STUDIES ON BACTERIEMIA*

III. The Blood Stream Clearance of Escherichia coli in Rabbits

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Recent studies by Hollingsworth and Beeson have shown that rats maintain a low grade bacteriemia following the intravenous injection of large numbers of *Escherichia coli* (9). Our increasing interest in the phenomenon of persistence of staphylococci within the circulation led to comparative studies on the behavior of this strain of *E. coli* in the blood stream of rabbits.

The present paper reports observations on the rates of clearance, and the sites of trapping of circulating *E. coli* following their intravenous injection. The behavior of circulating leukocytes during *E. coli* bacteriemia, and *in vitro* observations on the survival of this microorganism within rabbit polymorphonuclear leukocytes are also reported. These results are compared with our previous findings in experimental staphylococcal bacteriemia in the body of this paper.

Materials and Methods

The experimental methods outlined in the preceding paper were used in the performance of these studies (15). A single strain of *E. coli*, kindly supplied by Dr. Paul B. Beeson, Yale University, was used in these experiments. This microorganism was originally isolated from the urine of a patient with pyelonephritis. The biochemical characteristics of this strain have been reported in detail by Hollingsworth and Beeson (9). Stock cultures were maintained in peptone infusion broth, and an appropriate aliquot of an 18 hour culture was diluted to 2.0 cc. in normal saline for use as the intravenous inoculum.

Preliminary studies indicated that this strain was not susceptible to bacteriocidal factors present in normal rabbit serum (21). No loss in culturable *E. coli* occurred on incubation in fresh rabbit serum for as long as 24 hours, and luxuriant growth was obtained in whole plasma or 25 per cent rabbit serum.

In vitro studies of intraleukocytic survival of *E. coli* were performed in a manner previously described in detail (16). In brief, citrated rabbits' blood was mixed with equal amounts of 6 per cent bovine fibrinogen and allowed to sediment for 30 to 60 minutes at 37°C. The plasma

layer containing leukocytes was removed, the leukocytes concentrated by brief centrifugation, and the plasma-leukocyte mixture used directly for phagocytic studies. Washed suspensions of *E. coli* were added to plasma-leukocyte mixtures in capped, sterile 12 × 100 mm. tubes. These tubes were placed on a rotating drum at 37°C. Aliquots were removed at appropriate intervals, and coverslip preparations pulled for stained slides and slide cultures. At each sampling period, the percentage of polymorphonuclear leukocytes containing microorganisms were enumerated on Wright's or Giemsa stained smears. The percentage of polymorphonuclear leukocytes subsequently growing colonies of *E. coli* were counted microscopically in slide cultures prepared from the other coverslip pulled at the same time.

EXPERIMENTAL

When approximately 5 × 10^8 *E. coli* were injected into the ear veins of rabbits, the curve of clearance initially resembled that obtained after the intravenous injection of similar numbers of staphylococci. For the first 20 minutes the rates of disappearance of *E. coli* and staphylococci were essentially equal. Certain differences in the clearance of *E. coli* became apparent, however, 20 to 30 minutes following injection of bacilli.

At this point the removal of circulating staphylococci had ceased abruptly, resulting in a fairly static bacteriemia of approximately 2,000 microorganisms per milliliter. In contrast, no sharp alteration in the rate of removal of circulating *E. coli* was consistently noted, and a gradual reduction in clearance rate generally continued over 40 to 90 minutes. The number of *E. coli* in the blood stream fell progressively during this period until interrupted by an increasing bacteriemia.

Thus, 40 to 60 minutes following injection, the number of *E. coli* remaining in circulation averaged 200 microorganisms per milliliter or approximately $\frac{1}{10}$ that obtained in animals receiving similar numbers of intravenous staphylococci.

Fig. 1 summarizes the 3 hour clearance curves obtained in 6 animals receiving 5 × 10^8 *E. coli*, and 14 animals receiving equal numbers of staphylococci. Each shaded curve includes the quantitative observations made on all animals.

