacerbation of the disease, more than 20% of all clones had the $CD3^+$ $WT31^-$ $CD4^-$ $CD8^-$ 'NK-like' phenotype. All $CD8^+$ clones had cytotoxic activity and approximately 50% of all $CD8^+$ clones showed specific cytotoxicity against autologous fibroblasts infected with hepatitis A virus. The $CD8^+$ cells also produced IFN-γ in response to these target cells. Variable IFN-γ production was observed with all types of T-cell clones. These results suggest that the liver injury in hepatitis A is not caused by a viral cytopathogenic effect but is due to an immunopathological reaction of sensitized cytotoxic T lymphocytes against infected hepatocytes. In addition, these studies show an enrichment of $CD4^-8^-$ T-cell receptor $\alpha\beta$-chain-negative T lymphocytes at the site of an inflammation and suggest a role of these cells in an anti-viral reaction.

INTRODUCTION

Hepatitis caused by hepatitis A virus (HAV) is the most common liver disease in developing countries. After an incubation period of 4 weeks, clinical symptoms and elevated serum transaminases are found for approximately 3 weeks. Thereafter clinical and laboratory findings return to normal. The pathogenetic mechanisms leading to inflammation and destruction of liver tissue are unknown (Zuckerman, 1988). *In vitro*, HAV causes a persistent infection in fibroblasts without any cytopathogenic effects (Vallbracht *et al.*, 1984). Virus can be eliminated from such persistently infected cells by treatment with interferon (Vallbracht & Flehmig, 1985). The role of different immune mechanisms in the elimination of the virus and in the inflammatory reaction are unclear. Recently we have been able to demonstrate virus-specific cytotoxic T lymphocytes (CTL) in the peripheral blood of patients with HAV infection (Vallbracht *et al.*, 1986). Surprisingly, maximal cytotoxicity was detected in peripheral blood lymphocyte after normalization of laboratory findings (Vallbracht *et al.*, 1986). The contribution of these CTL to virus elimination and their possible role in liver cell destruction is unclear. It is possible that virus-specific CTL are generated too late to be of any significance for recovery from HAV infection. Alternatively, it is possible that during the hepatitis CTL are localized in the affected organ and therefore can not be detected in the periphery.

In this study we have performed a clonal analysis of the infiltrating T cells at the site of infection in four patients with hepatitis A. We show that during the acute infection virus-specific $CD8^+$ interferon-producing CTL are enriched at the site of inflammation. These results point to the destruction of infected hepatocytes by specifically sensitized CTL as the immunopathological mechanism in hepatitis A.

Abbreviations: B-LCL, B-cell line; CTL, cytotoxic T lymphocyte; HAV, hepatitis A virus; IFN, interferon; TCR, T cell receptor.

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MATERIALS AND METHODS

Patients

Diagnosis of acute hepatitis A was based on the detection of HAV-specific IgM in all patients. Patients Be, St and Sch (adult men) showed a typical monophasic course of hepatitis A. Fine-needle biopsies were taken under sonographic control a few days after onset of clinical symptoms from patients Be and St. A biopsy was obtained from patient Sch 6 weeks after onset of hepatitis, when all clinical and laboratory parameters had returned to normal values. Hepatitis in patient W (a 7-year-old girl) had a biphasic course. The high activity of transaminases in

the serum at admission decreased and clinical findings were nearly normal 10 days after hospitalization. Ten days later liver enzymes in the serum increased again in values higher than before and peaked at 4 weeks after the first admission with AST and ALT values higher than 1000 U/ml. A biopsy was taken for diagnostic purposes at this time. The serum was still positive for anti-HAV IgM at this time and no antibodies to other hepatotropic viruses were found. For technical reasons the biopsy of patient W was cultured for 1 week in recombinant IL-2 before the T cells were cloned.

From patient Sch a skin biopsy was available allowing the cultivation of fibroblasts that could be used as target cells. The liver biopsies of patients Be, St and Sch were obtained with written informed consent. Each biopsy was approved by the Ethics Committee of the University of Tübingen. These patients were out-patients and came to hospital for the biopsy of their own free will.

