NON-SPECIFIC RESISTANCE TO EXPERIMENTAL CHOLERA IN EMBRYONATED EGGS

By RICHARD A. FINKELSTEIN, Ph.D., and JOHN P. RANSOM,* Ph.D.

(From the Division of Immunology, Walter Reed Army Institute of Research, Washington, D. C.)

(Received for publication, March 22, 1960)

Microbial antagonism has been suggested as a mechanism of natural resistance to enteric disease although documentation for this hypothesis is scarce. The evidence is derived from experiments in which suppression or alteration of the normal microbial flora has resulted in increased susceptibility of the host.

Thus, Bohnhoff *et al.* (1) made mice more susceptible to oral challenge with a streptomycin-resistant strain of *Salmonella enteritidis* by pretreatment with streptomycin. They concluded that this increase in susceptibility resulted from a disturbance of the normal intestinal microflora. Using a similar method, Freter (2–4) was able to induce an asymptomatic carrier state in guinea pigs and mice with drug-resistant strains of *Shigella* and *Vibrio cholerae.* Feeding an *Escherichia coli* strain together with the pathogen prevented this carrier state and also increased the resistance of guinea pigs to a fatal enteric infection established by oral challenge following fasting.

The developing chick embryo has found wide application in the study of experimental infections and, as emphasized by Buddingh (5) and Cox (6), much may yet be learned from this experimental system. While the chick embryo appears to be an ideal tool for the study of combined infections, it seems to have been applied only, in this regard, by Bang (7) in the swine influenza system.

The susceptibility of the chick embryo to infection with *Vibrio cholerae* was first demonstrated by Wilson (8). His observations were extended by Lankford and his students (9–11) who studied the chick embryo virulence of a large series of cultures, including colonial and antigenic variants of recent isolates from a cholera outbreak in Calcutta. Reproducible LD_{50} values were obtained by intra-allantoic inoculation of 13-day-old chick embryos provided that only deaths occurring within 24 hours are considered. There was some indication that virulence of cholera strains for the chick embryo may be correlated with pathogenicity for man.

The present investigation was undertaken to determine whether the pattern of *V. cholerae* infection in the chick embryo can be influenced by the presence of other organisms. Because of previous information indicating an effect of *E. coli*, this organism was selected for this study.

* Lieutenant Colonel, Medical Service Corps, United States Army.

Materials and Methods

Bacterial Strains.—The major portion of this work was performed with *Vibrio cholerae* strains 20-A-10 (Inaba) and 20-A-11 (Ogawa) from the Walter Reed Army Institute of Research (WRAIR) stock culture collection. These strains are derived from the National Institutes of Health reference strains 35 and 41 respectively. They have been maintained by lyophilization and, for short terms, by periodic transfer on laboratory media with special precautions to maintain colonial homogeneity as described by Lankford (12). The results have been confirmed, particularly with strains N-1 (Ogawa) and D344 (Inaba) isolated from patients in Bangkok, Thailand, during an epidemic in early 1959 and transmitted to this laboratory by Lt. Col. Oscar Felsenfeld, Medical Corps, Division of Preventive Medicine, WRAIR. Strains 20-A-10-OR_1, 20-A-10-OR_2, and 20-A-11-OR were isolated from chick embryos which survived challenge with 20-A-10 and 20-A-11 following passive immunization with rabbit antibody against the parent cultures. These strains are essentially avirulent for chick embryos and for mice (mucin challenge). Although they have retained their ability to agglutinate in type-specific sera, they now also agglutinate in anti-rough cholera sera obtained from Dr. C. E. Lankford as well as in anti-rough sera prepared in this laboratory. Their colonial morphology on 2/1 agar (12) is indistinguishable from that of their parents with the exception that the edges of their colonies tend to maintain their integrity when pushed with an inoculating needle.

A representative series of *E. coli* serotypes was obtained from Dr. Viola Mae Yong, Division of Communicable Diseases, WRAIR. Most of the work was performed with strain 9 (our designation) serotype 0111-B4. The *E. coli* stocks were maintained on meat extract agar (MEA)[1] slants under sterile mineral oil and on fresh MEA plates.

Suspensions (both of *V. cholerae* and *E. coli*) for inoculation into chick embryos were prepared by suspending the 18 to 20 hour growth from a heavily streaked Petri dish of MEA or brain-heart infusion agar in 10 ml. sterile saline followed by serial decimal dilutions. Viable counts were determined by the pour plate technique with MEA or trypticase soy agar or by a modification of the drop count method (13).

