GROWTH AND LETHAL EFFECTS OF THE PSITTACOSIS AGENT IN
CHICKEN EMBRYOS OF DIFFERENT AGES

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ABSTRACT

Stewart, Robert B. (University of Rochester, Rochester, N. Y.). Growth and lethal effects of the psittacosis agent in chicken embryos of different ages. J. Bacteriol. 83:423–428. 1962.—Inoculation of the psittacosis agent (strain 6BC) into the yolk sacs of chicken eggs in different stages of embryonic development resulted in a steady decrease in LD₅₀ titer as the embryos increased in age from 7 to 10 days. Correlated with the decreased LD₅₀ titers in older eggs was the finding that the titers of the organism were lower in the yolk-sac tissues of the older eggs. The embryos themselves also gained appreciably in resistance with increase in age, since comparable titers of psittacosis organisms were found in embryonic tissues of differing ages at the time of inoculation but the mortality of older embryos was considerably less. This resistance of older embryos to the lethal effects of the psittacosis agent could not be demonstrated when the organism was introduced intravenously instead of into the yolk sac.

The method most frequently used for the titration of agents of the psittacosis-lymphogranuloma venereum group is LD₅₀ assay in chicken embryos, using the yolk-sac route of inoculation. Cox (1938) described this method for the growth of a variety of rickettsiae, and reported that 7-day eggs gave more consistent results than did 5- to 6-day eggs, which were too readily killed, or 8- to 9-day eggs, which gave lower yields of rickettsia. Rake and Jones (1942, 1944) and Hamre and Rake (1944) used 6-day eggs for the titration of lymphogranuloma venereum and feline pneumonitis agents, and noted an increase in the time of death of embryos inoculated with decreasing amounts of these organisms. This finding formed the basis for the single dilution titration method described by Golub (1948), who showed that a linear relationship existed between the LD₅₀ of the inoculum and the average day of death of the inoculated eggs. The age of the eggs used for the titration of these agents has been empirically selected and ranges usually from 6 to 9 days. The observation in this laboratory of differing titers of a single suspension of psittacosis organisms assayed in 7- and 8-day eggs led to an investigation of the effect of embryo age on this assay system.

MATERIALS AND METHODS

Eggs. White Leghorn (strain cross-Queen) eggs were from an antibiotic-free flock; a single supply was used throughout this study.

Organism. A single frozen lot of the 6BC strain of psittacosis organisms, prepared as a 10% yolk-sac suspension as described by Morgan (1956) and maintained at −60 C, was used.

Egg inoculation. Standard titrations were carried out in 7-day eggs, using the method described by Golub (1948) and adequate controls (Dougherty, McCloskey, and Stewart, 1960). Inoculation of eggs of varying age was done in the same fashion. Intravenous inoculation was carried out using a technique described by Beveridge and Burnet (1946).

RESULTS

Determinations of LD₅₀ in eggs at various stages of embryonic development. Eggs varying from 6 to 10 days of incubation (at 37 C) were inoculated as previously described with serial tenfold dilutions of the organism, using 12 eggs per dilution. The eggs were candled daily, and LD₅₀ determinations were made using the method of Reed and Muench (1938). There was a progressive drop in LD₅₀ titer with increasing age of the embryos at the time of inoculation (Table 1). Since the titers are based on death of embryos, it would appear that older embryos are more resistant to the lethal effects of this organism. The following experiments were carried out in an attempt to determine the mechanism of this increase in resistance with age of the embryos, and are based
on a hypothesis of decreased multiplication of the organism in the yolk sacs of older eggs or increased resistance of the embryos to its lethal effects.

Titration of the psittacosis agent in yolk sacs and embryos in eggs of different ages at time of inoculation. This experiment was carried out in a manner similar to the preceding except that a single dilution of psittacosis organisms, which titered at 2.6 log LD_{50}, was inoculated. Five days after inoculation, the yolk sacs were harvested and 10% suspensions prepared from the yolk sac of each egg. Similar preparations were made from each embryo. These materials were then assayed for content of psittacosis agent. The titer of the yolk-sac preparations decreased with an increase in age of the embryo at the time of inoculation (Table 2). The content of psittacosis agent in the embryo suspensions does not show the same degree of decrease in titer with increasing age of inoculated embryos, suggesting that the embryo may be more resistant with age while the yolk sac is becoming a less favorable system for the growth of the agent.

Table 1. Log LD_{50} titers of psittacosis agent in eggs at different ages of embryonic development

<table>
<thead>
<tr>
<th>Day of embryonic development at time of inoculation</th>
<th>Expt. no.</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>6.7</td>
<td>4.9</td>
<td></td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.3</td>
<td>4.8</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>5.6</td>
<td>4.8</td>
<td></td>
<td>1.0</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>5.5</td>
<td>6.6</td>
<td>4.5</td>
<td>3.4</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 2. Log LD_{50} titers of psittacosis agent in yolk sac and embryo suspensions inoculated at different days of embryonic development and harvested 5 days later

<table>
<thead>
<tr>
<th>Age of eggs at time of inoculation*</th>
<th>Mean log LD_{50} of 10% suspensions</th>
<th>No. suspensions titered</th>
<th>Embryos dead at 5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryo</td>
<td>Yolk</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.3</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.8</td>
<td>6.6</td>
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<td>9</td>
<td>3.2</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>4.1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Inoculum: 2.6 log LD_{50}.