As noted in Fig. 1, clearance rates were parallel during the initial 20 to 30 minutes following injection. In general, animals receiving *E. coli* cleared more bacteria from the circulation during the 40 to 90 minute period, athough overlapping was noted in one animal.

In all animals receiving *E. coli*, there was a more rapid resurgence of circulating bacterial populations which in part accounted for the wider variations in the levels of bacteriemia obtained at sampling periods beyond 60 minutes. In some animals, an increase in number of circulating bacteria could be noted as early as 60 minutes after injection, and increasing bacteriemia was noted in all animals by 3 hours (see Fig. 1, Tables I and II). This resulted in a V shaped curve of disappearance and return of circulating bacteria in animals receiving *E. coli*, in contrast to the plateau of constant bacteriemia seen in animals injected with staphylococci (Fig. 1). In animals receiving staphylococci, increasing bacteriemia was uncommon before 3 to 4 hours had elapsed.

Removal of E. coli in the Splanchnic Bed.—A series of experiments were performed in which quantitative cultures were obtained on simultaneous superior vena cava and hepatic vein blood specimens. In such experiments, an average of 66 per cent (range 30 to 80 per cent) of the circulating *E. coli* were removed in transit through the splanchnic viscera. Splanchnic trapping continued without notable change throughout the period of decreasing *E. coli* bacteriemia. This differed from the findings in staphylococcal bacteriemia where splanchnic
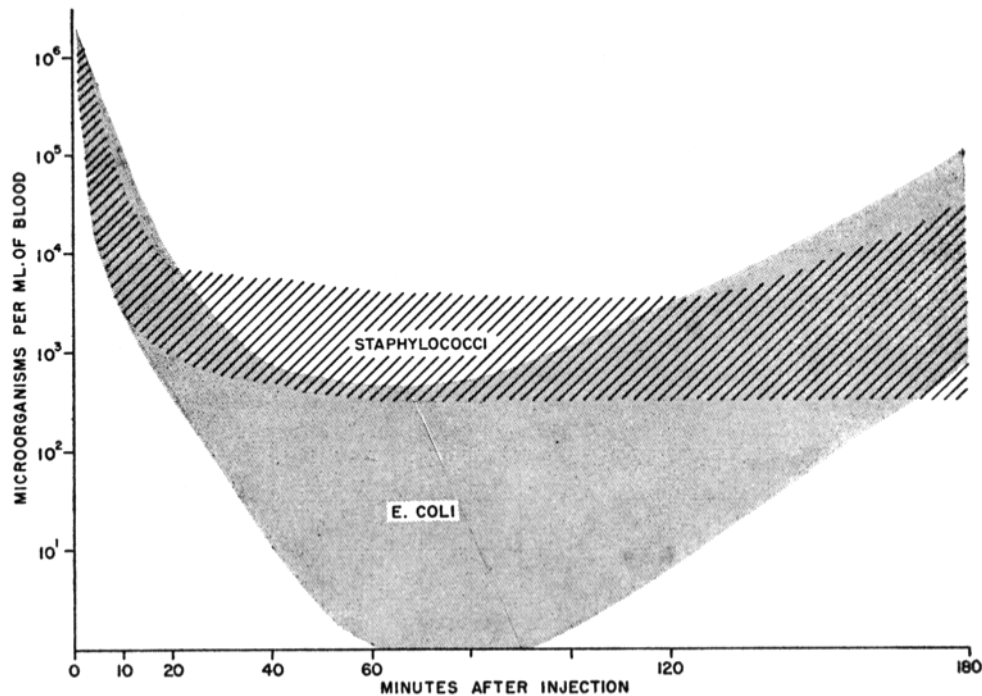

Fig. 1. The removal of equal numbers of *E. coli* or staphylococci from the blood stream of rabbits. Six rabbits received *E. coli*, 14 rabbits received staphylococci. Each shaded curve includes all quantitative observations made in each series of animals.

trapping characteristically declined, or virtually ceased, 20 to 40 minutes after injection of bacteria.

