Functional Role of Antibody against "Core" Glycolipid of *Enterobacteriaceae*

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ABSTRACT Antibodies against the "core" glycolipid of *Enterobacteriaceae* (2-keto, 3-deoxyoctonate-Lipid A) have been associated with protection against the sequelae of gram-negative rod bacteremia. To investigate the nature of this protection, dogs and rabbits were immunized with purified glycolipid prepared by phenol-chloroform-petroleum ether extraction of the "Re" mutant of *Salmonella minnesota* 595 and opsonophagocytic and bactericidal tests were carried out using lapine peritoneal granulocytes and serum factors. Whereas 1–4 μg/kg of glycolipid i.v. produced hypotension in dogs and 8 μg/kg i.v. was lethal, a rising dosage schedule of immunization with an average total dose of 1 mg/kg produced striking protection against shock and death against challenge with heterologous organisms. 20 control dogs, given approximately 10^{10} live, serum-resistant *Escherichia coli* 0.85:H9 or *Serratia marcescens* 03 during a continuous intra-arterial pressure transducer recording, showed a drop in mean systolic pressure from 186 (±6 SE) to 101 (±12 SE) mm Hg and a fall in mean diastolic pressure from 118 (±3 SE) to 64 (±8 SE) mm Hg within 60-120 min. Minor pressure changes (average < 12% of prechallenge levels) were seen in the same number of immunized dogs. In contrast, no significant difference was noted in the bloodstream clearance of these serum-resistant organisms over a period of 4–6 h between immunized and control dogs. Intravascular clearance was greater in animals immunized with the challenge strain or in glycolipid-immunized animals challenged with highly serum-sensitive *E. coli* 0.14:K7. Antibody against core glycolipid protected against the hemodynamic sequelae of bacillemia, augmented intravascular clearance of serum-sensitive organisms, and abrogated the pyrogenic response to enteric bacilli, but did not enhance clearance of serum-resistant organisms. Although canine and lapine antiserum against core glycolipid passively protected mice against a heterologous challenge, opsonophagocytic and bactericidal activity was at least 100-fold less than type-specific antiserum.

INTRODUCTION

The increasing incidence of nosocomial gram-negative rod bacteremia and its high mortality have led to renewed consideration of active or passive immunization as one method of preventing this disease in humans (1, 2). Active immunization against prevalent serotypes of *Pseudomonas aeruginosa* has been extensively studied (3, 4), but these organisms account for a relatively small percentage of systemic bacterial infections compared to the *Enterobacteriaceae* (5, 6). It is generally recognized that a formidable barrier to immunoprophylactic approaches against the latter family of organisms is the multiplicity of O-antigenic serotypes. Within the last decade, however, it became apparent that most *Enterobacteriaceae* share a "core" glycolipid (CGL)[1] antigen consisting of 2-keto, 3-deoxyoctonate (KDO) linked to Lipid A (7, 8). The "Re" chemotype mutant of *Salmonella minnesota* R595 has an outer cell wall layer consisting almost entirely of this biochemical moiety and antibody directed against this widely shared antigen (so-called "cross-reactive antibody") appears to be protective in human (9) and experimental murine enterobacterial infections (10).

Recognizing that there may be several mechanisms by which cross-reactive antibody against CGL is pro-

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[1] *Abbreviations used in this paper:* CGL, core glycolipid; FAS, fresh absorbed serum; GVB, veronal-buffered saline, pH 7.4, with 0.1% gelatin; HA, hemagglutination; HBSS, Hanks' balanced salt solution; KDO, 2-keto, 3-deoxyoctonate; PBS, phosphate-buffered saline; PH, passive hemolysis; SRBC, sheep red blood cells.

tective, we undertook these studies with the following major goals: First, we wished to evaluate the protective role of antibodies against CGL in mammalian systems in which complement may have an important role in the expression of antibody activity, since mice have relatively immature complement systems (11, 12). Secondly, we wanted to evaluate the activity of this antibody as an opsonin, bactericidin, and antitoxin. Third, we wished to assess the relative protective role of type-specific vs. cross-reactive antibody in animal model systems, inasmuch as one study has suggested that cross-reactive antibody appears more protective than type-specific antibody (9). Fourth, we felt there was need to assess the role of antibody using a variety of heterologous challenges including serum-resistant and serum-sensitive strains, since there is little information on host protection after challenge with both types of organisms. Accordingly, we approached these goals by carrying out intravascular bacterial challenge studies in canines and rabbits, passive protection studies in mice using normal, immune, and absorbed serum, and in vitro opsonophagocytic and bacterial studies using rabbit leukocytes and serum factors. In the animal challenge studies we sought to obtain, in addition to survival data, quantitative measurements of bacterial clearance from the bloodstream of immunized and control subjects and the effects of CGL immunization on systolic and diastolic blood pressure and protection against pyrexia. Our in vitro studies sought to measure the opsonizing and bactericidal activities of antibodies engendered by CGL immunization and compare these to type-specific antibodies.

METHODS

Bacterial strains and antigens

S. minnesota R595, chemotype Re (strain KNV 497), was obtained from Dr. Otto Westphal, Max Planck Institute für Immunobiologie, Freiburg, Federal Republic of West Germany. *Escherichia coli* 014:K7 was supplied by Dr. Erwin Neter and Dr. H. Y. Whang, Childrens Hospital of Buffalo, N. Y., and found to be serum-sensitive by the method of Goldman et al. (13). *Serratia marcescens* serotype 03 is a serum-resistant strain isolated from a bacteremic patient at the Memorial Sloan-Kettering Cancer Center, New York, and typed with monospecific antiserum derived from prototype strains provided by Dr. William H. Ewing, Center for Disease Control, Atlanta, Ga. *E. coli* 085:H9 is a serum-resistant, enteropathogenic, toxigenic strain provided by Dr. Sherwood Gorbach, Sepulveda Veterans Administration Hospital, Sepulveda, Calif. Purified CGL antigen was prepared by the phenol-chloroform-petroleum ether extraction method of Galanos et al. (14) from *S. minnesota* R595. After extraction the antigen was solubilized in 0.01 M phosphate, 0.15 M NaCl, pH 7.4 (phosphate-buffered saline PBS) using the "micro-tip" of a Branson ultrasonic cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). To characterize this antigen, gel-column chromatography on Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was carried out after the method described by Raynaud et al. (15). 10 mg of CGL was dissolved in 5 ml of 0.9% NaCl, placed on a column (2.5 × 40 cm, type K 25/45, Pharmacia Fine Chemicals, Inc.), and eluted with 0.9% NaCl at a flow rate of 0.08 ml/min. 4-ml fractions were obtained and analyzed for KDO. Only one peak was obtained at fractions 13–25 which were pooled and lyophilized. Since this peak was essentially at the exclusion limits of the chromatographic matrix, the molecular weight of the antigen was estimated at 5×10^6 daltons. KDO content of this antigen was 14.7% by weight using the thiobarbituric acid method of Aminoff (16). The phosphorus content of this preparation was 1.06% by weight by the method of Chen et al. (17). No heptose could be detected using the cysteine H_2SO_4 method of Dische (18) and no protein or nucleic acids were present as determined by ultraviolet scanning.

