Inhibition of Lymphocyte Cytotoxicity by Serum from Patients with Alcoholic Liver Disease: Partial Characterization of Serum Inhibitors

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Studies were performed to explore the effect on normal lymphocyte function of serum derived from patients with alcohol-induced liver injury and healthy controls. We examined the effect of such serum on the generation of both spontaneous and Concanavalin A (Con A)-induced lymphocyte cytotoxicity for Chang target cells. Normal lymphocytes, when incubated in the presence of 5% serum from patients with alcoholic liver disease, showed a marked (20.75 ± 5.1% mean ± SEM) reduction in the capacity to generate spontaneously cytotoxic cells compared to 5% control serum (3.2 ± 1.9%) (p < 0.001). Similar results were found in studies of Con A-stimulated cytotoxicity (36 ± 7.2% vs. 5 ± 2.3%; p < 0.001). Fractionation of serum by gel chromatography demonstrated the presence of inhibitory activity of various molecular weights, although a major peak of inhibitory activity (approximately 270,000 daltons) was identified in severe alcoholic hepatitis. Thus, this study demonstrates the presence of serum inhibitors in alcoholic liver disease which influence normal lymphocyte function.

INTRODUCTION

Alcoholic liver disease and, in particular, acute alcoholic hepatitis have been associated with a number of immunologic abnormalities. In vivo studies of delayed hypersensitivity skin testing have shown depressed thymus-derived or T-lymphocyte function in patients with alcohol-induced liver injury [1,2]. In such patients, both a peripheral T-lymphopenia and marked accumulation of T-cells at the site of liver injury have been described [3,4] and in vitro studies of lymphocyte activity have demonstrated depressed lymphocyte responsiveness to antigens and mitogens [5]. Furthermore, lymphocyte reactivity to liver tissue antigens and alcoholic hyalin have been demonstrated in some individuals [5–7]. More important, mononuclear cells from patients with alcoholic hepatitis but not inactive Laennec's cirrhosis exhibit cytotoxicity to autologous and heterologous cell lines, suggesting prior in vivo lymphocyte activation [8–10]. It is uncertain, however, whether these immunologic abnormalities persist in alcoholic liver disease patients who are in remission.

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abnormalities are primary and of pathogenetic significance or represent secondary and non-specific changes due to the disease process or a direct alcohol effect on immunologic responsiveness.

The importance of external modulating factors on in vitro lymphocyte function has only recently been appreciated. In patients with alcoholic liver disease, serum factors have been shown to play an important role in certain aspects of cell function. For example, DeMeo and Andersen [11] studied neutrophil chemotaxis in patients with alcoholic liver disease and found that the cirrhotic patients had marked impairment of chemotaxis compared to normal controls when neutrophils were incubated with autologous but not homologous normal serum. Of particular interest were investigations in which normal leukocytes exhibited defective chemotaxis in the presence of alcoholic liver disease serum but not in the presence of normal serum. Thus, this study represents an example of an external modulating serum factor(s) on a particular cell function in patients with alcohol-induced liver injury.

In the present investigation, we examined the effect of serum obtained from patients in various stages of acute alcoholic liver injury on the generation of cytotoxic lymphocytes. Our results indicated that there are potent serum inhibitors which depress the generation of cytotoxic lymphocytes derived from normal individuals.

MATERIALS AND METHODS

Patients

Fifteen patients with alcohol-related liver disease were investigated. Twelve of the 15 underwent liver biopsy; 7 demonstrated morphologic features consistent with alcoholic hepatitis [12], 4 with Laennec's cirrhosis, and 1 with fatty liver. Ten milliliters of venous blood was collected from each patient and the separated serum stored at -70°C for subsequent studies. All patients were hospitalized and studied between 3 days and 2 weeks following admission. This protocol has been approved by the Human Studies Committee at the Massachusetts General Hospital.

Preparation of Lymphocytes

Mononuclear cells obtained from normal individuals were prepared from 40 to 50 ml of fresh heparinized venous blood by dilution with an equal volume of isotonic saline and centrifugation through a Ficoll/Hypaque sedimentation gradient 400 × g for 35 min; the mononuclear cell band at the saline-Ficoll/Hypaque interface was removed. Cells were washed three times (400 × g for 10 min) in RPMI 1640 medium (Microbiological Associates, Bethesda, MD) enriched with 10% heat-inactivated fetal calf serum, 20 mM glutamine, penicillin (1000 units/ml) and streptomycin (100 μg/ml) (complete medium). Cells were resuspended in complete medium and adjusted to a concentration of 1 × 10⁶ cells/ml. Under these conditions cell viability was > 95% by trypan blue exclusion.

