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THE ROLE OF TYPE 1 PILI IN HEPATIC CLEARANCE OF *SALMONELLA TYPHIMURIUM*

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ABSTRACT

THE ROLE OF TYPE 1 PILI

IN HEPATIC CLEARANCE OF SALMONELLA TYPHIMURIUM

By

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The role of type 1 pili in the adhesion of Salmonella typhimurium strain SR-11 to hepatic sinusoidal cells was investigated using the perfused mouse liver model and an in vitro adherence assay. In the perfused liver model, an average of 67% of type 1 piliated S. typhimuruim were cleared from the perfusion medium on a single pass. Mannose and α-methyl-mannoside inhibited trapping in a dose-dependent manner. Preincubation of the bacteria, but not the liver, with either saccharide also inhibited trapping suggesting the saccharide binds to bacterial and not hepatic receptors. Significant numbers of previously trapped bacteria could be eluted by adding mannose to the perfusion medium. Bacteria with reduced piliation, obtained by growth on agar or by use of a nonpiliated variant of the parent strain, were trapped to a significantly lesser extent than was the parent strain. The liver appears to selectively trap heavily piliated organisms since reperfusion of singly passed bacteria through a second liver resulted in significantly less trapping than occurred with the first perfusion. In vivo, the nonpiliated variant strain was cleared much more slowly than the parent strain. Mannose and α-methyl-mannoside, but not

glucose, decreased clearance rates of piliated organisms. The data suggest that type 1 pili mediate trapping of S. typhimurium by the perfused mouse liver.

An in vitro adherence assay was developed to confirm and extend the results with the perfused mouse liver model. A nonparenchymal cell suspension consisting of approximately 35% Kupffer cells, 45% endothelial cells, and 20% lymphocytes was prepared from enzymatically disaggregated mouse livers. ^{51}Chromium-labeled S. typhimurium were mixed with nonparenchymal cells and nonadherent bacteria were separated from cells and adherent bacteria by filtration through a 5 micron pore size Nucleopore filter. At 37C, bacterial adhesion to nonparenchymal cells was maximal at 10 minutes and did not substantially change thereafter up to 60 minutes incubation. Lowering incubation temperature to 4C slowed the rate of bacterial adhesion but did not change the amount of bacterial adhesion at 60 minutes. Mannose and α-methyl-mannoside inhibited bacterial adhesion while glucose and α-methyl-glucoside had no inhibitory effect. As initial bacteria to nonparenchymal cell ratio increased from 10:1 to 1000:1, adhesion of type 1 piliated phase S. typhimurium greatly increased while adhesion of nonpiliated phase S. typhimurium remained low and did not substantially increase. The nonparenchymal cell population was fractionated by centrifugal elutriation to yield three subpopulations enriched for Kupffer cells, endothelial cells, or lymphocytes. Type 1 piliated S. typhimurium adhered to cells of all three fractions and adhesion was mannose-sensitive. Cumulatively, the data suggest that type 1 pili are a major determinant in hepatic clearance of S. typhimurium and that trapping involves pilus-mediated adhesion to both

Kupffer and endothelial cells.

To Thea and Elissa

Did you ever think I'd finish?

Now the good life begins ??

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INTRODUCTION

It has been known for decades that the liver is very effective in bloodstream clearance of a variety of particulate and colloidal substances as well as bacteria (82). After intravenous injection, the number of bacteria in the bloodstream decreases rapidly and bacterial numbers increase in the liver (5, 6, 81). Microscopic examination reveals cleared bacteria inside Kupffer cells, fixed macrophages of the reticuloendothelial system (RES) which reside in hepatic sinusoids (40).

The perfused liver model has proven useful to investigate hepatic clearance of bacteria. The perfused liver clears many bacteria from perfusion medium without serum (59, 62, 100) and serum acts opsonically to increase clearance (9, 40, 58, 100). The ability of the liver to kill bacteria has also been demonstrated with the perfused liver model (9, 35). Since cleared bacteria have been observed in Kupffer cells (40) and since phagocytic cells are known to engulf and kill bacteria *in vitro* (100), it has generally been assumed that phagocytosis is the mechanism responsible for hepatic clearance of bacteria.

It was not until 1975 that trapping and killing functions of hepatic tissue were distinguished in the perfused liver and some suggestion was made that hepatic clearance is not necessarily synonomous with phagocytosis. Phase and electron microscopy reveals that, in livers perfused with *Salmonella typhimurium*, bacteria are

visible lying in sinusoids associated with endothelial cells as well as Kupffer cells (62). More recent data showing that livers of mice treated with silica, a specific macrophage toxin, still retain significant ability to trap S. typhimurium (34) is further evidence that hepatic trapping involves liver components other than Kupffer cells.

Recent studies indicate that tissue tropisms of bacteria can sometimes be explained by their posession of adhesins enabling them to specifically attach to host tissues containing a receptor (27, 47). One such adhesin is type 1 pili. Many different species of bacteria posess type 1 pili including most Salmonella isolates (14, 17, 18, 20, 21). There is considerable evidence that type 1 pili mediate adhesion of piliated bacteria to eucaryotic cells in vitro (21, 32, 87, 88) and this mechanism is also thought to operate in vivo (1, 33).

The overall objective of this study is to determine the mechanism by which Salmonella typhimurium associates with hepatic tissue in bloodstream clearance. The first aim is to determine whether type 1 pili mediate clearance of S. typhimurium by the perfused liver. The second aim is to determine if S. typhimurium can associate with liver sinusoidal cells other than Kupffer cells as recent evidence has suggested.

LITERATURE REVIEW

The Role of the Liver in Vascular Clearance of Bacteria

Anatomy of Liver Sinusoids. The liver is an organ uniquely adapted to aid the host in filtration of the blood. First, its position in the bloodstream is such that it receives portal venous blood on its way to the heart. The liver can thus absorb nutrients and clear invading pathogens which have entered the circulation across the gastrointestinal tract. Second, blood percolates through liver sinusoids of relatively narrow diameter. Sinusoidal structure allows for close contact of substances in the bloodstream with liver sinusoidal and parenchymal cells. Third, the liver contains Kupffer cells, fixed macrophages of the RES, which are highly phagocytic. In addition, endothelial cells are actively endocytic by pinocytosis and together these cells have tremendous capacity to remove particulate, colloidal, and soluble substances from the bloodstream.

Blood entering the liver flows through a highly branched sinusoidal network before it is collected in the central veins. With the use of the electron microscope, the anatomy of liver sinusoids has been elucidated (34, 61, 63, 103, 104, 108). The sinusoids are lined by endothelial cells which have a flat, streamlined shape with processes extending out to form the lining. This lining is fenestrated, allowing fluid to pass through pores into the space of Disse beneath

the endothelial lining. Hepatic parenchymal cells beneath the endothelium have microvilli which extend into the space of Disse. Kupffer cells lie on the endothelial lining and stretched across the sinusoids. They are anchored by filamentous pseudopodia extending to the sinusoidal endothelium.

The morphology and function of liver sinusoidal cells have been characterized and compared in in situ studies as well as in studies of disaggregated liver cells in suspension. Kupffer cells are fixed macrophages belonging to the RES. The Kupffer cell surface is quite irregular with numerous microvilli (106) and contains both F_c and C3b receptors (64, 65). These large cells (about 10 microns in diameter) have abundant cytoplasm and a low nuclear to cytoplasmic ratio (92, 102). The nucleus is eccentrically located and the cytoplasm contains a large number of vacuoles and lysosomal structures (31, 51, 104, 106, 108). Kupffer cells are actively endocytic, both by phagocytosis and pinocytosis (108), and contain large amounts of several different lysosomal enzymes (52). Cytochemically, Kupffer cells show diffuse cytoplasmic staining for nonspecific esterase activity and positive staining for peroxidase activity in the nuclear envelope and endoplasmic reticulum (31, 65, 106).

Endothelial cells are more numerous and generally smaller than Kupffer cells(15, 31, 50, 92). These flat, streamlined cells round up on isolation and retraction of cellular processes containing the fenestrae forms characteristic sponge-like seive plates visible with transmission electron microscopy (TEM) (31, 105). Endothelial cells have a higher nuclear to cytoplasmic ratio than do Kupffer cells and their cytoplasm contains fewer vacuoles and organelles (31, 102). Although reported to have F_c receptors (15), endothelial cells are not

phagocytic. They are able to ingest particles up to 0.1 micron in diameter by pinocytosis (108). Endothelial cells are cytochemically positive for esterase and negative for peroxidase (31, 92).

Two other sinusoidal cell types have been identified, both of which are present in very small numbers in the liver and in isolated cell suspensions (63, 92, 107, 108). Fat-storing cells, so named because of their characteristic cytoplasmic fat droplets, reside in the space of Disse, under the endothelial lining. These cells are larger than Kupffer cells and stain with the oil red O stain because of the fat droplets (92). The cytoplasm also has a characteristically high content of granular endoplasmic reticulum. Fat-storing cells are nonphagocytic and are thought to play a role in Vitamin A metabolism and storage (12, 102). They reportedly constitute less than 5% of the total number of sinusoidal cells and are only observed in cell suspensions from livers of old rats (92).

Pit cells reside on the sinusoidal wall and in peripheral blood. These cells are smaller than endothelial cells and amount to less than 5% of sinusoidal cells. Pit cells contain characteristic granules which are grouped on one side of the nucleus with organelle-free cytoplasm on the other side. These cells show no endocytic activity and their function is unknown (107, 108).

The particular characteristics mentioned above for Kupffer and endothelial cells are the ones most useful for identification of these cell types. Almost all of this work has been done with rat liver cells. The few studies which have been done with mouse liver cells (15, 16, 51, 96) indicate much similarity exists between sinusoidal cells of mouse and rat livers, however some important differences have been recognized.

Morphologically, mouse Kupffer cells are slightly smaller than rat Kupffer cells and contain more mitochondria and smaller lysosomal structures than do rat Kupffer cells (51). Furthermore, some mouse endothelial cells, unlike rat endothelial cells, exhibit abundant lysosomal structures causing them to be less easily distinguished from mouse Kupffer cells (16). While positive peroxidase staining is characteristic of rat Kupffer cells only (106), 30% of mouse Kupffer cells show no peroxidase activity (16) and 57% of mouse endothelial cells demonstrate a positive peroxidase reaction (96). Finally, while 92% of rat Kupffer cells phagocytose IgG-coated sheep red blood cells, only 50% of mouse Kupffer cells do so (16). Such differences must be considered when applying techniques and procedures established with the rat to a new species and comparison of data from one species to another should be done with caution.

The Reticuloendothelial System and Clearance of Bacteria. The RES consists of phagocytic cells of mononuclear origin including fixed macrophages of liver (Kupffer cells), spleen, lung, bone marrow, and lymph node as well as wandering macrophages of blood and tissue (84). The RES acts as a host defense mechanism against bacteria which enter the bloodstream (41, 82). In addition to clearance of invading bacteria, the RES clears foriegn colloidal and particulate material from the bloodstream (82, 95).

Rogers (82) identified three phases of bloodstream clearance of bacteria. In the first phase, lasting 10 minutes to five hours, bacteria are cleared rapidly and logarithmically from the circulation. This is independent of the particular host and parasite and of the final outcome of infection. In the second phase, the number of circulating

organisms remains constant or declines slowly. This phase lasts several hours or days. The third and final phase lasts from 6 hours to several days. Depending on the virulence of the particular bacterium, organisms will completely disappear from the circulation or bacteriemia will increase resulting in the death of the host.

Many in vivo studies indicate that during the initial phase, when the vast majority of organisms is cleared, most organisms are sequestered in the liver and spleen. For example, Biozzi et al. (6) report that in mice, 70-80% of intravenously injected Salmonella enteritidis are taken up by the liver after 30 minutes. Although only 2-5% of the organisms are located in the spleen, on a weight basis the spleen contains half as many organisms as the liver. Other organs, (lungs, kidneys, blood) contain very few bacteria. In another study, the majority of both Escherichia coli and Staphylococcus aureus are recovered in liver and spleen after intravenous injection into mice (5). Rogers and Melly (81) injected E. coli intravenously into rabbits and obtained simultaneous blood specimens from the portal vein and superior vena cava at various times. By quantitating bacteria in each specimen, they determined that liver and spleen trap an average of 66% of the organisms entering these organs at any time during a 60 minute period following injection. These in vivo studies clearly demonstrate that injected organisms disappear from the circulation and can be recovered, either as viable counts or as radioactive counts, from liver and spleen. They do not, however, clarify the mechanism by which this initial clearance occurs or identify exactly where cleared bacteria reside in the organ.

The perfused liver model has been used for a number of years and provides a unique and simple model for the study of bloodstream

clearance of bacteria. Manwaring and Coe (58) perfused rabbit livers with pneumococci and found that when the bacteria are suspended in Ringer's solution with or without normal rabbit serum, there is no clearance of bacteria from the perfusion medium. When immune serum is added, virtually all of the bacteria are retained by the liver in three passages. Manwaring and Fritschen (59) perfused canine organs with a variety of bacteria in Ringer's solution. Forty percent of the E. coli perfused are cleared, 80% of S. aureus, 25% of Bacillus anthracis, 10% of Bacillus bisepticus, and 4% of Bacillus lactis aerogenes. This study supports the role of liver and spleen as the organs with the greatest "microbic-tissue affinity" although hepatic clearance varies widely for different bacterial species.

