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Effect of Immune Serum or Polymyxin B on *Escherichia coli*-Induced Inflammation and Vascular Injury

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Bacterial invasion of the tissues often stimulates a vigorous inflammatory reaction, which may limit the spread of microorganisms but may also be accompanied by serious vascular injury and tissue damage. We previously studied the inflammatory reaction induced by the injection of killed *Escherichia coli* into rabbit skin, a model suitable for the quantitation of various parameters of inflammation. Here we report the effect of immune serum treatment of the *E. coli* on their capacity to induce inflammation and vascular injury. Injection of killed *E. coli* treated with immune serum elicited a reaction which had a smaller increase in vascular permeability (protein exudation), measured with ^{125}I-labeled albumin, less increase in blood flow, measured with ^{86}RbCl, less leukocyte infiltration, measured with ^{51}Cr-labeled leukocytes, and a lesser degree of hemorrhage, measured with ^{59}Fe-labeled erythrocytes, than *E. coli* treated with nonimmune serum. Crossover experiments with four different *E. coli* serotypes and four different antisera indicated that antibody to specific O antigens or a related antigen, but not to K or H antigen, was important for modifying the inflammatory response. Treatment of four different *E. coli* serotypes with antiserum to "core" glycolipid, produced by immunization with the *E. coli* J5 mutant, inhibited the inflammatory response to all four *E. coli* serotypes. Finally, treatment of killed *E. coli* with polymyxin B also inhibited their inflammation-inducing potential. These results suggest that it may be possible to diminish the magnitude of local vascular and tissue injury associated with *E. coli* infections by the use of antisera or polymyxin B, which bind to endotoxin on the *E. coli*.

Bacterial invasion of tissues often evokes a severe inflammatory response. Such a response and the subsequent phagocytosis and killing of the bacteria by the infiltrating polymorphonuclear leukocytes (PMNLs) are important factors limiting the spread of the microorganisms (1, 24). However, this type of a response may be accompanied by local edema, vascular injury, and hemorrhage (4, 21). When these processes occur in a vital organ such as the central nervous system, serious tissue damage and neurological sequellae may result (7, 9). We have recently studied the inflammatory reaction elicited by *Escherichia coli*, a bacterium that is a major cause of newborn infection and meningitis. Meningitis caused by this organism is accompanied by a high incidence of central nervous system sequellae even with appropriate antibiotic therapy (2, 19).

We have found, in a rabbit model particularly suitable for quantitating inflammatory reactions, that injection of killed *E. coli* into rabbit skin induces severe inflammation accompanied by local protein exudation (permeability), increase in local blood flow, massive PMNL infiltration, and, shortly thereafter, extensive vascular injury with hemorrhage (17). Because of the seriousness of *E. coli* infections in humans and the severe inflammatory reaction induced by this pathogen under clinical and experimental conditions, we have investigated approaches which may modify this reaction and perhaps shed light on the bacterial and host factors that influence the reaction. Here, using the rabbit model, we report the effects of immune serum and polymyxin B on *E. coli*-induced inflammation and vascular injury.

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MATERIALS AND METHODS

Bacteria and antisera. All *E. coli* strains used were clinical isolates. Three of the strains were serum resistant (30% serum) and possessed K1 capsular antigen (O1:K1:H7, O:18_{ac}:K1:H7, and O7:K1), and one was a serum-sensitive stool isolate (O55:K59). A spontaneous mutant of O1:K1:H7, which lacked K1, that is, O1:K_{neg}:H7, was a kind gift from F. Orskov (Serumstaatsinstitut, Copenhagen, Denmark). These bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C

and then used live, Formalin killed (0.5% for 4 h), or boiled for 2.5 h in phosphate-buffered saline (PBS) as indicated. To raise antisera to "core" glycolipid, the UDP-galactose epimerase-deficient *E. coli* J5 mutant (kind gift from Peter Elsbach, New York University, New York, N.Y., and Loretta Leive, National Institutes of Health, Bethesda, Md.), which does not synthesize O antigen (10), was used. This strain was cultured in Davis minimal medium (6) to grow the rough mutant or in Davis medium with 5 mM galactose to grow the O111 phenotype (34). The bacteria were washed twice in PBS, and the number was adjusted spectrophotometrically at 540 nm, using a standard curve that was verified by pour plate colony counts.

Antisera to the various serotypes were raised by immunizing rabbits intravenously (i.v) at weekly intervals with 2.5×10^7 Formalin-killed *E. coli*, boiled J5, or boiled O111:B4 for 3 weeks, then 5×10^7 colony-forming units (CFU) at week 4, and 5×10^7 CFU of live *E. coli*, boiled J5, or boiled O111:B4 for 2 more weeks. One week later, serum was collected and tested for antibody as described below. All sera were heat inactivated (56°C, 30 min) before use. Antiserum specific for the K1 polysaccharide was produced by adsorption (90 min, room temperature) of anti-O1:K1:H7 serum with an excess (0.3 ml/10^{10} CFU) of the Formalin-killed K1-deficient mutant (O1:K_{neg}:H7).