Chick Embryos.—Antibiotic-free, pullorum clean White Leghorn chick embryos were used. The eggs were incubated at 37°C. on their sides on racks in a walk-in incubator room humidified by means of open basins of water. Despite these crude methods, control eggs maintained their viability and hatched under these conditions if kept to the end of the incubation period. The spontaneous death rate during the experimental periods was insignificant. Usually the embryos were 13 days old at time of challenge with cholera vibrios. They were inoculated allantoically with 0.1 ml. of the challenge suspensions through a small hole punched in the shell over an avascular area by means of a dental pick ground to a point or a diamond point scriber. Ordinarily, ten eggs were inoculated with each dilution for LD_{50} determinations. After inoculation, the holes were sealed with collodion or vaspar. Samples from representative infected embryos were streaked as routine on 2/1 agar or other media when indicated, to verify the purity of the infecting strain.

For differential viable counts, the whole egg was dipped in ethanol, flamed, and dropped into a Waring blendor container and homogenized. Samples removed from the blendor were diluted and plated. Since, in certain cases, an overwhelming ratio of *E. coli* to *V. cholerae* was expected, counts of both organisms were accomplished simultaneously by plating appropriate dilutions on eosin–methylene blue agar for the coli count and on the medium described by Felsenfeld and Watanabe (14) at pH 9.0 for counting the *V. cholerae*. Preliminary experiments revealed that *V. cholerae* counts were equivalent on this medium and a non-selective medium (MEA) tested in parallel, while the *E. coli* strains tested did not form visible colonies on

[1] MEA, meat extract agar

Felsenfeld and Watanabe's medium in 2 days. The presence of large relative numbers of *E. coli* had no significant effect on the cholera viable count and *vice versa*.

Endotoxin.—Shigella flexneri 2a endotoxin was obtained from Dr. S. B. Formal, Division of Immunology, WRAIR and *E. coli* endotoxin preparation 36 was obtained from Dr. Howard Noyes, Division of Surgery, WRAIR. Both preparations were extracted from acetone-treated cells with trichloracetic acid and further purified by NaCl-ethanol fractionation (15). The LD_{50} of the former for Bagg strain mice by the intraperitoneal route was approximately 132 γ; the latter was 290 γ. An additional sample of *Shigella flexneri* 2a endotoxin, prepared by pyridine extraction, was obtained from Dr. Eugene LaBrec, Division of Immunology, WRAIR.

EXPERIMENTAL

Characteristics of V. cholerae Infection in Embryonated Eggs.—The 13-day-old embryo was selected after preliminary trials as being most suitable for subsequent experiments. At this age, the chick embryo occupies a position of intermediate susceptibility to death following allantoic inoculation with *V. cholerae*

TABLE I

Virulence of V. cholerae, 20-A-10, for 11-, 13-, and 15-Day-Old Chick Embryos

Age of embryo	LD_{50}	
	After 1 day	After 3 days
11	$<2 \times 10^1$	$<2 \times 10^1$
13	6.6×10^3	$<6 \times 10^1$
15	$>5 \times 10^8$	2.75×10^7

(Table I). The 11-day-old embryo is extremely susceptible and dies within 24 hours following minimal challenge. From a number of experiments, it appears that one vibrio of the tested strains is capable of infecting and killing embryos of 11 and 13 days of age within 3 days. However, the 13-day egg is considerably more resistant than the 11-day egg to death during the 1st day after challenge. The 15-day egg is markedly resistant; deaths within 3 days occur regularly only at high levels of inoculum and frequently are not in proportion to the inoculum size at lower levels. These observations are in good accord with those of Lankford and his associates (9–11).

Despite the difference in the outcome of the infection, there is little difference in the growth pattern of *V. cholerae* in the eggs of different ages (Fig. 1). There appears to be a slightly increased lag in 15-day eggs and the generation time (34 minutes) of the vibrios during the logarithmic phase in the 15-day egg is somewhat slower than that in the 13-day egg (24 to 27 minutes), but the maximal cell yield is comparable in eggs of all tested ages. Likewise, there is little, if any, difference in count in eggs which succumb to the infection and eggs which would be expected to survive. Thus it appears that the response or

state of the host may have greater weight in determining the outcome of the infection than the bacterial population *per se*, although it is possible that there may be differences in the actual site of vibrio localization in the embryonated eggs. Occasionally, individual 13-day embryos survive which have received many lethal doses of vibrios. Eggs which survive usually contain large numbers