Wardlaw and Howard (100) similarly observed different hepatic clearance rates of the perfused rat liver for different bacterial species. Three nonflagellated Gram negative species and two Gram positive species are not cleared when suspended in Ringer's solution while three flagellated Gram negative species and four Gram positive species are cleared to varying degrees. Addition of human serum to the perfusion medium increases hepatic clearance for all Gram negative species and two Gram positive species and reduces clearance for four Gram positive species. One encapsulated Gram positive species is not cleared either in the presence or absence of serum.

Jeunet et al. (45, 46) observed that the perfused rat liver clears Salmonella typhosa and Brucella melitensis well either with or without plasma while Brucella abortus is not cleared at all. With addition of specific antibody, however, B. abortus is cleared.

Bonventre and Oxman (9) found that for S. aureus, clearance by the perfused liver is essentially the same in the presence of

normal or immune rat serum. For S. enteritidis, however, immune serum increases the clearance rate over that seen with normal serum.

Because these studies employed different perfusion procedures, different perfusion media and serum preparations, different preparations of bacteria, and different animal species, direct comparison of results is difficult. Yet two points become evident from early work with the perfused liver model. First, the liver behaves differently with respect to clearance of different bacterial species. It is possible that different mechanisms of clearance may be operative under different conditions. The surface which the bacterium presents to the host also likely makes a difference. Second, serum is generally opsonic for clearance of certain bacteria. Inclusion of normal serum (40, 100) or immune serum (9, 45, 58, 59) in the perfusion medium generally increases clearance.

Howard and Wardlaw (40) demonstrated that the opsonic activity of normal serum for E. coli is heat-labile (56C, 30 minutes), absorbable by homologous but not heterologous E. coli, and absorbable by zymosan. They conclude that specific antibody, complement, and probably properdin contribute to the opsonic activity of serum. It should be noted that in other instances, serum has no opsonic activity (45, 46, 58, 100).

Most of these studies use the term "clearance" synonomously with "phagocytosis". This notion is based on disappearance of bacteria from the perfusion medium with their subsequent accumulation in the liver (9, 40, 45, 59, 100). However, the observation that organisms do not wash out of the perfused liver does not mean that they have been phagocytosed. One study does indicate that cleared bacteria can be observed adhered to and inside Kupffer cells by light microscopic

examinations of liver sections (40). Also, the number of bacteria recoverable from the liver decreases with time indicating that they are being killed (9). Other literature confirms that phagocytes can engulf and kill bacteria *in vitro* (100).

While the association of phagocytosis with clearance was certainly not unreasonable at the time, further work suggests that there may be more to clearance than phagocytosis. Jeunet et al. (45, 46) provided the first suggestion that clearance involved a "recognition function" as well as phagocytosis. Their hypothesis is based on the observation that *B. melitensis* and *S. typhosa* are readily cleared by the perfused liver while *B. abortus* is not. This reflects *in vivo* clearance in that intravenously injected *S. typhosa* localizes primarily in the liver while *B. abortus* localizes primarily in the spleen with only a small amount in the liver. Plasma factors are not required for recognition because perfused liver clearance occurs in the absence of plasma and does not significantly increase with its addition. It is postulated that liver RES cells lack a receptor for *B. abortus* which accounts for the failure of recognition.

More direct evidence that liver clearance is not synonomous with phagocytosis is provided by Moon et al. (62) who distinguish trapping and killing functions of the perfused liver. When *Salmonella typhimurium* are perfused in Medium 199, approximately 70% of the organisms are recoverable from the liver indicating that they have been trapped but not killed. Phase and electron micrographs show many trapped bacteria lying in liver sinusoids. When whole blood or plasma is included in the perfusion medium, the number of organisms trapped is similar and almost 75% of the trapped bacteria are killed. Plasma alone is not responsible for the killing.

A subsequent study provides further evidence that bacterial trapping by the perfused liver is at least partially independent of Kupffer cell function (34). Mice were treated with crystalline silica, a specific macrophage toxin, to damage Kupffer cells both structurally and functionally (impaired Carbon clearance). Normal mouse livers trap about 67% of perfused S. typhimurium while silica-treated livers trap about 31% of a similar number of perfused bacteria. Although maximal trapping occurs when intact Kupffer cells are present, significant trapping still occurs in livers with damaged Kupffer cells suggesting some non-Kupffer cell component is involved.

The above work was done in the absence of plasma. In the presence of serum or plasma, other factors may contribute to the clearance of S. typhimurium by the perfused liver. Trapping is not enhanced in the presence of plasma though killing occurs only in the presence of plasma (35, 62). Addition of specific immune plasma to the perfusion medium enhances perfused liver trapping of S. typhimurium (35). This is suggested to be due to F_c receptor binding of antibody-coated bacteria. Both Kupffer cells (64, 65) and endothelial cells (15) reportedly posess IgG F_c receptors. In the presence of immune serum, bacterial trapping by the perfused liver may thus be partially F_c receptor-mediated.

The Biology of Type 1 Pili

Structure. Pili are nonflagellar filamentous appendages of bacteria and were first identified by Houwink and Van Iterson in 1950 (39). They occur as a variety of morphological types with a variety of functions. The various kinds of pili have been classified in

different schemes (11, 21, 78) although these schemes are not inclusive of all pilus types presently known. Perhaps the most widely occurring type of pilus is the type 1 pilus. These pili are defined by their filament width of 7 nanometers and by mannose-sensitive (mannose inhibitable) hemagglutination and adhesion (55). Type 1 pili are also referred to as type 1 fimbriae, common pili, somatic pili, mannose-sensitive pili, mannose-sensitive hemagglutinin, and mannose-sensitive adhesin. Much disagreement remains as to the correct name and none, as yet, has received universal acceptance (11, 21, 27, 80).

Type 1 pili are prevalent among members of the Enterobacteriaceae. They have been found on most strains of E. coli. (17, 26), Enterobacter (14), Klebsiella (20), Shigella (18), and Salmonella (19, 21, 24). Within the genus Salmonella, about 85% of wild type strains produce type 1 pili (21). The majority of S. paratyphi A, S. sendai, and S. pullorum strains are nonpiliated and most nonpiliated strains belong to the socalled FIRN biotype (fimbriae, inositol, rhamnose negative) which do not ferment inositol or rhamnose and do not spontaneously mutate to produce type 1 pili (21).

Type 1 pili are peritrichously arranged on the surface of the bacterium and there can be as many as 400 pili per cell (11, 54). They are characteristically 7 nm in width and range from 0.5-2.0 microns in length (11, 54, 55, 87). The native pilus is composed of a single protein subunit, pilin, arranged in a helical array to form a filament with an axial hole (11). The pilus is stable to extremely harsh procedures (60) although some treatments which break hydorgen and hydrophobic bonds depolymerize the filament. This process is reversible under appropriate conditions (11).

The molecular weight of pilin from E. coli has been estimated at 16,600 - 17,500 daltons. The amino acid composition shows 40-45% hydrophobic amino acids (11, 55, 87) and the amino acid sequence is known for the amino terminal end of the E. coli pilin subunit (38). Pilin from S. typhimurium type 1 pili has a molecular weight of about 21,000 daltons and an amino acid composition slightly different than that of E. coli pilin, although the two are similar in morphology, isoelectric point, and degree of hydrophobicity (54).

The way type 1 pili are assembled and attached to the cell is not known. Pilin can not be detected in isolated inner or outer membrane fractions (60). Pili are very securely attached to the cell and high speed blending is required to detach or shear them (66). After depiliation, regeneration of pili is rapid. Regeneration is not inhibited by chloramphenicol and is therefore assumed to be assembly of presynthesized subunits rather than de novo synthesis (11).

Antigenicity. As one might expect based on the known differences in pilin, type 1 pili from different species are not antigenically homologous. Shigella type 1 pili are antigenically distinct from those of Salmonella, Enterobacter, and Proteus (37). Shigella flexneri strains all have antigenically identicle pili containing a major group-specific antigen shared with E. coli. E. coli type 1 pili have type-specific major antigens which are shared among related strains as well as the shared flexneri-coli antigens. Salmonella type 1 pili share a common antigen also present in Arizona and Citrobacter but not E. coli, Enterobacter, Klebsiella, or Shigella. In addition to the common Salmonella antigen, each serotype posesses some common antigens distinct from those of other serotypes (23).

A partial basis for antigenic crossreactivity and variability is provided by Soderstrom et al. (94). Two monoclonal antibodies produced to E. coli type 1 pili from a particular strain were tested for crossreactivity against type 1 pili of other E. coli strains. One antibody shows broad crossreactivity and its precipitaion of type 1 pili is blocked by α-methyl-mannoside. The other antibody shows no crossreactivity and its precipitation of the homologous antigen is not blocked by α-methyl-mannoside. This suggests that the common antigenic determinants are related to the mannose-binding site while the variable antigenic determinants are not.

The antigenic relatedness among pili is an important consideration in host immunity and in vaccine production. When reactivity of antiserum to purified pili of a single E. coli strain is tested against a variety of E. coli strains, various degrees of crossreactivity are observed (36). The pilus of a particular strain is called a senior pilus if it raises an antiserum having broad crossreactivity, and is called a junior pilus if its antiserum has only narrow crossreactivity. It is obvious that senior pili will be the most useful for vaccine development.

Genetics. Genetic studies of type 1 piliation in E. coli indicate that the gene(s) for piliation are chromosomal and are located at 98 minutes on the linkage map (10, 14). This was determined by transductional analysis and interupted matings. In S. typhimurium, the pili gene(s) map at 23 minutes on the chromosome (75).

The type 1 pili genes of E. coli have been cloned onto a plasmid and introduction of this plasmid into a previously nonpiliated host strain confers piliation and mannose-sensitive adhesive ability (42). A probe constructed from this plasmid was used to screen other type 1

piliated Enterobacteriaceae and pili genes of most species tested show a high degree of conservation of gene sequence and arrangement (13).

Complementation analysis of type 1 pilus mutants indicates that the pilus genes at 98 minutes in E. coli are composed of three cistrons: pil A, pil B, and pil C. Pil A mutants and pil B mutants complement pil C mutants but many pil A and pil B mutants do not complement one another. It is suggested that C and A-B are separate operons with C exerting positive control over A-B (98).

The gene products of the cloned type 1 pilus genes have recently been analyzed and mapped by expression in minicells with Tn 5 mutagenesis (76). Five proteins are encoded by the three cistrons. The structural gene for pilin is encoded in pil C. Another protein in pil C evidently has a regulatory function because mutants at this locus show constitutive production of pili, producing 10-40 times the amount of pili as the parent strain.

Phase Variation and Expression. In genotypically piliated organisms, phenotypic expression of type 1 pili is regulated by at least two mechanisms: 1) growth cycle variation and 2) phase variation (97).

Growth cycle variation is responsible for changes in the number of pili per cell under various growth conditions. Factors such as temperature, medium, and stage of the growth cycle affect both the number of cells which are piliated and the number of pili per cell in E. coli (97). Serial culture in static aerobic broth favors growth of piliated organisms and serial growth on agar favors growth of nonpiliated or lesser piliated organisms (18, 21). Culture in 1% glucose broth, anaerobic culture in nutrient broth and culture at 44C all favor growth

of nonpiliated organisms (21). During the first six hours of growth in static aerobic broth, the proportion of piliated organisms and the degree of piliation sharply declines after which pili reappear and piliation returns to or exceeds the original proportion (21).

Phase variation is the spontaneous, all-or-none variation between piliated and nonpiliated states. Each phase can give rise to the other at a particular rate which is much higher than the mutation rate (28, 97). The ability to undergo phase variation may be lost and such phase-locked mutants are either stably piliated or nonpiliated (97).

There is some disagreement about the mechanism of phase variation. Some contend that synthesis of type 1 pili is dependent on the concentration of 3'5'-adenosine monophosphate (cAMP) and that synthesis is subject to catabolite repression (85). The evidence for this is that adenylate cyclase-deficient mutants of S. typhimurium do not synthesize type 1 pili unless small amounts (less than 1 mM) of cAMP are added to the culture. In addition, growth of wild type S. typhimurium in any of several sugars represses synthesis of type 1 pili. Because repression of synthesis is not observed in mutants deficient in transport of these sugars, repression of pili synthesis is clearly dependent on metabolism of the sugar.

Others contend that expression of type 1 pili is cAMP independent. They report that cAMP can either supress or enhance piliation depending on the particular E. coli strain (29). Furthermore, transcription of the pilus gene is not repressed by growth in glucose and therefore not subject to catabolite repression. Instead a transcriptional control mechanism is postulated because expression of pili is found to oscillate randomly (28, 30). It is suggested that the transcriptional control mechanism may be similar to the mechanism of flagellar

phase variation in Salmonella (91) in which an invertible DNA sequence puts the flagellar promoter in either the "on" or the "off" position.

A third factor which can influence the amount of piliation in a bacterial culture has little to do with gene expression but is instead dependent on growth rates. Type 1 piliated organisms selectively outgrow nonpiliated organisms in static aerobic broth (22, 73). Piliated organisms grow as a pellicle at the broth-air interface in static aerobic broth culture. The pellicle typically forms at 6-12 hours of culture just after the logarithmic phase of growth (18, 22). It is believed that either the hydrophobic nature of pili keeps bacteria suspended at the air-liquid interface or pili enable bacterial cells to adhere to one another and form a pellicle (27). Piliated organisms thus grow on the broth surface where oxygen tension is greatest, hence energy production is more efficient and growth is more rapid. Nonpiliated organisms must rely on dissolved oxygen in the medium or grow anaerobically, hence energy production is less efficient and growth is slower. Piliated cells thus outgrow nonpiliated cells and the proportion of piliated cells increases in the broth culture.