Cloning and propagation of T lymphocytes

T lymphocytes were cloned either from cells grown out of the biopsy material in medium containing recombinant IL-2 or directly from minced and filtered tissue. Cloning was performed by limiting dilution in histoplates as described (Fleischer & Kreth, 1983). Each well received irradiated (35 Gy) allogeneic PBMC (10^6/ml) and supernatant of PHA-stimulated PBMC at a final concentration of 50%. PHA was not removed from this supernatant in order to activate resting T cells for growth. Growing colonies were transferred to larger wells and expanded in medium containing recombinant IL-2. Cloning efficiency was around 20% and usually about 90% of the colonies could be expanded to more than 5×10^5 cells. For long-term propagation the clones were restimulated weekly with surface-oxidized PBMC as described (Fleischer, 1988).

Phenotypes of T-cell clones were determined by a sensitive single-cell ELISA using mAb and subsequent peroxidase staining with modifications according to Holzmann & Johnson (1983). Cells immobilized on poly-L-lysine-treated Terasaki plates were incubated with the mAb solution for 1 hr, washed with PBS and fixed for 4 min with 0·05% glutaraldehyde. Thereafter, a peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Denmark) was added at 1:50 for 10 min. After washing, the substrate carbazole (Sigma, Munich) was added until the positive controls were stained. Negative and positive controls were included in every plate. The mAb used were OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), BMA031 and WT31 (anti-TcR $\alpha\beta$-chain-associated determinants; Lanier *et al.*, 1987), and NKH-1 (anti-NK cells). Some of the clones were also typed with a fluorescence-activated cell sorter, with identical results.

Assay for cytotoxic activity

Spontaneous, antibody-dependent (ADCC), and lectin-dependent cytotoxicity were determined in a standard 4-hr ^{51}chromium release assay in 96-conical-well microtitre plates as described (Fleischer, Schrezenmeier & Wagner, 1986). Target cells for spontaneous cytotoxicity were K562 and JM, targets for ADCC were P815 cells in the presence of a rabbit anti-P815 serum (diluted 1:200). The cytotoxic potential of a given T-cell clone was determined by measuring its ability to kill Fc receptor-bearing P815 in the presence of anti-CD3 (0·1 μg/ml) or to kill B-LCL cells in the presence of PHA-P (75 μg/ml). In the absence of anti-CD3 or PHA both P815 and B-LCL cells are relatively resistant to lysis by NK cells. HAV-specific cytotoxicity was determined by incubating effector cells with ^{51}chromium-labelled HAV-infected fibroblasts in 96-flat-bottomed microtitre wells for 16 hr. Details of this assay have been described (Vallbracht *et al.*, 1986). Spontaneous release from infected and uninfected fibroblasts was usually below 15% of the maximal release and effector: target cell ratios of 10:1 and 5:1 were used.

Determination of IFN-γ production

Interferon was measured in the supernatants of clones after stimulation with PHA-P (10 μg/ml, 18 hr) by a plaque reduction assay in WISH cells with vesicular stomatitis virus (Vallbracht *et al.*, 1982). The characterization of the interferon produced was carried out by a neutralization assay using rabbit polyclonal antibodies to human IFN-α and human IFN-γ (Paesel, Frankfurt) as described previously (Vallbracht *et al.*, 1981). The detection limit was approximately 16 IU/ml.

Northern and Southern blot analysis

RNA was isolated from cloned T cells as described (Chirgwin *et al.*, 1979). Total RNA (10–20 μg/lane) was separated on a 1·5% agarose gel with 7% formaldehyde in MOPS-buffer. RNA was transferred to Genescreen filters (New England Nuclear, Dreieich) by electroblotting (200 mA overnight). The filter was hybridized as described (Snodgrass *et al.*, 1985) with $1–5 \times 10^6$ c.p.m./ml oligo-labelled (Feinberg & Vogelstein, 1983) human TcR γ-chain probe (Yoshikai *et al.*, 1987) and after washing off this probe with a human TcR β-chain probe (Yanagi *et al.*, 1984).

DNA was isolated from cell suspensions according to Blin & Stafford (1976) and digested to completion with ECoRI and BamH1. Approximately 6 μg of DNA per lane were subjected to electrophoresis through 0·6% agarose gels, blotted onto filters and hybridized as described (Snodgrass *et al.*, 1985).

