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Lymphocyte cytotoxicity to autologous hepatocytes in HBsAg-negative chronic active hepatitis

GIORGINA MIELI VERGANI, D. VERGANI, P. J. JENKINS, B. PORTMANN, A. P. MOWAT, A. L. W. F. EDDLESTON & R. WILLIAMS *The Liver Unit and Department of Immunology, King's College Hospital and Medical School, London*

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SUMMARY

In a micrototoxicity assay, lymphocytes from ten out of sixteen patients with HBsAg-negative chronic active hepatitis have been shown to be cytotoxic to autologous hepatocytes isolated from percutaneous liver biopsies. This cytotoxicity was demonstrable in all six untreated patients but in only four out of ten receiving immunosuppressive treatment, the presence of cytotoxicity showing a significant association with the activity of the disease assessed histologically. The addition of excess purified lipoprotein (LSP), derived from the hepatocyte plasma membrane, blocked the reaction in all cytotoxic cases, indicating that LSP was the major target antigen. Enriched fractions of T cells were cytotoxic in only one case, whereas non-T cell fractions were cytotoxic in the other ten cases investigated in this way. For optimum T cell cytotoxicity, effector and target cells must share histocompatibility determinants and the results of this study using an autologous system show conclusively that the lymphocyte cytotoxicity found in HBsAg-negative chronic active hepatitis is mediated by a non-T cell population.

INTRODUCTION

Peripheral blood lymphocytes from patients with chronic active hepatitis (CAH) have been shown to be cytotoxic for autologous liver cells (Wands & Isselbacher, 1975; Paronetto & Vernace, 1975; Geubel *et al.*, 1976), Chang cells (Wands *et al.*, 1975; Jacques *et al.*, 1976) and rabbit hepatocytes (Thomson *et al.*, 1974) in various microcytotoxicity assays. The effector cells in cytotoxicity to rabbit hepatocytes are non-T lymphocytes, bearing receptors for complement and immunoglobulin (Cochrane *et al.*, 1976a), although T cell involvement has not been excluded. Indeed, the test system in which rabbit liver cells are incubated with human lymphocytes is unsuitable for demonstrating T cell cytotoxicity, since it has been shown that for optimum T cell cytotoxicity the target cells and the effector cells must share histocompatibility determinants (Zinkernagel & Doherty, 1974). We therefore decided to investigate further the cytotoxicity of subpopulations of lymphocytes from patients with HBsAg-negative chronic active hepatitis using autologous hepatocytes isolated from percutaneous liver biopsies carried out for diagnostic or follow-up reasons.

PATIENTS AND METHODS

The sixteen patients with HBsAg-negative chronic active hepatitis (CAH) comprised twelve adults (mean age 53·9, range 33–63 years) and four children (mean age 4·8, range 2–9 years). Diagnosis at the time of presentation was according to internationally agreed criteria (Leevy, Popper & Sherlock, 1976). Six patients were untreated; patients 1 and 2 were on long-term maintenance treatment with penicillamine (1 g and 750 mg/day) and patients 3–10 were receiving prednisone (7·5–12·5 mg/day) and azathioprine (75 mg/day).

Correspondence: Dr R. Williams, The Liver Unit, King's College Hospital & Medical School, London, SE5.

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TABLE 1. Clinical data and cytotoxicity results in the sixteen patients with chronic active hepatitis

Case	Sex	Age (yr)	Serum AST (iu/l)	Serum Albumin (g/l)	Serum Globulin (g/l)	Serum Autoantibodies ANF†/SMA‡	Liver biopsy score	Total cytotoxicity (%)
On treatment								
1	F	58	14	44	33	+	1	31
2	F	50	8	43	24	—	1	41
3	F	53	20	39	29	—	2	36
4	F	56	17	43	25	—	1	18
5	M	48	26	45	31	+	3	36
6	F	62	22	47	35	—	1	27
7	F	33	9	38	23	+	1	5
8	M	56	21	40	29	+	1	22
9	M	53	23	48	31	—	2	27
10	F	63	32	43	28	+	3	19
Untreated								
11	F	53	488	33	67	+	4	47
12	F	62	219	38	53	+	5	55
13	F	5	650	33	37	+*	6	46
14	M	2	150	43	n.a.	n.a.	3	52
15	F	3	66	26	52	—	4	50
16	F	9	481	31	>50	—	6	37

* Liver-kidney microsomal antibodies.
† Anti-nuclear factors.
‡ Smooth muscle antibody.
n.a. = Not available.

