Pulmonary Infection Due to Disruption of the Pharyngeal Bacterial Flora by Antibiotics in Hamsters

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An animal model was used to determine the effect of oxacillin on the pharyngeal bacterial flora and the relationship of this flora to pneumonia. The pharyngeal bacterial flora of 68 healthy Golden Syrian hamsters was determined. A quantitative comparison between *Streptococci* and *Escherichia, Proteus, Klebsiella* and *Enterobacter* from 70 hamsters was made before and at 4, 24, 48 and 72 hours after oxacillin administration. Lung cultures were positive in 22 of 25 hamsters, yielding *K pneumoniae* type 1 most frequently. Lung histology from 25 hamsters revealed bronchopneumonia. Intestinal postmortem cultures of treated and untreated animals were similar. The importance of throat cultures in diagnosing pneumonia and the value of the hamster model to study the effect of other antibiotics on the temporary flora are demonstrated (Am J Pathol 76:469–480, 1974).

COLONIZATION AND SUPERINFECTION due to Gram-negative bacilli are intimately associated with antibiotic therapy as many investigators have shown that there may be a disruption of the normal pharyngeal bacterial flora following antibiotic usage.[1–3] It has been suggested that the pharyngeal flora may be responsible for Gram-negative bacterial infection of the lungs and that treatment with nafcillin predisposes to overgrowth with *Escherichia, Klebsiella, Serratia* and *Enterobacter*.[3,4] In the present study, a hamster model was used to determine the effect of oxacillin on the pharyngeal bacterial flora and the relationship of this flora to the development of bacterial pneumonia.

Materials and Methods

Hamsters

Adult Golden Syrian hamsters of both sexes were purchased from the same dealer (Flow Laboratories) and housed in wire cages. They were fed Ralston Purina laboratory chow during the entire experiment.

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Sampling Technics

The pharyngeal bacterial flora of 68 hamsters was determined before and after antibiotic therapy by the following method: 1 ml of Difco trypticase soy broth (TSB) was placed in a Bio-Cul disposable sterile culture unit (Medi, Inc, Holbrook, Mass) containing a swab. The swab was pressed against the sides of the tube to release excess broth. The hamster was then grasped by the skin of the neck and back in order to expose the mouth. The moistened swab was gently inserted into the open mouth, pressed back against the pharyngeal area, and rotated there for 5 seconds. The swab was then removed and returned to the TSB. All tubes were shaken using a Vortex, Jr. mixer (Scientific Industries, Inc, Queens Village, NY) for 10 seconds. The excess fluid was extracted from the swab and the latter was discarded. The tubes were shaken for an additional 5 seconds immediately before plating. This process and the planting of the specimen, which took approximately 1 minute, were completed on each animal before the next one was sampled.

Culturing Technics

The cultures were plated semiquantitatively as follows: 0.01 ml of the original broth suspension was inoculated on a plate of Difco blood agar (BAP), Difco phenyl ethyl alcohol (PEA) and Difco eosin methylene blue (EMB) and spread with a bent glass rod. The PEA plates were placed in a candle jar, and the EMB and BAP plates were incubated aerobically. All cultures were held at 37 C. The plates were read the next day, and the number of colonies for each species was counted.

The environment of the hamster was monitored daily by culturing food, water and swabs of the cage surfaces on BAP and EMB plates.

Bacterial Identification

All organisms were identified according to conventional technics.[5] *Klebsiella pneumoniae* strains were typed according to directions by the manufacturers (Difco) of the antiserum. The antiserum pools used were 1, 2, 3, 4, 5, 6 and 14.

Antibiotic Treatment

The antibiotic treatment consisted of two equal doses of 0.1 ml of oxacillin (Bristol Laboratories) given intramuscularly to each hamster. The total amount of antibiotic used was approximately 250 mg/kg of body weight. A 4-hour interval elapsed between injections.

Necropsy Procedure

When an animal was found dead or was sacrificed with Diabutal (0.8 ml intraperitoneally), the necropsy was conducted in the following manner: The pelt was decontaminated with 95% alcohol. The thoracic cavity was opened aseptically, and the heart exposed. Approximately 0.1 to 0.2 ml of heart blood was collected with a sterile tuberculin syringe and inoculated in 10.0 ml of TSB. The lungs were then removed, and a small (approximately 0.5 cm) wedge of tissue was taken from the apex of the left upper left lobe and placed in 10.0 ml of TSB. The remaining portions of lung were placed in 10% formalin for histologic studies. Standard hematoxylin and eosin sections were prepared from paraffin blocks.

