INCREASED RESISTANCE TO CHLORAMPHENICOL IN RICKETTSIA PROWAZEKII WITH A NOTE ON FAILURE TO DEMONSTRATE GENETIC INTERACTION AMONG STRAINS

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ABSTRACT

Weiss, Emilio (Naval Medical Research Institute, Bethesda, Md.) and Harry R. Dressler. Increased resistance to chloramphenicol in Rickettsia prowazekii with a note on failure to demonstrate genetic interaction among strains. J. Bacteriol. 83:400-414. 1962.—A further effort to produce genetically labeled Rickettsia prowazekii was made by serially passing the Madrid E strain in eggs in the presence of increasing concentrations of chloramphenicol. After 40 serial and 1 dilution passages, a strain, called C', was isolated, which appeared to be the result of a two-step change, each involving a twofold increase in resistance to the antibiotic. Chloramphenicol resistance was retained after 10 drug-free egg passages. Cross-resistance to thioacetimidate, but not to unrelated drugs, was shown.

Several attempts were made to demonstrate genetic interaction, in eggs or endodermal cell cultures, among strains resistant to chloramphenicol, erythromycin, and p-aminobenzoic acid. They have not been successful.

Rickettsial strains of increased resistance to the inhibitory compounds p-aminobenzoic acid, quinoloxaline oxide, and erythromycin have been selected and studied (Weiss, Dressler, and Suitor, 1957, 1959a, b; Weiss and Dressler, 1960a; Weiss, 1960). These resistant strains have provided valuable information on the physiology of the microorganisms, as several unselected changes were found to accompany these mutations. However, further study of rickettsial genetics, and, more specifically, the investigation of the possibility of genetic interaction among strains, required the selection of additional genetic markers. Tetracycline and chloramphenicol were used for this purpose, but only a strain of moderate resistance to chloramphenicol was recovered and is here described. A few experiments in which mixtures of two strains were used are also reported and discussed.

MATERIALS AND METHODS

The materials and methods were for the most part described previously (Weiss et al., 1957, 1959a, b; Weiss and Dressler, 1960a). The essential information is summarized below.

Rickettsiae. The Madrid E strain (Perez Gallardo and Fox, 1948) of Rickettsia prowazekii was grown in the yolk sacs of chick embryos, and suspensions were diluted in the isotonic solution of Bovarnick, Miller, and Snyder (1950). The following substrains were used: parent, i.e., the Madrid E strain as received in our laboratory and reisolated by limit-dilution (Weiss et al., 1957); P', and Q', resistant to p-aminobenzoic acid and quinoloxaline oxide, respectively (Weiss et al., 1959b); E', resistant to erythromycin (Weiss and Dressler, 1960a).

Antibiotics. Chloramphenicol solution, 0.250 g per ml in a 50% aqueous solution of N,N-dimethylacetamide, was obtained from Parke, Davis & Co., Detroit, Mich. Thioacetimidate (d-threo-2-dichloroacetamido-1,4-methylthiobenzoxazol-1,3-propanediol) was a gift from Sterling-Winthrop and was kindly sent to us by Randall Thompson.

Chick embryos. The eggs (7 days old) were from White Leghorn chickens maintained on an antibiotic-free diet. All injections were made into the yolk sacs. Volumes of 0.1 ml of antibiotic, or diluent in the case of the controls, were immediately followed by 0.4 ml of the rickettsial suspensions. The eggs were then incubated at 35°C and observed for 13 days.

Determinations of the effect of the antibiotics were made by comparing mean survival times of control and treated groups of 12 to 15 embryos. Yolk sacs from approximately one-third of the embryos were smeared and checked for the
presence of rickettsiae. Embryos surviving at 13 days were arbitrarily given 14 as the day of death. (This is indicated in the tables by addition of a + sign to the figure expressing the mean.) With concentrations of rickettsiae sufficient to kill embryos in less than 7 days, an increase in mean survival time of a group of drug-treated embryos of 1.0 day or greater was considered as indicating significant protection (Weiss et al., 1959b). Rickettsial titers were in most cases estimated from the mean survival times of embryos that had received single dilutions of the pools (Weiss et al., 1957, 1959a).