Isolated hepatocytes were prepared from 2–3 mm of liver tissue obtained with a needle biopsy performed either at time of diagnosis or during follow-up assessment. 48–96 hr after the hepatocytes were established in a microculture plate, a blood sample was taken from the patients and normal subjects. Lymphocytes isolated from these samples were then cultured with the patient hepatocytes in a microcytotoxicity assay. The major portion of each biopsy specimen was prepared for light microscopy and the activity of histological lesions was assessed by a pathologist (B.P.) without prior knowledge of the biochemical or cytotoxicity results. The appearance of liver architecture was recorded (normal, multilobular collapse, bridging hepatic necrosis, fibrosis and cirrhosis) and the biopsy was scored for mononuclear cell portal tract infiltrate and piecemeal necrosis (absent = 0, mild = 1, moderate = 2, severe = 3). If the total score was 2 or greater the biopsy was considered to be showing active disease.

Preparation of Lymphocytes. Lymphocyte-rich plasma was obtained from 20–30 ml of peripheral venous blood by sedimentation with 2–3 ml of 6% dextran containing 200–300 u of preservative-free heparin. After dilution 1 : 1 with RPMI 1640 medium with glutamine (Flow Laboratories Ltd.) plasma was incubated in a cotton wool column at 37°C for 15 min to remove macrophages (Rocklin, Mayer & David, 1970). The leucocytes were then centrifuged over a Ficoll–Triosil density gradient to remove the remaining neutrophils and red blood cells (Böyum, 1968). After washing, the interface lymphocytes excluded trypan-blue and contained less than 1% neutrophils and less than 5% macrophages. Subpopulations of lymphocytes were obtained using the technique described previously (Cochrane *et al.*, 1976a). A T cell-enriched lymphocyte subpopulation was obtained by rosetting the cells bearing receptors for complement with sheep red blood cells previously coated with antibody and complement. The non-rosetted T cells were collected after centrifugation over a Ficoll–Triosil density gradient. In a similar way, a non-T cell-enriched subpopulation was obtained by rosetting the T lymphocytes with sheep red blood cells and collecting the non-T lymphocytes by Ficoll–Triosil centrifugation. The purity of the enriched population of lymphocytes was checked by rosetting with both E and EAC cells (Stjernsward *et al.*, 1972) and counting the percentage of T and non-T lymphocytes. T cell-enriched fractions were contaminated with between 1 and 9% of non-T cells (mean ± s.d. = 3·4 ± 2·3%), and non-T cell-enriched fractions with between 1 and 25% of T cells (mean ± s.d. = 9·6 ± 6·5%).

Microcytotoxicity assay. The methods used were similar to those described previously for rabbit hepatocytes (Thomson *et al.*, 1974). Two to 3 mm of human liver biopsy, obtained for diagnosis or follow-up purposes, were used for the preparation of isolated hepatocytes. The liver was incubated at 37°C for 4–5 hr in RPMI 1640 containing 10% foetal calf serum, 0·01%

collagenase, 1M HEPES 2·3%, penicillin 200 u/ml, streptomycin 100 μg/ml and Amphotericin B 2 μg/ml, adjusted to a pH of 7·35 in an atmosphere of 95% O_2 and 5% CO_2. After washing with RPMI 1640 medium, the isolated hepatocytes were placed into each well of a microculture plate (Falcon 3034) to achieve a final concentration of approximately 100 cells per well and incubated in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. After 48–96 hr, the supernatant was aspirated from each well and replaced in at least ten test chambers with 10 μl of lymphocytes suspended in RPMI 1640 medium with 10% foetal calf serum. The concentration of lymphocytes was adjusted to produce a lymphocyte to hepatocyte ratio of 400 : 1. 1 μg of liver-specific lipoprotein (LSP), prepared as described previously (McFarlane *et al.*, 1977), was added to ten test wells. Controls were at least ten wells with hepatocytes plus medium alone and ten wells with hepatocytes plus lymphocytes from normal individuals. After incubation at 37°C for a further 48 hr, the plates were inverted for 2 hr and then carefully washed with medium. The number of hepatocytes remaining in each well was counted at × 60 magnification using a graticule eyepiece. The difference between the mean number of cells in control and test wells expressed as a fraction of the former gave the percentage cytotoxicity. For obvious reasons it was impossible to obtain a normal range using an autologous system in entirely healthy subjects. As an alternative control system lymphocytes from normal volunteers were tested against hepatocytes from patients in whom a liver biopsy was carried out for diagnostic investigation but which had only shown minor histological abnormalities. The upper limit of normal in such a system was 29%, 2 standard deviations (18) above the mean (11).

