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The Chick Embryo Neutralization Test in the Assay of Meningococcal Antibody*

1. Infection of the Embryo with *Neisseria meningitidis*

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Present methods of controlling meningococcal cerebrospinal meningitis have failed to contain the disease. This has led to the search for effective vaccines and to the development of methods for assaying the potency of these vaccines, as well as for measuring the immune response of the individual. The feasibility of using the sero-protection test in embryonated eggs for the assay of meningococcal antibody has been investigated. The paper describes detailed investigations of the infectious process established by injecting Neisseria meningitidis *by various challenge routes. Embryos, even 10–12 days old, showed high susceptibility to meningococci injected* via *the yolksac or chorioallantoic vein, and lesser susceptibility when injected* via *the intra-allantoic and chorioallantoic routes. The deaths of intravenously inoculated embryos coincided with the multiplication of the organisms and ensuing septicaemia. A study of the infection in embryos which had died, or were killed, 18–24 hours after inoculation showed a specific localization of the organisms in the brain.*

Epidemics of meningococcal cerebrospinal meningitis are still of considerable importance in some parts of the world (Lapeyssonnie, 1963); elsewhere, sporadic cases occur in special-risk groups, despite the general effectiveness of chemotherapy (Feldman, 1966; Vedros, Hunter & Rust, 1966). Present methods of control have to date failed to contain the disease and this has led to the search for an effective vaccine and to the development of methods to assay the potency of vaccines and to measure the immune response of the individual. The adoption of a sero-protection test in embryonated eggs for the assay of meningococcal antibody was one of the recommendations made at a meeting convened by the World Health Organization in 1965 to discuss research on *Neisseria*. The feasibility of using this technique has been investigated in our laboratory and this report outlines the first part of the study and deals with the infection of the chick embryo with *N. meningitidis*.

MATERIALS AND METHODS

Chick embryo

Fertile eggs were incubated in a Brower [4] egg incubator at a temperature of 38.5°C–39.5°C, with adequate humidity and air circulation.

Strain of Neisseria

N. meningitidis strain 1027 (group A) was used (see Greenberg & Cooper, 1965). The strain was maintained by daily transfer on nutrient blood agar plates (5% sheep red blood cells), incubated at 36°C–38°C in a carbon dioxide incubator.[5]

Diluent for bacterial suspensions

Sorensen's phosphate-buffered saline, pH 7.2, containing 1% medium 199 and 0.1% heat-inactivated absorbed guinea-pig serum, was the diluent. The serum component was prepared by pooling normal guinea-pig sera and absorbing the pool overnight in the cold with heat-killed (at 65°C for 1 hour) *N. meningitidis* (100 mg wet weight meningococci to

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[4] Manufactured by the Brower Manufacturing Co., Quincy, Illinois, USA.

[5] Manufactured by Hotpack, Waterloo, Ontario, Canada.

1 ml of serum). The absorbed pooled serum was then inactivated at 56°C for 30 minutes and kept frozen.

Inoculation and observation of embryos

Intra-yolksac (IYS), intra-allantoic (IAC), chorioallantoic membrane (CAM) and intravenous (IV) inoculations were made according to the methods of Goodpasture & Buddingh (1948) with the following modifications: IAC inoculation was made *via* the air-sac through a slit in the shell, and CAM inoculation was performed by making an artificial air-sac through a slit on the side of the shell. All shell openings were sealed with cellulose tape. The eggs were candled daily for 1 week and observed for viability.

Viable counts of organs and embryonic fluids

Allantoic fluid, blood, amniotic fluid and yolk were withdrawn at appropriate intervals (see below) by syringe, and 10-fold dilutions were made with the diluent and cultured on blood-agar medium. Pieces of organs (left lobe of the liver, a longitudinal half of the brain, including the mid-brain and cerebellum, one side of the mesonephros and of the lung, small pieces of the chorioallantoic membrane and yolksac) were weighed and homogenized in glass tissue-grinders (100 mg of tissue per 1 ml of diluent). Appropriate dilutions of these homogenates were plated for counts on the same media.