RESULTS

Serial passage and isolation of C' strain. The Madrid E strain of R. prowazekii was passed serially in eggs in high concentration in the presence of increasing amounts of chloramphenicol. Amounts of antibiotic administered were as follows: 0.10 to 0.15 mg/egg (passages 1 to 6); 0.20 to 0.25 mg (passages 7 to 12); 0.30 mg (passages 13 to 20); and 0.40 mg (passages 21 to 40). A first change in susceptibility became apparent during the 7th passage, a second one during the 16th to 19th passages, but impressions that other changes had occurred during further passages were not later substantiated. Following the 40th drug passage, a strain called C' was obtained by limit-dilution isolation, i.e., from a single infected egg of a group having a low (5%) incidence of infection (see Weiss et al., 1957, for details of procedure).

Comparison of C' to other strains. The effects of chloramphenicol on the parent strain, the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean chick embryo survival time (days)</th>
<th>Increase (days) after treatment (mg/egg)</th>
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<tbody>
<tr>
<td></td>
<td>Un-treated</td>
<td>0.1</td>
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<tr>
<td>Parent</td>
<td>6.1</td>
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<td>10th Drug</td>
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<td>20th Drug</td>
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<td>passage</td>
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<tr>
<td>C'</td>
<td>6.0</td>
<td>0.1</td>
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<td>C' + 10 drug-</td>
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harvests from two passage levels (10th and 20th), and C' are shown in Table 1. With the parent strain, 0.1 mg per egg was the smallest amount to produce a significant increase in chick embryo survival time (>1 day), and 0.4 mg was sufficient to protect a majority of the embryos to the end of the period of incubation (13 days). These results are in agreement with those of other investigators who employed similar methods (Jackson, 1951; Ormsbee, Parker, and Pickens, 1955).

By the 10th passage, the minimal effective dose had shifted to an amount intermediate between 0.2 and 0.3 mg, and by the 20th passage to approximately 0.4 mg. Serial drug passages were continued, but the results obtained with C' were identical to those of the harvest from the 20th passage. The difference between parent and C' can thus be defined as a fourfold increase in the amount of chloramphenicol required to produce a minimal inhibitory effect.

A more detailed representation of the difference between a susceptible strain, E', and C' is shown in Fig. 1. It was previously shown (Weiss and Dressler, 1960a) that E' has the same virulence for the chick embryo and the same susceptibility to chloramphenicol as the parent strain. E' and
C were injected into eggs, which had received either diluent or 0.5 mg of chloramphenicol per egg. At various intervals of time, duplicate pools of four yolk sacs each were obtained from living embryos and titrated. Ten eggs were set aside in each group for the determination of mean survival times of the embryos. As shown in Fig. 1, the growth rate of either strain in the absence of the antibiotic was approximately the same as that obtained with the other rickettsial strains (Weiss et al., 1957). Peak or near peak titers were obtained on the 6th day. With chloramphenicol, the titer of C was appreciably reduced by the 4th day; growth then proceeded, first slowly to reach the level of the inoculum by the 7th day, and then rapidly to a peak on the 10th to 12th day. The growth of C, on the other hand, was only slightly delayed by the antibiotic, and peak titer was reached only 1 or 2 days later than in the untreated controls.

C retained its full level of drug resistance after 10 drugless egg passages (Table 1). The virulence for the chick embryo, as determined by the difference between LD and LD (Weiss, 1960), was the same as that of the parent strain.

Cross-resistance. Ormsbee et al. (1955) have shown that thiocymetin is a very effective inhibitor of R. prowazekii. The close similarity of this compound to chloramphenicol (Cutler, Stenger, and Suter, 1952) suggested the possibility that increased resistance to one antibiotic may extend to the other. The results of such a test are shown in Table 2. While the parent strain was inhibited to the same extent as in the experiments of Ormsbee et al., C was relatively resistant. A stepwise increase parallel was the increased resistance to chloramphenicol was suggested by the results with the rickettsiae from the 10th passage (Table 2).

The susceptibility of C to p-aminobenzoic acid, quinoline oxide, and erythromycin remained unchanged.

Mixed infections. 1) Experimental models:— With the production of C, several genetically labeled strains became available for the study of genetic interaction among rickettsiae. Our first attempt to study genetic interaction, using p-aminobenzoic acid and quinoline oxide resistance as markers (Weiss et al., 1959a), were encouraging. Later investigations, however, indicated that quinoline oxide resistance was acquired by mutation too rapidly to be of value in experiments of genetic interaction (Weiss, 1960).

In addition to our own previous work, two other models have been followed for the conduct of such experiments, both dealing with viruses of the psittacosis group. Gordon and Mamay (1957) and Gordon, Mamay, and Trimmer (1960) used strains of Ab and aB type and showed that strain AB appeared more frequently in the presence of both parents than in the presence of only one. Greenland and Moulder (1961) followed the classical criteria for the demonstration of genetic interaction, i.e., they employed strains AB and abCD and used A and C as selected and B and D as unselected markers. Their results, however, did not provide an indication of genetic interaction.