Results

The normal pharyngeal flora of the hamsters consisted of α-hemolytic, nonhemolytic and enteric streptococci, *Neisseria* species, *Corynebac-*

terium, Staphylococcus aureus and *S epidermidis*. The Gram-negative bacillary flora was sparce and fluctuated considerably consisting of *E coli, Proteus mirabilis* and *P morganii*. In untreated animals, Gram-negative rods were never cultured in excess of 3.5×10^3 bacteria/ml of TSB. Table 1 lists the distribution of the Gram-negative rods and the average number of organisms per hamster.

The environmental testing of the cages, food and water revealed *Bacillus* species, diphtheroids and fungi. There were no Gram-negative enteric bacteria isolated from these sources. Fecal cultures revealed the expected types and numbers of Gram-negative enteric organisms.

The first experiment attempted to demonstrate a correlation between the effect of oxacillin on the animal's α-hemolytic streptococci and the appearance of Gram-negative rods. Cultures were taken at 0-, 4-, 24- and 48-hour intervals after the last antibiotic injection. The comparison between the approximate number of α-hemolytic streptococci and that of Gram-negative rods from 9 animals is shown in Tables 2 and 3. They consistently had counts of α-hemolytic streptococci in excess of 1×10^5 colonies/ml before injection. The effect of oxacillin on these bacteria was rapid, as 7 animals showed diminished numbers of streptococci after 4 hours. At 24 hours, all animals had had a significant decrease. However, the loss of α-hemolytic streptococci was not immediately consistent with an increase in Gram-negative rods. At this time, Gram-negative bacilli were isolated from 3 animals, but at 48 hours all

Table 1—Gram-Negative Flora of 68 Healthy Golden Syrian Hamsters

	No. of positive cultures	
No. of bacteria/ml	**Escherichia**	**Proteus**
<100	132	119
100	8	8
200	2	3
300	2	8
400	1	2
500	1	2
600	0	2
800	1	0
1,000	0	1
1,300	1	0
1,400	1	0
1,800	1	0
2,000	0	1
3,000	0	3
3,500	0	1
Total No. of cultures	150	150

Table 2—Comparison of Approximate Colony Counts Between α-Hemolytic Streptococci and **Escherichia, Proteus, Klebsiella** and **Enterobacter** in Hamsters Before Oxacillin Administration

Animal	No. of colonies/ml				
	α-Hemolytic streptococci	**E coli**	**Proteus**	**Klebsiella**	**Enterobacter**
1	$>10^5$	10^2	—	—	—
2	$>10^5$	—	—	—	—
3	$>10^5$	—	—	—	—
4	$>10^5$	—	—	—	—
5	$>10^5$	—	—	—	—
6	$>10^5$	—	—	—	—
7	$>10^5$	—	—	—	—
8	$>10^5$	—	10^2	—	—
9	$>10^5$	—	—	—	—

— = No organisms isolated.

hamsters had these organisms in their cultures. It should be noted that *K pneumoniae,* rarely recovered before 24 hours, was predominant at 48 hours. At 72 hours, 3 of the animals had died.

In the next experiment, no samples were taken at 4 hours, as it was felt that at least a 24-hour period was needed for overgrowth of Gram-negative bacilli to take place; instead, cultures were taken at 24, 48 and 72 hours. Results of samples taken from 61 animals treated with oxacillin are shown in Table 4. The percentage of positive cultures for Gram-negative rods appears to be a function of time. By 72 hours, 91 and 100% of all cultures showed growth of *Escherichia* and *Klebsiella,* respectively, and approximately 30% of the animals died.

Blood cultures were randomly taken at 72 hours from 14 of the 61 animals. Of these, 28% were positive for one or more types of bacteria. *E coli* and *Proteus* species grew from 2 of 14 animals and *K pneumoniae* in 3 of 14. No *Enterobacter* species were cultured. Six untreated hamsters were used for control purposes, and their blood cultures were sterile.

At 72 hours, 25 of the animals were sacrificed and lung cultures taken. These cultures were positive for one or more types of bacteria in 22 animals, as shown in Table 5. *K pneumoniae* was recovered most frequently (16/25 or 64%) followed by *E coli* (14/25 or 56%), *Proteus* species (9/25 or 36%) and *Enterobacter* species (8/25 or 32%). Of the 22 positive lung cultures, the frequency of occurrence and combinations of organisms were as follows: 36% of the cultures grew only one species, while 27% yielded two species, with the combination of *Klebsiella* and

Escherichia occurring most frequently. Six control lung cultures from untreated animals were sterile.