As γ-chain probe, a 1·6 kb insert containing VJCγ1 (Yoshikai *et al.*, 1987) was used, and as β-chain probe the Bgl II-EcoRV fragment of the human cDNA clone YTC (Yanagi *et al.*, 1984). Both probes were kindly supplied by Dr T. W. Mak, Ontario Cancer Institute.

RESULTS

Phenotypes of T-cell clones derived from liver tissue

Two protocols were used to clone liver-infiltrating T cells: (i) cultivation of a piece of liver tissue in mitogen-free recombinant IL-2 for 48 hr with subsequent cloning of T cells grown out of the biopsy, and (ii) cells directly seeded from minced and filtered tissue in mitogen-containing PHA-supernatant of PBMC with less than 1 cell/well. These two protocols were compared using the material of patient Be and appeared to give similar results; the ratio of CD4$^+$ clones:CD8$^+$ clones derived from patient Be was 0·41 after cloning with protocol 1 and 0·58 with protocol 2.

All clones were analysed in a micro-cell ELISA for the expression of T-cell markers CD3, CD4, CD8 and the TcR$\alpha\beta$ chain dimer using mAb BMA031. Table 1 summarizes the results obtained with the four patients. In all cases at least 90%

Table 1. Phenotypes of liver-derived T-cell clones in hepatitis A

Phenotype	% positive T-cell clones			
	Patient Be (acute disease)	Patient St (acute disease)	Patient Sch (reconvalescence)	Patient W (relapse)
$CD4^+8^-$ $TcR\alpha\beta^+$	32	21	42	51
CD^-8^+ $TcR\alpha\beta^+$	62	72	35	26
$TcR\alpha\beta^-$	4	5	2	22
$CD4^+8^+$ $TcR\alpha\beta^+$	1·5	2	2	0
$CD3^-$	0·5	0	0	1
CD4:CD8	0·5	0·3	1·2	1·9
Number of clones analysed	257	88	145	88

T-cell clones were tested in a single cell-ELISA for the expression of CD3, CD4, CD8 or TcR$\alpha\beta$-chain. Each clone was stained twice independently.

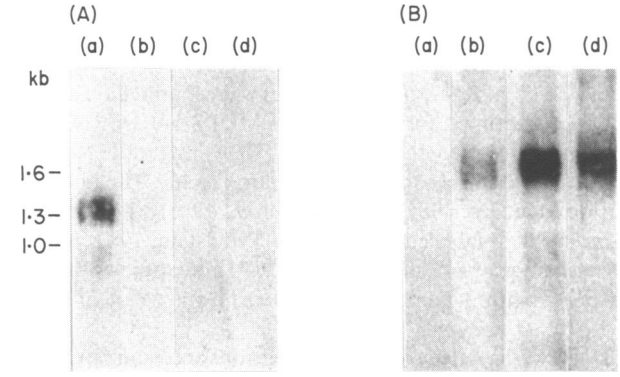

Figure 1. Northern blot analysis of TcRβ- (A) and TcRβ-chain mRNA expression in liver-derived T-cell clones (B). RNA from a $CD4^+$ clone of patient Be (a), or from the $CD4\ 8^-$ clones Be2 (b), W12 (c) and W83 (d) was subjected to electrophoresis agarose gel, blotting and hybridization as described in Materials and Methods.

of the clones grown in the initial cloning could be expanded to cell numbers sufficient for the assay of surface markers.

During the acute phase of the disease $CD8^+$ T cells dominated in the liver tissue (CD4:CD8 ratios of 0·5 and 0·3). In the biopsy of patient Sch taken after recovery, the CD4:CD8 ratio was back to values above 1·0. Similarly in the T-cell clones obtained from patient W, who had a second exacerbation of the disease, a high proportion of CD4 cells was found. In all four biopsies a fraction of $TcR\alpha\beta^-$ ($BMA031^-$) T-cell clones was detected. In the biopsy of patient W a surprisingly high proportion (22%) of these cells were found. Between 1% and 2% of the clones showed unusual phenotypes ($CD4^+8^+TcR\beta^+$ and $CD3^-TcR\beta^-$) as shown in Table 1.

