IV. PHAGOCYTOSIS OF ANTIBiotic-RESISTANT STRAINS OF Escherichia coli

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The widespread use of antibiotics as therapeutic agents against pathogenic bacteria sometimes has presented a problem as the result of the development of drug resistance by the organism involved (Finland, 1956). Although the level of an antibiotic necessary to elicit a growth response in young animals is many times less than the therapeutic dose, drug-fast organisms have been found among the intestinal bacteria isolated from animals fed nutritional levels of an antibiotic (Radisson, Smith, and Ward, 1956; Ingram et al., 1958). Developed resistance to antibiotics by microorganisms may pose serious problems. The antibiotic may become ineffective against the microorganism toward which the drug is directed, or the organism which is inhibited may be replaced by another pathogen that is resistant to the drug.

Radisson et al. (1956a) and MacFadden and Bartley (1959) showed that low level feeding of chlortetracycline to dairy calves aids the host defense mechanism of phagocytosis by reducing the virulence of the pathogen. Also, McKee and Houck (1943), Blair, Carr, and Buchman (1946), and Radisson et al. (1956b) observed that certain biological changes, such as in morphology and physiology, occurred in drug-resistant bacteria and that these changes were often accompanied by a reduction in virulence.

This investigation was conducted to determine what effect induced resistance to chlortetracycline in vitro would have on growth and sensitivity to phagocytosis of Escherichia coli that had been shown to be pathogenic in calves.

MATERIALS AND METHODS

Cultures. The two strains used in this study were obtained through the courtesy of Paul J. Glantz, Pennsylvania State University, where they had been identified as Escherichia coli of the following "O" groupings:

<table>
<thead>
<tr>
<th>Strain</th>
<th>O Group</th>
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<tbody>
<tr>
<td>P. S. 175M</td>
<td>O26:B6 (anaerogenic)</td>
</tr>
<tr>
<td>P. S. 56</td>
<td>O8</td>
</tr>
</tbody>
</table>

These organisms had been isolated from calves with infectious diarrhea and, under carefully controlled conditions, had reproduced the diarrhea when fed back to healthy calves (Dunne et al., 1956).

Cultural technique. Stock cultures were carried on nutrient agar slants, transferred at monthly intervals and stored at 5° C after initial development. An incubation temperature of 39° C was used throughout the study for both culture development and phagocytosis tests.

Determination of initial resistance to the antibiotic. To study initial resistance to chlortetracycline, the stock cultures were first transferred to nutrient broth and incubated for 24 hr. From this medium, 0.01 ml was inoculated into broth containing each of the following concentrations of chlortetracycline hydrochloride in μg per ml: 0.4, 0.3, 0.2, 0.1, 0.08, 0.06, 0.05, 0.04, 0.02, 0.01, and 0. Growth curves were determined by measuring with a Bausch and Lomb photometer the per cent light transmitted by broth cultures. Readings were made every hour for 8 hr and plotted against time.

Method of inducing resistance to the antibiotic. Induced drug-fastness was established by growing...
the bacteria in a concentration of antibiotic which would alter the normal growth curve but would not inhibit growth for an extended period of time. Daily transfers were made into media containing the above concentrations of the drug and growth curves were determined daily (photo-
metrically) until the organism had regained the same growth curve in the presence of antibiotic as in its absence, when it was assumed that drug-fastness had been established. If drug-fastness to a higher concentration of antibiotic was desired, the process was repeated, using a medium containing the next higher concentration of chlortetracycline hydrochloride.

To determine if induced drug resistance persisted when the organism was cultured in the absence of the antibiotic, transfers were made each day after washing the bacteria each time into nutrient broth containing no chlortetracycline. Susceptibility to phagocytosis as described below was then tested following both three or five such daily transfers.

Source of leucocytes. Leucocytes were obtained by centrifugation of blood (MacFadden and Bartley, 1959) from a healthy 2-month-old calf which had not received any antibiotic previously. The cells were adjusted to a concentration of 40,000 cells per mm³ with bovine blood plasma as determined by counting in a hemocytometer.

Method of determining the phagocytic index. The ability of drug-resistant coliform bacteria to resist phagocytosis was determined by measuring the phagocytic index of calf leucocytes exposed to antibiotic-treated bacterial cells or untreated (control) cells. Treated cells were removed from the nutrient broth containing the chlortetracycline concentration being studied, washed in sterile saline, and exposed to the phagocytes.