It is evident then that phenotypic expression of type 1 pili is very complex. It is likely that several mechanisms interplay to result in qualitative and quantitative variation in piliation.

Hemagglutinating Activity and Mannose Inhibition. While protein structure and antigenicity of type 1 pili may vary among species, the unifying characteristic defining type 1 pili is their mannose-sensitive hemagglutination and adhesion activities.

The hemagglutinating activity of type 1 pili was identified very soon after their discovery (14, 17, 18, 19, 20).

Hemagglutinating activity associated with type 1 pili shows red blood cell species specificity with agglutination being strongest for erythrocytes from guinea pig, horse, fowl > human > sheep > goat > ox (17, 18, 19, 20). Evidence that it is pili which are responsible for hemagglutination comes from several studies. First, in surveys of numerous strains of bacteria, the presence of pili on a strain correlates with hemagglutinating activity (14, 17, 18). Second, as a culture undergoes phase variation such as with changing from broth to agar culture, hemagglutinating activity fluctuates with piliation (17, 18, 21). Third, antibodies to pili can inhibit hemagglutination (17,37), and finally, purified pili have hemagglutinating activity (86). The strength of the hemagglutination reaction is dependent on the degree of piliation of a bacterial culture (18). Thus a hemagglutination assay can be used to quantitatively estimate the degree of piliation of a bacterial culture (2, 20).

Similar to erythrocyte agglutinating ability is the ability of type 1 piliated bacteria and purified pili to agglutinate yeasts (18, 20, 53, 68).

Yeast and erythrocyte agglutination as well as adhesion to other cell types by piliated bacteria is inhibited by mannose (18, 20, 53, 86). The specificity of this effect was investigated most completely by Old (74) who tested a wide variety of saccharides for ability ot inhibit hemagglutination. Mannose and certain mannose derivatives (α-methyl-mannoside and yeast mannan) are strongly inhibitory, while fructose is slightly inhibitory. Modification of hydroxyl residues at C-2, C-3, C-4, or C-6 on the mannopyranosyl ring abrogates inhibitory activity. The alpha configuration a C-1 is also important for maintaining inhibitory activity, although the

substituent group at C-1 is irrelevant.

When bacteria and erythrocytes are pretreated with mannose, excess sugar is washed away, and bacteria and cells resuspended together in saline, hemagglutinating activity is normal indicating that binding of mannose to its reaction site is rapidly reversible (18). Further evidence for the reversiblilty of mannose binding is provided by the finding that mannose can elute adherent bacteria from epithelial cells (67, 68).

The finding that addition of α-methyl-mannoside to the culture of piliated bacteria inhibits pellicle formation suggests that mannose acts on the bacteria to inhibit type 1 pilus-mediated activities (72, 73). In addition,pretreatment of type 1 piliated E. coli with yeast mannan (rather than mannose) followed by washing does inhibit their adhesion to leucocytes (3) while pretreatment of cells does not inhibit their adhesion.

The current hypothesis is that mannose binds to pili in a lectin-like reaction such that pili can no longer bind mannose-containing residues on the cell surface (67). Other possible explanations are that mannose covers hydrophobic groups on pili rendering tham more hydrophilic and thus repellent of cells or that pili are allosteric proteins which change from hydrophobic and adhesive to hydrophilic and nonadhesive when mannose binds.

Cell Adhesion. In addition to agglutinating activity for erythrocytes and yeasts, a second activity associated with type 1 pili is bacterial adhesion to other eucaryotic cell types. The cell types to which piliated bacteria adhere include buccal (21, 56, 67, 77), intestinal (18, 20, 21, 43), tracheal (21), and bladder (32, 56, 88, 89)

epithelial cells, leucocytes (20, 21), monkey kidney cells (87), plant cells (20), and fungal cells (21). Although there are minor differences in bacterial adhesion to different cell types, most characteristics of type 1 pilus-mediated adhesion are demonstrable in a variety of systems.

The role of type 1 pili as mediators of bacterial adhesion has been established using in vitro assays. Bacteria and cells are mixed and incubated after which nonadherent bacteria are removed from adherent bacteria and cells. The amount of bacterial adhesion is then determined, typically by microscopic observation and counting (32, 43, 56, 67, 87) or by determination of radioactive counts on bacteria associated with cells (79, 88, 89). Type 1 piliated bacteria readily adhere to cells while nonpiliated strains exhibit very low or no adhesion (32, 43, 87). Adhesion is a saturable phenomenon (43, 67, 88) and is independent of temperature (32, 88). Only a single, long incubation time has standardly been observed so no conclusion can be made regarding the effect of temperature on the rate of bacterial adhesion. When bacterial adhesion to urinary bladder epithelial cells is studied, the optimum pH for adhesion is pH 4-5 which approximates bladder pH in vivo (32, 88).

Mannose and related saccharides inhibit in vitro adhesion of type 1 piliated bacteria to cells (32, 56, 67, 87, 88, 89) in a way identicle to hemagglutination. This effect is concentration dependent (67, 89) and higher concentrations of mannose are required to inhibit bacterial adhesion to cells which are highly receptive for bacteria than to cells of low receptivity (89). Similar to the observations in the hemagglutination system, mannose elutes adherent bacteria (67, 68, 87) and pretreatment of bacteria, but not cells, with mannose inhibits bacterial adhesion (89). Pretreatment of cells with the plant lectin

Concanavalin A, which specifically binds mannose residues, inhibits bacterial adhesion to them (87, 89). Such findings suggest that type 1 pili bind a mannose-containing ligand on the cell surface to affect specific adhesion.

Other findings also support the role of type 1 pili in adhesion. When nonpiliated bacteria are cultured in broth, the appearance of pili correlates with increasing adhesion (32). Treatment of piliated bacteria with trypsin or pepsin eliminates hemagglutinating activity and eliminates adhesion (32). Specific antipilus antibody or Fab fragments thereof inhibit adhesion of piliated bacteria (32, 43, 67, 87). Finally, purified type 1 pili competitively inhibit adhesion of type 1 piliated bacteria to cells (43).

Ofek and Beachey (68) showed that epithelial cells selectively adsorb piliated organisms from a culture containing both piliated and nonpiliated cells. Bacteria and epithelial cells were mixed and adherent bacteria were eluted from cells by treatment with α-methyl-mannoside. The eluted adherent bacteria showed much greater piliation than the nonadherent bacteria by electron microscopy and by ability to agglutinate yeasts.

In addition to type 1 pilus-mediated adhesion observed *in vitro*, there is evidence that bacteria are piliated *in vivo* and attach to cells and mucosal surfaces via type 1 pili. First, the majority of isolates of Enterobacteriaceae from natural sources are piliated (21). Second, antibody to type 1 pili developes with experimental infection. In a rabbit model of pyelonephritis, serum IgG and IgM titers to type 1 pili persist for four months after injection of piliated *E. coli*. IgG anti-pilus antibody is also detectable in the kidneys (93). Third, administration of α-methyl-mannoside can lessen the severity of

an experimental infection with piliated organisms. In one study, injection of E. coli into mouse bladders in 10% α-methyl-mannoside rather than saline decreases the number of animals developing bactiuria (1). Microscopy shows fewer bacteria adherent to the bladder surface in the α-methyl-mannoside-treated group than in the control group. In a similar study, injection of piliated Klebsiella pneumoniae into rat bladders in 5% α-methyl-mannoside abrogates most of the pathology and bacterial adhesion which occurs when piliated bacteria are injected in saline (33). Injection of nonpiliated K. pneumoniae also results in less ulceration and bacterial adhesion in the bladder.

Binding of Purified Pili. Several studies indicate that binding of purified type 1 pili to yeasts, erythrocytes, and other eucaryotic cells is similar in several ways to adhesion of the intact, piliated bacteria to these cells (53, 54, 86, 87). Time, temperature, and pH optima of binding are similar for purified pili and intact bacteria, as are the saccharide pattern of inhibition and inhibition by anti-pilus antibody. In addition, metabolic inhibitors have no effect on the binding of purified type 1 pili to Vero cells (87). Concentrations as low as 1-3 micrograms of pure pilus protein per milliliter are able to agglutinate yeasts and erythrocytes (53, 86), confirming the role of pili in hemagglutination and adhesion.

Little is known about the binding site on the pilus or the nature of the cellular receptor molecule. The cellular receptor has been studied with little success by attempting to change receptive properties of cells with enzyme treatments. Treatment of Vero cells with neuraminidase, trypsin, or protease increases binding of radiolabeled type 1 pili while treatment with α-mannosidase has no effect on

pilus binding (87). If a mannose-containing residue on the cell surface is indeed the receptor molecule, these studies suggest it is either shielded or subterminal. The increase in pilus binding could be attributable to uncovering of the hidden sites, decreasing net surface charge such that cell-pilus repulsion is decreased, or changing mobility of receptors in the membrane. In another study, treatment of erythrocytes with α-mannosidase does increase the amount of pili required for hemagglutination (86), which is consistent with a mannose-containing cell receptor molecule.

Mannose-containing residues on the cell surface are postulated to be the receptor based largely on the specific stereochemistry required for mannose inhibition of bacterial adhesion (74). This is the most plausible explanation although other possibilities are tenable. The wide variety of receptive cell types necessitates a wide distribution of mannose-containing groups on cell surfaces.

The only direct evidence for a carbohydrate-containing molecule as the cell receptor comes from agglutination studies with piliated E. coli and liposomes. Piliated E. coli can agglutinate liposomes constructed with mannose-containing glycolipids but not liposomes constructed with lactose-containing glycopilids (71).

Less clear than the cellular receptor is the nature of the binding site on the pilus. Brinton (11) observed that purified E. coli type 1 pili bind to polystyrene latex beads in end-on fashion. This suggests a terminal location for the binding site. Others observed that bound pili contact the cell membrane along considerable portions of their length, suggesting multiple, lateral binding sites (87). Evidence for lateral binding sites is provided by studies with sonicated type 1 pili (99). Sonication sharply decreases the length

of purified pili and rapidly destroys hemagglutinating activity. When radiolabeled, cell-bound pili are sonicated (and their length presumably substantially shortened) the amount of bound pili falls only slightly. Such results are more consistent with multiple, lateral binding sites than with a terminal binding site.

The amino acid sequence of pilin is presently being investigated. This and studies on secondary and tertiary pilin structure may be helpful in the future to identify the chemical moieties of the binding site.

Some consideration must be given as to whether binding is indeed specific or whether it is nonspecific and can be explained in terms of hydrophobic interactions. In a survey of E. coli clinical isolates, all strains interacting with a hydrophobic gel posess type 1 pili (44). In a similar study, increasing capacity of E. coli strains for mannose-sensitive hemagglutination correlates with a greater degree of hydrophobicity as determined by hydrophobic interaction chromatography (71). The hydrophobicity of pili may allow piliated bacteria to penetrate an electrostatic barrier caused by repulsion of negatively charged bacterial and cell surfaces. Alternatively, hydrophobicity of pili may favor their embedding in the lipid interior of cell membranes. It is likely that some combination of nonspecific hydrophobic interaction and specific mannose-binding results in cell adhesion of piliated bacteria. Studies indicating that hydrophobic interaction can not account for adhesion (71, 99) do not preclude its involvement. They may simply indicate that hydrophobicity alone is not sufficient for adhesion. Nonspecific hydrophobic interactions may be the initial events preceeding specific chemical interactions.

Type 1 Pili and Virulence. Whether type 1 pili determine virulence is a complex question. Inasmuch as pathogenesis of disease is a multifactorial process involving several variables on the part of both host and pathogen, it is unlikely that simply infecting animals with piliated and nonpiliated organisms will provide satisfactory answers.

Certain observations indicate clearly that type 1 pili are not required for virulence. Some pathogenic strains of Shigella (18) and Salmonella (21) are stably nonpiliated, nonhemagglutinating, and nonadhesive. In a survey of Klebsiella isolates, pathogenic strains are nonpiliated and saprophytic strains are piliated (20). Yet the finding that most isolates of Enterobacteriaceae posess type 1 pili (14, 17, 18, 19, 20, 21, 24, 26) suggests that pili confer some advantage to parasites.

That type 1 pili may be useful for maintaining commensalism in the gut is suggested in a study by Duguid et al. (25). A nonpiliated strain of S. typhimurium and a piliated strain derived from it were compared for mouse pathogenicity. Intraperitoneal or conjunctival inoculation results in similar numbers of infections and deaths among mice given the two strains, suggesting no role in infectivity by these routes. When organisms are given orally, however, significantly more infections and deaths result from inoculation with piliated rather than nonpiliated organisms. This study suggests that type 1 pili may contribute to pathogenicity by promoting colonization in the intestine and fecal dissemination.

Results of a study by Orskov et al. (77) suggest type 1 pili mediate adhesion of E. coli to urinary mucus, while a different, mannose-resistant pilus type mediates adhesion of E. coli to uroepithelial cells. Bacteria with only type 1 pili would then associate with mucus covering the

urinary epithelium and be swept away with micturition. In this case, posession of type 1 pili would clearly be disadvantageous for the bacterium.

The above studies point out the difficulty in associating type 1 pili with overall virulence and the differing conclusions that have been reached. They also indicate the necessity of evaluating the role of pili at single, particular steps or events in the process of pathogenesis rather than in overall "infection".

This more narrow approach has been used with success in two areas: 1) in assessing the role of type 1 pili in adhesion to phagocytic cells and 2) in studying the effect of anti-type 1 pilus antibody in infection.