Sixty-seven strains of *K pneumoniae* were isolated from the respiratory tract during this study, and 50 of these were processed for typing. Forty-six of these 50 reacted with "pool 1," which included antisera to types 1, 2, 3 and 24. Four strains reacted with type 2, and 44 with type 1. Two agglutinated in "pool 6," which included antisera to types 11, 21, 22 and 23. Two other strains were nontypable because of autoagglutination.

Histologic studies were done on lung tissue from 25 hamsters, and all samples showed microscopic evidence of bronchopneumonia. The lung

Table 3—Comparison of Approximate Colony Counts Between α-Hemolytic Streptococci and **Escherichia, Proteus, Klebsiella** and **Enterobacter** in Hamsters 4, 24 and 48 Hours After Oxacillin Administration

		No. of colonies/ml				
Time	Animal	α-Hemolytic streptococci	E coli	Proteus	Klebsiella	Enterobacter
4 hrs	1	10^3–10^4	—	—	—	—
	2	10^3–10^4	—	—	—	—
	3	$>10^5$	—	—	—	—
	4	10^3–10^4	10^2	—	—	—
	5	10^2–10^3	—	—	—	—
	6	$>10^5$	—	—	—	—
	7	10^4–10^5	—	—	—	—
	8	10^3–10^4	—	—	—	—
	9	10^2–10^3	—	—	—	—
24 hrs	1	10^3–10^4	—	—	—	—
	2	10^3–10^4	—	—	—	—
	3	10^4–10^5	—	—	—	—
	4	10^2–10^3	10^2	10^3	—	—
	5	10^2–10^3	—	—	—	—
	6	10^2–10^3	—	10^2	10^3	—
	7	10^3–10^4	—	—	—	—
	8	10^2	—	—	—	—
	9	10^2–10^4	10^3	—	—	—
48 hrs	1	10^3–10^4	—	—	—	10^4
	2	$<10^2$	10^3	—	10^4	$<10^2$
	3	10^4–10^5	10^2	—	10^4	$<10^2$
	4	10^3–10^4	$<10^2$	—	—	10^4
	5	10^2–10^3	$<10^2$	—	10^4	10^4
	6	10^2–10^3	$<10^2$	10^3	10^3	10^4
	7	10^3–10^4	$<10^2$	—	10^3	$<10^2$
	8	$<10^2$	$<10^2$	—	10^3	$<10^2$
	9	10^3–10^4	$<10^2$	10^3	$<10^2$	$<10^2$

— = No organisms isolated.

Table 4—Comparison of the Gram-Negative Rods Isolated from Oropharyngeal Samples of 61 Hamsters 24, 48 and 72 Hours After Oxacillin Administration

	Escherichia coli			**Proteus** species			**Klebsiella pneumoniae**			**Enterobacter** species		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	24 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
No. of animals												
Positive	16	33	19	18	15	3	14	56	21	17	29	12
Tested	61	61	21	61	61	21	61	61	21	61	61	21
Percent with positive culture	26	54	91	30	24.5	14.3	23	92	100	27.8	47.5	57
Mean value per animal*	1,290	2,852	2,462	752	413	333	1,061	10,079	14,305	300	2,563	1,719
Mean value per control animal†	50	42	30	160	140	150	0	0	0	0	0	0

* Expressed as number of colonies/ml

† The number of untreated control animals was 6.

Table 5—Results of Lung Cultures Taken from Hamsters Given Oxacillin 72 Hours Previously

Animal	Escherichia	Proteus	Klebsiella	Enterobacter
1	—	—	—	—
2	—	—	—	—
3	—	—	—	—
4	+	+	—	+
5	+	+	—	+
6	+	+	—	+
7	+	—	—	—
8	—	+	+	—
9	+	—	—	—
10	+	+	+	+
11	—	+	+	+
12	+	+	+	+
13	+	+	+	+
14	+	+	+	—
15	—	—	+	—
16	+	—	—	+
17	—	—	+	—
18	+	—	+	—
19	—	—	+	—
20	+	—	+	—
21	+	—	+	—
22	—	—	+	—
23	—	—	+	—
24	—	—	+	—
25	+	—	+	—
Total No. of positive cultures	14	9	16	8
Percent	56	36	64	32

