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Effects of Localized *Pasteurella haemolytica* Infection on Erythromycin-Binding Properties of Bovine Alpha-1-Acid Glycoprotein, Albumin, Serum, and Tissue Chamber Fluids†

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The in vitro erythromycin-binding properties of bovine alpha-1-acid glycoprotein (AAG) and albumin were studied by using equilibrium dialysis. In addition, the proportions of free erythromycin in bovine serum and tissue chamber fluid before and 4 days after inoculation of subcutaneous tissue chambers with *Pasteurella haemolytica* were measured. At a concentration of 5 μg/ml, erythromycin was moderately bound to AAG (39% ± 4% free) and was only slightly bound to albumin (86% ± 2% free). Scatchard analysis of the data describing binding to pure bovine AAG indicated that erythromycin was bound to a single high-affinity (6.45×10^4 M^{-1}) site on the protein. At lower total concentrations of erythromycin, the free concentrations of the antibiotic were lower in serum samples collected after infection (49% ± 3% at 5 μg of erythromycin per ml) than in those collected before inoculation (55% ± 3% at 5 μg of erythromycin per ml). Inoculation had no effect on binding to macromolecules in chamber fluids. Inoculated tissue chambers served as a convenient model for studying the effect of infection on drug-macromolecule interactions in interstitial fluid.

In vivo antibacterial efficacy is determined by a number of factors including the level of penetration of the antibacterial agent into tissue and the antibacterial activity at the site of infection. Binding to serum proteins usually results in relatively higher total concentrations of drug in plasma because the area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) of free drug in plasma is equivalent to the $AUC_{0-\infty}$ of free drug in the periphery. Protein-bound drugs are too large to pass through capillary endothelial layers and are therefore restricted to the circulatory compartment, away from the site of most infections. Furthermore, there is reliable evidence indicating that drugs that are protein bound are not pharmacologically active (22, 26, 27).

Albumin is quantitatively the most important serum protein, and its effect on the disposition of acidic antibacterial agents is well established (20). Although alpha-1-acid glycoprotein (AAG) has a high affinity for basic drugs, the concentrations of this acute-phase protein in healthy animals are usually too low to affect drug disposition or efficacy. Consequently, basic drugs are usually poorly protein bound in healthy animals. For example, the organic base erythromycin has been reported to be only 20% bound to bovine serum (28). However, the concentrations of AAG in serum may increase considerably during periods of inflammation or infection, and this may cause an associated increase in the binding of basic drugs (21). During these periods, when antibacterial agents are frequently used, AAG-drug interactions may be of therapeutic importance.

Although binding to serum proteins may affect the disposition and tissue penetration of antibacterial agents (26), the detrimental effects of protein binding on antibacterial activity are primarily due to drug-protein interactions at the site of infection, which is usually the interstitium. Study of these interactions is frustrated by an inability to sample sufficient quantities of interstitial fluid. Tissue chambers are hollow, perforated devices that have been used as models to study interstitial fluid and tissue penetration of xenobiotics (6). After subcutaneous implantation, chambers fill with transudate, which is in direct communication with the interstitial space (9). Except for chloride levels in the tissue chamber, which are higher than the corresponding levels in serum the electrolyte composition of tissue chamber fluid approximates the hypothesized composition of true interstitial fluid (12, 18). Both chamber fluid and interstitial fluid have protein concentrations that are considerably lower than the corresponding concentrations in serum, although the concentrations in chamber fluid are reported to be slightly greater than those in interstitial fluid (18). Implanted tissue chambers therefore provide a model which can be used to investigate the interactions between proteins and drugs in the interstitial space. Experimental infections established by inoculation of subcutaneous tissue chambers (10, 11) provide a convenient source of infected tissue fluid which can be subjected to in vitro analysis.

Pasteurella haemolytica is considered to be the most important cause of bovine respiratory disease (8), which is responsible for substantial economic losses to the cattle industry. Selection of appropriate antimicrobial agents for treatment of this disease is usually based on in vitro antimicrobial susceptibility data (19). These data indicate that most isolates of *P. haemolytica* are susceptible to erythromycin (17), a macrolide antibiotic approved for this use by the U.S. Food and Drug Administration. However, results of clinical trials have indicated that the response of *P. haemolytica* to erythromycin may be poorer than that predicted by in vitro data (23). Evidence that raised concentrations of AAG in serum have been de-

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tected in calves following intratracheal inoculation of *P. haemolytica* (13) suggests that erythromycin may be inactivated in vivo by binding to AAG and other macromolecules in infected interstitium.