Inflammation in the rabbit skin was induced by intradermal (i.d.) injection, on the clipped back of a rabbit, of 10^8 Formalin-killed *E. coli* or 3×10^7 live *E. coli*. These doses were previously determined to induce a reproducible reaction, with significant vascular injury and hemorrhage (unpublished data). Bacteria for skin injections were preincubated with saline or heat-inactivated nonimmune serum (prebleed) or immune serum (approximately 3 agglutinating units or 0.5 to 2% serum) for 60 min at room temperature. They were then washed with 50 volumes of pyrogen-free saline ($3,000 \times g$, 20 min), resuspended in saline by using a Vortex mixer, and injected i.d. in 0.2 ml with a 30-gauge by 0.5-in. (1.27-cm) needle.

Measurement of hemorrhage. Hemorrhage was quantitated with ^{59}Fe-labeled transfused erythrocytes (RBCs) as described previously (16). Briefly, RBCs were labeled with ^{59}Fe by i.v. injection of 300 μCi of ^{59}Fe-ferrous citrate (New England Nuclear Corp., Lachine, Quebec). Three days later the blood obtained from such a "donor" rabbit contained 99% of the radioactivity in the RBC fraction. Blood was then collected into acid-citrate-dextrose anticoagulant, and approximately 12×10^6 cpm of RBCs, equivalent to 15 to 20 ml of blood, was transfused i.v. into blood group-compatible 2- to 2.5-kg female New Zealand white rabbits, which were bled for the same volume immediately before transfusion. A few hours after transfusion, the skin sites were injected i.d. Before sacrifice, 1 ml of venous blood was collected to determine the hematocrit and the amount of radioactivity circulating per milliliter of blood. After the rabbit was killed by an i.v. overdose of sodium pentobarbital, the skin of the back was removed and the blood in the large vessels was drained. Radioactivity in these sites was measured by a Packard gamma counter. The quantity of blood in the sites was calculated (microliters of blood equivalents per site) and adjusted to an average hematocrit of 40%.

Measurement of leukocyte infiltration. For the quantitation of leukocyte infiltration at the skin injection sites, ^{51}Cr-labeled leukocytes were used as described previously (12). Briefly, the rabbit receiving the skin injections was bled from the central ear artery for 30 ml of blood into 0.2% EDTA and 3 ml into acid-citrate-dextrose. One volume of 1% hydroxyethylcellulose (Polysciences Inc., Warrington, Pa.) was mixed with 4 volumes of EDTA-blood at 37°C to sediment the RBCs. The leukocyte-rich plasma was harvested and centrifuged ($200 \times g$, 10 min), and the leukocyte-RBC pellet was incubated for 30 min at 37°C in 4 ml of Ca^{2+}-Mg^{2+}-free Tyrode solution containing 10% autologous acid-citrate-dextrose plasma and 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear). After the incubation, the ^{52}Cr-labeled leukocytes were washed in Ca^{2+}-Mg^{2+}-free Tyrode solution and injected i.d. back into the donor rabbit. It has been shown that this type of leukocyte preparation gives results comparable to those of a highly purified PMNL preparation isolated by hydroxyethylcellulose and Percoll (Pharmacia Fine Chemicals, Dorval, Que.) density gradient separation (12). This is true for inflammatory reactions in which histology confirms that more than 90% of the infiltrating leukocytes are PMNLs, as was the case in the lesions studied here. For kinetic experiments, the rabbit was injected i.d. with *E. coli* at various times before labeled leukocyte injection. For cumulative measurements of leukocyte infiltration, the i.d. *E. coli* injections were given at the time of leukocyte injection (see below).

Measurement of vascular permeability. Exudation due to enhanced vascular permeability was quantitated with rabbit serum albumin (Sigma Chemical Co., St. Louis, Mo.) labeled with ^{125}I (New England Nuclear) as described previously (32). Skin sites were injected at various times (see below), and 20 min before sacrifice, the animals were injected i.v. with 5 μCi of ^{125}I-labeled albumin per kg.

Measurement of blood flow. Blood flow was measured with ^{86}Rb as previously described (11). Briefly, at the time of sacrifice, 75 μCi of ^{86}RbCl (New England Nuclear) was injected i.v. Forty-five seconds later an overdose of sodium pentobarbital and 10 ml of saturated KCl solution were injected by the same route.

***E. coli* i.d. injection protocol.** Two injection protocols were used for studying the inflammatory reaction to *E. coli*. The kinetics of the development of the reaction was followed by giving i.d. injections in quadruplicate at various times before sacrifice in the same rabbit. For example, sites were injected 6.5, 4.5, 3, 1.5, and 0.75 h before sacrifice so that at death lesions of different ages, and thus various stages of evolution, were present. In the kinetic experiments, leukocyte infiltration was measured by administering the ^{51}Cr-labeled leukocytes i.v. 60 min before sacrifice, permeability was measured by injecting the ^{125}I-labeled albumin 20 min before sacrifice, and blood flow was measured by injecting ^{86}RbCl in the last 45 s. In this way, in the lesions the content of ^{51}Cr was the rate of accumulation of labeled leukocytes per hour, the content of ^{125}I was the rate of albumin extravasation in 20 min, and the content of ^{86}Rb was the blood flow at the time of sacrifice. With the kinetic protocol, these three parameters of inflammation were measured simultaneously in the same animal, and the age of the lesions was expressed as the mean to the nearest

