THE INHERITANCE OF RESISTANCE, DEMONSTRATED BY THE DEVELOPMENT OF A STRAIN OF MICE RESISTANT TO EXPERIMENTAL INOCULATION WITH A BACTERIAL ENDOTOXIN

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Introduction

In a previous study of the problem of the inheritance of resistance to bacterial infection in animal species (Bradford Hill, 1934) it was suggested that in endeavouring to breed a resistant strain, and in measuring the average degree of immunity attained by any generation, it might be more profitable to work with a bacterial toxin rather than with a living bacterium. Any experiments in this field require that the animals employed should not be naturally infected with the bacterial parasite against which resistance is to be developed. If they are, the well-known immunizing effects of non-fatal infections will come into play in an unknown and uncontrollable fashion, and will make it difficult or impossible to distinguish clearly between an increased average resistance due to this factor and a similar increase resulting from selective breeding. This difficulty will become greater if, after starting with an uninfected stock, we infect some animals with living bacteria and breed the next generation from the survivors, breeding from the uninfected stock to obtain our unselected controls.

Many of the survivors to infection will remain infected, and infective, for a considerable time. Many of them, too, will have developed protective antibodies, and these antibodies the females will transmit to their young, transplacentally or in their milk, giving rise to a transient congenital passive immunity. Since a proportion of the young are likely to become infected from their infected parents, this temporary passive immunity may be followed by a persistent active immunity. The controls, on the other hand, bred in each generation from non-infected parents will not be subjected either to passive or to active immunization. Apart altogether from genetic factors the offspring of parents selected by survival to experimental infection with living bacteria are, therefore, likely to show a higher average resistance than the offspring of unselected and uninfected controls.

It is true, of course, that the intervention of passive or active immunization in this method of selective breeding can be tested in various ways. Such tests indicate very definitely that at least a large part of the increased resistance

which has been frequently demonstrated in this way is genetic in origin. But it is clearly an advantage to eliminate such disturbing factors altogether if this can be done. The use of a bacterial toxin as the selective agent, in place of living bacteria, allows us to eliminate the factor which is most difficult to control, namely the active immunization of the young by an infection acquired from their parents. We cannot be certain that we are eliminating entirely the effects of congenital passive immunity. The females that have survived an injection of toxin will have developed antibodies which they will transmit to their offspring. Since these offspring will not themselves be tested till they reach breeding age, it is probable that little of their passive immunity will remain; but the small rodents of the laboratory mature quickly, and it would be unsafe to assume that any observed increase in resistance owed nothing to residual antibodies. We can, however, easily eliminate the effect of this passive immunization by breeding, without further selection, from a resistant generation. The parents will, in this case, have received no injection of toxin, and the young can receive from their mothers only a proportion of the residuum of the passively received antibody that may have remained in their blood at the time of pregnancy. Moreover, the young themselves will have three months or so to eliminate this fraction of a fraction before their resistance is tested. If they prove as resistant as their grandparents it can safely be assumed that their resistance depends on genetic factors, not on any form of specific immunization.

Many workers have studied the inheritance by mice of resistance to infection with *Bact. typhi-murium*, and this a problem of particular interest to students of experimental epidemiology, since mouse typhoid is an infection which spreads readily by contact. Some six years ago Boivin & Mesrobeanu (1933) and Raistrick & Topley (1934) independently described the isolation from *Bact. typhi-murium* (*Bact. aertrycke*) of an antigenic fraction consisting of a complex polysaccharide linked to a component containing nitrogen, phosphorus and fatty acids. It has since been shown that this substance, which is highly toxic for mice, corresponds to the somatic antigen, or antigens, of the bacterial cell. The active immunity to which its injection gives rise is, therefore, at the same time antitoxic and antibacterial. As is so often the case with the toxic cell constituents of bacteria, as compared with the filtrable exotoxins, the antitoxic immunity induced is relatively feeble; but the antibacterial immunity, as shown by an increased resistance to the injection of living bacteria, though by no means absolute, attains a high level. This toxic fraction appeared to be a particularly suitable reagent for studies on the inheritance of resistance, since it would be possible to determine (1) whether selective breeding through a succession of generations would greatly increase the average resistance to its toxic effect, and (2) if such an increase occurred, whether it would be associated with an equal increase in the resistance of the selected mice to infection with the living organism.