RESULTS

Age susceptibility of the chick embryo to N. meningitidis *following inoculation by different routes*

Results of several experiments are listed together in Table 1. Chick embryos 5 and 8 days old were very susceptible by all the routes tested. Ten-day-old embryos showed high susceptibility when inoculated by either the IYS or IV routes but lower susceptibility to IAC and none at all to CAM injections. Twelve-day-old embryos were susceptible only to IV inoculations in the doses used.

In order to study the optimal conditions for the establishment of meningococcal infection, the following inoculation routes were studied in greater detail because they provided the best relationship between the infecting dose of meningococci and the death of the embryos: IYS route in 10-day-old embryos, and the IV route in the 12-day-old embryos.

TABLE 1
AGE SUSCEPTIBILITY OF EMBRYOS TO *N. MENINGITIDIS* INFECTION

Age of embryo (days)	No. of micro-organisms in dose	Route of injection [a]			
		IYS	IAC	CAM	IV
5	1.4×10^{3}	5/5			
	10^{2}	5/5			
	10^{1}	5/5			
	10^{0}	1/5			
	— (diluent only)	0/5			
8	1.2×10^{7}		5/6	6/6	
	10^{6}		2/6	5/6	
	10^{5}		3/6	5/6	
	10^{4}		2/6	4/6	
	10^{3}	6/6	4/6	5/6	
	10^{2}	6/6	3/6	4/6	
	10^{1}	4/6	4/6		
	10^{0}	6/6	2/6		
	—	1/6	0/6	2/6	
10	2.5×10^{7}		4/6		
	10^{6}		4/6	0/10	
	10^{5}	5/5	2/6	0/8	8/8
	10^{4}	5/6	2/6	0/10	7/8
	10^{3}	4/6	1/6	0/10	7/8
	10^{2}	4/6	1/6		4/8
	10^{1}	3/6	2/6		4/8
	10^{0}	1/6	0/6		
	—	1/6	2/6	1/10	2/6
12	1.3×10^{7}				
	10^{6}		0/10	0/10	8/10
	10^{5}		0/8	0/8	6/10
	10^{4}		0/10	0/10	8/10
	10^{3}		0/10	0/10	7/10
	10^{2}				0/6
	—		0/10	1/10	2/10
14	1.6×10^{8}				5/10
	10^{7}				2/10
	10^{6}				2/10
	10^{5}				2/10
	—				0/6

[a] Results expressed as dead/tested.

TABLE 2

ISOLATION OF *N. MENINGITIDIS* FROM ORGANS OF CHICK EMBRYOS AT VARIOUS TIMES AFTER IYS INOCULATION [a]

Time after inoculation	Egg No.	Killed (K) or died (D)	Recovery of organisms [b] from:			
			Blood [c]	Liver [c]	Brain [c]	Yolk [c]
1 hour	1	K	−	±	+	+
	2	K	+	−	−	+
	3	K	+	±	±	+
24 hours	4	D	Not done	+++	+++	+++
	5	D	Not done	+++	+++	+++
	6	D	Not done	+++	+++	+++
	7	K	−	++	++	+++
	8	K	−	+++	++	+++
	9	K	−	+++	+++	+++
48 hours	10	K	+++	+++	++	+++
	11	K	−	+++	+	++
	12	K	−	+++	+	+++

[a] Dose: 1.8×10^4 organisms per egg. Age of embryo: 10 days.

[b] Key to symbols: − = no growth; ± = a few colonies; + = less than 50 colonies; ++ = isolated colonies, more than 50; +++ = confluent colonies.

[c] Blood (0.1 ml of the blood sample from the chorioallantoic vein) was inoculated on a plate. Liver and brain: a cut surface was stamped on a plate. Yolk: a loopful was streaked on a plate.