Numerous studies indicate that phagocytes posess receptors for type 1 pili and that piliated bacteria are readily phagocytosed and killed in the absence of serum opsonins. The phagocytes used are typically rodent peritoneal macrophages (3, 4, 8) or human PMN (3, 7, 8, 57, 70, 83, 90). Mannose and related saccharides inhibit phagocytosis of piliated bacteria in a dose-dependent manner while other sugars do not (3, 4, 8, 57, 70, 90). These studies indicate that pilus-mediated association of bacteria with phagocytes does lead to ingestion (57, 83, 90) and killing (8, 57, 70, 90), and does stimulate phagocyte chemiluminescence (7, 8), oxygen consumption (83), release of lysosomal enzymes (57), and myeloperoxidase iodination of proteins (69). Nonpiliated organisms either do not attach to phagocytes or attach in far fewer numbers than piliated organisms (7, 70). In a survey of E. coli strains, those with mannose-sensitive pili attach to phagocytes and those with mannose-resistant pili do not (8). Also, strains of E. coli having mannose-sensitive

hemagglutinating activity stimulate a much greater chemiluminescent response than strains with adhesins other than type 1 pili (7).

Other observations are at variance with those mentioned above. Pretreatment of phagocytes with purified type 1 pili does not inhibit pilus-mediated phagocytosis of bacteria or stimulate phagocyte lysosomal enzyme release (57). Some contend that type 1 pili only mediate attachment of piliated bacteria to phagocytes, while other surface characteristics such as negatively charged capsular K antigens or smooth lipopolysaccharide lead to ingestion of attached organisms (70). Some claim that phagocytosis is mannose inhibitable even in the presence of normal serum (3, 4) while others report no difference between phagocytosis of piliated and nonpiliated organisms in the presence of serum or inability of mannose to inhibit phagocytosis in the presence of normal serum (70, 90).

Evidence also exists suggesting that type 1 pili play little role, if any, in internalization by a nonprofessional phagocyte, the HeLa cell. Entry of S. typhimurium into the HeLa cell resembles phagocytosis in many ways (49). In this case, bacterial adhesion is not mannose-sensitive and does not correlate with type 1 pilus production (48). It may be that in different systems, different adhesins are more or less important in mediating attachment depending on the conditions of the particular system.

A majority of the evidence favors an important role for type 1 pili in mediating phagocytosis. One study suggests it is as efficient as anti-pilus antibody in doing so (69). These findings argue against type 1 pili as a virulence determinant, at least in tissues where piliated bacteria can contact phagocytes.

A second instance where posession of type 1 pili by bacteria might be a liability rather than an asset is in the immune host, depending on the antibacterial properties of anti-pilus antibody.

The role of anti-type 1 pilus antibody in host defense has been studied only recently. One study shows that, in a phagocytosis assay, coating piliated bacteria with anti-pilus antibody results in a large increase in protein iodination by PMN. This effect, however is not mediated by the F_c portion of the antibody molecule but rather the pili, since Fab fragments are as effective as whole antibody molecules in stimulating iodination and iodination is inhibited by α-methyl-mannoside but not by staphylococcal protein A (69). It is postulated that the effect of anti-pilus antibody is to aggregate pili making them more active in stimulating the phagocyte. In another instance, however, phagocytosis of antibody-coated E. coli by macrophages was not mannose inhibitable (4) suggesting that pili do not mediate adhesion to phagocytes in the presence of specific antibody.

The antibacterial properties of monoclonal antibodies specific for type 1 pili have also been studied (94). They are not effective in increasing phagocytic killing of piliated organisms, nor are they capable of protecting neonatal rats or adult mice from infection or death after intraperitoneal challenge with piliated bacteria. Two antibodies of differing antigenic specificity were used in this study. It might be that monoclonal antibodies of still different antigenic specificity might give different results.

In agreement with these findings are those of Weinstein and Silverblatt (101) that anti-type 1 pilus antibody is not effective in increasing vascular clearance or in mediating complement-dependent

bacteriolysis of piliated bacteria. It does not increase opsono-phagocytosis of piliated bacteria and may even inhibit phagocytosis. This antiserum, however, does inhibit bacterial adhesion to buccal epithelial cells, suggesting that the principal antibacterial mechanism of anti-pilus antibody is inhibition of attachment to epithelial cells.

Anti-type 1 pilus antibody may thus be beneficial to the host on mucosal surfaces but inconsequential or even detrimental to the host in the vasculature or in tissues. Once again the particular event or location is important in determining the pathogenic significance of type 1 pili or protective significance of anti-pilus antibody.

The role for type 1 pili in pathogenesis which emerges is one which depends on several variables: the particular pathogen and host, the site of the pathogen, and the immune status of the host, among others. Depending on the microenvironment, it might be advantageous for the pathogen to express or not express type 1 pili. Given this, phase variation may provide an adaptive mechanism whereby at least a portion of the infecting population might evade host defense. In that sense, phase variation has pathogenic significance.

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ARTICLE 1

ASSOCIATION OF TYPE 1 PILI WITH THE ABILITY OF LIVERS TO CLEAR *SALMONELLA TYPHIMURIUM*

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Association of Type 1 Pili with the Ability of Livers to Clear *Salmonella typhimurium*†

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The role of type 1 pili in the adherence of *Salmonella typhimurium* strain SR-11 to hepatic sinusoidal cells was investigated. An average of 66.7% of piliated organisms was cleared by perfused livers on a single pass. Mannose and α-methyl-D-mannoside inhibited such trapping in a dose-dependent manner. Preincubation of the bacteria, but not the liver, with either sugar also inhibited trapping, suggesting that the sugar binds to bacterial, not hepatic, receptors. Significant numbers of previously trapped bacteria could be eluted by adding mannose to the wash medium. Bacteria with reduced piliation, obtained either by growing bacteria on agar or by using a nonpiliated variant of the parent strain, were trapped to a significantly lesser extent than the parent strain. The liver appears to selectively trap heavily piliated organisms since reperfusion of bacteria through a second liver results in significantly less trapping than occurs with the first perfusion. In vivo, the nonpiliated variant strain was cleared much more slowly than the piliated parent strain. Mannose and α-methyl-D-mannoside, but not glucose, decreased clearance rates of piliated organisms. Cumulatively, the data suggest that type 1 pili are a major factor in hepatic clearance of *S. typhimurium*.

Many different species of bacteria possess type 1 pili on their surfaces (4, 16), and most *Salmonella* isolates from natural sources possess type 1 pili (6, 7). Although the particular biological functions of type 1 pili remain controversial, considerable evidence exists that pili mediate bacterial attachment to eucaryotic cells, particularly gastrointestinal and genitourinary epithelial cells. Piliated bacteria also adhere to monkey kidney (18) and epithelial cells in vitro (9, 19). Organisms with substantially reduced or damaged pili have a reduced adherence capacity for epithelial cells (5, 6, 8, 13).

Purified type 1 pili (17) and piliated bacteria (5, 6, 15) both agglutinate guinea pig erythrocytes. D-Mannose and certain related saccharides, including α-methyl-D-mannoside, can inhibit type 1 pilus-mediated adherence and hemagglutination (13, 15, 17, 18).

Little of the above information helps clarify a role, if any, for type 1 pili in adherence of bacteria to deep tissues or to vascular endothelium. The primary aim of this study is to determine whether type 1 pili mediate adherence of *Salmonella typhimurium* to hepatic sinusoidal cells. To accomplish this aim we used both in vitro and in situ experimental models. The in situ studies involved a perfused liver model which has previously allowed us to experimentally distinguish between bacterial trapping and killing mechanisms of hepatic tissue (12). Using this model, we have also shown that hepatic trapping of *S. typhimurium* involves adherence to both endothelial and Kupffer cells (11). In this study the model was employed to demonstrate a role for type 1 pili in adherence of *S. typhimurium* to hepatic endothelium.

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MATERIALS AND METHODS

Bacteria. *S. typhimurium* SR-11 and a spontaneously arising nonpiliated, nonflagellated variant of strain SR-11 were used in most experiments. They were subcultured daily in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C.

Mice. Female CD-1 mice (24 to 28 g) were purchased from Charles River Laboratories, Portage, Mich. They were given food and water ad libitum.

Liver perfusions. The liver perfusion technique has been described in detail elsewhere (12). Briefly, a mouse was heparinized and anesthetized. The abdomen was opened, the portal vein was cannulated, and sterile medium 199 (M-199; GIBCO Laboratories) was allowed to flow through the liver. The inferior vena cava was cut below the kidneys to allow the medium to drain. The chest cavity was then opened, and the vena cava was cannulated. The inferior vena cava was tied off just above the kidneys. The liver was washed free of blood with 40 to 50 ml of M-199.

Bacteria were harvested by centrifugation, washed once in M-199, and resuspended in M-199 to 10^9 colony-forming units per ml. One milliliter of the bacterial suspension was perfused and then washed

TABLE 1. Saccharide inhibition of trapping of *S. typhimurium* by perfused livers

Saccharide[a]	% Recovery in:[b]		Total recovery
	Liver	Perfusate	
None	66.7 ± 10.6	25.8 ± 7.7	92.5 ± 8.8
Mannose	16.2 ± 9.2[c]	82.3 ± 16.6[c]	98.5 ± 9.5
α-Methyl-D-mannoside	23.2 ± 6.1[c]	74.8 ± 10.9[c]	98.0 ± 9.1
Fructose	40.4 ± 13.4[c]	50.1 ± 13.4[c]	90.7 ± 11.6
Galactose	73.4 ± 8.1	25.3 ± 2.3	98.9 ± 7.2
Glucose	69.7 ± 6.5	36.4 ± 6.5	106.6 ± 5.4
Lactose	82.8 ± 2.2	22.6 ± 4.7	105.4 ± 5.9
Mannitol	62.5 ± 6.3	33.8 ± 5.0	96.2 ± 6.5
Ribose	63.1 ± 12.2	27.5 ± 4.3	90.5 ± 14.1
Sucrose	69.9 ± 6.9	25.3 ± 7.9	95.2 ± 8.8

[a] All sugars were present at 1% (wt/vol) in M-199 for the entire perfusion.

[b] Mean ± standard deviation of at least six experiments.

[c] $P < 0.001$ versus control (no sugar) by the White rank order method (21).

with 50 ml of sterile M-199 over a 30-min period. The liver was then excised and homogenized, and the bacteria in the homogenate were counted by diluting in distilled water and plating on tryptose agar pour plates. The perfusate and the initial bacterial suspension were blended and plate counted in a similar way.

Data are expressed as the percentage of bacteria in the liver homogenate, the percentage in the perfusate, and the percent total recovery, using the initial bacterial count as 100%. Percent killing equals 100% minus the percent total recovery. Percent trapping equals the percentage in the liver homogenate plus the percentage killed.

Hemagglutination assay. Hemagglutination assays were performed as previously noted (2, 5) with slight modification. Guinea pig erythrocytes were washed three times in sterile saline and suspended to 3% (vol/vol) in either saline alone or saline plus 0.5% D-mannose. Bacteria were harvested by centrifugation (8,000 rpm for 10 min) and washed once with sterile distilled water. They were plate counted as above to determine the cell concentration. Bacteria were serially diluted (1:2) in saline (0.1-ml amounts) in wells of a porcelain-enamel plate. To each well was added 0.1 ml of 3% Guinea pig erythrocytes in saline. After a 10-min incubation at room temperature with mixing, the wells were read for agglutination. If necessary, 0.1 ml of the suspension was spread on a glass slide to facilitate reading. Guinea pig erythrocytes plus saline and guinea pig erythrocytes in 0.5% mannose plus bacteria were always included as controls. The minimum hemagglutinating concentration was the smallest number of bacteria per milliliter which caused hemagglutination.

Transmission electron microscopy. Both bacteria from cultures and bacteria prepared for perfusion were observed. A drop of bacterial suspension was placed on a Formvar-coated copper grid, allowed to stand for 2 min, and blotted off with filter paper. A drop of 1% phosphotungstic acid was then put on the grid, and the excess was drawn off with filter paper. The specimen was then viewed by transmission electron microscopy (TEM).

TABLE 2. Dose-dependence of inhibition of trapping by D-mannose.

Mannose concn % (wt/vol)	% Recovery in:[a]		Total recovery
	Liver	Perfusate	
None	66.7 ± 10.6	25.8 ± 7.7	92.5 ± 8.8
1.0	16.2 ± 9.2	82.3 ± 16.6	98.5 ± 9.5
0.1	37.1 ± 9.6	62.5 ± 11.2	99.6 ± 6.1
0.01	50.6 ± 8.0	42.7 ± 8.0	93.3 ± 6.4
0.001	58.1 ± 4.9	37.8 ± 8.8	96.0 ± 5.4

[a] Mean standard deviation of at least seven experiments.

Blood clearance of bacteria. Mice were injected intravenously in a tail vein with approximately 10^9 bacteria in 0.2 ml of saline or with 10^9 bacteria plus either 0.125 g of D-mannose or 0.125 g of glucose in 0.35 ml of saline. At timed intervals after injection, 0.1 ml of blood was obtained from the retroorbital plexus and plate counted in tryptose agar. The number of bacteria remaining in the bloodstream was calculated by assuming the blood volume to be 8% of body weight. The phagocytic index (K) and biological half-life ($t_{1/2}$) of intravenously injected bacteria are calculated by the method of Biozzi et al. for carbon clearance analysis (3).

