The Journal of Medical Microbiology

Vol. 3 No. 1

INTRACEREBRAL INFECTION OF MICE WITH HIGH- VIRULENCE AND LOW-VIRULENCE STRAINS OF *BORDETELLA PERTUSSIS*

G. J. ADAMS*

Department of Applied Immunology, Wright-Fleming Institute, St Mary's Hospital Medical School, London

THE mouse was used as an experimental model for whooping-cough by Burnet and Timmins (1937), who used the intranasal route of challenge. Ten years later Kendrick *et al.* (1947) introduced the intracerebral route and this was adopted for the potency testing of *Bordetella pertussis* vaccines. Many investigations have been concerned with the high-virulence strains no. 18/323 (Berenbaum, Ungar and Stevens, 1960; Dolby and Standfast, 1961; and Iida *et al.*, 1962), 2-Atox (Andersen, 1957) and G353. However, only a few brief reports have appeared concerning strains of low intracerebral virulence, which constitute the great majority of *Bord. pertussis* strains isolated from whooping-cough patients (Andersen; Brown, 1958; Iida *et al.*). For a general account of the subject see Adams (1968).

MATERIALS AND METHODS

The strains of *Bord. pertussis* investigated were the high-virulence strains no. 18/323, G353 and 2-Atox, having an LD50 of 30–60 viable bacteria, and the low-virulence strains no. B2288, B1772 and B124, having an LD50 of $>10^6$ viable bacteria by the intracerebral route. All these strains were grown from freeze-dried cultures on Bordet-Gengou (BG) medium at 35°C for 72 hr, and the second 18-hr subculture from this was used for the preparation of the challenge suspensions, which were made up in 1 per cent. (w/v) Casamino Acids solution.

Mice lightly anaesthetised with ether were given an intracerebral injection of 0.025 ml of the bacterial suspension through a 0.25 in. (6 mm) 26-SWG needle.

Three strains of mice were used; most of the experiments were performed with white Theiler's Original female mice (Scientific Animal Supplies, Elstree, Herts.) weighing 14–18 g at challenge. Two strains of black mice were also used: B10.D2 "Old Line" and B10.D2 "New Line" mice, which are genetically identical except that the "Old Line" are deficient in a component of complement probably corresponding to human C'5 (Terry, Borsos and Rapp, 1964).

Received 16 Jan. 1969; revised version accepted 26 June 1969.

* Present address: c/o Dr L. B. Holt, National Institute for Medical Research (Hampstead Laboratories), Holly Hill, London, N.W.3.

For examination, each brain was removed aseptically and added to 2 ml of 1 per cent. Casamino Acids solution, homogenised in a MSE homogeniser for 1 min. at half speed, and then centrifuged at 200*g* for 90 s; decimal dilutions of the supernatant, which contained the majority of organisms, were made in Casamino Acids solution and bacterial counts were performed on BG medium.

Viable counts of *Bord. pertussis* were made on BG medium. The results were expressed as numbers of colony-forming units per mouse brain. When no colonies were present the count was taken to be 50 organisms per brain. The figures (see *Results*) show counts on individual mouse brains, and the geometric mean of counts, on a log scale. In figs. 1–6 the means were of all counts from each group of mice, but in figs. 7–10, where a distinction was being drawn between mice with infected brains and mice with approximately sterile brains, the means were of counts exceeding 50 organisms per brain.

Mice were bled from the brachial artery with a siliconised dropping pipette and the blood was transferred to tubes at 0°C containing a few polystyrene crystals, which enhanced the formation of fibrin clots when the tubes were shaken. The cells and serum were separated by centrifugation and the serum could then be pooled without undue loss of activity of haemolytic complement.

The bactericidal power of mouse serum was determined by incubating suspensions containing 10^6 organisms per ml with serum for 30 min. at 37°C. The bacterial counts were made on BG medium.

