

Lymphocyte cytotoxicity to autologous hepatocytes in alpha₁-antitrypsin deficiency

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SUMMARY To investigate the possible role of cell mediated cytotoxicity in the pathogenesis or perpetuation of liver damage associated with alpha₁-antitrypsin (AAT) deficiency, peripheral blood lymphocytes (PBL) from 13 children with PiZZ phenotype who had had liver disease in infancy were incubated with autologous hepatocytes in a microcytotoxicity assay. There was a clear trend for cytotoxicity to increase with age and thus significantly increased cytotoxicity was found in four cases older than 2 years, while intermediate values were recorded in three children between 6 months and 2 years and normal values in children younger than 6 months. Fractionation of PBL into T and non-T-enriched subsets showed that both were involved in the cytotoxic reaction. A purified liver membrane lipoprotein preparation (LSP) inhibited both T and non-T-cell cytotoxicity suggesting that LSP is a major target antigen in this system. Control experiments performed in infants with neonatal hepatitis syndrome, but with normal Pi phenotype, showed consistently increased cytotoxicity values. Cell-mediated immune reactions directed against autologous hepatocytes develop late in the course of the liver disease associated with AAT deficiency. While this reaction cannot be involved in the pathogenesis of the initial liver lesion, it may contribute to perpetuation of liver damage.

The association between alpha₁-antitrypsin (AAT) deficiency and childhood cirrhosis was first described in 1969¹ and since confirmed in numerous studies,²⁻⁴ although only about 10% of the children with PiZZ phenotype develop liver disease. This characteristically presents in the first four months of life as an acute hepatitis often with cholestatic features. A further 40% of PiZZ children have anicteric hepatic involvement with abnormal liver function tests.⁴ The clinical severity of hepatitis is variable and in one follow up study of 66 children presenting with liver disease in infancy and re-evaluated in the second decade of life, approximately one quarter had died of cirrhosis, about 50% had histologically proven cirrhosis or persistently abnormal liver function tests, while the remaining 25% appeared to have made a complete clinical and biochemical recovery.⁵ The reasons for this variable outcome are largely unknown, as well as the pathogenesis of liver damage associated with AAT deficiency. Storage of AAT within the liver cells is

unlikely to be the cause as periodic-acid Schiff-positive globules are found in patients without liver involvement.^{6,7}

In earlier experimental work we have shown that peripheral blood lymphocytes (PBL) from children with AAT deficiency and liver disease were cytotoxic *in vitro* for isolated rabbit hepatocytes⁸ and, on the basis of observations in adults with liver disease, it was postulated that cytotoxicity could be mediated by non-T lymphocytes bearing receptors for complement and IgG Fc.⁹ Detection of cytotoxic T cells with the xenogeneic system used in those studies is not always possible as the experiments of Zinkernagel and Doherty have emphasised the requirement for histocompatibility between T-effector cells and target cells for optimal cytotoxicity.¹⁰ We have therefore developed a technique using as target cells autologous hepatocytes isolated from a small portion of a liver biopsy obtained from patients at the time of diagnosis or follow up assessment: such a system has the theoretical advantages that antigen presentation on the target cell surface should be equivalent to the *in vivo* situation and that effector and target cells are fully

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histocompatible. The aim of the present study was to investigate, using the autologous system, the possible role of cell-mediated cytotoxicity in the pathogenesis or perpetuation of liver damage associated with AAT deficiency and to analyse both the antigenic specificity of the cytotoxic reaction and the type of lymphoid cell responsible.

Methods

PATIENTS

All the 13 children studied (eight boys and five girls, median age 14 months, range 1 month–8 years), had the PiZZ phenotype of AAT as determined by isoelectric focusing¹¹ and were investigated one month to eight years after the initial presentation with neonatal hepatitis. All had abnormal serum aminotransferase levels. Histological examination of the liver biopsy showed hepatitis with features of bile duct obstruction in four, mild inflammation with portal tract fibrosis in three, more severe portal tract fibrosis in two, and active cirrhosis in four. To test the possibility that the results obtained in infants with AAT deficiency were simply a reflection of age related changes in immunocompetence, five infants (two boys and three girls, median age 1 month, range 1 month–2 months), who presented with conjugated hyperbilirubinaemia, but had normal Pi phenotype (PiM), were also studied. Two of them had extrahepatic biliary atresia, two showed histological features of giant cell hepatitis of unknown aetiology, and one had severe acute hepatitis with bridging necrosis.