Chemicals. All sugars used in perfusion media were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Inhibition of bacterial clearance by the perfused liver in the presence of selected sugars. Various sugars were added individually to the perfusion medium at a concentration of 1% (wt/vol). Table 1 shows that of nine sugars tested, only mannose, α-methyl-D-mannoside, and to some extent fructose were able to significantly inhibit trapping. Mannose and α-methyl-D-mannoside decreased trapping to very low levels. A dose-dependent relationship existed between mannose concentration and inhibition of clearance (Table 2).

Mechanism of mannose inhibition of trapping. In the above experiments, mannose was present in the perfusion medium throughout the perfusion. To investigate more specifically where and when mannose exerts its inhibitory effect, we removed the mannose from the medium used to wash the liver before bacteria were perfused (prewash) or from the medium used to wash the liver after bacteria were perfused (postwash). For maximal inhibition of clearance to occur, mannose need not be present in the postwash after bacteria have been perfused (data not shown). If mannose is also removed from the prewash and only perfused with the bacteria, slightly less than maximal inhibition of clearance is observed (data not shown). To determine

TABLE 3. Liver clearance of piliated *S. typhimurium* after preincubation with sugars

Experimental[a]	% Recovery in:[b]		Total recovery
	Liver	Perfusate	
Control (mock-treated)	85.3 ± 6.2	12.0 ± 7.9	98.9 ± 9.1
Mannose (1%)	22.8 ± 8.7[c]	71.6 ± 12.1	93.9 ± 7.9
α-Methyl-D-mannoside (1%)	22.5 ± 4.5[c]	85.2 ± 13.6	107.8 ± 13.1
Glucose (1%)	80.5 ± 6.8[d]	17.0 ± 3.4	97.4 ± 7.9

[a] Bacteria were incubated with the appropriate sugar in M-199 for 30 min at 37°C followed by centrifuge washing in M-199 before perfusion. Percentages are the concentrations of the sugars used.
[b] Mean ± standard deviation of at least six experiments.
[c] $P < 0.001$ versus control (mock-treated) by the White rank order method (21).
[d] Not statistically significant versus control.

more precisely the mechanism of mannose inhibition of trapping, we separately pretreated the liver and the bacteria with mannose. When livers were washed with 30 ml of M-199 containing 1% mannose followed by 20 ml of M-199 alone, bacterial clearance was not significantly different than that of normal control livers (liver, 61.0% ± 6.2; perfusate, 34.0% ± 7.0; total recovery, 95.0% ± 6.9). By contrast, when piliated bacteria were preincubated with M-199 containing carbohydrate for 30 min at 37°C followed by washing in M-199 alone, both mannose and α-methyl-D-mannoside significantly reduced trapping (Table 3). Pretreatment with glucose had no effect.

Various sugars were tested for their abilities to elute bacteria previously trapped in the liver. In this case the sugar was included in the perfusate only after bacteria had been perfused (Table 4). Both mannose and α-methyl-D-mannoside were able to elute significant numbers of trapped bacteria, whereas glucose and mannitol were ineffective at similar concentrations. A higher concentration of mannose resulted in increased elution of trapped bacteria.

Perfusion of a mutant strain and agar-grown *S. typhimurium* SR-11. *S. typhimurium* SR-11 was both flagellated and piliated when viewed by TEM (Fig. 1A and 1C). Strain SR-11 showed motility in motility test medium and was able to agglutinate guinea pig erythrocytes (Table 5). When transferred to a tryptose agar slant and incubated at 37° with daily subculture for 2 weeks, strain SR-11 retained its motility and flagella, but showed a sharp decrease in piliation and ability to cause guinea pig erythrocyte agglutination (Table 5). Agar-grown bacteria showed identical reactions to broth-grown bacteria in biochemical tests, in sensitivity to antibiotics, and in O and H serotypes.

A spontaneously arising variant strain of SR-11 was isolated. The isolate was identical to the parent *S. typhimurium* strain in standard biochemical tests, in sensitivity to antibiotics, and in O antigen serotype. It was nonpiliated and nonflagellated by TEM with negative staining (Fig. 1B and 1D). It was nonmotile when tested in motility test medium and did not cause hemagglutination of guinea pig erythrocytes. This organism was originally believed to be a true genetic mutant because of the absence of both pili and flagella, but after several months of laboratory subculture, a few pili could be observed on some cells and slight hemagglutinating ability was detectable. Thus, this organism might be a phase variant rather than a true mutant.

Table 5 compares these three strains with

TABLE 4. Ability of sugars to elute trapped bacteria from the liver

Experimental[a]	% Recovery in:[b]		Total recovery
	Liver	Perfusate	
None	69.1 ± 8.3	17.1 ± 4.2	86.8 ± 6.7
Mannose (2%)	37.6 ± 11.6[c]	54.5 ± 14.2	92.1 ± 11.9
Mannose (1%)	54.4 ± 10.3[d]	37.9 ± 10.3	92.2 ± 9.1
α-Methyl-D-mannoside (2%)	33.7 ± 13.8[c]	63.7 ± 15.3	97.3 ± 8.4
Glucose (2%)	60.6 ± 11.8[c]	40.2 ± 11.3	99.4 ± 4.1
Mannitol (2%)	77.5 ± 4.9[c]	15.3 ± 6.0	92.8 ± 6.8

[a] The sugar, at the indicated concentration, was in the perfusate only after the bacteria had been perfused.
[b] Mean ± standard deviation of at least six experiments.
[c] $P < 0.001$ versus control (no sugar) by the White rank order method (21).
[d] $P < 0.05$ versus control.
[e] Not statistically significant versus control.

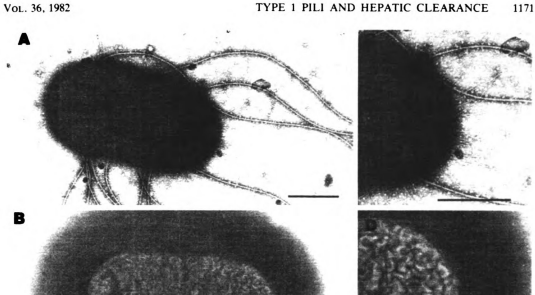

FIG. 1. Piliated and nonpiliated *S. typhimurium* by TEM. A and C: Broth-grown, piliated *S. typhimurium* strain SR-11. Bar, 500 nm. B and D: Nonpiliated, nonflagellated variant. Bar, 500 nm.

respect to hepatic trapping. Trapping of agar-grown strain SR-11 and the nonpiliated mutant are both significantly reduced compared with that of the parent strain. When agar-grown strain SR-11 was returned to broth and subcultured daily, within 3 to 5 days it recovered its pili (as determined by TEM) and its ability to be cleared by the perfused liver (data not shown).

Clearance of previously perfused bacteria. Not every cell in a bacterial population is piliated, and the proportion of piliated cells within a given population varies (6). To determine if clearance was selective for a portion of the bacterial population, we harvested bacteria from a perfusate by centrifugation and perfused them through a second liver at a concentration of 4×10^7 to 9×10^7 bacteria per ml. Control perfusions used freshly grown bacteria at the same concentration. Table 6 shows that previously perfused bacteria are trapped to a significantly lesser degree than are freshly grown bacteria. In addition, perfused bacteria, harvested by centrifugation, were tested for the ability to cause guinea pig erythrocyte agglutination. Before perfusion, the minimal hemagglutinating concentration of bacteria for guinea pig erythrocytes was 1.8×10^8 bacteria per ml. The minimal hemagglutinating concentration of bacteria harvested from

TABLE 5. Perfusion of broth and agar-grown *S. typhimurium* SR-11 and a nonpiliated variant strain

Bacterial strain	MHC for guinea pig erythrocytes[a]	% Recovery in:[b]		Total recovery
		Liver	Perfusate	
Broth-grown *S. typhimurium* (piliated phase)	8.6×10^7	66.7 ± 10.6	25.8 ± 7.7	92.5 ± 8.8
Agar-grown *S. typhimurium* (nonpiliated phase)	3.9×10^9	8.4 ± 2.4[c]	87.2 ± 7.3	95.6 ± 7.1
Nonpiliated variant	Negative[d]	1.2 ± 0.6[c]	98.7 ± 9.2	99.8 ± 9.2

[a] MHD, Minimum hemagglutinating concentration (bacteria per ml) calculated as described in the text.
[b] Mean ± standard deviation of at least seven experiments.
[c] $P < 0.001$ versus broth-grown *S. typhimurium* (piliated phase) by the White rank order method (21).
[d] Bacteria did not cause hemagglutination at doses of 2.2×10^{10} bacteria per ml.

TABLE 6. Trapping of previously perfused piliated *S. typhimurium* by mouse livers

Bacteria	% Recovery in:[a]		Total recovery
	Liver	Perfusate	
Control[b]	60.1 ± 11.2	46.2 ± 10.5	106.2 ± 8.7
Previously perfused[c]	39.4 ± 8.4[d]	66.2 ± 9.5[d]	105.6 ± 10.3

[a] Mean ± standard deviation of at least seven experiments.

[b] Freshly grown bacteria prepared for perfusion at a concentration of 6×10^7 to 8×10^7 bacteria per ml, as described in the text.

[c] Bacteria perfused through one liver, harvested from the perfusate by centrifugation, and resuspended to 4×10^7 to 9×10^7 bacteria per ml.

[d] $P < 0.001$ versus control by the White rank order method (21).

perfusates had increased to 9.1×10^8 bacteria per ml. Perfused bacteria were less able to cause hemagglutination than the same preparation of bacteria before perfusion.

In vivo bacterial clearance. In vivo bacterial clearance rates were determined in mice given piliated *S. typhimurium* SR-11 in the presence and absence of mannose, α-methyl-D-mannoside, or glucose and in mice given the nonpiliated variant. Figure 2 shows that normal mice cleared greater than 90% of the piliated bacteria within 30 min in the absence of added mannose (phagocytic index, $K = 0.053$; biological half-life, $t_{1/2} = 5.7$ min). In the presence of mannose, clearance of piliated *S. typhimurium* was slowed significantly ($K = 0.022$, $t_{1/2} = 13.7$ min). α-Methyl-D-mannoside had a similar effect (data not shown). Clearance in nonpiliated variant was significantly slower than clearance of the piliated strain ($K = 0.010$, $t_{1/2} = 29.2$ min). Clearance in the presence of glucose was not significantly different from clearance of the piliated organism alone ($K = 0.055$, $t_{1/2} = 5.4$ min).

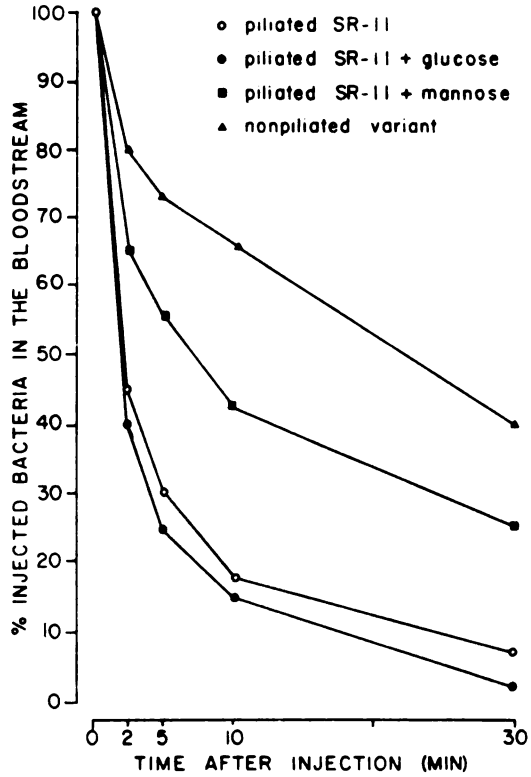

FIG. 2. In vivo clearance of intravenously injected bacteria. Piliated *S. typhimurium* in the presence (■) and absence (□) of mannose and in the presence of glucose (●). Nonpiliated *S. typhimurium* (▲).

DISCUSSION

Saccharide inhibition of *S. typhimurium* trapping by the perfused liver is dose-dependent for mannose and also occurs with α-methyl-D-mannoside and, to a lesser extent, fructose (Tables 1 and 2). This same pattern of saccharide inhibition has been observed for other activities believed to be mediated by type 1 pili such as hemagglutination (15, 17) and in vitro cell adherence (18). Old (15) emphasized the importance of unmodified hydroxyl groups at C-2, C-3, and C-4 of the six carbon chain and a specific requirement for the α configuration at C-1 for a saccharide to inhibit type 1 pilus-mediated hemagglutination. This could explain why mannose and α-methyl-D-mannoside inhibit, whereas the closely related sugars glucose and mannitol do not.

The sugar need not be present in the perfusion medium to inhibit bacterial clearance if the bacteria have previously been incubated with the sugar (cf. Table 3). Coupled with the observation that saccharide pretreatment of the liver has no effect on bacterial trapping, these data suggest that mannose binds to the bacteria rather than to the liver. This is similar to pilus-mediated epithelial cell adherence (19) and consistent with the hypothesis that the pilus is involved in a lectin-like surface reaction with a mannose-containing ligand on the eucaryotic cell (14, 18). The data showing that mannose specifically elutes trapped bacteria (Table 4) lend support to the possibility that mannose is a competitive inhibitor. They also indicate that, at least initially, liver trapping of bacteria is reversible.

