

Deficient Serum Bactericidal Activity Against *Escherichia Coli* in Patients with Cirrhosis of the Liver

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ABSTRACT The serum bactericidal activity (SBA) of cirrhotic patients was compared with that of normal individuals using the release of ^{51}Cr from radiolabeled *Escherichia coli* as the assay method. 80% (22/27) of patients were found to have deficient SBA against at least one of three smooth, serum-sensitive test strains of *E. coli*. Cirrhotic patients were found to have normal levels of serum lysozyme. Although some patients were mildly hypocomplementemic, this abnormality did not correlate with the presence of a bactericidal defect. Bactericidal antibody in normal and cirrhotics' sera was limited to the immunoglobulin (Ig)M class. Purified IgM from patients with deficient SBA against *E. coli* 0111 had lower concentrations of bactericidal antibody for that *E. coli* than did IgM from normal sera; the calculated bactericidal activity of total serum IgM was also lower. The bactericidal defect in cirrhotic serum could be completely corrected by either human antiserum to the homologous strain of *E. coli* or by purified, normal human IgM. However, because higher concentrations of IgM were required to restore normal SBA to a cirrhotic's serum than to agammaglobulinemic serum, there may be an inhibitor of bactericidal antibody in addition to a deficiency of bactericidal IgM antibody to *E. coli* in the serum of patients with cirrhosis. The bactericidal activity of the alternative complement pathway was also assessed. Sera from cirrhotic patients had no deficit in SBA attributable to the alternative complement pathway. In fact, in some, the activity of the alternative complement pathway was supernormal, compensating in part for the deficit in IgM-mediated SBA.

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INTRODUCTION

Patients with Laennec's cirrhosis are unusually susceptible to infections with Gram-negative bacilli: the syndromes of spontaneous peritonitis and spontaneous colon bacillus bacteremia are commonly associated with this disease (1-4). The high incidence of infection with both commensal and uncommonly isolated Gram-negative bacteria (5, 6) suggests that patients with cirrhosis have abnormalities in their host defenses against Gram-negative bacteria. Although the presence of ascites itself may contribute to the high incidence of peritonitis and bacteremia, the high frequency of infections at other sites in patients with Laennec's cirrhosis suggests a more general abnormality in host defenses. Defective chemotaxis because of a circulating inhibitor is, to date, the only such abnormality of host defenses that has been documented (7). In the presence of cirrhosis, clearance of radioactive particles by the liver is abnormal (8), but it has not been shown that the clearance of bacteria is similarly depressed.

Normal serum is bactericidal for a wide variety of Gram-negative bacteria (9); normal bactericidal activity may be an important host defense against tissue invasion and septicemia (10). However, serum bactericidal activity has not been systematically studied in patients with cirrhosis. In this paper we report that the majority of patients with severe cirrhosis have diminished serum bactericidal activity against *Escherichia coli*. This abnormality is associated with decreased immunoglobulin (Ig)M bactericidal activity and not with deficiencies of complement of lysozyme.

METHODS

Patients. All patients studied were hospitalized at the Veterans Administration Hospital, San Diego because of ad-

vanced liver disease. All patients had ascites and/or pedal edema. None of the patients had received blood transfusions in the month before the study, and none was infected or receiving systemic antibiotics. The diagnosis of Laennec's cirrhosis was confirmed by biopsy or autopsy in 50% of the patients. One patient had biliary cirrhosis. The majority of the patients had evidence of diminished hepatic protein synthesis, such as hypoalbuminemia and prolonged prothrombin time. Hyperglobulinemia, which is characteristic of this disease, was found in one-half of the patients. Many also had hyperbilirubinemia.

Control serum was obtained from healthy volunteers who were in their third or fourth decade of life. Sera from 10 individuals were combined to make a serum pool. Serum from patients and the control serum pool were frozen at -70° in 1-ml aliquots.

Bacteria. *E. coli* 0111:B4 is a smooth, serum-sensitive, laboratory strain. *E. coli* 06 was isolated from the urine of a patient with pyelonephritis, and *E. coli* 04 was isolated from the blood of a patient with Laennec's cirrhosis. Both of the clinical isolates are smooth, serum-sensitive, motile organisms. Their K and H antigens have not been characterized. All bacteria were stored in trypticase soy broth at -70° .

Bactericidal assay. Bactericidal activity was determined by measuring the release of ^{51}Cr from bacteria as previously described (11). For each experiment, 1 ml of the frozen stock was added to 10 ml of trypticase soy broth and grown overnight at 37°C . 0.1 ml of the overnight growth was subcultured into 50 ml of trypticase soy broth and incubated in a shaking water bath at 37°C for 150 min before labeling with ^{51}Cr . The exponentially growing bacteria were then labeled with $150\ \mu\text{Ci}$ ^{51}Cr (as sodium chromate, Amersham Corp., Arlington Heights, Ill.) for 45 min at 37°C , washed free of unbound radioactivity with cold, 0.1 M phosphate-buffered saline (PBS),¹ pH 7.4, and then resuspended in PBS at a concentration of $2\text{--}5 \times 10^8$ colony-forming units per milliliter. The serum to be tested was mixed with the bactericidal suspension in a ratio of 9:1 (vol/vol) and incubated at 37°C . Aliquots were removed at intervals and filtered through 0.45-micron filters (HAWP, Millipore Corp., Bedford, Mass.). As a control for non-specific release of ^{51}Cr , labeled bacteria were suspended in PBS and processed as above. Radioactivity in the filtrates and in the unfiltered sera was measured in an automatic gamma counter (Nuclear-Chicago, Des Plaines, Ill.). The amount of ^{51}Cr specifically liberated by serum was expressed as a percentage of the total radioactivity in the serum after subtracting radioactivity released spontaneously by bacteria incubated in PBS. % ^{51}Cr release = (cpm serum filtrate - cpm PBS filtrate/cpm unfiltered serum - cpm PBS filtrate) \times 100.