— = Culture negative for the organism, + = culture positive for the organism.

sections were classified arbitrarily according to the degree of pneumonia. Those animals whose lungs showed only minimal pneumonia were rated as 1+; those with 50% of the lung tissue diseased were rated as 2+. Those animals whose lung sections showed almost complete consolidation of the lung were rated as 3+. Ten of these 25 hamsters had a 1+ reaction; 10, 2+; and 5, 3+. The lung pathology is sufficient to account for the death of the animals from pneumonia. Fifty percent of those hamsters which died had a terminal diarrhea which could be a result of toxicity to oxacillin. Rectal and bowel postmortem cultures of these hamsters revealed the same flora as that associated with healthy animals, with no increase in the incidence of *Klebsiella*.

Discussion

Hamsters with altered pharyngeal flora due to oxacillin treatment demonstrated Gram-negative rods in the oropharynx in concentrations

of 1×10^3 or greater bacteria/ml before developing pneumonia. Our results indicate that the Gram-negative bacilli appeared initially in the throat and later overcame the mechanical defense barriers as described by Kass.[6] The results also confirm Weinstein's observation that a marked increase in the numbers of organisms in the nasopharynx frequently precedes their invasion of the lung by at least 24 hours.[7] In this study, a hamster was considered to be infected when its posttherapy throat and necropsy lung cultures grew the same organisms and tissue sections revealed evidence of pneumonia.

Alpha-hemolytic streptococci have been regarded as the bacteria which regulate the flora of the upper respiratory tract, and a correlation exists between the presence of α-hemolytic streptococci and the absence of Gram-negative rods in the pharynx.[3,8,9] This may be due either to antibacterial substances produced by α-hemolytic streptococci or to differential clearing by host defenses which favor streptococci. Although Meads *et al*[2] indicated that short courses of therapy with oral penicillin seem to have little effect on α-hemolytic streptococci, the two injections of oxacillin used in this study resulted in a threefold decrease in these organisms and an increased incidence of Gram-negative rods. It appeared that once the Gram-negative rods became established as the predominant organisms, the inhibition previously exerted by the α-hemolytic streptococci had little effect on colonization reversal. The predominant organism was *Klebsiella*. At the end of 48 hours *Klebsiella* had outnumbered *Proteus* by 9:1 and *Escherichia* by 4:1. By 72 hours, *Klebsiella* outnumbered *Escherichia* by 5:1. With such a rapid proliferation, the inhibition process, whatever its mechanism, was incapable of retarding and/or eliminating the Gram-negative rods.

It is interesting to note that *Klebsiella*, the only organism not detected in the untreated hamsters prior to antibiotic therapy, was the most efficient colonizer afterwards. This fact has also been noted by Tillotson and Finland in humans.[3] Types 1, 2 and 4 are most commonly associated with the respiratory tract of humans, and destructive lung diseases are three times as common when *Klebsiella* is among the lower serologic types. Weiss *et al*[10] have postulated that when types 1, 2 and 4 are isolated during or after penicillin therapy, they were probably present prior to therapy. If higher types are isolated, they may have been exogenously acquired. This latter possibility was considered early in the experiment, when the cultures consistently grew *Klebsiella*. Consequently, environmental checks were made on the hamsters' water supply and food. No Gram-negative bacilli were cultured from these areas. The interiors of the cages were repeatedly swabbed, but no Gram-

negative organisms were isolated. However, *Klebsiella* was infrequently recovered from the feces along with other Gram-negative enteric bacteria.

Since an environmental source of *Klebsiella* was tentatively eliminated, and since rare *Klebsiella* of the high serologic types were isolated from the treated animals, the hamsters apparently carried these bacteria in concentrations of less than 100/ml. Solomon[11] indicated that *K pneumoniae* is a normal inhabitant of the upper respiratory tract in a small percentage of normal individuals, while Lampe[12] reported a 2 to 25% carrier rate for this organism. It would appear that a high percentage of hamsters in this study were carrying *Klebsiella* type 1, but the exact site of origin is unknown since they were not demonstrated in oropharynx cultures prior to treatment.

Tillotson and Finland reported 12.1% incidence of positive blood cultures in their study of primary pneumonia, with *K pneumoniae* representing 8% and *E coli* 20% of the bacteremias.[8] In the present study, 28.5% of the blood cultures were positive for one or more types of bacteria. The pathogenicity of *Klebsiella* was again demonstrated, as this organism was grown from three of the four positive cultures, thus representing 21% of the total blood cultures.