The experimental procedure

The experiment was begun on 30 April 1935 with a heterogeneous population of mice known to be free from infection with *Bact. typhi-murium*, and all subsequent generations, both treated and control groups are the descendants of this original stock.

Diet. For some weeks before mating the mice were fed on the following diet (Watson, 1937):

Coarse oatmeal	40 parts
Dried separated milk ...	25 ,,
Dextrine	23 ,,
Coco-nut oil	4 ,,
Cod liver oil	1 part
Yeastrel (dry weight) ...	2 parts
Wheat bran	5 ,,

This diet was fed to all later generations. In addition the original stock and the first eight generations of their descendants were given to drink water and pasteurized milk in equal proportions. In the last two generations bred, F_9 and F_{10}, the pasteurized milk was excluded and they had water alone to drink. Some experiments had indicated that this change had an immaterial effect upon fertility, rate of growth and mortality.

Breeding. The offspring of the original random matings were divided into two groups. The larger group was to be inoculated with the partially purified toxin and from the survivors to that procedure—presumably the more resistant components of the stock—the next generation was to be bred. The small group was to serve as the producer of control mice of the same generation as the toxin-treated animals. Thus in each generation a proportion of these control mice were inoculated, their reaction serving as the standard against which to weigh the experience of the mice with toxin-resistant ancestors; another proportion was retained untested for the production of the next generation of control stock.

The number of females mated with one male varied from generation to generation, according to the number of males available (from 1 buck to 1 doe to 1 buck to 6 does). Toxin-inoculated mice and control mice of the same generation were, however, always mated in the same proportion of males to females. To avoid infertile matings does were given the opportunity to mate with more than one buck. Brother-sister matings were not made.

Pregnant does were isolated in breeding cages and remained there until the young had been weaned—usually at about 28 days of age. After weaning the young mice were housed in cages containing 8–12 animals of the same sex. At this point of time the death rate (i.e. before inoculation) was small in all generations. Before weaning the customary losses took place, mice dying or being eaten shortly after birth both in the control groups and in those with

inoculated ancestors. All such casualties, excluding, of course, the eaten or too decomposed, were submitted to bacteriological examination, but with the exception of *Bact. morgani* which was found on rare occasions no pathogenic organism was ever isolated. It must be noted that for some unexplained reason, which never recurred subsequently, over half the young mice of the second generation died suddenly at an early age.

Inoculation. At approximately 12 weeks of age the resistance of the mice of each generation was tested by the intraperitoneal inoculation of the partially purified toxic fraction isolated from *Bact. typhi-murium.* The dose administered varied, for reasons discussed later, from 1 to 4 mg. suspended in 0·5 c.c. of sterile normal saline.

Of the first generation, bred from the original untested stock, a large and randomly selected group was inoculated (600 mice in all). In the second, third, fourth, fifth, sixth and eighth generations all offspring from previously inoculated parents were submitted to test, i.e. all those that had survived to the age of inoculation. The only exclusions were mice that were obviously dwarfed (under 10 g. in weight), deformed or injured, the number of which were negligible (rarely more than one or two and never more than five in a generation). In the seventh, ninth and tenth generations from inoculated stock a proportion of the mice only was inoculated, the remainder being retained for special investigations.

Of the control groups of the corresponding generations a random selection of mice for inoculation was made from their card records. Usually a hundred was thus chosen, the remainder being required for the production of the next generation of control animals.