If the above experiments are carefully analyzed, several differences can be noted in the two investigations, even though Greenland and Moulder in some cases attempted to reproduce most of the details of Gordon’s experiments. The following are the two main differences: (i) Gordon et al. inoculated eggs with 10 to 10 infectious microorganisms and applied selective agents which did not entirely inhibit growth. Thus, they depended primarily on the second cycle of infection for genetic interaction. Greenland and Moulder, on the other hand, used higher inocula, approximately 10, and leaned more heavily on the first cycle. (ii) Gordon et al. tested their harvests from the first egg passage, prior to limitation isolation. Their limit-dilution strains, which confirmed their results, were obtained only after a second egg passage in the presence of the screening agents. Thus, they allowed two egg passages for the population shift to take place from Ab and aB to predominantly AB. Greenland and

<table>
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<th>Table 2. Effect of thiocymetin on rickettsial strains</th>
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<td>Parent</td>
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<tr>
<td>10th Chloramphenicol passage</td>
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<td>Parent</td>
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<tr>
<td>C</td>
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Moulder, on the other hand, exposed their harvests to selective action for only one passage prior to limit-dilution isolation, and their tests were done exclusively with limit-dilution isolates. Thus, success in their experiments required a much higher rate of population shift than was needed in those of Gordon et al.

Our experiments resembled those of Gordon et al. (1960). Our strains were of Ab, aB type. If genetic interaction did occur, it would have readily provided the AbBed, abCD strains, which would have been advantageous in further studies of genetic interaction. Although rickettsiae are not known to undergo cycles of infection similar to those of psittacosis virus, the inoculum in our experiments was relatively small and interaction was sought in the cells which were secondarily invaded, rather than in the first sites of infection. The harvests from the first egg passages were studied prior to limit-dilution isolation.

2) Experiments with eggs:—A total of 10 mixed-infection experiments was carried out with eggs and tissue culture, including one already described in detail (Weiss et al., 1959a) and four others briefly mentioned (Weiss, 1960). Since the results failed to provide any clear indication of genetic interaction, only one egg and one tissue culture experiment will be reported.

The experiment performed with eggs is illustrated in Fig. 2. Eggs were inoculated each with $3 \times 10^4$ E' and $2.1 \times 10^4$ Cr rickettsiae. A larger number of Cr than E' rickettsiae was used, because erythromycin has a more effective selective action than chloramphenicol (Weiss and Dressler, 1960a). Unselected growth was allowed to proceed for 90 hr. Erythromycin (1 mg/egg) and chloramphenicol (0.5 mg/ml) were then injected via the air sac (Bloom and Gordon, 1955). Because of the delay in drug administration, only about 70% of the embryos were protected. Duplicate pools of four yolk sacs each were prepared from living embryos, immediately prior to antibiotic administration, at 2-day intervals thereafter, and at the end of the period of incubation, 13 days. Figure 2 illustrates the total titers of the pools and the relative concentrations of the two strains. The pattern of rickettsial titers is remarkably similar to that of the E' strain in the presence of chloramphenicol. It is apparent that Cr, the preponderant strain at the beginning of the experiment, was gradually replaced by E'. By the 4th day after drug administration, E' were more numerous than Cr rickettsiae, and, by the 8th or 9th day Cr rickettsiae were rare or had completely disappeared. There was no indication that a strain with dual drug resistance had emerged.

3) Experiments with tissue cultures:—In this and the other tissue culture experiments, Prr was used instead of Cr. Because of the large size of the yolk sac, it is not known whether a sufficient number of cells was dually infected in the above-described experiment to provide a true test of genetic interaction. Some of the uncertainty about rickettsial multiplicity was eliminated by using tissue cultures instead of eggs. Entodermal cell cultures (Weiss and Huang, 1954; Weiss

![Fig. 2. Mixed infection with E' and Cr (see text for description of experiment). Large circles represent titers of individual pools of four eggs. The continuous line connects the means of duplicate pools. Dotted line represents estimate of the relative concentration of E' in the mixture. This estimate was based on a comparison of survival times of embryos inoculated with the various pools and receiving either diluent, erythromycin (1 mg/egg), or chloramphenicol (0.5 mg/egg). Erythromycin, in the concentration used, completely inhibits susceptible strains (Weiss and Dressler, 1960) and has no effect whatever on E'. Thus, E' titers were estimated by the same method employed for the mixture. Chloramphenicol, on the other hand, while effectively protecting embryos infected with E' to the end of the period of observation, also prolonged the survival time of embryos inoculated with Cr by 2.0 to 2.5 days, and this delay was taken into account. The consistency of the results (i.e., approximation of titers of E' + Cr to titer of mixture) fell well within the limits of experimental error.](http://jb.asm.org/)