Therefore, the objective of the study was to determine the effect of *P. haemolytica* infection on the proportion of erythromycin bound to macromolecules in serum and tissue fluid and to estimate the binding parameters describing binding between erythromycin and bovine AAG and albumin.

MATERIALS AND METHODS

Animals. Six Hereford cross-bred calves, 4 to 8 months of age and weighing from 100 to 225 kg, were used in the experiment. Prior to use, the animals were dewormed with fenbendazole (5 mg/kg of body weight; Safeguard; Hoechst-Roussel Agri-Vet Co., Somerville, N.J.) and were observed on a daily basis during a 2-week acclimation period to establish that they were in good health. They were maintained in small pens for the duration of the study and were fed grass and alfalfa hay supplemented with a commercial grain mixture containing 14% protein.

Implantation of tissue chambers. Two cup-shaped tissue chambers (Delrin; E. I. du Pont de Nemours & Co., Wilmington, Del.) measuring 46 mm (inner diameter) and 15 mm in depth were implanted subcutaneously in each calf, one chamber in each paralumbar fossa. Perforations in the walls and bases of the chambers allowed unrestricted exchange of solutes and cells between tissue chamber fluid and the surrounding interstitial fluids. Chamber fluid could be aspirated by inserting a needle through the skin and the silicone rubber (Silastic; Dow Corning Corp., Midland, Mich.) membrane, which covered the top of the chamber. Tissue chamber assembly, sterilization, surgical implantation, and collection of chamber fluid samples have been described previously (12). The sterility of the surgical technique was monitored approximately 1 month after implantation by culturing an aspirate of chamber fluid aerobically and anaerobically on 5% sheep blood agar.

Inoculation and sample collection. Approximately 2 months after implantation, preinoculation blood and chamber fluid samples were collected. Five days later, all chambers were inoculated with 1 ml of a 6-h culture of a field isolate of *P. haemolytica* serotype 1 (14). The MIC of erythromycin for this isolate has previously been determined to be 2 μg/ml (11). Inocula were prepared by culture on supplemented brain heart infusion agar; this was followed by suspension of bacteria in phosphate-buffered saline (PBS) at a concentration of 3×10^6 CFU/ml as described previously (11). Four days after inoculation, samples of blood and tissue chamber fluid were collected, clarified through centrifugation and filtration, and frozen at −20°C until analysis.

Dialysis experiments. The proportion of erythromycin bound to proteins was determined by equilibrium dialysis. Plexiglas dialysis chambers, each containing eight compartments (200-μl capacity on each side), were custom constructed. Reconstituted cellulose membranes, 0.073 mm thick and with a 6,000-molecular-weight cutoff (BelArt, Pequannock, N.J.), separated protein-free and protein-containing compartments. Two hundred microliters of sample (serum or chamber fluid collected before and 4 days after inoculation) were dialyzed for 48 h against 200 μl of 0.15 M phosphate buffer (pH 7.2) containing different concentrations (0.5, 5.0, and 50.0 μg/ml) of erythromycin in duplicate. The duration of incubation was chosen on the basis of preliminary studies that investigated the time necessary to achieve equilibrium. The concentrations of erythromycin were within the range of those expected in serum and tissues after intravascular (4) or intramuscular (5) administration of a recommended dose to cattle. Additional equilibrium controls (50 μg of erythromycin per ml) were included in each assay and were dialyzed for 72 h.

Affinity- and capacity-binding parameters were determined by dialyzing (48 h) different concentrations of erythromycin (0.5, 5, 20, 40, 60, 80, 100 μg/ml) in phosphate buffer against 1 mg of AAG (Sigma Chemical Co., St. Louis, Mo.) per ml or 3 g of albumin (Sigma Chemical Co.) per ml in buffer. Similar concentrations of these proteins have been reported in the sera of cattle inoculated with *P. haemolytica* (13, 25). The purity of bovine AAG was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples of albumin in PBS were assayed by radial immunodiffusion assay (25) and were found to be contaminated with very low levels of AAG (0.034 ± 0.019 mg of AAG per ml in samples containing 300 mg of albumin per ml). These levels were considered inconsequential. An equilibrium control (100 μg of erythromycin per ml) was included in each assay and was dialyzed for 72 h. Each assay, which included all concentrations of erythromycin, was repeated six times.