All patients were negative for hepatitis B surface antigen and antibody by radioimmunoassay (Travenol). Three patients in the AAT deficiency group were receiving D-penicillamine (20 mg/kg body weight) in an attempt to delay fibrous tissue deposition and one of them was also taking prednisolone (7.5 mg/daily) because a previous liver biopsy had shown features of chronic active hepatitis.

PREPARATION OF PBL AND PBL SUBSETS

Peripheral blood lymphocytes were prepared from venous blood by Ficoll-Isopaque gradient centrifugation¹² and monocyte depletion by plastic adherence¹³ and, after washing in HBSS, resuspended in HEPES – buffered RPMI 1640 medium (GIBCO) supplemented with 10% heat inactivated fetal calf serum (GIBCO), 2 mM glutamine and antibiotics (complete medium), pH 7.4 as previously described.¹⁴ Viability of the lymphocytes always exceeded 98%, and less than 3% were macrophages as assessed by peroxidase staining.¹⁵ Whenever possible subpopulations of

lymphocytes were prepared using the technique described previously.¹⁴ Briefly, a T cell-enriched subset was obtained by removing the lymphocytes forming EAC rosettes on Ficoll-Isopaque gradient. Similarly, a non-T cell-enriched subset was obtained by removing the lymphocytes forming rosettes with sheep erythrocytes on Ficoll-Isopaque gradient. We have previously shown, using this technique, that the T cell enriched fraction was contaminated with 1 to 9% (mean $3.4 \pm 2.3\%$) of non-T cells and the non-T cell-enriched fraction with 1 to 25% of T cells (mean $9.6 \pm 6.5\%$).¹⁴

AUTOLOGOUS MICROCYTOTOXICITY ASSAY

Two to 3 mm of the patient's own liver biopsy performed for diagnostic or follow up purposes, were used for the preparation of isolated hepatocytes as described in detail previously.¹³ Briefly, the liver was cut into small pieces and incubated in complete medium containing 0.01% collagenase in 5% CO₂ in oxygen at 37°C for four hours under gentle agitation. After being washed in collagenase free medium, the isolated liver cells retained membrane integrity when evaluated by phase contrast microscopy. Examination by transmission electron microscopy showed that less than 2% of the isolated liver cells were of sinusoidal origin. Ten microlitres of the hepatocyte suspension were subsequently dispensed into each well of a microcytotoxicity plate (Falcon 3034F) to achieve a final concentration of approximately 100 cells per well. After overnight incubation at 37°C in 5% CO₂ in oxygen, the supernatant was aspirated from each well and replaced with the lymphocyte suspension at an effector to target ratio of 300:1. Controls were at least 10 wells containing hepatocytes plus medium alone. After a further 48 hours incubation at 37°C in oxygen-CO₂, the plates were inverted for one hour, washed, and stained. The difference between the mean number of hepatocytes remaining adherent in control wells and that in test wells expressed as a fraction of the former gave the percentage cytotoxicity.

The normal range was calculated using lymphocytes and hepatocytes from six children and 11 adults in whom a liver biopsy was performed for diagnostic reasons, but failed to show significant histological abnormalities. Four of these patients had recovered from a previous episode of acute hepatitis, two were thought to have Gilbert's disease, two mild steatosis, one Dubin-Johnson syndrome, one diabetes, one had benign familial recurrent cholestasis, one Crigler-Najjar disease, one congenital hepatic fibrosis, one had a liver biopsy before starting methotrexate therapy for psoriasis, one had previous granulomatous disease,

one patient was referred with an increased alcohol intake but had normal liver histology, and finally one patient had a liver biopsy because his brother died of fulminant hepatic failure owing to Wilson's disease but his liver histology and liver copper content were found to be normal. As there was no significant difference in cytotoxicity between adults and children controls, they have been considered together. The upper limit of normal in such a system was 35% (n=16; mean 15+20 2SD) for undivided lymphocytes, 33% (n=14; mean 11+22 2SD) for T-enriched lymphocytes, and 26% (n=11; mean 9+17 2SD) for non-T-enriched lymphocytes.