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and Dressler, 1960b), containing approximately 2 × 10^4 cells were inoculated with a mixture of equal numbers of P'E' and E'T rickettsiae. Control groups contained parent rickettsiae in place of one or the other strain. Rickettsial multiplicity was approximately 10, as determined by infectivity in eggs, and agent-host cell contact was insured by centrifuging the inoculum onto the cells, as previously described (Weiss and Dressler, 1906b). p-Aminobenzoic acid was introduced at the time of inoculation, because of its delayed effect on growth of susceptible strains in eggs (Weiss et al., 1957) and in tissue culture (Weiss and Dressler, unpublished data). Erythromycin was added to the cultures 3 days later. Seven days after inoculation the intracellular and extracellular rickettsiae were harvested and tested for drug resistance. Although the titers were greatly reduced by the presence of the two inhibitors, the parent, P'E', and E'T substrains were recovered. In this experiment, also, there was no indication that dual drug resistance had been produced.

**DISCUSSION**

The C' strain was most likely the product of a two-step increase in chloramphenicol resistance of the parent Madrid E strain. Each step involved approximately a twofold increase in resistance. Cavalli and Maccacaro (1950) showed that chloramphenicol resistance in *Escherichia coli* K12 developed in several steps, each involving, on the average, a two-fold or lower increase in resistance. In view of the more complex growth requirements of rickettsiae, it is not surprising that only two steps in increased resistance could be obtained by our procedures, while numerous steps are obtained in bacteria.

It was shown with bacteria that chloramphenicol resistance is often accompanied by other physiological changes (Coffey, Schwab, and Ehrlich, 1950; Weiner and Swanson, 1960), but in our limited experience this did not occur in C'. Because of the similarity between chloramphenicol and thiocymetin in chemical structure, the two-step increase in resistance to the latter compound was not unexpected, although, to our knowledge, this is the first instance in which cross-resistance between these two drugs has been demonstrated in the laboratory. Finland, Hirsh, and Wallmark (1960), in their study of pathogenic staphylococci isolated at Boston City Hospital, noted that the strains that were resistant to chloramphenicol were usually resistant to thiocymetin also.

In bacteria, chloramphenicol resistance is often lost in media free of the antibiotic because of the selective advantage of the susceptible back-mutants (Herrmann and Steers, 1953). De Carneri (1957) obtained a chloramphenicol-resistant mutant from a B+E-requiring strain of *E. coli* and showed that full resistance was maintained in drugless passages. C' resembled the auxotrophic *E. coli* strain and apparently was not at a selective disadvantage to any susceptible back-mutants which may have arisen in the drugless passages in the chick embryo yolk sac.

The failure to demonstrate genetic interaction among rickettsiae is difficult to interpret. The experimental designs followed in this investigation are just a few of the many that could have been devised. The fact that some of our experiments were modeled after one (Gordon et al., 1960) which has yielded excellent evidence of genetic interaction in psittacosis virus is of limited significance, because of the wide difference in the mechanisms of growth of rickettsiae (Schaechter, Bozeman, and Smadel, 1957) and viruses of the psittacosis group (Litwin, 1959).

A complicating factor is that in entodermal cell cultures (Weiss and Dressler, 1958) and most likely in the intact yolk sac the host cells are not uniformly infected by the rickettsiae, even when the multiplicity is high. Furthermore, even if a cell is infected with two agents, contact between these two agents is not insured. It has been shown, at least with viruses of the psittacosis group (Weiss and Huang, 1954), that separate loci of infection can occur within one cell. Thus, it can be concluded that some of the more obvious experiments have not yielded any evidence of genetic interaction, but the possibilities of demonstrating such interaction have by no means been exhausted. These experiments, however, strengthen the indications that genetic interactions among rickettsiae are not frequent occurrences. This is suggested by the report of an "interference phenomenon" which, presumably, is responsible for the maintenance of strain characteristics of *R. rickettsii* in nature (Price, 1953; Price et al., 1954), and from the survey of epidemiological data, which indicates that genetic variation does not play an important role in rickettsial ecology (Weiss, 1960).
ACKNOWLEDGMENT

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LITERATURE CITED