Chromate release was compared with a standard bactericidal assay in several experiments. The radiolabeled bacteria were removed from serum at zero time and at intervals thereafter. Each aliquot was serially diluted in sterile saline, and the appropriate dilutions were spread, in duplicate, over the surface of the trypticase soy agar culture plates which were then incubated overnight. Colonies were counted, and the number of surviving colony-forming units (CFU) was determined.

Antibody. Antiserum to *E. coli* 0111 with a hemagglutinating (HA) titer of 1:4,000, prepared by hyperimmunizing human volunteers with boiled bacteria, was kindly provided by Dr. Elizabeth Zeigler, University of California, San Diego

(12). Antiserum to *E. coli* 04 (HA titer, 1:2,000) was prepared by injecting rabbits intravenously with boiled bacteria every other day for 2 wk. Animals were bled to obtain serum 1 wk after the last injection.

Complement. Total hemolytic complement was measured by the method of Kent and Fife (13). Concentrations of C3 and C4 were measured by radial immunodiffusion (14) with reagents purchased from Hyland Diagnostics Div., Travenol Laboratories, Costa Mesa, Calif. C3PA (factor B) concentrations were measured similarly with reagents purchased from Calbiochem-Behring Corp., San Diego, Calif.

Serum from a patient (J.L.) with acquired common variable hypogammaglobulinemia was used as the complement source for ^{51}Cr -release studies. This serum had the following characteristics: no measurable isohemagglutinins; IgG and IgM concentrations of <50 mg/dl and <5 mg/dl, respectively; normal total hemolytic complement activity; and normal C3, C4, and factor B concentrations. Neither diluted guinea pig serum nor diluted rabbit serum were adequate complement sources for the ^{51}Cr release assay.

Immunoglobulin separation. Lipoproteins were precipitated from serum with dextran sulfate (500,000 mol wt) (15). The supernate was dialyzed against a 0.1-M Tris buffer (pH 7.5), and 8 ml was added to a $2.5 \times 100\text{-cm}$ column of Bio-Gel A-5M (Bio-Rad Laboratories, Richmond, Calif.). The protein was eluted with Tris buffer in 0.15 M NaCl at 30 ml/h. The effluent was monitored continuously at a wavelength of 280 nm. Individual protein peaks were pooled and concentrated with an ultrafiltration cell (Amicon Corp., Lexington, Mass.). The IgM in the first peak was further purified and separated from the dextran sulfate by use of concanavalin A Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (16). IgG from the second peak was separated from contaminating IgA by use of DEAE-Sephadex (Pharmacia Fine Chemicals) (17). The concentrations of IgA, IgG, and IgM in each of the purified fractions were determined by radial immunodiffusion. The IgM fractions were free of measurable IgG, and vice versa. IgM fractions did contain IgA in approximately equal concentrations.

IgM bactericidal activity. The separated IgM fractions were serially diluted in Dulbecco's buffered saline (18) containing 1% bovine serum albumin, and 0.1-ml aliquots added to an equal volume (0.1 ml) of complement in $12 \times 75\text{-mm}$ glass tubes. ^{51}Cr -labeled *E. coli* 0111 (0.5×10^8 organisms in 0.02 ml of PBS) were added and the tubes incubated stationary at 36°C for 20 min. The percentage of ^{51}Cr release was determined as usual and compared with a maximum ^{51}Cr release (^{51}Cr release by pooled, normal human serum). A unit of IgM bactericidal activity is defined as the amount which induces 50% of maximum ^{51}Cr release. The reciprocal of the concentration of the IgM determined to contain one bactericidal unit per milliliter is a measure of the concentration of specific bactericidal antibody in that IgM fraction. That value (units per microgram IgM), multiplied by the concentration of IgM present in the starting serum, gives the total serum IgM bactericidal activity.

Hemagglutination titer. Human O erythrocytes were sensitized with alkali-treated lipopolysaccharide which was extracted from *E. coli* 0111 by the method of Westphal et al. (19). $25\ \mu\text{l}$ of a heat-inactivated serum was serially diluted in microdilution trays ($25\ \mu\text{l}$ /well), and $25\ \mu\text{l}$ of a 1% suspension of sensitized erythrocytes was added to each well. The mixture was incubated at 37°C for 2 h and then held overnight at 4°C . Hemagglutination was then read macroscopically. All HA activity was eliminated when serum was treated with 2-mercaptoethanol, indicating that the HA antibody was IgM (20).

¹Abbreviations used in this paper: CFU, colony-forming units; HA, hemagglutinating; PBS, phosphate-buffered saline; SBA, serum bactericidal activity.

RESULTS

We compared the kinetics of ^{51}Cr release from *E. coli* 0111 with the kinetics of bacterial killing (as measured by change in CFU) in both normal and cirrhotic sera. As shown in Fig. 1, release of ^{51}Cr paralleled bacterial killing in both sera. In the normal serum, maximum ^{51}Cr release from *E. coli* 0111 was achieved after 7 min, by which time >95% of the inoculum had been killed. In the cirrhotics' serum, the rates of ^{51}Cr release and bacterial killing were both decreased compared with normal serum. Nevertheless, ^{51}Cr release paralleled bacterial killing. For each of the three test strains of *E. coli*, we compared bacterial killing with ^{51}Cr release by normal (pooled) serum and serum from a patient with cirrhosis. In every instance, normal ^{51}Cr release was indicative of normal bactericidal activity and decreased ^{51}Cr release reflected diminished bactericidal activity. We therefore used ^{51}Cr release as our assay method to compare the bactericidal activity of different sera.