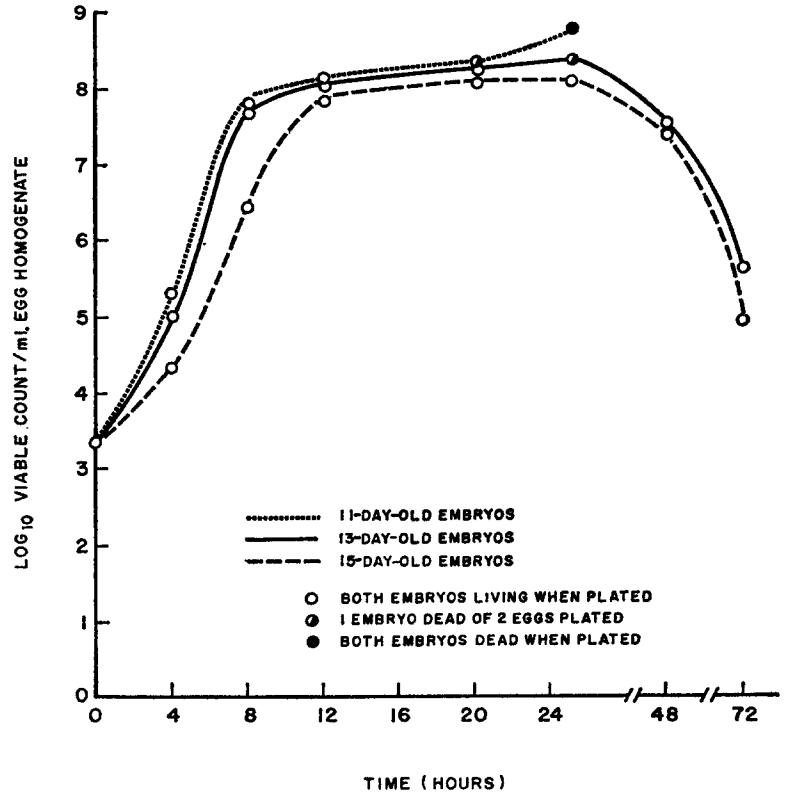

FIG. 1. Growth of *V. cholerae* in 11-, 13-, and 15-day-old embryonated eggs (each point is the log of the mean of determinations on two embryos per time interval except points at 48 and 72 hours which are derived from one egg each).

of viable vibrios, although occasionally they appear to have disposed of the infection.

Virulence of E. coli Strains for Chick Embryo.—Initially, twenty strains of *E. coli* including several "enteropathogenic" serotypes were screened for virulence for the 11-day-old chick embryo. Eggs were inoculated allantoically with approximately 10^5 cells of each strain. The results (Table II) indicate that there is no correlation between the ability of a strain to cause death of the 11-day-old chick embryo and its ability to cause disease in human beings (on

the basis, at least, of possessing antigenic formulae associated with enteropathogenicity).

Effect of E. coli on V. cholerae Infection in the Chick Embryo.—The embryos surviving 48 hours after challenge with the relatively avirulent *E. coli* strains (indicated by § in Table II) were inoculated with approximately 10^5 *V. cholerae* (20-A-10). In each case, some embryos survived for 3 days following the cholera challenge while control eggs inoculated at the same time with *V. cholerae* alone were all dead by the 2nd day. Strain 9 seemed particularly effective in protecting against the cholera challenge in this preliminary trial and was selected for further study. This strain occasionally kills some chick

TABLE II

*Virulence of E. coli strains for 11-day-old chick embryos**

Strain No.	Serotype	Chick embryo mortality			Strain No.	Serotype	Chick embryo mortality		
		1 day	2 day	3 day			1 day	2 day	3 day
1	08	3/5‡	5/5		14	07	5/5		
2	06	5/5			15	09	0/5	1/5§	—
6	055:B5:2	5/5			16	076	2/5	4/5	5/5
7	055:B5:2	3/5	4/5	5/5	17	0126	1/5	4/5	4/5
8	0111:B4:2	1/5	2/5	3/5	18	086:B7	0/5	2/5	5/5
9	0111:B4:2	0/5	0/5§	—	19	03	4/5	5/5	
10	0127:B8	2/5	3/5	3/5	20	0112	0/5	1/5§	—
11	0127:B8	1/5	3/5	3/5	21	07	0/5	1/5§	—
12	0114	0/5	3/5	3/5	22	018	2/5	3/5	3/5
13	02	5/5			23	019	0/5	3/5	4/5

* Inoculated allantoically with approximately 10^5 cells.

‡ No. dead/no. challenged.

§ Surviving embryos challenged with *V. cholerae* (see text).

embryos. This effect varies somewhat from experiment to experiment and is not dependent on dosage. Generally, less than 10 per cent of the inoculated eggs are killed and no attempt has been made to interpolate this factor in subsequent results.