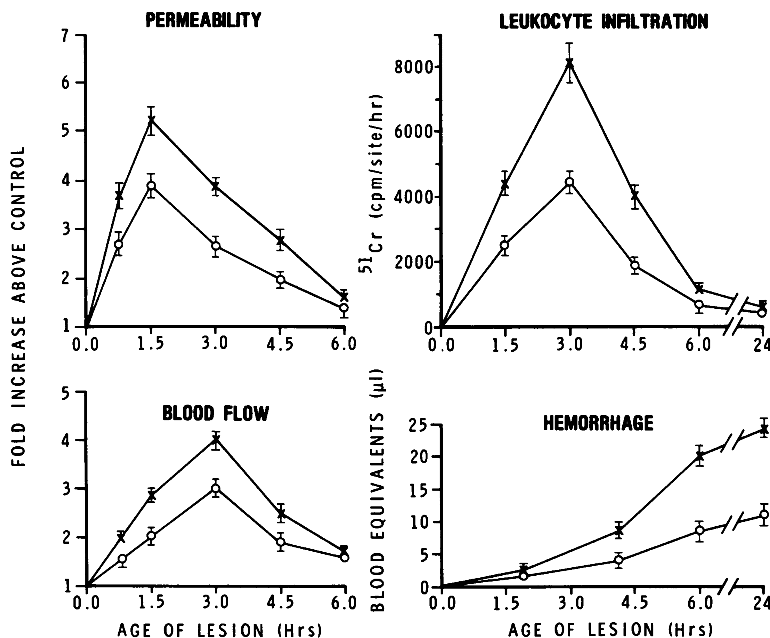

FIG. 1. Effect of immune serum on *E. coli*-induced inflammation. Killed *E. coli* O1:K1:H7 bacteria were treated (60 min, room temperature) with nonimmune (prebleed) serum (×) or immune (anti-O1:K1:H7) serum (O) and then washed. These *E. coli* were then injected i.d. in saline (10^8 CFU/0.2 ml) at various times so that lesions of different ages were present at the time of sacrifice. The parameters were measured as described in the text. Control sites were injected with saline. These sites contained 80 cpm of ^{51}Cr, 280 cpm of ^{125}I, 235 cpm of ^{86}Rb, and 150 cpm of ^{59}Fe, equivalent to 1.2 µl of blood, and these values were not influenced by the time of saline injection. The blood content of control sites were subtracted from the hemorrhage values of the lesions. Points are means ± standard errors of quadruplicate sites in one representative experiment of three.

15 min at the time that the measurements were performed.

Hemorrhage was measured as a cumulative parameter rather than as a rate because, unlike the case with ^{51}Cr-labeled leukocytes, ^{125}I-labeled albumin, or ^{86}RbCl, the specific activity of the ^{59}Fe-labeled RBCs in the circulation of these animals did not change significantly over the 24-h period of the experiment. Thus, the ^{59}Fe content of the lesions was a measure of the labeled RBCs accumulating during the whole course of the reaction. In addition to the kinetic experiments, leukocyte infiltration could be measured in a cumulative fashion simultaneously with hemorrhage by injecting the sites with the *E. coli* at the time of i.v. ^{51}Cr-labeled leukocyte injection. The reactions were allowed to develop for 4.5 h, after which time rabbits were sacrificed.

In all of these experiments, 36 to 40 skin sites were injected in a random fashion so that control *E. coli* and test *E. coli* lesions were present on the same animal for the same time periods. Control experiments showed that this many *E. coli* injections did not alter the reaction in any individual lesion regardless of the time of injection. The content of ^{51}Cr, ^{125}I, ^{86}Rb, and ^{59}Fe in the lesions was analyzed with a Packard Auto-Gamma spectrometer. Corrections were made for the spill of radioactive emissions into adjacent channels. ^{86}Rb and ^{59}Fe were not used in the same animal because of spectral overlap.

Antibody determinations. The antibody titer in the antisera was determined by bacterial agglutination, performed by serial 50-µl dilutions of antiserum in PBS in U-bottom microtiter plates (Falcon Plastics, Oxnard, Calif.) and addition of 50 µl of 1.5×10^8 CFU of Formalin-killed (containing K, O, and H antigens) or boiled (lacking K and H) *E. coli* per ml (22). Readings were performed after 18 h at 4°C. In addition, a modification of an enzyme-linked immunoassay described by Polin and Kennett (23) was used to detect antibodies to core glycolipid. Disposable flat-bottom 1.5-ml polyvinylchloride vials (Chester Plastics, Chester, N.S.) were treated sequentially with 0.75 ml of poly-L-lysine (0.001% × 2 h, room temperature) and washed in PBS. Boiled or live *E. coli* (4×10^7 CFU) in 1 ml were then added, centrifuged at 2,000 rpm for 20 min, fixed by addition of 1 ml of 1% glutaraldehyde (Polysciences Inc.), and incubated for 30 min at room temperature. The glutaraldehyde was decanted and followed by addition of 1% bovine albumin (Sigma Chemical Co.) in 0.1 M glycine buffer, pH 7.6. Finally, the vials were washed and allowed to dry. Testing of antisera was performed by making threefold dilutions in 0.1% bovine serum albumin–PBS and incubating 0.75 ml in the bacteria-coated vials for 60 min at room temperature. After extensive washing with PBS, 0.75 ml of a 1:1,000 dilution of peroxidase-labeled goat anti-rabbit gamma globulin or goat anti-rabbit immunoglobulin G (IgG) (gamma chain specific) or IgM (mu chain

specific) (Cappel Laboratories, Cochranville, Pa.) was added and incubated for 60 min at room temperature. After extensive washing, the peroxidase activity was determined, using O-dianisidine substrate (Sigma Chemical Co.) (28). The reaction was linear up to an absorbance of 0.350 at 460 nm. Therefore, titers are extrapolated and expressed as the greatest dilution giving 0.150 absorbance.

