STUDIES ON MARKERS OF HEPATITIS B VIRUS REPLICATION IN MAN.

Eric James GOWANS
M. App. Sc

Division of Medical Virology
Institute of Medical and Veterinary Science
ADELAIDE.
South Australia

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy.

January 1986.
CHAPTER 1: INTRODUCTION
Preface
1. Sources of hepatitis B virus-infected tissues.
2. Possible mechanisms of persistent virus infection.
3. Clinical and epidemiological aspects of persistent hepatitis B virus infection.
4. HBV particles.
5. The HBV genome and products.
6. HBV products in infected cells.
7. Viral antigen expression in infected tissues and cells.
8. Persistent infection with HBV.
9. Sequelae of persistent HBV infection.
10. Animal models of HBV.
11. HBV replication mechanisms.

CHAPTER 2: MATERIALS AND METHODS
1. Tissue collection and storage.
2. Preparation of microscope slides and coverslips.
3. HBV antigen detection in livers.
5. Probe preparation.
6. In situ hybridisation.
7. Autoradiography.
8. In situ hybridisation interpretation.

CHAPTER 3: DETECTION OF HBsAg and HbcAg IN FIXED TISSUES
1. Introduction.
2. Experimental design.
3. Results.
   a. Fixation of frozen sections.
   b. Comparison of direct and indirect IF for detection of HBsAg in frozen sections.
   c. Comparison between frozen and fixed tissue sections for HBsAg detection.
   d. Comparison between frozen and fixed tissue sections for HbcAg detection.
   e. Examination of biopsy specimens.
4. Conclusions and discussion.
CHAPTER 4: THE DEVELOPMENT OF METHODS TO DETECT HBV DNA - AND HBV DNA AND VIRAL ANTIGEN SIMULTANEOUSLY - IN FIXED, PARAFFIN EMBEDDED TISSUE.
1. Introduction.
2. Experimental procedures.
3. Results.
   a. The development of in situ hybridisation with good histological detail.
   b. The development of a method for the simultaneous detection of HBeAg and HBV DNA.
4. Conclusions and discussion.

CHAPTER 5: DEMONSTRATION AND ANALYSIS OF HBV DNA IN THE CYTOPLASM AND NUCLEUS OF INFECTED CELLS.
1. Introduction.
2. Experimental design.
3. Results.
   a. Characterisation of cytoplasmic HBV nucleic acid sequences.
   b. Detection of nuclear HBV nucleic acid sequences.
   c. Examination of ethanol acetic acid fixed, paraffin embedded tissue.
4. Conclusions and discussion.
   a. Identification of replicative intermediates.
   b. Different forms of HBV DNA detected.
   c. Permissive and non permissive HBV infection.

CHAPTER 6: CORRELATION BETWEEN HBeAg EXPRESSION AND THE PRESENCE OF CYTOPLASMIC HBV DNA.
1. Introduction.
2. Experimental design.
3. Results.
4. Conclusions and discussion.

CHAPTER 7: LIVER HISTOLOGY AND MARKERS OF HBV INFECTION
1. Introduction.
2. Anti-d FITC conjugate preparation and evaluation.
3. Experimental design.
4. Results.
   a. HBV.
   b. Presence of d-Ag.
   c. Cumulative results.
5. Conclusions and discussion.

CHAPTER 8: CONCLUDING REMARKS
1. Introduction.
2. In situ hybridisation.
3. HBV replication.
5. Conclusions.

REFERENCES

APPENDIX - Publications resulting from this work.
The relationship between different markers of hepatitis B virus (HBV) infection and virus replication in HBV infected human liver tissue was examined, with particular emphasis on the relationship between hepatitis B virus core antigen (HBCAg), which is the main virus capsid antigen, and replicative forms of HBV DNA.

Viral antigen detection in liver tissue

Examination of HBV infected livers by the indirect and direct immunofluorescence methods showed the classical distribution pattern of cytoplasmic hepatitis B surface antigen (HBsAg) and nuclear HBCAg respectively. However, when the livers were re-examined with a more sensitive assay, HBCAg was detected in the cytoplasm of a population of cells previously thought to contain only nuclear HBCAg and in the cytoplasm of a population of cells previously negative for HBCAg. Thus, in the samples examined, the majority of cells positive for HBCAg expressed the antigen in the cytoplasm.

