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Experimental Production of Lethal *Escherichia coli* Bacteremia of Pelvic Origin

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To reproduce the syndrome of overwhelming *Escherichia coli* bacteremia and shock after pelvic instrumentation, a model was developed by feeding *E. coli* via drinking water to coliform-free rabbits, injecting nitrogen mustard intravenously, and inserting a temperature probe into the rectum. The temperature probe was inserted to mimic pelvic instrumentation of patients and to detect fever. Rabbits fed invasive serotypes of *E. coli* all suffered overwhelming bacteremia with high fever and fatal vascular collapse secondary to invasion of pelvic veins as the granulocyte count approached zero. In the absence of granulocytopenia, the rectal temperature probe produced an intensive inflammation with numerous polymorphonuclears and bacteremia did not develop. In the absence of rectal probing, granulocytopenic rabbits developed high fever without bacteremia. This model resembles human bacteremic shock with respect to the endogenous source of the bacteria, the high frequency of bacteremia due to *E. coli* and other enteric bacilli, the importance of pelvic instrumentation, and the associated immune disturbances such as granulocytopenia.

The study of bacteremic shock needs an experimental model. Bacteremic shock in man most often develops after pelvic instrumentation and invasion of the blood stream by *Escherichia coli* or other bacilli normally resident in the colon (10). Until now, studies of bacteremia and bacteremic shock have been conducted in experimental animals by the intravenous injection of laboratory strains of living bacteria, dead bacteria, or their endotoxins. The purpose of the present work was to reproduce in animals the human form of bacteremic shock that follows pelvic instrumentation. The experiments were based on the discovery that many rabbits are naturally free of coliform bacteria (2, 15). By introducing strains of *E. coli* of known serotype and pathogenicity into the intestinal tract, it was possible to study their role in the production of fever, bacteremia, and shock of intestinal origin.

MATERIALS AND METHODS

The technique for inducing fatal bacteremia consists of three steps: feeding of *E. coli* in the drinking water of rabbits, intravenous injection of nitrogen mustard, and insertion of a temperature probe into the rectum. The *E. coli* organisms were fed to coliform-free rabbits by placing 1 ml of an 18-hr Trypticase Soy Broth (BBL) culture in the drinking water daily at 9:30 AM and 4:00 PM. The animals ingested the bacteria readily and their feces became heavily populated with *E. coli*.

The nitrogen mustard was given to lower the rabbits' resistance to infection by producing granulocytopenia and impaired serum bactericidal activity (6). Immediately after it was dissolved, nitrogen mustard was injected at 3 mg/kg into the marginal vein of the ear of albino rabbits weighing 3 to 4 kg. The rabbits were purchased locally and housed in air-conditioned quarters.

The temperature probe was inserted to mimic pelvic instrumentation of patients as well as to detect the development of fever. The glass probe of the thermistor apparatus (Fig. 1) was inserted into the rabbit's rectum for a distance of 10 cm and always removed in less than 30 sec, a period just long enough to record the temperature. The procedure was performed daily five or six times over a period of 6 hr, beginning 48 hr after the injection of nitrogen mustard. Rabbit temperatures were also recorded by placing the "banjo" probe (Fig. 1) of the thermistor apparatus against the skin of the groin. The inguinal temperatures were found to correspond almost exactly to the rectal temperatures taken simultaneously and provided a technique for measuring febrile response to nitrogen mustard without rectal trauma. Rectal temperatures were also taken with a flexible esophageal-rectal probe with a vinyl plastic tip (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) inserted 10 cm and kept in place for 6 hr daily while the rabbits sat quietly, with only slight restraint, in a wooden frame. To avoid cross-contamination, each

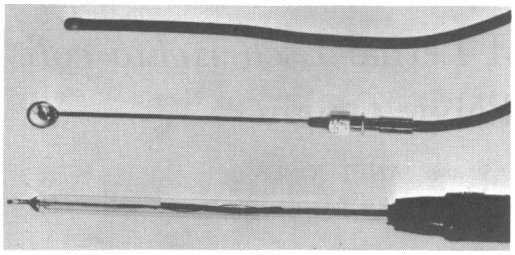

FIG. 1. *(top) Internal (esophageal-rectal) probe: 10-ft (3 m) vinyl-covered shield with phone plug tip approximately 0.5 cm in diameter. Inserted into rabbit's rectum approximately 100 mm. Probes used with model 43-TD single-channel and 44-TA multichannel telethermometers. Manufactured by Yellow Springs Instrument Co., Inc. (middle) "Banjo" surface temperature probe for taking skin temperature. Used with 43-TD single-channel telethermometer. Manufactured by Yellow Springs Instrument Co., Inc. (bottom) Thermometric element (glass-enclosed): overall length, 150 mm. Inserted approximately 100 mm into rabbit's rectum. Used with thermistor thermometer. Manufactured by by E. H. Sargent Co.*

temperature probe was sterilized in 70% alcohol before insertion.

Blood cultures were obtained by cardiac puncture through the chest wall, after sterilization of the fur and skin with tincture of iodine and alcohol; 4 ml of heparinized blood was cultured aerobically in 10 ml of Trypticase Soy Broth, and another 4 ml was cultured anaerobically by inoculation into evacuated rubber-stoppered bottles containing 10 ml of thioglycolate broth. Intestinal contents and feces were inoculated in duplicate on 10% rabbit blood-agar and on E M B Agar; one plate was incubated aerobically and the other was incubated anaerobically in a Brewer jar after it had been evacuated with a vacuum pump, filled with illuminating gas, and depleted of residual oxygen by an electric charge through the element under the lid. Aerobic cultures were incubated for 48 hr and anaerobic cultures for 5 days at 37 C.

Total and differential leukocyte counts were made from the marginal vein of the ear not used for injection of nitrogen mustard. Antibody response to endotoxin of *E. coli* was measured by a modification of the hemagglutination technique of Neter (3). Serum complement (C′) levels were assayed in duplicate by the method of Osler, Strauss, and Mayer (13).

Blood pressures were measured on the tail of the unanesthetized rabbit by the indirect method of Prioli and Winbury (14). For this we used a Brecht-Boucke (4) blood pressure marker (model FBR-2A; Biophysical Electronics Development Co., New York, N.Y.). A circular pressure cuff, originally designed for rattail blood pressures, was slipped over the tail to the base after the hair had been cut close with scissors. The condenser-microphone pulse pickup was attached to the ventral surface with moderate pressure by adhesive tape. An electrocardiograph was used as the amplifier-recorder.