RESULTS

Survival rates in different generations

The basic data and the results of the experiment are set out in Table 1. From initial small scale trials it had been concluded that a dose of 1 mg. of toxin would lead to a sufficiently high death rate for the effective selection of the more resistant animals. Applied on a large scale this belief was not borne out. Of 600 mice bred from the originally imported stock two-thirds survived the inoculation with this amount (F_1, 66·8% survived). These survivors were mated, but owing to the abnormal rate of infant mortality in their offspring, previously referred to, only 429 mice in the F_2 generation came to inoculation. In testing their resistance the dose was increased to 2·5 mg. The result was, as expected, a substantially higher death rate, only 41% of the mice surviving. Of the control group of the same generation 43% survived. Clearly the elimination of one-third of the parental population had been insufficient to raise the average resistance of the offspring, as tested by this higher dosage, above that possessed by the young of unselected parents (though the odd conditions of life observed in infancy may somehow have obscured the issue, e.g. by eliminating the weaker animals in both groups).

Table 1

No. of generation	Date on which parents of generation were mated	Date on which generation was tested	Dosage of toxin given mg.	Number of mice inoculated: Offspring of inoculated parents (1)	Number of mice inoculated: Offspring of uninoculated parents (2)	Percentage of mice surviving inoculation: Offspring of inoculated parents (3)	Percentage of mice surviving inoculation: Offspring of uninoculated parents (4)	Difference between survival rates, (3)–(4), and standard error of difference
F_1	30. iv. 35	4. x. 35	1	♂ — ♀ —	310 + 290 = 600	— —	62·9 + 71·0 = 66·8	— —
F_2	10. x. 35	26. ii. 36	2·5	♂ 213 + ♀ 216 = 429	50 + 50 = 100	30·5 + 50·5 = 40·6	42·0 + 44·0 = 43·0	−11·5±7·7 + 6·5±7·8 = − 2·4±5·5
F_3	2. iii. 36	23. vii. 36	3	♂ 256 + ♀ 213 = 469	50 + 50 = 100	46·9 + 54·5 = 50·3	16·0 + 40·0 = 28·0	+30·9±6·1 + +14·5±7·7 = +22·3±5·0
F_4	28. vii. 36	15. xii. 36	4	♂ 271 + ♀ 298 = 569	50 + 50 = 100	22·1 + 35·2 = 29·0	4·0 + 12·0 = 8·0	+18·1±3·7 + +23·2±5·4 = +21·0±3·3
F_5	22. xii. 36	1. vi. 37	4	♂ 194 + ♀ 171 = 365	50 + 50 = 100	45·4 + 50·3 = 47·7	20·0 + 14·0 = 17·0	+25·4±6·7 + +36·3±6·2 = +30·7±4·6
F_6	7. vi. 37	2. xi. 37	4	♂ 167 + ♀ 174 = 341	50 + 50 = 100	35·3 + 37·9 = 36·7	8·0 + 0·0 = 4·0	+27·3±5·3 + +37·9±7·3 = +32·7±3·3
F_7*	9. xi. 37	29. iii. 38	4	♂ 70 ♀ —	40 —	71·4 —	20·0 —	+51·4±8·3 — = +51·4±8·3
F_8*	4. iv. 38	26. vii. 38	4	♂ 306 + ♀ 309 = 615	50 + 50 = 100	53·6 + 65·0 = 59·3	22·0 + 12·0 = 17·0	+31·6±6·5 + +53·0±5·3 = +42·3±4·2
F_9	3. viii. 38	6. xii. 38	4	♂ 150 + ♀ 181 = 331	50 + 50 = 100	52·7 + 58·0 = 55·6	10·0 + 8·0 = 9·0	+42·7±5·9 + +50·0±5·3 = +46·6±4·0
F_{10}	13. xii. 38	9. v. 39	4	♂ 124 + ♀ 110 = 234	50 + 50 = 100	76·6 + 76·4 = 76·5	18·0 + 20·0 = 19·0	+58·6±6·6 + +56·4±7·0 = +57·5±4·8

* Only a small proportion of the F_7 generation was inoculated and F_8 was bred from the remaining uninoculated mice.