In the experiments on modulation, the bacteria were grown (*a*) on BG medium containing 6 per cent. of blood at 35°C or 25°C, (*b*) on blood agar (BA) medium containing 6 per cent. of blood at 35°C or (*c*) on modified Cohen and Wheeler medium (Cohen and Wheeler, 1946) containing 6 per cent. of horse blood and an amount of magnesium sulphate equivalent to the sodium chloride that it replaced.

RESULTS

Infections with low virulence strains

The bacterial counts and geometric means of counts observed after intracerebral challenge with 10^3 – 10^4 viable bacteria of the three low-virulence strains of *Bord. pertussis* are shown in fig. 1. In no case was the bacterial count in the brain 1 hr after challenge greater than about 10 per cent. of the number of organisms inoculated. After this time it increased to reach a maximum usually between 1 and 2 days after challenge; from the 2nd day onwards it declined rapidly until the majority of brains examined on the 4th day contained less than 50 organisms.

When graded doses of one of these strains (no. B2288) were injected intracerebrally, three distinct courses of infection became evident (fig. 2). With a challenge dose of 5.6×10^3 viable bacteria the course of infection was similar to those described above, but when the inoculum was increased to 5.6×10^5 viable bacteria the period of infection was significantly extended and all brains were still highly infected at the 5th day. With a challenge dose of 5.6×10^7 viable bacteria the course of infection for 48 hr was very similar to that obtained with the smaller doses, but by the 3rd day all the mice had died of infection.

Up to 24 hr after challenge with all doses of the low-virulence strains the bacterial counts from the individual brains were scattered over a very small range, nearly always less than 1 on a log₁₀ scale. However, from the 2nd day onwards in the surviving mice the counts were very scattered, and by the

4th day there was sometimes a 500-fold difference between the most and least heavily infected brain.

Infection with high virulence strains

Typical intracerebral infections in mice challenged with 5.7×10^3 organisms of no. 18/323 and 3.6×10^3 organisms of no. G353 are shown in figs. 3 and 4. Again, about 90 per cent. of the inoculum disappeared within the 1st hr

FIG. 1.

FIG. 2.

FIG. 1.—Bacterial counts after intracerebral injection of the low-virulence strains of *Bordetella pertussis* no. B2288, B1772 and B124. The challenge inocula were 6.4×10^3 organisms of strains no. B2288 and B1772 and 8.7×10^3 organisms of strain no. B124.

FIG. 2.—Bacterial counts after intracerebral injection of graded doses of the low-virulence strain *Bord. pertussis* no. B2288. The challenge inocula were 5.6×10^3 , 5.6×10^5 and 5.6×10^7 organisms.

[Figs. 1–6: Individual brain counts and geometric mean of all counts for each group of mice.]

after inoculation. By 24 hr the number of bacteria had reached or even exceeded the number inoculated, and during the next 2 days the count increased about 10-fold daily.

From the 3rd day onwards the rate of increase fell slightly with no. 18/323, but with no. G353 a stationary phase was noted when the count exceeded 10^6 viable bacteria. This stationary phase was recorded on all four occasions in replicate experiments with this strain, but was not seen in a similar number of experiments with no. 18/323. However, it was only transitory, and the count increased again after the 4th day. Mice challenged with approximately 5×10^3 viable bacteria of no. G353 died on average 1 day later than mice challenged with an equal number of no. 18/323. Infections with no. 2-Atox were very similar to those with no. 18/323.

Bacterial counts from individual brains were spread over a very small range, rarely greater than 1 on a $\log_{10}$ scale throughout the course of infections

with no. 18/323 and no. 2-Atox, but there was some spread in counts in the terminal stages of infection with no. G353.

FIG. 3.

FIG. 4.

FIG. 3.—Bacterial counts after intracerebral injection of 5.7×10^3 bacteria of the high-virulence strain no. 18/323.

FIG. 4.—Bacterial counts after intracerebral injection of 3.6×10^3 bacteria of the high-virulence strain no. G353.

FIG. 5.—Bacterial counts at short time-intervals after challenge with the high-virulence strain no. 18/323 and the low-virulence strain no. B1772.