Characteristics of TcR $\alpha\beta^-$ T-cell clones

All $CD4^-8^-$ T-cell clones expressed CD3 but did not react with the TcR$\alpha\beta$-chain-specific mAb BMA031 (Lanier *et al.*, 1987) and not with mAb WT31 specific for an $\alpha\beta$-chain-associated CD3 epitope. In three out of four clones tested, these cells expressed mRNA for the γ-chain but not for the β-chain of the TcR (Fig. 1). A $CD4^+\alpha\beta^+$ clone was included as a control. Southern blot analysis showed a re-arranged TcR β-chain gene in one of these four clones (data not shown).

Expression of other markers was heterogeneous in these cells. Several TcR $\alpha\beta$-chain-negative clones were $CD8^+$ and a few expressed the NKH-1 marker. All clones could be induced to kill P815 cells with anti-CD3 or PHA and approximately 60% had NK activity as they killed K562 and JM targets spontaneously. Upon stimulation with PHA, two-thirds of these clones produced IFN-γ (Tables 2 and 3). The findings with 12 representative clones are summarized in Table 2.

Two $CD4^-8^-$ clones were tested for cytotoxicity against autologous and allogenic HAV-infected fibroblasts. No lysis was observed.

Functional characteristics of TcR $\alpha\beta$-expressing $CD4^+8^-$ and $CD4^-8^+$ clones

Essentially all $CD8^+$ clones had cytotoxic activity as determined in a lectin-dependent cytotoxicity assay against B-LCL cells. From patients Be and Sch the autologous B-LCL was available. In the absence of lectin, none of the $CD8^+$ clones of these patients showed cytotoxicity against the autologous cells but all clones could kill these B-LCL cells in the presence of lectin (data not shown). Thus, there was no autoreactivity among these clones. Cytotoxic activity was also present in 50–80% of $CD4^+$ clones, but was much lower than that of the $CD8^+$ clones. The same findings were made with the clones of patients St and W but we had to use an allogeneic B-cell as target.

As the persistent infection of fibroblasts with HAV can be cured by the addition of interferon (Vallbracht & Flehmig, 1985) it was of interest to determine the ability of clones with various phenotypes to produce IFN-γ. Since the antigen-specificity of most of the clones was not known (see below) we used mitogen as a stimulus for IFN production. Thirty-one clones from patient Be and 48 from patient W were tested. Ninety per cent of these clones with various phenotypes produced detectable amounts of IFN-γ (Table 3).

Table 2. Characteristics of T-cell clones with unusual phenotypes from patients W and Be

		Phenotype of T cell clone						Cytotoxicity (% lysis)			IFN-γ	mRNA	
Clone		CD2	CD3	αβ	CD4	CD8	NKH-1	K562	anti-CD3	ADCC	(IU/ml)	TcRβ	TcRγ
W	3	+	+	−	−	+	NT	32	56	NT	25	NT	NT
W	10	+	+	−	−	−	NT	2	37	NT	25	NT	NT
W	12	+	+	−	−	−	−	0	62	0	100	−	+
W	63	+	+	−	−	−	−	22	48	NT	25	−	+
W	83	+	+	−	−	−	−	30	69	15	25	−	+
Be	2	+	+	−	−	−	−	27	11	NT	50	−	+
Be	3	+	+	−	−	−	+	NT	14	NT	NT	NT	NT
Be	4	+	+	+	+	+	−	0	70	NT	NT	NT	NT
Be	6	+	+	+	+	+	−	0	38	NT	NT	NT	NT
Be	13	+	+	−	−	−	−	NT	51	NT	NT	NT	NT
Be	15	+	+	−	−	−	−	0	32	NT	NT	NT	NT
Be	16	+	−	−	−	+	+	20	0	NT	NT	NT	NT

Table 3. IFN-γ production by T-cell clones from patients Be and W

Patient	Phenotype of clones	Number of clones producing/tested	Range (IU/ml)
Be	$\alpha\beta^+$ $CD4^+$ 8^-	6/8	22–55
	$\alpha\beta^+$ $CD4^-$ 8^+	21/22	22–176
	$\alpha\beta^-$ $CD4^-$ 8^-	1/1	44
W	$\alpha\beta^+$ $CD4^+$ 8^-	41/44	24–400
	$\alpha\beta^+$ $CD4^-$ 8^+	22/23	25–800
	$\alpha\beta^-$ $CD4^-$ 8^-	12/17	25–200
	$\alpha\beta^-$ $CD4^-$ 8^+	3/3	25

T-cell clones (5×10^4/well) were stimulated with PHA for 24 hr. The supernatants were assayed for the presence of IFN-γ.

