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ACQUIRED RESISTANCE TO PENICILLIN AND TO NEOARSPHENAMINE IN *SPIROCHAETA RECURRENTIS*

BY

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WITH A STATISTICAL ADDENDUM BY

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The problem of acquired resistance to organometallic drugs in experimental spirochaetal infections has been investigated frequently (Gonder, 1912 ; Akatsu and Noguchi, 1917 ; Klauder, 1924 ; Feldt, 1932 ; Fischl and Singer, 1934 ; Probey, 1948) since the introduction of arsphenamine as a spirochaeticide by Ehrlich and Hata (1910). Much of this work is difficult to interpret with certainty, but it appears that resistance to arsenical drugs can be produced in some experimental spirochaete infections, though much more slowly than in experimental trypanosomiasis.

The demonstration of the therapeutic activity of penicillin in experimental spirochaetal infections of mice by Lourie and Collier (1943), and the introduction of penicillin for the treatment of syphilis in man by Mahoney, Arnold, and Harris (1943), raised the question of acquired resistance to penicillin also, and the treatment of two offshoots of a strain of *S. recurrentis* with penicillin and with neoarsphenamine was begun in 1944 at the Liverpool School of Tropical Medicine by Dr. E. M. Lourie and Dr. H. O. J. Collier.

Existing evidence for penicillin resistance in experimental spirochaete infections is equivocal. Dunham, Hamre, McKee, and Rake (1944) have reported that the Nichols strain of *Treponema pallidum* in an " insufficiently treated " rabbit gave rise to a variant which was more resistant to penicillin *in vitro* than the parent strain. Tung and Frazier (1946), however, failed to induce resistance to penicillin in cultures of the Reiter strain of *T. pallidum* after fifteen passages in penicillin-containing media, and Kolmer and Rule (1946) have shown that the Nichols-Hough strain of *T. pallidum* in rabbits acquired no increased tolerance to penicillin after three serial treated passages.

Development of Resistant Strains

Parent strain.—Spirochaeta recurrentis (*S. duttoni*) was maintained continuously in mice by blood passage since it was obtained in 1939 from Dr. C. M. Wenyon, who received it as *S. duttoni* in 1935 from Dr. Stefanopoulo of the Institut Pasteur, Paris. Its previous history is not known.

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Three mice were infected intraperitoneally from the tail blood of a mouse infected with the parent strain ; 48 hours later, when microscopic examination of a drop of tail blood showed parasites present, the three mice were treated subcutaneously with graded doses of the drug. On the following day, the strain was passaged from the mouse that had received the highest dose which failed to clear the blood of parasites into three further uninfected mice, in which the procedure was repeated. No control of the size of the infective inoculum was made. Drug dosage was increased when decreased therapeutic activity appeared, as shown by consistently high parasite counts (i.e. parasites/microscope field, 1/6th obj. 10× oc.) on the day following treatment. Drug solutions were prepared in distilled water and the injections made in 0.5 ml./20 g. body weight.

Penicillin-resistant strain.—The penicillin used was sodium penicillin (TRC batches) in the form of tablets containing 10–20 per cent " pure " penicillin (8–9,000 units/tablet) ; the samples used were manufactured in 1944–5 and were stored in a refrigerator immediately on receipt. The strain was passaged 324 times, including 30 untreated passages, over a period of 3 years 2 months, from December 11, 1944, to March 6, 1948. The distribution of penicillin dosage in six-monthly periods is indicated in Table I.

TABLE I

History of *S. recurrentis* strain in mice treated with penicillin

Dates		11.12.44 to 10.6.45	10.6.45 to 10.12.45	10.12.45 to 9.6.46	9.6.46 to 9.12.46	9.12.46 to 11.6.47	11.6.47 to 8.12.47	8.12.47 to 6.3.48
Interval (months)		0–6	6–12	12–18	18–24	24–30	30–36	36–39
No. of passages		40	51	50	51	54	54	24
No. of treated passages ..		37	50	47	49	48	41	22
No. of passages treated with penicillin at dose (units/20 g. mouse, s.c.) ..	250	2	0	0	0	0	0	0
	350	1	0	0	0	0	0	0
	500	26	5	7	5	0	0	0
	1,000	5	31	15	12	2	0	0
	1,250	3	0	0	0	0	0	0
	2,000	0	14	25	22	3	1	1
	3,000	0	0	0	7	5	1	1
	4,000	0	0	0	3	8	10	9
	5,000	0	0	0	0	0	1	1
	6,000	0	0	0	0	8	11	10
	8,000	0	0	0	0	9	16	0
	10,000	0	0	0	0	9	1	0
	12,000	0	0	0	0	4	0	0

Neoarsphenamine-resistant strain.—The strain was developed as above ; a commercial preparation of neoarsphenamine (ampoules, for clinical use) supplied by Messrs. May and Baker, Dagenham, was used. The strain was passaged 312 times, including 17 untreated passages, over a period of 2 years 11 months, from April 6, 1945, to March 6, 1948. The distribution of neoarsphenamine dosage in six-monthly periods is indicated in Table II.