On the other hand this rate of mortality of nearly 60% in the F_2 generation should, it seemed, be fairly effective in picking out the resistant stock, and this was confirmed by the results given by the F_3 generation, to which a still higher dose, 3 mg., was given. In spite of this increase in dosage 50% of the experimental group survived, or $9{\cdot}7 \pm 3{\cdot}3$% more than in the previous generation when the slightly lower dosage was used. Still more striking was the difference between the young of the parents surviving previous inoculation and those from unselected stock of the same generation. The survival rate of the former was 50·3% and of the latter only 28%, the difference being $4\frac{1}{2}$ times its standard error.

To keep the number of animals within manageable bounds the dose was raised to 4 mg. in the F_4 generation and kept at this level subsequently. The immediate effect was, naturally, a high rate of mortality. Of the 100 control mice only 8 survived but the young of the toxin-selected parents gave a survival rate of 29%. There was no doubt that by these means a more resistant stock had been developed.

In ensuing generations the survival rates in the control groups fluctuated between 4 and 19%, and as, generally, these fluctuations uniformly affected both sexes they must be attributed to unknown influences rather than to chance. These influences seem also to have affected the mice bred from the toxin-resistant animals as their survival rates for each sex show corresponding movements. Not only, however, does each selected generation show a substantial advantage in power of survival over its control group but there is a clear indication of an increasing degree of resistance. In the first generation inoculated with the maximum dose of 4 mg. (F_4) 21% more of the offspring of selected parents survived than did the offspring of unselected parents. In generations F_5 and F_6 the excess was 31–33%, in F_8 and F_9 it was 42–46% (omitting the small experience of F_7) and in the final generation it was nearly 58%.

Combining two generations to give a more substantial basis gives the following figures:

	Percentage survival rates		
Generations	Experimental group	Control group	Difference
F_4 and F_5	36·3	12·5	23·8
F_6 and F_8	51·3	10·5	40·8
F_9 and F_{10}	64·2	14·0	50·2

The control group shows negligible changes while the survival rate of the selected animals increases from 36·3 to 64·2%, or by 77% of the former rate.

It was obviously desirable to see to what limits this improvement could be raised (the figures above suggest a slackening rate in such improvement) but the outbreak of war unfortunately brought the experiment to an end.

Sex differences

Before turning to other points it may be noted that the figures of Table 1 show a higher rate of survival in females than in males in the experimental groups though not consistently in the control groups. It has been previously observed that female mice appear to be more resistant than males, on the average, to *Bact. typhi-murium* and perhaps to other bacteria (Watson, Wilson & Topley, 1938). In the present experiment, taking all generations F_2 to F_{10} combined (excluding F_7 in which only males were tested), the survival rates were 43·4 for males and 52·2 for females, the difference of 8·8 being significant (S.E. 1·72). On the other hand, in the corresponding control series, the difference between the rates, 17·5 for males and 18·8 for females, is negligible and technically insignificant.

The control group was, it will be observed, equally balanced by sex in each generation while in the experimental groups there were sometimes more males than females, sometimes less. The differences were, however, so small that no fallacy is involved in utilizing for comparison between the control and experimental generation the total rates for both sexes combined. (Comparing these rates with the unweighted averages of the male and female rates gives a maximum difference of only 0·4 in the resulting survival rates.)

Effects of "skipping a generation"