*Comparison of infections with a high- and a low-virulence strain
at short time-intervals after challenge*

When groups of mice were challenged with either the low-virulence strain no. B1772 or the high-virulence strain no. 18/323 and bacterial counts were

made at short time-intervals up to 24 hr afterwards, little difference was observed between the course of infection with either strain (fig. 5). As early as 3 min. after challenge only 11·5 per cent. of the inoculum of no. 18/323 and 23 per cent. of the inoculum of no. B1772 were detected. By 1 hr the percentages remaining were 8·5 of no. 18/323 and 11·5 of no. B1772, and they fell later to 6·8 and 8·7 respectively. The multiplication phase seemed to begin about 6 hr after challenge, and by 24 hr the count had reached or even exceeded the number of organisms in the inoculum.

TABLE I

Percentage of the inoculum of various strains of Bordetella pertussis remaining in the mouse brain 1 hr after challenge

Strain	Number of experiments	Percentage of inoculum detectable at 1 hr
18/323	19	11·7
G353	3	11·0
2-Atox	2	10·5
B2288	17	12·0
B1772	15	12·9
B124	7	11·7
Total	63	11·7

The percentage of bacteria remaining 1 hr after challenge was independent of the challenge strain (table I), and fell within a narrow range (10·5–12·9 per cent.). When these results were rearranged as a function of challenge size, there was no evidence that the proportion of the challenge remaining was dependent on dose.

Sensitivity of Bordetella pertussis to complement

The possibility that the difference in virulence was due to varying sensitivity of the strains to bacterial complement was examined *in vitro* in tests with normal mouse serum. No bactericidal activity was demonstrable against strains no. 18/323, G353, B2288 and B124.

Intracerebral injection of the low-virulence strain no. B1772 into Theiler's Original mice, and into B10.D2 "Old Line" and "New Line" mice (fig. 6) showed that there was no significant difference between the course of infection in complement-deficient and normal mice.

Intracerebral infection with modulated organisms

The self-limiting nature of infections with the low-virulence strains of *Bord. pertussis* might be attributable to changes that they underwent after the

introduction of the organism into the brain. Lacey (1960) showed that the growth of a *Bord. pertussis* strain H5 on a medium in which the sodium chloride was replaced by magnesium sulphate led to an increase in the intranasal LD50 from 10^7 to 10^{10} organisms. Later he pointed out that the surface characters of *Bord. pertussis* could be changed by varying the growth medium or incubation temperature (Lacey, 1961) and described this non-genetic variation as "modulation".

In preliminary experiments, modulation was produced in high-virulence strains, and the variant organisms were injected intracerebrally into mice. The bacterial strains were serially subcultured from freeze-dried stocks on BG medium at 35°C and at 25°C, on blood agar at 35°C and on modified Cohen and Wheeler medium with a high magnesium content. The 4th subculture was used for injection into mice.

FIG. 6.—Bacterial counts after intracerebral injection of no. B1772, a low-virulence strain, into Theiler's Original, B10.D2 "Old Line" and B10.D2 "New Line" mice.

Table II shows that throughout the experiments growth on BG medium at 35°C produced highly virulent organisms, doses as low as 400 viable bacteria killing all the mice. Thus there was no spontaneous modulation to low virulence under these growth conditions. Cultures grown on BA medium at 35°C were also highly virulent and as agglutinable as those grown on BG medium at 35°C.

However, when both no. G353 and no. 2-Atox were subcultured for 7 days on BG medium at 25°C they became inagglutinable by type antisera and were less virulent; challenge doses greater than 2000 viable organisms killed only 1 or 2 mice of each group. No such change occurred in no. 18/323 under similar conditions. On the modified Cohen and Wheeler medium both no. G353 and no. 2-Atox lost their agglutinability and their virulence was reduced, but again no. 18/323 maintained its agglutinability and virulence.

