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α-6-Deoxyoxytetracycline IV. Penetration and Concentration within a Localized Area of Inflammation (33008)

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In bacterial infections, the host responds to the presence of the microorganism by dynamic, complex, and variable processes referred to as the inflammatory reaction. Some of the biochemical properties of the tissues and body fluids within the resulting zone of inflammation are markedly different from normal. For example, as a result of cell injury, various chemical factors or mediators, including polypeptides (leukotaine), euglobins (necrosin), are liberated and changes may also occur in pH and glycolytic activity (1,2). In the treatment of disease, therefore, it is most likely that the interaction between an antibiotic and microbe takes place in tissues or fluids quite unlike those in the normal *in vivo* environment. Thus, the chemotherapeutic efficacy of an antibiotic, in addition to its inherent antimicrobial activity, depends upon its ability to reach and to maintain effective concentrations in such modified physiological environments instead of in normal tissues and fluids.

The present studies were initiated to determine the efficacy of α-6-deoxyoxytetracycline [DOOTC,[1](3,4,5)] in an experimental situation that mimics the modified physiological environment in which therapeutic agents are expected to function. Antibiotic concentrations were determined in the inflammatory exudate produced within a granuloma pouch as well as in the serum of rats. For comparative purposes, 3 other commercially-available tetracyclines were included in the studies.

[1] Vibramycin, Chas. Pfizer and Co., Inc.

Methods. The antibiotics, DOOTC, 6-methylene oxytetracycline (MOTC)[2] 6-demethylchlortetracycline (DMCT)[3] and tetracycline (TC)[4], used in these studies were research quality hydrochloride salts.

The granuloma pouch was produced in male rats weighing about 100 gm (Blue Spruce Farms, Altamount, New York) utilizing, in general, the technique of Robert and Nezamis (6). Briefly, the pouch was formed by injecting 25 ml of air under the dorsal skin in the midline of the neck. The chemical irritant, 0.8 ml of turpentine, was next introduced into the pouch. Pouches were partially deflated 2 days after production by the removal of 10 ml of air. Three days later, groups of 3 rats were dosed orally with an antibiotic and sacrificed at various times later; blood was collected and pouch contents were aspirated with a sterile pipette. Blood and pouch contents, respectively, from a group of 3 rats were pooled and treated as single samples. Each time sample, therefore, is represented by groups of different rats. Serum was removed after allowing blood to clot at refrigerator temperature. Antibiotic concentrations in both serum and inflammatory exudate were assayed using the *Bacillus cereus* var. *mycoides* plate technic (7). Standard curves for the assay of antibiotic in rat serum and in inflammatory exudate were prepared, respectively, in normal rat serum or in inflammatory exudate from control rats or in a solution containing sufficient bovine albumin to compensate for either serum protein or inflammatory exudate binding. Any dilutions necessary of test samples also utilized control sera, inflammatory exudate, or the albumin solution mentioned above. Each experiment was replicated 3 times. Assays were carried out and replicated as often as possible on portions of the same samples by 2 different Pfizer laboratories. All assay values, usually 8 or 9, were added and the mean and standard error of the mean were calculated. These values are represented as total antibiotic concentration in sera and in inflammatory exudate.

TABLE I. Percentage Binding of DOOTC and Other Tetracyclines in Inflammatory Exudate of the Granuloma Pouch and in Serum of Rats.

	Binding (%, mean ± SE)	
Tetracycline	Inflammatory exudate	Serum
DOOTC	67.7 ± 6.3	63 ± 2.6
MOTC	56 ± 4.0	64 ± 2.4
DMCT	64.6 ± 1.3	71.4 ± 2.1
TC	67 ± 8.7	67.3 ± 2.6

The percentage binding of each antibiotic to rat serum proteins and in inflammatory exudate was determined by a microbiological plate procedure described by Scholtan and Schmid for tetracyclines (8) and recently utilized by this laboratory with mouse sera for the same antibiotics used in the present study (5). Scholtan and Schmid reported that their data obtained by this procedure for several tetracyclines, in general, compared favorably with literature values from several other methods (8). As an example, a comparison was made between zone sizes produced against *B. cereus* var. *mycoides* by equal concentrations of each antibiotic in, respectively, pooled normal rat serum (pH 7.4), and pH 7.4-buffered-saline. The inhibition zone produced by a tetracycline in buffered saline is larger than that produced when in rat serum and such difference in inhibition area is interpreted to be a consequence of serum binding, i.e., the tetracycline in buffer, which is present as unbound antibiotic, has a faster diffusion rate than the serum-tetracycline combination when tested in agar.