Preparation of Target Cells

Chang cells (Microbiological Associates) were continuously cultivated in our laboratory and a single cell suspension was harvested by brief treatment with trypsin (5 min). Cells were washed three times in RPMI medium, resuspended in 5 ml of complete medium, and incubated with 300 μCi of ⁵¹Cr (New England Nuclear, Boston, MA) for 30 min at 37°C. Radiolabeled Chang cells were washed X3 with 10 ml of complete medium, counted, and rechecked for viability with trypan blue. The final concentration of target cells was adjusted to 1 × 10⁴ cells/ml.
**Cytotoxicity Studies**

In both the spontaneous and Con A-induced lymphocyte cytotoxicity studies, done in triplicate, 2 ml of complete medium containing $1 \times 10^6$ mononuclear cells/ml were added to $1 \times 10^4$ labeled Chang cells in 1 ml of complete medium to yield a lymphocyte to target cell ratio of 200:1. In spontaneous lymphocyte cytotoxicity studies $2 \times 10^6$ lymphocytes were incubated in complete RPMI medium for 18 hours with $1 \times 10^4$ Chang cells on a rocker platform (6 cycles/min at 37°C in a 95% air and 5% CO₂ atmosphere). At the end of this incubation period, cell suspensions were transferred from 35 × 25 mm plastic petri dishes to small test tubes and centrifuged at 450 × g for 15 min. The supernatant was carefully decanted into counting tubes and counted in a three-channel Packard gamma counter (Packard Instrument Co., Inc., LaGrange, IL). The mean counts per 5 min release from $1 \times 10^4$ Chang cells and standard error of the mean were calculated. Cytotoxicity was measured by $^{51}$Cr release into the medium after incubation with mononuclear cells. Percent lymphocyte cytotoxicity was calculated from the following formula:

$$\text{% cytotoxicity} = \frac{X - SR}{TRC - SR} \times 100\%$$

where  
$X =$ mean cpm measured in tubes containing $^{51}$Cr-labeled Chang cells and lymphocytes  
$SR =$ cpm in tube containing $^{51}$Cr-labeled Chang cells without lymphocytes (spontaneous release)  
$TRC =$ total releasable counts of $1 \times 10^4$ $^{51}$Cr-labeled Chang cells by freeze-thawing three times

Concanavalin A (Con A) was a thrice-crystallized preparation obtained from Miles-Yeda (Elkhart, IN). For Con A-stimulated cytotoxicity studies, mononuclear cells prepared in an identical fashion were incubated at a concentration of $1 \times 10^6$ cells/ml in complete medium (total volume 2 ml) with 10 μg/ml Con A for 72 hr in a 5% CO₂ incubator at 37°C. At the end of this incubation period, cell suspensions were added to $1 \times 10^4$ $^{51}$Cr-Chang cells and incubated for 6 hr as described above and the percent cytotoxicity determined.

**Inhibition of Lymphocyte Cytotoxicity by Serum**

Serum from patients with alcohol-related liver injury and from normal control individuals was heat-inactivated at 56°C for 30 min. Such serum at a concentration of 5% was added to duplicate cultures at the beginning of the 18 hr period in the spontaneous lymphocyte cytotoxicity experiments and the 72 hr incubation in the Con A-stimulated studies. Serum from normals was always present in parallel experiments as a control. To assure that the added serum was not cytotoxic toward normal mononuclear cells, we evaluated cell viability at 12, 24, 48, and 72 hrs by trypan blue exclusion. There was no significant difference in cell viability between cultures with or without serum addition. To assure that serum from normals and patients with alcoholic liver disease did not effect $^{51}$Cr release from Chang cells, parallel experiments were performed with 5% serum added to $1 \times 10^4$ $^{51}$Cr-labeled Chang cells during the 18 and 6 hr incubations. There was no increased $^{51}$Cr with such addition compared to spontaneous $^{51}$Cr release from Chang cells cultured under
identical conditions. Percent inhibition of both spontaneous and Con A-stimulated cytotoxicity was calculated according to the following formula.

\[
\% \text{ inhibition of cytotoxicity} = 1 - \frac{\% \text{ cytotoxicity with serum addition}}{\% \text{ cytotoxicity without serum addition}} \times 100
\]