RESULTS

Syndrome of experimental bacteremia. Coliform-free rabbits were divided into six groups and a different strain of *E. coli* was fed to each group (Table 1). After ingesting *E. coli* for 3 to 5 days, all rabbits received nitrogen mustard. Starting 48 hr after injection of nitrogen mustard, rectal temperatures were measured six times at hourly intervals with the glass thermistor probe; the measurements were repeated the next day.

On the second day of rectal trauma with the thermistor probe, most rabbits had fever of 1.5 F or higher. On the morning of the third day (72 hr after the start of rectal temperature measurement), when the granulocyte count approached zero, all rabbits had high fevers, usually 3 or 4 F above their base-line temperatures, and those with bacteremia suffered fatal vascular collapse. The vascular collapse was characterized by weak thready pulse, cold pale ears, hypotension (frequently), and weakness. Diarrhea was also prominent. When rectal trauma was combined with nitrogen mustard, all rabbits fed serotypes *O*:H11, O1:H4, and O7:H7 developed fever and overwhelming bacteremia due to *E. coli* (Table 1). In the absence of rectal trauma, *E. coli* O7:H7 produced slight, inconstant bacteremia and did so in only a small proportion of rabbits given nitrogen mustard.

The different serotypes varied in their ability to establish *E. coli* bacteremia (Table 1). This difference is attributed to the relative invasiveness of the organisms. Their ability to establish themselves in the rectum could not account for differences in ultimate occurrence of bacteremia because all animals developed equally heavy rectal cultures for the respective serotype of *E. coli*. Heavy growth of *E. coli* was also obtained from the liver, kidney, spleen, and lung of bacteremic animals at 96 hr, and *E. coli* was shown to be the dominant aerobic organism in the colon, ileum, and duodenum. *Bacteroides funduliformis* appeared in the blood of 32% of the rabbits. It was recovered in large numbers from the colons of those rabbits whose blood contained *Bacteroides*, but not from the colons of the remaining rabbits.

To determine whether the depression of C′ that occurs during lethal endotoxemia (8, 16) also occurs in lethal bacteremia, blood was obtained for determining C′ levels before injecting nitrogen mustard and when the temperature spiked. The blood for the C′ determinations was placed in an ice bath immediately; the serum was stored in a freezer at −70 C and later thawed at 4 C.

Animals that were fed *E. coli* O:113, and three groups that were fed no *E. coli*, were examined

TABLE 1. *Influence of serotype on development of bacteremia in rabbits fed E. coli and subjected to rectal temperature measurement after injection of nitrogen mustard*

Serotype of *E. coli* fed in water	Origin of *E. coli*	Rabbit	Degrees of fever (F) at time of blood culture	Blood culture 96 hr after injection of HN_2 (colonies of *E. coli*/ml)
O74:H19	Rabbit feces	A	5.0	24
		C	4.2	6
		D	3.4	0
		E	4.4	7
O39:H48	Rabbit feces	F	3.3	3,600
		G	3.3	0
		H	0.2	0
		I	4.0	0
		J	3.5	50
O1:H4	Rabbit feces	K	4.3	2
		L	3.1	1,500
		M	4.8	780
		N	4.3	120
		O	3.9	140
O undetermined:H11	Rabbit feces	P	4.4	780
		Q	5.6	3,660
		R	3.8	13,500
		S	5.7	14,400
		T	3.7	420
O:113	Human blood	AA	4.5	Innumerable
		BB	4.8	480
		DD	5.5	2
		FF	3.0	600
		GG	4.4	3,000
		HH	0	0
O7:H7	Rabbit blood (septicemia)	A2	5.0	Innumerable
		B2	4.0	Innumerable
		I2	5.2	900
		V2	3.5	Innumerable
		K2	4.2	60
		O2	5.0	16,000
		T2	3.6	Innumerable

for C′ levels after neutropenia was produced by nitrogen mustard (HN_2). The first group, fed *E. coli* O:113, were subjected to rectal probing. The three additional groups comprised rabbits with naturally heavy populations of *E. coli* in their stools, subjected to rectal probing; coliform-free rabbits; and coliform-free rabbits subjected to rectal probing.

The average change in C′ level in rabbits (no. 1 through 15A) undergoing lethal *E. coli* bacteremia and shock was a small rise: 14.56% (Table 2). In controls that received rectal probing but had no *E. coli* in the stool and no *E. coli* bacteremia, the average rise in C′ was 78.4% (group 4, animals 21–29). These elevated control values suggest that the proctitis of rectal probing produced a marked rise in C′ levels since such C′ elevations were not present in group 3 rabbits given nitrogen mustard without rectal probing. The mean difference in C′ levels in the bacteremic and nonbacteremic animals was: 78.4 − 14.56 = 63.84% (significant at $P < 0.001$, as calculated by Student's t test). Experimental lethal bacteremia due to *E. coli*, like lethal endotoxemia, is thus associated with low C′ levels (8).

The chief finding at autopsy was massive engorgement and thrombosis of the pelvic vascular plexes just above the rectum, with hyperemia and edema of the rectal mucosa. The pelvic engorgement and thrombosis were observed only in rabbits subjected to rectal probing. Edema and hyperemia of the entire bowel was also present but grossly suppurative, and necrotic lesions were not found in any organs.

Microscopically, numerous large bacterial colonies were found to invade the rectal wall and vessels, and to be accompanied by necrosis of the mucosa, interstitial edema, and a complete absence of polymorphonuclears (Fig. 2 and 3). This is a striking example of how the deficiency of granulocytes permits unrestricted growth of bacteria and invasion of the blood. In the ab-

TABLE 2. *Changes in serum complement levels during lethal E. coli bacteremia*

Group	Rabbit	No. of colonies *E. coli*/ml of blood	Change in C' level
			%
1. Overwhelming bacteremia due to *E. coli* O:113	1	Innumerable	+15.7
	2	480	0
	3	2	+35.9
	4	600	+21.8
	5	3,000	+11.3
	6	720	+ 8.9
	7	1,320	−11
	8	22	+70
	9	11,880	−20
	10	16,200	+75
2. Overwhelming bacteremia due to *E. coli* normally present in rabbits' own stools	11	600	+20.4
	12	3,000	0
	13	5	+12
	14	300	+30.6
	15	4,500	+29
	15A	1,800	− 8.4
3. Neutropenia 4 days after HN_2 in coliform-free rabbits not fed *E. coli*; no rectal probing	16	0	+20
	17	0	+16
	18	0	+33
	19	0	0
	20	0	0
4. Neutropenia 4 days after HN_2 in coliform-free rabbits not fed *E. coli* but given rectal probing	21	0	+50
	22	0	+90
	23	0	+60
	24	0	+89
	25	0	+60
	26[a]	0	+46
	27	0	+89
	28[a]	0	+183
	29	0	+29

[a] Forty-nine colonies of Viridans group streptococci were isolated from rabbit 26, and 56 colonies of *S. faecalis* were isolated from rabbit 28.

sence of granulocytopenia, rectal probing induced an intense inflammatory reaction with numerous polymorphonuclears, and bacteremia did not develop (Fig. 4).