When graded doses of *E. coli* (strain 9) were administered to 13-day-old chick embryos immediately prior to challenge with *V. cholerae*, there was no evidence of protection. This was true even when the coli:cholera inoculum ratio was as high as 10^4. However, a definite relationship was found between the time of administration of the preparatory *E. coli* inoculation (approximately 5×10^4 cells) and subsequent resistance to challenge with *V. cholerae* (Table III). The data show: (*a*) a trend toward increasing resistance with increasing time before the cholera challenge; and (b) a significant degree of resistance when coli was administered 24 hours prior to cholera challenge. This appears to

diminish with a further increase (to 48 hours) in the interval before cholera challenge.

Groups of twenty 12-day-old embryos were inoculated with approximately 10^5 cells of six additional *E. coli* strains of low virulence, including a streptomycin-resistant variant (9SR) of strain 9, as well as three avirulent "OR" cholera strains. The eggs which died during the first 24 hours were discarded and those remaining for each strain were divided into two groups. One group was not further treated (to serve as a control on virulence of the preparatory infection) while the other was challenged with approximately 4.6×10^4 *V. cholerae*, 20-A-10.

It is evident (Table IV) that protection against cholera challenge was afforded by all the strains tested. It is likely that the degree of protection was greater

TABLE III

Mortality of 13-Day-Old Chick Embryos Challenged with V. cholerae at Intervals Following E. coli Infection

Hrs. after *E. coli* infection	Experiment 1*		Experiment 2‡	
	Mortality§	Per cent	Mortality	Per cent
0 (cholera control)	10/10	100	15/20	75
0	15/15	100	20/20	100
3	7/10	70	17/20	85
6	6/10	60	17/20	85
12	n.d.	n.d.	12/20	60
24	1/15	6.7	1/20	5
48	3/5	60	3/20	15

* Cholera inoculum approximately 10^5.

‡ Cholera inoculum approximately 7×10^3.

§ No. dead at 3 days following cholera challenge/total.

than is revealed by this data since a proportion of the doubly infected eggs undoubtedly succumbed to the preparatory infection.

Magnitude of Protection Afforded by Previous E. coli Infection.—The LD_{50} of *V. cholerae* strain 20-A-10 was compared in eggs infected 24 hours prior to challenge and in normal controls. The LD_{50} of this strain in coli-infected eggs was 4.15×10^5 while simultaneously the 50 per cent lethal dose for normal 13-day eggs was less than 2.5 organisms. Thus the magnitude of the protective effect is of the order of 10^5 and possibly greater since a proportion of the eggs infected with both organisms died of the coli infection.

Growth of V. cholerae in E. coli-Infected Eggs.—

Differential viable counts were performed at intervals on three groups of 13-day eggs inoculated as follows:

1. *V. cholerae*, 20-A-10.
2. *V. cholerae* in eggs infected 24 hours previously with *E. coli*.
3. Eggs inoculated only with *E. coli*.

The results, plotted in Fig. 2, which are based on two eggs harvested from each group at the indicated intervals, disclose that growth of the vibrios (in eggs previously infected with *E. coli*) is suppressed for a period of about 18 hours. The count then rose until, at 48 hours, it was equal to (or somewhat greater than) that in control eggs which all succumbed to the infection before 48 hours had elapsed. The *E. coli* count had reached a level of about 10^8/ml. before the cholera inoculum was added and remained relatively constant thereafter in both singly infected eggs and the eggs superinfected with cholera and, for this reason, is not indicated in Fig. 2. These results indicate that the protective effect of *E. coli* infection is associated with a temporary suppression of the cholera infection.

TABLE IV

Protective Effects of Additional E. coli Strains of Low Embryo Virulence and "OR" V. cholerae Variants

Preparatory infection	Chick embryo mortality*	
	Cholera challenged	Control
None	14/15	—
E. coli 9SR	2/10	1/5
E. coli 15	3/10	3/9
E. coli 18	6/10	5/8
E. coli 20	4/10	1/8
E. coli 21	4/10	2/8
E. coli 23	5/10	1/7
V. cholerae, 20-A-10-OR_1	0/10	0/10
V. cholerae, 20-A-10-OR_2	1/10	3/9
V. cholerae, 20-A-11-OR_1	2/10	2/8

* No. dead 3 days' postchallenge/No. challenged.

Failure of V. cholerae to Develop Resistance to the Protective Mechanism of the Embryo.—Cholera strains were isolated from eggs which died following the combined infection of the previous experiment. Their virulence was compared in coli-infected and control eggs with that of cholera strains isolated previously from singly infected eggs. These experiments showed that the secondary rise in cholera count (Fig. 2) was not attributable to the selection and emergence of a resistant population since in each case the protective effect of the *E. coli* infection was still manifested.