Intra-yolksac infection

Ten-day-old embryos inoculated *via* the IYS generally died in 1–5 days. An attempt was made to recover *N. meningitidis* from the blood, liver, brain and yolksac of these embryos in order to determine whether the tissues had been invaded and whether multiplication of the organisms had occurred. The results shown in Table 2 reveal that numerous meningococci can be recovered from the organs of dead or killed embryos 24–28 hours after the injection of 1.8×10^4 organisms.

Infection by the intravenous route

With an inoculum of 130 000–200 000 *N. meningitidis*, 12-day-old embryos began to die 6–8 hours after being inoculated. Mortality was 60% after 12 hours and 90% within 24 hours (Table 3). Here again, to determine whether multiplication of the organisms occurred before death, viable counts were made from the blood, liver, brain and allantoic fluid. Fig. 1 shows that multiplication occurred 2 hours after injection of the organisms, with peak incidence of mortality and bacterial multiplication by the

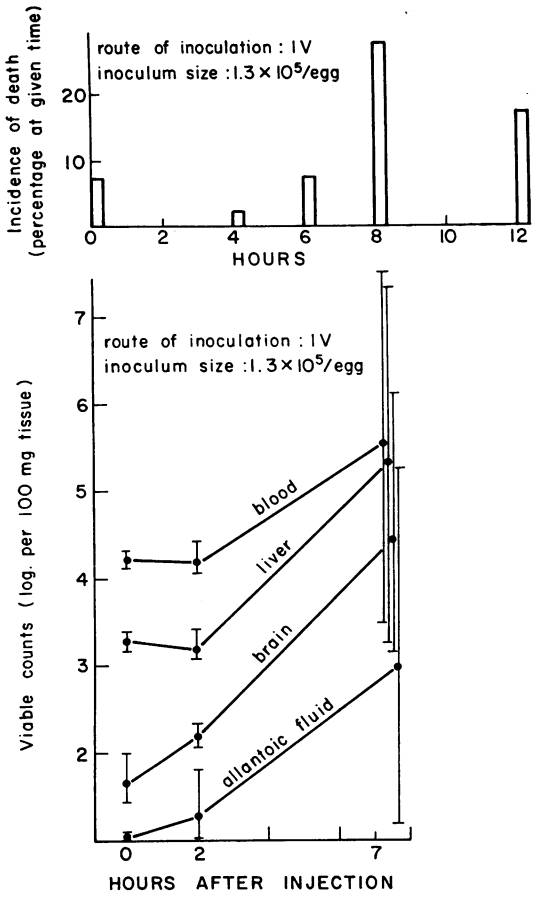
TABLE 3

TIME OF DEATH OF INFECTED EMBRYOS AFTER CHALLENGE

Experiment No.	*N. meningitidis* (inoculum size)	No. of eggs	Hours after inoculation:										
			0	3	5	7	9	11	13	15	17	21	25
I	(1.3×10^5/egg)	40	3	1	3	11	7				12		3
II	(2.0×10^5/egg)	48			22			6	4	7	6	2	1

FIG. 1

GROWTH OF *N. MENINGITIDIS* IN CHICK EMBRYO ORGANS, AND TIME OF DEATH AFTER CHALLENGE



7th hour. With one exception, the blood contained more bacteria than any other fluid or organ tested.

Distribution of N. meningitidis *in organs of intravenously inoculated embryos*

To assess more precisely the outcome of IV infections, embryos inoculated with 2.0×10^5 bacteria, and surviving until the 12th hour, were autopsied and viable counts were made (Fig. 2). A slight degree of congestion was noted in the spleen, liver and subcranial meninges; the counts indicated that the blood, liver and mesonephros contained more organisms than other fluids or organs. Embryos which survived more than 24 hours occasionally showed conspicuous haemorrhages in the subcranial meninges and mid-brain ventricles; the liver and the spleen were enlarged. Some embryos killed and autopsied after surviving for 18 hours contained more organisms in the brain than in the liver and mesonephros, which is the reverse of the finding at the 12th-hour stage of infection (Fig. 3).