Table 4. Virus-specific cytotoxicity and IFN-γ-production by liver-derived $CD8^+$ clones

Clone no.	Specific lysis of targets (%)		IFN-γ production (IU/ml)	
	Infected	Uninfected	Infected	Uninfected
1	71	5	64	<16
2	73	3	60	<16
5	76	3	70	<16
7	5	4	40	<16
12	4	5	<16	<16
13	9	3	<16	<16
16	92	8	280	<16
26	3	4	<16	<16
33	5	2	<16	<16
36	3	4	<16	<16
46	81	4	130	<16
50	4	4	68	<20

$CD8^+$ clones from patient Sch were incubated on autologous infected and uninfected fibroblasts. From the supernatants ^{51}chromium-release and IFN-γ concentration were determined.

The effector:target cell ratio was 10:1.

Presence of anti-viral cytotoxic T cells in liver tissue

HAV-infected and uninfected fibroblasts from patient Sch were available as target cells. These cells express HLA class I but not class II antigens. The $CD8^+$ clones of this patient could therefore be tested for virus-specific cytotoxicity and for production of IFN-γ after recognition of specific antigen. Table 4 shows an experiment in which several $CD8^+$ clones from patient Sch were tested for cytotoxicity against autologous infected and uninfected fibroblasts. The supernatant of these cultures was tested for the presence of IFN-γ. All CTL clones that showed specific lysis of targets also specifically produced IFN-γ upon target-cell recognition. Two clones (nos 7 and 50) produced IFN-γ specifically in response to the infected cells but did not lyse these target cells.

Of 26 $CD8^+$ clones of patient Sch, 11 (42%) showed cytotoxicity against the infected, but not the uninfected, fibroblasts similar to those shown in Table 4. The specificity of these clones is presently under investigation. Results with a few clones derived from patient Sch show that they are HAV-specific and HLA-restricted (Vallbracht *et al.*, 1989).

DISCUSSION

In contrast to most other enteroviruses that destroy their host cells, HAV establishes a persistent infection of fibroblasts and hepatoma cells without any apparent cytopathic effect (Vallbracht *et al.*, 1984). During acute viral hepatitis A signficant cell death occurs *in vivo*. Peak production and excretion of infectious virus, however, occurs before serum transaminases are elevated (Zuckerman, 1988). This indicates that the hepatocyte injury leading to disease is mediated by an immune mechanism. Antibody-dependent cytotoxic mechanisms appear not to have a major function in virus elimination and hepatocyte destruction (Gabriel, Vallbracht & Flehmig, 1984). Cytotoxic T cells lysing autologous but not allogenic HAV-infected fibroblasts could be detected in the peripheral blood of patients with acute

hepatitis A (Vallbracht *et al.*, 1986). However, such findings in the periphery may not necessarily be related to the events in the affected organ. In addition, these CTL reached their maximal activity after recovery from the disease.

In this report we describe the phenotypical and functional composition of the infiltrating T lymphocytes in different stages of hepatitis A and demonstrate the occurrence and enrichment of virus-specific CTL at the site of a virus-induced hepatitis. Several of our findings are relevant to the pathogenetic mechanisms leading to hepatitis A. First, $CD8^+$ T lymphocytes dominate in the infiltrate over $CD4^+$ cells during the acute phase of the disease, whereas after recovery the CD4:CD8 ratio was back to a normal value. Secondly, a high percentage of virus-specific CTL able to kill autologous virus-infected fibroblasts could be demonstrated among the liver-derived $CD8^+$ clones. It should be noted that the actual fraction of virus-specific CTL is possibly even higher, since the virus used for infection of target cells is a virus adapted to growth in fibroblasts for several years. This virus has possibly experienced changes in antigenicity during the process of adaption (Cohen *et al.*, 1987). Similar host cell-dependent changes in antigenicity have been described for several other viruses (Schild *et al.*, 1983).

