Effect of Mouse Peritoneal Fluid on Strains of Enteric Bacilli.* (27025)

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The widespread occurrence throughout the subphylum Vertebrata of serum complement of very similar character suggests to us that the presence of this group of proteins may be of survival value to the animal species possessing it(1). One property of serum which may have survival value and is dependent upon complement is its ability to kill certain strains of gram-negative enteric bacilli *in vitro*(2). Evidence has been presented that enteric bacilli sensitive *in vitro* to the action of the complement-dependent bactericidal system of serum (i.e. serum-sensitive strains) are less likely than resistant strains to elicit bacteremia in man(3). Serum-sensitive strains do not survive as well as serum-resistant strains in the blood stream of the rabbit(4). Also it has been shown that serum-sensitive bacilli placed in intraperitoneal diffusion chambers are more likely to be killed by guinea pig peritoneal fluid than serum-resistant strains(5).

One serious objection to the proposal that the bactericidal activity of serum may be of survival value is the fact that, unlike man, the guinea pig, rabbit, and other vertebrates tested, the mouse does not possess a humoral bactericidal system which can be demonstrated by usual techniques *in vitro*(6). McGhee(7) was able to demonstrate *in vitro* a very small degree of hemolytic activity in normal mouse serum. Nevertheless several investigators(8,9) have confirmed the finding of Brown(10) that C'2 is missing in the mouse. Conclusions regarding C'3 and C'4 are not, however, in agreement. In the past, therefore, the lack of bactericidal activity of mouse serum has generally been attributed to an absence or relative deficiency of one or more of the major components of complement. Very recently a reëvaluation of this problem by Borsos and Cooper(11) indicated the presence of all 4 components of complement in mouse serum. They suggest that the lack of complement activity *in vitro* may be attributed to a serum substance which is perhaps actually one of the group of C'3 factors. Upon reaction of this substance with the C'1, 4, 2 complex a stable successor is formed.

In spite of this inability to demonstrate complement activity in mouse serum *in vitro*, the work of Carey *et al.*(12) indicates that complement may, nevertheless, be active *in vivo*. They reported that when gram-negative enteric bacilli are injected into the peritoneal cavity of the mouse, these bacilli undergo spheroplast formation and ultimate lysis. Under these circumstances complement may even play a rôle in determining the outcome of the host-parasite relationship. When an LD 50 inoculum of gram-negative enteric bacilli was injected into the peritoneal cavity, Carey *et al.* could, by determining the percentage of inoculated organisms undergoing spheroplast formation, predict whether the inoculum would be fatal.

Jeter and McKee(13) studied changes in resistance of mice to bacterial infections following intravenous injection of rabbit antiserum containing antibodies to mouse complement. Although they found decreased resistance to several gram-positive cocci, they found no discernible effect on resistance to gram-negative bacilli.

The Work of Rowley(14), while not discounting humoral factors, suggests that cellular activity plays the major rôle in removal of enteric bacilli from the mouse peritoneal cavity.

The present study was initiated to evaluate further the possible bactericidal property of mouse peritoneal fluid. In this study a modified Algire diffusion chamber provided an environment in which the fate of a bacterial inoculum could be determined when it was subjected *in vivo* to humoral defense mechanisms, yet remain relatively protected from cellular elements. It was of interest to compare, using the

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same test organisms, these results with those obtained in the guinea pig, which has an active complement system *in vitro*. The diffusion chamber also provided a means for the collection of peritoneal fluid for bactericidal studies done *in vitro*.

Materials and Methods. All seven organisms used were strains recovered from human cases of bacteremia or bacilluria with the exception of the 2 strains of *Salmonella typhimurium*.‡ These 2 typhimurium strains differ considerably in their virulence for the mouse. The less virulent strain (172) was derived from the more virulent strain (173) through passage on agar by Jensen(15). All mice used in these experiments were adult CF#1. The LD_{50} for CF#1 mice, when injected intraperitoneally, was between 9 and 90 organisms for typhimurium strain 173, and between 10^6 and 10^7 organisms for typhimurium strain 172. The sensitivities to active normal human and guinea pig sera were known for all the organisms used. In our experience, strains of bacteria sensitive to guinea pig serum have been sensitive in slightly varying degree to human serum; those strains resistant to killing by guinea pig serum have been resistant to human serum.