We measured the capacity of normal and cirrhotics' sera to release ^{51}Cr from three serotypes of *E. coli*. Normal ^{51}Cr release for each microorganism was defined as ± 2 SD from the mean of ^{51}Cr release by neat, normal sera. As illustrated in Fig. 2, the percentage of ^{51}Cr released from *E. coli* 0111 was below normal in 44% (12/27) of the cirrhotics' sera; from *E. coli* 06, in 36% (9/25); and from *E. coli* 04, in 61% (8/13). All sera were tested against *E. coli* 0111 but, because we had limited quantities of sera from some patients, not all were tested against the other two serotypes of *E. coli*.

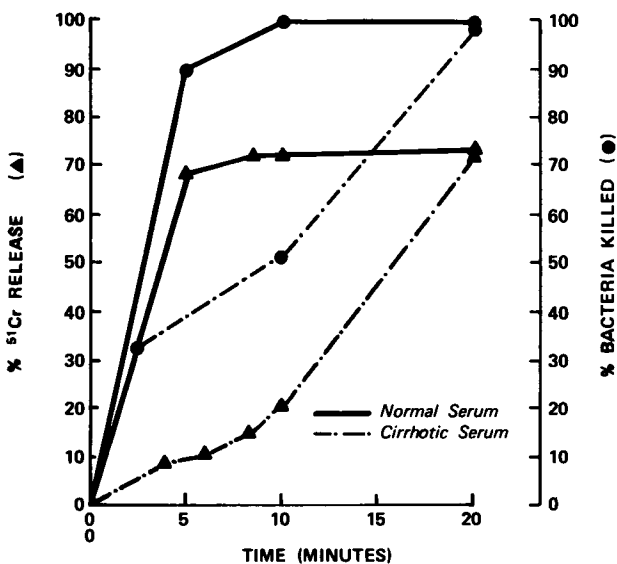


FIGURE 1 ^{51}Cr release and surviving CFU were determined at timed intervals after ^{51}Cr -labeled *E. coli* 0111 were added to a normal serum and to serum from a cirrhotic patient. 0.1 ml of saline containing 5×10^7 CFU was added to 0.9 ml of neat serum.

The kinetics of ^{51}Cr release by sera obtained from normal volunteers and from patients with cirrhosis were also compared. Four representative sera from cirrhotic patients are compared with normal serum (pooled) in Fig. 3. There is very little variation in the rate of ^{51}Cr release by normal serum, and the activity of the pooled serum was representative of ^{51}Cr release by individual normal sera (11). While ^{51}Cr release from *E. coli* 0111 was accomplished rapidly by the normal serum, reaching a plateau within 10 min, the rate of ^{51}Cr release by the cirrhotics' sera was so slow that after 20 min the percentage of ^{51}Cr released was still significantly less than that released by normal serum. In fact, ^{51}Cr release by two of the four sera did not achieve control values even after 60 min. In sera with less pronounced defects, ^{51}Cr release reached control values after 15–20 min.

To determine whether this decreased rate of ^{51}Cr release was a result of a low molecular weight inhibitor, serum was dialyzed overnight in Dulbecco's buffered saline at 4°C. After dialysis, there was a slight decrease in ^{51}Cr release by both the normal and the patients' serum, probably because of some loss of complement activity (Fig. 4). Significantly, there was no increase in the bactericidal activity of a cirrhotics' serum after dialysis, suggestive that the abnormality was not a result of a dialyzable inhibitor.

Sera from cirrhotic patients and normal serum were mixed together in varying proportions to test for the presence of an inhibitor in the cirrhotics' sera (Table I). As much as 0.5 ml of cirrhotics' sera did not inhibit ^{51}Cr release by 0.4 ml normal serum. Furthermore, preincubation of bacteria with cirrhotics' sera did not prevent the subsequent release of ^{51}Cr when the preincubated bacteria were washed and then resuspended in normal serum (Table II).

After failing to demonstrate an inhibitor, we examined the functional activity of each of the known components of the serum bactericidal system. Serum lysozyme was measured spectrophotometrically with *Micrococcus lysodeikticus* as the substrate (21) and crystalline egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) as the standard. Serum lysozyme values ranged from 6 to 31 U/ml in patients with Laennec's cirrhosis (Table III). The normal range was 6–12 U/ml. No cirrhotic patient had a low level of serum lysozyme and one-half of those tested had concentrations which were higher than normal.

