Sensitive avidin biotin based technique for identifying liver membrane antigens in tissue sections

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SUMMARY An enzyme immunohistochemical technique for the localisation of liver membrane antigens in tissue sections by antisera raised in guinea pigs against the liver preparation known as "liver specific membrane lipoprotein (LSP)" was developed, based on the alkaline phosphatase avidin biotin complex (ABC AP) system. Of a wide range of fixatives and fixation conditions investigated, a short (five minute) exposure of cryostat sections to Bouin's fluid provided the most satisfactory results and—together with procedures to block endogenous biotin and alkaline phosphatase—yielded clear sections with no background staining or other artefacts to interfere with specific staining patterns. The sensitivity of the technique approaches that of a radioimmunoassay, as shown by the staining of the sinusoidal domains of hepatocellular plasma membranes by the guinea pig anti-LSP antisera at dilutions up to 1/50 000. Apart from its reliability and sensitivity the procedure offers additional advantages over techniques such as indirect immunofluorescence in that it provides a permanent preparation with well defined morphological details which can be seen by ordinary light microscopy.

Liver specific membrane lipoprotein (LSP) is a preparation from normal liver that contains various membrane fragments and other macromolecular liver cell constituents.¹ A proportion of the membrane fragments derive from the hepatocellular plasma membrane and clinical interest in LSP has been stimulated by the numerous reports of cellular and humoral immune reactions against different antigens in this preparation in patients with autoimmune chronic active hepatitis and certain other liver diseases.¹⁻⁵ Current evidence suggests that the antigens in LSP that are of greatest importance, as targets of autoreactions are both liver specific and immunologically cross reactive among species^{1,4} and, if the autoreactions are associated with pathogenesis, the relevant antigens must presumably be located on hepatocellular surfaces.

The immunochemical heterogeneity of LSP and the comparative lability of many of its constituent antigens have presented important technical obstacles to defining the targets of these autoreactions and in particular to locating them in the liver by histochemical techniques. Lebowitz and Gerber were unable to obtain any liver specific staining of unfixed frozen sections of human liver by indirect immunofluorescence with a rabbit antiserum to

human LSP and speculated that the relevant antigens may have been present in amounts that were too small to be detected by this technique.⁶ Lee *et al* suggested that the antigens might have been lost during preliminary washing of the sections and showed that previous fixation with Bouin's fluid permitted detection of linear polygonal immunofluorescence staining of hepatocyte plasma membranes in rat liver sections by serum samples from patients with various liver diseases.^{7,8}

Helin *et al* used the more sensitive peroxidase linked avidin biotin complex (ABC) system to investigate reactions against deparaffinised sections of paraffin wax embedded normal human liver with rabbit anti-human LSP antisera.⁹ They were able to stain hepatocyte plasma membranes with some antisera but the results were variable and it was difficult to interpret the findings. The present study was therefore undertaken to define more precisely the conditions required to identify the location of the relevant target antigens of anti-LSP antibodies in liver sections by using the highly sensitive alkaline phosphatase ABC system.

Material and methods

PREPARATION OF RAT TISSUES

Wistar rats were killed by cervical dislocation and

tissues were excised into ice cold (4°C) phosphate buffered saline (PBS), pH 7.4, then immediately snap frozen in isopentane liquid nitrogen according to the method of Sheibani and Tubbs.¹⁰ Five micron sections of composite (liver, adrenal cortex, kidney, pancreas, and lung) tissue blocks were cut in a cryostat at -20°C, mounted on glass slides, air dried for one hour at room temperature, and then stored at -20°C.

IMMUNOHISTOCHEMICAL PROCEDURES

The procedures described here are the optimal conditions derived from the investigations detailed in the *Results* section. The reagents for the avidin biotin complex (ABC) system (Vector Laboratories Peterborough, UK) were: avidin biotin blocking kit number SP-2001; ABC alkaline phosphatase conjugated antiguinea pig IgG Vectastain kit number AK-5007; and alkaline phosphatase substrate kit number SK-5100. Unless otherwise indicated, all preparations and dilutions of these reagents were performed strictly according to the manufacturer's instructions. Thawing of tissue sections and all other procedures were carried out at room temperature.

Sections were fixed by immersion in Bouin's fluid (75 ml saturated aqueous picric acid, 24 ml formalin, and 5 ml glacial acetic acid) for five minutes. Slides were then transferred directly to PBS and washed thoroughly for 15 minutes with three changes of the buffer.