Sterile diffusion chambers were constructed from lengths of Tygon tubing, the ends of which were covered with Millipore membranes having a pore diameter of 0.45μ(5). These chambers have a capacity of between 0.3-0.5 ml.

To determine the permeability of the diffusion chambers to cells of the host we examined the fluid contents from the chambers for mammalian cells using phase microscopy. None were found. Upon removal of the chamber from the peritoneal cavity, the diffusion membranes were cut from the chamber after the host cells had been meticulously cleaned from the outer surface. These membranes were then stained with hematoxylin and eosin utilizing a procedure which rendered the membrane transparent. As demonstrated by others(16) a membrane with 0.45 μ average pore size is usually impermeable to cells of the host. Of 55 membranes examined at 24, 48, and 72 hours after the diffusion chamber had been implanted, five showed histologic evidence of host cell penetration. When host cells were present these were fewer than several hundred and these were located usually in a discrete cluster on the inner surface of the membrane. Chambers filled with inoculum showed no greater penetration by host cells than did chambers filled with saline. The percentage of membranes showing penetration by host cells did not increase when the chambers remained in the peritoneal cavity for several weeks. Fibrin clots removed from the diffusion chamber at termination of the experiment were fixed, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined for host cells. None were found.

Paper electrophoretic analysis of contents from chambers containing fluid in equilibrium with the peritoneal fluid showed a total protein concentration approximately 50% of that in normal mouse serum. The gamma globulin fraction in 5 samples of fluid removed from diffusion chambers averaged 13% of total proteins as compared to an average gamma globulin fraction of 9% for 6 samples of serum. Twenty-four hours after implantation of a diffusion chamber filled with 0.85% sodium chloride, total protein concentration was approximately one-third that in peritoneal fluid; 48 hr after implantation, total protein concentration was 85% of that in peritoneal fluid. Electrophoretic patterns of contents from diffusion chambers implanted filled with saline showed the appearance of some protein fractions at 8 hours. By 12 hr after implantation all major protein fractions of serum could be identified by paper strip electrophoresis. Fibrin clots appeared within the chambers filled with saline and occupied approximately 25%-50% of the volume of the chamber. No such clots were found in chambers which were implanted filled with air and allowed to fill with peritoneal fluid. The latter procedure was used to collect peritoneal fluid for the tests carried out *in vitro*.

A saline suspension of bacteria obtained from an 18 hour brain-heart broth culture was placed within the chamber before it was implanted in the peritoneal cavity. In further experiments an empty chamber was placed in the peritoneal cavity, and several weeks later, after the chamber had filled with peritoneal fluid, a small amount of this fluid was with-

‡ The 2 strains of *Salmonella typhimurium* were obtained through the courtesy of Dr. D. Rowley.

TABLE I. Relationship of Sensitivity of Bacillary Strains to Bactericidal Effect of Normal Sera and Their Survival in Diffusion Chambers in the Guinea Pig and Mouse Peritoneum

Bacterial Strain	N Bacteria Killed/ml Serum in 2 hrs G. Pig	Mouse	Size of Inoculum	Growth in I.P. Chambers N Positive Cultures/N Trials G. Pig	Mouse
Klebsiella 15	160 x 10^6	0 (Growth)	<100	—	2/3
			100- 500	0/2	5/5
			1000-1500	0/5	—
Shigella 141	110 x 10^6	0 "	<100	—	0/3
			100- 500	0/3	5/5
			1000-1500	0/4	—
E. coli A9	170 x 10^6	0 "	<100	0/2	3/3
			100- 500	1/1	3/3
			1000-1500	0/4	—
E. coli 108	140	0 "	<100	—	5/5
			100- 500	—	3/3
			1000-1500	14/15	—
Sal. typhosa 202	400	0 "	<100	—	4/4
			100- 500	1/2	1/1
			1000-1500	1/1	—
Sal. typhimurium 172 (Sensitive)	95 x 10^2	0 "	<100		3/3
			100- 500	2/6*	3/3
			1000-1500		—
Sal. typhimurium 173 (Resistant)	650	0 "	<100	—	3/3
			100- 500	3/3	3/3
			1000-1500	—	—