Bacterial Antagonism.—

Thirty-four strains of *E. coli*, including strain 9 and a streptomycin-resistant variant derived from it, were tested by the technique of Fredericq (16) for their ability to produce factors *in vitro* which would inhibit the growth of *V. cholerae*, 20-A-10. The strains were "spot inoculated" on MEA plates which were incubated for 24, 48, and 72 hours. The plates were than exposed to chloroform vapor for 1 hour to kill the bacteria. After further incubation (2 hours) to dispel absorbed chloroform, a layer of MEA seeded with *V. cholerae* was added to each plate.

No antagonistic factors produced by strain 9 or any of the other coli strains could be demonstrated, nor did two "OR" strains of *V. cholerae* produce inhibitors. These results are reminiscent of those of Freter (3) who likewise detected no *in vitro* inhibitory factor with his *E. coli* strain.

Resistance-Promoting Effect of Heat-Killed E. coli Cells and Purified Endotoxins.—

Twelve-day-old eggs were inoculated with varying amounts of heat-killed (60°C./30 minutes) *E. coli* cells or endotoxin (No. 36) and were challenged the next day with approximately 10^4 *V. cholerae*, 20-A-10.

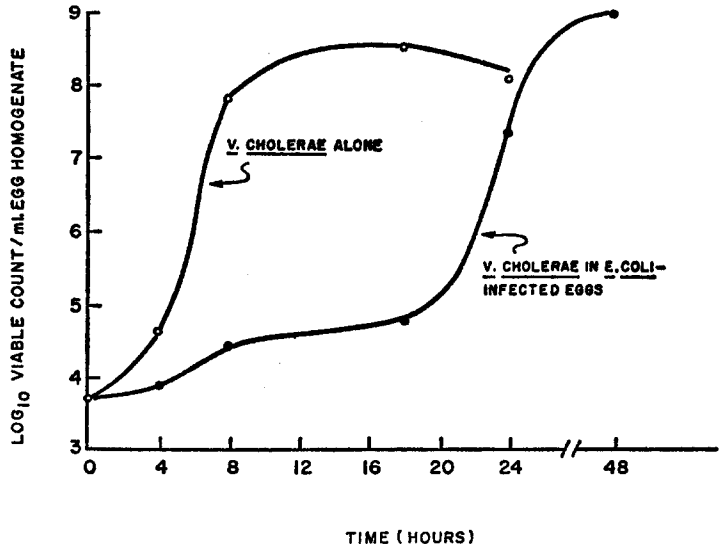

FIG. 2. Effect of *E. coli* infection on growth of *V. cholerae* in 13-day-old embryonated eggs (each point is the log of the mean of determinations on two embryos per time interval).

The composite results of three experiments, plotted in Fig. 3, indicate that the optimal level of endotoxin for protection is approximately 1 microgram. Less protection is obtained when higher or lower levels of endotoxin are used. The killed coli elicited a similar response. Maximal protection was obtained with approximately 3×10^7 dead cells.

Based on the assumption (17) that endotoxin constitutes 10 per cent of the dry cell weight (10^9 cells = approximately 400 μg. (18)), calculations indicate that the effect of heat-killed cells can be correlated closely with their endotoxin content. Two *Shigella* endotoxin preparations also enabled chick embryos to survive cholera challenge. Of these, a pyridine extract preparation was somewhat less effective than the *E. coli* endotoxin and required about 10 γ to elicit maximum protection. A clear-cut dose-response relationship could not be

established with the other *Shigella* preparation produced by the same technique as the *E. coli* endotoxin.

To determine the most favorable time for the administration of endotoxin, groups of ten 13-day-old embryos were challenged with 10^3 vibrios at 0, 3, 6, 12,

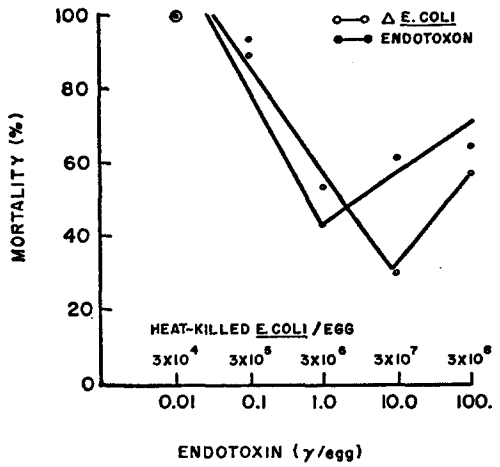

FIG. 3. Effect of heat-killed *E. coli* cells and endotoxin on mortality of 13-day-old chick embryos inoculated with *V. cholerae*.