DISCUSSION

Infection of embryos with *N. meningitidis* by various inoculation routes was investigated to establish the method best suited to the development of an assay for meningococcus vaccines. Embryos 5–8 days old were killed by small challenge doses, regardless of the route of administration. However, these younger embryos may have acted simply as a nutrient medium, which would therefore considerably limit the usefulness of this type of test.

Increasing the age of the embryo led to a more specific infection. Ten-day-old embryos are very susceptible to meningococci injected into the yolksac, as has been shown by Schoenback (1948), and the infection so produced has some characteristics in common with the disease in man. Multiplication of bacteria in the embryo was suggested by our experiments because of the presence of numerous organisms in the liver and brain, in spite of the low level of bacteraemia. However, it is probable that the organisms may multiply in the yolk before they reach the tissue of the developing egg. If such is the case, the use of this infection route for an effective passive protection test would be ruled out.

The IV infection route was next studied, and meningococcal infection was successfully established. Buddingh & Polk (1939) found that IV injection of *N. meningitidis* kills almost all the embryos within 24 hours, but they were unable to demonstrate the presence of organisms in the blood in the 18–24-hour period following such injection. On the basis of these results, they suggested that the meningococci do not survive for any length of time *in vivo* and that death is presumably caused by toxic products released by disintegrating organisms. Although in our experiments the embryos also died within 24 hours, multiplication of the organisms occurred before death. This is a point of considerable interest for the establishment of a sero-protective test in the chick embryo because it shows that an actual infection had in fact been established, even though the immediate cause of death might be attributed to toxic substances released by the meningococci.

Blood from embryos inoculated intravenously and killed within 12 hours contained more organisms

FIG. 2

DISTRIBUTION OF *N. MENINGITIDIS* IN THE CHICK EMBRYO 12 HOURS AFTER CHALLENGE

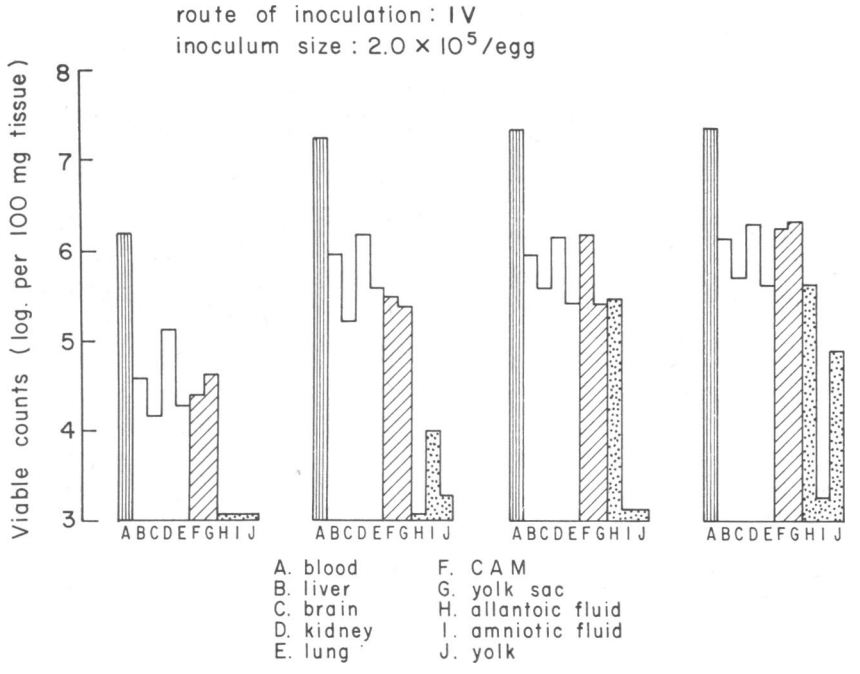
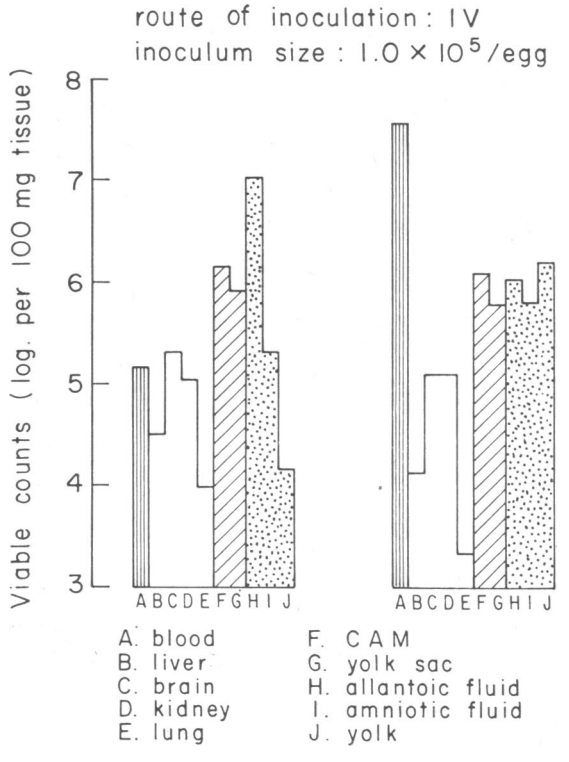