In certain animals it was apparent that the splanchnic viscera were actively returning *E. coli* to the blood stream during the later period of increasing bacteriemia. In other animals, splanchnic trapping of *E. coli* continued, despite an increasing bacteriemia. Two experiments illustrating the splanchnic removal, and subsequent variations in the behavior of the splanchnic tissues following the injection of *E. coli*, are presented in Table I.

As noted in Table I, the splanchnic tissues were delivering *E. coli* into the general circulation within 90 minutes after injection in Experiment E-12. In

contrast, the animal studied in Experiment E-13 continued to trap *E. coli* in the splanchnic bed, despite a rapid increase in the number of circulating bacteria apparent beyond 90 minutes after injection.

Behavior of Circulating Leukocytes Following the Injection of E. coli. Changes in the numbers of circulating granulocytes following the intravenous injection of *E. coli* differed from the changes noted following the injection of staphylococci. When 5×10^8 bacterial units were injected, a progressive leukopenia ensued which reached a maximum in 10 to 20 minutes. Again differential smears revealed that changes in total leukocyte counts were primarily due to changes in circulating polymorphonuclear leukocytes. Granulocytopenia per-

TABLE I

The Splanchnic Removal of E. coli Following the Intravenous Injection of 5×10^8 Microorganisms

Time after injection	Experiment E-12			Experiment E-13		
	Microorganisms per ml.		Splanchnic removal of bacteria	Microorganisms per ml.		Splanchnic removal of bacteria
	Superior cava	Hepatic vein		Superior cava	Hepatic vein	
			per cent			*per cent*
1 min.	1,800,000	1,100,000	39	1,800,000	700,000	62
5 "	400,000	165,000	59	550,000	136,000	75
10 "	44,000	18,400	58	115,000	33,000	71
20 "	3,600	2,400	33	10,700	1,850	83
40 "	445	140	68	880	140	84
60 "	610	430	30	—	75	—
90 "	615	2,000	Seeding	1,730	340	80
3 hrs.	3,700	4,600	Seeding	>100,000	30,500	70
5 "	50,000	65,000	Seeding	192,000	173,000	10
20 "	Dead ⟶			299,000	490,000	Seeding
				Died, 22 hrs.		

sisted for 4 to 5 hours in animals receiving *E. coli*, in contrast to the rapid return to preinjection granulocyte levels noted in animals receiving staphylococci. Prolonged granulocytopenia was followed by a slow rise to granulocyte counts exceeding control levels in 8 to 24 hours in *E. coli*-injected animals. Protracted leukopenia following the injection of Gram-negative bacteria or endotoxins has also been noted by many other investigators (1, 18, 20).

Fig. 2 shows the circulating granulocyte levels obtained in 4 animals receiving injections of 5×10^8 *E. coli*. The average curve of the granulocyte response in a series of 14 animals receiving similar numbers of staphylococci is superimposed for comparison.

Trapping of Leukocytes Following the Injection of E. coli.—Differential arterial-venous samples across the liver and the lung revealed that small numbers of granulocytes were continuously removed in these capillary beds through-

out the period of leukopenia. Again, this contrasted with the results noted following the injection of staphylococci, in which capillary bed release of leukocytes from the liver and lung was generally noted beyond 20 to 40 minutes.