All of the following procedures were carried out in the dark in a humidified chamber. Endogenous biotin in the fixed sections was inactivated by using the Vector avidin biotin blocking kit. Thus sections were incubated for 20 minutes with the avidin solution provided, washed with PBS (pH 7.4) for five minutes, then incubated with the biotin solution for 20 minutes. After a further five minutes wash in PBS sections were incubated with a 1/50 dilution (in PBS) of normal goat serum for 20 minutes to block any sites that might bind goat proteins non-specifically. Excess normal goat serum was removed by blotting and the sections were incubated for 30 minutes with the required dilution (1/50 or greater, in PBS) of the guinea pig antirat LSP antisera washed for 10 minutes in PBS, incubated with the goat antiguinea pig IgG for 30 minutes, washed (for 10 minutes with PBS) and then incubated with the Vectastain ABC alkaline phosphate reagent. After a further 10 minute wash in PBS the sections were incubated with the Vector Red alkaline phosphatase substrate solution to which had been added levamisole (1mM final concentration), washed for five minutes in tap water, counterstained with Mayer's haemalum for 10 seconds and rinsed in tap water. Finally, the sections were dehydrated by absolute ethanol, cleared by xylene, and mounted in distrene plasticiser xylol.

Antisera were raised in guinea pigs against rat LSP

that had been prepared from 100 000 × g supernatants of normal rat livers by gel filtration over sepharose 6B in 0.1 M boric acid/sodium borate buffer, pH 8.5, containing 1 mM disodium edetic acid as previously described.¹¹ The immunisation programme which entailed subcutaneous injections of LSP with and without Freund's complete adjuvant over five weeks has been previously described.¹¹ Production of anti-LSP antibodies was monitored by radioimmunoassay and titres of the antisera determined by doubling dilution titration.¹²

Results

At the beginning of the study preliminary investigations were undertaken to test each component of the system for non-specific reactivity, starting with the alkaline phosphatase substrate and working backwards through the procedure. As described by the manufacturers, it was found that when unfixed liver sections were incubated with the alkaline phosphatase substrate alone a definite positive reaction was obtained (an intense red staining mainly of bile duct epithelial cells and indicating pronounced endogenous alkaline phosphatase activity) and that this could be completely abolished by including levamisole (at 1 mM) in the substrate solution.

When the Vectastain ABC reagent was used without any antiserum, fine, granular staining of the cytoplasm was seen (fig 1). That this could be abolished by using the avidin biotin blocking procedure (fig 2a) indicated that it was due to binding of the ABC to endogenous biotin in the cells. It should be noted that a quick rinse after performing this procedure is inadequate, and a minimum of five minutes washing after each step is essential.

Fig 1 Definite granular staining of the cytoplasm of hepatocytes seen when endogenous biotin was not blocked before incubation with the ABC reagent.

Fig 2 Staining mainly of sinusoidal surfaces of hepatocytes by guinea pig anti-LSP antiserum. (a) Control (normal guinea pig serum at 1/50 dilution); (b) strong granular staining with guinea pig anti-LSP at 1/1000 dilution; (c) fine granular staining with antiserum at 1/50 000 dilution. Developed with Vector Red alkaline phosphatase substrate and counterstained with Mayer's haemalum.

Incubation of sections with undiluted guinea pig serum (after blocking endogenous biotin) also led to fine granular staining. To investigate the possibility that this might have been due to ionic interactions with basic proteins (which have the ability to bind ABC) or to interaction with lectin like molecules in the liver that may have affinity for carbohydrate residues in guinea

pig serum glycoproteins, the procedure was carried out using carbonate buffer, pH 9.4, and (in further experiments) after pretreatment of the sections with 0.05 M α -methyl-D-mannoside. Neither of these modifications had any effect, but it was found that a completely clear section with no residual staining was obtained if the normal guinea pig serum was applied at