Immunization with a given *E. coli* serotype resulted in agglutinating antibody titers to K and H antigens of 1:64 to 1:256. The titer to the O antigen of the immunizing strain, as determined by agglutination of boiled *E. coli* (lacking K and H), was in the range of 1:2,500 to 1:10,000. The agglutination titer in nonimmune (prebleed) sera to any of these antigens did not exceed 1:4 to 1:16. However, in several instances, immunization with one serotype caused a significant (fourfold or greater) rise in agglutinating titer against one or more of the other three boiled *E. coli* serotypes. Therefore, these "cross-reacting" antibodies were removed by a single adsorption (90 min, room temperature) of the antiserum with an excess (0.3 ml per 3×10^9 boiled *E. coli*) of the boiled cross-reacting strains. Such adsorption did not significantly decrease (less than fourfold) the titer against K, H, and O antigens of the serotype used for immunization. The sera of rabbits used for the *E. coli* skin injections were screened for agglutinating titers against the test *E. coli*. Animals with titers in excess of 1:16 were not used.

Fractionation of antiserum. Some antisera were fractionated by sucrose density gradient centrifugation (kindly performed by R. S. Faulkner), as previously described for the separation of 7S from 19S rubella antibodies (8), except that the sera were not adsorbed with chicken RBCs. Removal of IgG from the antiserum was also performed by passing 2 ml through a 2-ml column of protein A-Sepharose 4B (Pharmacia Fine Chemicals) as recommended by the manufacturer.

RESULTS

Effect of immune serum on the kinetics of *E. coli* inflammation. Figure 1 shows the kinetics of four parameters of inflammation during the reaction induced by the intradermal injection of Formalin-killed *E. coli* O1:K1:H7. Bacteria pretreated with nonimmune serum evoked a rapid increase in the vascular permeability, blood flow, and leukocyte infiltration within the first hour after injection. These parameters peaked in 1.5-h-old (permeability) to 3-h-old (blood flow and for leukocyte infiltration) lesions. Changes in all three of these parameters then diminished and were close to base-line values when the lesions were 6 h of age. Between 2 and 6 h after injection of *E. coli*, RBC extravasation and hemorrhage developed. The maximum degree of hemorrhage was reached by 6 h. These results are similar to the reaction elicited by killed *E. coli* that were not serum treated, as described in a previous study (17). Figure 1 also shows the effect of pretreating *E. coli* with approximately 3 agglutinating units (1%) of heat-inactivated antiserum to O1:K1:H7. The antiserum-treated bacteria induced milder reactions in permeability, blood flow, and leukocyte infiltration, and, most important, significantly less vascular injury and hemorrhage was observed.

Effect of antibody to K, H, or O antigens on inflammatory response induced by killed *E. coli*. The antiserum used in Fig. 1 contained antibodies reactive with at least three known antigenic structures on the *E. coli*, namely, the capsular polysaccharides (K), flagellum (H), and endotoxin (O) (22). To determine whether antibody to one of these components of the bacterial surface was responsible for the modification of the inflammatory reaction, various antisera were used to treat a variety of *E. coli* serotypes before injection (Table 1). Measurements were limited to leukocyte infiltration and hemorrhage, because previous work had shown a direct correlation between the degree of PMNL infiltration and the severity of the vascular injury (13, 14; H. Z. Movat, M. M. Kopaniak, A. C. Issekutz, and B. J. Jeynes, Proc. 4th Int. Congr. Immunol., abstr. 15.8.10, 1980). In contrast, the increases in vascular permeability and blood flow are transient and reversible phenomena, which are less clearly associated with irreversible vascular damage.

It can be seen from Table 1 that inhibition of PMNL infiltration (by 36 to 54%) and hemorrhage (by 55 to 72%) occurred only when the challenge *E. coli* was treated with antiserum containing antibody directed at the specific O antigen. It should be noted that this was the case even though all of the K1 challenge strains were agglutinated by each of the three anti-K1-specific antisera.

The effect of anticapsular or flagellar antibody was further investigated by adsorbing out the K and H antibodies with Formalin-killed *E. coli* containing K1 and H7 but differing at the O antigen. Table 2 shows the effect of such adsorption on the ability of the antisera to modify the PMNL infiltration and hemorrhage induced by two K1- and H7-bearing *E. coli* strains. It can be seen that the adsorption of K and H antibodies to the point where none were detectable by agglutination (<1:2) did not alter the capacity of the serum to inhibit PMNL infiltration (40 to 45%) and hemorrhage (60 to 75%) when the antiserum contained antibodies to the O antigen of the challenge *E. coli* strain. The effect of antibody to K1 capsular antigen was tested directly by treating the bacteria with an anti-K1 antiserum. This antiserum was prepared by adsorbing antiserum raised against O1:K1:H7 with a Formalin-fixed mutant of this strain, which appears to differ only in that it does not synthesize K1 antigen (O1:K_{neg}:H7). After such adsorption, the antiserum retained an agglutination titer of 1:128 against Formalin-fixed O1:K1:H7, but it did not agglutinate (<1:2) these *E. coli*

