Leading article

Lymphocyte cytotoxicity for autologous hepatocytes

The histological finding of lymphocyte and plasma cell inflammatory infiltrates in many patients with chronic liver disease has prompted clinical immunologists to study immune responses which may be relevant to the pathogenesis of hepatocyte damage. Despite much experimental work in vitro and even more theoretical speculation, however, no firm conclusions can be reached at present concerning the role of the host's immune response in causing liver cell damage in vivo. Initial studies were designed to explore leucocyte sensitisation to ‘liver specific’ and hepatitis B virus (HBV)-associated antigens, but the diversity of the techniques used and differences in the preparation of antigenic material lead to somewhat conflicting results.

As no consensus emerged from these studies, attention turned towards cell-mediated cytotoxicity experiments. A variety of target cells were used including Chang,1,2 EL-4 mouse sarcoma,2 mammalian hepatocytes3-5 as well as avian,6,7 or rabbit8 erythrocytes coated artificially with liver specific protein or hepatitis B surface antigen and, more recently, human hepatoma cells lines.9,10 A number of caveats, however, apply to such studies. Firstly, most of the target cells are not derived from liver and may therefore be unsuitable to study specific effector cell/target interactions in chronic liver disease. Secondly, although the detection of optimal T-cell cytotoxicity requires histocompatibility between effector and target cells,11 the constraints of HLA restriction are not met in most studies. Thirdly, the antigenic specificity of the effector cells has been rarely established. Finally, tumour cell lines may induce production of interferon in human lymphocytes, which may stimulate cytolytic cells endowed with natural killer properties.12

Another approach is to use an autologous cytotoxicity assay whereby patients' peripheral blood lymphocytes are incubated with hepatocytes isolated from a portion of the patient's own liver biopsy. This method has the advantages that antigenic expression on the target cell surface should be closer to the in vivo situation and that effector and target cells are fully histocompatible. In two early studies,13,14 hepatocytes were labelled with 51Cr: the release of this marker is a sensitive indicator of target cell lysis. It soon became clear, however, that the freshly isolated liver cells released the isotope at such a high and variable spontaneous rate, that the assay system was insensitive and unreliable15 (and unpublished data). Furthermore, the relatively large number of target cells needed to get sufficient counts for reliable measurement isotope levels, together with the relatively small number of autologous human hepatocytes available from a fragment of a diagnostic liver biopsy specimen, limited severely the
number of experiments which could be done in each case. For these reasons, we and others have used the microcytotoxicity assay originally developed by Takasugi and Klein,\textsuperscript{16} which is based on the detachment of adherent target cells after exposure to mononuclear cells. Briefly, hepatocytes isolated enzymatically, or mechanically from a small portion of a diagnostic liver biopsy core are plated into wells of a microtest plate and allowed to attach during an initial 24 hour period. Mononuclear cells are subsequently added and, after further incubation (usually 18 or 48 hours), the plates are inverted to allow detachment of damaged liver cells. The remaining target cells are then counted and, by comparing the values found with those from control wells (containing hepatocytes plus medium alone), the percentage cytotoxicity can be calculated. Advantages of this technique are, that it is relatively simple to do and that many replicates can be obtained, despite the low number of cells (20 to $150 \times 10^6$) available from a small portion of a liver biopsy. The disadvantages are that the visual counting procedure is tedious and that adherence to plastic may not represent viability of target cells, while detachment may not reflect cell death.\textsuperscript{17} Comparative studies, however, have shown a good correlation between the detachment and the $^{51}$Cr-release assay in similar experimental conditions.\textsuperscript{18}

Viability of hepatocytes is also difficult to define precisely. The dye exclusion test may not be an accurate estimate because trypan blue can be endocytosed complexed with albumin by undamaged liver cells.\textsuperscript{19} In one study viability of rat hepatocytes in primary monolayer cultures was evaluated by the more sensitive measurement of LDH release and by trypan blue uptake.\textsuperscript{20} Although there was a good correlation between these two variables during the initial 24 hours of culture, after 48 hours the percentage of LDH release was less than the percentage of trypan blue uptake, suggesting that factors other than liver cell membrane damage may be responsible for the dye uptake. Indeed, morphological studies of freshly isolated liver cells show good preservation of the plasma membrane by scanning\textsuperscript{19,20} and by transmission\textsuperscript{22} electron microscopy.

Another problem which is often encountered when using established cell lines, is that the target cells may proliferate during incubation and that the diminished number of target cells in wells containing lymphocytes may be due either to a ‘crowding’ effect, or to cytostasis, rather than cytolysis.\textsuperscript{23} This phenomenon, however, is unlikely to be important when using hepatocytes as target cells, because well differentiated hepatocytes maintained in long term tridimensional cultures do not appear to divide.\textsuperscript{24}

The autologous microcytotoxicity assay has been used during the last six years by our group to explore lymphocyte/hepatocyte interactions, principally in ‘autoimmune’ and HBsAg positive chronic active hepatitis. Using blocking studies to define the specificity of the reactions detected, the evidence strongly suggests that in HBV infection, cytotoxic T cells are directed against HBV core antigen on the hepatocyte plasma membrane and that circulating anti-core antibodies may modulate the T cell attack by covering the viral antigens on the liver cell surface.\textsuperscript{25–27} Non-T-cells are also cytotoxic to autologous hepatocytes in many of these patients and this may be because of an antibody dependent cell mediated reaction rather than a reflection of NK cell activity.\textsuperscript{26} In ‘autoimmune’ chronic active hepatitis, this non-T-cell cytotoxicity predominates and seems to be
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directed to autoantigens present in a membrane derived lipoprotein complex.\textsuperscript{29}

Poralla and coworkers, in the paper published in this issue of Gut, have used a similar assay system to study lymphocyte cytotoxicity to autologous hepatocytes in acute and chronic non-A non-B (NANB) hepatitis.\textsuperscript{25} In view of the difficulties in establishing the specificity of the lymphocyte/hepatocyte interactions in HBV infection where the viral antigens are well defined, it is not surprising that there are several problems in interpreting the results of these studies.

It is always difficult to establish the diagnosis of NANB hepatitis, as this is invariably done by exclusion of known viral infections. In chronic active hepatitis this problem is compounded by the fact that all the ‘autoimmune’ cases would, by this simple exclusion criterion, also be defined as NANB. Poralla \textit{et al}, have, therefore, additionally excluded all cases with non-organ specific autoantibodies in their serum and those with liver membrane antibodies. These exclusions are only valid if NANB infection is never associated with production of these autoantibodies, but in the absence of reproducible laboratory tests for markers of NANB viruses this is impossible to prove. The finding of liver membrane antibodies in a significant proportion of patients with chronic NANB hepatitis in two independent studies,\textsuperscript{30, 31} suggests that Poralla \textit{et al} may have restricted the patients studied to a discrete subgroup. Another consequence of the lack of serological tests for NANB hepatitis is that antibodies to defined antigenic components of the virus are not available and it is therefore not possible to explore the specificity of the T-cell cytotoxicity, or entirely exclude the possibility that NK cells may be involved.

It will by now have become clear that while direct examination of lymphocyte/hepatocyte interactions promises to provide much information relevant to the immunopathogenesis of acute and chronic viral hepatitis, there are major problems in the design of appropriate assay systems, in interpretation of the results and in the relevance of the findings to the sequence of events \textit{in vivo}. These should not, however, deter potential investigators, but rather stimulate them to provide more rigorous proof (or repudiation) of their hypotheses. We could then proceed with some confidence to the next stage, namely the design and testing of logical therapeutic strategies to prevent or inhibit those reactions which are responsible for progressive liver injury.

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