Table I shows that it was possible to increase the dosage of penicillin within 6–12 months of the start of continuous treatment, for, in 31 of 37 treated passages in the first six months, the strain withstood 500–1,000 units/20 g., compared with 1,000–2,000 units in 45 of 50 treated passages in the second six months. In the

TABLE II

History of *S. recurrentis* strain in mice treated with neoarsphenamine

Dates		6.4.45 to 4.10.45	4.10.45 to 4.4.46	4.4.46 to 3.10.46	3.10.46 to 3.4.47	3.4.47 to 2.10.47	2.10.47 to 6.3.48
Interval (months)		0–6	6–12	12–18	18–24	24–30	30–35
No. of passages		51	52	50	54	54	51
No. of treated passages		51	51	50	52	48	45
No. of passages treated with neoarsphenamine at dose (mg./20 g. mouse, s.c.) ..	0.5	24	10	1	0	0	0
	1.0	24	17	13	5	0	0
	2.0	3	24	30	15	7	1
	3.0	0	0	6	14	2	0
	4.0	0	0	0	18	17	15
	5.0	0	0	0	0	15	27
	6.0	0	0	0	0	0	2

ensuing twelve months this level appeared to be maintained, for in 74 out of 96 treated passages the strain continued to withstand doses of 1,000–2,000 units. Thereafter, in the period between 24 and 39 months after beginning treatment, a more variable response appeared at a higher level (4,000–6,000 units).

These findings are open to the two following interpretations:

(i) The penicillin may have deteriorated during the 2–3 years of the experiment; this possibility is borne out by the experiments, described in a later section, with fresh crystalline sodium penicillin G, in which the treated strain, in tests done over a 5-week period immediately after the end of treatment, proved to be only twice as resistant as the parent strain instead of 10 times as resistant, which Table I would suggest.

(ii) Penicillin-resistance may, in fact, have been produced and may already have developed within 6–12 months after starting the continuous treatment. That this early development of acquired resistance is likely is supported by Lourie and Collier, who tested the parent and treated strains after exactly 6 months of continuous treatment of the latter. Mice were infected intraperitoneally, and two days later, when microscopic examination of coverslip preparations of tail blood showed 1–20 parasites/field (1/6th obj.), penicillin was administered subcutaneously in doses of 500 and 1,000 units/20 g. mouse, the same penicillin solution being used simultaneously for both the parent and treated strains. On the day after treatment, microscopic examination of coverslip preparations of tail blood was again carried out to determine the number of animals in which parasites could not be detected after examination of 50–100 microscope fields. By this criterion, after a dose of 500 units, 9/10 animals infected with the parent strain, and 0/10 animals infected with the treated strain, showed parasite-free blood. After a dose of 1,000 units, 10/10 animals infected with the parent strain, and 4/10 animals infected with the treated strain, showed parasite-free blood.

From Table II, resistance to neoarsphenamine as estimated tentatively by inspection appears to develop gradually until the levels of the M.T.D. are reached (4.0–6.0 mg./20 g.) in 24–30 months. Since no question of drug deterioration arises in this

series, this conclusion is probably valid and is in fact borne out by the experiments described below.

Determination of Optimal Assay Conditions

An attempt has been made, in the experiments below, to define the conditions for a more accurate statistical evaluation of therapeutic efficacy than is possible from the data above or than has hitherto been achieved with this strain.

Richardson, Walker, Loeb, and Miller (1945), using a strain of *B. novyi*, were able to produce a standard infection level in mice of about 11,000 parasites per 10,000 red blood cells 48 hours after intraperitoneal inoculation of 10^{10} parasites per kg., and this degree of infection was used in their therapeutic tests. With the strain used here and with convenient amounts of blood for the inoculation of sufficient numbers of mice, it was not possible consistently to reproduce peak levels of more than about 3,000–5,000 parasites per 10,000 red blood cells. Infections of this degree were produced in the experiments to be described below by diluting heavily infected blood 1 in 5 or more in citrated saline, and inoculating 0.2 ml. containing $1–2 \times 10^8$ parasites per 20 g. mouse, i.e., $0.5–1 \times 10^{10}$ parasites per kg. The parasite numbers in the inocula were estimated by relating differential spirochaete/red cell counts in dry films to red cell counts made with a standard counting chamber.