Certain experiments suggested that trapping of significant numbers of *E. coli* could take place within the pulmonary capillary bed late in the course of bacteriemia. Such pulmonary trapping of *E. coli* appeared to follow the pulmonary sequestration of large numbers of leukocytes.
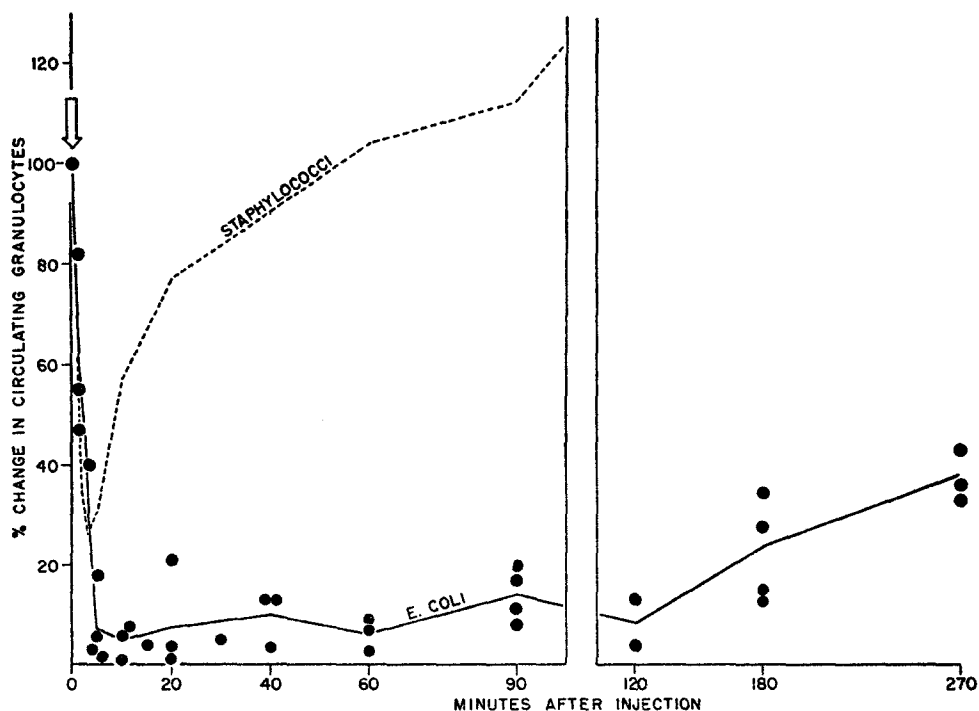

Fig. 2. Changes in the number of circulating granulocytes in 4 rabbits receiving 5 × 10⁸ *E. coli* intravenously. The average curve of leukopenia produced by the injection of equal numbers of staphylococci is superimposed for comparison.

In Table II such an experiment is pictured. As noted in comparison of columns 1 and 2, femoral artery granulocyte counts were consistently lower than those obtained from the pulmonary artery during the initial 3 hour period following the injection of bacteria. In columns 3 and 4 the numbers of *E. coli* cultured from the same samples are also recorded. During the initial 10 minutes of clearance comparative pulmonary artery and femoral artery cultures showed only slight evidence of trapping of *E. coli* across the lung. Later samples, taken 3 and 5 hours after injection, suggested that 90 to 95 per cent of the circulating *E. coli* failed to traverse the lung at this time.

In all simultaneous studies of leukocyte behavior and clearance, no correla-

tions could be obtained between the return of polymorphonuclear leukocytes to the circulation and resurgence of bacteriemia. This differed from the findings in staphylococcal bacteriemia, in which the return of leukocytes to the circulation coincided with a diminution in splanchnic trapping of bacteria, and a phase of relatively static bacteriemia.

Survival of E. coli in Polymorphonuclear Leukocytes.—*In vitro* experiments were performed to determine whether phagocytized *E. coli* could multiply after residence within the cytoplasm of rabbit leukocytes. Leukocytes containing *E. coli* were incorporated in slide culture preparations, and the ability of phagocytized *E. coli* to initiate growth was quantitated microscopically after 18 hours incubation at 37°C.

TABLE II

The Trapping of Polymorphonuclear Leukocytes and E. coli in the Pulmonary Tissues Following the Injection of 5 × 10⁸ Microorganisms

Time after injection	Granulocytes per c.mm.		E. coli per ml.	
	Pulmonary artery	Femoral artery	Pulmonary artery	Femoral artery
0	5,800	5,240		
1 min.	4,920	3,670	1,940,000	1,550,000
3 "	2,360	2,400	—	—
5 "	1,100	280	412,000	342,000
10 "	370	0	46,600	40,500
15 "	250	75	—	—
30 "	290	90	4,900	1,040
60 "	400	70	100	80
90 "	640	170	150	100
3 hrs.	780	900	920	90
5.5 "	2,130	3,500	20,200	570

In such preparations there was little or no evidence of survival of intracellular *E. coli*. In different experiments, colonies developed in less than 1 to a maximum of 9 per cent of leukocytes containing bacteria after 1 hour or less of intracellular residence. Microscopic examination of smears prepared from phagocytic preparations showed that phagocytized *E. coli* faded rapidly and showed little signs of viability after a few minutes within granulocytes. This contrasted sharply with our previous experiments on the survival of coagulase-positive staphylococci within cells. Under similar experimental conditions, 33 to 100 per cent of polymorphonuclear leukocytes containing staphylococci were the site of colonial growth after prolonged periods of intracellular residence (16).