The percentage binding in inflammation exudate was done in identical manner except exudate was substituted for serum. The pH of the exudate in these studies was consistently 7.3–7.4, therefore, the pH 7.4 buffer used in serum binding was also used in the exudate binding studies. The percentage values presented in Table I for both serum and inflammation exudate are means of 5 different experiments. The standard error of the

[2] Rondomycin, Chas. Pfizer and Co., Inc.

[3] Declomycin, Lederle Laboratories, American Cyanamid Co., Inc. I wish to thank Dr. B. W. Carey, Lederle Laboratories, Pearl River, New York for my supply of DMCT.

[4] Tetracyn, Chas. Pfizer and Co., Inc.

TABLE II. Total Antibiotic Concentration in Inflammation Exudate and Sera after Oral Administration to Rats.

Tetracycline	Oral (mg/kg)	Total antibiotic conc at hours (μg/ml mean ± SE)											
		Inflammation exudate						Serum					
		1	2	4	6	8	24	1	2	4	6	8	24
DOOTC	12.5	0.22 ±.11	0.36 ±.10	0.59 ±.07	0.80 ±.11	0.55 ±.04	0	1.47 ±.11	1.37 ±.09	1.43 ±.16	1.16 ±.14	0.51 ±.03	0
MOTC	12.5	0	0	0.14 ±.09	0.26 ±.17	0	0	0.47 ±.11	1.04 ±.22	0.71 ±.12	0.74 ±.35	0	0
DMCT	12.5	0	0.14 ±.13	0.48 ±.16	0.14 ±.09	0	0	0.72 ±.12	0.91 ±.18	0.82 ±.09	0.18 ±.11	0	0
TC	12.5	0	0	0.10 ±.10	0.21 ±.14	0	0	1.43 ±.05	1.92 ±.33	1.24 ±.08	0.70 ±.33	0.33 ±.01	0
DOOTC	25	0.34 ±.10	0.78 ±.07	1.77 ±.07	1.74 ±.09	1.20 ±.12	0.08 ±.05	3.33 ±.22	3.64 ±.22	3.30 ±.22	2.01 ±.17	1.24 ±.14	0.08 ±.05
MOTC	25	0	0.23 ±.14	0.94 ±.08	0.73 ±.31	0.34 ±.11	0	1.26 ±.20	0.96 ±.19	1.52 ±.07	1.60 ±.55	0.39 ±.20	0
DMCT	25	0	0.35 ±.09	0.87 ±.13	0.51 ±.04	0.25 ±.12	0	2.22 ±.46	1.94 ±.36	1.87 ±.10	0.72 ±.06	0.72 ±.14	0.06 ±.06
TC	25	0.18 ±.11	0.97 ±.12	1.32 ±.22	0.97 ±.17	0.50 ±.10	0	2.30 ±.17	4.0 ±.20	3.95 ±.16	1.76 ±.21	0.75 ±0	0
DOOTC	50	0.73 ±.44	1.10 ±.10	1.45 ±.07	1.86 ±.14	1.53 ±.25	0.36 ±.06	2.04 ±.41	5.29 ±.64	3.63 ±.53	3.06 ±.17	2.38 ±.28	0.22 ±.05
MOTC	50	0	0.84 ±.32	0.40 ±.14	0.52 ±.29	0.30 ±.16	0	1.32 ±.58	2.83 ±1.24	0.71 ±.17	1.54 ±.30	0.41 ±.28	0
DMCT	50	0	0.52 ±.18	1.06 ±.20	0.82 ±.17	0.22 ±.11	0	1.12 ±.07	2.55 ±.56	1.46 ±.10	1.55 ±.77	0.38 ±.14	0
TC	50	0.16 ±.16	1.67 ±.55	1.12 ±.18	1.52 ±.45	0.95 ±.10	0	3.24 ±.78	4.13 ±1.2	3.28 ±.16	2.78 ±.29	1.63 ±.19	0

mean is also presented. Unbound antibiotic in sera and inflammatory exudate was calculated from total antibiotic minus the amount bound based on the percentage binding determined in sera or the exudate.