The desired erythromycin concentrations were obtained by combining a constant amount of *N-methyl*-^{14}C-labeled erythromycin of 99% radiochemical purity (0.013 μg of 55 mCi/mmol; New England Nuclear, Boston, Mass.) with various amounts of unlabeled erythromycin (Gallimycin; Sanofi Animal Health, Overland Park, Kans.). Dialysis was performed at 37°C for 48 h (72 h for equilibrium controls) in an oscillating incubator (Gallenkamp Plus No. 1; Curtin Matheson, Carrollton, Tex.). After dialysis, the radioactivity of 150 μl aspirated from each compartment was measured by adding 5 ml of scintillation cocktail (Atomlight; New England Nuclear) and counting on a liquid scintillation counter (LS5000 TDC; Beckman Instruments, Fullerton, Calif.) for 5 min. Quench correction was achieved by the automatic external standard method.

Erythromycin stability. The stability of erythromycin under dialysis conditions was monitored by bioassay by using *Sarcina lutea* ATCC 9341 and the techniques described by Bennett et al. (1). Standard concentration curves were constructed by using 20, 10, 7, 5, 1, and 0.5 μg of erythromycin per ml and were used to assay the test standard before and after incubation. A 5-μg/ml test standard was divided into 30 aliquots; 10 aliquots were analyzed immediately and the concentrations were determined from a standard curve, 10 aliquots were incubated at 37°C in an oscillating incubator for 48 h, and the remaining aliquots were placed in a refrigerator at 4°C for the same period. In addition, fresh test standard (5 μg/ml) was prepared and assayed concurrently with the postincubation samples. After 48 h, the samples refrigerated at 4°C and the samples incubated at 37°C were analyzed for their erythromycin concentrations. The average concentration in the fresh samples was 5.07 μg/ml, while those in the refrigerated and incubated samples were 4.98 and 4.94 μg/ml, respectively. An analysis of variance indicated that there was no significant difference between the means of the data for the three treatment groups. From these results, it was concluded that, over a period of 48 h, the degradation of erythromycin activity was not significant at either 4 or 37°C.

Protein assay. Albumin and AAG concentrations were assayed by the bromcresol green method (Centrifichem; Baker Instruments Corp., Allentown, Pa.) and radial immunodiffusion (25), respectively.

Data analyses. The degree of protein binding was calculated by using the following equation: percent binding = (100 × difference in activity between chamber sides)/activity on the protein-containing side. The number of binding sites for

TABLE 1. Proportional binding of erythromycin to serum or tissue chamber fluid before and after inoculation of *P. haemolytica*

Binding site and erythromycin concn (μg/ml)	% Free erythromycin[a]	
	Before inoculation	After inoculation
Serum		
0.5	0.52 ± 0.04	0.45 ± 0.02[b]
5	0.55 ± 0.03	0.49 ± 0.03[b]
50	0.73 ± 0.03	0.71 ± 0.04
Chamber fluid		
0.5	0.66 ± 0.04	0.66 ± 0.04
5	0.68 ± 0.07	0.65 ± 0.03
50	0.84 ± 0.04	0.81 ± 0.04

[a] Data are means ± standard deviations.
[b] Significantly different from preinoculation value.

TABLE 2. Proportional binding of erythromycin to AAG and albumin

Erythromycin concn (μg/ml)	% Free erythromycin bound to[a]:	
	AAG	Albumin
0.5	38 ± 3	85 ± 2
5	39 ± 4	86 ± 2
20	49 ± 5	86 ± 3
40	59 ± 3	89 ± 2
60	65 ± 4	91 ± 2
80	71 ± 5	92 ± 1
100	73 ± 3	93 ± 2

[a] Data are means ± standard deviations.