Studies kindly performed by Dr. James G. Hirsh indicated that this strain of *E. coli* was susceptible to the bacteriocidal action of phagocytin, a substance derived from rabbit polymorphonuclear leukocytes (7, 8). Incubation of *E. coli*

in systems containing phagocytin in dilutions of less than 1 to 100 resulted in total destruction of bacilli in less than 1 hour.

Examination of stained smears of peripheral blood taken 1 to 20 minutes after the injection of *E. coli* showed occasional bacilli within polymorphonuclear leukocytes. These intracellular microorganisms stained poorly and raggedly, suggesting rapid intracellular destruction.

DISCUSSION

In these experiments, *E. coli* were cleared from the rabbit blood stream in a manner which differed in certain respects from the clearance of staphylococci. Removal of circulating *E. coli* continued over longer periods of time, without the abrupt transition to the constant bacteriemia commonly noted 20 to 30 minutes following the injection of staphylococci. There was a more rapid resurgence of circulating *E. coli* populations, resulting in a V shaped curve of clearance and return of bacilli to the blood stream. This differed from the low grade bacteriemia produced by staphylococci, which generally persisted for 3 to 5 hours before an increasing bacteriemia was noted.

Splanchnic tissues removed approximately two-thirds of the circulating *E. coli* in transit through the splanchnic organs. Trapping within the splanchnic viscera appeared to continue relatively unaltered during *E. coli* bacteriemia, unless the splanchnic tissues themselves were clearly reseeding the systemic circulation. This differed from the characteristic cessation of splanchnic removal of bacteria consistently noted 20 to 40 minutes after the injection of staphylococci.

A marked granulocytopenia followed injection of *E. coli* and persisted for 3 to 5 hours. Leukocytes were removed from the blood stream within the lung and liver for as long as 3 hours following the injection of *E. coli*, in contrast to the early trapping and subsequent release of granulocytes which followed the injection of staphylococci.

Studies by Wood and his associates have clearly demonstrated that leukocytes attached to vascular endothelium can actively engulf circulating bacteria (22). In the present studies, certain experiments showed that the continued sequestration of granulocytes in the lung was associated with increasing trapping of *E. coli* in transit through the pulmonary vascular bed. That sequestered leukocytes can remove circulating *E. coli* is also suggested by recent ingenious experiments of Hollingsworth, Finch, and Beeson (10). In their studies, irradiated, leukopenic rats receiving transfused leukocytes cleared more *E. coli* from the blood stream than did non-transfused leukopenic controls. Improved clearance was evident despite disappearance of the transfused leukocytes from the peripheral blood, suggesting that sequestered leukocytes were active in trapping microorganisms.

In vitro phagocytic studies indicated that this particular strain of *E. coli* did

not survive within rabbit polymorphonuclear leukocytes. It was also independently noted that this strain was extremely susceptible to destruction by phagocytin, a bacteriocidal substance derived from rabbit granulocytes (7, 8).

In considering the possible reasons for the differences in the intravascular behavior of these two microorganisms, certain general facts relating to the blood stream clearance of microorganisms bear consideration. Review of the literature on clearance suggests that the removal of circulating bacteria follows a characteristic pattern, regardless of the species of microorganism injected or the experimental animal under study (2).

In general, the injection of bacteria into the vascular system is immediately followed by a phase of extremely rapid removal of microorganisms lasting 20 to 40 minutes. The phase of rapid clearance is then followed by a period of relatively constant bacteriemia, or in the case of certain microorganisms, continued clearance at significantly slower rates. This phase may be followed by complete and permanent sterilization of the blood stream, or by a resurgence of circulating microbial populations and eventual death of the animal.

