Lymphocyte cytotoxicity in chronic active hepatitis: effect of therapy and correlations with clinical and histological changes

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SUMMARY A study of lymphocyte cytotoxicity for rabbit hepatocyte cultures in 15 patients with untreated chronic active hepatitis showed positive results in all cases, both HBsAg positive and negative. After immunosuppressive therapy cytotoxicity became negative and remained negative, in four of nine patients followed serially. In 51 patients established on therapy for periods from three months to 12 years, cytotoxicity was negative in 19 and all patients are currently alive. However, in the remaining 32 patients in whom cytotoxicity was positive there has been a 34% mortality. Cytotoxicity remained persistently positive in 12 of 15 patients followed serially, and persistently negative in seven of nine. Cytotoxicity showed a significant association with histological disease activity, especially the extent of piecemeal necrosis, but not with biochemical tests of liver function, immunoglobulins, or autoantibodies. The basis of this cytotoxicity test is an antibody dependent cell-mediated autoimmune reaction directed against a liver specific protein, and the results suggest that in some cases immunosuppressive therapy is followed by control of this reaction. It may be possible to stop therapy in these patients, but in those in whom the reaction continues, as shown by continuing cytotoxicity, the prognosis is not as good and the use of other drug schedules would seem worthy of trial.

There is now considerable evidence to suggest that cell-mediated autoimmune reactions are involved in the pathogenesis of chronic active hepatitis (Cochrane et al., 1976). Such patients have been shown to be sensitised to a liver specific membrane lipoprotein (LSP) (Miller et al., 1972; Thestrup-Pedersen et al., 1976), and their lymphocytes to be cytotoxic for autologous liver cells (Wands and Isselbacher, 1975; Geubel et al., 1976), Chang cells (Wands et al., 1975; Jacques et al., 1976), and rabbit hepatocytes (Thomson et al., 1974) in tissue culture. In the latter system the reaction can be blocked by LSP, suggesting that it is the major target antigen involved (Thomson et al., 1974) and the ability to demonstrate cytotoxicity after the removal of T cells, and not with T cell enriched fractions, suggests that the reaction is mediated by Fc receptor bearing cells and is therefore another example of antibody dependent cell-mediated cytotoxicity (Cochrane et al., 1976). Antibodies reacting with the antigen on the surface of rabbit hepatocytes have now been demonstrated in the serum of some patients with antigen negative chronic hepatitis (Tage-Jensen et al., 1977), and repeated immunisation of rabbits with LSP has produced histological changes very similar to chronic aggressive hepatitis (Meyer zum Büschenfelde et al., 1972).

The improvement in survival time following immunosuppressive therapy has been well demonstrated (Cook et al., 1971; Soloway et al., 1972; Murray-Lyon et al., 1973) and in the present study we report the effects of such treatment on the cytotoxicity of peripheral blood lymphocytes for isolated hepatocytes in relation to biochemical histological features.

Methods

Patients
Sixty-six patients with chronic active hepatitis, diagnosed at the time of presentation according to the criteria of de Groote et al. (1968), were investigated. Levels of serum aspartate transaminase,
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bilinear, alkaline phosphatase, albumin, total globulin, immunoglobulins, and autoantibodies were determined at the time of the cytotoxicity assay. HBsAg was detectable in the serum of 16 patients by radioimmunoassay (Professor A. Zuckerman, Austria II, Abbott Laboratories). A liver biopsy had been performed within two weeks of cytotoxicity testing in all 15 patients who were untreated at the time of the initial test, and in 42 of the 51 who were already established on therapy (prednisone 7-5-20 mg daily with or without azathioprine 75 mg or penicillamine 900 mg daily). Each biopsy was assessed by a pathologist as active or inactive without knowledge of the result of cytotoxicity or liver function tests. In addition, the major abnormalities seen in this condition, including those used in the assessment of disease activity, piecemeal necrosis, spotty parenchymal and bridging necrosis, rosette formation, portal infiltration with plasma cells and lymphocytes, Kupffer cell hyperplasia, and bile reduplication were scored from 0-3 (0 absent, 1 minimal, 2 moderate, 3 abundant) and the scores summed to provide an index of total activity.

