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Observations on the growth of trachoma and inclusion blennorrhoea viruses in embryonate eggs

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INTRODUCTION

Many strains of trachoma and inclusion blennorrhoea viruses have been isolated in several countries, but their similarities appear to be more pronounced than their differences. Although serological differences between trachoma strains isolated in Saudi-Arabia (Bell, Snyder & Murray, 1959) were demonstrated by a toxin-protection test in mice, none were found between a Chinese and an Israeli strain in a neutralization test (Bernkopf, 1959). However, strains vary in biological characteristics, for instance in pathogenicity for the chick embryo, man, simians, mice and cell cultures and in their susceptibility to antibiotics.

Since 1958, seven strains of trachoma virus and four strains of inclusion blennorrhoea have been maintained in this laboratory in the yolk sac of embryonate hens' eggs. Comparison of dose-response curves obtained for these strains titrated in chick embryos revealed differences in growth characteristics.

METHODS

Virus strains

Trachoma. G 1, G 17, isolated in the Gambia, West Africa (Collier & Sowa, 1958; Sowa & Collier, 1960). Gambia 062(t), and Gambia 221(t), referred to as G 062 and G 221, isolated by J. Sowa (unpublished). SA 2, isolated in Saudi Arabia (Murray, Bell, Hanna, Nichols & Snyder, 1960). TE 55, isolated in China (T'ang, Chang, Huang & Wang, 1957). BOUR and ASGH, isolated in U.S.A. (Hanna, Jawetz, Thygeson & Dawson, 1960).

Inclusion blennorrhoea. LB 1 (Jones, Collier & Smith, 1959) and LB 4 (Jones, 1961) isolated in London.

Lymphogranuloma venereum (LGV). JH strain in its 264th egg passage, obtained from Dr Sylvia Reed, London Hospital.

Serial passage

Heavily infected yolk sacs were homogenized or shaken to make 10–25 % (w/v) suspensions in sucrose potassium glutamate solution (SPG) (Bovarnick, Miller & Snyder, 1950), containing 1 mg./ml. of streptomycin, and 0·2–0·5 ml. amounts injected into the yolk sacs of eggs which were then incubated at 35° C. The eggs were candled daily; yolk-sac smears made from dead embryos were stained with

Giemsa and examined for the presence of virus. The day after inoculation on which each embryo died was recorded.

Infectivity titration in eggs

Serial tenfold dilutions of virus suspension were made in SPG and 0·5 ml. of each dilution was injected into the yolk sacs of at least five 7-day embryonate eggs. Dead embryos were examined for elementary bodies and the LD 50 per ml. of the original suspension calculated (Reed & Muench, 1938).

Infectivity titrations in HeLa cells

These were done as described by Furness, Graham & Reeve (1960).

Particle counts

The elementary bodies in films of infected yolk sac suspensions were stained with Giemsa and counted under dark ground illumination (Reeve & Taverne, 1962).

RESULTS

Table 1 gives the days on which embryos died in representative titrations, and in Fig. 1 the arithmetic mean death time of each group of embryos is plotted against dose of virus inoculated expressed as a multiple of the $\log_{10}$ LD 50 (the dose killing 50 % of eggs). At least two titrations were done for strains LB 1, TE 55, G 17 and SA 2 in this laboratory; the curves shown for strain BOUR and ASGH are taken from Jawetz & Hanna (1960). Titrations of G 221 and G 1 gave points fitting the curve drawn for G 17 and BOUR; for clarity they are omitted from the figure. In many infections caused by either bacteria or viruses, log dosage, within limits, is inversely proportional to mean death time (see review by Meynell & Meynell (1958), who discuss the general characteristics of such curves). This is also true of trachoma virus (Jawetz & Hanna, 1960; Watkins, 1961) and we have observed it for all the strains tested. The dose-response curves shown in Fig. 1 are sigmoid, so that three portions can be distinguished: (1) With very large doses ($> 10^5$ LD 50 for strains TE 55 and LB 1) the curve becomes roughly parallel to the dose axis, presumably because even the largest doses take a minimum time to kill the embryo. (2) At intermediate doses, the mean death time is inversely proportional to log dose. The most probable interpretation of this is that the virus responsible for the death of the embryo increases exponentially to a critical level at which death occurs. The slope of this part of the curve is proportional to the rate of increase of the virus; since the slopes of the curves are parallel, all strains of virus examined increased at the same rate (Fig. 1). The rate estimated from the slope is 30 LD 50 per day, and the doubling time calculated on the basis of this constant is 6 hr. Jawetz, Hanna, Chino & Zichosch (1962) concluded from similar curves that strains ASGH, BOUR, APACHE and TANG in their laboratory had similar growth rates and increased by '1·3 logs of infective virus per day per g. of yolk sac'. (3) At doses < 1 LD 50 the mean death time becomes constant. Meynell & Meynell (1958) and Plus (1954) suggest that this occurs because at