Table 1 Details of the fixatives and fixation conditions investigated

Fixative	Buffer	Temperature	Time (minutes)
Acetone	None	-20°C	1 and 5
Acetone	None	+4°C	10
Ethanol	None	-20°C	1 and 5
Ethanol	None	+4°C	10
Methanol	None	+4°C	10
Paraformaldehyde 2.0%	Aqueous, pH 7.5	Room temperature	30
Formalin 3.7%	Phosphate, pH 7.4	Room temperature	0.5, 2, 10, 30
Formalin 3.7%	Tris, pH 7.6	Room temperature	15
Formalin 3.7% with 0.1% Triton X-100	Phosphate, pH 7.4	Room temperature	30
Formalin 3.7% with 0.1% Brij 35	Tris, pH 7.6	Room temperature	20
Glutaraldehyde 1.0%	0.1M cacodylate, pH 7.4	+4°C	15
Glutaraldehyde 1.0% with 0.1% saponin	0.1M cacodylate, pH 7.4	+4°C	5, 10, 15
Glutaraldehyde 1.0% with 3.7% formalin	0.05M Tris/HCl, pH 7.4	Room temperature	20
Glutaraldehyde 1.0% with 3.7% formalin	0.1M cacodylate, pH 7.4	+4°C	15
Glutaraldehyde 1.0% with 3.7% formalin	PBS, pH 7.4	+4°C	1, 3, 10
Glutaraldehyde 1.0% with 3.7% formalin	PBS, pH 7.4	Room temperature	3
Bouin's fluid	None	Room temperature	5 and 10

dilutions of 1/50 or greater. Thus 1/50 was adopted as the lowest dilution at which guinea pig serum could be used in the system.

To examine the effects of fixation of the sections on specific staining by anti-LSP antibodies, a wide range of fixatives and conditions was investigated (table 1). It was found that with the precipitating fixatives, acetone, ethanol, or methanol, some membrane staining with anti-LSP could be obtained but this was generally weak and the signal: noise ratio was too great to permit confident interpretation of staining patterns. Similarly, paraformaldehyde fixation resulted in only weak staining of liver cell surfaces and was associated with various artefacts. Among the other crosslinking fixatives, formalin proved to be fairly effective—resulting in stronger membrane staining than paraformaldehyde, with few artefacts—particularly when it was buffered with phosphate and at shorter (0.5 to 2 minute) fixation times, but when detergents (Brij 35 or Triton X-100) were added to the formalin almost no staining was obtained with the anti-LSP antisera.

In sections fixed with glutaraldehyde alone there was no staining of cell membranes by anti-LSP. Addition of formalin to the glutaraldehyde resulted in a fine membrane staining pattern, the intensity of which varied inversely with fixation time but was independent of the buffer used. The inclusion of saponin (without formalin) in the glutaraldehyde yielded the strongest staining of membranes but some staining of control sections with normal guinea pig serum (1/50) was also obtained.

The most satisfactory result was obtained when sections were fixed with Bouin's fluid, especially when exposure to the fixative was kept to five minutes. Clear, strong staining of the sinusoidal portions of hepatocellular plasma membranes and (to a lesser extent) of basolateral and cytoplasmic membranes was evident (figs 2a, b, and c), at anti-LSP dilutions of 1/50 to 1/50 000. In contrast, sections incubated with normal guinea pig serum at 1/50 dilution were completely negative. The sensitivity of the procedure is shown by comparison with the radioimmunoassay titres of the four anti-LSP antisera used (table 2).

Table 2 Comparison of titres obtained by radioimmunoassay and by the ABC immunohistochemical technique with four guinea pig anti-LSP antisera

Anti-LSP antiserum	ABC immunohistochemical titre*	Radioimmunoassay titre
GP 9	1/20 000	1/160 000
GP 10	1/50 000	1/160 000
GP 11	1/5 000	1/100 000
GP 12	1/50 000	1/220 000

*End point = highest dilution at which a definite positive reaction could be detected.

Of the various tissues other than liver that were examined in this study, positive reactions with the anti-LSP antisera under these conditions were seen only with kidney. Staining of rat renal tubular epithelium, with prominent epithelial surface membrane and weak cytoplasmic staining of proximal tubular cells, was seen when low dilutions (less than 1/400) of the antisera were used. At higher dilutions (1/400 or greater), the antisera stained only liver. This suggests that the highest titres of antibodies in these antisera were directed at liver specific determinants.

Discussion

The present study shows that an enzyme immuno-histochemical technique based on the alkaline phosphatase avidin biotin complex (ABC AP) system is a reliable method of detecting liver plasma membrane antigens by specific antisera. The technique has sensitivity approaching that of radioimmunoassay and offers a number of advantages over other methods. In contrast to indirect immunofluorescence, which—although comparatively insensitive—is widely used for tissue localisation of antigens, the ABC AP system provides a permanent preparation (thereby avoiding the need for photographic records) with well defined morphological details that can be seen by ordinary light microscopy.