Total hemolytic complement activity was measured in sera from 16 cirrhotic patients. Immunoreactive C3, C4, and factor B (C3PA) concentrations were also measured in most of these sera. As shown in Table III, only 3 of 16 had abnormally low CH_{50} . C3, C4, and factor B concentrations also were in the normal range in most of the cirrhotics' sera, and the few abnormal values were not <75% of normal. Furthermore, there was no correla-

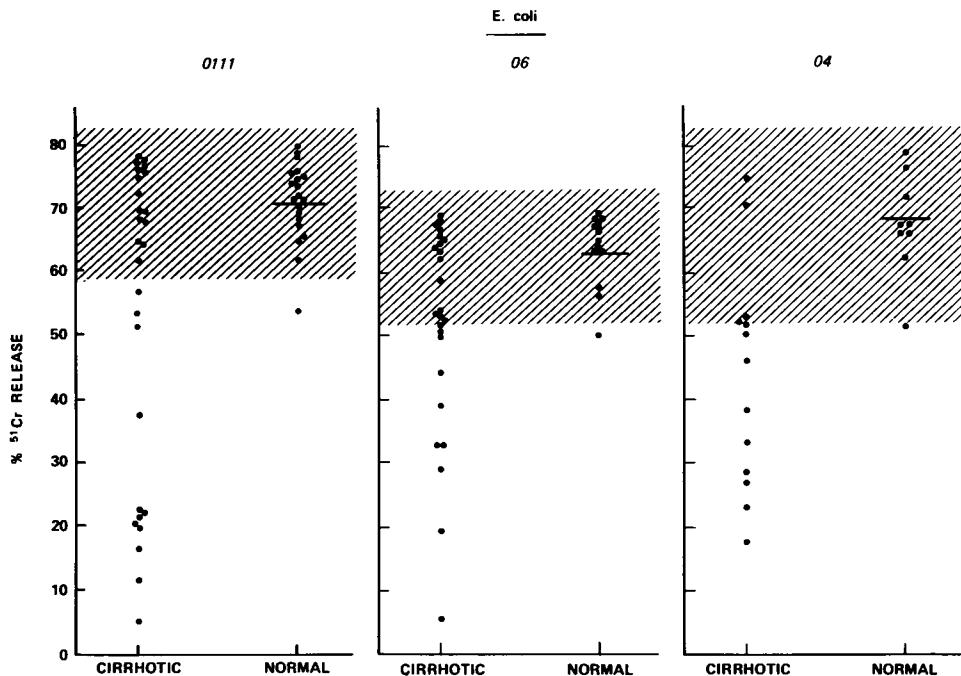


FIGURE 2 0.05 ml of saline containing 1×10^7 CFU of labeled *E. coli* was added to 0.45 ml of neat serum, and the suspensions were incubated at 37°C. Percentage of ⁵¹Cr released from *E. coli* 0111 was determined after 10 min incubation and from *E. coli* 06 and 04 after 15 min incubation. The mean percentage of ⁵¹Cr release was calculated for the normal sera, and the hatched areas show ± 2 SD from the mean for each organism.

tion between deficiencies in bactericidal activity and a low CH_{50} or low concentrations of C4, C3, or C3PA (factor B).

The activity of the alternative complement pathway

can be measured by chelating Ca^{++} with Mg-EGTA, which inactivates C1, the first component in the classical complement pathway (22). We used this technique to compare the bactericidal function of the alternative complement pathway in normal and cirrhotics' sera.

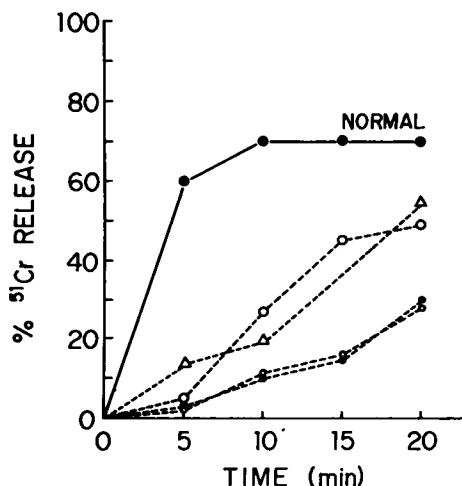


FIGURE 3 ⁵¹Cr-labeled *E. coli* 0111 were added to neat, pooled, normal serum and to neat serum from four patients with cirrhosis (2×10^7 CFU/ml). Percentage of ⁵¹Cr release was determined at the designated intervals after incubation at 37°C. Percentage of ⁵¹Cr released by sera from cirrhotic patients is illustrated with dashed lines; percentage of ⁵¹Cr released by normal serum is indicated by the solid line.

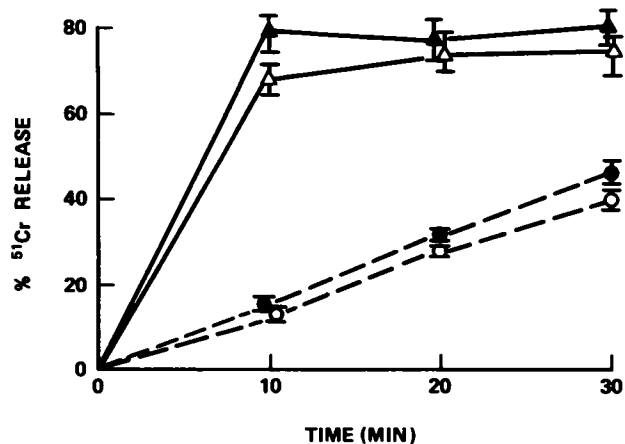


FIGURE 4 Pooled, normal serum and a cirrhotic serum were dialyzed against 100 times the volume of Dulbecco's buffered saline for 18 h at 4°C with three changes of the dialysate. ⁵¹Cr release from *E. coli* 0111 by dialyzed (open symbols) and undialyzed (closed symbols) serum was determined in parallel. Normal serum is represented by solid lines, the cirrhotics' serum by broken lines.