Techniques

MICROCYTOTOXICITY ASSAY
This assay was carried out using hepatocytes prepared by enzyme digestion of freshly killed rabbit livers and maintained in tissue culture flasks for up to three weeks as described in detail elsewhere (Cochrane et al., 1977). The morphological appearance of the cells, both on light and electron microscopy, their ability to synthesize albumin and to incorporate 14C leucine into acid precipitable material suggested that they were hepatocytes (Hughes et al., 1976). Further evidence of their viability has been shown using metabolic inhibitors and a 51Cr release assay (Hughes et al., 1976). Lymphocytes were prepared from peripheral venous blood by dextran sedimentation, filtration through a cotton wool column (Rocklin et al., 1970), followed by centrifugation over Ficoll triosil (Böyum, 1968). After adjustment to the required concentration, lymphocytes were added to hepatocytes in microwell plates and incubated for 48 hours at 37°C in a 5% CO2 atmosphere. Detached hepatocytes and lymphocytes were then gently washed clear and the percent cytotoxicity calculated from the formula:

\[
\text{Percent Cytotoxicity} = \frac{\text{average number cells in control well} - \text{average number in test well}}{\text{average number in control well}} \times 100.
\]

The upper limit of the normal range for cytotoxicity (32%) has been taken as two standard deviations above the mean value (2-9%) obtained in 35 normal controls (age 17-36 years).

Reproducibility of results
In 120 tests cytotoxicity was estimated from two separate microtest plates using the same preparation of lymphocytes. In 56 (47%) cases the difference in the cytotoxicity values was less than 10% and in 96 (80%) less than 20%. With respect to positive or negative results, 56 of the 120 tests were positive on both occasions and in 50 both fell within the normal range, giving an overall reproducibility rate of 88%. In one normal subject four separate lymphocyte preparations were processed simultaneously from a single blood sample and used in four microtest plates. The mean cytotoxicity of the 16 tests was 7.7 ± 9% and all fell below 32%, the upper limit of the normal range.

Results

All 15 patients who were untreated at the time of first testing showed significant cytotoxicity. Although the values of cytotoxicity covered a considerable range (from 44-91%) there was no correlation with liver function tests, serum immunoglobulin levels, presence or absence of serum autoantibodies, total biopsy score, or HBsAg status (Table 1).

Serial cytotoxicity tests were performed in nine of these patients following immunosuppressive therapy. Cytotoxicity became negative in four cases within three to six months (Fig. 1) and has remained negative for up to 22 months. In each case the value of aspartate aminotransferase (AST) has remained below twice normal and repeat liver biopsies were considered to be inactive. In the remaining five cases cytotoxicity remained positive. Three of the patients in this group, with persistently abnormal liver function tests, died in liver failure, two of them two months and one two years after treatment was begun. The two remaining cases are still alive at 14 months and 24 months, both having AST values twice normal, although they continue to show histological activity.

Of the 51 patients receiving immunosuppressive therapy at the time of initial testing, 32 (63%) gave positive and 19 (37%) negative cytotoxicity tests. Eleven (34%) of the 32 patients showing cytotoxicity have so far died, whereas there have been no deaths in those with negative results (\(\chi^2 = 5.0, p < 0.025\). The five year survival (Fig. 2) in patients showing cytotoxicity on the initial test was 55% compared to 100% for those in whom cytotoxicity was negative.

Comparison of patients with positive and negative cytotoxicity at the time of the first test did not
Table 1  Clinical details of patients with untreated chronic active hepatitis