Since the results of these experiments were obtained at a single time after the inoculation of the organism, it was considered possible that changes in titers might have occurred, during the 5-day incubation period, that would influence the interpretation of data, particularly if titers of embryo and yolk-sac suspensions had fallen from a higher peak. The following experiment was, therefore, carried out so that material could be collected for assay at different time periods after the inoculation of psittacosis organisms. The results of such an experiment would provide a growth curve of the agent in yolk sacs and embryos in eggs of different ages.

Growth of the psittacosis agent in yolk sacs and embryos inoculated at different days of embryonic development. This experiment was carried out in a manner similar to the preceding, except that only 7- and 10-day eggs were used; of the different aged eggs used, they represented the extremes in susceptibility. The titer of the inoculum was 3.6 log LD_{50}, and yolk sacs and embryos were collected for individual assay at different time periods after the inoculation of the organism. Eight to ten eggs were taken from each group at each time period. At comparable times, the mean titer of psittacosis organisms in the yolk sacs of eggs inoculated at 10 days of embryonic development was consistently lower than in those from eggs inoculated at 7 days (Table 3), as was expected from the results of the preceding experiments. As in the preceding experiment, the titers of the embryo suspensions from eggs inoculated at 7 and 10 days did not differ greatly. The
relationship between titers from embryos and the cumulative percent mortality does, however, provide more direct evidence that the older embryos are more resistant to the lethal effects of the agent. It can be seen in Table 3 that at 4 days after virus inoculation the titer of psittacosis organisms in the 10-day embryo suspension was slightly higher than in the 7-day suspension; however, none of the older embryos had died by this time, whereas 12.5% of the younger embryos were dead. At 10 days after inoculation, 100% of the younger embryos were dead, contrasted to 31% of the older. Yet the content of psittacosis agent in the embryo suspensions was again similar.

Effect of intravenously inoculated psittacosis agent on the survival of 7- and 10-day embryos. The preceding experiment demonstrated that older embryos were more resistant to the lethal effects of organisms reaching them via the yolk-sac route of inoculation. It then became of interest to determine whether this resistance was still evident when the organism was inoculated directly into the embryo by an intravenous route of inoculation. Seven and 10-day eggs were selected for uniformity of size and well-developed veins on the chorioallantoic membrane. Serial tenfold dilutions of psittacosis organisms were made in Hanks' balanced salt solution (Hanks and Wallace, 1949), and the eggs were inoculated using the technique described by Beveridge and Burnet (1946). Eggs were discarded if there was any doubt of the success of the inoculation technique. The 7-day eggs were particularly difficult in this respect, since the veins were not well developed, were poorly embedded in the membrane, and had a tendency to fall free into the allantoic cavity when attempts were made to insert a needle. The eggs were candled daily, and the dead embryos removed. A 10% suspension from each dead embryo was titrated for content of psittacosis agent. The results of this experiment are shown in Fig. 1, where the number of embryos dead on a given day and the mean log LD_{50} of the embryo suspensions are plotted against the day of death and the titer of the inoculum. It can be seen that the psittacosis agent titers from embryos inoculated as 7- or 10-day eggs are grouped about a mean of 3.6 log LD_{50}. The standard deviation of titers of embryo suspensions from 7-day embryos was 0.7 log LD_{50}, and was 0.3 log LD_{50} for the suspensions from 10-day embryos. Since these standard deviations approximate the limits of error of the assay, there does not appear to be a significant difference in the titers of the agent in the embryo suspensions whether from embryos inoculated at 7 or 10 days.

It would also appear that the titer of the inoculum had no effect on the titer obtained from the suspensions of dead embryos. The effect of differences in titer of the inoculum appeared to be on the time of death of the embryo, with those receiving the least amount of agent dying later. In this regard, the age of the embryo at the time of inoculation appeared to have no effect on the average day of death, since at comparable levels of inocula the average day of death in eggs inoculated at 7 and 10 days was nearly identical. Embryos dead on the first day were excluded from the calculations, since these early deaths are thought to be the results of toxin (Cox, 1953), or are nonspecific, probably associated with traumatization by the inoculation procedure since a period of 24 to 48 hr followed without any deaths. The failure to get early deaths after the intravenous inoculation of 7-day embryos is difficult to explain, since one would not expect them to be more resistant to psittacosis toxin or traumatization than the 10-day embryos. The possibility that the inoculation was not intravenous is excluded since the 6BC strain of psittacosis agent maintained in this laboratory is not efficient in killing chick embryos when introduced into the allantoic cavity, which would have been the inoculation route had the inoculum missed the vein.