A typical infection with the modulated organisms is shown in fig. 7. Here mice were challenged with 2.2×10^3 *Bord. pertussis* no. G353 cultured on BG medium at 25°C; 24 hr later the brains of 3 of the 5 mice contained less than

50 organisms. In the other 2 brains the count was only marginally greater than the count at 1 hr, but later as with normal virulent strains it increased about 10-fold daily. Presumably the lag between 1 and 24 hr was due either to the time taken for reversion of the modulated organisms to their normal state or to the fact that the inoculum contained a few organisms that had not undergone modulation.

TABLE II

Effect of growth medium and incubation temperature on the agglutination and virulence of high-virulence strains of Bordetella pertussis

Strain no.	Medium	Incubation temperature	Degree of agglutination* with antisera for types			Number of viable bacteria injected intracerebrally	S/T
			1	2	3		
18/323	BG	35°C	...	...	...	400	2/8
18/323	BG	25°C	...	...	...	400	0/8
18/323	BG	35°C	+++	++	++	1150	0/11
18/323	BG	25°C	+++	+++	+++	1400	0/10
18/323	BA	35°C	+++	++	+	1900	0/12
18/323	CW High Mg	35°C	+++	++	++	1300	1/10
G353	BG	35°C	...	...	...	450	0/8
G353	BG	25°C	...	...	...	450	8/8
G353	BG	25°C	...	...	...	2200	9/10
G353	BG	35°C	+++	++	—	500	2/10
G353	BG	25°C	—	—	—	1000	9/10
G353	BA	35°C	+++	++	+	400	5/10
G353	CW High Mg	35°C	—	—	—	600	10/10
2-Atox	BG	35°C	+++	+++	+++	1150	2/10
2-Atox	BG	25°C	—	—	—	1450	9/11
2-Atox	BA	35°C	+++	++	++	1500	2/9
2-Atox	CW High Mg	35°C	—	—	—	3000	7/10

* Degree of agglutination determined by the semi-quantitative method of Preston (1963).

S/T = Ratio: no. survivors/no. mice challenged.

BG = Bordet-Gengou medium.

BA = Blood agar medium.

CW High Mg = Cohen and Wheeler medium with added magnesium sulphate.

Intracerebral challenge with infected brain emulsions

To investigate the possibility that modulation occurred in low-virulence infections with *Bord. pertussis*, bacteria from the brains of infected mice were transferred to previously uninfected mice. Mice were challenged with 2×10^7 viable bacteria of the low-virulence strain no. B2288; a peak of infection was

reached 24 hr later and from this time the bacterial count declined. The brains of infected mice were removed $2\frac{1}{2}$ days after challenge, and 0.025 ml of the macerated brain suspension containing 1.45×10^4 viable bacteria was injected into another group of mice.

One hour after challenge only 10 per cent. of the inoculum was detectable in the brains. From this time the count increased to a maximum at the 2nd day and then declined rapidly. By the 4th day 2 of 4 brains contained less than 50 organisms. This resembled the normal course of infection with a low-virulence strain, and a continuous decrease in count after the transfer of the bacteria, which might have been expected if modulation of the organisms had taken place, did not occur.

FIG. 7.—Bacterial counts after intracerebral injection of the high-virulence strain no. G353 grown *in vitro* on Bordet-Gengou medium at 25°C.

[Figs. 7–10: Geometric means are of all counts exceeding 50 organisms per brain.]

Successive intracerebral inoculations of Bordetella pertussis

The possibility that high-virulence and low-virulence strains of *Bord. pertussis* differed in the response that they elicited in brain tissue was next examined. Mice originally infected with a low-virulence strain were subsequently challenged with a low-virulence or a high-virulence strain.