In the F_7 generation only a few spare bucks were tested and showed a substantial advantage to the offspring of toxin-resistant animals. The remainder of this generation were mated without test with the object of assessing the possibility of the transference of a passive immunity from the inoculated parents to their offspring, even though the latter were not inoculated in their turn until the age of some 12 weeks. F_8 were, therefore, the offspring of uninoculated parents, but the descendants of inoculated generations. In spite of this breaking of the chain of inoculation the F_8 mice show an average power of resistance considerably above that of the corresponding control mice, the comparative survival rates being 59·3 and 17·0. The difference is far beyond the play of chance and it follows that the advantages shown by the experimental groups cannot be attributed to the passage of passive immunity from their inoculated parents. It also seems likely that the average degree of immunity reached by the F_7 generation was maintained unaltered in the F_8 generation. The survival rate fell, it is true, from 71·4% in F_7 to 59·3% in F_8 but it fell also in the control groups from 20% to 17%. In the latter the survival power of F_8 was 85% of that of F_7, while in the experimental group the corresponding percentage was 83. This suggests that the relative position was little changed although the absolute difference between the experimental and control groups was rather less in F_8 than in F_7 (42·3 to 51·4%). The number tested in F_7 was, however, very small and the difference of 51·4% is out of keeping with what went both before and after. On the whole it seems

likely that by the F_7 generation a population had been developed with a fairly stable degree of resistance and that it would have maintained its advantage by continued inbreeding without further recourse to inoculation. But for the war the effect of omitting inoculation for a series of generations would have been tested.

Time to death

The great majority of mice that succumbed to inoculation died within the first 24 hr. Table 2 shows the distribution in time of deaths for the experimental and control groups for generations F_4 to F_{10}, during which the dose given was stabilized at 4 mg. (Two generations have been combined as the numbers in the control groups are relatively small. F_8 has been kept separate as its parents were not inoculated.) It is clear that of the dying offspring of inoculated mice a slightly smaller proportion succumbed within the first 24 hr. The result is uniform at each stage of the development of the generations and the differences are significant ($-7{\cdot}6 \pm 3{\cdot}54$, $-10{\cdot}7 \pm 3{\cdot}86$, $-11{\cdot}1 \pm 4{\cdot}74$ and $-16{\cdot}2 \pm 4{\cdot}47$). In those doomed to die, therefore, there was a slightly greater degree of resistance in the offspring of inoculated parents, leading to some delay in the time at which they succumbed.

Table 2. *Time to death of mice succumbing to inoculation (in generations given 4 mg. of toxin)*

	Offspring of inoculated stock				Offspring of control stock			
		Percentage of deaths falling on				Percentage of deaths falling on		
Generation	Total deaths	1st day	2nd day	3rd or later days	Total deaths	1st day	2nd day	3rd or later days
F_4 and F_5	595	75·8	17·8	6·4	146	83·4	10·9	5·7
F_6 and F_7	236	79·2	16·1	4·7	115	89·9	7·8	2·3
F_8	250	75·6	23·2	1·2	72	86·7	13·3	0·0
F_9 and F_{10}	201	68·1	24·9	7·0	145	84·3	14·5	1·2

On the other hand there is no evidence that death was further delayed with the development of later generations. Generations F_4 to F_8 show only immaterial changes in the distribution of the days upon which death took place. F_9 and F_{10} do show some decline in the percentage dying rapidly and a corresponding rise in the percentages dying on the 2nd and later days, but, taking the extremes (F_4 and F_5 against F_9 and F_{10}) the changes are not more than might be due to chance ($\chi^2 = 5{\cdot}08$, $P > 0{\cdot}05$). It is reasonable to suppose that such changes might take place but the evidence is not sufficient to substantiate the belief.

Inoculation with the living organism

Having reached the stage, in F_8, at which rather more than half the experimental group was capable of surviving inoculation with 4 mg. of toxin, compared with less than one-fifth of the control group, it was decided to test the extent to which this enhanced resistance would extend to inoculation with the living organism, *Bact. typhi-murium*, from which the toxic fraction was

derived. For this purpose some of the F_9 and F_{10} mice were selected at random from the total populations of those generations available for inoculation with toxin, and were inoculated with the living organism, one group by the intraperitoneal route and one group *per os* (the respective doses being 100,000 and 2500×10^6).