Normal bacterial flora of the intestine. The intestinal flora of rabbits (2.5 to 3.5 kg), rats (350 g; Holtzman Lab Animals, Madison, Wis.), and mice (25 g; Carworth Farms, Inc., New City, N.Y.) was determined by culturing feces aerobically and anaerobically on tryptose-agar containing 10% rabbit blood, on tomato juice-agar, and on E M B Agar. All animals were albino and had reached maturity; they were housed in air-conditioned quarters and fed commercial pellets (Wayne Rabbit Ration, Allied Mills, Chicago, Ill.). The pellets contained no antibiotics and were composed of corn, oats, soybean, wheat, whey, alfalfa, cane molasses, vitamin supplements, and calcium supplements. Whole fecal masses were removed from the rectums, placed directly in sterile petri dishes, and homogenized in an equal volume of Trypticase Soy Broth before streaking on culture plates with a cotton swab saturated with the fecal suspension.

Table 3 shows from how many animals *E. coli* was isolated, and the amount of growth from each animal; Table 4 lists the other species recovered from rabbits. The results indicate that coliform bacteria were rarely present in rabbits, even though they were readily isolated from rats and mice fed the same diet and in the same environment.

The absence of coliform bacteria was observed repeatedly in other groups of rabbits. Thus, in nine groups of rabbits, housed in different parts of the University of Pittsburgh Medical School and examined over a period of 9 months, coliform bacilli could not be recovered from 60.5% of 248 animals. Antibiotics in the feed were carefully excluded as the cause for their absence.

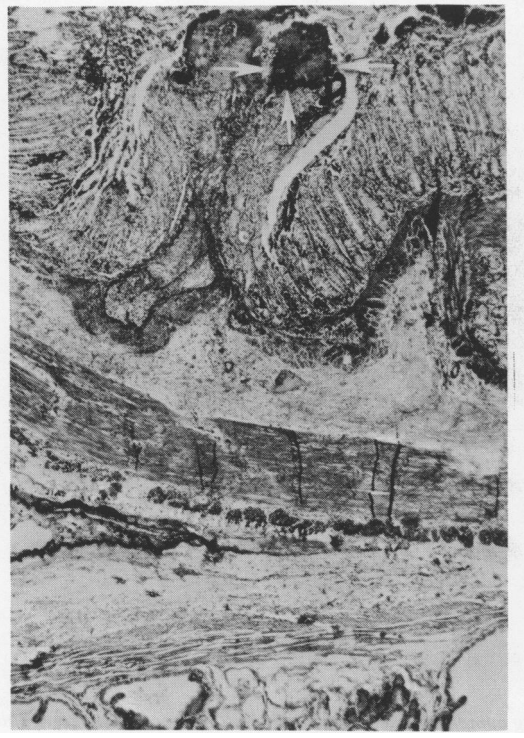

FIG. 2. *Rectum of rabbit with E. coli bacteremia given 3 mg of HN_2 96 hr previously and subjected to rectal temperature measurement. Arrows point to invasion of rectal mucosa by fecal bacterial colony (top center). Hematoxylin-eosin. × 40).*

Effect of feeding E. coli to rabbits naturally free of intestinal E. coli and coliform bacilli. Ten coliform-free rabbits were divided into two groups (of six and four rabbits). Blood was obtained from each (on 25 May) by cardiac puncture for base-line hemagglutination titers, and the serum was stored immediately after separation at −20 C. Duplicate leukocyte counts were taken from the marginal veins of the ears. Three days after the bleeding, the 10 rabbits were weighed and each of the six in one group was given 1 ml of an 18-hr Trypticase Soy Broth culture of *E. coli* (serotype O:113) daily for the next 25 days. Rectal cultures promptly became positive for that organism. The weights and leukocyte counts were determined periodically; blood was obtained on 19 June by cardiac puncture for determination of hemagglutinating antibodies to endotoxin extracted from the strain of *E. coli* fed to the rabbits. The antibody response noted in the two groups is given in Table 5.

Except for the development of antibody, the animals fed *E. coli* could not be distinguished from controls. Both groups gained weight (by an average of approximately 15%) during the 28 days of observation and there was no difference in leukocyte counts to indicate that infection had occurred in the group fed *E. coli*. The stools in the two groups also retained the same normal consistency.

The results of these experiments strongly indicate that the presence of exogenous *E. coli* in the gut can stimulate antibody formation to its endotoxin without producing infection or illness. In animals given nitrogen mustard and rectal trauma, however, the same strain of *E. coli* (O:113) produced overwhelming bacteremia (Tables 1, 2, and 9b).

Role of nitrogen mustard in production of leukopenia. The blood leukocyte response to an intravenous dose of 3 mg of nitrogen mustard per kg in rabbits is shown in Fig. 5. These rabbits were given no feedings of *E. coli* and were subjected to no rectal temperature probing. The polymorphonuclears in the circulating blood fell to their lowest concentration 72 to 96 hr after injection of nitrogen mustard. In rabbits with *E. coli* bacteremia, the circulating polymorphonuclears always fell below 100/mm³ by 72 to 96 hr and frequently reached counts of zero.

Effect of nitrogen mustard on bactericidal power of serum. Blood obtained from the hearts of 12 rabbits before and 4 days after the injection of 3 mg of nitrogen mustard per kg was allowed to clot at room temperature. After 1 hr at 4 C, it was centrifuged and then stored at −70 C. Of the 12 rabbits, 4 (C, G, H, and K) had *E. coli* in their stool cultures and these four strains were used for determining the bactericidal action of the rabbit sera. The remaining eight rabbits were coliform-free on stool culture.

The sera were thawed in a refrigerator, and the bactericidal test was performed by inoculating 0.1 ml of a 1:10,000 dilution of an 18-hr broth culture into 0.9 ml of serum. Plate counts of viable bacteria were made immediately after inoculation and again after incubation of the inoculated sera for 24 hr at 37 C. The results (Table 6) indicate that nitrogen mustard markedly impaired the bactericidal power of 4 of the 12 sera (B, E, I, and J) and seemed to improve the serum bactericidal power markedly in two others (A and H).