BLOCKING EXPERIMENTS

When a sufficient number of target cells was available, blocking experiments have been performed in which 0.8 μ g of liver specific lipoprotein (LSP), purified as previously described,¹⁶ was added to the culture wells. In addition, hepatocytes were preincubated in the microwells with 13 μ g of an F(ab')₂ fragment anti-human IgG Fc, prepared as described elsewhere,¹⁷ at 37°C for two hours, before addition of the effector cells. This manoeuvre would prevent Fc receptor-bearing lymphocytes to mediate antibody-dependent cytotoxicity as shown previously.¹⁸

Results

Four (31%) of the 13 children with AAT deficiency showed significantly increased lymphocyte cytotoxicity to autologous hepatocytes (Fig. 1). There was no relationship between the degree of cytotoxicity *in vitro* and the severity of liver damage as assessed histologically (Fig. 2), but cytotoxicity values did appear to be related to increasing age. Thus four of the five children tested when older than 2 years showed significant cytotoxicity, the only one showing normal cytotoxicity being on corticosteroid treatment. Intermediate values, although not significantly raised, were obtained in children between 6 months and 2 years of age, while normal cytotoxicity was recorded in those younger than 6 months (Fig. 2). The results of sequential estimation in one child were in agreement with this: cytotoxicity values were 0% at the time of the first biopsy (1 month of age) and 29% at 12 months of age.

Subpopulations of lymphocytes were prepared in eight patients. Three of the four children who showed raised cytotoxicity values with undivided lymphocytes had also increased cytotoxicity with both lymphocyte subsets, and in one with the non-T-cell fraction only (Fig. 3). One patient exhibited a mild degree of cytotoxicity with the non-T-cell subpopulation only (Fig. 3).

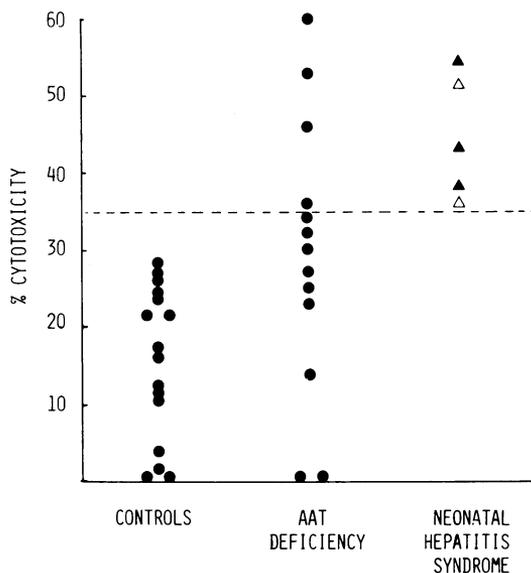


Fig. 1 Percentage cytotoxicity values found when autologous hepatocytes were incubated with undivided PBL from the three groups of patients studied. Dashed line represents the upper limit of the normal range for percentage cytotoxicity. \blacktriangle idiopathic neonatal hepatitis; \triangle extra-hepatic biliary atresia.

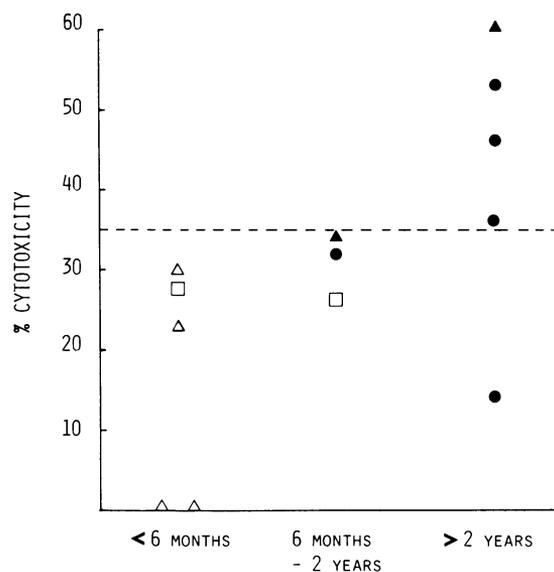


Fig. 2 Relationship between age of patients with AAT deficiency and percentage cytotoxicity values using undivided PBL in an autologous cytotoxicity assay. \triangle hepatitis; \blacktriangle mild changes; \square fibrosis; \bullet cirrhosis.