* Growth or killing here was not related to size of inoculum. Organisms isolated from chambers in which growth occurred showed increased resistance *in vivo* to the bactericidal effect of guinea pig peritoneal fluid. This suggests that within the chamber in this situation a mutant more similar in sensitivity to the parent strain (173) may have been selected from the inoculum.

drawn under sterile conditions at laparotomy and replaced with an equal volume of saline containing a known number of bacteria. The fate of each bacterial inoculum was then determined at the end of the experiment by agar pour plates prepared from the chamber fluid. Most chamber experiments were terminated at 24, 48, or 72 hours.

The 5 enteric strains showing the greatest sensitivity to the bactericidal effect of normal human and guinea pig sera were used for experiments to determine the fate of such organisms *in vitro* in peritoneal fluid from the mouse. An inoculum of less than 150 organisms in 0.1 ml was added to 0.4 ml of peritoneal fluid. This was maintained at 37 °C, and 0.1 ml aliquots were removed at intervals and agar pour plates made.

Results. Table 1 shows comparative results for survival of enteric bacilli in intraperitoneal diffusion chambers in the guinea pig and mouse. Note that of 47 experiments in the mouse the inoculum failed to multiply in only 4 experiments. In these 4 chambers the inoculum was either 30 or 35 organisms. The same strain grew when inoculated in greater numbers. It is possible that in the 4 chambers where growth failed to occur, the organisms failed to survive that interval of time in the saline before sufficient nutrients from the host had diffused into the chamber. Note also that size of inoculum was considerably smaller in the mouse than that used in the guinea pig. When the inoculum consisted of fewer than 75 organisms, the increase after 24 hours was in the order of 10^4 organisms. Regardless of size of inoculum, the increase after 48 or 72 hr within the chamber was in the order of 10^6 or 10^7 organisms.

To be certain that the time required for concentration of host proteins to reach effective levels within the chamber was not a critical factor in the fate of the inoculum, chambers already containing fluid in equilibrium with that of the peritoneum were inoculated as described under materials and methods. In such chambers inoculated with 32 organisms of *Salmonella typhimurium* strain 172, or 100 organisms of *Salmonella typhimurium* strain 173, growth at the end of 4 hours was almost 4 divisions per inoculated organism for strain 172, and about 4½ divisions per inoculated

organism for strain 173.

Peritoneal fluid from the mouse failed to show bactericidal activity *in vitro* against 5 gram negative enteric bacilli tested. In most of these experiments unpooled samples of peritoneal fluid were used. No significant difference was noted between growth in normal peritoneal fluid which had been freshly collected, and that which had been heated at 56°C for 20 minutes. Growth in the peritoneal fluid was essentially as found in normal mouse serum which had been freshly collected, and in that which had been heated to destroy the activity of complement.

Discussion. Although it has been impossible to demonstrate bactericidal activity of mouse serum *in vitro*, Carey *et al.*(12) recently showed intraperitoneal formation of spheroplasts in the non-immunized mouse. This suggests that factors responsible for spheroplast formation, namely, natural antibody, complement, and perhaps lysozyme are active *in vivo*. It is possible, although unlikely, that peritoneal exudate of the mouse may possess properties not shared with serum. We have been unable to show *in vivo* or *in vitro* that peritoneal exudate of mouse possesses bactericidal properties. More likely, it may be that under the conditions in which Carey *et al.* demonstrated spheroplast formation, participation of peritoneal cells of the host was necessary. Our failure to find spheroplasts in diffusion chambers suggests this latter possibility.

For a given organism the correlation between the sensitivity to serum *in vitro* and survival within intraperitoneal diffusion chambers in the guinea pigs provides evidence that diffusion of humoral elements into this type of chamber occurs sufficiently quickly under these conditions to affect the fate of the inoculum (5). The techniques used in the mouse were essentially the same as those used in the guinea pig. In addition, in some of the mouse experiments an effort was made to demonstrate bactericidal activity by placing the bacterial inoculum into chambers containing fluid already in equilibrium with the peritoneal fluid, and further, by using a very small inoculum.