TABLE V

Effect of Time of Administration of Endotoxin on Mortality of Chick Embryos Challenged with V. cholerae, 20-A-10‡*

Time of administration (hrs. before challenge)	Deaths/total (3 days' postchallenge)
0	9/10
3	7/10
6	9/10
12	2/10
24	4/10
(Saline control) "	10/10

* Preparation 36, 1 γ/egg.

‡ Approximately 10^3 cells.

and 24 hours after they had received 1.0 γ of *E. coli* endotoxin. This dose of vibrios again caused 100 per cent mortality of control eggs (Table V). Marked protection was obtained when endotoxin was administered 12 hours prior to challenge and this time appeared to be optimum.

It was of interest to determine whether cells of a Gram-positive species could elicit a similar protective response. There was no evidence of protection in eggs

which received preparatory injections of a staphylococcus vaccine (heat-killed cells of a serological Type I strain, 8530, which was obtained from Dr. Betty Hobbs, Colindale, England) at two dosage levels 24 hours prior to challenge.

Effect of Embryonic Age on Endotoxin Response.—Groups of 8- and 10-day-old embryos given 2 γ of coli endotoxin (No. 36) were challenged the next day with graded doses of cholera vibrios which were simultaneously inoculated into parallel groups of control eggs of equal age. The results depicted in Fig. 4 indicate that the 8-day-old embryo is capable of a response to endotoxin which delays but does not prevent the lethal outcome of subsequent cholera infection. Endotoxin-treated 10-day-old embryos reacted similarly.

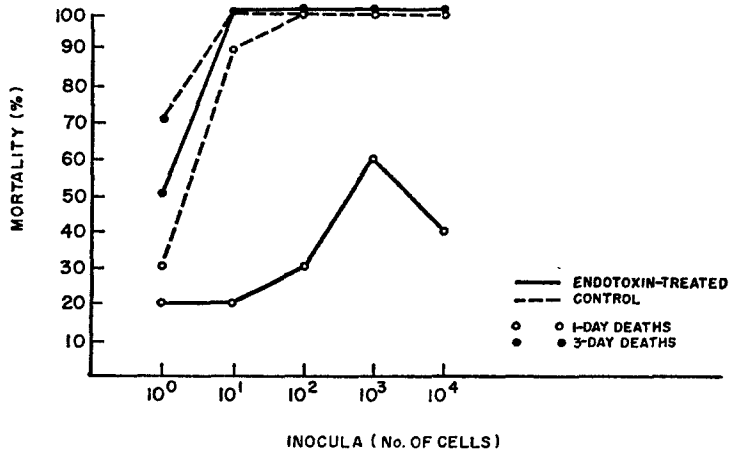

FIG. 4. Effect of previous administration of endotoxin on mortality of 9-day old embryos inoculated with *V. cholerae.*

DISCUSSION

This investigation has shown that prior infection with *E. coli* has a marked effect on subsequent cholera infection of the chick embryo. In the course of establishing this information, a number of points have been revealed which merit further consideration.

The development of resistance to fatal cholera infection with increasing age of the embryo poses an interesting problem which is presently being investigated. A number of anatomical, physiological, and metabolic changes are occurring (19, 20) which may be related to the resistance or reduced susceptibility of the 15-day embryo. Smith and Thomas (21) demonstrated that the age of the embryo has an effect on its sensitivity to endotoxin. They found that sensitivity to endotoxins from several sources was maximal in the 10-day-old embryo, while younger or older embryos showed a lesser or no response. However, endotoxin does not appear to be the primary cause of death in the cholera infection described here, since (*a*) chick embryos challenged with