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Table 1. *Survival times of embryos infected with different doses of virus. Results of some representative titrations*

Strain	LD 50/ml.	Virus dilution	Day of specific death
G 17	2×10^5	10^{-1}	6, 6, 6, 7, 8
		10^{-2}	6, 7, 7, 8, 8, 9
		10^{-3}	8, 8, 8, 9, 9, 9
		10^{-4}	9, 9, 9, 9, 9, 9
		10^{-5}	9, 9, 9, 10, 11, 11, 11, 11, 12
		10^{-6}	9, 10, 11
TE 55	6×10^6	10^{-1}	4, 4, 4, 4, 5, 5
		10^{-2}	4, 4, 4, 4, 5, 5, 6
		10^{-3}	4, 4, 5, 5, 5, 6, 6
		10^{-4}	6, 6, 6, 7, 7, 8
		10^{-5}	5, 6, 6, 7, 7, 8, 8
		10^{-6}	8, 8, 9, 9, 10, 11
		10^{-7}	9, 10
SA 2	2×10^3	Undiluted	5, 5, 5, 5, 5, 6
		10^{-1}	5, 5, 5, 5, 5, 5
		10^{-2}	5, 5, 5, 6, 8, 7
		10^{-3}	7, 7, 9, 8
		10^{-4}	7, 8, 8, 8, 9
		10^{-5}	10, 10, 7
LB 1	4×10^7	10^{-2}	5, 5, 5, 5
		10^{-3}	5, 5, 5, 5, 6
		10^{-4}	6, 5, 7
		10^{-5}	5, 7, 8, 9
		10^{-6}	7, 8, 9, 9
		10^{-7}	9, 9, 9

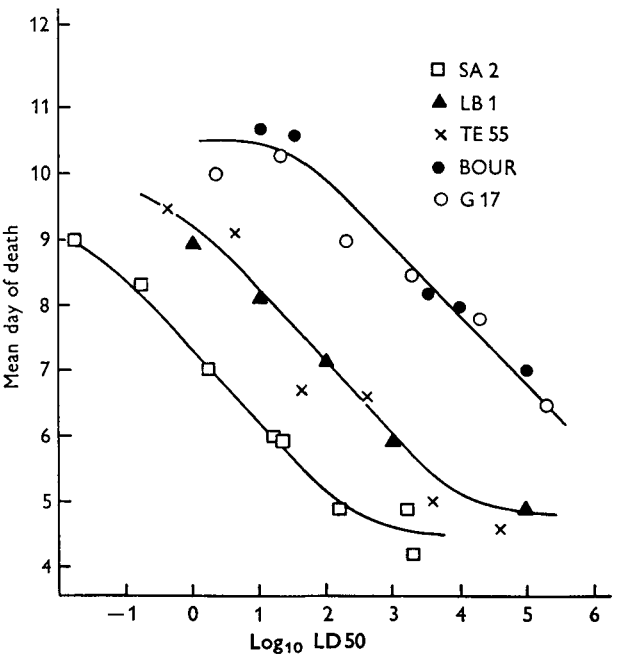

Fig. 1. Dose-response curves for different strains of trachoma and inclusion blennorrhoea in chick embryos.

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doses < 1 LD 50 each response is usually caused by the multiplication of 1 particle, although the average dose may have contained many particles, each potentially capable of causing a lethal infection (Meynell & Stocker, 1957). On re-examination the data given by Jawetz & Hanna (1960) clearly fit a sigmoid curve better than a straight line.