In addition to the purified CGL, whole bacterial vaccines were prepared from overnight growth of organisms on trypticase soy agar which was removed and washed three times in distilled water, boiled for 1 h, and washed twice, followed by resuspension in phosphate-buffered saline (PBS) by matching to a turbidity standard corresponding to 5×10^8 bacteria/ml.

Animal immunizations

Male or female mongrel dogs averaging 20 kg were immunized using the following dosage regimen of CGL: 1 μg/kg of antigen was injected as a test dose either i.v. or i.m. This was followed by progressively increasing doses of 2, 4, 8, 16, 32, 64, 128, and 256 μg/kg every 5–7 days with the last dose repeated two or three times at the same interval. The average dog received 1 mg/kg of antigen in 11 divided doses. Immunization of rabbits (average weight = 3 kg) proceeded in a similar manner except that the largest dose given was 64 μg/kg and this was also repeated an additional two times for an average total dose of 255 μg/kg. Immunization using whole cell vaccines proceeded on a weekly basis with a test dose of 0.5 ml of canines and 0.1 ml to rabbits i.m. with four additional 3-ml injections given each time to dogs and 0.5 ml to rabbits i.v. All animal challenge experiments were carried out more than 1 wk after completion of immunization to minimize "nonspecific" protective effects secondary to administration of lipopolysaccharides (10, 19, 20).

Serologic studies

Antibodies against purified CGL antigen were measured by complement-dependent passive hemolysis (PH) using a modification of the method of Phillips et al. (21) and by passive hemagglutination (HA) according to the method of Nowotny (22). For PH tests, purified CGL (5 mg/ml) was dissolved in 0.1% triethylamine (Fisher Scientific Co., Pittsburgh, Pa.) in 0.01 M PBS, pH 7.4, further diluted in veronal-buffered saline with 0.1% gelatin, pH 7.4 (GVB), to a concentration of 500 μg/ml, and heated at 100°C for 1 h. A 10% solution of sheep red blood cells (SRBCs) was gently agitated with an equal volume of antigen for 2 h at 37°C, washed three times in GVB, and resuspended at a final concentration of 0.8%. Serum was heat-inactivated at 56°C for 30 min and absorbed with ½ vol of 50% washed SRBCs in physiologic saline at 37°C for 2 h, then held overnight at 4°C. After centrifugation, the supernatant serum was removed, diluted 1:4 in GVB, and serial twofold dilutions of 0.025 ml in disposable "microtiter" trays with U-shaped wells were carried out

using an automatic microtiter diluter (Cooke Engineering Co., Alexandria, Va.). 50 μl of GVB was added followed by addition of 0.025 ml of guinea pig complement (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) at a dilution of 1:20 which was selected because it did not promote hemolysis of sensitized SRBCs per se (the initial dilution of serum in this system was thus 1:20). Finally, 0.025 ml of sensitized SRBCs was added, plates were incubated at 37°C for 1½ h, and the titer determined as the dilution associated with complete hemolysis. Controls including unsensitized SRBCs and GVB substituted for complement were always used.

In micro-HA tests correspondingly sensitized SRBCs were exposed to serial twofold dilutions of serum beginning with a dilution of 1:4; 0.025-ml vol, microtiter plates, and dilution apparatus were identical to those used in PH tests. HA patterns were read after 2 h incubation at 37°C and 18 h incubation at 4°C; end points were complete agglutination of the SRBC "button."

Antigenic extracts of *E. coli* 014:K7, *S. marcescens* 03, or *E. coli* 085:H9 were prepared by removing overnight growth on agar surfaces with cotton swabs, washing three times in normal saline, followed by resuspension at a concentration of 5×10^8 organisms/ml, and boiling for 2 h. Suspensions were centrifuged at 1,200 *g* for 10 min and supernatant fluids used to sensitize SRBCs.

Animal studies

Response to i.v. CGL antigen and clearance of i.v. injected bacteria. Canine experiments were always carried out with an immunized animal paired with a control animal. After i.v. sodium pentobarbital anesthesia, a femoral artery was cannulated with a 14-gauge polyethylene catheter linked by saline infusion line to a Statham P23AA pressure transducer (Statham Instruments, Inc., Oxnard, Calif.); this in turn was connected to a Sanborn recorder (model 7702B Hewlett-Packard Co., Waltham, Mass.). Intra-arterial pressure was calibrated with a mercury sphygmomanometer at the beginning and conclusion of each experiment and all results were recorded on no. 651-52 two-channel recording paper. Base-line continuous intra-arterial pressure recordings were obtained for both control and immunized animals for 20 min after which purified CGL antigen or log-phase organisms were injected i.v. over a period of 5 s. In bacterial clearance studies, serial colony counts in peripheral venous blood were obtained by venapuncture of peripheral limb veins. Blood was heparinized and instantly chilled in an ice bath after which 1-ml aliquots of heparinized peripheral blood was lysed in 9 ml sterile distilled water, serially diluted in trypticase soy broth, and incorporated into trypticase soy agar pour plates. Intravascular clearance of organisms was determined by quantitative colony counts 5, 10, 15, 30, 60, 120, 240 min, and in some animals 6 h after injection of bacteria during simultaneous measurement of arterial systolic and diastolic pressure. At the conclusion of the experiment in canines, the cannulated artery was repaired and animals were subsequently followed for a period until death or for 3 wk thereafter.

Some dogs were studied after pretreatment with 10 mg/kg of cyclophosphamide (Mead Johnson Laboratories, Evansville, Ind.) given i.v. 3–6 days before live bacterial challenge.

Rabbit experiments were carried out in animals acclimatized for at least 72 h. Animals were restrained on holding boards in the same room after insertion of a rectal thermal probe. After a base-line observation period of 1 h, an intracardiac injection of approximately 10^7 organisms was given over 5 s and peripheral blood was serially obtained from a marginal ear vein. Rectal temperatures were recorded for the ensuing 6 h and surviving animals were observed for an additional 3 wk.