TABLE 1. Modification of the inflammatory response to various strains of killed *E. coli* by various antisera[a]

Antiserum treatment	Inhibition (%) of response to killed *E. coli*[c]				
	Response[b]	O1:K1:H7	O18$_{ac}$:K1:H7	O7:K1	O55:K59
Anti-O1:K1:H7	PMNL	48 ± 5	10 ± 8	10 ± 5	ND
	Hemorrhage	72 ± 6	5 ± 3	15 ± 5	8 ± 3
Anti-O18$_{ac}$:K1:H7	PMNL	8 ± 8	54 ± 5	9 ± 3	ND
	Hemorrhage	10 ± 6	55 ± 5	2 ± 1	0 ± 2
Anti-O7:K1	PMNL	0 ± 3	14 ± 7	40 ± 7	ND
	Hemorrhage	8 ± 5	23 ± 7	62 ± 6	8 ± 4
Anti-O55:K59	PMNL	8 ± 3	3 ± 5	ND	36 ± 3
	Hemorrhage	12 ± 5	15 ± 7	5 ± 5	56 ± 9

[a] Formalin-killed *E. coli* (10^9/ml) were incubated (60 min, room temperature) with 3 agglutinating units (0.5 to 2%) of specific antiserum, of cross-reacting (with "K" or "H" antigens) antiserum (5 to 15%), or the corresponding nonimmune (prebleed) serum at the same concentration. The *E. coli* isolates were washed, and 10^8 (0.2 ml) were injected i.d. into rabbits.

[b] PMNL infiltration was measured cumulatively during the first 4.5 h, using ^{51}Cr-labeled leukocytes. Hemorrhage was measured simultaneously in 6.5-h-old lesions with ^{59}Fe-labeled RBCs (see text). Control (nonimmune) serum *E. coli* lesions contained an average of 6,110 cpm of ^{51}Cr and 1,805 cpm of ^{59}Fe, equivalent to 18 μl of blood. Saline-injected sites contained 80 cpm of ^{51}Cr and 110 cpm of ^{59}Fe.

[c] Results are expressed as percent inhibition due to antiserum treatment relative to nonimmune serum treatment of the *E. coli*. Values are means ± standard errors of at least three experiments performed in quadruplicate. ND, Not determined.

isolates after they were boiled to destroy K1 and H7 (O1). Table 2 shows that this anti-K1 serum did not modify the PMNL infiltration and hemorrhage induced by O1:K1:H7 even though the equivalent of 10 agglutinating units of antiserum was used to treat the bacteria.

Class of antibody influencing the inflammatory response to *E. coli*. Antiserum to O1:K1:H7 was fractionated into 7S and 19S antibodies by sucrose gradient centrifugation to determine the type of antibody responsible for inhibiting the inflammatory reaction induced by the *E. coli*. Antibody to O antigen, as detected by agglutination of boiled O1:K1:H7, was present in both the 7S and 19S fractions (Table 3). Not shown is that two fractions collected from the middle of the gradient, corresponding to the region with less than 19S but greater than 7S sedimentation, had agglutinating titers of only 1:16, indicating relatively good separation of the 7S and 19S peaks. When 7S and 19S fractions were used to treat O1:K1:H7 *E. coli* before injection, both fractions were found to inhibit PMNL infiltration and the hemorrhagic reaction. Because it was thought important to ensure that the observed effect of the 19S IgM antibodies was not due to

TABLE 2. Effect of antiserum adsorption on modification of the inflammatory response to killed *E. coli*[a]

Antiserum	Adsorbing strain	Response[b]	Inhibition (%) of response to killed *E. coli*[c]	
			O1:K1:H7	O18$_{ac}$:K1:H7
Anti-O1:K1:H7	O18$_{ac}$:K1:H7	PMNL	40 ± 5	
		Hemorrhage	60 ± 8	
Anti-O18$_{ac}$:K1:H7	O1:K1:H7	PMNL		45 ± 4
		Hemorrhage		75 ± 5
Anti-O1:K1:H7	O1:K1:H7 (K-negative mutant of O1:K1:H7)	PMNL	5 ± 5	
		Hemorrhage	7 ± 7	

[a] Antisera were adsorbed (90 min at 4°C) until no agglutinating antibody against the adsorbing strain was detectable. Killed *E. coli* O1:K1:H7 or O18$_{ac}$:K1:H7 were treated with similarly adsorbed nonimmune serum (control) or 3 agglutinating units of adsorbed antiserum before i.d. injection (10^8/0.2 ml), as in Table 1.

[b] Responses were measured simultaneously, as in Table 1.

[c] Results are percent inhibition relative to control, nonimmune serum-treated *E. coli*. Values are means ± standard errors of three rabbits performed in quadruplicate.

TABLE 3. Effect of antiserum fractions on inflammatory response to killed *E. coli*[a]

Anti-O1:K1:H7 fraction	O-antigen agglutination titer	Inhibition (%) of response to *E. coli* O1:K1:H7[b]	
		PMNL	Hemorrhage
7S	1:256	39 ± 4	76 ± 3
19S	1:128	47 ± 13	68 ± 8
19S after protein A-Sepharose[c]	1:64	41 ± 5	52 ± 6

[a] Anti-O1:K1:H7 serum and nonimmune serum were fractionated on linear sucrose gradients. The fractions were then used to treat (60 min, room temperature) killed *E. coli* O1:K1:H7 as in Table 1.