RESULTS

Lymphocyte cytotoxicity to autologous hepatocytes was detected in all six untreated patients, but in only four of the ten receiving immunosuppressive therapy (Fig. 1). The total biopsy score in those showing positive cytotoxicity was significantly higher than in those with cytotoxic values within the normal range ($P < 0.05$, Rank sum test). The addition of LSP to the microtest wells reduced cytotoxicity values into the normal range in all cases showing positive results ($P < 0.01$, Rank sum test) (Fig. 2). Subpopulations of T and non-T lymphocytes were prepared in twelve cases. In eleven out of the twelve cases the results obtained using the subpopulations were very similar, with cytotoxicity restricted to the non-T cell preparation (Fig. 3). In one case, however, cytotoxicity was observed in both of the lymphocyte subpopulations.

Control lymphocytes obtained from normal subjects were cytotoxic to hepatocytes from some of the patients with CAH and overall there was some correlation with the cytotoxicity results in the autologous system. Thus, cytotoxicity was detected in seven out of ten tests in which normal lymphocytes were added to hepatocytes prepared from patients with CAH who gave increased cytotoxic values in the

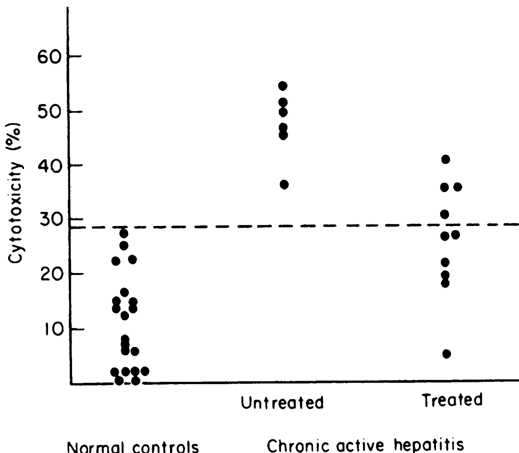

Fig. 1.

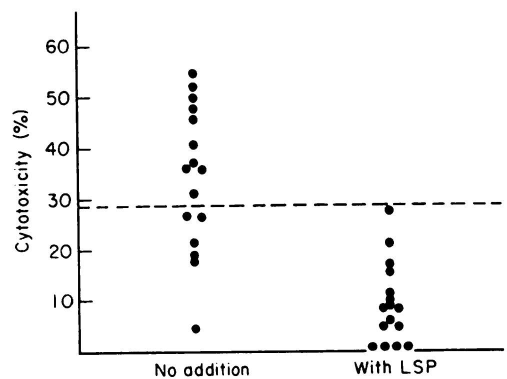

Fig. 2.

FIG. 1. Percentage cytotoxicity for normal lymphocytes against hepatocytes from patients with minor histological abnormalities compared to that found in an autologous system in which lymphocytes from patients with chronic active hepatitis were incubated with autologous hepatocytes. The patients were divided into two groups: (1) those untreated at the time of testing and (2) those receiving immunosuppressive therapy.

FIG. 2. The effect on lymphocyte cytotoxicity against autologous hepatocytes in chronic active hepatitis of the addition of 1 μg of LSP to the microculture wells.

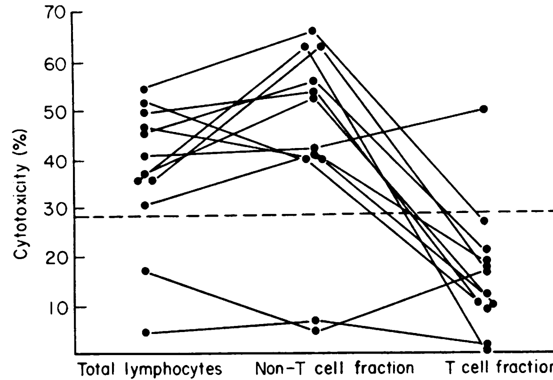

FIG. 3. The cytotoxic effect of two different fractions of peripheral blood lymphocytes, enriched in non-T or T cells respectively, on autologous hepatocytes in patients with chronic active hepatitis.

autologous system. In contrast, in the seven tests in which the hepatocytes were obtained from patients who did not show cytotoxicity in the autologous system, normal lymphocytes were cytotoxic on only two occasions. There was a significant difference in the cytotoxic values in these two groups ($P < 0{\cdot}01$, Rank sum test) (Fig. 4). This effect was not due to non-specific changes in the viability of the hepatocytes from these different populations since the difference between the groups in the mean number of hepatocytes remaining in control wells after culture was small and not statistically significant.

DISCUSSION

The significant correlation between cytotoxicity and histological evidence of disease activity in this autologous system is similar to previous findings from this Unit using a xenogeneic system in which rabbit hepatocytes were used as target cells (Cochrane *et al.*, 1978). In that system the cells responsible for the cytotoxicity were shown to be non-T lymphocytes bearing receptors for complement and immunoglobulins (Cochrane *et al.*, 1976a). However, the detection of cytotoxic T cells in such a heterologous system may be difficult or impossible since the full expression of T cell cytotoxicity has been shown to be dependent upon histocompatibility between target and effector cells (Zinkernagel & Doherty, 1974).