Systemic effects of nitrogen mustard in the presence and absence of intestinal E. coli. Two groups of rabbits with intestinal *E. coli* were examined for the effects of nitrogen mustard (3.0 mg/kg). Nitrogen mustard was injected into the ear veins of 10 rabbits that carried *E. coli* as part of their normal intestinal flora. *E. coli* O7:H7 was introduced into the bowel of eight others by feeding after the animals had initially been shown to be

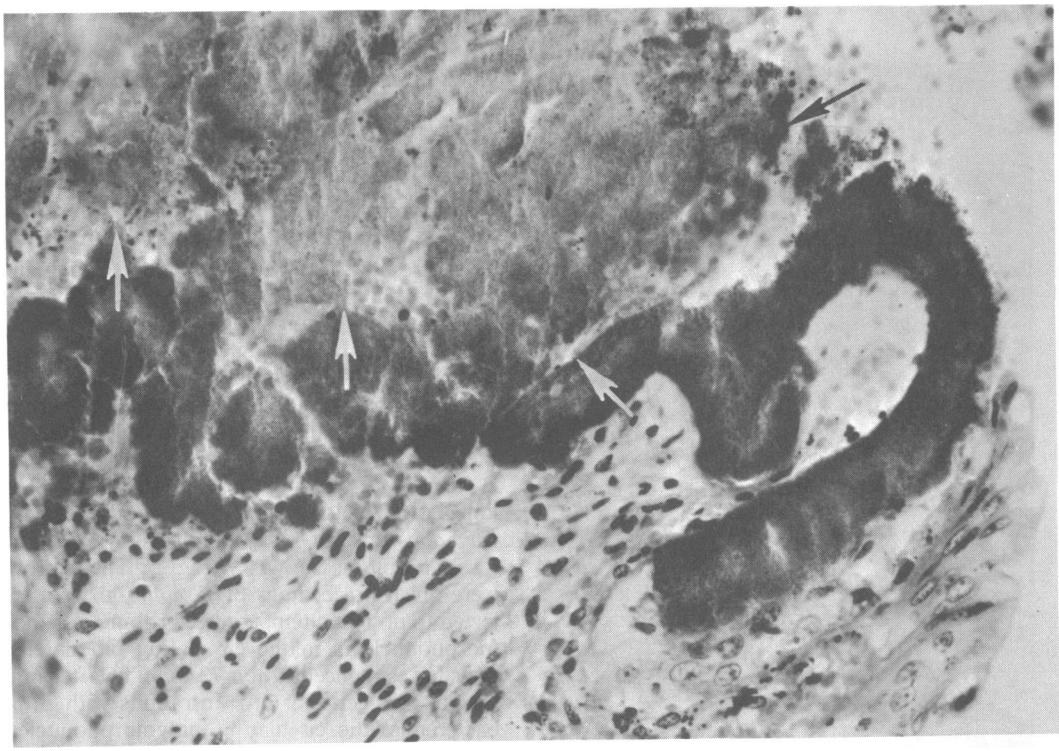

FIG. 3. *High-power view of a portion of Fig. 2, showing complete absence of inflammatory reaction. Arrows point to bacterial colony extending across the top.*

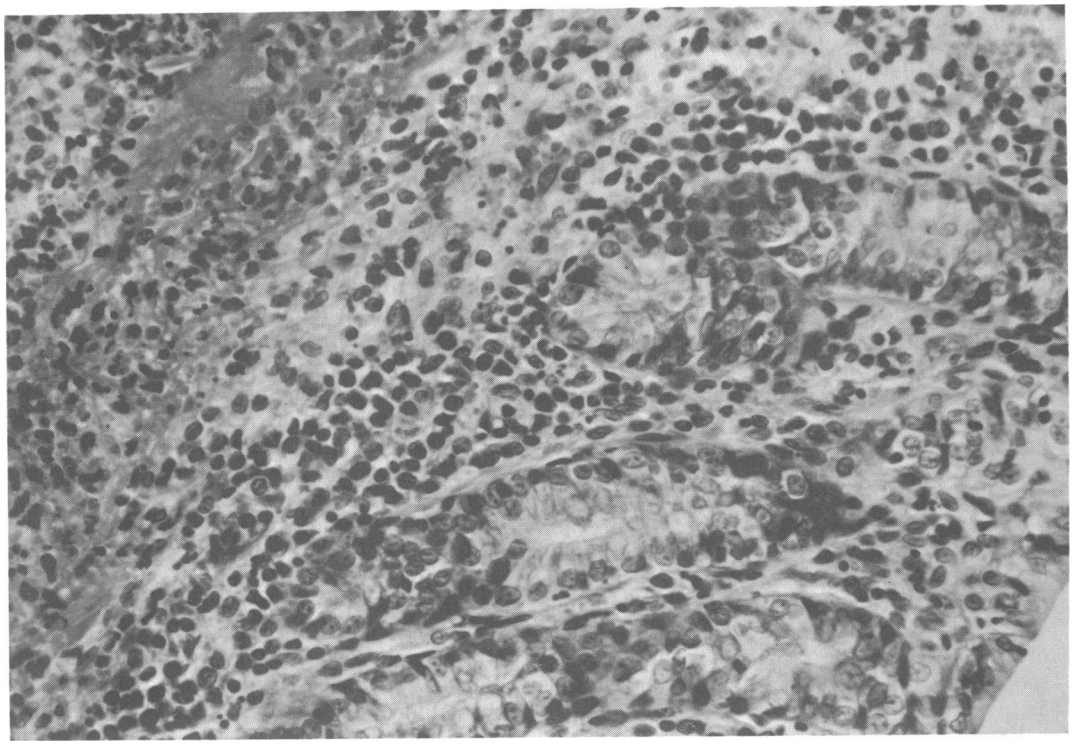

FIG. 4. *Rectum of normal rabbit after rectal temperature probing. Note intense granulocytic infiltration but no bacteria. Hematoxylin-eosin.* ×410.

TABLE 3. *Amount of growth of E. coli obtained from a fecal pellet of laboratory animals*

Total no. of animals	Innumerable (confluent) colonies	More than 100 colonies	Less than 100 colonies	No Colonies
15 rats	15	0	0	0
14 mice	3	8	2	1
20 rabbits	1	3	2	14

TABLE 4. *Bacterial species in feces of 20 rabbits*

Species	No. of rabbits	Per cent
Streptococcus faecalis	16	80
Diphtheroids	14	70
Haemophilus sp.	11	55
Bacteroides	10	50
Anaerobic streptococci	8	40
Proteus vulgaris	1	5

free of fecal *E. coli* and other coliforms. The nitrogen mustard was also injected into the second group 72 hr after the first feeding of *E. coli*. Nitrogen mustard was also injected into a third group of 16 control rabbits that contained no *E. coli* or other coliforms in their fecal cultures. The temperatures of all rabbits were measured four times daily by placing a banjo surface probe against the skin of the groin. Before the experiment, the animals were conditioned so that their temperatures did not fluctuate by more than 0.5 F. Blood was obtained for culture 96 hr after the injection of nitrogen mustard.