The role of pili in trapping was studied more directly by comparing piliated and nonpiliated organisms derived from our parent piliated strain. The degree of piliation of broth-grown and agar-grown phases and the nonpiliated vari-

ant of *S. typhimurium* were compared by TEM and guinea pig erythrocyte agglutination. Although the exact number of pili were not quantitated, in general, the extent of piliation as seen by TEM and the activity as measured by hemagglutination correlated with the amount of trapping by the perfused liver (Table 5). Cultivation on agar medium significantly reduced, but did not eliminate, pili on *S. typhimurium* SR-11. Since we cannot rule out differences other than a lack of pili between the agar-grown and broth-grown phases, the data must be interpreted with caution. Duguid et al. observed that cultivation of piliated *Salmonella* sp. on agar decreased the proportion of cells which possessed pili to a very low percentage (6). Fader et al. used this property to obtain piliated and nonpiliated phases of *Klebsiella pneumoniae* and observed that piliation correlated with in vitro adherence to rat bladder epithelial cells (9).

The variant of *S. typhimurium*, confirmed as nonpiliated by TEM and hemagglutination, was trapped by the perfused liver only in very low numbers (cf. Table 5) and is consistent with pilus-mediated liver clearance of bacteria. Though similar to the parent strain in many characteristics, this nonpiliated variant is also nonflagellated. There might also be differences in properties not studied or other subtle surface differences between the parent and daughter strains. As such, these data do not conclusively establish that pili mediate liver trapping, but do imply that they are important. The relatively small amount of these nonpiliated bacteria which were trapped (1.2%) might represent an additional pilus-independent mechanism for liver clearance of bacteria.

Two possibilities exist with respect to trapping of individual organisms within the liver. Individual cells in a bacterial population might be randomly cleared by the liver, each cell having an equal probability of being trapped, or certain cells might be more likely to be cleared because of certain unique surface characteristics which enhance their probability of attachment. If liver clearance is random, than equal percentages of bacteria should be cleared with each pass through the liver. If certain bacteria are more likely to be trapped than others, then a population less likely to be trapped will be selected by one passage through the perfused liver. The data in Table 6 regarding previously perfused bacteria favor this latter possibility. Previously perfused organisms were not trapped as well as control organisms (Table 4), and they were less able to cause hemagglutination. These results are similar to those of Ofek and Beachey (13), who showed that an *Escherichia coli* subpopulation which initially adheres to buccal epithelial cells and then is displaced by incubation with α-methyl-D-mannoside is high in the number of heavily piliated organisms and possesses considerable ability to aggregate yeast cells. The subpopulation which does not initially adhere does not contain piliated organisms and does not aggregate yeast cells. Thus, it might be that liver clearance of *S. typhimurium* is similar to buccal epithelial cell adherence in that the portion of organisms which is trapped or adheres is piliated to a higher degree than that of the subpopulation which is nonadherent.

In vivo bacterial clearance was studied to determine if the mannose-sensitive trapping observed in situ occurred in vivo as well and to determine if blood clearance was affected by the presence or absence of pili. In previous studies we showed that the liver is the major clearance organ in vivo for both live bacteria (11) and chromium-labeled endotoxin (22). A major concern of our perfusion studies is that they reflect in vivo realities. The results suggest that the in situ system does reflect the in vivo system. Piliated bacteria are readily cleared from the bloodstream and trapped by the perfused liver. D-Mannose and α-methyl-D-mannoside, but not glucose, retard clearance in both systems. The mutant strain is not readily cleared in vivo or in the perfused liver system. Hence, both in vivo and in situ, the presence of type 1 pili can be associated with increased clearance or trapping.

There is growing evidence that pilus-mediated attachment phenomena observed in vitro actually reflect in vivo events and that pili do mediate attachment in vivo. Silverblatt observed by electron microscopy that *Proteus mirabilis* attaches to epithelial cells of the renal pelvis via pili in early experimental pyelonephritis (20). Aronson et al. reported that α-methyl-D-mannoside given with bacteria inhibited *E. coli* adherence to rat bladder epithelial cells in experimental cystitis (1). Fader and Davis also observed that piliated-phase *Klebsiella pneumoniae* was less able to adhere to the rat bladder surface in vivo in the presence of α-methyl-D-mannoside (10).

The data in this study provide evidence suggesting that type 1 pili mediate trapping of bacteria by the liver. Subsequent experiments will investigate the nature of this binding and expand upon the in vivo relevance of these observations.

ACKNOWLEDGMENTS

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ARTICLE 2

IN VITRO ADHESION OF *SALMONELLA TYPHIMURIUM* TO MOUSE LIVER NONPARENCHYMAL CELLS

ABSTRACT

An assay was developed to study adhesion of Salmonella typhimurium to mouse liver nonparenchymal cells in vitro. A nonparenchymal cell suspension consisting of approximately 35% Kupffer cells, 45% endothelial cells, and 20% lymphocytes was prepared from enzymatically disaggregated livers. ^{51}Chromium-labeled S. typhimurium SR-11 were mixed with nonparenchymal cells and nonadherent bacteria were separated from adherent bacteria and cells by filtration through a 5 micron pore size Nucleopore filter. At 37C, bacterial adhesion to nonparenchymal cells was maximal at 10 minutes incubation and did not substantially change thereafter up to 60 minutes incubation. Incubation at 4C slowed the rate of bacterial adhesion but did not substantially change the amount of bacterial adhesion at 60 minutes. Mannose and α-methyl-mannoside inhibited bacterial adhesion while glucose and α-methyl-glucoside did not. As initial bacteria to nonparenchymal cell ratio increased from 10:1 to 1000:1, adhesion of type 1 piliated S. typhimurium greatly increased while adhesion of nonpiliated S. typhimurium remained low. The nonparenchymal cell population was fractionated by centrifugal elutriation to yield three subpopulations enriched for Kupffer cells, endothelial cells, or lymphocytes. Type 1 piliated S. typhimurium adhered to cells of all three fractions and adhesion was mannose-sensitive. The data suggest that type 1 pili are a major determinant in adhesion of S. typhimurium to mouse liver nonparenchymal

cells *in vitro* and confirm previous observations made with the perfused mouse liver.

INTRODUCTION

The liver, containing cells of the reticuloendothelial system, is an important host organ for bloodstream clearance of many bacteria (25), including Salmonella typhimurium (9). As a result of observations of bacterial killing by the liver and opsonic activity of serum in the perfused liver model, phagocytosis was assumed to be the mechanism by which bacteria were cleared (3, 10, 12, 25). Yet, just because bacteria can not be washed out of the perfused liver does not mean that they have been phagocytosed. Some investigators have suggested a recognition function of hepatic tissue apart from phagocytosis (12).

Although phagocytosis may contribute to clearance or be an event subsequent to clearance, previous work in our laboratory suggests that clearance (trapping) and killing (phagocytosis) are separate functions of hepatic tissue, at least for Salmonella typhimurium. Such a conclusion is based on electron microscopic observations of organisms trapped by the perfused liver showing bacteria in the sinusoids apparently associated with both Kupffer and endothelial cells (9, 20) and on data showing that perfused livers of mice treated with silica, a specific macrophage toxin, still retain significant ability to trap S. typhimurium (9).

Type 1 or mannose-sensitive pili have been implicated as mediators of bacterial adhesion to mammalian epithelial cells (6, 8, 26, 27) and previous work in our laboratory suggests that type 1 pili of S. typhimurium facilitate bacterial clearance by the perfused mouse liver

(18). Perfused mouse livers trap approximately 67% of type 1 piliated S. typhimurium strain SR-11 on a single pass while only 1.2% of a nonpiliated phase variant of S. typhimurium SR-11 is trapped. Inclusion of mannose in the perfusion medium specifically inhibits liver trapping of piliated bacteria in a dose-dependent manner. Because incubation of type 1 piliated bacteria in mannose before perfusion is sufficient to inhibit trapping by the perfused liver, it is believed that the pilus binds a mannose-containing ligand on the eucaryotic cell surface to effect adherence. This is analogous to other models in which type 1 pili mediate bacterial adhesion to eucaryotic cells (23,26).

The association of type 1 pili with liver trapping in the perfused liver model is also observed in vivo in that piliated phase S. typhimurium are cleared from the bloodstream both faster and to a greater extent than are nonpiliated phase bacteria. In addition, injection of mannose with piliated organisms significantly slows their disappearance from the circulation (18).

For this study, a radiometric in vitro adherence assay has been developed using ^{51}Chromium-labeled S. typhimurium and isolated mouse liver nonparenchymal cells. It allows for greater manipulative control over bacteria and nonparenchymal cell incubation conditions than does the perfused liver model. The in vitro assay is used here to confirm the observations made with the perfused liver model. An attempt is also made to determine to which liver sinusoidal cells bacteria adhere.

MATERIALS AND METHODS

Preparation of Radiolabeled Bacteria. S. typhimurium strain SR-11 was maintained in piliated phase by serial subculture in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37C. Nonpiliated phase S. typhimurium SR-11 was maintained by serial subculture on Tryptose Agar (Difco) at 37C.

For use, piliated and nonpiliated organisms were grown in BHI broth for 24 hours at 37C and washed twice by centrifugation in Medium 199 (M199, Difco). Forty to fifty microCuries of ^{51}Chromium (as $Na_2{}^{51}CrO_4$ in saline, 1 mCi/ml, New England Nuclear, Boston, MA) was added to the bacteria and the mixture incubated at 37C for 4-5 hours with stirring. Labeled bacteria were washed twice in M199 and re-suspended to 1-2 X 10^{10} colony-forming units (cfu)/ml in suspension buffer (see below). Labeling efficiency averaged 40%. Cfu per milliliter were determined by standard plate count and specific activity was determined by gamma counting an aliquot of labeled bacteria. A ratio of 1 count per minute (cpm) to 5000 cfu was typical (assuming random incorporation of label).

To assess piliation of ^{51}Chromium-labeled bacteria, a yeast agglutination assay was performed as previously described for a hemagglutination assay (18), substituting a glutaraldehyde-fixed Saccharomyces cerevisiae suspension (6) for guinea pig red blood cells. The minimal agglutinating concentration (MAC) was the smallest number of bacteria per milliliter which had yeast

agglutinating activity.

Preparation of Mouse Liver Nonparenchymal Cells. Female CD-1 mice (18-24 grams, Charles River Laboratories) were used in all experiments. Mouse liver cells were suspended by the procedure of Seglen (28) with some modification. Surgical technique for mouse liver perfusion has been described in detail elsewhere (20). The liver was washed free of blood by perfusion with 50 ml of perfusion buffer (28) at 37C. It was then perfused with 30 ml of enzyme buffer at 37C which was recirculated over a 30 minute period by means of a polystaltic pump. Enzyme buffer consisted of calcium and magnesium-free Hank's Balanced Salts Solution (HBSS) containing 0.75% Collagenase (Type IV, Sigma Chemical Co., St. Louis, MO) and 0.1 % Hyaluronidase (Sigma) (11), substituting 75 mM HEPES (Sigma) for $NaHCO_3$ and supplementing with 0.05% Bovine Serum Albumin, 100 U penicillin G/ml, 100 micrograms streptomycin sulfate/ml, and 5.5 mM glucose. Following perfusion, the liver was transferred to a Petri dish containing suspension buffer (28) at 4C and gently teased to disperse cells. This initial cell suspension was filtered through 250 micron nylon mesh to remove clumps of undigested material and contained both liver parenchymal and several types of nonparenchymal cells.

The larger parenchymal cells were removed by centrifugation at 50 x g for 1 minute (22) and remaining nonparenchymal cells were harvested by centrifugation at 500 x g for 7 minutes. Contaminating red blood cells were lysed with Gey's Balanced Salts Solution containing 130 mM NH_4Cl (21). Remaining perenchymal cells and cell debris were removed by centrifugation in 17.5% Metrizamide (Grade II, Sigma), leaving nonparenchymal cells in the supernatant while debris pellets (14). After washing to remove Metrizamide, the resulting nonparenchymal cell

preparation was resuspended to the desired concentration in suspension buffer.

Characterization of Nonparenchymal Cells. Cell number and viability were determined by counting in 0.2% Trypan Blue in saline. Nonparenchymal cell morphology was observed by light microscopy. Cytocentrifuge preparations of cell suspensions were made using a Cytospin 2 (Shandon Inst.). Slides were air-dried, fixed in methanol for 10 minutes, and stained by the May-Grunwald-Giemsa method. At least 200 cells of each suspension were observed.

Cytochemical staining for nonspecific esterase was performed by the method of Li et al.(7, 19) using naphthyl-AS-acetate as substrate.

The ability of cells to phagocytose latex beads was assessed in vitro by mixing 1 X 10^6 cells with two drops of a 1:10 dilution of latex spheres (0.81 microns diameter, Difco) in M199 plus 10% newborn calf serum. After incubation at 37C for one hour with 5% CO_2 atmosphere and agitation, the mixtures were filtered through Nucleopore membranes of 5 micron pore size, rinsed with 5 ml medium, and the cells were washed off the filters. Cytocentrifuge preparations were made as described above. At least 200 cells of each suspension were examined for the presence of intracellular latex spheres at 1000X magnification.

Centrifugal elutriation. For some experiments, the nonparenchymal cell preparation was fractionated using centrifugal elutriation as outlined by Knook and Sleyster (14) for rats, using the modifications these authors have suggested for mice (16). A JE6-B rotor with a Sanderson chamber in a J2-21 centrifuge (Beckman Inst., Palo Alto, CA) was employed. For all separations, HBSS was the medium used, rotor speed was 2500 rpm, and temperature of the entire system was 4C.