cholera on the 13th day die during the ensuing 3-day period while their resistance to endotoxin is becoming maximal; (*b*) while Smith and Thomas found that cortisone would protect the chick embryo against endotoxin, cortisone accelerates the death of 13-day-old embryos challenged with *V. cholerae* (22); and (*c*) large doses of killed vibrios (11, 22), and culture filtrates (22) fail to kill 13-day-old chick embryos. An indirect role of cholera endotoxin is indicated, however, by the avirulence of serologically rough cholera vibrios. It appears likely that the ability to kill a chick embryo is an attribute of the living smooth cholera vibrio and its products. Since the resistance of the 15-day embryo is apparently not antibacterial (Fig. 1), one may infer that it is founded on lack of sensitivity to products of the growing vibrios. In this regard, it is attractive to consider that the younger-than-15-day-old embryo is in a "sensitive period of development" (23) with respect to cholera infection. For example, the data of Romanoff and Bauernfeind (24) indicate that chick embryos from riboflavin-deficient hens exhibit three defined peaks of mortality, during which the vital processes of the developing embryo are apparently most sensitive to the insult of riboflavin deficiency. By analogy, it is conceivable that the processes of the younger embryo are sensitive to the insult of cholera infection, while the older embryo is relatively unaffected. On this basis, one should search for metabolic differences between the 13-day- and 15-day-old embryos. It is also possible, however, that the 15-day embryo has developed a "detoxification" mechanism. The observation of Green and Lorincz (25) that γ-globulin containing natural antibody (to mouse tumor cells) appears in significant concentration in the embryonic circulation about the 17th day does not seem to be related to the present problem since there is no agglutinin detectable in the serum of hatched chicks (22) and neither complement nor properdin could be detected in a single pool of serum of 16-day-old embryos (26).

The original premise that *E. coli* might alter the course of *V. cholerae* infection in the chick embryo by a direct mechanism of microbial antagonism was not substantiated in this study. The absence of detectable antagonistic factors in *in vitro* tests and the necessity for a preliminary incubation period, even when overwhelming numbers of coli were administered, indicated rather that the protective effect was mediated by the host. This was further substantiated by the observations that heat-killed coli cells and endotoxin in a calculable quantitatively similar fashion elicited the same protective response. It is uncertain what bearing these observations may have on the systems described by Freter (4). It has been well established (27, 28), however, that small amounts of microbial endotoxins have profound effects on the susceptibility of experimental animals to bacterial infections. The present study reveals that the chick embryo has the capacity to respond to small doses of endotoxin with a phase of enhanced resistance similar to that reported for mature animals. This may actually have been the basis for the experimental observation of Barnes and Stacy (29) that chick embryos showed increased resistance to *Shigella flexneri 3* infection following administration of homologous vaccines. Unfortunately, the latter workers did not fully explore the nature of this response.

The protective effect of *E. coli* or endotoxin appears to be due, at least in part, to the induction of a phase characterized by vibriostasis. Whether this is due to inhibition of growth or an enhanced clearance is still under study. In

the case of the 13-day-old embryo, the vibrios eventually do emerge and develop to essentially the same number as in control eggs, but the embryos survive. The prolonged lag may serve to allow the embryo to pass into the stage of increased resistance associated with increasing age, although it is possible that additional detoxification mechanisms are evoked. It is of interest in this regard that the response of the 8- and 10-day-old embryo is limited to an increase in survival time.

The ability of many strains of *E. coli* to kill chick embryos was an ancillary finding which was not surprising since the organism is capable of causing fatal infections in animals and man under unusual conditions. It is unfortunate that the chick embryo apparently does not discriminate between enteropathogenic strains and other varieties. However, such features as the pathology of the infection, possible differences in LD_{50} of virulent strains, and comparisons of properties of embryo-virulent and avirulent strains do merit more detailed investigation.

This investigation emphasizes the opportunities still offered by the embryonated egg as a tool for the study of infectious processes, the host-parasite relationship and the mechanisms of natural resistance.

SUMMARY

It has been confirmed that 11- and 13-day-old chick embryos are susceptible to lethal infection with minute inocula of *V. cholerae*, while 15-day-old embryos are relatively resistant.

Twenty strains of *E. coli* were found to vary in their capacity to kill chick embryos, without relationship to their human enteropathogenicity. Prior infection of 13-day-old embryonated eggs with *E. coli* strains selected for low embryo virulence had a marked protective effect against superinfection with *V. cholerae*.

This effect was duplicated by pretreatment of embryos with killed *E. coli* cells or endotoxin preparations but not with a suspension of killed cells of a Gram-positive species. Preparatory *E. coli* infection induces a phase of vibriostasis in the 13-day-old egg which may be sufficient to tide the embryo over into the phase of relative insusceptibility associated with age. Younger embryos exhibit only a lag in death under similar conditions.

The chick embryo is presented as a potentially valuable tool for the study of combined infections and of the mechanisms of natural resistance.

The authors acknowledge, with gratitude, the excellent technical assistance of Mr. Calvin Powell, Mr. Giles White, and Mr. Howard Bohner during phases of this investigation. In addition, we wish to thank Lt. Col. A. S. Benenson, Medical Corps, and Dr. S. B. Formal, Division of Immunology, Walter Reed Army Institute of Research for their constructive criticism of the manuscript.