The influence of intestinal *E. coli* on the occurrence of fever, bacteremia, and death after injection of nitrogen mustard (without rectal trauma) is shown in Table 7. The blood cultures of six of the seven bacteremic rabbits with *E. coli* in the bowel were positive for *E. coli* alone; one culture was positive for both *E. coli* and *Streptococcus faecalis*. Two rabbits that died with bacteremia had innumerable colonies of *E. coli* in the pour plates of their blood, but blood from the five surviving bacteremic rabbits contained, respectively, 6, 6, 24, 99, and 120 colonies/ml. Diphtheroids were isolated from the blood of one of the coliform-free rabbits that developed bacteremia, and a *Haemophilus* sp. was isolated from the blood of the other; these organisms were in such small numbers that they appeared only in the broth cultures.

Nine of the animals with *E. coli* in the bowel died before fever developed in them or in the others, so that they cannot be used for calculating the incidence of fever. Of the rabbits with *E. coli*

TABLE 5. *Antibody response to rabbits fed E. coli O:113*

Group	Serum hemagglutinins to human O erythrocytes sensitized with endotoxin from *E. coli* O:113 (range of reciprocal titer)	
	Before feeding on 25 May	After feeding on 19 June
Fed *E. coli*	4–16	128–256
Not fed *E. coli*	8–16	8–1

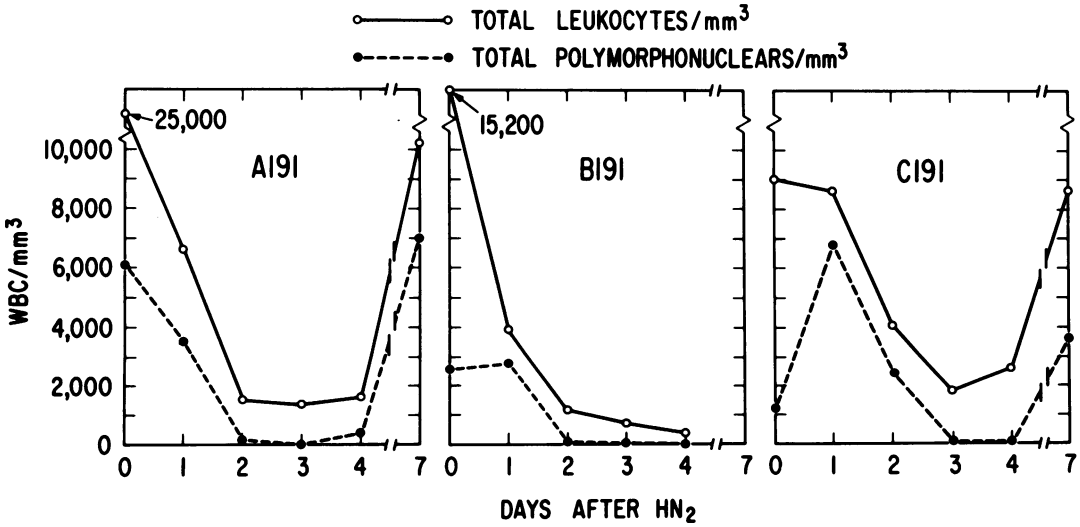

FIG. 5. *Blood leukocyte changes after intravenous injection of HN_2 (3.0 mg/kg) in rabbits. (A191, B191, and C191 are identifying numbers for the rabbits.)*

TABLE 6. *Bactericidal action of serum against E. coli before and 4 days after treatment with nitrogen mustard*[a]

Origin of *E. coli* (rabbit)	Origin of serum (rabbit)	No HN_2		Four days after HN_2	
		0 hr	24 hr	0 hr	24 hr
C	A	1,560	Innumerable	1,620	0
	B	2,040	0	2,640	13,140
	C	1,800	0	1,740	0
G	D	1,980	360	2,820	0
	E	2,100	0	2,820	Innumerable
	G	2,580	0	2,940	0
H	F	1,800	240	1,980	35
	H	1,980	27,000	1,800	0
	I	2,400	0	1,800	Innumerable
K	J	2,100	480	1,860	Innumerable
	K	1,980	36,000	2,100	Innumerable
	L	1,600	18,000	1,800	Innumerable

[a] Results are expressed as the number of colonies of *E. coli* per milliliter after incubation at 37 C.

TABLE 7. *Incidence of bacteremia, fever, and death in the presence and absence of E. coli in the bowel of rabbits*[a]

Presence of *E. coli* in bowel	No. of rabbits	No. of bacteremic rabbits	No. of febrile rabbits	No. of deaths
Normally	10	2	4	6
Through feeding	22	5	22	4
Absent	16	2	11	4

[a] Given nitrogen mustard (3.0 mg/kg) intravenously.

in the bowel, 12 of 13 that survived long enough developed fever. In the coliform-free group, 11 of 16 developed fever. Fever never appeared before 72 hr after nitrogen mustard was given and then rose from 1 to 4 F above normal. The presence of *E. coli* in the bowel did not seem to influence the time of appearance or the height of the fever. The fever of nonbacteremic animals may be due to the pyrogens of noncoliform bacteria in the bowel (streptococci, *Bacteroides*, diphtheroids, and *Haemophilus*). To examine this possibility, the pathogenesis of fever from nitrogen mustard (3 mg/kg) in a group of nonbacteremic rabbits was investigated by passive transfer of serum taken at the onset of the first spike of fever. The blood was obtained for culture and passive transfer when the fever had reached 2.4 to 3.2 F, as measured by the banjo probe in the skin of the groin. Each rabbit was bled and the spleen was removed aseptically for aerobic and anaerobic culture on blood-agar, E M B Agar, and Trypticase Soy Broth. Serum was removed from clotted blood that had been refrigerated at 4 C overnight. The sera from five animals whose blood and spleen cultures were sterile were pooled for subsequent inoculation into four normal rabbits and stored at 4 C. Each normal rabbit received in a marginal ear vein 30 ml of pooled serum after it had reached room temperature. None of the four recipients developed fever, as measured rectally; it appeared, therefore, that a circulating pyrogen was not present in the blood of febrile animals at the time of their initial spike in fever.

These results demonstrate that nitrogen mustard can produce *E. coli* bacteremia without rectal trauma in about one-fourth of animals with *E. coli* in the bowel, and that the bacteremia may be low grade and not lethal. To produce overwhelming bacteremia and death in more than 80% of the rabbits, it is necessary to combine nitrogen mustard with rectal temperature measurement.