Opsonophagocytic studies

Rabbit peritoneal granulocytes were obtained for in vitro studies after the method of Hirsch and Strauss (23). Approximately 250 ml of saline containing 0.1% shellfish glycogen (Sigma Chemical Co., St. Louis, Mo.) was injected i.p. 4 h later a no. 14 Bardic inside needle-plastic catheter (C. R. Bard, Murray Hill, N. J.) was inserted in the abdomen and the inner catheter advanced. Approximately four small perforations were made in the catheter before insertion. Leukocyte-rich peritoneal fluid was readily drained by gentle pressure and massaging the abdomen. Up to 150 ml of peritoneal exudate per animal was collected in heparinized plastic tubes (10 U heparin/ml, Riker Laboratories, Northridge, Calif.). The cell suspension was filtered through sterile gauze to remove fibrin clots, centrifuged at 500 *g* at 4°C, and the pellet was washed three times with heparinized Hanks' balanced salt solution without bicarbonate (HBSS, Microbiological Associates, Bethesda, Md.) supplemented with 0.1% gelatin (Difco Laboratories, Detroit, Mich.). More than 95% of recovered cells were typical rabbit granulocytes and more than 95% were viable as determined by trypan blue exclusion. For phagocytosis tests, the standard mixture consisted of 0.5 ml of the leukocyte suspension (10^7 cells/ml, thus a final concentration 5×10^6 granulocytes/ml were present), a 0.1-ml suspension of log-phase growth organisms (final concentration 2×10^6 organisms/ml), with the balance of the volume to 1 ml consisting of various combinations of serum (usually 0.1 ml), a source of complement-like factors (0.1 ml), and HBSS with 0.1% gelatin in solution. These mixtures were tumbled at a rate of 12/min in sterile plastic screw-cap tubes (size 13 × 100 mm, Falcon Plastics, Oxnard, Calif.) on a Labtek aliquot mixer (Miles Laboratories Inc., Elkhart, Ind.) at 37°C. Samples were taken at 0 time and at 120 min. 0.1-ml aliquots were serially diluted in sterile distilled water and each dilution incorporated into trypticase soy agar plates to determine residual bacterial viability of the test suspension. The percent of organisms in each suspension which remained viable after 2 h was calculated on the basis of initial inoculum size.

To assess the relative role of heat-stable antibody in normal and immune serum, specimens were heat-inactivated at 56°C for 30 min. Heated serum was titered in the presence of fresh normal serum absorbed of "natural" antibodies using washed, live bacteria (fresh absorbed serum, FAS). The bacteria were log-phase organisms washed twice in PBS (0.01 M, pH 7.4), and absorptions (approximately 10^9 organisms/ml of serum) were carried out twice at 4°C with gentle agitation for 1 h each (24).

Bactericidal tests

Log-phase growth of *E. coli* 014:K7 in trypticase soy broth was harvested by centrifugation at 4°C and washed twice in GVB. The test system consisted of 0.1 ml of approximately 2×10^7 or 2×10^5 organisms (as determined by serial dilutions from a suspension matched to a turbidity standard), 0.1 ml (10% final vol) of fresh serum, heat-inactivated serum (normal or immune) or dilutions thereof, and when indicated, 0.1 ml of FAS. In preparing FAS for bactericidal tests it was found necessary to ab-

TABLE I

Mortality in Canine Challenge Experiments

Challenge	Dogs immunized against*	Total number of animals	Survived	Mortality
				%
(1) *S. minnesota* R595 ($\sim 2.06 \times 10^8$)	NA	5	5	0
Purified CGL, 1 μg/kg	NA	6	6	0
Purified CGL, 4 μg/kg	NA	4	2	50
Purified CGL, ≥8 μg/kg	NA	4	0	100
(2) *E. coli* 085:H9	CGL	10	9	10
E. coli 085:H9	*E. coli* 085:H9	5	5	0
E. coli 085:H9	NA	10	4	60
(3) *S. marcescens* 03	CGL	10	8	20
S. marcescens 03	*S. marcescens* 03	5	5	0
S. marcescens 03	NA	10	5	50
(4) *E. coli* 014:K7	CGL	6	6	0
E. coli 014:K7	NA	6	1	84

Pooled mortality for CGL-immunized animals in groups 2, 3, and 4 was 12% contrasted with 62% for control animals. *E. coli* and *Serratia* challenges were approximately 10^{10} live organisms.

* NA, No antigen, normal dog.

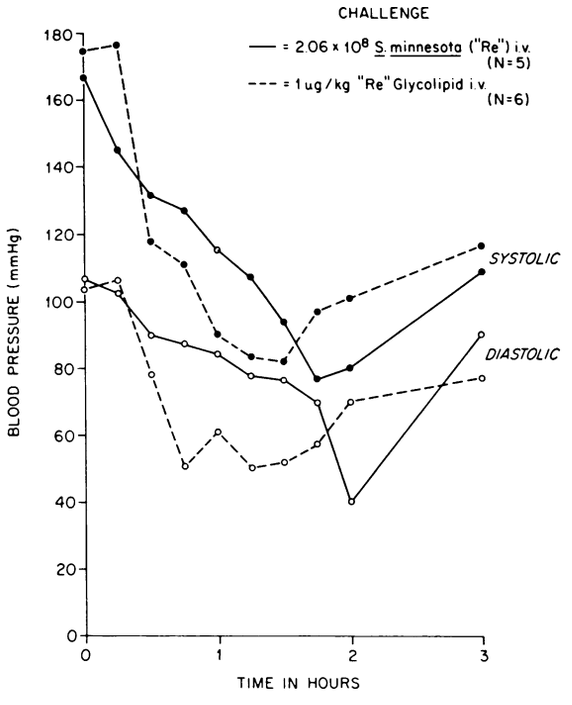

FIGURE 1 Mean arterial blood pressures (mm Hg) after i.v. administration of an average of 2.06×10^8 live *S. minnesota* R595 (chemotype Re) to five dogs or 1 μg/kg of purified CGL antigen to six dogs. Within 2 h mean systolic pressure fell to 53% of prechallenge values and mean diastolic pressure declined to less than half of base-line levels.

sorb fresh serum of properdin after the method of Pillemer et al. (25) to provide a source of heat-labile factors which did not kill the test strain per se. Accordingly, zymosan (Sigma Chemical Co.) was boiled for 1 h in PBS and the centrifuged pellet (10 mg dry wt/ml of serum) was resuspended in fresh serum twice absorbed with *E. coli* 014:K7. This mixture was shaken for 1 h at 17°C, centrifuged, and the supernatant filter-sterilized. Residual total hemolytic complement activity (26) was 68% of preabsorption serum. Surviving bacteria after 2 h of incubation at 37°C were enumerated by the pour plate technique.