Although the dose-response curves shown in Fig. 1 are similar in shape, for a given number of LD 50 of some strains the mean death time was less with some strains than with others. For instance, embryos receiving 10^4 LD 50 of strain

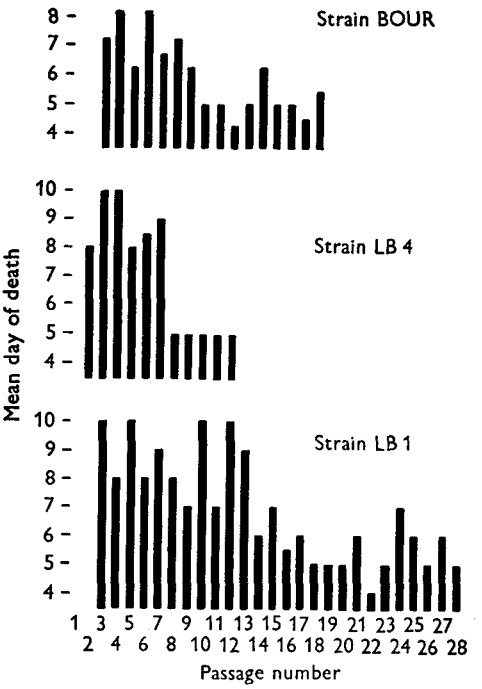

Fig. 2. The mean day of death of groups of chick embryos inoculated with three different strains of virus, showing changes occurring during routine passage.

TE 55 died 5 days after inoculation while embryos receiving the same dose of the Gambian strains died 9 days after inoculation. Similarly, strain ASGH killed embryos one day earlier than the same dose of strain BOUR (Jawetz & Hanna, 1960). Their dose-response curve for strain ASGH lies between those of the Gambian strains and TE 55. The differences in behaviour in the chick embryo existing between the Gambian strains and the others are also apparent in routine passages using undiluted yolk-sac suspensions as inocula.

We analysed the records of eggs inoculated in this laboratory for each passage of each strain and noted the total numbers of embryos dying with virus in them and the day after inoculation on which they died. The total number of embryos dying without detectable virus in them, including those dying from non-specific causes, was much the same for each strain, and no strain killed more embryos within the first 48 hr. of inoculation than any other. Most embryos died on one day which was characteristic of the strain inoculated: thus, strains SA 2, LB 1,

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TE 55 and also LGV killed embryos most quickly, the majority dying on the 5th day, whereas embryos inoculated with the Gambian strains G 1, G 17 and G 221 died 3–4 days later on the 8th or 9th day. Embryos infected with Gambian strains rarely died before the 6th day and if they did, contained no virus.

Three strains changed in their growth characteristics during passage: LB 1 after the 13th passage, LB 4 after the 7th passage and BOUR after the 9th passage. In each case, before the change most embryos died on the 8th day, like those inoculated with Gambian strains, but thereafter the majority died on the 5th day after inoculation. With all three strains the change appears to have been abrupt (Fig. 2).

Table 2. *Infectivity titres and particle counts of different suspensions of trachoma viruses*

Virus suspension					
Strain	Pass. no.	*IFU per ml.	Particles per ml.	LD 50 per ml.	Particles per LD 50
G 1	42 (*a*)	0	$3{\cdot}0 \times 10^{9}$	$2{\cdot}0 \times 10^{2}$	$1{\cdot}0 \times 10^{7}$
G 1	42 (*b*)	0	$9{\cdot}5 \times 10^{8}$	$2{\cdot}0 \times 10^{2}$	$4{\cdot}5 \times 10^{6}$
G 1	32	0	$2{\cdot}0 \times 10^{10}$	$9{\cdot}0 \times 10^{4}$	$2{\cdot}0 \times 10^{5}$
G 221	5	0	$1{\cdot}5 \times 10^{10}$	$3{\cdot}0 \times 10^{3}$	$5{\cdot}0 \times 10^{6}$
G 062	4	0	$1{\cdot}5 \times 10^{10}$	$1{\cdot}5 \times 10^{4}$	$1{\cdot}0 \times 10^{6}$
BOUR	8	0	$1{\cdot}5 \times 10^{9}$	$2{\cdot}0 \times 10^{3}$	$7{\cdot}5 \times 10^{5}$
BOUR	19	$1{\cdot}0 \times 10^{6}$	$2{\cdot}5 \times 10^{8}$	$1{\cdot}0 \times 10^{6}$	$2{\cdot}5 \times 10^{2}$
ASGH	16	$4{\cdot}3 \times 10^{5}$	$4{\cdot}0 \times 10^{7}$	$2{\cdot}0 \times 10^{5}$	$2{\cdot}0 \times 10^{2}$
SA 2	13	ND	$8{\cdot}0 \times 10^{8}$	$3{\cdot}0 \times 10^{5}$	$2{\cdot}0 \times 10^{3}$
SA 2	14	$2{\cdot}4 \times 10^{7}$	$4{\cdot}0 \times 10^{9}$	$4{\cdot}0 \times 10^{5}$	$1{\cdot}0 \times 10^{4}$
SA 2	16	$2{\cdot}5 \times 10^{6}$	$1{\cdot}0 \times 10^{9}$	$2{\cdot}5 \times 10^{6}$	$4{\cdot}0 \times 10^{2}$
TE 55	63	$3{\cdot}0 \times 10^{6}$	$9{\cdot}0 \times 10^{8}$	$1{\cdot}0 \times 10^{4}$	$9{\cdot}0 \times 10^{4}$
TE 55	65	ND	$9{\cdot}0 \times 10^{9}$	$3{\cdot}0 \times 10^{6}$	$3{\cdot}0 \times 10^{3}$