Effect of rectal trauma without nitrogen mustard after coliform-free rabbits were fed E. coli. *E. coli* O7:H7 was fed twice daily to eight coliform-free rabbits in the manner described above. All developed strongly positive rectal cultures for *E. coli* on the third day of the feeding of *E. coli*. At this time, the rectal temperature of each animal was measured five times daily with the thermistor probe at 60- to 90-min intervals. This traumatic procedure was carried out on three consecutive days. Rectal temperatures were likewise measured in four coliform-free rabbits that received no feedings of *E. coli* and thereby served as controls.

No fever developed in any rabbit despite the occurrence of rectal inflammation and pelvic vas-

cular congestion (Fig. 4). The dominant features of the rectal reaction to the thermistor probe in normal rabbits without granulocytopenia were an intense infiltration by polymorphonuclear leukocytes and the absence of bacteria. This local leukocyte reaction stood in sharp contrast to the mustard-treated rabbits whose rectal mucosa exhibited intense bacterial invasion, severe necrosis, and a complete absence of inflammatory cells after rectal probing. In the absence of rectal probing, nitrogen mustard produced no injury to the rectum. One month after the feedings with *E. coli* and the rectal probings had been discontinued, the animals had virtually eliminated *E. coli* from their rectums.

These results demonstrate that, in the absence of nitrogen mustard, coliform-free rabbits fed *E. coli* will not develop fever (or bacteremia, presumably), even when subjected to rectal trauma, and that coliform-free rabbits will spontaneously eliminate *E. coli* that have been artificially introduced into their bowels. (Note: antibiotics were strictly excluded from the feed of these animals.)

Protection against overwhelming E. coli bacteremia by streptomycin. Twenty rabbits with rectal cultures free of *E. coli* were fed *E. coli* O7:H7. Thereafter, a heavy growth of *E. coli* was recovered from the rectum of each animal. Each was given 3 mg of nitrogen mustard per kg intravenously 4 days after the first feeding of *E. coli*; 48 hr later, each of 10 rabbits received 100 mg of streptomycin intramuscularly (hip) twice daily for the duration of the experiment. At the same time, all animals were subjected to rectal temperature measurement with the thermistor probe six times daily at 90-min intervals. Within 24 to 48 hr after rectal temperature measurements were started, the untreated rabbits (given no antibiotics) developed high fevers and died (Table 8).

These results demonstrate that streptomycin can prevent bacteremia of intestinal origin. In the control group, all blood cultures contained innumerable colonies of *E. coli*; in three, *Bacteroides* was also isolated. In the treated group, the animal developing bacteremia had only 13 *E. coli* colonies/ml of blood. The low incidence of fever in the treated group suggests that streptomycin also prevents fever from noncoliform intestinal bacteria. Nitrogen mustard usually causes fever in 70% of coliform-free rabbits, even without trauma (*see* Table 7).

TABLE 8. *Effect of streptomycin on lethal bacteremia in neutropenic rabbits subjected to rectal trauma*

Effect	Treated (streptomycin)	Untreated (controls)
Deaths	2/10	10/10
Fever	4/10	7/7[a]
Bacteremia	1/10	9/10

[a] Three animals died with *E. coli* bacteremia before fever developed.

Protection against overwhelming bacteremia by prolonged feeding of E. coli. The observation that prolonged feedings of *E. coli* also stimulated antibody formation to *E. coli* endotoxin suggested that resistance to lethal *E. coli* bacteremia might be increased by feeding that organism. We attempted to increase resistance in that way.

Twenty-four coliform-free rabbits were fed *E. coli* by placing 1 ml of an 18-hr broth culture of *E. coli* O:113 in their drinking water daily for 3 weeks before injection of nitrogen mustard (3 mg/kg). A control group of 24 rabbits was fed *E. coli* in the same fashion but for only 3 days before receiving nitrogen mustard. Blood for antibody titers to *E. coli* endotoxin was taken by cardiac puncture before feeding was started and again just before nitrogen mustard was injected; 48 hr after each animal had received nitrogen mustard, the rectum was traumatized by taking continuous temperature measurements with the internal soft vinyl probe. The probe was washed first with Zephiran solution, rinsed in tap water, dried with gauze, and sterilized with 70% alcohol before temperature measurements were started each day. These precautions prevented transfer of intestinal bacteria from test animals to control animals. Fecal cultures showed that all rabbits had heavy populations in the stools when rectal temperature measurements were started. As soon as each animal developed fever, it was bled to obtain enough blood for both culture and antibody titer determination. The fever usually took the form of a sudden spike 48 hr after rectal probing began. Because of the bleeding procedure, it was not possible to compare mortality rates in the two groups.

Tables 9a and 9b compare the incidence of bacteremia, the height of fever, and the antibody titers in the two groups of rabbits. The animals given *E. coli* orally for 21 days developed the same degree of granulocytopenia as the controls 96 hr after injection of nitrogen mustard. The total count of circulating polymorphonuclears in the blood of controls then was 0 to 44/mm^3, and in those fed *E. coli* for 21 days it was 0 to 27/mm^3.

The results summarized in Table 10 demonstrate that the incidence of overwhelming bacteremia can be reduced by oral immunization.

DISCUSSION

The discovery that many rabbits normally contain no coliform bacilli has provided a unique

TABLE 9a. *Prevention of E. coli bacteremia in rabbits by oral immunization with E. coli O:113 fed for 21 days*[a]

Rabbit	Maximum fever (F)	*E. coli* in blood (colonies/ml)	Reciprocal hemagglutinin titer to *E. coli* 00:113 endotoxin				
			Before feeding *E. coli*	After feeding *E. coli*		Titer ratio (after/before)	
				A	B	A	B
A-249	0	P	4	32		8	
B-249	3.8	12	ND	16		ND	
C-249	4.5	Sterile	2	8	8	4	4
D-249	2.8	Sterile	2	32	8	16	4
E-249	3.5	Innumerable	0	16	4	>16	>4
F-249	3.5	2	0	32	8	>32	>8
G-249	D	P	4	8		2	
H-249	3.1	Sterile	4	32	16	8	4
I-249	3.0	1	0	4	2	>4	>2
J-249	2.6	Sterile	0	16	8	>16	>8
K-249	D	Sterile	0	8		>8	
L-249	D	Sterile	2	4		4	
A-253	2.5	Sterile	8	128	16	16	2
B-253	1.5	Sterile	4	16	8	4	2
C-253	1.4	Sterile	8	128	128	16	16
D-253	3.0	Sterile	2	256	256	128	128
E-253	1.5	Sterile	4	16	16	4	4
F-253	1.9	Sterile	2	32	32	16	16
M-253	2.2	Sterile	2	32	4	16	2
N-253	2.5	Sterile	16	512	128	32	8
O-253	3.1	Sterile	0	16	4	>16	>4
P-253	D	Sterile	0	32		>32	
R-253	3.6	Sterile	0	8		>8	
S-253	D	Sterile	0	32		>32	