We detected several $CD8^+$ clones that were able to produce IFN-γ but not to exert cytotoxicity in response to HAV-infected fibroblasts. All these clones had lectin-dependent cytotoxic activity. It is possible that such $CD8^+$ cells—that are apparently virus-specific— may well be able to lyse hepatocytes infected with the wild type virus *in vivo*. Several reasons for their failure to lyse fibroblasts are conceivable: different CTL clones may have different avidities and some may require high levels of adhesion molecules such as LFA-3 or ICAM-1 on target cells; and in addition, evidence has been presented that triggering of cytotoxicity has a higher threshold than triggering of lymphokine release (Blanchard, de Vries & Spits, 1986). Taken together, these findings indicate that the percentage of virus-specific CTL from the biopsy is a minimal estimate of the fraction of specific T cells at the site of infection.

A third noteworthy finding is the ability of the majority of T cells present at the site of infection to produce IFN-γ. Since the antigen specificity of the $CD4^+$ and $CD4^-8^-$ $TcR\gamma^+$ clones could not be tested we had to use mitogen to test for the capacity to produce IFN-γ. Persistent infection of fibroblasts by HAV is terminated by the addition of IFN-γ (Maier *et al.*, 1988). As HAV-infection does not induce production of IFN-α or IFN-β (Vallbracht *et al.*, 1985), IFN-γ production appears to be particularly important as an anti-viral mechanism. On the other hand, in contrast to normal hepatocytes (Flehmig, McMichael & Morton, 1981), hepatocytes in hepatitis A have been shown to express HLA-class I antigens (Maier *et al.*, 1988) possibly induced by T cell-derived IFN-γ. The production of IFN-γ by infiltrating T cells may thus make infected hepatocytes recognizable for CTL and may thus contribute to the immunopathology of the disease.

Although we could not demonstrate the specificity of the $CD4^+$ clones it is likely that they also contain a fraction of virus-specific proliferative and lymphokine-producing cells. Most of these clones had lectin-dependent cytotoxic activity. Whether this cytotoxic potential is of any significance for the disease is unclear in view of the fact that $CD4^+$ T cells can acquire cytotoxic capacity during *in vitro* cultivation (Fleischer, 1984).

A surprising finding was the enrichment of TcR $\alpha\beta$-chain-negative T lymphocytes in the tissue of patient W that express the TcR γ-chain. The CD4:CD8 ratio among $\alpha\beta^+$ clones of this patient was normal, but 22% of all clones were TcR $\alpha\beta$-chain negative. It is unlikely that these cells were preferentially expanded in recombinant IL-2 during the time of bulk cultivation because we did not observe a better growth of these cells during the later period of cultivation after cloning. Indeed, these cells appeared to grow slower and required stimulation more often than $CD4^+$ or $CD8^+$ clones. The role of these T lymphocytes in the usual course of hepatitis A in patient W is unclear. Lymphocytes expressing the γ-TcR have been postulated to play a role in the defence of epithelia (Janeway, 1988). Remarkably, $\gamma\delta^+$ T cells comprise 5% of T cells in joint fluids of patients with juvenile rheumatoid arthritis (De Maria *et al.*, 1987) and such cells have been detected in the cerebrospinal fluid of patients with subacute sclerosing panencenphalitis, a measles virus-induced demyelinating disease (Ang *et al.*, 1987). These reports support the notion that the accumulation of $\gamma\delta^+$ cells could be of significance in an immune-mediated inflammatory process.

In several other viral infections of liver tissue a cytotoxic mechanism of liver cell injury by virus-specific CTL has been proposed, a paradigm being hepatitis B virus-induced acute and chronic hepatitis. Although this assumption is supported by immunohistological findings, showing infiltration of $CD8^+$ T cells in liver tissue, the functional analysis of these T cells has been hampered by the lack of appropriate target cells expressing viral antigens and autologous HLA-antigens. The availability of such a system makes hepatitis A a unique model for the study of immunopathological mechanisms leading to inflammation and virus elimination.

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