TABLE I
Lack of Inhibition of Normal ⁵¹Cr Release
from *E. coli* 0111 by Cirrhotics' Sera*

| Volume of normal serum | + | Volume of cirrhotic serum | + | Volume of PBS | Percentage of ⁵¹ Cr release | |
|---------------------------|---|------------------------------|---|------------------|-------------------------------------------|--------|
| | | | | | Time of incubation | |
| | | | | | 10 min | 20 min |
| ml | | ml | | ml | % | |
| 0.4 | | 0 | | 0.5 | 70 | 73 |
| 0.6 | | 0 | | 0.3 | 77 | 74 |
| 0.9 | | 0 | | 0 | 75 | 74 |
| 0 | | 0.9‡ | | 0 | 26 | 53 |
| 0.4 | | 0.5‡ | | 0 | 75 | 80 |
| 0.6 | | 0.3‡ | | 0 | 75 | 75 |
| 0 | | 0.9§ | | 0 | 47 | 79 |
| 0.4 | | 0.5§ | | 0 | 75 | 75 |
| 0.6 | | 0.3§ | | 0 | 78 | 79 |

* 2×10^7 CFU of ⁵¹Cr-labeled *E. coli* 0111 in 0.1 ml of saline was added to each mixture.

‡ Serum from G.P.

§ Serum from J.S.

As has been reported (23), Mg-EGTA slows the bactericidal activity of normal serum. We confirmed this with both ⁵¹Cr release and the enumeration of surviving CFU to measure serum bactericidal activity. As shown in Fig. 5, the rate of ⁵¹Cr release and bacterial killing by normal serum were markedly decreased by Mg-EGTA. In contrast, although the rates of ⁵¹Cr release and bacterial killing by the patients' serum were already substantially lower than normal, there was no further decrease when Mg-EGTA was added. In fact, the Mg-EGTA-resistant bactericidal activity of the patients' serum (which constituted virtually all of the serum bactericidal activity [SBA] of this serum) was greater

TABLE II
Effect of Preincubation in Cirrhotics' Sera on ⁵¹Cr Release
from *E. coli* by Normal Serum

| Preincubation* | Percentage of ⁵¹ Cr release | |
|----------------|----------------------------------------|--------|
| | Time of incubation | |
| | 10 min | 20 min |
| | % | |
| Normal serum | 70 | 68 |
| R.W.‡ | 69 | 72 |
| G.P.‡ | 64 | 65 |
| I.Z.‡ | 64 | 65 |

* Bacteria were preincubated in heat-inactivated serum for 10 min at 24°C, then sedimented, washed in PBS, and added to normal serum at 37°C.

‡ Serum from patients with cirrhosis.

than the Mg-EGTA-resistant activity of the normal serum. With *E. coli* 04, we compared the effect of 10 mM Mg-EGTA on ⁵¹Cr release by normal and cirrhotics' sera. Bacteria were incubated in sera for 30 min. The addition of Mg-EGTA decreased the percentage of ⁵¹Cr released by 7 of 9 normal sera, but only 2 of 11 sera from cirrhotics showed a measurable decrease in ⁵¹Cr release (data not shown). This is further evidence that nearly all of the SBA against *E. coli* of most patients with cirrhosis is a result of the activity of the alternative complement pathway.

We compared the bactericidal activity of 28 sera from cirrhotic patients for two serotypes of *E. coli* (Table IV). In 12 of 28 instances there were discordant results: 12 sera had decreased bactericidal activity against either *E. coli* 0111 or *E. coli* 06, but not both. This suggested that the bactericidal defect in these patients had antigenic specificity which varied from serum to serum. To further examine this possibility, we measured the bactericidal titers of antibodies isolated from abnormal sera. Normal and cirrhotics' sera were fractionated to separate IgG and IgM as described in Methods. Each fraction was concentrated so that the concentration of the immunoglobulin was equal to that in the starting serum. The bactericidal antibody titer of the IgG and IgM from all of the fractionated sera was measured with *E. coli* 0111. When added to complement, there was no release of ⁵¹Cr by IgG from any serum except hyperimmune serum. All of the bactericidal antibody activity in both normal and cirrhotics' sera was found in the IgM fraction. Fig. 6 illustrates the relationship of IgM concentration to ⁵¹Cr release for purified IgM from two normal sera and one cirrhotic's serum. The concentration of IgM which induced 50% of normal ⁵¹Cr release was determined by interpolation. This value was used to determine the bactericidal activity of the purified IgM (units per microgram) and to determine the total serum IgM bactericidal activity (units per milliliter of serum). These values for four cirrhotics' and four normal sera are shown in Table V, where they are compared with the bactericidal activity and the HA titers of the corresponding whole sera. In the cirrhotics' sera, serum bactericidal activity was proportional to both the bactericidal activity of purified IgM and the total serum IgM bactericidal activity. All four normal sera had essentially identical serum bactericidal activity, even though total serum IgM bactericidal activity ranged from 10.5 to 129.5 U/ml. Apparently, all of these values were above the threshold of specific bactericidal IgM activity needed for maximum ⁵¹Cr release from *E. coli* 0111. Total IgM bactericidal activity was roughly correlated with HA titer, at least at the extremes. However, HA titers did not discriminate between sera with normal and decreased SBA as well as did the IgM bactericidal titers.