Data and Discussion. Mean percentage binding values for DOOTC and the other tetracyclines are presented in Table I for rat serum and inflammatory exudate. The data, with the exception of tetracycline, for binding in rat serum are quite comparable to those reported earlier for mouse serum. (5). Tetracycline is bound to a greater extent in rat serum (67.3% ± 2.6%) than in mouse serum (54.8% ± 2.2%).

Percentage binding values in inflammation exudate with their standard error are quite similar for these 4 tetracyclines. In addition, the binding values of each tetracycline are similar for serum and inflammation exudate. Thus, although the composition of serum and exudate may be quite different, these data indicate that relevant to binding, the two environments appear similar. Ascheim and Zweifach (9) have demonstrated that the protein concentration in edema fluids during inflammation is essentially the same as that of the plasma. In agreement with this, electrophoresis studies made on representative inflammation exudates and

TABLE III. Unbound Antibiotic Concentrations in Inflammation Exudate and Rat Sera after Oral Administration.[a]

Drug	Dosage (mg/kg)	Antibiotic at hours (μg/ml)											
		Inflammation exudate						Serum					
		1	2	4	6	8	24	1	2	4	6	8	24
DOOTC	12.5	0.07	0.12	0.19	0.26	0.18	0	0.54	0.51	0.53	0.43	0.19	0
MOTC		0	0	0.06	0.11	0	0	0.17	0.37	0.26	0.27	0	0
DMCT		0	0.05	0.17	0.05	0	0	0.20	0.26	0.23	0.05	0	0
TC		0	0	0.03	0.07	0	0	0.47	0.63	0.40	0.23	0.11	0
DOOTC	25	0.11	0.25	0.57	0.56	0.39	0.03	1.23	1.35	1.22	0.74	0.46	0.03
MOTC		0	0.10	0.41	0.32	0.15	0	0.45	0.34	0.55	0.58	0.14	0
DMCT		0	0.12	0.31	0.18	0.09	0	0.63	0.55	0.53	0.20	0.20	0.02
TC		0.06	0.32	0.43	0.32	0.16	0	0.75	1.31	1.29	0.58	0.24	0
DOOTC	50	0.24	0.36	0.47	0.60	0.50	0.12	0.75	1.96	1.34	1.13	0.88	0.08
MOTC		0	0.37	0.18	0.23	0.13	0	0.48	1.02	0.26	0.55	0.15	0
DMCT		0	0.18	0.38	0.29	0.08	0	0.32	0.73	0.42	0.44	0.11	0
TC		0.05	0.55	0.37	0.50	0.31	0	1.06	1.35	1.07	0.91	0.53	0

[a] Calculated from mean values for percentage binding to rat serum and inflammation exudate (Table I) and mean value for total antibiotic in rat serum and inflammation exudate after oral administration (Table II).

serum from the same rats in the present studies showed a very similar band pattern.[5]

Mean concentrations, expressed as total antibiotic, of the tetracyclines detected in inflammation exudate and sera of rats after oral drug administration are presented in Table II. It is generally believed that only the unbound portion of the drug exerts direct chemotherapeutic activity (5); therefore, these data serve primarily as a reference point for calculation of unbound antibiotic. Emphasis is placed in the present report on the unbound drug concentration.

Mean values of DOOTC and the other antibiotics, expressed as unbound antibiotic, in inflammation exudate and serum are presented in Table III. In an overall summation of the 3 dosages used, DOOTC and the other antibiotics readily appeared in the localized area of inflammation. The concentrations of DOOTC, expressed as mean values of unbound antibiotic in inflammation exudate or serum, were consistently greater than the values for MOTC and DMCT. Although DOOTC concentrations were generally greater than those of TC in both inflammation exudate and serum, the differences were not as striking as when DOOTC is compared with MOTC and DMCT.