Groups of mice were inoculated with 2×10^4 viable *Bord. pertussis* of the low-virulence strain no. B2288 and killed at daily intervals to establish the course of the primary infection. After $4\frac{1}{2}$ days the remaining mice and a control group were challenged with 6.9×10^3 viable bacteria of the same strain and bacterial counts were determined after 1 hr and then at daily intervals. At 1 hr there was little difference between the bacterial counts in the control and the rechallenged groups of mice (fig. 8), but after 1 day a clear difference had appeared. In the control group the count at this time exceeded the inoculum and on the 2nd day the geometric mean count was slightly greater than 10^4

organisms. In the reinfected group, however, the count after 1 day was only marginally greater than at 1 hr, and on the 2nd day it had declined to 3.2×10^2 organisms, less than 1/30th of the count in the control mice; the brains of 3 of 5 mice contained less than 50 organisms.

FIG. 8.—Bacterial counts after reinfection with the low-virulence strain no. B2288 in mice infected 4 days previously with the same strain.

When mice were challenged with the low-virulence strain no. B1772 and rechallenged 4½ days later with the high-virulence strain no. 18/323, no decline in the intracerebral viable count was detected and the course of infection in the rechallenged mice was very similar to that in the control group (fig. 9).

FIG. 9.

FIG. 10.

FIG. 9.—Bacterial counts after reinfection with the high-virulence strain no. 18/323 in mice infected 4 days previously with the low-virulence strain no. B1772.

FIG. 10.—Bacterial counts after reinfection with the high-virulence strain no. 18/323 at the peak of an infection with the low-virulence strain no. B2288.

In the two previous experiments the second inoculation was made at a time when most of the mice had few of the original organisms remaining in the brain. However, when mice were challenged with the high-virulence strain at the peak of an infection with the low-virulence strain, many mice survived. A dose of

2.0×10^3 viable bacteria of strain no. 18/323 was given 2 days after infection with strain no. B2288. One day later 3 out of 5 brains contained less than 50 organisms and the bacterial counts in the other 2 were far lower than those in the control infection. The number of nearly sterile brains recorded daily was fairly constant until the termination of the experiment, and the mean viable count in the infected rechallenged brains always lagged behind the mean count in the control mice (fig. 10).

DISCUSSION

The courses of infection with the two high-virulence strains no. 18/323 and 2-Atox were very similar; the rate of increase of the viable count was about 10-fold daily for the first 3 or 4 days, but later fell slightly to between 3- and 8-fold daily, mice dying from the 5th day onwards with viable counts of approximately 5×10^7 viable bacteria. Almost identical results were obtained by Dolby and Standfast (1961), Berenbaum *et al.* (1960) and Iida *et al.* (1962) for the strain no. 18/323 and by Andersen (1957) for the strain no. 2-Atox.

With the high-virulence strain G353, however, the viable count increased 10-fold daily until it reached approximately 10^6 , when there was a 24-hr stationary phase. After this it increased 3- to 8-fold daily, and the mice died with viable counts of 5×10^7 bacteria about 1 day later than with the other two high-virulence strains.

The stationary phase occurred when the count reached the level indicated by Holt *et al.* (1961) as being that necessary for breakdown of the blood-brain barrier. In this laboratory, strain no. G353 proved to be a very potent vaccine strain (Holt, unpublished) and hence a continuous spill-over of organisms from the brain to the meninges might lead to the formation of small amounts of antibody. This entry of antibody when the intracerebral viable count exceeds 10^6 organisms may account both for the stationary phase in the multiplication of G353 and for the small decrease in multiplication rate in the terminal stages of infections with no. 18/323 and 2-Atox.

The use of no. G353 in place of no. 18/323 as challenge strain in active immunisation tests with vaccines (Eldering, Holwerda and Baker, 1966) and in passive immunisation tests with antisera (Eldering, Holwerda and Baker, 1967; Preston, 1967) led to much lower ED₅₀ values irrespective of the serotype of the immunising strain. Both groups of workers also reported that the time taken to death was longer with no. G353 than with similar doses of no. 18/323, although the LD₅₀ of the cultures was similar.

The infections resulting from high- and low-virulence strains behaved very similarly up to 24 hr after challenge, but differences appeared later. The three low-virulence strains all gave very similar courses of infection and, with small challenge doses, most mice had sterile brains by the 5th day; with high doses the infection time was extended, and with very high doses the mice died from the 2nd day onwards with terminal viable counts very similar to those infected with high-virulence strains.