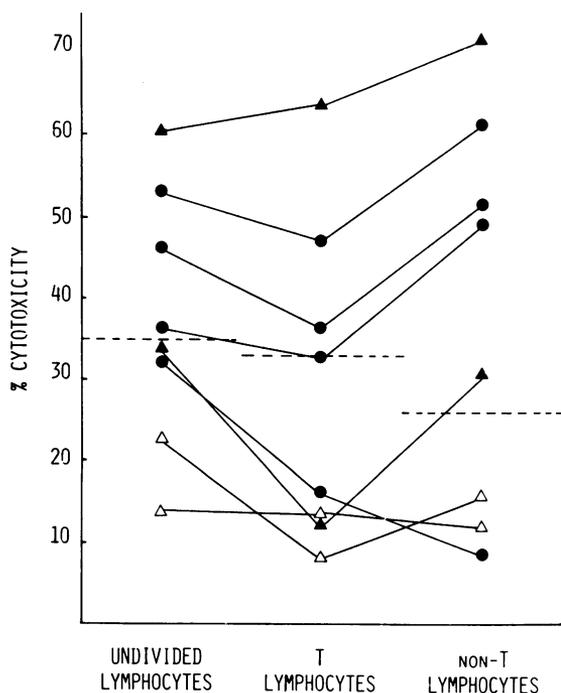


Fig. 3 Cytotoxic effect of undivided PBL and fractions enriched in T or non-T cells on autologous hepatocytes in patients with AAT deficiency. Dashed lines represent upper limit of normal range for each lymphocyte fraction. Continuous lines join results obtained in each patient. Δ hepatitis; \blacktriangle mild changes; \bullet cirrhosis.

Blocking experiments were performed in five patients of whom two showed significant cytotoxicity. The addition of LSP to the culture system inhibited both T and non-T cell cytotoxicity in the patients in whom cytotoxicity was initially significantly increased (Table), whereas preincubation of the target cells with $F(ab')_2$ anti-human IgG Fc was only effective in blocking the cytotoxic effect on non-T-cells (Table).

All five children with neonatal hepatitis syndrome and the normal PiM phenotype exhibited significant

Table The blocking effect on cytotoxicity of the addition of LSP (0.8 μ g/well) or preincubation of the hepatocytes with an $F(ab')_2$ fragment of anti-human IgG (13 μ g/well)

	LSP	$F(ab')_2$ anti-IgG Fc
T	2/2*	0/2
non-T	2/2	2/2

* Number blocked/total number.

cytotoxicity (Fig. 1). The very limited number of lymphocytes and the small size of the biopsy available from the majority of these infants did not allow the production of sufficient replicates to adequately investigate the cytotoxicity of lymphocyte subsets or to perform blocking experiments.

Discussion

The finding that PBL from a proportion of children with liver disease associated with AAT deficiency are cytotoxic to the patient's own liver cells *in vitro* confirm those previously obtained using a xenogeneic system where positive cytotoxicity results were obtained from all of seven children, who were older than 2 years of age.⁸ In the present study we have, in addition, been able to investigate affected subjects during infancy and we have shown that cytotoxicity is not significantly increased in infancy, in spite of severe liver damage, but appears with increasing age. It was clearly important to evaluate the possibility that the finding of normal cytotoxicity in infancy was not merely because of inefficiency of the effector lymphocytes at this age. Cord blood T cells sensitised *in vitro* with hapten-labelled autologous target cells or with allogeneic target cells show decreased cytotoxicity when compared with T cells from adults.¹⁹ Our finding of increased cytotoxicity in age-matched infants with idiopathic neonatal hepatitis or extrahepatic biliary atresia, however, strongly suggests that the normal cytotoxicity values recorded in infants with AAT deficiency are not the result of an age related impaired function of the effector cells, at least in this *in vitro* system.