Another factor which had to be considered was the total amount of agent per embryo at the time of death. If, for example, an embryo inoculated at 10 days had the same titer per gram of tissue at a time of death 5 days after inoculation as did an embryo inoculated at 7 days and dead 3 days later, one would expect that the total amount of psittacosis organisms in the 15-day embryo might be significantly greater than in the 10-day embryo, because of the greater weight of the older embryo. This would lead to the conclusion that the older embryo was more resistant, since more of the agent would have accumulated before death. When the data from these experiments were considered from that point of view, it was found that the total psittacosis agent content of the older embryos...
was of the order of 0.5 log \( L_{D50} \) higher than in the younger embryos. When these differences were considered statistically, a very low level of significance was found, as was to be expected from the accuracy limits of the titration system (about 0.5 log \( L_{D50} \)). Greater accuracy in the assay system would, therefore, be required before one could determine whether older embryos were more resistant to intravenously inoculated psittacosis organisms.

**DISCUSSION**

The findings presented in this study provide an explanation for the greater degree of inaccuracy of psittacosis agent titrations in eggs when low concentrations of the organism are used, and particularly when the single dilution method of Golub (1948) is the technique employed. It would seem logical to imagine that, when low concentrations of organisms are inoculated into the yolk sacs of 7-day eggs, the developing resistance mechanisms involving both yolk sac and embryo reach levels which result in a failure of the agent to attain a concentration that will result in embryo death. Evidence of variation in susceptibility among chick embryos, as well as increasing resistance with age, are to be found in a number of studies using agents of the psittacosis-lymphogranuloma venereum group. Rake and Jones (1942) and Dougherty et al. (1960), using staining techniques, noted the presence of elementary bodies in yolk-sac preparations from eggs which had survived well beyond the time of death of most of the embryos similarly inoculated, indicating a degree of enhanced resistance in the surviving embryos. Nadel and Fellowes (1953), by measuring the psittacosis agent content of living and dead embryos which had been inoculated with psittacosis organisms via the yolk sac, found high concentrations of the agent in a significant percentage of embryos surviving 7 days after the inoculation of \( 50 \) \( L_{D50} \). This again indicates a higher resistance of these embryos to the lethal effects of the agent. Davis and Vogel (1949) demonstrated persistence of psittacosis agent in hatched chicks which had been inoculated as embryos; they utilized eggs ranging from 0 to 18 days of embryonic development. The dose of psittacosis organisms inoculated was adjusted so that about 50% of the inoculated embryos would survive. When mortality is related to the age of the embryo at time of inoculation, however, there is a mortality of about 80% up to 9 days of embryonic development; after this time, the mortality falls to about 50%, showing...
an increased resistance to the lethal effects of psittacosis organisms accompanying an increase in age of the embryo. This phenomenon of altered susceptibility to infection has been described for a number of host-virus systems (Sigel, 1952). It is apparent that the phenomenon is a complex of many factors, and its expression may be dependent not only on host factors but also on the experimental techniques used, such as differing routes of inoculation, and on evaluation criteria such as severity of infection, dosage required to establish infection, and multiplication of virus.

The finding that an increased resistance of chick embryos to psittacosis organisms occurs with age when the agent is introduced via the yolk sac, but not when it is introduced intravenously, provides an example of the importance of the route of inoculation in detecting changes in susceptibility. A somewhat similar finding was reported by Crawley (1948), who found that chick embryos became increasingly resistant with age to Eastern equine encephalomyelitis virus introduced into the allantoic sac; however, they became increasingly susceptible when the agent was inoculated into the yolk sac.

Decreased amounts of psittacosis agent in yolk sacs when eggs were inoculated at later stages of embryonic development were interpreted as indicating that the yolk-sac tissue becomes less favorable for the growth of psittacosis organisms as embryonic development progresses. The possibility of this interpretation not being wholly justified on the basis of greater yolk-sac weight of older eggs was considered. The weight of the yolk sac increases about fourfold from the 7th to 15th days of embryonic development, while there is a concomitant decrease in surface area due to folding and thickening of the inner membrane and the inclusion of larger amounts of yolk material within the cells and blood vessels (Romanoff, 1952). Thus, if 10% suspensions of yolk sac are prepared for assay, and assuming the same number of cells capable of supporting psittacosis growth in the younger and older membranes, there would be a greater dilution factor applied to the agent in membrane suspensions of older eggs. When these factors were applied to the data obtained in this study, it was found that there was still considerably less psittacosis agent present in the yolk sacs from older embryos. The possibility is suggested that the individual cells in older yolk-sac membranes are not less susceptible to psittacosis or less efficient for its synthesis, but rather that fewer susceptible cells are available for infection due to folding and thickening of the inner membrane. A similar concept, proposed by McLaren and Sanders (1959) to explain the increased resistance of mice to encephalomyocarditis virus accompanying ageing, was based on changes which resulted in susceptible cells being less accessible to the virus.

ACKNOWLEDGMENT

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