Because we had found that cirrhotics' sera were de-

TABLE III
 Values for Serum Lysozyme and Complement in Cirrhotic Patients with Normal and Decreased SBA

| Cirrhotic patient | Lysozyme activity | CH ₅₀ | C3 | C4 | C3PA | SBA | |
|-------------------|-------------------|------------------|--------------|--------------|--------------|---------------------|-------------------|
| | | | | | | <i>E. coli</i> 0111 | <i>E. coli</i> 06 |
| | <i>U/ml</i> | <i>U/ml</i> | <i>mg/dl</i> | <i>mg/dl</i> | <i>mg/dl</i> | | |
| R.B. | 6 | 161 | ND* | 21.5 | 23.0 | N† | N |
| G.S. | 31 | 179 | 80§ | 12.0 | 11.0 | N | N |
| W.C. | ND | 215 | 160 | 27.0 | 33.0 | N | N |
| C.D. | 11 | 156 | 110 | 11.4 | 9.0 | ↓ [‡] | N |
| G.P. | 12 | 179 | 105 | 20.0 | 11.0 | ↓ | N |
| J.S. | 15 | 227 | 225 | 19.0 | ND | ↓ | N |
| R.W. | 6 | 227 | 140 | 26.0 | 18.0 | ↓ | N |
| R.N. | 14 | 117 | 120 | 23.0 | 14.4 | N | ↓ |
| I.Z. | 6 | 137 | 85 | 11.5 | 13.0 | ↓ | ↓ |
| R.H. | 6 | 169 | 150 | 19.0 | ND | ↓ | ↓ |
| L.C. | 15 | 120 | 79 | 21.0 | 20.8 | ↓ | ↓ |
| B.C. | 19 | 277 | 245 | 70.0 | 32.8 | ↓ | ND |
| F.D. | ND | 179 | 190 | 32.0 | 22.0 | ↓ | ND |
| R.S. | ND | 111 | 120 | 16.2 | 15.2 | ↓ | ND |
| R.W. | 6 | 227 | 140 | 26.0 | 18.0 | ↓ | ND |
| J.S. | 15 | 222 | 225 | 19.0 | ND | ↓ | ND |
| Normal values | 6-12¶ | 125-175¶ | 88-252** | 12-72** | 12-30** | | |

* ND = not done.

† N = normal SBA against that strain of *E. coli*.

§ Italicized values are below the lower limit of normal.

‡ ↓ = decreased SBA against that strain of *E. coli*.

¶ Published values. Sera from 20 normal volunteers were within this range when tested in parallel with cirrhotic sera.

** As determined by manufacturers. Sera from 20 normal volunteers were within this range when tested in parallel with cirrhotics' sera.

ficient in bactericidal antibody, we attempted to correct the defect with antiserum to boiled bacteria. Antiserum against the homologous strain (*E. coli* 0111 in this case) increased ⁵¹Cr release to normal values. Antiserum to the heterologous strain (*E. coli* 04) did not increase ⁵¹Cr release at concentrations which were effective when the test bacteria was *E. coli* 04. IgM but not IgG from a normal serum (L.T.) also completely restored the SBA of a cirrhotic's serum (R.S.). However, it required 1,100 μg/ml of the IgM to correct the bactericidal deficiency of the cirrhotic's serum whereas 200 μg/ml was sufficient to correct the defect in agammaglobulinemic serum. These results suggest that, in addition to the deficiency in bactericidal IgM, there may also be a "blocking" factor in cirrhotic sera, such as noncomplement-fixing antibody (24). Further work is needed to clarify this point.

DISCUSSION

In this study we found that most patients with advanced cirrhosis of the liver had diminished SBA against at least one of the three test strains of *E. coli*. This ab-

normality was manifest primarily as a kinetic defect, i.e., *E. coli* were killed more slowly in cirrhotic than in normal serum. At its extreme, the bactericidal reaction was so slow that the cirrhotics' sera essentially lacked bactericidal activity. 80% (22/27) had diminished bactericidal activity against at least one of the three *E. coli* strains (Fig. 2). We consider 80% to be a minimal estimate of the prevalence of the bactericidal defect in cirrhosis because we used only three test strains of *E. coli* and it was not unusual for a serum to have decreased SBA against only one strain. Had we used more strains, we might have identified a bactericidal defect in all patients with cirrhosis. Because all sera were tested undiluted, we believe that these in vitro measurements of SBA are true estimates of in vivo bactericidal capacity.

The measurement of SBA was facilitated by the use of the ⁵¹Cr-release assay. We have found that with each strain of *E. coli*, the percentage of ⁵¹Cr released after exposure to human serum is proportional to bacterial killing as measured by decrease in CFU. We have previously shown that in normal serum ⁵¹Cr is released from bacteria almost simultaneously with cell death,

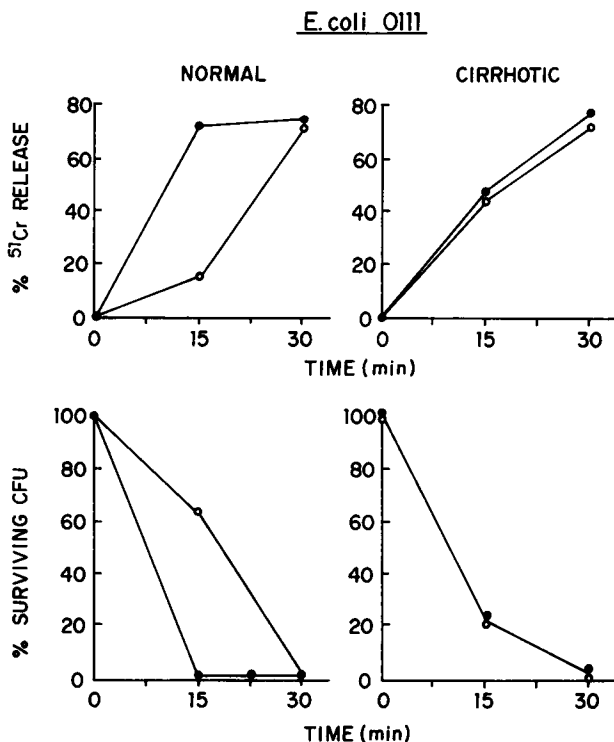


FIGURE 5 Percentage of ^{51}Cr release and surviving CFU were determined after 5×10^7 radiolabeled *E. coli* 0111 in 0.1 ml of saline was added to 0.9 ml of normal serum and of serum from a cirrhotic patient. The open circles indicate serum to which 10 mM Mg-EGTA had been added. An equal volume of PBS (0.1 ml) was added to the control serum (closed circles).