[b] Results are expressed as the percent inhibition of the responses relative to *E. coli* treated with fractions of nonimmune serum. Values are means ± standard errors of four experiments performed in quadruplicate.

[c] Antiserum was passed through a column of protein A-Sepharose 4B and the 19S fraction was separated by sucrose gradient centrifugation.

contamination by small amounts of 7S IgG, these experiments were repeated by using procedures to minimize this possibility. Two milliliters of this antiserum was therefore first passed through a 2-ml column of protein A-Sepharose 4B to adsorb out nearly all of the IgG. The material that passed through the column was then applied to the sucrose gradient. Agglutination titers performed with these sucrose fractions indicated no agglutinating antibody in the 7S fraction, whereas the 19S fraction contained a titer of 1:64. This doubly fractionated 19S antibody still inhibited the two inflammatory parameters under study (PMNL infiltration, 41%; and hemorrhage, 52%).

Effect of polymyxin B on *E. coli*-induced inflammation. The results of the above experiment suggested that antibodies reacting with the O antigen of the endotoxin moiety on the *E. coli* surface could diminish the severity of inflammation induced by Formalin-killed *E. coli*. This prompted us to test the effect of polymyxin B, a drug that is known to bind to the lipid A moiety of endotoxin and neutralize many of the biological actions of these molecules (5, 20, 25, 26). Figure 2 compares the development of the four parameters of inflammation after the injection of Formalin-killed $O18_{ac}$:K1:H7 *E. coli* treated with either saline or polymyxin B (50 μg/ml, 60 min, room temperature) and washed. It can be seen that the polymyxin B treatment of the *E. coli* diminished the degree of vascular permeability, blood flow, leukocyte infiltration, and

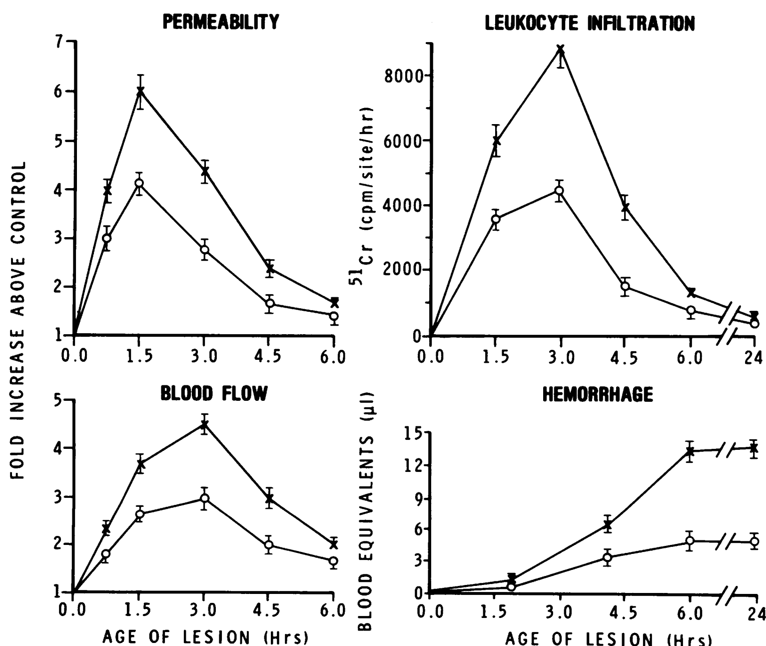

FIG. 2. Effect of polymyxin B on *E. coli*-induced inflammation. Killed *E. coli* $O18_{ac}$:K1:H7 bacteria were treated for 60 min at room temperature with saline (×) or 50 μg of polymyxin B per ml (O) and then washed. The bacteria were then injected i.d. in saline (10^8 CFU/0.2 ml) at various times so that lesions of differing ages were present at the time of sacrifice. Inflammatory parameters and control injections were described in the text. Control sites contained 260 cpm of ^{125}I, 210 cpm of ^{86}Rb, 75 cpm ^{51}Cr, and 125 cpm ^{59}Fe, equivalent to 1.1 μl of blood. Points are means ± standard errors of quadruplicate sites in one representative experiment of three.

TABLE 4. Modification of the inflammatory response to various strains of killed *E. coli* by antiserum to core glycolipid and by polymyxin B[a]

Treatment	Response[b]	Inhibition (%) of response to killed *E. coli*[c]			
		O1:K1:H7	$O18_{ac}$:K1:H7	O7:K1	O55:K59
Polymyxin B (50 μg/ml)	PMNL	49 ± 6	30 ± 4	40 ± 5	37 ± 5
	Hemorrhage	68 ± 4	60 ± 5	72 ± 6	58 ± 7
Anti-J5 serum	PMNL	29 ± 3	37 ± 5	39 ± 6	18 ± 5
	Hemorrhage	59 ± 5	39 ± 8	57 ± 8	30 ± 8
Anti-O111 serum	PMNL	5 ± 3	6 ± 5		
	Hemorrhage	8 ± 5	5 ± 2		

[a] Killed *E. coli* (10^9/ml) were incubated (60 min, room temperature) with 4% anti-J5 serum, anti-O111 serum, or the corresponding nonimmune serum as a control, or polymyxin B (50 μg/ml) or saline as a control. The bacteria were washed, and 10^8 (0.2 ml) were injected i.d.