The course of infection in 65 control mice infected at different times in batches of five for the test experiments below, and with inocula prepared as above, is shown in Table III. The variation may reasonably be ascribed to variable host susceptibility.

Apart from the inconvenience of using larger amounts of blood for the inoculations, with the object of obtaining heavier infections, the use of inocula more heavily charged with spirochaetes was not, in fact, found to result in higher peak infections than were produced by the standard inocula described immediately above. The heavier inocula tested were diluted mouse blood containing up to 1.6×10^{11} parasites/kg., and diluted rat blood containing up to 1.0×10^{11} parasites/kg. The peak infections in rats were not increased by splenectomy one to two hours before inoculation.

Experimental Assessment of Drug Resistance

Usually batches of 25 mice were used ; one batch was infected with the normal parent strain of *S. recurrentis* and another with the drug-fast strain, using the standard inocula described above. Exactly one hour after inoculation, groups of five mice were treated subcutaneously (0.5 ml./20 g.) with appropriate dilutions of the drug in 0.85 per cent saline containing 1 per cent sodium citrate. Five mice in each batch served as controls. Blood films were made at intervals of 24, 48, and 72 hours after treatment, and infection levels estimated as described earlier. The experiments were repeated later at least once, and the separate results were recombined to obviate differences due to irregular response. The drug samples used were (i) crystalline sodium penicillin (Glaxo), of potency 1,640 units/mg., containing 90 per cent penicillin G (II) (benzyl penicillin), and (ii) neoarsphenamine (ampoules for clinical use, as supplied by Messrs. May and Baker, Ltd.). A preliminary experiment on the effect on the course of infection of the length of the interval between infection and treatment showed that with doses of penicillin of 1,000 units/20 g. the peak infection levels were not significantly different after treatment intervals of one, three,

TABLE III

The early course of infection in mice infected intraperitoneally with a standardized inoculum ($0.5–1.0 \times 10^{10}$ parasites/kg.)

Inoculum: Parasites $\times 10^{10}$/kg.	Mean infection level (parasites/10,000 RBC) for batch of five mice	
	Hours after inoculation	
	24	48
0.5	1,400	6,528
0.5	106	2,088
0.5	253	2,059
0.5	930	3,860
0.7	324	2,751
0.8	502	2,142
0.8	2,312	1,369
0.8	982	1,661
0.9	325	4,104
1.0	1,712	6,376
1.0	365	2,604
1.0	638	1,443
1.0	920	6,936

and six hours after infection. Early treatment, which may be considered as suppressive rather than therapeutic, was used throughout to avoid error in the assessment of response caused by spontaneous remission of the infection.

For each drug, individual log parasite counts at 48 hours were plotted against dosage for the normal and resistant strains, and the responses of each strain were compared by a variance analysis method applied to the regression lines thus obtained, in order to assess the significance of the difference in response of (1) the penicillin-resistant and (2) the neoarsphenamine-resistant strains. The response of strain (1) was significantly different from the normal parent strain (variance ratio $F=3.83$, $P<0.01$); the response of strain (2) showed a more markedly significant difference from the parent strain (variance ratio $F=10.53$, $P<0.001$).

TABLE IV

Suppressive effect of penicillin on the parent and penicillin-resistant strains of *S. recurrentis* in mice

Penicillin (units/20 g. mouse, s.c.)	Mean $\log_{10}$ (No. of parasites/ 10,000 RBC)		Mean parasite count as proportion of control mean	
	Parent strain	Resistant strain	Parent strain	Resistant strain
0	3.61 (8)	3.40 (20)	1.00	1.00
250	3.23 (10)	3.02 (10)	0.42	0.42
500	3.21 (10)	3.23 (15)	0.40	0.68
1,000	2.53 (10)	2.99 (14)	0.08	0.39
2,000	1.15 (5)	2.58 (13)	0.003	0.15
4,000	—	1.50 (15)	—	0.013

Figures in parentheses indicate number of mice.