and that ^{51}Cr release is therefore a useful method for studying the kinetics of the bactericidal reaction (11). The ease with which serial measurements of ^{51}Cr release can be made allowed us to compare the initial rates of bactericidal reactions in serum from cirrhotic and normal individuals (Fig. 3). This is quite difficult to do with viability assays. Furthermore, the use of ^{51}Cr release made it practical to measure the SBA of serum from large numbers of cirrhotic patients using three different strains of *E. coli*.

TABLE IV
 ^{51}Cr Release from Two Strains of *E. coli**
by 28 Cirrhotic Sera

| <i>E. coli</i> 06 | <i>E. coli</i> 0111 | | Total |
|-------------------|---------------------|--------|-------|
| | Decreased | Normal | |
| Decreased | 4† | 4 | 8 |
| Normal | 8 | 12 | 20 |
| Total | 12 | 16 | |

* With *E. coli* 06, ^{51}Cr release was measured after 15 min incubation in neat serum; with *E. coli* 0111, after 10 min incubation.

† Number of patients.

The abnormality of SBA observed in cirrhotics' sera might have been a result of an abnormality of antibody, complement, or lysozyme. We found that patients with cirrhosis had a normal or increased activity of lysozyme, but some were hypocomplementemic, as has been reported (7). In our patients, the complement abnormalities were quite mild and were not well correlated with deficiencies in SBA. We therefore sought to measure bactericidal antibody in these patients.

Antibody-mediated humoral defense mechanisms have not been studied previously in patients with cirrhosis, perhaps because it has been shown that these patients have high titers of agglutinating antibacterial antibodies (25). Antibodies to Gram-negative bacteria may promote immunity in two ways: They may be opsonic or they may be bactericidal. The latter function always requires complement. In normal individuals, bactericidal antibodies are limited to the IgM class of immunoglobulins (26). After immunization or infection it is possible to detect bactericidal antibody of the IgG class, even though these are much less efficient bactericidal antibodies than are IgM antibodies (27). In cirrhotics, we found that bactericidal antibodies to *E. coli* were also limited to the IgM class.

More surprising than the absence of bactericidal IgG in the serum of cirrhotic patients is the relative deficiency of bactericidal IgM against *E. coli* 0111. Despite

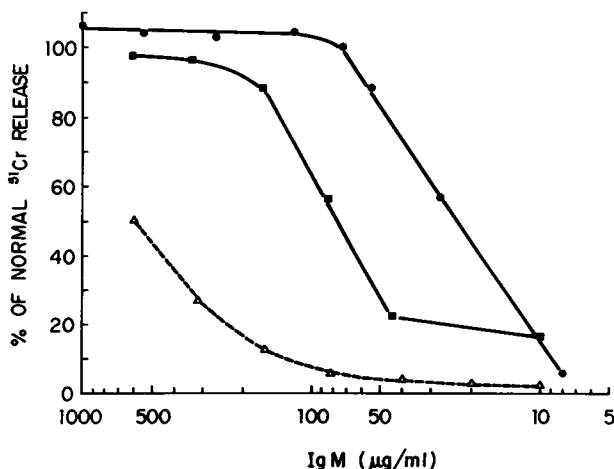


FIGURE 6 IgM was purified as described in Methods. 0.1 ml of saline was serially diluted in Dulbecco's buffered saline plus 1% bovine serum albumin, and 0.1 ml of human complement was added to each dilution. Radiolabeled *E. coli* 0111 (final concentration 2.5×10^7 ml) were added, and the tubes were incubated at 37°C for 20 min. The percentage of ^{51}Cr released by pooled, normal serum was used as the 100% standard, and percentage of ^{51}Cr release induced by the IgM was compared with that value. There was no specific release of ^{51}Cr by the IgM fractions in the absence of complement. IgM concentrations were determined by radial immunodiffusion. This figure illustrates the bactericidal activity of IgM from two normal sera (solid lines) and a cirrhotic's serum (dashed line).

TABLE V
Comparison of SBA against *E. coli* 0111 with IgM Bactericidal Activity and HA Titers

| Patient | Bactericidal activity of whole serum | IgM bactericidal activity | Serum IgM | Total serum IgM bactericidal activity | HA titer (whole serum) | |
|------------|--------------------------------------|---------------------------|-----------|---------------------------------------|------------------------|---------------------------------------|
| | | | | | | % of normal ⁵¹ Cr release* |
| Cirrhotics | J.F. | 69 | 2.7 | 5,300 | 14.3 | 1:16 |
| | I.Z. | 46 | 3.1 | 2,700 | 8.3 | 1:32 |
| | G.P. | 32 | 2.2 | 1,500 | 3.3 | 1:4 |
| | R.S. | 21 | 0.4 | 8,200 | 3.2 | 1:4 |
| Normals | I.K. | 97 | 37.0 | 3,500 | 129.5 | 1:128 |
| | D.C. | 96 | 11.9 | 1,150 | 13.6 | 1:32 |
| | L.T. | 95 | 11.1 | 1,500 | 16.6 | 1:64 |
| | H.L. | 90 | 4.1 | 2,700 | 11.7 | 1:16 |

* (% ⁵¹Cr release [patient]/% ⁵¹Cr release [pooled, normal serum]) $\times 100$. Bacteria were incubated in serum for 20 min.