PASSIVE PROTECTION EXPERIMENTS IN MICE

Female CD albino mice weighing 18–22 g (Carworth Div., Becton, Dickinson & Co., New York) were used in groups of 10. "Checkerboard titration" of 10-fold serial dilutions of 6–8-h broth cultures of the challenge strain (*E. coli* 085:H9) and hog gastric mucin (granular type 1701-W, lot 144917, Wilson Labs, Chicago, Ill.) determined that the smallest uniformly lethal dose (LD_{100}) was 1.53×10^7 organisms in 0.4% (final concentration) mucin. 0.3-ml aliquots of serial twofold dilutions of heat-inactivated (56°C for 30 min) serum were injected i.p. into groups of 10 mice followed within ½ h by injection of an equal volume of the challenge inoculum, five LD_{100}. Animals were observed for 1 wk after challenge, and the titer for each serum that protected 50% of animals (PD_{50}) was calculated using the Reed-Muench technique (27).

STATISTICAL ANALYSIS

The statistical significance of differences in survival rates and intravascular bacterial clearance between immunized and control animals was determined by the chi square test without Yates correction, and differences in reciprocal geometric mean antibody titers was assessed using Student's *t* test (28). The SEM arterial blood pressure was determined using a Hewlett-Packard HP-46 calculator (Hewlett-Packard Co.).

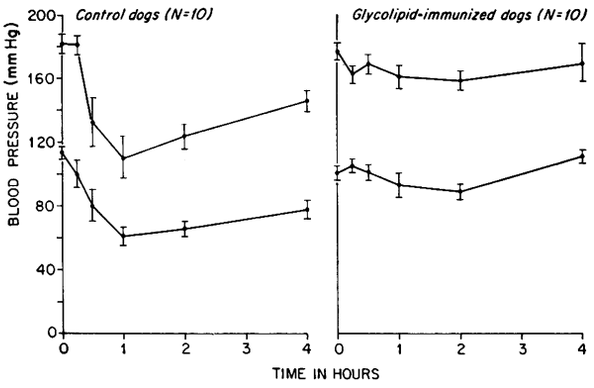

FIGURE 2 Mean arterial blood pressure levels (upper lines, systolic; lower lines, diastolic) after i.v. injection of approximately 10^{10} live, serum-resistant *E. coli* 085:H9 to 10 dogs immunized with approximately 1 mg/kg of purified CGL antigen and the same number of control animals. By 1 h the control group showed a decline in mean systolic pressure from 182 (±8 SE) to 111 mm Hg (±11 SE) while diastolic pressure was almost halved. Arterial pressures in the immunized group varied no more than ±12% of prechallenge values.

RESULTS

Arterial blood pressure changes after i.v. challenge with live S. minnesota R595 or CGL. Fig. 1 summarizes the changes in arterial blood pressure in 11 normal dogs, 5 that were challenged with an average of 2.06×10^8 variable *S. minnesota* R595 i.v., and 6 that were given 1 μg/kg of CGL i.v. Although transient, sudden declines in blood pressure of the range of 40–60 mm Hg in both systolic and diastolic pressure were noted in seven animals 5–10 min post-injection (not shown), such changes reversed spontaneously (returned to within±11% of prechallenge values) within 5 min. This was followed by a more gradual and consistent decline in arterial pressures within 1–2 h postinjection, during which mean systolic blood pressure fell to 53% of prechallenge levels. Purified CGL had an earlier effect on the diastolic blood pressure in these animals. For both CGL and *S. minnesota* R595-challenged animals, the mean diastolic arterial pressure eventually declined to less than half of prechallenge values. Blood pressures gradually returned to normal beginning by the 3rd h and by 6 h, both mean systolic and diastolic pressures were within 15% of control values. All animals survived these experiments. Additionally, another four animals were given 4 μg/kg of CGL and only two survived while a third group of four animals challenged with 8 or more μg/kg died during or within 48 h of the experiment (Table I).

i.v. challenge with serum-resistant organisms, E. coli 085: H9 and S. marcescens 03. Continuous intra-arterial pressure recordings in 10 normal control animals challenged with approximately 10^{10} serum-resistant *E. coli* 085:H9 demonstrated a significant drop in mean systolic and diastolic pressures by 30 min with the greatest decline noted at 1 h (Fig. 2). Four animals again showed transient declines in blood pressure immediately postinjection which corrected spontaneously, but for this group as a whole, mean systolic pressure fell from 182 (±8 SE) to 111 mm Hg (±11 SE) while diastolic pressure was almost exactly halved by 1 h. In contrast, CGL-immunized animals experienced no significant change in mean diastolic pressure and systolic pressures as the latter varied within 12% of prechallenge values. Similarly, 10 nonimmunized canines challenged with a similar inoculum of another serum-resistant strain, *S. marcescens* 03, demonstrated a mean 52% decline in systolic pressure and a mean 46% decline in diastolic pressure (Fig. 3). An average 14% decline in systolic pressure was noted in 10 CGL-immunized dogs, but diastolic pressures fluctuated no more than 7% of control values. After challenge with both serum-resistant bacteria, diastolic and systolic blood pressures usually returned to within 15% of initial values between the 6th and 8th h after challenge. However, 7 of 20 control animals and 1 of 20 glycolipid-immunized animals developed irreversible blood pressure changes and died of respiratory arrest during the course of the experiment and the mean blood pressures that are shown are for the survivors at each point in time. The overall mortality in glycolipid-immunized animals was 15% but 11 of 20 or 55% of control animals died, usually within the first 48 h after challenge ($P < 0.0001$) (Table I).

Five animals were immunized with heat-killed *E. coli* 085:H9 and an equal number with similarly treated

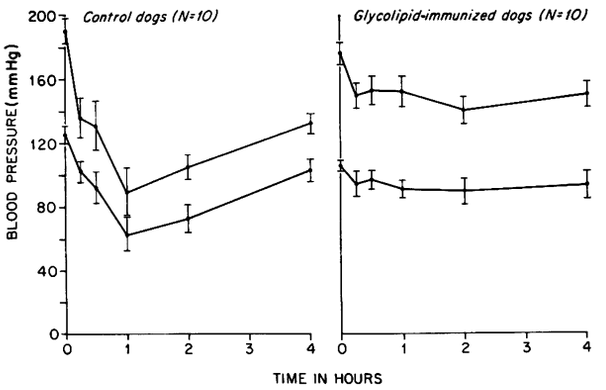

FIGURE 3 Mean arterial blood pressures (upper lines, systolic; lower lines, diastolic) after i.v. injection of 10^{10} live, serum-resistant *S. marcescens* 03. 10 control animals showed a mean 52% decline in systolic pressure and a mean 46% decline in diastolic pressure while 10 CGL-immunized dogs experienced a mean 14% fall in systolic pressure and a mean 7% fall in diastolic pressure.