Rowley(14) has found that both virulent and avirulent strains of bacteria are killed by mouse peritoneal macrophages at similar rates. He feels that the limiting factor in the interaction of bacteria and monocytes is the rate of phagocytosis. Thus if *Salmonella typhimurium* strain 172 (avirulent) and *Salmonella typhimurium* strain 173 (virulent) are each placed in separate diffusion chambers, and rate of phagocytosis is the predominent determining factor as to outcome (i.e. virulence), then rate of growth within the chamber should be approximately the same for both these organisms. Our data confirm this. Additional support in favor of the importance of cellular mechanisms in resistance of the mouse to gram-negative enteric bacilli is the absence of killing in the diffusion chambers where all major serum protein fractions were present in concentrations comparable to those in the chambers in the guinea pig where killing did take place.

Further experiments are in progress to determine the effect of active immunization upon the fate of bacterial inocula in intraperitoneal diffusion chambers. Also, the fate of bacteria in the mouse is being determined after injection of the inoculum into diffusion chambers already containing mouse peritoneal macrophages.

Summary and Conclusions. Millipore diffusion chambers filled with gram-negative enteric bacilli and placed in the mouse peritoneum have been used to determine the fate of these bacilli in a cell-free environment. In contrast to findings in the guinea pig, all 7 strains of bacteria tested in the mouse grew in such diffusion chambers. These chambers also served to collect cell-free peritoneal fluid for bactericidal studies *in vitro*. Such studies showed mouse peritoneal fluid to have no bactericidal activity against gram-negative enteric bacilli.

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Antiproteolytic Power of Blood and Body Weight in the Albino Rat. (27026)

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The manifest antiprotease power (AP) of plasma as measured by its ability to inhibit tryptic digestion depends not only on the amount of antiproteases present but also on the amount of circulating proteases, on the physico-chemical state of the blood, and on the different affinities of the various inhibitor substances for trypsin.

This is a report on plasma AP of 143 albino rats (71 males and 72 females).

Method. 20 cmm of tail blood was withdrawn into a haemoglobin pipette and expelled into 10 ml of Sorensen's Phosphate Buffer pH 7.1. At the same time some blood was withdrawn into a heparinized capillary tube for determination of the packed cell volume (PCV). The blood-buffer mixture was immediately centrifuged for 15 minutes at 3,000 r.p.m. and the supernatant fluid used for antiprotease assay.

The assay was based on the determination of the optical density produced by the residual protein after incubation with protease, both with and without addition of plasma. The reaction mixture consisted of 1 mg Casein, 5 units Tryptar Armour (Lyophilized Crystalline Trypsin) and 0.001 ml plasma, made up to a total volume of 4 ml with Sorensen's Phosphate Buffer pH 7.1. The mixture was incubated for 15 minutes at 37.5°. The results are expressed in mg Casein saved from tryptic digestion by 1 ml plasma, this being the form of expression involving the least number of assumptions.

Results. Results are shown in Table 1. They were analysed from the point of view of sex, age, weight and PCV. Irrespective of sex, animals of the same weight show the same average plasma AP values. The PCV of these rats is low at birth and rises rapidly to reach a plateau of about 44% at the age of 8 weeks. The rising PCV correlates with the rising AP but thereafter the AP continues to rise while the PCV remains constant. It would therefore seem that concomitant rises during the first 8 weeks are not causally related. Plasma AP rises with age but the increase follows weight and not age.

When the weights of the animals regardless of sex, age or PCV are plotted against the AP, the data fit the quadratic equation: $AP = 252.7922 + 2.7083\ (G) - 0.004548\ (G)^2$. This is a statistically significant fit ($F = 10.4$; $n_1 = 1$, $n_2 = 136$; $P = 0.002$). Over the range of weights observed the data agree with the hypothesis that the change in antiproteolytic power is related to weight by a quadratic equation. It follows that there will also be a correlation with body surface.

Discussion. Plasma AP results from the actions of a number of inhibitor substances each having different affinities for at least 5 distinct proteases; (1,2,3,4,5). Changes in the plasma AP occur in response to a variety of distinct though not necessarily unrelated processes including absorption into the blood of pancreatic trypsin and antitrypsin, blood clot-

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