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Duration of illness (yr)</th>
<th>Drug</th>
<th>AST (U/l)</th>
<th>Bilirubin (µmol/l)</th>
<th>Alkaline phosphatase (U/l)</th>
<th>Albumin (g/l)</th>
<th>IgG (g/l)</th>
<th>IgM (g/l)</th>
<th>ANA SMAs titre</th>
<th>HBSAg positive</th>
<th>Total biopsy score</th>
<th>% cytotoxicity</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>36</td>
<td>60</td>
<td></td>
<td>2250</td>
<td>154</td>
<td>207</td>
<td>34</td>
<td>57</td>
<td></td>
<td>1000</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>39</td>
<td>60</td>
<td></td>
<td>1100</td>
<td>170</td>
<td>96</td>
<td>43</td>
<td>42</td>
<td></td>
<td>2000</td>
<td>640</td>
<td>160</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>49</td>
<td>3</td>
<td></td>
<td>161</td>
<td>10</td>
<td>125</td>
<td>34</td>
<td>60</td>
<td></td>
<td>1080</td>
<td>20</td>
<td>20</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>51</td>
<td>9</td>
<td></td>
<td>1300</td>
<td>240</td>
<td>167</td>
<td>31</td>
<td>51</td>
<td></td>
<td>2270</td>
<td>20</td>
<td>40</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>55</td>
<td>48</td>
<td></td>
<td>300</td>
<td>170</td>
<td>151</td>
<td>28</td>
<td>44</td>
<td></td>
<td>1800</td>
<td>20</td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>62</td>
<td>6</td>
<td></td>
<td>1600</td>
<td>78</td>
<td>282</td>
<td>19</td>
<td>62</td>
<td></td>
<td>4200</td>
<td>160</td>
<td>320</td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>
| 7    | M   | 30       | 3                        |      | 177       | 85                  | 82                          | 39           | 39        |             | 2940          | 640             | 160               | 20            | 45
| 8    | M   | 38       | 6                        |      | 260       | 53                  | 191                         | 26           | 40        |             | 1500          | 20              |                   |               | 8       |
| 9    | M   | 53       | 3                        |      | 187       | 113                 | 139                         | 39           | 31        |             | 1420          | 10              |                   |               | 14      |
| 10   | M   | 67       | 24                       |      | 280       | 80                  | 290                         | 20           | 60        |             | 1230          | 10              | 10                |               | 15      |
| 11   | M   | 55       | 12                       |      | 136       | 240                 | 135                         | 17           | 45        |             | 1100          | 10              |                   |               | 16      |
| 12   | M   | 59       | 48                       |      | 350       | 50                  | 201                         | 28           | 59        |             | 1770          | 10              |                   |               | 16      |
| 13   | M   | 52       | 20                       |      | 115       | 21                  | 122                         | 45           | 30        |             | 1980          | 10              |                   |               | 14      |
| 14   | M   | 41       | 12                       |      | 108       | 26                  | 102                         | 35           | 35        |             | 1690          | 10              |                   |               | 11      |
| 15   | M   | 41       | 6                        |      | 130       | 9                   | 68                          | 44           | 28        |             | 940           | 20              |                   |               | 14      |

Fig. 1  Lymphocyte cytotoxicity for isolated rabbit hepatocytes in patients with chronic active hepatitis, before treatment (time 0) and serially after immunosuppressive therapy. ———: upper limit of normal range. O: HBSAg positive. ●: HBSAg negative.

reveal any significant differences with respect to age, duration of therapy, ratio of females to males, or HBSAg status. Nor were there significant differences in the mean values of aspartate aminotransferase, bilirubin, alkaline phosphatase, albumin, globulin, immunoglobulin, or presence or absence of autoantibodies determined at the time of cytotoxicity testing (Table 2).

In the 43 treated patients in whom liver histology was available, positive cytotoxicity was associated with an active biopsy in 21 (84%) of 25 cases compared with only three (18%) of 17 negative cases. Similarly there was a close correlation

Fig. 2  Survival curves in patients with treated chronic active hepatitis who showed either positive or negative cytotoxicity.

Table 2  Mean values of liver function tests and immunoglobulins in treated patients with and without significant cytotoxicity

<table>
<thead>
<tr>
<th>Cytotoxicity</th>
<th>Positive (32)</th>
<th>Negative (19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yr)</td>
<td>43.1 ± 14</td>
<td>42.8 ± 14</td>
</tr>
<tr>
<td>Duration of therapy (mth)</td>
<td>48.7 ± 33</td>
<td>44.2 ± 33</td>
</tr>
<tr>
<td>Ratio male:female</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>HBSAg positive</td>
<td>7 (22%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/l)</td>
<td>93</td>
<td>79</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>125</td>
<td>130</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>35</td>
<td>38</td>
</tr>
<tr>
<td>Globulin (g/l)</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>IgA (g/l)</td>
<td>3.72</td>
<td>3.47</td>
</tr>
<tr>
<td>IgG (g/l)</td>
<td>15.28</td>
<td>14.16</td>
</tr>
<tr>
<td>IgM (g/l)</td>
<td>1.97</td>
<td>2.29</td>
</tr>
<tr>
<td>Autoantibodies (% positive)</td>
<td>65%</td>
<td>50%</td>
</tr>
</tbody>
</table>
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between positive cytotoxicity and a high total biopsy score, 19 of 25 patients with positive cytotoxicity scoring 7 or more compared to only three of 17 with negative cytotoxicity ($\chi^2 = 13.5$, $p < 0.005$). When cytotoxicity was compared with individual histological parameters (Fig. 3), those with positive results showed significantly more piecemeal necrosis ($p < 0.01$, Rank sum test), rosette formation, and spotty parenchymal necrosis ($p < 0.05$, Rank sum test) than those with negative results. There were no other significant differences with respect to other histological features examined.