It should be noted that the mean concentration of DOOTC as well as other tetracyclines expressed as unbound antibiotic in both inflammation exudate and serum (Table III), were generally above their minimal inhibitory concentrations for bacteria commonly associated with suppurative disease. In a previous study the MIC values of DOOTC, MOTC, DMCT, and TC ranged from 0.1 to 0.2 μg/ml against *Staphylococcus aureus* and 0.03—0.04 μg/ml against *Streptococcus pyogenes* (5). Thus, one could easily presume effective antimicrobial activity in the modified physiological environment, i.e., inflammation exudate, as well as in serum.

At all dosages studied, DOOTC persisted in inflammation exudate at higher concentrations for a longer period of time than did the other antibiotics. For example, on the 12.5 mg/kg regimen, 0.18 μg/ml of unbound DOOTC was present in the exudate at 8-hours post oral dosage. Activity was not detected with the other tetracyclines at this

[5] I wish to thank Dr. T. M. Lees for these electrophoresis studies.

time. Similarly, 24 hours after oral dosage of 50 mg/kg, 0.12 μg/ml of unbound DOOTC was present in inflammation exudate while activity was not detected for any of the other tetracyclines at this time period.

A time–dosage relationship exists between the unbound drug concentration in inflammation exudate and serum. As presented in Table IV, concentrations in the exudate in the early hours were considerably less than those in serum. The ratio gradually increased over the time period until the ratio between the exudate and sera approached 1 or the trend of the data indicated that 1.0 would be reached with the possible exception of tetracycline. It is noticeable that DOOTC, even on the low dosage of 12.5 mg/kg gave progressive ratios of 0.12 at 1 hour to 0.90 at 8 hours. In contrast, the other antibiotics on this dosage gave ratios in the range of 0.10 to 0.41 and only in the 4–6 hour samples. Another point of interest is that the exudate concentration of DOOTC at 24 hours after oral dosage of 50 mg/kg was higher than the serum level, hence the ratio was 1.5.

The presence of DOOTC, as well as the other tetracyclines, in inflammation exudate after oral administration, is the result of many complex pharmacodynamic processes. The final equilibrium distribution of the drug is determined by competitive forces and rates involving such factors as: (i) absorption from the intestinal tract, (ii) distribution in the animal body, (iii) binding to serum, tissue, and exudate, and (iv) rate of excretion. The phramacodynamic properties of DOOTC are such that an advantage is conferred so that DOOTC appears faster and persists longer and at higher concentrations in sera and exudate than do the other tetracyclines. As the degree of exudate and serum binding in the rat (Table I) are so similar for these tetracyclines, this offers little in the way of explanation. Probably the rapidity of DOOTC's appearance in inflammation exudate is associated with its more efficient absorption from the gastrointestinal tract. This has been shown experimentally for a variety of animal species (5, 10, 11) including man. The persistence of DOOTC in inflammation exudate probably is a consequence of its slower rate of urinary excretion and slow release from body compartments as has been shown in the dog (10) and postulated for man (11).

TABLE IV. Ratios of Unbound DOOTC and Other Tetracyclines in Inflammation Exudate and Sera.

Tetracycline	Sample time (hours)	Inflammation exudate conc./serum conc (on mg/kg oral dosage) 12.5	25	50
DOOTC	1	0.12	0.09	0.32
	2	0.24	0.19	0.18
	4	0.36	0.47	0.35
	6	0.60	0.76	0.53
	8	0.90	0.85	0.56
	24	0[a]	0.67	1.50
MOTC	1	0	0	0
	2	0	0.29	0.36
	4	0.23	0.74	0.69
	6	0.41	0.55	0.42
	8	0	1.07	0.87
	24	0	0	0
DMCT	1	0	0	0
	2	0	0.22	0.25
	4	0.10	0.56	0.90
	6	0.35	0.90	0.66
	8	0	0.45	0.72
	24	0	0	0
TC	1	0	0.08	0.06
	2	0	0.25	0.41
	4	0.10	0.33	0.35
	6	0.35	0.55	0.55
	8	0	0.66	0.58
	24	0	0	0

[a] Inflammation exudate concentration not detected.