† A bactericidal unit is that quantity of IgM which induces 50% of maximal ⁵¹Cr release (compared with pooled, normal serum). Bactericidal activity (U/ μ g) is the reciprocal of that concentration of IgM determined to contain 1 bactericidal U/ml.

‡ Measured by radial immunodiffusion.

^b Total serum IgM bactericidal activity (U/ml) = IgM bactericidal activity (U/ μ g) \times serum IgM concentration (μ g/ml).

slightly higher serum concentrations of IgM, 75% of patients with deficient SBA had less circulating bactericidal IgM antibody than did normals (Table V). Although we did not directly measure the concentrations of bactericidal IgM antibody against *E. coli* 04 and 06, we assume that they were also diminished because SBA against these strains was reduced.

There are several possible explanations for the relative deficiency of anti-*E. coli* bactericidal IgM antibody in patients with cirrhosis. It is possible that these patients preferentially synthesize noncomplement-fixing IgM antibody in response to bacterial antigens (28). If this were the case, they should have deficient SBA against all *E. coli*, not just against selected serotypes, the most common pattern observed in this study. Alternatively, it is possible that these patients are hyperimmunized by their current microflora because their livers do not remove and sequester bacterial antigens normally (29), and that the resultant hypergammaglobulinemia might suppress the synthesis of antibody to other bacterial antigens. Finally, the cirrhotic patient may make more IgG and less IgM antibody in response to chronic immunization by lipopolysaccharides (30). Because IgG is a relatively inefficient mediator of SBA and hemagglutination, we may not have detected such an increase in IgG antibody with our assays. Very little is known about the basis for the switch from IgM to IgG synthesis, but there is some evidence that IgG antibody can suppress the synthesis of IgM antibody (31). We did attempt to quantitate anti-*E. coli* antibody in each immunoglobulin class using indirect immunofluorescence with an antibody specific for each human Ig heavy chain (Meloy Laboratories, Inc., Springfield, Va.), but the technique was too insensitive. All sera, both normal and cirrhotic, had titers between 0 and 1:4.

In view of the deficiency in bactericidal antibody observed in cirrhotic patients, it was of interest to examine the functional activity of their alternative complement pathway, which does not use IgM antibody (32). We tested alternative pathway function by chelating Ca⁺⁺ with Mg-EGTA (21), which slows the bactericidal reaction in normal serum. However, Mg-EGTA did not have that effect on the bactericidal activity of sera from many cirrhotics. In many cirrhotics' sera, such as the one shown in Fig. 5, the SBA of chelated serum and neat serum was nearly identical. Thus, whereas most of the SBA of normal serum is a result of the activity of the classical C pathway, the SBA of many cirrhotic sera is a result of the activity of the alternative C pathway. Furthermore, their alternative C pathway is sometimes sufficiently active to produce normal SBA.

The exact role that serum bactericidal activity plays in immunity to Gram-negative infections is controversial. It has been shown that acquired immunity to *Haemophilus influenzae* (33) and to *Neisseria meningitidis* (34) correlates with the development of bactericidal antibody. Although normal adults have a high degree of "natural" immunity to *E. coli* and normal serum contains "natural" antibody that is bactericidal for many strains of *E. coli* (and other Gram-negative bacilli), the relationship between immunity to these Gram-negative bacilli and SBA has not been conclusively established. However, because most strains of *E. coli* isolated from the blood of bacteremic patients are serum resistant (10), whereas many fecal strains are serum sensitive, it is likely that SBA does play a role in natural immunity.

The importance of SBA has been demonstrated in experimental models. Durack and Beeson (35) have shown that the serum resistance of the bacteria and the

host SBA can both influence the outcome of an *E. coli* infection. In their experiments, rabbits with congenital C6 deficiency developed endocarditis after intravenous injections of either serum-sensitive or serum-resistant *E. coli*. Normal rabbits were resistant to infection with the serum-sensitive, but not the serum-resistant, *E. coli*. Similar results have recently been obtained with a model of *E. coli* meningitis (36). Because C6-deficient rabbits completely lack SBA but have normal opsonic capacity, these studies support the importance of SBA as a natural defense mechanism. Cirrhosis may be a disease which mimics these experimental models. Under normal circumstances, *E. coli* remain within the intestinal lumen and are not pathogenic. In the presence of portal hypertension, bacteria which are ordinarily "noninvasive" may pass out of the intestinal lumen and gain access to the circulation; this would be analogous to the direct injection of the bacteria in the experimental animal. These bacteria are more likely to multiply in the circulation if they are fully resistant to SBA or if the bacteria are serum sensitive but the host lacks normal SBA (which we have found to be the case in cirrhotics). In this regard, it is interesting that 3 out of 10 strains of *E. coli* isolated from the blood of patients with cirrhosis were very serum sensitive (i.e., they exhibited a 90% decrease in CFU after 20 min in normal serum), whereas 95% of blood-culture isolates from unselected patients are serum resistant (10).

We have established that there is a deficiency in bactericidal IgM antibody activity against one or more strains of *E. coli* in patients with advanced cirrhosis. It is possible that this deficiency has other ramifications besides the bactericidal defect; it may also affect cellular defenses. For example, while IgM alone is not opsonic, it becomes opsonic in the presence of complement (37). The possibility that cirrhotics have an opsonic defect in addition to their bactericidal defect is presently under investigation.

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