erythromycin on AAG was determined by Scatchard analysis (24), as were the initial estimates of binding-affinity constants. Binding parameters of individual data sets were then determined by using a microcomputer program (3) to solve the quadratic equation describing the relationship between total and free drug concentration:

$$C_{free} = \frac{Ka \cdot C_{tot} - 1 - K_a \cdot Pt + \sqrt{(Ka \cdot C_{tot} - 1 - Ka \cdot Pt)^2 + 4 \cdot Ka \cdot C_{tot}}}{2 \cdot Ka}$$

where K_a is the binding-affinity constant, P_t is the protein concentration, and C_{tot} and C_{free} are the total and free drug concentrations, respectively (2). Minimum sums of weighted residuals ($1/C_{free}$) were approached by using the Damping Gauss-Newton fitting algorithm. Data describing the effects of *P. haemolytica* on protein concentrations and the binding of erythromycin to serum and chamber fluid were analyzed by using paired *t* tests and two-factor (animal and chamber side) analyses of variance, respectively. Differences were considered significant at the $P < 0.05$ level.

RESULTS

Binding of erythromycin to macromolecules in serum and chamber fluid varied widely depending on the matrix and erythromycin concentration (Table 1). At two concentrations of erythromycin (0.5 and 5.0 μg/ml), binding in serum was significantly higher after inoculation, when the concentration of AAG was higher (0.54 ± 0.04 mg/ml) compared with preinoculation values (0.39 ± 0.11 mg/ml). Analysis of variance revealed no difference in binding to macromolecules in chamber fluids collected before and after inoculation, despite the significant difference in AAG concentration (0.12 ± 0.02 mg/ml in noninfected chamber fluid versus 0.24 ± 0.05 mg/ml in infected chamber fluid).

Binding of erythromycin to AAG was saturable between the concentrations of 0.5 and 100 μg/ml (Table 2) and varied between 38% free drug at 0.5 μg/ml and 73% free drug at 100 μg/ml. Scatchard analysis of the data indicated a single binding site with a mean binding-affinity constant of $6.45 \times 10^4 \pm 1.65 \times 10^4$ M^{-1} (Fig. 1). Equilibrium analysis of the binding by using the quadratic equation describing the relationship between C_{tot} and C_{free} yielded a similar mean estimate of binding affinity ($6.91 \times 10^4 \pm 3.83 \times 10^4$ M^{-1}) at a mean protein concentration of $2.74 \times 10^{-5} \pm 6.09 \times 10^{-5}$ M (Fig. 2).

Erythromycin was only slightly bound to albumin (Table 2), and no specific binding sites could be identified by Scatchard analysis.

DISCUSSION

Most infections are not limited to the blood vascular system, but are usually located in the interstitium. Therefore, although drug-protein interactions in vascular blood may affect drug disposition, interactions in tissues are most relevant to antimicrobial efficacy. Tissue chambers have been validated as a model for studying interstitial infections (6, 7, 10, 11) and contain fluid that is thought to be similar to the theoretical composition of interstitial fluid (12). This characteristic and the ease with which these chambers can be sampled make them convenient models for studying drug-protein interactions in interstitial fluid. Furthermore, the chamber model allows study of protein binding in infected animals, which may be particularly appropriate when a drug interacts with acute-phase proteins, such as AAG. Previous studies have shown that inoculation of *P. haemolytica* into subcutaneous tissue chambers causes inflammatory changes that are similar to those observed in the lungs of cattle with pneumonic pasteurellosis (10, 11, 25). These changes include rapid sequestration of degranulating neutrophils, deposition of fibrin, and leakage of plasma proteins. Subcutaneous tissue chambers are slowly equilibrating models and, therefore, represent inaccessible tissue sites such as abscesses or consolidated lung lesions. Such lesions are common in pneumonic pasteurellosis and are believed to protect bacteria from therapeutic concentrations of antibiotics.

Protein binding at the site of infection may be affected by several factors including temperature, pH, drug concentration, and protein concentration. Previous studies have indicated that inoculation of chambers causes increased concentrations of both AAG and albumin in chamber fluid and an increased concentration of AAG in serum (25). Thus, infection is likely to affect the binding of AAG to organic bases, such as erythromycin. This was confirmed by the results of the present study, in which a higher proportion of erythromycin was bound in serum after inoculation than before inoculation. Although a similar trend in binding was evident in chamber fluid, apparent differences were not large enough to be declared statistically significant. Assuming saturable binding, the effect of an increase in protein concentration has a greater effect on proportional binding when drug concentrations are relatively lower. This relationship may explain the failure to identify significant differences in binding in chamber fluids which contain less protein and relatively more erythromycin or in serum at the highest concentration of erythromycin.