Nonparenchymal cells were pumped into the separation chamber at a flow rate of 6 ml/min and 100 ml of outflow was collected. Three fractions of 100 ml each were collected at flow rates of 13.5, 21, and 40 ml/min. Fractions were harvested by centrifugation at 500 x g for 15 minutes and resuspended in suspension buffer. Cell types in the three fractions were characterized as described above.

In Vitro Bacterial Adherence Assay. An aliquot of ^{51}Chromium-labeled bacteria containing the desired number of organisms was mixed with 2-4 X 10^5 nonparenchymal cells. If present, saccharide was added to the desired final concentration and the volume brought to 0.2 ml total with suspension buffer. Typical experiments employed a bacteria to cell ratio in the assay tube of approximately 1000:1, unless otherwise stated. Each tube was set up in triplicate and control tubes containing bacteria only (and saccharide, if present) were incubated with experimental tubes. Standard incubation conditions were 37C for 30 minutes in a gyrotory water bath with shaker at high setting.

After incubation, the mixture was filtered through a Nucleopore polycarbonate filter of 5 micron pore size followed by a rinse with 10 ml of HBSS. Nonparenchymal cells with attached bacteria remained on the filter while unattached bacteria passed through. Filters were counted in a gamma counter (Beckman 5500) to determine counts per minute associated with the filters. Cpm due to unattached bacteria remaining on the filter were determined by filtration of bacteria alone. These cpm were subtracted from the number of cpm remaining on the filter when bacteria were mixed with cells. Data are expressed as mean number of bacteria adhered per nonparenchymal cell or, because this value showed considerable daily variation, as adherence relative to the designated control.

To determine the reliability of the radiometric adherence assay, it was initially compared with a similar assay in which bacteria on filters were enumerated by standard plate count. In this case, filters were blended for 30 seconds at high speed in a Waring blender, diluted in sterile distilled water, and plated on Tryptose Agar pour plates.

RESULTS

Cellular Composition of the Nonparenchymal Cell Suspension. About 30% of the nonparenchymal cells initially suspended from the liver could be recovered by this procedure and they were usually more than 93% viable. There was less than 1% parenchymal cell contamination and a small amount of subcellular debris. A few cell nuclei were visible by light microscopy.

The various cell types were identified using three methods: observation of cellular morphology by light microscopy, *in vitro* phagocytosis of latex beads, and cytochemical staining for nonspecific esterase. Results are shown in Table 1.

Kupffer cells exhibited an oval or elongated, often indented, nucleus which was usually located eccentrically. Their ropey chromatin stained lightly. The cytoplasm was lightly basophilic and highly vacoulated. Endothelial cells had a more darkly staining round or oval nucleus with densely packed chromatin. The slightly granular cytoplasm was basophilic. Lymphocytes had a round, deeply staining nucleus, often filling almost all of the cell. The cytoplasm was deeply basophilic and stained homogenously. Other cell types present included polymorphonuclear leucocytes (PMN) and eosinophils and these cells exhibited their typical morphology (described elsewhere, 29).

Latex positive cells exhibited several, often clumped, refractile latex spheres contained in the cell cytoplasm. Cellular morphology

Table 1. Cellular composition of the nonparenchymal cell population.

	Kupffer cells	Endothelial cells	Lymphocytes	Other cells
Morphology	34±1[a]	46±3	19±4	2±1
Latex phagocytosis	36±2 positive	64±2 negative		
Esterase cytochemistry	78±3 positive		22±3 negative	

[a]Mean percentage (±standard error) of at least 4 determinations.

could also be observed in these cytocentrifuge preparations. Kupffer cells are the only cell type which internalizes latex spheres of 0.81 microns in diameter (4, 5, 32). A low number of cells exhibited typical Kupffer cell morphology yet contained no latex spheres. Some PMN also had phagocytosed latex spheres. These were excluded from tabulations.

Esterase positive cells appeared blue while esterase negative cells were clear when viewed by light microscopy. Both Kupffer and endothelial cells are positive for nonspecific esterase (4, 7) and these cells exhibit diffuse cytoplasmic staining. Lymphocytes are esterase negative, except for a subclass of human T lymphocytes which reportedly contain a single perinuclear spot of esterase activity (17).

Reliability of the Radiometric Adherence Assay. Several controls were performed to ensure that the in vitro adherence assay was a reliable measure of bacterial adhesion to mouse liver nonparenchymal cells. Radiolabeled bacteria alone were incubated at assay conditions, filtered through a Millipore filter of 0.22 micron pore size, and the filtrate and filter were gamma counted. Up to 7% of the cpm in the initial labeled bacteria preparation dissociated from the bacteria during the incubation period. This dissociated label (as sterile filtrate of the labeled bacteria) did not associate with nonparenchymal cells over a standard incubation period and fewer than 5% of the counts associated with a Nucleopore filter. Taken together, these data indicate that, after assay, counts per minute observed on filters are representative of bacteria which have become trapped on the filter.

The radiometric adherence assay was compared with a similar assay in which bacteria were enumerated by standard plate count rather than gamma counting. The mean ($\pm$ standard error) number of bacteria adhered

per nonparenchymal cell was 75±32 when determined by the standard plate count method and 69±21 when determined by the radiometric assay (n=7). There was no significant difference in the number of bacteria adhered per nonparenchymal cell as determined by the two methods.

Bacteria alone or mixtures of bacteria and cells were prepared as for assay and bacteria were counted by standard plate count both before and after incubation to determine whether bacterial multiplication had occurred. For both piliated and nonpiliated phase S. typhimurium, recovery was not significantly different from 100%, indicating that bacteria did not multiply in the presence or absence of nonparenchymal cells nor were they killed in the presence of nonparenchymal cells.

Effect of Time and Temperature of Incubation on Adhesion of Piliated S. typhimurium to Mouse Liver Nonparenchymal Cells. To determine the effect of time and temperature of incubation on bacterial adhesion measured by this assay, bacteria and cell mixtures were incubated at 4C or 37C for 2, 10, 30, or 60 minutes. Results are shown in Figure 1. At 37C, bacterial adhesion was maximal at 10 minutes and did not substantially change as incubation time increased to 60 minutes. At 4C, bacterial adhesion reached a maximum similar to that observed at 37C but a longer incubation time was required to reach this level.

Effect of Saccharides on Adhesion of Piliated S. typhimurium. The specificity of saccharide inhibition of adhesion was studied by testing various saccharides for their ability to inhibit bacterial adhesion to mouse liver nonparenchymal cells. Saccharide was included in the incubation mixture at a final concentration of 0.1% (wt/vol). Table 2 indicates that both mannose and α-methyl-mannoside had

Figure 1. Effect of time and temperature of incubation on adhesion of piliated S. typhimurium to mouse liver nonparenchymal cells. Adherence is espressed relative to that observed at 30 minutes incubation at 37C (Control).

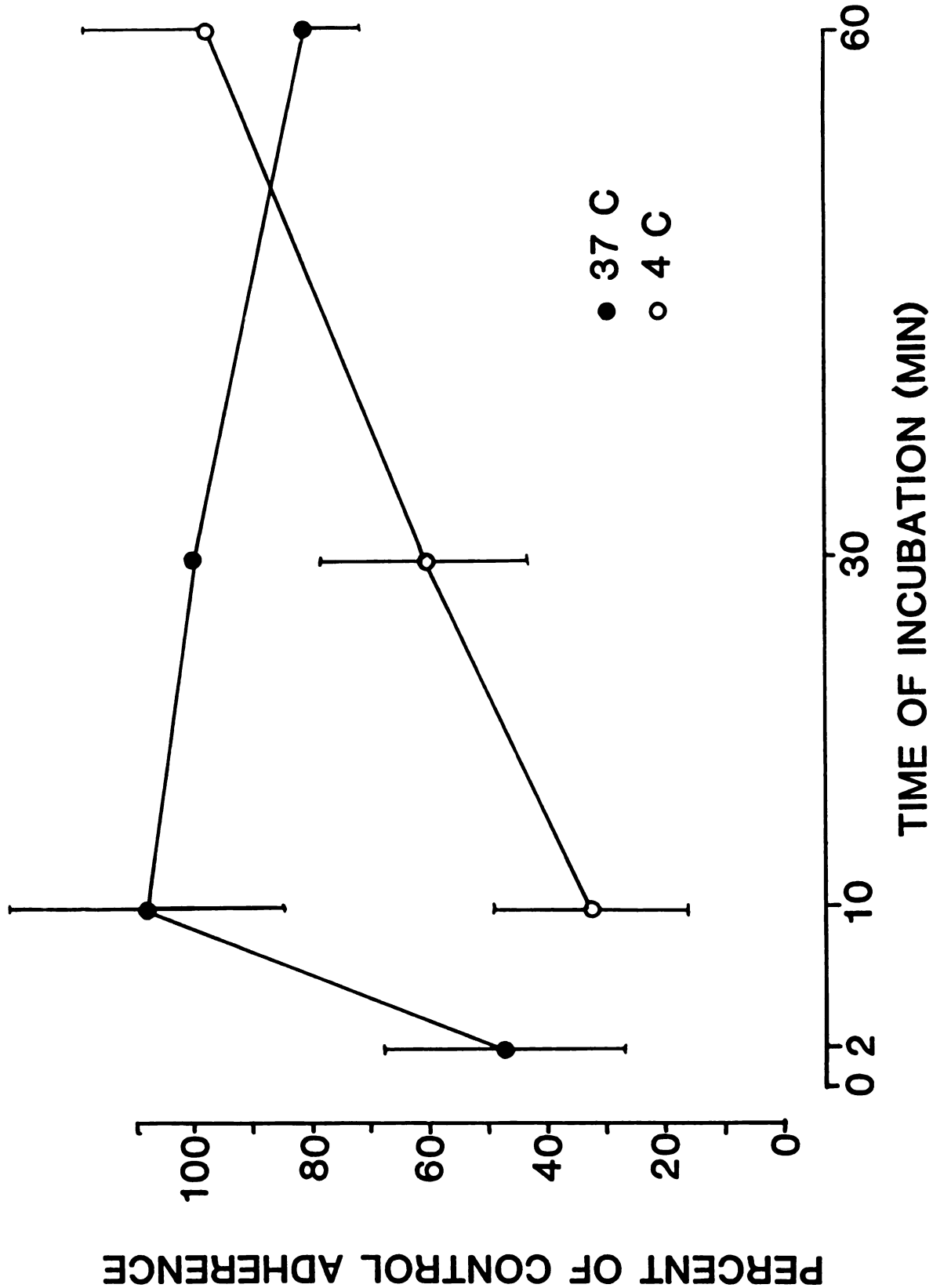
PERCENT OF CONTROL ADHERENCE
0
20
40
60
80
100
TIME OF INCUBATION (MIN)
0 2
10
30
60
● 37 C
○ 4 C

Figure 1.

Table 2. Effect of various saccharides on adhesion of piliated *Salmonella typhimurium* to mouse liver nonparenchymal cells *in vitro*.[a]

Saccharide	Percent of control adherence[b]
Control (none)	100
Mannose	8±6[c]
α-Methyl-mannoside	10±3[c]
Glucose	90±8[d]
α-Methyl-glucoside	98±8[d]

[a]Approximately 3 X 10^8 bacteria were mixed with 4 X 10^5 mouse liver nonparenchymal cells for assay. Saccharide was at a final concentration of 0.1% (wt/vol).

[b]Mean ± standard error of at least five experiments.

[c]$P<0.05$ versus control by paired t test.

[d]Not statistically significant versus control.

significant inhibitory activity and the closely related saccharides glucose and α-methyl-glucoside did not. Furthermore, a dose-dependent relationship existed between mannose concentration and inhibition of adhesion (Table 3).

Adhesion of Piliated and Nonpiliated Phase S. typhimurium. Immediately before use in the adherence assay, ^{51}Chromium-labeled bacteria were tested for piliation using a yeast agglutination assay. Piliated phase bacteria showed uniformly good ability to agglutinate yeasts (average MAC = 6 X 10^6 cfu/ml) while nonpiliated phase bacteria showed either very poor or no yeast agglutinating ability (MAC > 8.7 X 10^9 cfu/ml). Both piliated and nonpiliated phase bacteria were tested for nonparenchymal cell adhesion at initial bacteria to cell ratios ranging from 10:1 to 1000:1.

Piliated phase bacteria adhered in significantly greater numbers than did nonpiliated phase bacteria and adhesion of nonpiliated phase organisms was very low (Table 4). As initial bacteria to cell ratio increased, adhesion of piliated phase bacteria increased substantially, while adhesion of nonpiliated bacteria remained low.

Adhesion of piliated and nonpiliated phase organisms was visualized by May-Grunwald-Giemsa staining of filters after the adherence assay. Figure 2A shows a mixture of piliated phase S. typhimurium and nonparenchymal cells. Adherent bacteria can be seen on and around the nonparenchymal cells. Figure 2B shows a mixture of nonpiliated phase bacteria and nonparenchymal cells. In this case, very few bacteria are visible associated with nonparenchymal cells. Inclusion of mannose in mixtures of piliated phase S. typhimurium and nonparenchymal cells gave results similar to Figure 2B (not shown).

Table 3. Dose-dependent relationship of mannose and inhibition of adhesion.

Mannose concentration % (wt/vol)	Percent of control adherence[a]
Control (none)	100
0.1	10± 4
0.01	67±37
0.001	94±32

[a]Mean ± standard error of at least seven experiments.

Table 4. Adhesion of piliated and nonpiliated Salmonella typhimurium to mouse liver nonparenchymal cells in vitro.