[b] PMNL infiltration and hemorrhage were measured as in Table 1. Control *E. coli* lesions contained a mean of 5,210 cpm of ^{51}Cr and 1,650 cpm of ^{59}Fe, equivalent to 15 μl of blood.

[c] Results are expressed as percent inhibition relative to nonimmune serum or saline treatment. Values are means ± standard errors of five experiments.

vascular injury with hemorrhage much as did antiserum treatment of the bacteria (Fig. 1).

In control experiments, zymosan particles were treated with polymyxin B or injected together with 50 μg of polymyxin B per ml. These concentrations of polymyxin B did not alter the course of the reactions of zymosan (peak leukocyte infiltration in control zymosan lesions, 4,100 cpm per site per h; polymyxin B treated, 3,910 cpm; hemorrhage in control zymosan, 10.5 μl; polymyxin B, 9.7 μl). Since polymyxin B can bind to the lipid A of most endotoxins (20), we tested the effect of this drug on all four *E. coli* serotypes. Table 4 shows that polymyxin B treatment of any of the *E. coli* serotypes inhibited the degree of PMNL infiltration (by 30 to 49%) and hemorrhage (by 58 to 72%).

Effect of antiserum to core glycolipid on *E. coli*-induced inflammation. The core glycolipid or polysaccharide region of endotoxin from a wide variety of gram-negative bacteria is antigenically similar (reviewed in 3, 18). Therefore, antiserum to this region was produced by immunizing rabbits with the *E. coli* J5 mutant, which cannot synthesize O antigen (3, 10). The anti-J5 serum was tested for cross-reacting antibody to boiled O1:K1:H7, $O18_{ac}$:K1:H7, and O7:K1, using the enzyme-linked immunoassay. This antiserum exhibited cross-reacting antibody titers against these *E. coli* strains of 1:10,000 to 1:30,000, whereas nonimmune titers were 1:40 or less. The titer of cross-reacting antibody in the anti-J5 antiserum was approximately one-third to one-fourth as high as in the antiserum produced by specific immunization, e.g., anti-O1:K1:H7 against boiled O1:K1:H7 was 1:40,000 by enzyme immunoassay. In contrast to the anti-J5 serum, such specific antisera cross-reacted weakly (1:100 to 1:300) when measured by this method.

Table 4 shows the effect on PMNL infiltration and hemorrhage of anti-J5 serum treatment of the four *E. coli* strains before i.d. injection. This antiserum inhibited the leukocyte response (15 to 39%) and the degree of hemorrhage (30 to 59%) induced by any of the four *E. coli* serotypes. Antiserum was also raised against *E. coli* J5 grown in the presence of galactose to obtain the phenotypic O111:B4 parent (34). In contrast to the anti-J5 serum, treatment of *E. coli* with this anti-O111 serum did not inhibit PMNL infiltration or hemorrhage (Table 4). Anti-J5 serum was also tested and compared with specific O antiserum for modification of the inflammatory response induced by live *E. coli*. Table 5 shows that, although anti-J5 treatment of Formalin-killed *E. coli* diminished somewhat their ability to induce leukocyte infiltration and hemorrhage, this antiserum did not modify the response elicited by live *E. coli*. By contrast, specific O antiserum treatment diminished the reaction induced by live *E. coli*, as it did with killed *E. coli*; this effect appeared to be specific for the immunizing strain and is probably related to antibody to O antigen (not shown).

DISCUSSION

Invasion of host tissues by many types of bacteria elicits a vigorous inflammatory reaction, often accompanied by tissue injury. We previously found, in a rabbit model, that these reactions are also elicited even by killed bacteria such as *E. coli* (17). Here we investigated some factors which may modify the severity of the inflammatory reaction. In most of these experiments the reaction elicited by killed *E. coli* was

TABLE 5. Modification of the inflammatory response to live E. coli by specific and core glycolipid antisera[a]

Antiserum treatment	Response[b]	Inhibition (%) of response to live E. coli[c]			
		O1:K1:H7	$O18_{ac}$:K1:H7	O1:K1	O55:K59
Anti-J5	PMNL	16 ± 12	21 ± 14	5 ± 4	14 ± 8
	Hemorrhage	15 ± 11	12 ± 5	19 ± 8	14 ± 9
Anti-challenge strain	PMNL	40 ± 7	42 ± 3	58 ± 9	ND
	Hemorrhage	50 ± 6	78 ± 6	70 ± 8	ND

[a] Live E. coli (3×10^8/ml) were incubated (60 min, room temperature) with 4% anti-J5 serum, 3 agglutinating units of antiserum to the challenge strain (0.3 to 1%), or the corresponding nonimmune serum. The bacteria were then washed, and 3×10^7 (0.2 ml) were injected i.d.

[b] PMNL infiltration and hemorrhage were measured as in Table 1. Nonimmune serum-treated E. coli lesions (control) contained a mean of 6,910 cpm of ^{51}Cr and 1,918 cpm of ^{59}Fe, equivalent to 21 μl of blood.