It could be argued that detachment of isolated liver cells from plastic is mediated by production of proteases by monocytes during *in vitro* incubation and could therefore be non-specific. This seems unlikely, however, as monocyte contamination of the lymphocyte preparation was low and cytotoxicity was increased in only four of the 13 children with deficiency of protease inhibitor. This restriction of cytotoxicity to only a small subgroup within the AAT group also argues against the possibility that lack of protease inhibitor could decrease, in some non-specific way, the adherence of the hepatocytes to the plastic microcytotoxicity plates.

The lack of correlation between cytotoxicity and the severity of liver damage as assessed histologically, indicates that lymphocyte-mediated cytotoxic reactions do not play an important role in the pathogenesis of the initial liver injury associated with AAT deficiency. The cell-mediated immune reactions to autologous hepatocytes which develop

later in the course of the disease, however, may have a role in the perpetuation of the liver cell damage.

Fractionation of the effector lymphocytes into T and non-T cell subsets indicated that both subpopulations were responsible for the cytotoxic reaction. Although the blocking experiments using LSP showed that both lymphocyte subsets reacted with this normal component of the hepatocyte plasma membrane, the mechanism through which they mediated cytotoxicity was quite different. Only non-T lymphocytes were effectively blocked by preincubation of the target cells with an F(ab')₂ fragment of anti-human IgG Fc, indicating that the cytotoxicity was mediated by cells bearing receptors for the Fc portion of IgG and was probably of the antibody-dependent type (ADCC). The effector cells are likely to be K cells reacting with antibodies bound to the LSP complex, as already shown in other chronic liver diseases.^{14 18}

Previous experiments using an additional EA rosetting purification step have shown that the effector cells within the T-enriched subpopulation in this system were Fc γ -receptor negative¹⁸ suggesting that cytotoxicity is unlikely to be mediated by natural killer cells.

The inhibition of T-cell cytotoxicity by addition of LSP to the culture system indicates that in children with AAT deficiency and liver disease T cells are sensitised to normal hepatocyte membrane components. This phenomenon has never been shown in other types of chronic liver disease in man. In chronic hepatitis B virus infection, T cells cytotoxic for autologous hepatocytes can be detected but are directed at viral antigens¹³ and LSP has no effect on cytotoxicity.¹⁸ It is possible that in AAT deficiency the hepatocyte membrane becomes altered by the action of some still unknown factors and that T lymphocytes become sensitised to normal liver cell components exerting a direct cytotoxic effect and inducing B cells to produce antibody to an antigen in the LSP complex. Indeed it has been hypothesised that the increased transport of macromolecules, including proteases, from the intestinal lumen into the systemic circulation normally present in the newborn, when associated with deficiency of protease inhibitors as in AAT deficiency, leads to a greater concentration of proteases in the liver with consequent inflammation.²⁰ In the absence of an adequate catabolism, proteases might modify the normal hepatocyte membrane structure leading eventually to the exposure of normally hidden self or altered antigens to which lymphoid cells may become sensitised.

Several studies have shown that AAT play a modulating role in the immune system. Alpha₁-

antitrypsin suppresses antigen-dependent antibody production in mice both *in vivo* and *in vitro*²¹ and inhibits natural cell-mediated cytotoxicity in humans.²² In addition, it has been reported that concanavalin A-stimulated, but not resting, lymphocytes express AAT on their surface,²³ suggesting that control of proteolytic activity may be important in the regulation of lymphocyte blastogenesis and recently it has been shown that serum from AAT deficient patients induces enhanced lymphocyte responsiveness to phytohaemagglutinin²⁴ which can be suppressed by addition of purified AAT.²⁵

Lack of AAT could lead to abnormal immunoregulation and it is conceivable that this phenomenon associated with alteration of the liver cell membrane, predisposes PiZZ children to develop potentially damaging autoimmune reactions later in the course of the disease.

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