BIBLIOGRAPHY

1. Bohnhoff, M., Drake, B. L., and Miller, C. P., Effect of streptomycin on susceptibility of intestinal tract to experimental *Salmonella* infection, *Proc. Soc. Exp. Biol. and Med.*, 1954, **86,** 132.
2. Freter, R., The fatal enteric infection in the guinea pig, achieved by inhibition of normal enteric flora, *J. Infec. Dis.*, 1955, **97,** 57.
3. Freter, R., Experimental enteric Shigella and Vibrio infections in mice and guinea pigs, *J. Exp. Med.*, 1956, **104,** 411.
4. Freter, R., Coproantibody and bacterial antagonism as protective factors in experimental enteric cholera, *J. Exp. Med.*, 1956, **104,** 419.
5. Buddingh, G. J., The Pathological Effects of Viruses on the Chick Embryo, *Ann. New York Acad. Sc.*, 1952, **55,** 248. Bacterial and mycotic infections of the chick embryo, *Ann. New York Acad. Sc.*, 1952, **55,** 282.
6. Cox, H. R., Growth of viruses and rickettsiae in the developing chick embryo, *Ann. New York Acad. Sc.*, 1952, **55,** 236.
7. Bang, F. B., Synergistic action of *Hemophilus influenzae suis* and the swine influenza virus on the chick embryo, *J. Exp. Med.*, 1943, **77,** 7.
8. Wilson, A. T., Experimental vibrio infections of developing chick embryos, *J. Exp. Med.*, 1946, **84,** 293.
9. Hagens, S. J., Studies of variation of *Vibrio cholerae*, M.A. Thesis, Library of The University of Texas, Austin, 1953.
10. Gardner, E. W., Studies of the virulence of *Vibrio cholerae* for the chick embryo, M.A. Thesis, Library of The University of Texas, Austin, 1954.
11. Lyles, S. T., Studies of the antigenic structure of *Vibrio cholerae*, Dissertation, Library of The University of Texas, Austin, 1955.
12. Lankford, C. E., The serologic identification of *Vibrio cholerae*, *J. Microbiol. Soc. Thailand*, 1958, **2,** 93.
13. Miles, A. A., Misra, S. S., and Irwin, J. O., Estimation of bactericidal power of blood, *J. Hyg.*, 1938, **38,** 732.
14. Felsenfeld, O., and Watanabe, Y., An alkaline tellurite lauryl sulfate-salt plate for the isolation of *Vibrio comma*, *United States Armed Forces Med. J.*, 1958, **9,** 975.
15. Noyes, H. E., Pulaski, E. J., and Balch, H. H., The detection of bacterial endotoxins in the plasma of dogs with experimental peritonitis, *Ann. Surg.*, 1956, **144,** 51.
16. Fredericq, P., Colicins, *Ann. Rev. Microbiol.*, 1957, **11,** 7.
17. Burrows, W., Endotoxins, *Ann. Rev. Microbiol.*, 1951, **5,** 181.
18. Abrams, A., 1959, personal communication.
19. Hamilton, H. L., Lillie's Development of the Chick, New York, Henry Holt and Company, 3rd edition, 1952.
20. Conference on the Chick Embryo in Biological Research, *Ann. New York Acad. Sc.*, 1952, **55,** 37.
21. Smith, R. T., and Thomas, L., The lethal effect of endotoxins on the chick embryo, *J. Exp. Med.*, 1956, **104,** 217.
22. Finkelstein, R. A., unpublished data.

23. Hamilton, H. L., Sensitive periods during development, *Ann. New York Acad. Sc.*, 1952, **55**, 177.
24. Romanoff, A. L., and Bauernfeind, J. C., Influence of riboflavin deficiency in eggs on embryonic development (*Gallus domesticus*), *Anat. Rec.*, 1942, **82**, 11, (cited by Hamilton (23)).
25. Green, H., and Lorincz, A. L., The role of a natural antibody in the rejection of mouse tumor cells by the chick embryo, *J. Exp. Med.*, 1957, **106**, 111.
26. Hook, W. A., personal communication.
27. Landy, M., and Pillemer, L., Increased resistance to infection and accompanying alteration in properdin levels following administration of bacterial lipopolysaccharides, *J. Exp. Med.*, 1956, **104**, 383.
28. Dubos, R. J., and Schaedler, R. W., Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins, *J. Exp. Med.*, 1956, **104**, 53.
29. Barnes, L. A., and Stacy, I. B., Observations on immunity to *Shigella* infections in embryonated eggs, *Naval Med. Research Inst. Rep.*, 1955, **13**, 621.