S. marcescens 03. In identical challenge experiments using live organisms, antecedent immunization with the same strain afforded complete protection against hypotension (maximal blood pressure changes ±8% of base line) and death.

Intravascular clearance of serum-resistant strains. Quantitative cultures were performed on blood taken of all animals challenged with *E. coli* 085:H9 and *S. marcescens* 03, and the residual bacterial concentrations in the venous blood of animals challenged with *E. coli* 085:H9 as a function of time are depicted in Fig. 4. Of the 10 animals that were CGL immunized, a prompt decline after injection of approximately 10^{10} organisms was consistently noted. Within 1 h between 10^3 and 10^4 organisms/ml were found to be circulating in the peripheral venous blood and this degree of bacteremia remained fairly constant over the ensuing 3 h. More important, there was no significant difference in the magnitude of bacteremia at any point in time between the immunized and control animals. In contrast, five animals immunized with heat-killed *E. coli* 085:H9 showed a sustained decline in intravascular bacterial concentrations such that by 4 h fewer than 10^2 organisms/ml were recovered and bacteremia usually cleared by 6–8 h postchallenge. Similarly, of the 20 animals challenged with *S. marcescens* 03, no significant difference in intravascular bacterial clearance was observed between CGL-immunized animals and controls. All five animals previously immunized with heat-killed *S. marcescens* 03 demonstrated accelerated intravascular clearance compared to CGL-immunized animals and blood was usually sterile within 6 h.

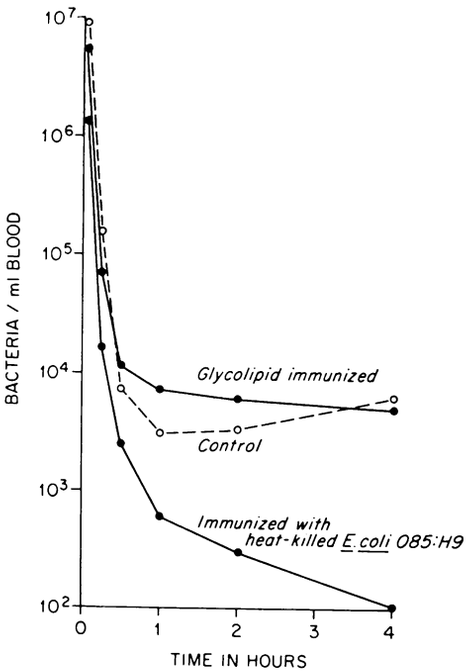

FIGURE 4 Intravascular clearance of serum-resistant *E. coli* 085:H9. Despite the differences in blood pressures between CGL-immunized and control animals shown in Fig. 2, quantitative cultures of venous blood failed to show a difference in bloodstream clearance of organisms. Enhanced intravascular clearance was demonstrable in five animals immunized with the challenge strain.

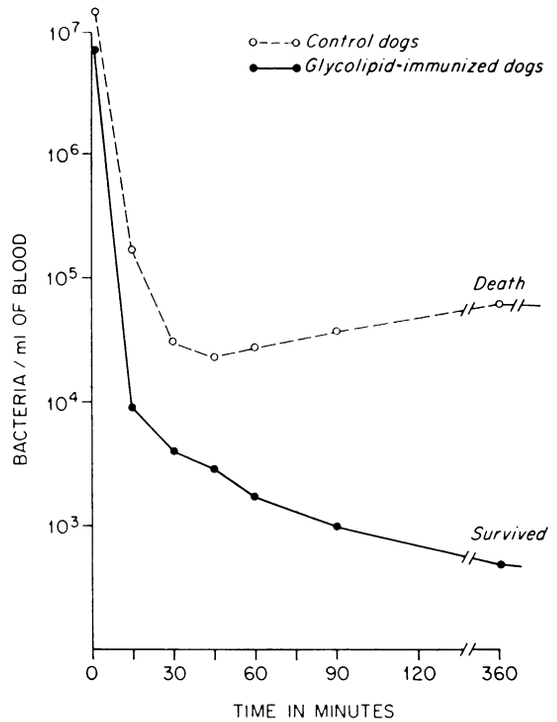

FIGURE 5 Intravascular clearance of serum-sensitive *E. coli* 014:K7. Six animals given approximately 10^{10} organisms survived; the decline in viable circulating organisms was significantly greater compared to six control animals, five of which died. Rate of bloodstream clearance was significantly greater in six animals immunized with the challenge strain (not shown).

Intravascular challenge with serum-sensitive E. coli 014:K7. The effect of CGL immunization on subsequent challenge with this highly serum-sensitive strain is summarized in Fig. 5. In vitro studies with 10% normal dog serum previously demonstrated better than 90% killing after 1 h exposure to a 10^4 inoculum. Of six immunized dogs that received an inoculum of approximately 10^{10} organisms, there was a progressive decline of bacterial counts in whole blood as well as complete protection against hypotension and death. Additionally, beyond 1 h, bacterial counts actually rose in the blood of control animals and the difference in bacterial clearance between immunized and control animals was significant. Five of the six animals in the control group died during or within 18 h of the experiment (Table I).

TABLE II

Prechallenge Antibody Titers (Reciprocal Geometric Mean) in Immunized and Control Animals

	Titer against antigen											
	CGL			*E. coli* 014:K7			*E. coli* 085:H9			*S. marcescens* 03		
	no.(s) tested	PH	HA	no.(s) tested	PH	HA	no.(s) tested	PH	HA	no.(s) tested	PH	HA
(1) Dogs immunized against												
CGL	(23)	85	14.6	(8)	ND*	80.6	(8)	219	32	(10)	341.4	48.4
E. coli 014:K7	(6)	19.8	2.8	(6)	ND	1,287			ND		ND	
E. coli 085:H9	(5)	21.8	3.2		ND		(5)	2,981	194.4		ND	
S. marcescens 03	(5)	23.4	2.9		ND				ND	(5)	3,229	198.3
Nonimmunized controls	(18)	21.6	2.3	(6)	ND	71.5	(9)	347	32	(10)	483	27.9
(2) Rabbits immunized against												
CGL	(10)	1,196	36.6	(6)	ND	5.05	(7)	107.8	2.69		ND	
E. coli 014:K7	(6)	149.6	2.8	(7)	ND	172.1			ND		ND	
E. coli 085:H9	(6)	225.9	2.3		ND		(7)	9,778	1,249		ND	
Nonimmunized controls	(8)	89.4	2.0	(5)	ND	8.0	(7)	194.4	11.9		ND	

Complement-dependent PH titers were determined using twofold serial dilutions beginning with a dilution of 1:20 whereas passive HA tests began with a dilution of 1:4. For purposes of calculation, a hemolysis titer of <1:20 was assumed to be 1:10 and an HA titer of <1:4 was assumed to be 1:2.