* Inclusion forming units (IFU) in HeLa cells.

Particle counts

Table 2 gives results of total particle counts on suspensions of different virus strains and the ratio of particles to LD 50. Strains which had a ratio of 10^4 or less could also be titrated in HeLa cells, whereas strains with a ratio of 10^5 particles/LD 50 or more did not form inclusions in HeLa cells. The strains with the higher ratio were those for which a given dose took longer to kill chick embryos. All the strains have a higher particle/LD 50 ratio than meningopneumonitis virus, another agent of the psittacosis-lymphogranuloma group (Manire & Smith, 1959). With care to avoid heat inactivation our ratios could probably be decreased but as all suspensions were made in the same way the results are comparable. The comparatively high particle/LD 50 ratio of some strains is not entirely due to their low infectivity titres; thus, suspensions G 1/passage 32 and G 221/passage 5 had infectivity titres of the same order as TE 55/passage 63 and ASGH/passage 16 but much higher particle/LD 50 ratios. Figures are given for two suspensions of strain BOUR. Before passage 9 this strain contained more than 10^5 particles/LD 50 and did not form inclusions in HeLa cells. By passage 19, when the survival time of the chick embryo had decreased, it contained 10^2 particles/LD 50 and the suspension had a titre in HeLa cells of $1{\cdot}0 \times 10^6$ inclusion forming units (IFU) per ml.

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DISCUSSION

Implications of the dose/death time curves

Golub (1948) observed the linear relationship between log. dose and day of death for psittacosis virus and suggested that provided large numbers of eggs were used a single dilution method could be used for titration; Jawetz & Hanna (1960) and Watkins (1961) have applied his method to trachoma virus. However, this method is valid only if the linear portion of the dose-response curve is used and if virus strains do not change their growth characteristics. Our results and those of Meynell & Meynell (1958) show that the linear relationship does not hold for doses $\leqslant$ 1 LD 50 or for very large doses ($\geqslant 10^5$ LD 50); in addition three of our strains altered their growth characteristics during passage. Furthermore, with strains killing embryos slowly there is greater variation about the mean survival time for embryos receiving the same dose and, for all strains, the less virus inoculated the greater is the scatter of embryo deaths. Our dose-response curves conform to the model proposed by Meynell & Meynell (1958) for the production of a response by the multiplication of micro-organisms for which average latent period is linearly related to logarithm of dose. The model postulates that the organisms causing the response increase *in vivo* at a constant rate so that their number rises exponentially and that the response (in this case death of the embryo) occurs when their total number reaches or exceeds a critical figure. For systems having the same growth rate, as have our strains, three situations could produce the observed regression lines:

(i) The same critical number of particles causes death, but either the strains have different lag phases before exponential growth begins or, after the lethal concentration of particles is reached, there is a delay, varying with strain, before the embryo dies. Our curves only show the exponential growth phase so cannot be used to demonstrate differences in lag phases. However, the numerous growth curves which have been published for trachoma and other viruses of the psittacosis-lymphogranuloma group are so similar (e.g. Litwin, Officer, Brown & Moulder, 1961) that such differences are unlikely.

(ii) The strains all kill when the total number of particles reaches the same critical figure though the numbers of infective particles are different.

(iii) The strains attain about the same infectivity titre but kill when the total number of particles reaches a different critical figure.

The total number of particles in an infected yolk sac from a dead embryo appears to be relatively constant, regardless of strain, and therefore on average the infectivity titres of the strains which kill more slowly would be expected to be lower than the rest, and the ratio of particles per LD 50 would be higher. Reference to Table 2 shows that the ratio of particles/LD 50 for the Gambian strains is in fact consistently higher than that for the more pathogenic strains, and the difference, which is of the order of 1000-fold, is of the magnitude which would be predicted from the growth rate and the different positions of the dose-response curves in relation to the mean death day axis. Our results therefore appear best explained by hypothesis (ii).