Serial cytotoxicity tests were performed in 24 treated patients. Of 15 with positive cytotoxicity at the initial test (Fig. 4), 12 (80%) continued to give positive results following continued therapy, although in two the test became negative on one occasion. In contrast, of the nine patients in whom cytotoxicity fell within the normal range on initial testing (Fig. 5), only two (22%), both with values close to the upper limit of the normal range, became positive, a significant difference ($\chi^2 = 5.5$, $p < 0.025$).
Discussion

Although cytotoxicity was demonstrated in all patients with untreated chronic active hepatitis, there was a wide variation in the values recorded. This is unlikely to be solely due to variations in the experimental technique as shown by the results of the reproducibility studies. The lack of a correlation between the degree of cytotoxicity and liver function tests or the degree of histological activity is not surprising. The explanation may relate to the fact that the ability to demonstrate cytotoxicity in vitro presumably depends on the in vivo absorption of complexes of liver-specific protein and its antibody to Fc-receptor bearing cells (Cochrane et al., 1976). Support for this concept comes from recent studies in which we have shown that normal lymphocytes develop cytotoxicity for hepatocytes after incubation in serum from patients with chronic active hepatitis (Gonzalez et al., 1978). Competition for these receptor sites by other immune complexes present in the serum, perhaps as a consequence of the liver damage, will therefore modify the degree of cytotoxicity.

Although in the patients already established on immunosuppressive therapy there was a close association between the ability to demonstrate cytotoxicity and an active liver biopsy (score of 7 or more), there was again no correlation between the degree of cytotoxicity and biopsy score in those showing positive cytotoxicity. As the cytotoxic reaction is thought to be antibody mediated (Cochrane et al., 1976), the striking association between the finding of cytotoxicity and the pressure of piecemeal necrosis may be due to periportal hepatocytes adsorbing the majority of antibody to liver-specific protein, and thus being the most susceptible to destruction by Fc-receptor bearing cells. This may be why periportal or piecemeal necrosis is such a hallmark of this disease in both HBsAg negative and positive cases.

In contrast with the association between positive cytotoxicity and certain histological features there were no significant differences in liver function tests, immunoglobulins, or frequency of autoantibodies between those with and without lymphocyte cytotoxicity. We have suggested that immunoglobulin levels may be largely genetically
Lymphocyte cytotoxicity in chronic active hepatitis
determined, while autoantibodies may be markers of
an inherited autoimmune diathesis (Galbraith et al.,
1976) and neither would therefore directly reflect
disease activity. As liver function tests reflect the
synthetic and metabolic capacity of the liver as a
whole and cytotoxicity reflects only one specific
immunological reaction, it would be surprising if
there were an association between any of these
parameters.

The factors determining the variability in the
response to treatment are largely unknown, but it
may be helpful in planning the management of
these patients to construct a working hypothesis.
Thus, it seems likely that the conventional immu
nosuppressive drugs in the doses used by most
authors are not truly immunosuppressive but are
principally exerting an anti-inflammatory action
(Denman et al., 1970). We have proposed that the
autoimmune reaction in chronic active hepatitis is
maintained by a constant helper T-cell stimulus of
autoreactive B cells and a partial defect in
suppressor T-cell activity (Eddleston and Williams,
1974). In HBsAg negative chronic active hepatitis
the latter defect would predominate, but Allison
et al. (1971) have pointed to the potential
importance of altered self antigens, released at the
site of tissue injury, acting to promote a helper
T-cell response. Corticosteroids with their action
to reduce lysozymal enzyme release would be potent
inhibitors of this process, and the reduction in the
formation of the altered self antigens could be
sufficient to allow a weak suppressor T-cell system
to regain control. In HBsAg positive chronic active
hepatitis, on the other hand, the self antigens
are altered by viral products and anti-inflammatory
drugs would not directly reduce formation, and the
stimulus for autoimmunity would persist. This may
explain the relatively poor response to treatment in
the HBsAg positive cases reported by Schalm
et al. (1976). We found all but one of our antigen
positive cases to be histologically active with
persistently positive cytotoxicity and half have so
far died. In this group, and in the HBsAg negative
cases with postulated severe suppressor T-cell
defects, more intense therapy with a truly immuno-
suppressive action might be needed and it is of
interest that Schalm was able to establish control
in some of his unresponsive cases by doubling the
dose of prednisone or azathioprine.

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