* ND, not done.

Effect of cyclophosphamide on i.v. challenge with E. coli 085:H9 or S. marcescens 03. Cyclophosphamide treatment induced leukopenia ranging from 800-1,700 granulocytes/ml in five CGL-immunized animals: three were challenged with *E. coli* 085:H9 and two with *S. marcescens* 03. These animals were protected against hypotension and death and there was no significant difference in the bloodstream clearance of organisms between these animals and nonleukopenic, unimmunized controls.

Intravascular clearance and rectal temperatures in rabbits challenged with E. coli 085:H9. Fig. 6 summarizes changes in average rectal temperatures as well as the degree of intravascular bacterial clearance after challenge of nine CGL-immunized rabbits and the same number of controls with approximately 10^7 *E. coli* 085:H9. Control rabbits showed an average 1.6°C elevation in rectal temperatures, usually between the 1st and 3rd h after challenge while a slight temperature fall of 0.4°C was observed in CGL-immunized animals. There was a consistent difference in intravascular clearance between control and immunized animals during the 4-h observation period but the differences between control and immunized animals were usually within one 10-fold dilution. Eight of nine rabbits in the control group died as opposed to one of nine in the CGL-immunized group.

Prechallenge antibody titers for animals used in clearance experiments. Table II summarizes prechallenge antibody titers for most of the immunized and control animals used in intravascular bacterial clearance experiments. Noteworthy are the almost negligible titers of CGL antibody in normal, unimmunized canines and rabbits. Despite the relatively intense immunization procedure, eight or more doses of purified antigen given on a rising dosage schedule with a final dose six to eight times the usual single lethal dose for an

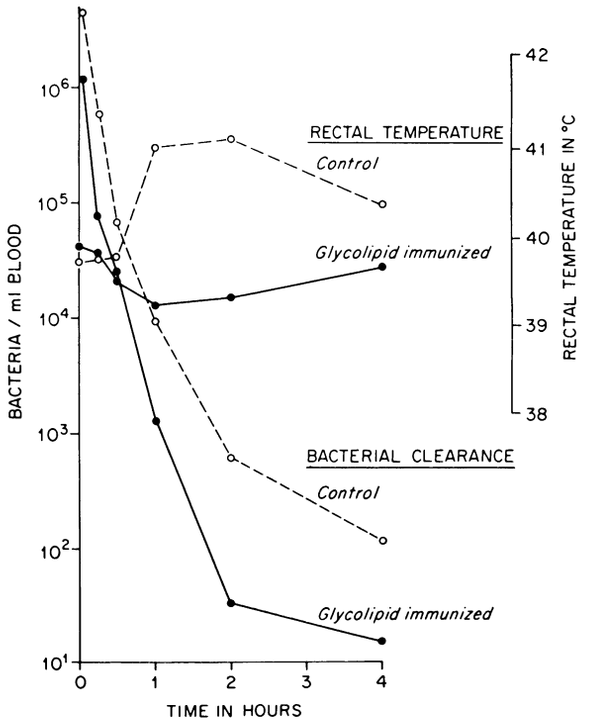

FIGURE 6 Intravascular clearance of *E. coli* 085:H9 and rectal temperatures in rabbits. Nine rabbits were CGL immunized and showed slightly greater bloodstream clearance of bacteria after injection of 10^7 organisms. Control animals ($n = 9$) showed a 1.6°C increase in rectal temperature in contrast to a mean 0.4°C fall observed in immunized rabbits.

unimmunized animal, only four to sixfold increments in anti-CGL titers were observed in dogs and 13- to 18-fold increments were noted in rabbits. For each antigen, complement-dependent PH titers were always greater than HA titers but the results tended to parallel each other. The crude *E. coli* 014:K7 antigen could not be used in PH tests due to the tendency of sensitized SRBCs to hemolyze with or without saponification of the antigen; the PH test worked well for the other antigens.

By these serologic measurements, it is apparent that considerable "natural" antibody against challenge strains bearing O-antigenic determinants was present in the serums of unimmunized canines and antibody levels in dogs and rabbits against such antigens were significantly enhanced by four dose courses of type-specific immunization. On the other hand, no significant difference in anti-CGL titer was observed between animals immunized with *E. coli* 014:K7, 085:H9, or *S. marcescens* 03 and unimmunized controls. Conversely, animals immunized with CGL showed no significant titer rises against the latter three antigens. The results of challenge experiments, specifically the rates of intravascular clearance in the immunized animals and the ability of serum from immunized animals to promote phagocytosis or killing of serum-sensitive organisms (see following experiments), was clearly better for animals immunized with O-antigen-bearing organisms. Nevertheless, anti-CGL titers in serum from heterologously immunized animals were negligible.

Opsonophagocytic tests with lapine granulocytes and S. marcescens 03. The results of opsonophagocytic tests using *S. marcescens* 03, rabbit peritoneal granulocytes induced by the glycogen injection technique, CGL antiserum, and type-specific antiserum against *S. marcescens* 03 are summarized in Table III. Bacterial survival at the end of 2 h of tumbling the granulocyte-bacterial suspension is expressed as the percent of the inoculum remaining viable and each result reported is the mean of at least four different experiments. Averaging was done because of variations in the exact inoculum size (usually within ±12% of the desired 2×10^6 organisms) due to use of the turbidity-matching technique in preparing the inoculum from log-phase growth; the exact number of bacteria added to each test suspension could only be determined in retrospect. Reaction mixtures, with the same components, yielded results which were within ±15% of the mean in 38 of 44 experiments and this range was used to assess the significance of differences in opsonic activity observed at different serum concentrations. Since granulocytes from normal animals were used, intracellular killing of phagocytized bacteria was presumably normal and the reduction in viable counts was interpreted as a reflection of the opsonic activity of serum (23, 24). This was confirmed by differential sedimentation of granulocytes and determining the bacterial viability of washed sediment after resuspension in HBSS. Cell-associated bacterial were always less than 10% of the total decrement in viable counts.

TABLE III

Opsonophagocytic Tests with S. marcescens 03

5×10^6 Leukocytes + serum factors	% of 2×10^6 Bacterial inoculum viable at 2 h
10% Fresh normal serum	18
10% FAS	> Inoculum
10% Heated normal serum (56°C × 30 min)	> Inoculum
10% Heated normal serum + 10% FAS	37
10% Heated CGL antiserum	87
10% Heated CGL antiserum + 10% FAS	48
10% Heated 03 antiserum	32
10% Heated 03 antiserum + 10% FAS	6
1% Heated normal serum + 10% FAS	> Inoculum
1% Heated CGL antiserum + 10% FAS	94
1% Heated 03 antiserum + 10% FAS	11

Glycogen-induced, rabbit peritoneal leukocytes (>95% granulocytes) were tested with serum from a normal rabbit, a rabbit immunized with CGL, and heat-killed *S. marcescens* 03. FAS was normal serum twice absorbed with 10^9 live *S. marcescens* 03. Percent survival is the mean of at least four determinations.