[c] Results are expressed as percent inhibition relative to nonimmune serum treatment of E. coli. Values are means ± standard errors of three experiments. ND, Not determined.

studied. The reason for this was because some of the modalities tested would have killed the E. coli or likely shortened their survival in vivo. Therefore, comparisons with live E. coli-injected control lesions could not have been valid. Moreover, any application of inflammation-modifying modalities under clinical conditions would likely accompany antibiotic therapy, which would rapidly kill the bacteria.

The results indicate that antiserum treatment of E. coli diminished their capacity to induce an inflammatory reaction. The magnitude of the response, using four separate parameters of inflammation (vascular permeability, blood flow, leukocyte infiltration, and hemorrhage) were significantly less than in controls treated with nonimmune serum. Experiments performed with various types of antisera and killed E. coli strains indicated that antibody to the O antigen or an antigen linked to specific O-antigenic determinants was important for the decrease in PMNL infiltration and hemorrhage. One possible explanation for these results may have been that the antiserum treatment caused agglutination of the E. coli, which was visible macroscopically, using 3 agglutinating units of antiserum. Agglutination of the bacteria would diminish the number and surface area of particles injected compared with nonimmune treated E. coli. This possibility was excluded by the finding that 3 to 10 agglutinating units of antiserum reacting with K or H antigens or both, a dose which caused similar degrees of agglutination of E. coli, did not modify the inflammatory response (Tables 1 and 2). An alternative explanation for these findings is that the O antiserum opsonized the E. coli for phagocytosis by PMNL and that this mechanism cleared the tissues of E. coli, thus diminishing the inflammatory reaction. However, antibody reacting with the K and H antigens of these E. coli is known to be opsonic as well (29, 30) and was found to promote phagocytosis and killing of E. coli by rabbit PMNL in a chemiluminescence assay (31; unpublished data). In addition, the fractionation of antiserum to O1:K1:H7 indicated that either IgG (7S) or IgM (19S) antibody treatment could modify the inflammatory response. This also would argue against the opsonization being a major factor since IgM is known to be a poorer opsonin for PMNLs than IgG (27). Thus, it appears that agglutination or opsonization of the E. coli played a minor role in the observed effect of antiserum treatment.

Since these results suggested that antibody binding to the O antigen of endotoxin on the E. coli was in some way modulating the inflammatory response, we examined the effect of modifying other regions of the endotoxin molecule in the cell wall. Polymyxin B is known to bind to the lipid A moiety of many endotoxins and neutralize many of the biological activities of this molecule (5, 20, 25, 26). Our results indicate that polymyxin B-treated E. coli of all four serotypes elicited a milder inflammatory reaction than control-treated organisms (Fig. 2; Table 4). The effect of antibody to the core polysaccharide region of the endotoxin molecule was also studied. These antibodies were produced by immunizing rabbits with the E. coli J5 mutant, which cannot synthesize O antigen but has core polysaccharide and lipid A in its cell wall (10). The core polysaccharide antigens are antigenically similar among gram-negative organisms (3, 18). Therefore, it was not surprising that anti-J5 serum cross-reacted well with the boiled E. coli of different serotypes as measured by enzyme-linked immunoassay (titer of 1:10,000 to 1:30,000). These cross-reactions have been described previously by Ito et al. (15). It was also found that anti-J5 serum treatment of any of the four E. coli serotypes resulted in inhibition of leukocyte infiltration and hemorrhage (Table 4). It thus appears that antibodies or polymyxin B

binding to three parts of the endotoxin molecule in the cell wall of *E. coli*, namely, "O" polysaccharide, core polysaccharide, or lipid A, decreases the inflammatory properties of these microorganisms. These observations suggest that endotoxin may be one factor eliciting or maintaining the inflammatory reaction to *E. coli*.

Experiments were also performed with live *E. coli*. It was found that the magnitude of leukocyte infiltration and hemorrhage induced by live *E. coli* was modified by antiserum reacting with the O antigen, as was the case with the killed *E. coli* (Table 5). However, anti-J5 serum did not reliably inhibit these parameters when live *E. coli* were tested. The reason for this discrepancy between specific and J5 antisera is not clear. It is likely that the live *E. coli* multiplied in the tissues after injection, and because anti-J5 serum is a poorer opsonin, as judged by chemiluminescence, than specific antiserum (unpublished data), the anti-J5-treated *E. coli* may have survived longer and replicated more during the 6.5 h of lesion development. This effect could overcome the inhibitory effect of anti-J5 serum.

The mechanism of action of antiserum in diminishing the magnitude of inflammation is not clear. However, a primary effect of decreasing PMNL infiltration may be responsible, because in several types of the neutrophilic inflammatory reactions the rate and degree of PMNL infiltration in tissues were found to be important determinants of the magnitude of vascular permeability and blood flow changes as well as of the severity of vascular injury and hemorrhage (13, 14, 33; Movat et al., Proc. 4th Int. Congr. Immunol., abstr. 15.8.10, 1980). The role of PMNLs and factors regulating PMNL infiltration in *E. coli* lesions is addressed in a separate report (11a). Nevertheless, our observations that or antiserum to O polysaccharide, core glycolipid, or polymyxin B can diminish the severity of inflammatory tissue damage induced by *E. coli* raise the possibility that therapy with one of these modalities in conjunction with bactericidal antibiotics may be beneficial in these types of human infection. In fact, recent clinical trials (35) using antibody to core glycolipid as an adjunct to the therapy of gram-negative bacterial infections support this view.

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