**Partial Characterization of Serum Inhibitory Factors**

In order to characterize serum inhibitory activity and to determine the approximate molecular weight of such inhibitor(s), we performed cytotoxicity studies with serum obtained from normal and alcoholic liver disease patients and fractionated by column chromatography. A 2.5 × 30 cm column was packed at 12 cm H₂O pressure with Sephadex G-200 (Pharmacia) dissolved in phosphate buffered saline (PBS) (pH 7.2) containing 0.01% sodium azide and had a flow rate of 12 ml/hr. This column was calibrated with blue dextran, bovine hemoglobin, human ¹²⁵I-IgG and ³H-glucose. Two ml fractions were collected. A similar column was prepared with Sepharose 4B but was packed at 30 cm of H₂O pressure with a measured bed volume of 96 ml and had a flow rate of 24 ml/hr. This column was calibrated with blue dextran, horse spleen ferritin, and ¹²⁵I-IgG.

Consecutive fractions were pooled to form 10 ml aliquots, concentrated 5X by the use of collodion bags (Arthur Thomas), dialyzed against PBS to remove sodium azide, lyophilized, reconstituted in PBS to a volume of 2 ml and filtered through a 0.45 μ Millipore filter. Fractions collected and prepared in this way were added in 0.2 ml aliquots (in triplicate) to lymphocyte suspensions at the time of Con A stimulation and were present for the entire 72 hr incubation period. The cytotoxicity assay was subsequently performed as previously described.

**RESULTS**

**Characteristics of the Cytotoxicity Assay**

Preliminary ⁵¹Cr labeling experiments demonstrated that Chang cells yielded 1 × 10⁵ to 2 × 10⁵ cpm/10⁴ cells. When labeling was less than 1 × 10⁵ cpm/10⁴ cells, the experiment was discarded. Spontaneous release was usually less than 15% of the total releasable counts as determined by freeze-thawing the ⁵¹Cr Chang cells. The releasable counts averaged 80 ± 5.6% of the total cpm/10⁴ cells. Studies on lymphocytes obtained from 15 normal control individuals yielded values of 26 ± 5.2 (mean ± SEM)% for spontaneous lymphocyte cytotoxicity and 38 ± 7.6 for Con A-stimulated cytotoxicity.

**Inhibition of Spontaneous and Con A-Induced Lymphocyte Cytotoxicity by Serum from Patients with Alcoholic Liver Disease**

Serum obtained from patients with alcohol-related liver disease had a profound effect on the generation of cytotoxic lymphocytes obtained from normal control individuals as shown in Figs. 1 and 2. As depicted in Fig. 1, there was a substantial difference in the inhibition of spontaneous cytotoxic lymphocytes when cells were incubated in the presence of 5% normal serum compared to 5% alcoholic liver disease serum (3.2 ± 1.9 vs. 20.75 ± 5.1%; \( p < 0.001 \)). Similarly, in Con A-stimulated experiments in which maximum cytotoxicity is generated, alcoholic liver disease serum inhibited cytotoxicity by 36 ± 7.2% as compared to 5 ± 2.3% inhibition by normal serum \( p < 0.001 \).

We compared the results obtained on serum inhibition of cytotoxicity exhibited in
FIG. 1. Effect of serum obtained from alcoholic liver disease patients and normal controls on spontaneous cytotoxicity exhibited by normal lymphocytes against $^{51}$Cr-labeled Chang target cells.

the spontaneous assay to that observed in the Con A-stimulated experiments as shown in Table 1. (Clinical and laboratory features of these patients are presented for reference in Table 2.) There was no relationship between the magnitude of depressed lymphocyte reactivity in the two assays. In fact, we observed in some patients a dissociation between the inhibitory effect of serum in the spontaneous assay compared to the Con A-stimulated studies (e.g., Patients 1 and 5). However, the greatest degree of serum inhibitory activity was observed in patients with severe alcoholic hepatitis.

FIG. 2. Inhibition of concanavalin A-stimulated lymphocyte cytotoxicity against $^{51}$Cr-labeled Chang cells by serum obtained from patients with alcoholic liver disease.
Representative Comparisons Between Inhibition of Spontaneous and Con A-stimulated Lymphocyte Cytotoxicity

| Patient No. | % Inhibition spontaneous* lymphocyte cytotoxicity | % Inhibition Con A-induced** lymphocyte cytotoxicity |
|-------------|-----------------------------------------------|-----------------------------------------------|
| 1           | 6                                             | 56                                             |
| 2           | 27                                            | 42                                             |
| 3           | 17                                            | 71                                             |
| 4           | 6                                             | 22                                             |
| 5           | 30                                            | 3                                              |
| 6           | 16                                            | 0                                              |
| 7           | 14                                            | 35                                             |
| 8           | 5                                             | 32                                             |
| 9           | 62                                            | 69                                             |
| 10          | 36                                            | 25                                             |
| 11          | 33                                            | 45                                             |
| 12          | 0                                             | 30                                             |
| 13          | 7                                             | 33                                             |
| 14          | 25                                            | -                                              |
| 15          | 21                                            | -                                              |

* Percent spontaneous lymphocyte cytotoxicity as measured on 15 controls was 26 ± 5.2%.
** Percent Con A-induced lymphocyte cytotoxicity as measured on 32 control subjects was 38 ± 7.6% (see Materials and Methods).