Fresh normal serum at a 10% concentration supported a greater than 80% reduction in the inoculum but fresh serum absorbed with bacteria (FAS) as well as normal heated serum at the same concentration possessed no opsonizing activity. However, FAS combined with both 10% (final concentration) heated normal serum or heated CGL serum had modest opsonic activity which effected a reduction in the inoculum of 63 and 52%, respectively. The difference between the latter results was not significant and suggested that both normal and CGL antiserum contain "natural" heat-stable antibodies against the test organism. Further, 10% heated CGL antiserum, 1% CGL antiserum combined with FAS, and 1% normal rabbit serum with FAS lacked significant opsonic activity.

In contrast, heated type-specific antiserum against *S. marcescens* 03 possessed opsonic activity alone and when combined with FAS, 94% of the inoculum was phagocytized and killed. Dilution of the type-specific antiserum 1:1,000 (final concentration) in combination with FAS still promoted an almost 90% reduction in viability of the inoculum, a dilution at which heated normal or CGL antiserum had no significant opsonic effect. The CGL antiserum used in these tests had a PH titer of 1:5,120 and HA titer of 1:256 against

TABLE IV
Bactericidal Tests with E. coli 014:K7

Serum factors	% of Inoculum viable after 2 h	
	2.06×10^4 inoculum	2.27×10^6 inoculum
10% Heated normal serum (56°C × 30 min)	>100	>100
10% Heated CGL antiserum	>100	>100
10% Heated *E. coli* 014 antiserum	>100	74
10% Fresh normal serum	4	12
10% FAS	94	>100
10% Heated normal + 10% FAS	19	29
10% Heated CGL antiserum + 10% FAS	5	9
10% Heated *E. coli* 014 antiserum + 10% FAS	2	4
1% Heated normal serum + 10% FAS	98	89
1% Heated CGL antiserum + 10% FAS	32	37
1% Heated *E. coli* 014 antiserum + 10% FAS	12	8

Bactericidal tests were carried out in gelatin veronal buffer using lapine serum and log-phase bacteria at two concentrations. Percent survival is the mean of three determinations. FAS = fresh normal serum absorbed twice with 014:K7 at 4°C and once with zymosan at 17°C.

CGL; it was 1:160 and 1:32 against *S. marcescens* 03. Type-specific antisera against the latter antigen had a PH titer of 1:20,480 and HA titer of 1:1,024, it was 1:160 (PH) and 1:4 (HA) against CGL. Three absorptions of antisera against *S. marcescens* 03 with 10^9/ml boiled, washed *S. minnesota* R595 organisms failed to remove significantly opsonic activity against *S. marcescens* 03. After three absorptions of CGL antiserum with 10^9 boiled, washed *S. marcescens*, a 10% concentration of this antiserum plus 10% FAS supported only an 11% reduction in the viability of the *S. marcescens* inoculum.

Bactericidal tests with E. coli 014:K7. Two inoculum sizes, 2.06×10^4 and 2.27×10^6 organisms (mean for three determinations each), were used to assess the bactericidal activity of normal serum, CGL antiserum, and homologous antiserum against this serum-sensitive strain (Table IV). Use of the lower inoculum size was intended to minimize the role of bacterial clumping on colony counts as determined by the pour plate technique. As anticipated, use of the larger inoculum against heated *E. coli* 014:K7 antiserum showed some reduction in bacterial counts, an effect which was related to microscopic clumping. There was no major inoculum effect in other serums.

Both heated normal and heated CGL antiserum had no bactericidal effect on this organism. Fresh normal serum was highly bactericidal for the test organism and to assess the relative bactericidal activity of heated serums (complement-dependent, antibody-mediated activity) a source of heat-labile factors (FAS) was prepared by absorption as for the opsonophagocytic tests. In addition, it was necessary to absorb with zymosan to produce an FAS which lacked bactericidal activity per se but promoted killing of heated serum-containing antibodies. In this system, CGL antiserum possessed bactericidal activity greater than heated normal serum, but this was always less than an identical dilution of type-specific antiserum. A 1:320 dilution of heated CGL antiserum was the highest dilution promoting 50% or more killing of the test organism, while a 1:40,960 dilution of type-specific antiserum achieved a similar effect. The source of CGL antiserum used in these tests was identical to that used in opsonophagocytic studies. Antiserum against *E. coli* 014:K7 had a 1:512 HA titer against the homologous antigen and 1:160 PH and 1:2 HA titer against CGL.

Passive protection experiments in mice. Normal serum, CGL antiserum, CGL antiserum four times absorbed with *S. minnesota* R595, and antiserum against *E. coli* 085:H9 from dogs and rabbits were compared for their ability to passively protect mice (Table V). Serum was heat inactivated (56°C for 30 min) and pooled from five animals; reciprocal geometric mean titers against CGL and the type-specific antigen are also summarized. The results demonstrate significant protection associated with CGL immunization, although this was still less than type-specific immunization. The absorptions removed most of the protective activity.

DISCUSSION

The initial work of Subbaiah and Stocker with natural and induced "rough" mutants of salmonella species (29, 30) was the beginning of intensive biochemical, genetic, and immunologic studies during the past decade that established that most lipopolysaccharides derived from *Enterobacteriaceae* have identical or very similar core

TABLE V

Passive Protection of Mice Challenged with E. coli 085:H9

Pooled serum	Reciprocal geometric mean titer against		Reciprocal PD_{50} titer
	E. coli 085:H9	CGL	
Canine serum			
Normal	25.4	2.1	16
CGL immune	34.1	17.1	135
CGL immune, four times absorbed with *S. minnesota* R595	28.2	8.9	31
E. coli 085:H9 immune	218	3.0	750
Rabbit serum			
Normal	14.6	2.2	3
CGL immune	3.6	45.8	78
CGL immune, four times absorbed with *S. minnesota* R595	3.2	7.7	18
E. coli 085:H9 immune	1,427	3.4	360

Each serum was pooled from five animals with known titers against CGL and *E. coli* 085:H9. PD_{50} titer was estimated by the Reed-Muench technique (27).

regions (7, 8). Antibodies raised against core antigens, particularly the lipid A component, appear to be the basis for cross-protective immunity as the "toxic" properties of lipopolysaccharides are likely due to Lipid A (31, 32).