[a] Abbreviations: D, died from HN_2 before rectal probing began; P, positive culture of heart swab postmortem (no plate count); ND, no determination because of accident; A, just before HN_2; B, 3 to 4 days after HN_2, when fever spike occurred.

opportunity to study the effects of intestinal *E. coli* on them, both under normal conditions and during induction of *E. coli* bacteremia. When a normal coliform-free rabbit is fed *E. coli*, the bacteria remain in the bowel for 1 month after feedings are discontinued, and do not disturb health. Although *E. coli* thus appears harmless in the bowel under ordinary conditions, it kills the animal if natural resistance is disturbed. In the presence of severe granulocytopenia induced by nitrogen mustard, approximately one-third of rabbits with intestinal *E. coli* develop bacteremia and an even larger percentage die, possibly because of the effects of the absorbed endotoxin in the blood. When rectal temperatures are measured, so that the rectum is traumatized, in animals given nitrogen mustard, all may develop overwhelming lethal *E. coli* bacteremia. The development of universal bacteremia after such manipulation depends on the strain of *E. coli*; some serotypes appear more invasive than others.

The bacteremia in these experiments is obviously secondary to the invasion of the rectal mucosa by fecal bacteria. Injury to the rectal mucosa by the temperature probing enables *E. coli* and certain anaerobic bacteria to invade and multiply there in the absence of neutrophils. The crucial role of the circulating granulocytes in preventing bacteremia is made dramatically evident by comparison with normal control animals subjected to rectal temperature measurement without nitrogen mustard; such controls manifested heavy granulocytic infiltration of the rectal mucosa but there was no bacterial invasion of the tissues, no bacteremia, and no fever. The importance of granulocytopenia in the development of *E. coli* bacteremia was also brought out by the facts that granulocytopenia never developed before 48 hr after injection of nitrogen mustard, and that fever, bacteremia, and death followed within the next 48 hr. During the first 48 hr, when the granulocyte counts were either high or normal, the animals remained perfectly well.

Other changes in the blood besides granulocy-

TABLE 9b. *E. coli bacteremia in nonimmunized rabbits: E. coli O: 113 fed for 3 days (cf. Table 9a)*

Rabbit	Maximum fever (F)	*E. coli* in blood (colonies/ml)	Reciprocal hemagglutinin titer to *E. coli* O:113 endotoxin	
			Before feeding *E. coli*[a]	After feeding *E. coli*[b]
M-249	4.3	720	8	4
N-249	D[c]	P[d]	4	
O-249	D	Sterile	16	
P-249	5.5	1,320	8	8
Q-249	S	Sterile	4	
R-249	5.3	22	2	4
S-249	D	Sterile	8	
T-249	5.0	11,880	2	0
U-249	3.8	16,200	2	2
C-249	4.1	Sterile	4	16
W-249	4.5	Sterile	4	2
X-249	D	Sterile	4	
G-253	3.0	Innumerable	2	2
H-253	3.5	Innumerable	4	2
I-253	4.2	Innumerable	8	16
J-253	3.9	Innumerable	4	4
K-253	D	Innumerable	2	
L-253	0.4	Innumerable	2	2
S-253	3.5	P	0	
T-253	3.5	2,040	0	0
U-253	D	Sterile	0	
V-253	D	P	2	
W-253	3.5	Innumerable	0	
X-253	D	Sterile		

[a] Just before HN^a.

[b] Three to four days after HN_2, when fever spike occurred.

[c] Died from HN_2 before rectal probing began.

[d] Positive culture of heart swab postmortem (no plate count).

topenia may contribute to the development of bacteremia and fever. One of these is the decline in bactericidal power reported by Donaldson and Miller (6) in rabbits given nitrogen mustard. They found that, regardless of the dose, nitrogen mustard depresses the serum bactericidal activity by the third day after injection in most animals. This fall, coinciding with a sharp fall in granulocytes, could thus abolish the other important process for destroying intestinal bacteria in the circulation. It should be emphasized, however, that Donaldson and Miller examined the action of bactericidins against *Bacillus subtilis* only; bactericidal activity against *E. coli* was reduced in only one-third of the sera obtained from mustard-treated rabbits in the present study.

The critical immunological importance of circulating phagocytic cells is emphasized by the observation that intact reticuloendothelial function alone cannot protect against bacteremia and endotoxemia in mustard-treated rabbits. Derby and Rogers (5) found that nitrogen mustard, in doses that produced granulocytopenia, did not consistently impair the clearance of *E. coli* from the blood. We have confirmed their findings and also found that the rate of removal of endotoxin from the blood was not reduced in mustard-treated rabbits (*unpublished data*).

In spite of the drastic loss of resistance to invasion of the blood by intestinal *E. coli*, it was possible to protect animals against overwhelming bacteremia by resorting either to streptomycin or to oral immunization. The effectiveness of specific antibiotic prophylaxis is not surprising, because antibiotics can prevent bacterial propagation in the blood without assistance from the immunological apparatus of the animal. The effectiveness of oral immunization, however, despite damage to defenses by nitrogen mustard, was not fully expected. This oral immunization is accompanied by a sharp rise in specific antibody titer to *E. coli* endotoxin but the mode of protection by this antibody has not been examined. The protection by antibody against endotoxin cannot be the result of better phagocytosis by polymorphonuclear leukocytes, inasmuch as blood counts showed that these cells had virtually disappeared from the blood of all animals in both groups on the fourth day after nitrogen mustard when fever spiked and blood cultures were obtained. Per-

TABLE 10. *Reduction of bacteremia by oral immunization (summary of Tables 9a and 9b)*

Determination	Orally immunized, fed *E. coli* 21 days before HN_2	Nonimmunized, fed *E. coli* 3 days before HN_2	Significant difference ($P <$ 0.01)
Total incidence of bacteremia	26%	67%	Yes
Corrected incidence of bacteremia[a]	21%	87%	Yes
Average fever maximum	2.6 F	3.9 F	No
Average antibody titer to *E. coli* O.113 endotoxin just before HN_2	1:59	1:3	Yes
Average antibody titer to *E. coli* O.113 endotoxin height of fever (3 to 4 days after HN_2)	1:40	1:5	Yes

[a] Incidence is corrected for animals that died from nitrogen mustard before rectal temperatures were taken.

haps antibody reduced the ability of *E. coli* in the rectum to invade the traumatized pelvic veins and to establish thrombophlebitis, the essential preliminary to overwhelming bacteremia. It is also possible that antibody increased the clearance of *E. coli* by the fixed macrophages of the reticuloendothelial system. The reticuloendothelial cells are resistant to nitrogen mustard, which does not impair clearance of *E. coli* or endotoxin from the blood (2). Finally, a rise in antibody titer to endotoxin may indicate a corresponding rise in bactericidal antibody. Whether or not any of these proposed mechanisms of protection is correct, the results suggest that antibody to endotoxin may help prevent infections in granulopenic individuals and emphasize the importance of prior contact with gram-negative bacteria (low-grade wound infection, intestinal colonization) in conferring resistance to bacteremia.