Partial Characterization of Serum Inhibitors

Figure 3 depicts a representative elution profile of serum inhibitory activity on Sephadex G-200 columns in two patients with severe alcoholic hepatitis (III and IV) compared to a patient with active Laennec's cirrhosis (II) and a normal control (I). All three patients had previously determined inhibitory activity when tested at a 5% serum concentration in the Con A-stimulated cytotoxicity assay. In the alcoholic

TABLE 2
Clinical and Laboratory Data on 8 Patients with Alcoholic Liver Disease

| Patient No. | Age/Sex | SGOT (IU/ml) | A/G (gm%) | PT*P/C (sec) | Total Bili (mg%) | Alk Phos (BU) | Liver Bx         |
|-------------|---------|--------------|-----------|--------------|-----------------|---------------|-----------------|
| 1           | 54M     | 87           | 2.1/5.2   | 16.0/11.6    | 14.2            | 8.6           | Alcoholic hepatitis |
| 2           | 43F     | 49           | 3.0/6.4   | 14.1/11.7    | 8.2             | 9.4           | Alcoholic hepatitis |
| 3           | 54F     | 106          | 2.9/3.2   | 12.1/11.6    | 2.4             | 11.6          | Alcoholic hepatitis |
| 4           | 18M     | 81           | 3.0/4.9   | 15.0/11.1    | 17.0            | 6.4           | Alcoholic hepatitis |
| 5           | 47F     | 31           | 3.1/3.6   | 13.0/11.9    | 0.8             | 6.8           | Laennec's cirrhosis |
| 6           | 28F     | 42           | 4.2/3.9   | 10.3/10.7    | 0.3             | 9.8           | Fatty infiltration |
| 7           | 38M     | 306          | 4.8/3.6   | 10.1/11.1    | 2.2             | 9.7           | -               |
| 8           | 60M     | 53           | 2.2/3.9   | 13.8/11.2    | 1.9             | 7.8           | "Active" Laennec's cirrhosis |
| 9           | 42M     | 86           | 2.1/5.4   | 14.5/12.0    | 12.0            | 8.6           | Alcoholic hepatitis |
| 10          | 37M     | 42           | 3.1/3.9   | 12.6/11.7    | 1.1             | 5.4           | -               |
| 11          | 55M     | 126          | 2.8/4.1   | 13.6/11.4    | 5.9             | 6.1           | Alcoholic hepatitis |
| 12          | 47F     | 56           | 3.7/3.9   | 12.0/11.9    | 1.4             | 3.9           | Laennec's cirrhosis |
| 13          | 62M     | 41           | 3.5/3.2   | 12.1/11.7    | 0.9             | 4.9           | Laennec's cirrhosis |
| 14          | 51F     | 129          | 2.8/4.6   | 15.1/11.9    | 9.6             | 8.2           | Alcoholic hepatitis |
| 15          | 29F     | 92           | 3.0/4.5   | 15.6/12.0    | 4.0             | 10.0          | -               |

*A/G = albumin/globulin; nl values for albumin > 3.5 gm%, globulin < 3.0 gm%
**PT = prothrombin time; P/C = patient/control
Normal values for total bilirubin < 0.8% mg% and alkaline phosphatase < 4.2 Bodansky Units (BU)
lymphocytosis inhibition in alcoholic liver disease

FIG. 3. Elution profile of serum inhibitory activity obtained after fractionation over a Sephadex G-200 column. I represents a control, II active Laennec's cirrhosis, III and IV severe alcoholic hepatitis (see Material and Methods.)

hepatitis patients there was a peak of serum inhibitory activity corresponding to a molecular weight of approximately 270,000 daltons when further analyzed by Sepharose 4B chromatography (data not shown). However, lower levels of inhibitory activity were observed in lower molecular weight fractions on Sephadex G200 as exemplified by patient II with active Laennec's cirrhosis.