Although there have been three other reports in which autologous hepatocytes have been used as

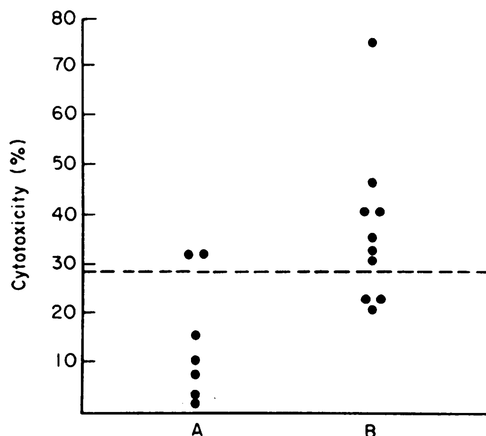

FIG. 4. The cytotoxic effect of normal lymphocytes against hepatocytes from patients with chronic active hepatitis. The results have been divided into two groups according to the source of the hepatocytes, being derived from patients having normal (A) or abnormal (B) cytotoxicity values in the autologous assay.

target cells (Wands & Isselbacher, 1975; Paronetto & Vernace, 1975; Geubel *et al.*, 1976), in none of these studies was there any attempt to analyse the nature of the lymphocyte–target cell interaction or to characterize the effector cells. Our results show that the cells responsible for *in vitro* cytotoxicity in an autologous system in HBsAg-negative chronic active hepatitis are almost always non-T lymphocytes, bearing receptors for complement. The mechanism involved is likely to be an antibody-dependent cell-mediated cytotoxicity in which K cells become attached to the hepatocyte membrane by an antibody reacting with an antigen on the liver cell surface. Such antibodies directed against hepatocyte membrane antigens have been identified in the serum of patients with CAH by employing a radioimmunoassay (Jensen *et al.*, 1978).

Other mechanisms, however, could be involved. For instance, it has been demonstrated in mice and man that unsensitized lymphocytes, called natural killers (NK) from normal individuals are cytotoxic for some target cell lines in culture (Herberman *et al.*, 1975; Kiessling *et al.*, 1975; Peter *et al.*, 1975; Trinchieri *et al.*, 1977). These NK cells show optimal cytotoxicity when effector and target cells share histocompatibility antigens (Anderson, 1978; Santoli *et al.*, 1976). In the animal studies NK cells have low adherent properties and morphological appearance of lymphocytes, but lack the surface markers for either B or T cells (Kiessling *et al.*, 1976). Since NK cells do not form E and EAC rosettes, they should equally contaminate the T and non-T-enriched populations used in the present study, and the finding of a clear difference in the cytotoxic activity in the two subpopulations therefore suggests that NK cells are not usually a major effector cell in this system. Such cells may have been involved in the one case in which cytotoxicity was detected in both lymphocyte subpopulations. Studies to assess the nature of NK cells in man have shown that at least some of these cells bear receptors for immunoglobulins (Peter *et al.*, 1975; Santoli *et al.*, 1978) and it has been suggested that they are the same type of effector cells involved in antibody-dependent cytotoxicity, activated through different and still unknown mechanisms (Trinchieri *et al.*, 1977). Such NK cells could be responsible for the low level of spontaneous cytotoxicity to normal hepatocytes detectable using lymphocytes from healthy subjects, but it is unlikely that they can explain the significant increase in cytotoxicity in CAH which shows antigen (Thomson *et al.*, 1974) and target cell specificity (Cochrane *et al.*, 1976b). In the present study, the addition of excess LSP blocked the cytotoxicity reaction in all cases, indicating that this lipoprotein, derived from the hepatocyte membrane (McFarlane *et al.*, 1977), is an important target antigen in the autologous system.

The finding that hepatocytes from patients with CAH who show positive cytotoxicity are more susceptible to damage by lymphocytes obtained from normal subjects was unexpected. One possible explanation is that hepatocytes from patients with ongoing hepatic damage and inflammatory infiltration are less viable and become easily detached from the culture wells. If there is reduced viability, this was not reflected by a lower number of cells remaining in the wells in which the hepatocytes were cultured alone. Another possibility is that hepatocytes from patients with CAH are coated *in vivo* with antibodies to which K cells present in the normal lymphocyte population bind *in vitro*. Previous studies have shown that isolated hepatocytes from rabbits with experimentally induced CAH have surface-bound immunoglobulins (Hopf & Meyer Zum Buschenfelde, 1974). Furthermore, membrane-fixed IgG have been demonstrated on isolated liver cells prepared from biopsy material of patients with CAH (Hopf *et al.*, 1975), and this we have also been able to confirm in some of our patients with CAH using a direct immunofluorescence technique.

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