FIG. 3

DISTRIBUTION OF *N. MENINGITIDIS* IN THE CHICK EMBRYO 18 HOURS AFTER CHALLENGE



than any other organs or fluids. This infection is similar to the " septicaemia " described by Buddingh & Polk (1938) in chick embryos infected by the CAM route with a larger challenge than that used in our experiments. The distribution of the organisms revealed that the liver and the mesophrenos harbour more organisms than either the brain or lung. This may be explained by preferential trapping of phagocytized *N. meningitidis* by liver and mesonephros.

When embryos were autopsied 18 hours after infection by the IV route, meningococci were found to be localized in specific areas, namely the cranial sinuses, lung, brain and meninges, and haemorrhages were seen in the meninges and mid-brain ventricles. The selective localization of the organisms in the brain at the later stage of infection is interesting in relation to the pathogenesis of meningococcal cerebrospinal meningitis.

In conclusion, the intravenous inoculation of 12-day-old embryos is considered to be the best method to establish *N. meningitidis* infection for a seroprotection test.

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RÉSUMÉ

ÉPREUVE DE NEUTRALISATION SUR EMBRYON DE POULET POUR LA MESURE DES ANTICORPS MÉNINGOCOCCIQUES: 1. INFECTION DE L'EMBRYON PAR *NEISSERIA MENINGITIDIS*

En deux articles, les auteurs exposent leurs recherches en vue de définir un test de séroprotection permettant d'évaluer l'activité des vaccins antiméningococciques et la réponse immunitaire postvaccinale. Ils décrivent ici la technique utilisée pour réaliser l'infection par *N. meningitidis* chez l'embryon de poulet.

Les embryons âgés de 10 à 12 jours sont très réceptifs à l'infection par le méningocoque, que l'inoculation ait lieu dans le sac vitellin ou par voie intraveineuse. Dans le premier cas, la mort survient en général après 1 à 5 jours; 24 à 48 heures après l'inoculation, de nombreux méningocoques peuvent être isolés des organes de l'embryon. Si *N. meningitidis* est injecté par voie intraveineuse, le taux de mortalité des embryons est de 60% après 12 heures et atteint 90% après 24 heures. La mort survient après une phase précoce de multiplication des micro-organismes. L'examen des embryons sacrifiés à la 21e heure décèle des méningocoques dans les divers organes et liquides organiques, avec une concentration maximale dans le sang. Si l'examen nécropsique est effectué 18 heures après l'inoculation, on constate des lésions cérébrales sélectives avec présence de méningocoques.

L'inoculation de *N. meningitidis* par voie intraveineuse à des embryons de poulet âgés de 10 à 12 jours semble s'affirmer comme la meilleure méthode pour obtenir une infection se prêtant à l'exécution d'un test de séroprotection.

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