DISCUSSION

In patients with alcohol-related liver injury we demonstrated the existence of serum factor(s) which inhibit the generation of cytotoxic activity in lymphocytes obtained from normal controls. What are the biological properties and significance of these inhibitory substances? In the first place, the biological potency varies from patient to patient as shown in some studies (Fig. 3, patient III) in which peak serum inhibitory activity almost completely abolished the Con A-generated response, while in others (Figs. 1 and 2) less inhibitory activity was found. These serum substances do not appear to be cytotoxic toward normal lymphocytes, as shown by trypan blue exclusion studies, or to the target cells, as illustrated by the lack of enhanced $^{51}$Cr release in the absence of effector cells. The mechanism(s) of this effect on normal lymphocyte reactivity, however, is unknown.

Although we observed the greatest degree of serum inhibition of lymphocyte
cytotoxicity in patients with severe alcoholic hepatitis, study of a larger group of patients in various stages of alcohol-induced injury would be needed before an association between such inhibitors and disease severity could be drawn. Our studies indicate that serum from such patients contains inhibitory activity over a broad molecular weight range. A major peak of inhibitory activity, however, was identified in the range of 270,000 daltons in the 5 patients studied thus far with severe alcoholic hepatitis.

Recent studies have demonstrated that Con A-stimulated lymphocyte cytotoxicity toward Chang cells is probably mediated by T-lymphocytes whereas spontaneous cytotoxicity directed against these same target cells is a property of a non-adherent, surface immunoglobulin-negative, Fc receptor-positive cell population [13-14]. Based on the dissociation noted in many patients between the degree of inhibition in the spontaneous and the Con A-induced cytotoxicity experiments, we are tempted to speculate that these serum inhibitors may influence lymphocyte subpopulations to variable degrees; however, lymphocyte separation experiments are necessary to explore this possibility.

In this study we chose to focus on the effects of serum inhibitory factors on normal lymphocyte reactivity, for patients with liver disease may have intrinsic as well as extrinsic defects in lymphocyte function. Indeed, three types of serum inhibitors (extrinsic factors) which influence various aspects of lymphocyte reactivity have been described. Several studies have shown that in patients with a variety of liver diseases (alcoholic hepatitis, viral hepatitis, primary biliary cirrhosis, halothane hepatitis, and extrahepatic biliary obstruction) there are serum inhibitors which depress phytohemagglutinin (PHA) stimulated blast transformation in lymphocytes obtained both from patients and normal individuals [15-20]. The properties of these inhibitors are unknown.

More recently, Chisari et al. have described a "regulatory" low density lipoprotein which inhibits T-lymphocyte binding to sheep red blood cells (SRBC rosette formation) in patients with acute viral hepatitis. This substance has been named rosette inhibitory factor or RIF [21,22]. Rosette inhibitory factor is biologically active at extremely low concentrations and is found in approximately 50 percent of patients with type A or B hepatitis during the active course of illness. Apparently RIF has unusual specificity for viral hepatitis and has not been detected in other viral diseases or in a variety of acute and chronic liver diseases of other etiologies. More important, the persistence of this factor in the blood following acute hepatitis has been associated with unresolved hepatitis and chronic active hepatitis. No correlation has been found, however, between RIF and the inhibitory factor(s), mentioned above, which inhibit the DNA synthetic response to PHA [22].

A third serum factor has been described in patients with acute and chronic active hepatitis which inhibits spontaneous and Con A-induced lymphocyte cytotoxicity [23]. These factor(s), like RIF, disappear with clinical recovery [24]; however, the biological properties differ from those of RIF in that the major peak of serum inhibitory activity determined by gel chromatography occurs in the 160,000–180,000 dalton range in contrast to the large molecular weight of RIF [22]. The above data suggest that there are multiple serum inhibitors of lymphocyte function in patients with acute and chronic active hepatitis, some of which may be more specific for the disease process than others.

We have shown, in addition, that in patients with alcoholic liver disease serum inhibitors occur which influence the generation of cytotoxic lymphocytes. These inhibitors were partially characterized and found to differ in molecular weight from
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those previously described in acute and chronic active hepatitis and primary biliary cirrhosis [25]. Similar to the inhibitors observed by DeMeo et al. [11], the serum factor(s) described here exert an inhibitory effect on normal cells. Finally, additional studies are required to define further the physical properties of these inhibitors, examine inhibitory effects of serum on lymphocyte subpopulations and other forms of lymphocyte reactivity, determine whether the activity of these inhibitors varies during the natural course of alcohol-induced liver injury, and explore the biological significance of these potential regulatory substances.

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