In this study we observed that administration of CGL antigen (KDO-Lipid A) alone triggers hypotension and can cause death, confirmed that active immunization will protect the host against death, hypotension, and pyrogenicity after a heterologous bacterial challenge, and demonstrated that this protection could be passively transferred by heated serum. These results are consistent with those previously reported in experimental studies of this or closely related core antigens (10, 33–37).

A remarkable finding, however, was that both immunized and control dogs challenged with large inocula of two serum-resistant strains rapidly cleared injected organisms at rates which were not significantly different. This finding was further confirmed when one of these strains was tested in rabbits. Nevertheless, a consistent and striking difference between control and immunized canines was the protection against hypotension after challenge. These differences were most apparent between the 1st and 3rd h of observation. It should be noted that after injection of live bacteria transient hypotension was often observed. This seemed anaphylactic in nature, as noted in other canine experiments (38, 39), and may have been related to the finding of high antibody titers against some of the challenge strains. After unassisted correction of the blood pressure within minutes, a more consistent decline in both systolic and diastolic blood pressure was observed between the 1st and 2nd h, as has also been noted in canine studies (39). Blood pressure tended to recover between the 4th and 6th h after challenge with some animals dying during the acute administration phase and the remainder dying approximately within the first 24-h period after challenge. Regardless of whether animals were rendered leukopenic with cyclophosphamide or had normal white counts, challenge with serum-resistant bacteria resulted in protection of CGL-immunized animals against the hemodynamic sequelae of bacteremia without evidence of enhanced intravascular clearance.

The protective effect of CGL immunization was additionally assessed after challenge with a serum-sensitive organism, *E. coli* 014:K7. Protection against hypotension and death was evident when inocula of this organism, comparable in size to *E. coli* 085:H9 or *S. marcescens* 03, were used. Additionally, intravascular clearance was consistently more rapid in the CGL-immunized group, though the difference was small. The release of endotoxins was suggested by the very precipitous decline in venous blood colony counts followed by rapid onset of hypotension in controls. After an initial decline, bacterial counts in blood of controls increased and all but one of control animals died, for the highest mortality observed in any group. Thus, while most organisms isolated from human bacteremic infections are serum-resistant (40), serum-sensitive organisms can cause hypotension and death and CGL immunization was also protective. The mechanism of this protection appeared to be both enhanced bactericidal activity and the ability to neutralize the effect of circulating endotoxin.

These animal experiments have provided us with the opportunity to assess the relative protective roles of type-specific vs. cross-reactive (anti-CGL) antibody. For the 26 CGL-immunized dogs challenged with *E. coli* 085:H9, *S. marcescens* 03, or *E. coli* 014:K7, the mortality was 11% compared to the 0% mortality for the animals immunized with challenge strains and 62% in control animals. Intravascular clearance was significantly more rapid in animals immunized with the challenge strains (type-specific protection).

The protective value of homologous immunization with O-antigen-bearing organisms has been supported by many animal experiments (35, 41, 42). The initial serologic studies of McCabe et al. in human gram-negative septicemia indicated that anti-0-antibody titers were not protective against shock and death in contrast to antibody against CGL (9). One problem in human studies is that it is not always possible to precisely date the onset of infection and it is uncertain how "initial" antibody titers are acquired, though presumably this is due to antecedent or chronic infection. This emphasizes the need for careful animal studies whereby the timing of immunization relative to challenge can be precisely

assessed. Thus, in mouse protection studies, McCabe also showed that active immunization with CGL derived from *S. minnesota* R595 was considerably less effective than type-specific immunization (10). Our data from canine, lapine, and murine experiments support the latter conclusion. Protection was greater in homologously immunized animals than in CGL-immunized subjects but the former had lower antibody titers against CGL antigen. The immunizing preparations were obviously not comparable and we did not establish whether repetitive immunization with CGL could approach the levels of protection afforded by type-specific immunization. We did note that many doses of CGL were needed to engender significant rises in antibody to a range which was reliably protective.

Our in vitro studies using lapine granulocytes and serum factors yielded information which was consistent with the results of animal challenge studies. When serum-resistant bacteria were used, the opsonic effect of CGL antiserum was negligible considering the inherent variability of results (±15%) using this technique. Although some evidence exists from experiments using mouse peritoneal exudates that antibodies against CGL (43) or the Lipid A (32) component of CGL enhance phagocytosis of bacteria, our opsonophagocytic system employing rabbit polymorphonuclear leukocytes and serum factors showed this to be a minor effect. There are obvious differences in experimental methods between this and preceding studies, particularly the cell type used (rabbit neutrophils vs. mouse peritoneal macrophages) and the fact that the rabbit has a more effective complement-mediated bactericidal system than the mouse (12). Further, other investigators used a system which measured disappearance of bacterial cells from the peritoneal cavity of infected animals (32) as opposed to these experiments which quantitated uptake and killing by phagocytic cells in vitro. We were able to demonstrate that high titered CGL antiserum has slight bactericidal activity against a serum-sensitive organism, but like the opsonic effect, this was present only at low dilutions. Perhaps more important, type-specific antiserum was more than 100 times more effective as an opsonin and bactericidin than CGL antiserum, while possessing a significantly lower titer of antibody against the CGL antigen.

Taken together these experiments suggest that CGL immunization does not have a marked effect in enhancing uptake of bacteria by host phagocytic cells or promoting complement-mediated bacterial killing, but acts directly to abort pathophysiologic events that may be triggered by endotoxin. Although we did not carry out studies to assess activation of the blood coagulation and kinin systems after administration of gram-negative bacteria, the role of antibody against CGL appears to be more that of an antitoxin rather than opsonin or bactericidin. While CGL antibody may neutralize some of the biologic effects of endotoxin, it seems to be of limited potential in preventing multiplication of gram-negative bacteria within the host, particularly those serum-resistant, O-antigen-bearing organisms which are usually invasive (40, 44).

Our findings do not mitigate against the potential protective role of cross-reactive antibodies, however, for even though they may be less potent on a relative basis than type-specific antibody, their protective ability has clearly been shown in these and other studies. Further, the finding that the bulk of CGL antibody in normal human serum or in patients convalescing from gram-negative bacteremia is IgG- (45) and IgG-specific, and that CGL antibody is more strongly associated with protection than IgM (46), gives encouragement to attempts at passive protection since available processes yielding gamma globulin for human use result in a product which is almost exclusively IgG rather than IgM. It seems clear from these studies that cross-reactive antibody is "second-best" antibody relative to type-specific antibody, but this should not detract the need to assess the feasibility of protecting high risk human patients by immunization with CGL or related antigens, for these antigens seem to be considerably more versatile immunogens.

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