TABLE V

Suppressive effect of neoarsphenamine on the parent and neoarsphenamine-resistant strains of *S. recurrentis* in mice

Neoarsphenamine (mg./20 g. mouse, s.c.)	Mean $\log_{10}$ (No. of parasites/10,000 RBC)		Mean parasite count as proportion of control mean	
	Parent strain	Resistant strain	Parent strain	Resistant strain
0	3.28 (15)	3.22 (20)	1.00	1.00
0.25	3.32 (10)	2.97 (5)	1.10	0.56
0.50	2.09 (15)	3.53 (5)	0.06	2.04
1.00	2.42 (13)	3.12 (14)	0.10	0.80
2.00	1.33 (5)	3.36 (10)	0.01	1.38
4.00	—	2.45 (9)	—	0.17

Figures in parentheses indicate number of mice.

In Tables IV and V, the infection levels at individual doses are tabulated and expressed as proportions of the infection level of the controls. From Table IV it appears that whereas a dose of *ca.* 1,000 units of penicillin will reduce the parasite density by 90 per cent in the normal strain, a dose of *ca.* 2,000 units is necessary to produce this effect in the resistant strain, i.e., *an approximately twofold increase in resistance to penicillin.*

From Table V it appears that a dose of 0.5 mg. neoarsphenamine will produce a 90 per cent decrease in parasite density with the normal strain, but, to produce the same effect in the resistant strain, a dose of at least 4 mg. is necessary, i.e., *an approximately eightfold increase in resistance to neoarsphenamine.*

Although some resistance to penicillin was developed by this strain of spirochaetes, it may perhaps be regarded as propitious, for the future clinical use of penicillin against spirochaetal diseases in general, that the increased resistance was no more than twofold, in spite of treatment continued for over three years. Syphilis has now been treated with arsenicals for about 40 years without any indications of an increased incidence of arsenic-resistant infections, and this work gives grounds for hoping that the widespread use of penicillin will equally not result in an increasing incidence of infections resistant to penicillin.

SUMMARY

1. Two strains of *Spirochaeta recurrentis* (*S. duttoni*) have been passaged over a long period in a series of mice subjected to treatment with penicillin and with neoarsphenamine, respectively, in an attempt to demonstrate acquired drug resistance.

2. After a period of three years, two and three-quarter months, involving 294 treated passages, acquired resistance to penicillin was observed, but the resistance was no more than twice that of the parent strain. After a period of two years eleven months, involving 295 treated passages, an approximately eightfold increase in neoarsphenamine resistance was observed.

3. The assay procedure was based on an estimation of infection level by a differential parasite/erythrocyte count on heavily infected mice inoculated, treated, and examined under controlled conditions.

4. The results have been subjected to statistical analysis for assessment of significance.

We wish to express our appreciation of the encouraging and continued interest taken in this work by Dr. E. M. Lourie, former Director of the Department of Chemotherapy, Liverpool School of Tropical Medicine. The work was carried out with a grant from the Medical Research Council to one of us (I. M. R.) and during the tenure of a May and Baker Research Fellowship held by the other (J. W.).

ADDENDUM

Statistical analysis of data

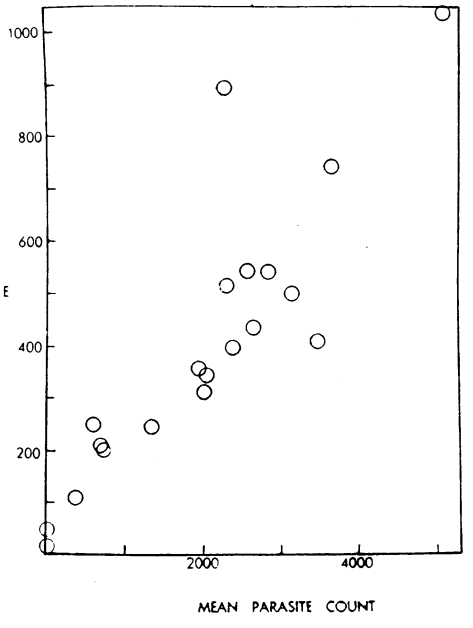

FIG. 1.—Relation between the means of untransformed parasite counts (x) and their corresponding standard errors (S.E.), for 20 experiments with normal, penicillin- and neoarsphenamine-resistant strains of *S. recurrentis* in mice.