The inability of other workers⁶ to detect plasma membrane antigens with anti-LSP antisera on unfixed cryostat sections of liver is probably the result of a combination of factors including the small amounts of antigens that are available for interaction with the antibodies and the loss of some of these antigens during the obligatory washing of the sections. The necessity for fixation was shown by Lee *et al.*^{7,8} who used Bouin's fluid with indirect immunofluorescence, and by Helin *et al.*⁹ on paraffin wax embedded material with an ABC peroxidase system. In the studies of Lee *et al.*^{7,8} staining of hepatocellular plasma membranes (with no cytoplasmic staining) was seen with serum samples diluted up to 1/320, but in that of Helin *et al.*⁹ animal antisera were used only at a dilution of 1/500. Helin *et al.* reported considerable variability in patterns of staining, including both hepatocellular plasma membrane and diffuse cytoplasmic staining (similar to that shown in fig 1) that made interpretation of the results difficult. From the present findings it seems that this variability may have been caused by the fixation conditions, by non-specific interactions between avidin and the peroxidase or because they did not block endogenous biotin before staining, or by a combination of these factors.

The importance of fixation conditions is underlined in the present study. The precipitating fixatives (acetone, ethanol, or methanol) are generally

inadequate for immobilisation of all protein antigens and do not stabilise surface membrane components sufficiently for immunohistochemical procedures that entail prolonged incubations or multiple washings of the sections. Cross linking fixatives have shown more promise in this regard but glutaraldehyde, which has proved useful in enzyme histochemistry, is often inadequate for immunohistochemistry¹³ and this was confirmed in the present study. Addition of formalin or saponin to the glutaraldehyde considerably improved stabilisation of the membrane constituents (especially when fixation time was kept short) but some non-specific staining with normal guinea pig serum was seen and the formalin glutaraldehyde system was not superior to formalin alone. We confirm the finding of other studies,¹⁴ that detergents should not be used. A short exposure to Bouin's fluid proved to be superior to all other fixatives and fixation conditions investigated in the present study. As noted by Lee *et al*⁷ Bouin's fluid has the added advantage when used for the study of circulating liver auto-antibodies in that it apparently denatures the target antigens of non-organ specific autoantibodies such as antinuclear, antismooth muscle, and antimitochondrial antibodies that are common in patients with various liver diseases and which would give staining patterns on liver sections that could make specific staining due to anti-LSP antibodies difficult to interpret. The mechanism of action of Bouin's fluid is unknown, but the possibility that this denaturing of certain proteins (for example, nuclear, smooth muscle, and mitochondrial antigens) may stabilise the hepatocyte surface as suggested by Lee *et al*⁷ seems untenable because the anti-LSP antisera were raised against a presumably undenatured antigen preparation. It is unlikely therefore that the antibodies would bind to antigens that had been appreciably altered by the fixative.

Although problems of non-specific reactions and background staining are common with immunohistochemical techniques,¹⁰ they were not encountered in the present study when optimal conditions prevailed. Specific inactivation of endogenous biotin (particularly in liver, which has a high content of the vitamin) is imperative, irrespective of which fixatives or fixation conditions are used. Non-specific binding of avidin (which has a high isoelectric point, pH 10.0) to certain classes of proteins can be a problem and can be avoided by using the ABC reagent at pH 9.4 (which does not interfere with binding of specific antibodies).¹⁵ We could, however, find no difference between PBS at pH 7.5 and carbonate buffer at pH 9.4. Endogenous alkaline phosphatase activity is effectively blocked by including levamisole in the substrate buffer, and presumably the acetic acid in the Bouin's fluid also contributes to blocking of alkaline

phosphatase isoenzymes.¹⁶ The possibility that guinea pig serum glycoproteins might bind to hepatocytes by the hepatic asialoglycoprotein receptor was considered but such binding requires Ca^{++} (which was not present in the buffers),¹⁷ and pretreatment of the sections with α -methyl-D-mannoside to block the receptor¹⁷ had no effect on the specific binding of anti-LSP antibodies to the hepatocyte membranes.

The ABC system is generally 20 to 40 times more sensitive than procedures such as the unlabelled peroxidase antiperoxidase method,¹⁸ and the present study indicates that the sensitivity of the ABC AP system in particular makes it eminently suitable for the identification of hepatocellular plasma membrane antigens with anti-LSP antisera. In addition to careful attention to washing protocols and incubation times, the important features of the technique include the use of Bouin's fluid for fixation and the absolute necessity for blocking endogenous biotin and alkaline phosphatase. Finally, we would recommend the Vector Red alkaline phosphatase substrate used in this study in preference to the "black" or "blue" substrates offered by the manufacturer because the red gives superior contrast with the counterstain and, unlike the "blue", permits subsequent dehydration and mounting of the sections as permanent preparations.

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