Summary. The presence of DOOTC, as well as MOTC, DMCT, and TC, in inflammation exudate within the granuloma pouch demonstrated that these tetracyclines can pass from the blood into localized areas of inflammation. In addition, unbound concentrations of DOOTC and the other antibiotic were generally greater than the MIC values for bacteria commonly associated with suppurative disease. One could readily presume effective antimicrobial chemotherapy under the condition of these modified physiological conditions. The DOOTC appeared in the

inflammation exudate more rapidly, to a greater concentration, and persisted longer than did the other antibiotics studied.

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1. Dubos, R. J. and Hirsch, J. G., eds., "Bacterial and Mycotic Infections of Man", 4th ed., Lippincott, Philadelphia, Pennsylvania, 1965.

2. Menkin, V., "Dynamics of Inflammation," p. 78. Macmillan, New York 1940.

3. English, A. R., Proc. Soc. Exptl. Biol. Med. **122**, 1107 (1966).

4. English, A. R. and Lynch, J. E., Proc. Soc. Exptl. Biol. Med. **124**, 586 (1967).

5. English, A. R., Proc. Soc. Exptl. Biol. Med. **126**, 487 (1967).

6. Robert, A. and Nezamis, J. E., Acta Endocrinol. **25**, 105 (1957).

7. Grove, D. C. and Randall, W. A., "Assay Methods of Antibiotics," Med. Encycl. New York, 1955.

8. Scholtan, W. and Schmid, J., Arzneimittel-Forsch. **13**, 288 (1963).

9. Ascheim, E. and Zweich, B. W., Am. J. Physiol. **202**, 554 (1962).

10. Schach Von Wittenau, M. and Delahunt, C. S., J. Pharmacol. **152**, 164 (1964).

11. Migliardi, J. R. and Schach Von Wittenau, M., "Intern. Congr. Chemotherapy," p. 165. Vienna, 1967.

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Growth Morphology of *Mycoplasma pneumoniae* Strain FH on Glass Surface (33009)

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The variable morphology of mycoplasma still evokes different views about growth and multiplication of these microorganisms (1). The results of experiments to determine morphology are further complicated by the possibility of artifacts due to the preparation. The ability of *Mycoplasma pneumoniae* to adhere on a glass surface (2), provides a chance of observing living organisms without further manipulation. The following experiments were undertaken to study the growth morphology of *M. pneumoniae* on a glass surface in a fluid environment.

Material and Methods. Mycoplasma. Mycoplasma pneumoniae strain FH used in the study was kindly supplied by Dr. Del Giudice, Baltimore Biological Laboratories (BBL).

Media. The growth medium consisted of PPLO broth or PPLO agar (Difco Lab.), both supplemented with 20% horse serum and 10% yeast extract (Microbiological Assoc.) (1). For biochemical tests 1% glucose was added together with final concentrations of 0.005% phenol red, 0.025% 2,3,-5-triphenyltetrazolium chloride (Tetrazolium) or 0.002% methylene blue. To demonstrate the lysis of red blood cells (RBC), 3% sheep RBC were added to PPLO agar or the enriched PPLO agar.

Growth chamber. A coverslip chamber described for the demonstration of cytophilic antibody (3) was used. Glass rings (9 mm i.d., 2–3 mm high) were mounted on 22-mm square coverslips by silicone grease. The chambers were autoclaved, inoculated with about 0.2 ml of 1:10 dilution of a 24-hour culture, sealed by a second sterile coverslip and incubated at 37°C. Growth was observed by phase contrast microscopy.

Identification tests. After opening and removal of broth, the chambers showing growth were either filled with the test medium, or, after removing the rings, the coverslips with the adherent organisms were placed upside down on a test agar plate. Serological identification by growth inhibi-

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