With data representing counts of parasites or bacteria it is usually found that the variability of the count increases with the count. This is clearly shown in Fig. 1, where the mean of a sample of counts obtained under the same experimental conditions is plotted against the standard deviation. Given a sample of n counts, x_1, x_2, . . ., x

calculate $$m = \frac{Sx}{n} \text{ and } \quad X = Sx^2 - \frac{(Sx)^2}{n}$$

the summation being taken over all counts. Sx^2 and Sx can be found in one operation on a calculating machine. The mean is then m and the standard deviation s, where

$$s^2 = \frac{X}{(n-1)}$$

In order to compare results obtained under different experimental conditions it is necessary that the variable concerned should have about the same standard deviation whatever the magnitude of the mean values; this is a prerequisite of analysis of variance. Evidently the count itself does not satisfy such a requirement, so it becomes essential to transform it into a new variable which does. The nature of the relation between mean and standard deviation indicates some sort of logarithmic transformation, but log (count) is not satisfactory, showing a decrease in standard deviation with increasing mean (see Fig. 2). Kleczowski (1949) has given reasons for using

$$y = \log\ (\text{count} + c)$$

where the best value of the constant c is obtained as follows.

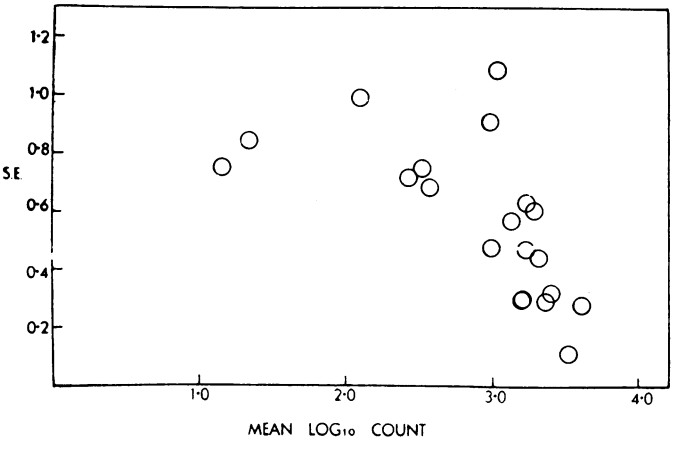

FIG. 2.—Relation between the means of the logarithms of untransformed parasite counts (x) and their corresponding standard errors, for 20 experiments with normal, penicillin- and neoarsphenamine-resistant strains of *S. recurrentis* in mice.

Each set of counts for which the experimental conditions are the same gives a pair of numbers m, s: the mean and standard deviation of the sample; suppose there are k such samples. Calculate

$$\bar{m} = \frac{Sm}{k}\ ;\ \bar{s} = \frac{Ss}{k}$$

$$M = Sm^2 - \frac{(Sm)^2}{k}\ ;\ T = Sms - \frac{SmSs}{k}$$

$$\text{then } c = \frac{M\bar{s}}{T} - \bar{m}$$

Here $c = 358$, so the analysis is continued with the new variable

$$y = \log_{10}\ (\text{count} + 358)$$

To check the usefulness of this transformation the sample means of y are plotted against standard deviations in Fig. 3, and the standard deviation is now seen to remain more or less constant as the mean increases.

The results of the ensuing analysis of variance are given in Table VI, with appropriate values obtained from Table V in Fisher and Yates (1948).

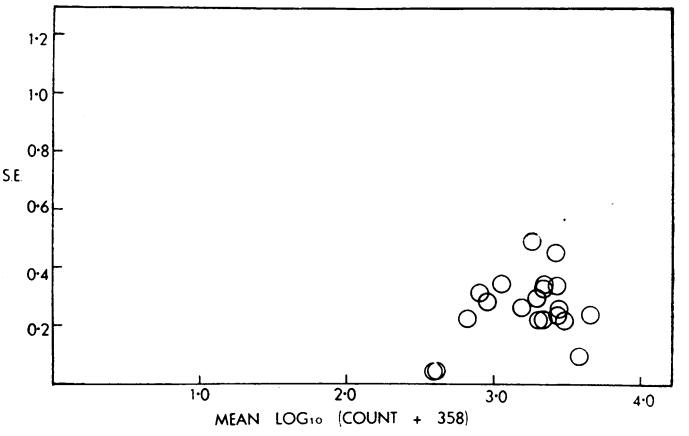

FIG. 3.—Relation between the means of the logarithms of transformed parasite counts (x + 358) and their corresponding standard errors, for 20 experiments with normal, penicillin- and neoarsphenamine-resistant strains of *S. recurrentis* in mice.

TABLE VI

RESULTS OF ANALYSIS OF VARIANCE

Source of variation	Sum of squares	d.f.	Mean square	F	P
Between strains (penicillin)	1.38	5	0.276	3.83	0.001–0.01
Within strains (error) ..	7.57	105	0.0721		
Between strains (neoarsphenamine)	5.53	5	1.106	10.53	<0.001
Within strains (error) ..	10.75	102	0.105		

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