Examination of the viable counts 1 hr after challenge confirmed the observation of Dolby and Standfast that there had been approximately a 90 per cent.

loss of organisms 2 hr after challenge. This loss was found to be independent of both the strain and challenge size. Similarly both Schlesinger (1949) using a virus suspension and Cairns (1950) using a bacteriophage suspension could recover only 2–10 per cent. of the inoculum after challenge. This loss of organisms is probably mechanical and due to washback along the needle-track.

Complement appeared to play no part in the defence of the mouse brain against low-virulence strains of *Bord. pertussis*. Mouse serum was without bactericidal action *in vitro* against *Bord. pertussis*, as against a number of other bacteria (Marcus, Esplin and Donaldson, 1954; Muschel and Muto, 1956). Glynn and Medhurst (1967) used B10.D2 mice to demonstrate the killing action of mouse complement on some strains of *Escherichia coli*, but when a low-virulence strain of *Bord. pertussis* was injected into these two strains of mice, no enhancement of infection was found in the mice deficient in the C'5 component.

Modulation of the bacteria was considered as a possible reason for the decline in infection with low-virulence strains of *Bord. pertussis*. I found that growing both no. G353 and 2-Atox either on Cohen and Wheeler medium containing an increased amount of magnesium sulphate at 35°C or on BG medium at 25°C increased the intracerebral LD50 of these strains from approximately 50 viable bacteria to more than 2000 viable bacteria. The brains of surviving mice were apparently sterile 24 hr after challenge. None of these treatments increased the LD50 of strain no. 18/323.

Now that it had been shown that modulation *in vitro* increased the intracerebral LD50 of the high-virulence strains, the brains of mice infected with a low-virulence strain were removed during the decline phase of infection and macerated, and the supernatant was injected into another group of mice. If modulation was the explanation of this decline, these bacteria, when injected into fresh brains, should have continued to decrease in numbers, but this did not occur.

The response of the mouse-brain to infections with low-virulence strains was next investigated. When the mice were rechallenged with the same low-virulence strain 4½ days after the original infection, at a time when the brain was sterile or nearly sterile, no increase in viable count was detected and most of the animals had less than 50 organisms in the brain 2 days after rechallenge. However, when mice were rechallenged after a similar time-interval with a high-virulence strain, the course of infection was little different from that in the control mice. Thus it seems that limited growth of a low-virulence strain in the mouse brain caused a resistance to reinfection with low-virulence strains that was evident even after the organism had been eliminated, but that this resistance did not affect a high-virulence infection. The evidence that this was a response of the cerebral glial cells is presented in the next paper in this issue (Adams and Hopewell, 1970).

However, when mice infected with a low-virulence strain were rechallenged with a high-virulence strain at the peak of the infection, many of them survived. This is analogous with the "interference" phenomenon described by Evans

and Perkins (1954), who found that intracerebral injections of heat-killed vaccines caused "interference" to infections with strain no. 18/323. Later Blyth (1955) showed that the brains of surviving mice were sterile 24 hr after challenge and that the proportion of sterile brains did not increase after this time. The most likely explanation of this "interference" phenomenon is that all strains have the same "infection sites" on the ciliated ependyma, and that prior injection of either a low-virulence strain or a killed vaccine takes up the majority of "sites", thus decreasing the probability of the high-virulence strain finding free "sites" on which to grow.

SUMMARY

The course of infection after the intracerebral inoculation of high-virulence and low-virulence strains of *Bordetella pertussis* into mice was followed by making serial viable counts of the number of bacteria in the brain.

With all strains, some 90 per cent. of the inoculum was lost within 1 hr, but by 24 hr the number of bacteria in the brain reached or exceeded the number inoculated.

When small doses ($c. 5 \times 10^3$) of high-virulence strains were given, the bacterial count increased continuously until death. With one strain (no. G353) there was a pause in multiplication when the viable count reached 10^6 , but this was not seen with 2 other high-virulence strains (no. 18/323 and 2-Atox).