The procedure for determining the phagocytic index (MacFadden and Bartley, 1959) consisted

![Figure 1. Normal growth curve, and altered growth curves of Escherichia coli strain 08, resulting from varying the level of chlortetracycline present in the media.](image-url)
of holding washed bacteria and leucocytes separately in a water bath at 39 C for 30 min. Then, 0.6 ml of leucocytic suspension with 40,000 cells per mm$^3$ were pipetted into a 5-ml test tube containing 0.4 ml of bacterial suspension with $3 \times 10^8$ cells per ml as determined photometrically. The mixed bacteria-leucocyte suspension was held at 39 C for 30 min with gentle shaking of the tubes every 5 min; then smears were prepared, stained with Wright's stain, and the number of bacteria engulfed by 50 phagocytes was counted under the microscope. The resulting number was divided by 50 to give the average number of bacteria engulfed per phagocyte—the phagocytic index. Four slides were prepared and counted for each treatment of each strain.

RESULTS

Growth curves of antibiotic-treated bacteria. Typical growth curves for strains O26:B6 and O8 are shown in figures 1 and 2. Very low concentrations of chlortetracycline were detectable, since levels in the range of 0.02 and 0.05 µg per ml delayed the growth of the test organisms slightly, and concentrations in the range of 0.1 to 0.2 µg per ml markedly delayed and reduced total growth up to 8 hr. Concentrations in the range of 0.3 and 0.4 µg per ml completely inhibited growth for 20 to 24 hr (data not shown). Curves were readily duplicated if conditions remained standardized and the organism had not been cultured in chlortetracycline previously.

Morphological studies. Observation of stained bacteria revealed somewhat smaller, more coccoid cells during the time the growth curve was altered by the antibiotic. Morphology appeared normal once the bacteria had become resistant, except for a few grossly irregular cells. No difference was noted in the degree of encapsulation before or after drug resistance was obtained.

Figure 2. Normal growth curve, and altered growth curves of Escherichia coli strain O26:B6, resulting from varying the level of chlortetracycline present in the media.
TABLE 1
Susceptibility to phagocytosis of pathogenic strains of Escherichia coli made resistant to chlortetracycline

<table>
<thead>
<tr>
<th>Concentration of chlortetracycline (µg/ml)</th>
<th>Phagocytic Index*</th>
<th>Treatment†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain Ø2:Ø6</td>
<td>Strain Ø8:Ø6</td>
</tr>
<tr>
<td>1.0</td>
<td>2.83</td>
<td>2.95</td>
</tr>
<tr>
<td>0.5</td>
<td>3.23</td>
<td>3.18</td>
</tr>
<tr>
<td>None (control 1)</td>
<td>1.62</td>
<td>1.61</td>
</tr>
<tr>
<td>None (control 2)</td>
<td>1.58</td>
<td>1.52</td>
</tr>
</tbody>
</table>

* Each value is an average of 4 replications. Least significant difference (P < 0.05) = 0.98.
† A: After resistance had been established bacterial cells were taken directly from the concentration of chlortetracycline indicated, washed in saline, and used in the phagocytic study.
B: After resistance had been established bacterial cells were transferred three times in the absence of chlortetracycline.
C: Same as B except that five transfers were made in the absence of chlortetracycline.

Establishing drug-fastness. Drug-fastness to chlortetracycline was not easily established in E. coli. Although a test organism placed directly into antibiotic concentrations of 0.1 µg per ml would finally grow, 10 or more transfers in this concentration were necessary before the growth curve began to approach normal. The best procedure appeared to be to start transfers of the bacteria in concentrations of chlortetracycline in which the growth curve was not markedly altered (0.02 to 0.04 µg per ml) and to repeat transfers (usually, 8 to 10) into this concentration until drug-resistance was established. Once drug-fastness had been established the organism had the same general growth curve as cells grown in the absence of chlortetracycline (figures 1 and 2), but establishing resistance at a higher level of the drug was just as difficult as inducing resistance at the lower level.