Lung cultures were taken from approximately 40% of the animals under study, and 92% of these cultures were positive for one or more types of organisms, a rate similar to that reported for humans by Klasteresky *et al.*[1] *Klebsiella* was recovered most frequently (64%), followed by *E coli* (56%), *Proteus* species (36%) and *Enterobacter* species (32%). *Klebsiella* was recovered three times as frequently as *E coli* from the cultures that grew only one type of organism, a fact which correlates well with the incidence of these two bacteria in throat cultures. Of the six cultures growing two types of organisms, four showed the combination of *Klebsiella* and *E coli* which might represent a synergistic effect.

The hematoxylin and eosin preparations of lung tissue taken from 25 hamsters all revealed bronchopneumonia. Attempts to correlate a particular animal with a particular microorganism failed to explain the diversity of responses to colonization and infection. When the pneumonia was classified as to severity, no one particular species appeared responsible for the disease pattern; however, *Klebsiella* and *Escherichia* were usually the bacteria most frequently detected.

One of the objectives of this investigation was to demonstrate the value of the hamster as a model for the study of bacterial overgrowth associated with antibiotic therapy. The similar findings from this study

and those reported in the literature indicate the value of the further use of this model to study the effect of other antibiotics on the respiratory bacterial flora.

Another objective was to further investigate the relationship between the results of cultures from the oropharynx and the predictive value of these results in diagnosing, or at least anticipating, the possibility of Gram-negative rod pneumonia. The finding of Gram-negative bacilli in the throat poses a problem of interpretation, as these isolates may not reflect conditions within the lungs. Klasteresky *et al* studied Gram-negative rod pneumonia in 37 patients and found that the ratio of clinical pneumonia to bacterial colonization was 1:3.6.[1] This means that there was approximately a one-in-four chance that bacteria cultured from the throat actually represented pneumonia. According to our data, the high correlation between pneumonia and colonization, on the basis of positive throat and lung cultures and tissue sections, would emphasize the value of throat cultures in diagnosing pneumonia. The lowest correlations achieved between pneumonia and colonization was for the *Enterobacter* species, a 1:3 ratio. The other bacteria varied from a rate of 1:1.4 for *Escherichia* to 1:1.6 for *Klebsiella* and *Proteus*.

References

1. Klasteresky J, Cappel R, Debusscher L, Stilmant M: Pneumonias caused by Gram-negative bacilli in hospitalized patients presenting malignant disease. Eur J Cancer 7:329–336, 1971
2. Meads M, Rowe WB, Haslam NM: Alterations in the bacterial flora of the throat during oral therapy with aureomycin. AMA Arch Intern Med 87:533–540, 1951
3. Tillotson JR, Finland M: Secondary pulmonary infections following antibiotic therapy for primary bacterial pneumonia. Antimicrob Agents Chemother 8:326–330, 1968
4. Johanson WG, Pierce AK, Sanford JP: Changing pharyngeal bacterial flora of hospitalized patients. N Engl J Med 281:1137–1140, 1969
5. Blair JE: Manual of Clinical Microbiology. Edited by JE Blair, EH Lennettee, JP Truant. Bethesda, Md, American Society of Microbiology, 1970, pp 61–344
6. Kass EH, Green GM, Goldstein E: Mechanism of antibacterial action in the respiratory system. Bacteriol Rev 30:488–497, 1966
7. Weinstein L, Goldfield M, Chang T: Infections occurring during chemotherapy: a study of their frequency, types, and predisposing factors. N Engl J Med 251:247–254, 1954
8. Tillitson JR, Finland M: Bacterial colonization and clinical superinfection of the respiratory tract complicating antibiotic treatment of pneumonia. J Inf Dis 119:597–624, 1969
9. Yow EM: Development of Proteus and Pseudomonas infections during antibiotic therapy. JAMA 149:1184–1188, 1952

10. Weiss W, Eisenberg GM, Spival A, Nadel J, Kayser HL, Sathavara S, Flippin JF: Klebsiella in respiratory disease. Ann Intern Med 45:1010–1026, 1956
11. Soloman S: Primary Friedlander's pneumonia. JAMA 108:937–946, 1937
12. Lampe WT: *Klebsiella pneumoniae:* a review of 45 cases and re-evaluation of the incidence of antibiotic sensitivities. Dis Chest 46:599–606, 1964

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