By using the quadratic equation describing the relationship

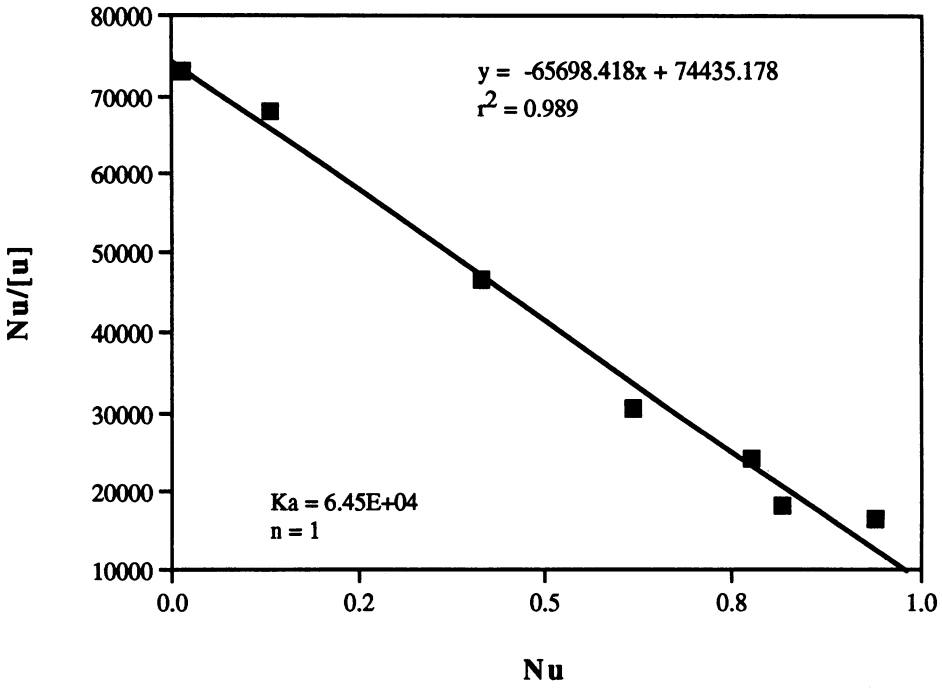

FIG. 1. Scatchard plot of mean data describing binding of erythromycin to AAG. The symbol [u] signifies the free concentration of erythromycin, and Nu is the ratio of [u] to the AAG concentration. The plot was constructed by using mean data derived from six separate assays, each of which included seven erythromycin concentrations (0.5, 5, 20, 40, 60, 80, and 100 μg/ml).

between total and free drug concentrations (2) and the affinity constant derived by using that equation (6.91×10^4 M^{-1}), the theoretical proportions of free drug present at each of the measured AAG concentrations in serum are similar to those estimated by dialysis; at 5 μg of erythromycin per ml, infection should cause a theoretical decrease in the proportion of free drug from 62 to 54%. The slightly lower values (55 and 49% for noninfected and infected sera, respectively) measured by dial-