Susceptibility of drug-fast bacteria to phagocytosis. Bacteria in which drug-fastness had been established were significantly more susceptible to phagocytosis than untreated bacteria (table 1). This statistically significant difference existed regardless of the strain used or the concentration of antibiotic employed. There was no significant difference between strains or concentrations of antibiotics employed in establishing drug-resistance.

Also, following the antibiotic treatment, susceptibility to phagocytosis was not markedly changed after the bacteria had been transferred 3 or 5 times in media containing no chlortetracycline (table 1).

DISCUSSION
The findings of this study are in agreement with those reported by other workers with respect to certain physiological changes that occur with drug-fastness (McKee and Houck, 1943; Blair et al., 1946; Gezon and Fasan, 1950). In most cases, drug-fastness of bacteria has been accompanied by many altered characteristics such as decreased viability, reduced growth rate, retarded metabolism, altered nutritional requirements, pleomorphic changes, altered staining, and a reduction in virulence (McKee and Houck, 1943; Blair et al., 1946; Gale and Rodwell, 1948; Ramsey and Padron, 1954; Radisson et al., 1956). Since no gross changes were observed in the morphology of the drug-fast organisms used in the present study it is conjectured that the susceptibility to phagocytosis of strains of E. coli may be explained by physiological changes in the organisms. Further studies are needed to confirm this hypothesis.

Radisson et al. (1956b) and Ingram et al. (1958), isolated drug-fast bacteria from antibiotic-fed animals. Also Johansson, Peterson, and Dick (1953) and Reid et al. (1957) have reported that nutritional levels of antimicrobial agents increased the appearance of resistant bacterial strains. The quantities of antibiotics found in the excretions of antibiotic-fed calves have been reported (Rusoff et al., 1954; Radisson et al., 1956b). Quantities found were from 0.05 to above 5.0 µg per ml which is considerably above the quantity necessary to induce drug-resistance in vitro in the present study. Further, it has been reported that the intestinal flora from animals not fed an antibiotic is responsible for a decrease in growth and well-being of young animals (Luckey, 1952; Coates, 1953; Dunne et al., 1956).
Yet the significance of the development of antibiotic-resistant flora in the intestines of animals fed nutritional levels of an antibiotic has not been considered with respect to growth stimulation.

In two previous studies (Radisson et al., 1956a; MacFadden and Bartley, 1959), it was shown that leukocytes from newborn calves had a lower phagocytic activity than did leukocytes from older calves. The greatest growth response to antibiotics was obtained during the early life of the calf when it was most susceptible to intestinal disturbances. Accordingly, the hypothesis was advanced that antibiotics, by decreasing the virulence of bacteria and making them more susceptible to body defense mechanisms, prevent the early depression of the rate of growth of animals soon after birth.

From evidence presented here it is postulated further that antibiotics fed at low levels, relative to therapeutic doses, select mutant strains of bacteria within the gastrointestinal tract, and that these mutants are able to proliferate in the concentration of the antibiotic present. The nature of these drug-fast organisms is markedly altered from their wild types in that the mutant is less able to resist phagocytosis. Thus, an increase in rate of growth and better health for the animal may be expected even after continued use of an antibiotic in specific locations where the intestinal bacterial flora may have become resistant to the antibiotic.

ACKNOWLEDGMENT

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SUMMARY

A study was made on the development of drug-fastness in two strains of *Escherichia coli* known to be pathogenic to calves. Drug resistance to chlortetracycline was difficult to induce initially in the organisms, and resistance was acquired in a step-wise progression from any given concentration of the drug to the next higher concentration. Low levels of chlortetracycline altered the growth curve of the bacteria, and repeated transfers in these low concentrations of antibiotic resulted in re-establishing a normal growth curve by the organism. The organisms which had become drug resistant were more susceptible to phagocytosis than their non-drug-resistant sister cells even after 5 transfers in the absence of the drug.

It was postulated that the feeding of nutritional levels of chlortetracycline to young calves contributes to the stimulation of the growth rate of the calves by reducing the resistance to phagocytosis of intestinal bacteria, thereby aiding the host defense mechanism. Such mode of action would result in a reduction in incidence of intestinal infections which may cause stress in the early weeks of growth of the calf.

REFERENCES


MacFadden, D. L., and E. E. Bartley 1959 Mode of action of antibiotics in the nutrition of the dairy calf. III. Relative effect of age, colostrum, and chlortetracycline feeding on