There is much evidence to indicate that the initial rapid removal of injected bacteria is accomplished by the trapping of microorganisms in fixed phagocytic cells of the reticuloendothelial system (2, 12, 19). The second phase of significantly slower clearance, or relatively constant bacteriemia, is less clearly understood. In the studies reported, we have directed our primary attention toward this phase of bacteriemia in which microorganisms appear to persist in the circulation, or are cleared at significantly slower rates.

The following possible mechanisms that might explain the phenomenon of reduced clearance or persistent bacteriemia have been considered:—

1. *The rate of clearance might abruptly decline or cease if reticuloendothelial removal mechanisms were fully saturated.*—There is abundant evidence to suggest that this is not the case. Clearing mechanisms have been shown to operate effectively after secondary injections of bacteria, or in the presence of overwhelming bacteriemia (6, 11, 13, 14).

2. *The small number of microorganisms remaining within the circulation after the initial rapid clearing might fail to be sequestered by the reticuloendothelial system because of infrequent reticuloendothelial cell-bacterium contact.*—Our studies with staphylococci suggest that it is not the low level of bacteriemia *per se* which inhibits clearance. Injections of small numbers of staphylococci were cleared at precisely the same rate as a large inoculum (14).

3. *The persistence of bacteriemia may represent a balance between reticuloendothelial trapping of circulating microorganisms and the simultaneous release of microorganisms from other tissue sites.*—We have evidence to suggest that this sequence of events may operate to maintain *E. coli* bacteriemia. In certain experiments, simultaneous differential arterial-venous samples across the splanchnic bed indicated that *E. coli* were released from the very site of initial

trapping in the splanchnic tissues 60 to 90 minutes after injection. The steady trapping of leukocytes across the lungs following E. coli injection, and the late pulmonary trapping of E. coli, suggested that sequestered leukocytes might act as a secondary clearing system during E. coli bacteriemia. These capillary bed trapping sites may also serve as a focus for subsequent bacillary release, thus maintaining the level of bacteriemia.

It appears unlikely that release of bacteria can explain the persistence of staphylococci within the circulation. First, the phase of steady staphylococcal bacteriemia begins within 20 to 30 minutes following the injection of cocci. *In vitro* observations have shown that the lag phase in growth of Staphylococcus MAM is always 3 to 4 hours when this microorganism is transferred abruptly from one media to another. Secondly, differential sampling across the splanchnic tissues has failed to demonstrate release of staphylococci before 3 to 5 hours have elapsed after injection. Similar differential sampling across the extremities, the pulmonary capillary bed, and the head have not shown either significant initial trapping of staphylococci or their subsequent release.

4. The intravascular localization of microorganisms may render them relatively insusceptible to trapping within the reticuloendothelial system.—We have reported observations which suggest that the localization of staphylococci may indeed have a bearing on the persistence of bacteriemia (14). Living staphylococci circulating within the leukocytes appear to offer a very different problem to the reticuloendothelial system. In contrast to unphagocyted staphylococci, intracellular staphylococci may apparently traverse the splanchnic bed without removal in reticuloendothelial cells.

There is no evidence to suggest that the persistence of E. coli is due to intracellular residence of bacilli in circulation. In phagocytic preparations, these microorganisms die swiftly within rabbit polymorphonuclear leukocytes. Furthermore, the leukopenia which follows the injection of E. coli is prolonged, suggesting that cells containing phagocyted bacilli do not return to the circulation. This differs sharply from the transient leukopenia and the return of circulating leukocytes which follows the injection of staphylococci.

5. There may occur within the animal body, changes in microorganisms which render them more resistant to clearance.—It seems reasonable to believe that the selection pressures obtaining within the animal body, and the removal of the majority of injected microorganisms, may selectively leave microorganisms in circulation which differ in subtle ways from their brethren grown *in vitro*. Studies by other investigators have shown that microorganisms growing *in vivo* may acquire resistance to phagocytosis, or may produce powerful toxins not elaborated during growth *in vitro* (4, 17). There may thus be alterations in some members of the injected population which render them more resistant to phagocytosis or reticuloendothelial uptake.