We do not know how the virus kills embryos, but since all strains have so much in common it seems likely that they all kill in the same way; it is possible that some strains are more virulent than others because their infective particles possess more of some lethal factor, or perhaps a lethal factor of a slightly different kind. Members of this group are known to produce a toxin (Rake & Jones, 1944; Bell, Snyder & Murray, 1959); a toxin may contribute to the death of the embryos, which show haemorrhagic lesions similar to those described for mice dying after an intravenous dose of live trachoma virus (Bell *et al.*, 1959).

It can be deduced from Fig. 1 that to kill an embryo within 24 hr. of inoculation at least 10^8 LD 50 of our most virulent strain (LB 1) would be required; and this is often more than is contained in a whole yolk sac. Therefore Watkins's (1961) argument that a toxic factor is not involved because there is no evidence that embryos die in the absence of multiplication may not be valid as, in the experiments he described, doses large enough to be immediately toxic were not administered.

Changes in behaviour on passage

T'ang *et al.* (1957) reported that 'with the increase of number of passages the lethal action of the virus for the embryos was increased', and that a steep rise in infectivity occurred between the 10th and 15th passage. We too observed some changes during passage, a given dose of three strains (LB 1, LB 4 and BOUR) now killing embryos more rapidly than originally. However, we only have the full histories of the Gambian strains: G 1, G 17 and G 221, which were received in their 3rd, 7th and 1st egg passage, have been passed in this laboratory 42, 49 and 7 times respectively and full records have been kept of the survival times. We do not have the full histories of strains which kill embryos more rapidly than the Gambian strains, i.e. TE 55, SA 2 and LGV. These may have been slow to kill the embryo immediately after isolation, and like LB 1, LB 4 and BOUR they may have increased in virulence on passage, but before we received them.

The pathogenicity of strain LB 1 for chick embryos changed in March 1959, that of LB 4 in June 1961 and of BOUR in February 1962. Since strict precautions are taken to avoid any confusion, LB 1, LB 4 and BOUR are unlikely to have become contaminated at different times with one of the strains killing embryos quickly. Furthermore, there is no evidence that any of the Gambian strains have become contaminated, although G 1 and G 17 have been passaged frequently for over 3 years. These changes are most reasonably explained by the appearance of a mutant which kills embryos more rapidly than the parent strain. The conditions of routine passage in this laboratory would especially favour its selection because high concentrations of fresh virus are inoculated into eggs with short intervals between passages.

All strains which kill chick embryos relatively quickly have the ability to induce inclusions in HeLa cells (shown for TE 55 and LB 1 by Furness, Graham, Reeve & Collier (1960), and for SA 2, LB 4, BOUR and ASGH by P. Reeve & D. M. Graham (unpublished observations)) and some have also been shown to grow in the mouse brain (Hurst & Reeve, 1960); but in their early passages strains LB 4 and BOUR

failed to infect HeLa cells (D. M. Graham & W. A. Blyth, unpublished observations). Collier (1961) noticed that after its 8th passage strain LB 1 lost the ability to infect the baboon conjunctiva and this was about the same time as the change in pathogenicity for the chick embryo occurred. There is thus some evidence for correlated changes in increased ability to kill chick embryos and to infect HeLa cells and the mouse brain, and loss of pathogenicity for the baboon. These events may also be accompanied by loss of specific antigen, as in smooth to rough variation in bacteria (Wilson & Miles, 1946). Mutation followed by selection of mutants serologically different from naturally occurring strains may account for our failure to extract specific complement-fixing antigen (Jenkin, Ross & Moulder, 1961) from virus grown in HeLa cells and to distinguish between strains by neutralization tests in HeLa cells (Reeve & Graham, 1961, and unpublished observations).

SUMMARY

When trachoma and inclusion blennorrhoea viruses were titrated in chick embryo yolk sacs and the average day of death was plotted against dose of virus inoculated, sigmoid curves were obtained. Although all strains tested had the same growth rate, a given dose of some killed embryos more quickly than others. Strains killing most rapidly had the fewest elementary bodies per LD 50 and were the only strains to form inclusions in HeLa cells. During passage in the chick embryo three strains changed in their behaviour, killing embryos faster and acquiring the ability to form inclusions in HeLa cells.

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