	Number of bacteria adhered per nonparenchymal cell[a]		
	Bacteria to cell ratio[b]		
Bacterial phase	10:1	100:1	1000:1
Piliated	2±1	13±2	66±12
Nonpiliated	0±0[c]	1±0[c]	3± 1[c]

[a]Mean ± standard error of five experiments.

[b]Nonparenchymal cell concentration remained constant at 2×10^5 cells per assay tube and bacterial cell number varied from 2×10^6 to 2×10^8 bacteria per tube to obtain the desired bacteria to cell ratio.

[c]$P<0.05$ versus adhesion of piliated phase organisms.

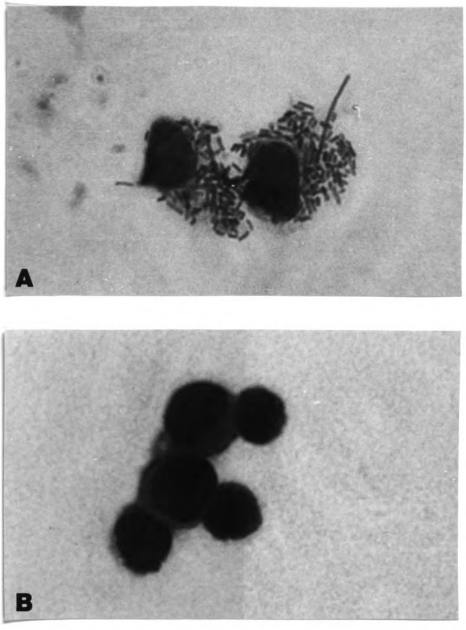

Figure 2. Adhesion of S. typhimurium to mouse liver nonparenchymal cells. A. Piliated S. typhimurium and nonparenchymal cells. Bar, 5 microns. B. Nonpiliated S. typhimurium and nonparenchymal cells. Bar, 5 microns.

Bacterial Adhesion to Subpopulations of Nonparenchymal Cells. To determine to what extent bacteria adhered to each of the major nonparenchymal cell types, the nonparenchymal cell preparation was fractionated using centrifugal elutriation. The cellular composition of the three fractions is given in Table 5. Fraction 1 was somewhat enriched for lymphocytes yet still contained significant numbers of other nonparenchymal cell types. Fraction 2 was enriched to approximately 70% endothelial cells and fraction 3 was enriched to about 65% Kupffer cells.

Bacterial adhesion to cells of the three fractions was tested in the presence and absence of 0.1% mannose. Results are shown in Table 6. Bacteria adhered in significant numbers to cells of each fraction. When expressed as relative adherence (number of bacteria adhered per fraction 2 cell set equal to 1.00), proportionately fewer bacteria adhered per fraction 1 cell and proportionately more bacteria adhered per fraction 3 cell. In all three cases, bacterial adhesion was mannose-sensitive.

Table 5. Cellular composition of nonparenchymal cell subpopulations produced by centrifugal elutriation.

		Kupffer cells	Endothelial cells	Lymphocytes	Other cells
Fraction 1	Morphology	8±2[a]	38±3	54±2	1±1
	Latex phagocytosis	3±1 positive	97±1 negative		
	Esterase cytochemistry	50±2 positive		50±2 negative	
Fraction 2	Morphology	19±2	72±4	10±3	1±0
	Latex phagocytosis	17±5 positive	83±5 negative		
	Esterase cytochemistry	88±3 positive		12±3 negative	
Fraction 3	Morphology	62±3	31±3	6±1	0
	Latex phagocytosis	53±3 positive	47±3 negative		
	Esterase cytochemistry	93±1 positive		7±1 negative	

[a]Value shown is mean percentage ± standard error of four determinations.

Table 6. Adhesion of Salmonella typhimurium to mouse liver nonparenchymal cell subpopulations.

Cells of	Relative adhesion[a]	Percent inhibition of adhesion with 0.1% mannose
Fraction 1 (lymphocyte-enriched)	0.80±0.05[b]	98±1[b]
	$p<0.05$[c]	
Fraction 2 (endothelial cell-enriched)	1.00	95±2
	$p<0.05$[c]	
Fraction 3 (Kupffer cell-enriched)	1.75±0.27	97±1

[a]Number of bacteria adhered per fraction 2 cell set equal to 1.00.

[b]Mean ± standard error of four determinations. Number of bacteria adhered per cell for fraction 1 cells = 32±6, for fraction 2 cells = 40±8, and for fraction 3 cells = 61±18.

[c]Statistical significance determined by Student's t test.

DISCUSSION

The nonparenchymal cell suspension is a heterogenous population consisting of cells resident in liver sinusoids and blood cells remaining after the organ has been perfused free of gross blood. Our nonparenchymal cell preparation contained approximately 35% Kupffer cells, 45% endothelial cells, and 20% lymphocytes. This distribution agrees well with other results reported for mice (4) and with results for rats (7, 16). In similar work done with mice, Knook and Sleyster (16) obtain a nonparenchymal cell suspension consisting of 54% Kupffer cells, 26% endothelial cells, and 20% lymphoid cells. Differences in mouse strain, in enzymes used to disaggregate liver cells, in method of cell preparation, or in criteria used for cell identification might account for this discrepancy. A ratio of about 5 endothelial cells to 4 Kupffer cells in the intact rat liver has been estimated by observation of tissue sections (32). Although lymphoid cells are not resident cells of the liver sinusoids, white blood cells are visible in sinusoids of perfusion-fixed specimens by electron microscopy (33), and lymphocytes are present in liver cell suspensions prepared by enzymatic disaggregation (4, 7, 16, 30).

Several controls upheld the reliability of the radiometric adherence assay in that radioactive counts on the filters were indicative of bacteria attached to nonparenchymal cells. This was an important consideration given the uncertainty of the mechanism by which Chromium associates with bacteria and the finding that a small

percentage of counts dissociated from labeled bacteria during assay incubation.

Data are sometimes expressed as adherence relative to a designated control because the calculated number of bacteria adhered per non-parenchymal cell showed considerable daily variation. This is typical of assays of this type (8, 27). To express data in terms of bacteria adhered per nonparenchymal cell is somewhat artificial in that microscopic observation of adherence mixtures revealed that nonparenchymal cells with attached bacteria tended to aggregate in clumps. This was true especially at very high bacteria to cell ratios. It is not known to what extent such aggregation might facilitate nonspecific trapping of unadhered bacteria on filters, if at all.

At 37C, bacterial adhesion was rapid and increased with time to a plateau after which it did not significantly change (Figure 1). This agrees in principle with the findings of others (26), although slightly longer times were required to reach maximal adhesion in that system.

The rate of bacterial adhesion to nonparenchymal cells decreased when incubation temperature decreased, however at 60 minutes incubation, temperature made no significant difference in the amount of bacteria adhered. This latter finding is consistent with other results for adhesion of type 1 piliated bacteria to various cells (8, 26, 27). The slower rate of bacterial adhesion at lower temperature may be the result of decreased nonparenchymal cell membrane fluidity, thereby decreasing mobility of receptors in the membrane and hence ability to be bound by pili.

Mannose and α-methyl-mannoside specifically inhibited bacterial adhesion to mouse liver nonparenchymal cells while glucose and

α-methyl-glucoside had no effect (Table 2). This is characteristic of type 1 pilus-mediated reactions and these observations confirm those of the perfused liver model (18). The concentration of saccharide required for maximal inhibition is about tenfold less in the in vitro assay system than in the perfused liver system. This probably reflects the differing number of bacteria and, especially, nonparenchymal cells in the two assay systems.

In contrast to the rapid adhesion of type 1 piliated bacteria to nonparenchymal cells, nonpiliated phase bacteria exhibit a very low degree of adhesion (Table 4). Furthermore, adhesion of piliated S. typhimurium to a constant number of nonparenchymal cells greatly increased as the number of bacteria increased. This was not true for nonpiliated bacteria. The nonpiliated variant thus seems genuinely unable to adhere and it is unlikely that the lack of adhesion is simply a problem of inappropriate conditions or concentration. This nonpiliated variant was derived from the piliated strain, lacked guinea pig erythrocyte agglutinating ability, and was not trapped by the perfused mouse liver (18).

The small amount of adhesion of the nonpiliated variant observed at higher bacteria to cell ratios (Table 4) as well as the failure of mannose to completely abolish adhesion (Table 2) may be indicative of a mechanism of association of bacteria and nonparenchymal cells other than type 1 pilus-mediated adhesion. It might also be accounted for by a few remaining pili on some cells of the nonpiliated phase culture.

Type 1 pilus-mediated adhesion could well be the mechanism which accounts for clearance of S. typhimurium by the liver. Determination of the liver sinusoidal cell type(s) to which bacteria adhere is important. If bacteria adhere both to phagocytic Kupffer and to nonphagocytic

endothelial cells, further evidence would be obtained for separation of trapping and killing functions of hepatic tissue.

Centrifugal elutriation was used to separate nonparenchymal cells into subpopulations. This technique yields highly purified preparations of rat Kupffer and endothelial cells (14, 15, 16, 30). Initial attempts to separate mouse sinusoidal cells seemed promising (16) although a subsequent report indicates that results with mouse cells are not as good as those with rat cells (5). The reason for this is less distinct differences between mouse Kupffer and endothelial cells than between rat Kupffer and endothelial cells (5).

The fractions obtained by centrifugal elutriation (Table 5) represent approximately twofold enrichment for the various cell types over their respective concentrations in the heterogenous nonparenchymal cell suspension. Since these fractions are by no means pure, the data must be interpreted with caution.

Mannose-sensitive adhesion of piliated S. typhimurium was observed to cells of each of the three fractions (Table 6) suggesting that these bacteria can adhere to all three cell types by a type 1 pilus-mediated mechanism. Adhesion of bacteria to cells in suspension indicates that trapping observed in the perfused liver is not mechanical, due to clogging of the sinusoids.

Relative adhesion differed for the various cell types (Table 6), being greatest to cells of the Kupffer cell-enriched fraction and least to cells of the lymphocyte-enriched fraction. This trend was statistically significant even though the cell subpopulations were somewhat less than pure. This might reflect real differences in receptor affinity or density on the different cell surfaces. Alternatively, it may simply be due to different cell size in the three

fractions. Centrifugal elutriation separates cells based on cell size and density and the average cell diameter increases for each fraction collected. The observed differences in relative adhesion could then be due to the capacity of larger cells to adhere greater numbers of bacteria. This would argue against large differences in receptor affinity or density for the three cell types.

The data in Table 6 suggest that bacteria can mediate adhesion of bacteria to Kupffer cells. This finding indicates that Kupffer cells do play a role in liver trapping. It is consistent with the finding that perfused livers of silica-treated mice trap fewer S. typhimurium than do normal livers (9). Adherence to the phagocyte cell membrane is considered to be an initial step in phagocytosis (31) and previous studies suggest type 1 pili facilitate phagocytosis of Gram-negative bacteria by mouse peritoneal macrophages and human PMN (1, 2). Other evidence suggests type 1 pili do not mediate phagocytosis of S. typhimurium by HeLa cells (13). It remains to be determined whether type 1 pili promote phagocytosis by Kupffer cells as well as mediate trapping.

The finding that S. typhimurium can adhere to endothelial cells by a type 1 pilus-mediated mechanism provides the strongest evidence to date that non-Kupffer cell components of the liver are involved in hepatic clearance of bacteria. The concept of hepatic clearance as the sole function of RES cells (Kupffer cells) may become obsolete as data is obtained suggesting that endothelial cells function in clearance of substances from the circulation even though they are only pinocytotic and not phagocytic. Consistent with this finding is that of Praaning-van Dalen et al. (24) that endothelial cells as well as Kupffer cells are responsible for clearance of several colloidal substances once

thought to be cleared only by Kupffer cells.

The significance of the apparent pilus-mediated adhesion to lymphocytes is unknown. Certainly a large part of the observed adhesion is to the contaminating Kupffer and endothelial cells. It might be that what apparently occurs *in vitro* does not occur *in vivo* where conditions such as the relatively short time that bacteria remain in the circulation or the presence of serum would tend to discourage bacterial association with lymphocytes even though there is a potential receptor on this cell's surface.

The data support the notion of mannose-sensitive bacterial adhesion to both mouse liver endothelial and Kupffer cells. They confirm and extend the findings with the perfused liver model (18) and provide further support for the hypothesis that type 1 pili can mediate liver clearance of *Salmonella typhimurium*.

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CONCLUSION

The overall objective of this study was to determine the mechanism by which Salmonella typhimurium associates with hepatic tissue in bloodstream clearance.

The first aim was to determine whether type 1 pili mediate clearance of S. typhimurium by the perfused liver. Mannose-sensitive trapping of piliated organisms and the inability of the perfused liver to trap nonpiliated organisms suggests that type 1 pili are a major factor in perfused liver clearance of S. typhimurium. Intravenous injection of piliated and nonpiliated S. typhimurium indicates that pili affect bloodstream clearance in vivo as well as in the perfused mouse liver.

The second aim of this study was to determine if S. typhimurium can associate with sinusoidal cells other than Kupffer cells. An in vitro adherence assay was employed to demonstrate that type 1 piliated S. typhimurium adhere to subpopulations of nonparenchymal cells enriched for Kupffer cells, endothelial cells, or lymphocytes and this adhesion is inhibited by mannose. Nonpiliated S. typhimurium do not adhere to nonparenchymal cells in suspension. Overall, the data indicate that type 1 pili mediate hepatic clearance of S. typhimurium and that this trapping involves both adhesion to Kupffer and to endothelial cells.