An inevitable result of the administration of nitrogen mustard in these experiments was fever, and it appeared whether or not bacteremia occurred. The pathogenesis of fever in rabbits given nitrogen mustard without rectal probing is less obvious than in those subjected to that procedure. Without rectal trauma, rabbits given nitrogen mustard occasionally developed low-grade bacteremia comparable to that observed in mice given nitrogen mustard by Hammond, Tompkins, and Miller (9). This light bacteremia was not responsible for mortality in mice, and was probably of little importance in the development of fever in our rabbits. The blood, spleens, and other tissues were often sterile in febrile rabbits subjected to nitrogen mustard alone, so bacteremia did not seem responsible for the fever. The inability to demonstrate a transferrable pyrogen in 30 ml of serum, just after the fever spiked, indicates that endotoxin from the bowel was not then present in the blood. It is also possible that the fever in these agranulocytic animals was produced by secondary infection of the bowel wall. Such fever might be expected to result from the elaboration of endogenous pyrogen, as reported in other experimental infections (12). The absence of transferable pyrogen in large volumes of serum cannot be attributed in our experiment to the deficiency of granulocytes, in view of the recent discovery that other cells may also release endogenous pyrogens (1). The alternative possibility, that granulocytes are needed somehow to prevent fever, must also be considered. It might be postulated, for example, that endotoxin periodically enters the blood, in normal individuals, and that granulocytes normally prevent its pyrogenic effect by removing it from the circulation.

Regardless of its pathogenesis, the occurrence of fever in rabbits given granulocytopenic doses of nitrogen mustard (even without rectal probing) helps to explain the conflicting results reported by various investigators on the response to bacterial pyrogen in such animals. The occurrence of fever from nitrogen mustard seems to have been obscured before now by the fever from injected pyrogens. It is of interest that Freedman (7) observed higher fever in mustard-treated rabbits than in normal controls on injection of pyrogens; the fever from the pyrogens might have been superimposed on that just then developing from the effects of nitrogen mustard. However, it is surprising, in view of our results, that Herion et al. (11) obtained no fever in rabbits given pyrogens after they had been made granulocytopenic with large doses of nitrogen mustard.

Future studies are needed to unravel entirely the complex series of reactions leading to fever and overwhelming bacteremia in the experimental model described in this report. The results of such studies may aid in the understanding and management of human bacteremic shock, inasmuch as the experimental and clinical syndromes are similar. They resemble each other with respect to (i) the endogenous source of the bacteria; (ii) the high frequency of bacteremia due to *E. coli* and other enteric bacilli; (iii) the importance of pelvic instrumentation; and (iv) the associated disturbances in immune mechanisms, such as granulocytopenia. The last point deserves emphasis because of the high incidence of bacteremic shock in patients with leukemia and neoplasms for which irradiation or radiomimetric agents are administered. Similar effects might be anticipated in radiation casualties following atomic explosions.

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LITERATURE CITED

1. Bodel, P., and E. Atkins. 1967. Release of endogenous pyrogen by human monocytes. New Engl. J. Med. 276:1002.
2. Braude, A. I. 1962. Mechanism of action of endotoxin. Annual report to Research and Development Division, Office of the Surgeon General, Department of the Army, Washington, D.C., 31 October 1961 to 31 October 1962. AD 207655. Defense Documentation Center, Cameron Station, Alexandria, Va.
3. Braude, A. I., J. L. Jones, and H. Douglas. 1963. The behavior of *Escherichia coli* endotoxin (somatic antigen) during infectious arthritis. J. Immunol. 90:297–311.
4. Brecht, K., and H. Boucke. 1953. Ein neuer Pulsabnehmer für Kleintiere. Arch. Exp. Pathol. Pharmakol. 217:399–400.
5. Derby, B. M., and D. E. Rogers. 1961. Studies on bacteremia. V. The effect of simultaneous leukopenia and reticuloendothelial blockade on the early blood stream clearance of staphylococci and *Escherichia coli*. J. Exp. Med. 113:1053–1066.

6. Donaldson, D. M., and M. L. Miller. 1959. Depression on normal serum bactericidal activity by nitrogen mustard. J. Immunol. **82**:69–74.
7. Freedman, H. H. 1960. Further studies on passive transfer of tolerance to pyrogenicity of bacterial endotoxin. The febrile and leucopenic responses. J. Exp. Med. **112**:619–634.
8. Gilbert, V. E., and A. I. Braude. 1962. Reduction of serum complement in rabbits after injection of endotoxin. J. Exp. Med. **116**:447–490.
9. Hammond, C. W., M. Tompkins, and C. P. Miller. 1953. Occurrence of generalized infection of enteric origin in mice poisoned by nitrogen mustard. Proc. Soc. Exp. Biol. Med. **82**:137–140.
10. Heineman, H. S., and A. I. Braude. 1961. Shock in infectious diseases. DM (Disease-a-Month), October.
11. Herion, J. C., R. I. Walker, and J. G. Palmer. 1961. Endotoxin fever in granulocytopenic animals. J. Exp. Med. **113**:1115–1125.
12. King, M. K., and W. B. Woods, Jr. 1958. Studies on the pathogenesis of fever. V. The relation of circulating endogenous pyrogen to the fever of acute bacterial infection. J. Exp. Med. **107**:305–318.
13. Osler, A. G., J. H. Strauss, and M. M. Mayer. 1952. Diagnostic complement fixation; method. Amer. J. Syph. **36**:140–153.
14. Prioli, N. A., and M. M. Winbury. 1960. Indirect method for blood pressure determination in the dog. J. Appl. Physiol. **15**:323–324.
15. Smith, H. W. 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. **90**:495–513.
16. Spink, W. W., R. B. Davis, R. Potter, and S. Chartrand. 1964. The initial stage of canine endotoxic shock as an expression of anaphylactic shock: studies on complement titers and plasma histamine concentrations. J. Clin. Invest. **43**:696–704.