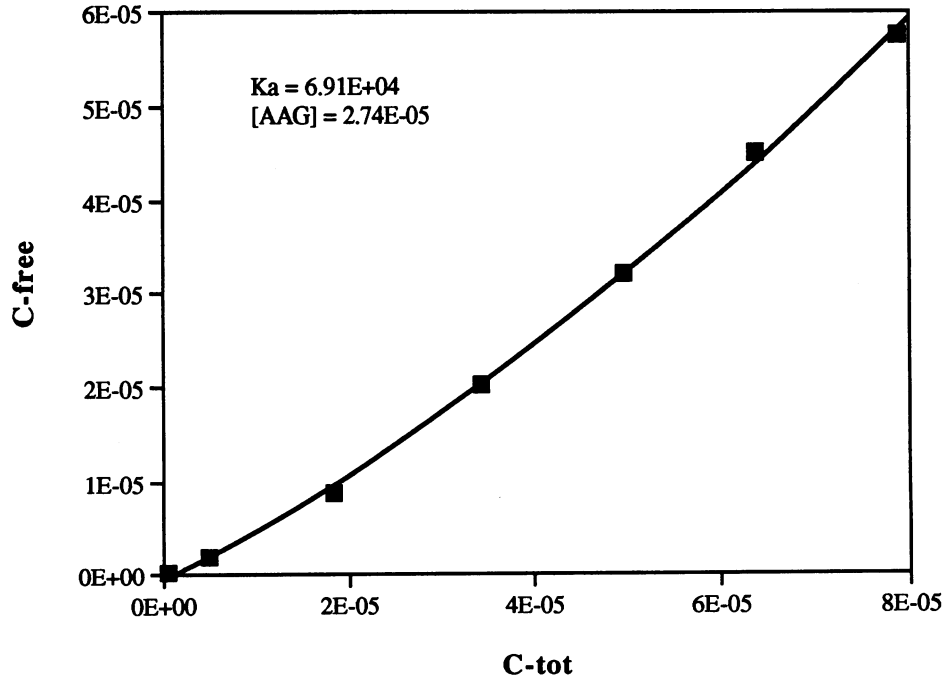

FIG. 2. Quadratic relationship between free (C-free) and total (C-tot) concentrations of erythromycin. The plot was constructed by using mean data derived from six separate assays, each of which included seven erythromycin concentrations (0.5, 5, 20, 40, 60, 80, and 100 μg/ml).

ysis can be explained by the additional drug-binding activity of albumin in serum. In contrast to serum, calculation of free drug proportions at concentrations of AAG measured in chamber fluids yields theoretical values of 84% for noninfected chamber fluids and 73% for infected chamber fluids versus corresponding measured values of 68 and 65%. Thus, theoretical calculation of free drug concentrations underestimated binding in chamber fluids and predicted a greater effect of inoculation than was experimentally evident. Factors other than changes in AAG concentration must have affected the binding of erythromycin in chamber fluids after inoculation. Previous studies have shown that inoculation of tissue chambers with *P. haemolytica* causes a decrease in pH from 7.2 to 7.0 (10). Considering that binding of erythromycin is reported to decrease at lower pH (16), the lower pH of infected chamber fluid would oppose the effects of the higher AAG concentration and may explain why the free concentration of erythromycin in infected chamber fluids was higher than expected.

Intramuscular administration of a recommended dose of 15 mg/kg to cattle results in concentrations in serum that fluctuate between 3.5 and 0.5 μg/ml (5). Concentrations in tissue, measured by using lung homogenates from animals sacrificed at intervals for 24 h after drug dosing varied between 6 and 1 μg/ml. Considering that concentrations in serum and tissue barely exceeded the MIC for the *P. haemolytica* isolate used in the present experiment (2 μg/ml), changes in protein binding could decrease the free and pharmacologically active proportion of erythromycin to concentrations that are lower than the MIC. Therefore, although erythromycin is only moderately protein bound, small changes in the concentration of AAG could have significant effects on the in vivo efficacy of erythromycin. This is especially true at low and therapeutically relevant concentrations of erythromycin.

Depending on the drug concentration, erythromycin was moderately bound to AAG but was only slightly bound to albumin. These data are consistent with those presented in previous reports indicating that AAG is the major binding protein for erythromycin in human serum (15). Thus, erythromycin follows the same pattern as many other basic xenobiotic compounds in that it has a higher affinity for AAG than for albumin, which binds acidic molecules more readily (15, 20).

The nonlinear regression analysis served to verify the Scatchard analysis and identify the possible existence of binding sites other than those on AAG. A close correlation between binding-affinity constants and protein concentrations confirmed that binding to other macromolecules was not substantial, thus validating the dialysis methodology. Furthermore, the nonlinear relationship between C_{tot} and C_{free} confirmed the saturable nature of protein binding at the erythromycin concentrations tested.

In summary, values describing the binding of bovine AAG and albumin to erythromycin approximate those reported in humans. Differences in pre- and postinoculation binding are significant at lower, therapeutically relevant concentrations of erythromycin, probably because of the increased concentration of AAG in serum. The soft-tissue infection model, established by inoculation of subcutaneous tissue chambers, served as a useful model for studying the effect of infection on drug-protein interactions in interstitial fluid.

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