The detection of viral antigens in frozen sections was compared with their detection in fixed, paraffin embedded tissue from the same liver samples. HBsAg and HBCAg, localised in the cytoplasm and nucleus respectively, were detected in the fixed tissue, but additional membranous HBsAg and membranous or cytoplasmic HBCAg were generally only detected in frozen sections. This problem could be overcome with respect to HBCAg by use of the
sensitive peroxidase-antiperoxidase (PAP) method, which allowed satisfactory detection of HBeAg in ethanol:acetic acid fixed, paraffin embedded tissue.

As a result of the improved histological appearance of the tissue after this method, HBeAg detected by PAP was shown to be usually restricted to foci of infected cells. Two types of foci were seen. Type 1 foci contained cells positive for nuclear and cytoplasmic HBeAg as well as cells positive for only cytoplasmic HBeAg, while Type 2 foci contained cells positive for only cytoplasmic HBeAg.

Correlation of replicative HBV DNA with HBeAg

The distribution of HBV DNA in different infected livers was examined by in situ hybridisation using recombinant HBV DNA probes, radiolabelled usually by nick translation. These experiments demonstrated vastly differing levels of HBV nucleic acid within different infected cells. A spectrum of HBV DNA levels, ranging from high to intermediate, was seen in hepatocytes usually in a cytoplasmic location. In contrast, low levels of HBV nucleic acid sequences were detected in the nuclei of cells that were shown to be hepatoma cells and lymphocytes.

Detailed investigation of the HBV DNA detected in the cytoplasm of hepatocytes demonstrated that this contained regions of single stranded virus DNA that was normally double stranded in mature virions, consistent with the properties of a DNA
replicative intermediate. It was concluded that the hepatocyte cytoplasm was the likely intracellular site for HBV DNA synthesis.

Analysis of the intracellular locations of HBV DNA and HBeAg (detected by direct immunofluorescence) in different infected cells showed that the presence of cytoplasmic HBV DNA was more closely correlated with cytoplasmic rather than nuclear HBeAg. These studies were extended to examine HBV DNA and HBeAg (detected by PAP) in sequential sections or simultaneously in the same tissue section. These experiments demonstrated cytoplasmic (replicative) HBV DNA in cells which were either positive or negative for nuclear HBeAg but which always contained cytoplasmic HBeAg. The levels of cytoplasmic HBV DNA and HBeAg were directly proportional; thus, cytoplasmic HBeAg was a more reliable indicator of HBV DNA replication than nuclear HBeAg at the level of the single cell.

Cells containing the above cytoplasmic replicative HBV DNA always contained HBsAg and HBeAg, while cells with low level, nuclear HBV nucleic acid sequences did not contain detectable viral antigens. In addition, HBsAg-positive, HBeAg-negative hepatocytes were frequently seen in which HBV nucleic acid could not be demonstrated with the techniques used. It is proposed that the above observations identify (i) permissively infected cells (hepatocytes) containing HBV DNA replicative intermediates and both HBsAg and HBeAg (ii) cells undergoing restricted infection, demonstrated by either the possession of HBsAg
without replicative HBV DNA (hepatocytes) or by the presence of
nuclear HBV nucleic acid sequences without detectable viral
antigen expression (lymphocytes and tumour cells).

Association of HBV with hepatocyte injury

A number of liver samples were also examined for HBV markers and
these results compared with the histological diagnosis and
serological markers of infection. In comparing different
patients, the presence of replicative HBV DNA in liver cells was
associated with liver HBeAg and usually with serum HBeAg, while
absence of replicative HBV DNA was associated with the absence
of liver HBeAg and usually with serum anti-HBe.

In livers containing foci of hepatocytes with high levels of
cytoplasmic (replicative) HBV DNA, histological evidence of
injury, viz. vacuolation and hydropic degeneration, was seen in
these foci. However, in different patients, progressive liver
damage (chronic active hepatitis) was seen both in those
patients with or without evidence of HBV replication; in
contrast, cirrhosis was strongly correlated with either HBV
replication or the presence of delta-Ag. It was concluded that
more than one mechanism for hepatocyte damage may operate, that
one of these mechanisms was related to HBV replication, and that
cirrhosis was an especially common finding in those patients
with chronic active hepatitis who also had evidence of either
HBV replication or delta infection.