Challenge doses of low-virulence strains smaller than 10^5 organisms gave rise to a transient infection, the maximum count was reached on the 1st or 2nd day, and most brains examined after 4 days contained less than 50 *Bord. pertussis* bacteria.

Resistance of the mouse brain to low-virulence strains was not due to the action of mouse complement or to the occurrence of modulation. The intracerebral injection of a small dose of a low-virulence strain made the mouse brain resistant to reinfection $4\frac{1}{2}$ days later by a low-virulence but not by a high-virulence strain.

When the interval between injections was reduced to 2 days, however, mice infected with a low-virulence strain were somewhat resistant to reinfection with a high-virulence strain.

I am very grateful to Dr L. B. Holt for the help and guidance involved in the experimental work and to Professor R. E. O. Williams for constructive criticism of the text.

REFERENCES

- ADAMS, G. J. 1968. Ph.D. Thesis, Univ. London.
 ADAMS, G. J., AND HOPEWELL, J. W. . 1970. *J. Med. Microbiol.*, **3**, 15.
 ANDERSEN, ELSE K. 1957. *Acta path. microbiol. scand.*, **40**, 227.
 BERENBAUM, M. C., UNGAR, J., AND STEVENS, W. K. . 1960. *J. Gen. Microbiol.*, **22**, 313.
 BLYTH, W. A. 1955. M.Sc. Thesis, Victoria Univ. of Manchester.
 BROWN, ANNE M. 1958. *J. Gen. Microbiol.*, **18**, 48.
 BURNET, F. M., AND TIMMINS, CECILY . 1937. *Br. J. Exp. Path.*, **18**, 83.

- CAIRNS, H. J. F. 1950. *Nature, Lond.*, **166**, 910.
- COHEN, S. M., AND WHEELER, M. W. . . 1946. *Amer. J. Publ. Hlth*, **26**, 506.
- DOLBY, JEAN M., AND STANDFAST, A. F. B. 1961. *J. Hyg., Camb.*, **59**, 205.
- ELDERING, GRACE, HOLWERDA, J., AND 1966. *J. Bact.*, **91**, 1759.
- BAKER, JULIA
- " " " . 1967. *Ibid.*, **93**, 1758.
- EVANS, D. G., AND PERKINS, F. T. . . 1954. *Br. J. Exp. Path.*, **35**, 603.
- GLYNN, A. A., AND MEDHURST, FIONA A. 1967. *Nature, Lond.*, **213**, 608.
- HOLT, L. B., SPASOJEVIĆ, VERA, DOLBY, 1961. *J. Hyg., Camb.*, **59**, 373.
- JEAN M., AND STANDFAST, A. F. B.
- IIDA, T., KUSANO, N., YAMAMOTO, A., AND 1962. *Jap. J. Exp. Med.*, **32**, 471.
- SHIGA, H.
- KENDRICK, P. L., ELDERING, GRACE, 1947. *Amer. J. Publ. Hlth*, **37**, 803.
- DIXON, M. K., AND MISNER, J.
- LACEY, B. W. 1960. *J. Hyg., Camb.* **58**, 57.
- " " " " " . 1961. *Symp. Soc. Gen. Microbiol.*, **11**, 343.
- MARCUS, S., ESPLIN, D. W., AND DON- 1954. *Science, N. Y.*, **119**, 877.
- ALDSON, D. M.
- MUSCHEL, L. H., AND MUTO, T. . . . 1956. *Ibid.*, **123**, 62.
- PRESTON, N. W. 1963. *Br. Med. J.*, **2**, 724.
- " " " " " . 1967. *Nature, Lond.*, **213**, 830.
- SCHLESINGER, R. W. 1949. *J. Exp. Med.*, **89**, 491.
- TERRY, W. D., BORSOS, T., AND RAPP, 1964. *J. Immun.*, **92**, 576.
- H. J.