We have just begun to investigate this possibility. Studies are now in prog-

ress to determine whether staphylococci or *E. coli* remaining in the blood stream 90 minutes to 3 hours after injection are detectably different than the microorganisms injected.

6. There may be changes in the host secondary to infection which render host cells and body fluids incapable of destroying circulating bacteria.—Alterations in local biochemical conditions, depletion of essential host metabolites, or changes in host metabolic pathways may cripple host mechanisms which initially destroy invading bacteria.

To date, these possibilities have been incompletely explored. Dubos has emphasized the rapid changes in local *milieu* which may follow infection (5). Such changes may impair host enzyme systems which prevent microbial growth *in vivo*. Woodward, Sbarra, and Holtman have shown that infection with *B. tularense* may produce a profound depletion of certain blood amino acids in rats (23). It is conceivable that depletion of host metabolites essential to phagocytic cell function may occur during certain infections. The studies of Berry and Mitchell indicate that administration of certain inhibitors of the Krebs cycle allows greater bacillary multiplication *in vivo* (3). These observations suggest that infection *per se* may sometimes derange normal host metabolic systems essential to the destruction of bacteria.

The current studies indicate that differences in initial removal of circulating bacteria may in part be determined by the host granulocyte-bacterial cell relationships. The strain of *E. coli* studied does not appear to survive within polymorphonuclear leukocytes. The capillary bed sequestration of leukocytes associated with *E. coli* injection appears to aid in removing *E. coli* from the circulation, and probably in their eventual destruction.

In contrast, the ability of staphylococci to survive polymorphonuclear leukocyte ingestion, and the transient sticking and subsequent return of granulocytes containing staphylococci to the circulation appear to contribute to the maintenance of a relatively constant staphylococcal bacteriemia.

Obviously unanswered is the mechanism of late resurgence of bacteriemia. Clearly many *E. coli*, as well as staphylococci, survive initial removal from the blood stream. Whether such survival is due to inherent changes in the bacteria *in vivo* which render them resistant to destruction, or progressive damage to the metabolic processes of host cells which destroy bacteria, remains to be determined.

SUMMARY

Large numbers of *E. coli* were rapidly removed from the blood stream of rabbits at rates which initially paralleled the removal of similar numbers of staphylococci. Splanchnic tissues removed approximately two-thirds of the circulating bacilli in transit through the liver and spleen.

In contrast to the cessation of splanchnic trapping noted 20 to 40 minutes following the injection of staphylococci, splanchnic trapping of *E. coli* con-

tinued unchanged for 3 to 5 hours unless the splanchnic tissues were clearly reseeding the blood stream. This resulted in the continued disappearance of *E. coli* over a 60 to 90 minute period, and differed from the constant bacteriemia maintained beyond 20 minutes after the injection of staphylococci.

Some of the differences in the initial clearance of these two microorganisms appeared to relate to differences in host leukocyte-bacterium relationships. *In vitro* studies indicated that *E. coli* were rapidly killed following ingestion by rabbit polymorphonuclear leukocytes. Staphylococci survive such ingestion. The injection of *E. coli* was followed by a prolonged granulocytopenia with evidence of sequestration of granulocytes within the pulmonary vascular bed. The injection of staphylococci was followed by a transient leukopenia, with rapid return of granulocytes to the circulation (15).

It appears probable that *E. coli* ingested by sequestered leukocytes are destroyed within the cell, and that leukocytes do not reenter the circulation containing living *E. coli*. Such intraleukocytic residence in the blood stream has been shown to be of possible importance in the maintenance of staphylococcal bacteriemia (14).

An increasing *E. coli* bacteriemia occurred rapidly after the initial clearance period, indicating that many sequestered bacilli remained viable. Increasing bacteriemia also occurs 3 to 5 hours after the injection of staphylococci. The bacterial or host cell mechanisms which allow this secondary resurgence of bacterial populations are currently under investigation.

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