The results, set out in Table 3, were very surprising. Not only were the toxin-resistant mice of toxin-resistant ancestry no more resistant to the living organism than the control mice, but they were on the average uniformly more susceptible to it. The differences between the survival rates after intraperitoneal inoculation are not technically significant but they are, in both generations, in keeping with the corresponding differences observed with inoculation *per os*, both of which are more than would be attributable to the play of chance. (Calculating χ^2 from fourfold tables, with Yates's correction, the figures are: intraperitoneal inoculation, F_9, $\chi^2 = 1{\cdot}63$, $P = 0{\cdot}20$; F_{10}, $\chi^2 = 2{\cdot}56$, $P = 0{\cdot}11$; *per os* inoculation, F_9, $\chi^2 = 5{\cdot}14$, $P = 0{\cdot}02$; F_{10}, $\chi^2 = 4{\cdot}23$, $P = 0{\cdot}04$.)

Table 3. *Resistance to inoculation with living* Bact. typhi-murium *as compared with resistance to toxin*

	Intraperitoneal inoculation						*Per os* inoculation					
	Offspring of toxin-inoculated ancestry			Control group			Offspring of toxin-inoculated ancestry			Control group		
Generation	No. inoculated	No. surviving	% surviving	No. inoculated	No. surviving	% surviving	No. inoculated	No. surviving	% surviving	No. inoculated	No. surviving	% surviving
F_9	50	3	6% (55·6)*	50	8	16% (9·0)*	50	25	50%	50	37	74%
F_{10}	80	1	1·3% (76·5)*	80	3	3·8% (19·0)*	80	32	40%	80	46	57·5%

* These figures are the percentage survival rates of the same generations to inoculation with toxin given for comparison with those derived by the two methods of inoculation with the living organism.

We can offer no explanation of so curious and unexpected a result but it clearly raises questions of great interest. It affords additional evidence, if such were needed, that the increased resistance to toxin of the later selected generations was in no way due to specific antibodies. Had it been, it can safely be assumed that the selected mice would have been more rather than less resistant to infection with living bacteria.

The only conclusion that can, at this stage, safely be drawn from the experimental data is that the heritable mechanism which rendered the selected mice resistant to the toxin did not suffice to render them resistant to the living cells from which the toxin was derived. It is impossible to say whether the observed decrease in resistance to experimental infection resulted from the same change in genetic characters that determined the increased resistance to toxin, or whether some different genetic factors, not necessarily related to the method of selection adopted, more than counterbalanced the effect which an increased resistance to the toxin might have been expected to exert on resistance to the living bacteria.

There is a suggestion, too, in the figures of Table 3, that the toxin-resistant mice may have differed from the control mice in their relative susceptibility to infection with the living organism according to whether that organism was introduced intraperitoneally or by the mouth. If this were borne out in experiments on a larger scale, we should have to assume a further complexity in the genetic factors on which resistance to infection depends.

SUMMARY

Ten generations of mice (with one exception) were inoculated with a partially purified toxic fraction isolated from *Bact. typhi-murium*, the next generation being bred each time from the survivors to the test. By this selective process a stock was produced with a substantially increased power of resistance to the toxin. For instance, in the fourth and fifth generations (combined), at which point of time the dose inoculated was stabilized at 4 mg., 36% of the mice with resistant ancestors survived inoculation compared with 12% of normal mice derived from the same original stock; in the ninth and tenth generations (combined) 64% of the resistant stock survived and only 14% of the controls. Also, those mice which failed to survive inoculation took slightly longer to die, on the average, in the resistant stock than in the normal stock.

The mice of each generation were not inoculated until about the age of 12 weeks, but to test the possibility of the transference of a passive immunity from mother to young the seventh generation was bred from without previous inoculation. The eighth generation was composed therefore of descendants of animals selected for resistance by inoculation but its own parents had not been tested. Its survival rate, 59%, was substantially greater than that of the normal stock, 17%, proving that the differences observed in previous generations were not the results of a transference of passive immunity.

Some mice of the ninth and tenth generations were inoculated with the living organism *Bact. typhi-murium* in place of the toxic fraction derived from it. Although these generations were relatively highly resistant to inoculation with the toxin itself they were less resistant than normal mice to the living organism.

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