AN UNIDENTIFIED PLEUROPNEUMONIALIKE ORGANISM ISOLATED DURING PASSAGES IN CHICK EMBRYOS[1]

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Since a large number of developing eggs are employed in the making of vaccines and the propagation of viruses and rickettsiae, it is of primary importance to detect any spontaneous infection occurring in chick embryos, especially with those agents that are filterable. In the course of passing a strain of the virus of primary atypical pneumonia amniotically in the developing chick embryo (Eaton, Meiklejohn, and van Herick, 1944) an unrelated organism filling many of the criteria for the pleuropneumonia group has been isolated. A suspicion that a contaminating agent had been encountered was raised by the following evidence: (a) sudden increase in the number of deaths of the chick embryos; (b) marked changes of the amnion, amniotic fluid, and embryo itself not previously seen in infections with the primary atypical pneumonia virus; (c) the sudden increase in virulence of the virus for the cotton rat and loss of virulence for the hamster; and (d) the finding of the organisms on microscopic examination of stained preparations from the embryos.

The organism has been isolated on only one occasion, and therefore it is difficult to state its origin with certainty. It has not been isolated during passages of other strains of the same virus and was not isolated again after repeated passages when the earlier "uncontaminated" passages of the strain with which it was originally associated were used as starting material.

Cultural Methods and Characteristics

In adapting the organism from the amniotic membrane of the developing chick embryo to horse serum broth, a 0.5 per cent glucose beef heart infusion hormone broth with a 10 per cent concentration of filtered horse serum was used. This medium was employed for all subsequent passages. The first passages were allowed to incubate for 4 to 6 days before being passed, at which time there was no visible turbidity; in fact, there sometimes was none for 2 or 3 passages. However, when turbidity became visible passages could be made at 24 to 48 hours. When the organism was inoculated into glucose hormone broth without added horse serum, no growth occurred. In all passages an uninoculated horse serum infusion hormone broth was incubated along with the inoculated media, serving as a comparison for turbidity and as a test for the sterility of the added horse serum.

With well-adapted cultures in glucose hormone infusion horse serum broth,

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incubated at 37 C for periods longer than 4 days, the organisms tended to become nonviable. This may, in all probability, have been due to the fermentation of the glucose with a consequent reduction in pH. This phenomenon has been observed with type A pleuropneumonialike organisms of mice (Sabin, 1941). The organisms retained their viability for longer periods of time in plain hormone infusion horse serum broth. They remained viable in broth cultures at 4 C for at least 2 months, and as suspensions of infected embryo tissue at −70 C for at least 11 months.

For plate growth, 15 per cent horse serum agar plates were used and incubated in tight containers to prevent loss of moisture from the media. Minute colonies best seen with a hand lens made their appearance in approximately 5 days. Colonies were opaque, with convex smooth edges and raised rhizoid centers. Passages were made by cutting small squares of agar with colonies present, transferring to a new plate, and streaking back and forth.

Morphology in Cultures

The organisms stained by the Giemsa method from glucose beef heart horse serum broth had, generally, the appearance of distinct elementary bodies. As the culture aged the elementary bodies tended to form large colonies or clusters which at times were interwoven with faintly visible, short, fine filaments. Other polymorphic structures, such as commas and short bacillary forms, sometimes were encountered. Rarely there were a few larger spherical or ring forms.

Only with a heavy suspension of the organism was the Gram method of staining effective and then poorly, with the great majority of the organisms not stained at all. Those that were stained were gram-negative. The organisms stained slightly with methylene blue, but were difficult to differentiate from the amorphous material present. Direct microscopic examination of unstained cultures revealed amorphous clumps without any differentiated morphology. However, under dark-field illumination the colonies could be seen to comprise numerous elementary bodies.

Appearance and Transmission in the Developing Chick Embryo

The agent was readily transmitted by the amniotic route in the developing egg. The optimum incubation period was 5 days, using 12-day incubated eggs for inoculation. Infection was manifested by small opaque areas throughout the amniotic membrane and by thickening of the amniotic membrane with white granular formations, incorporating an abundance of organisms, adhering to the membrane or the embryo. The embryos were often stunted and the amniotic fluid cloudy. One or more of these changes were manifested in the infected egg.

Filtration Experiments

Repeatedly the organism has been successfully passed through a Berkfeld N filter which retained a suspension of *Chromobacterium prodigiosum*. Inoculum

in amounts of 0.5 ml was used in passages to horse serum broth cultures. However, repeated attempts to pass the organism through a Seitz filter have failed.

Pathogenicity for Animals

The animals used for susceptibility experiments were cotton rats (*Sigmodon hispidus eremicus*, *Sigmodon hispidus hispidus*, and *Sigmodon hispidus littoralis*), Syrian hamsters (*Cricetus auratus*), white rats, white mice, guinea pigs, and

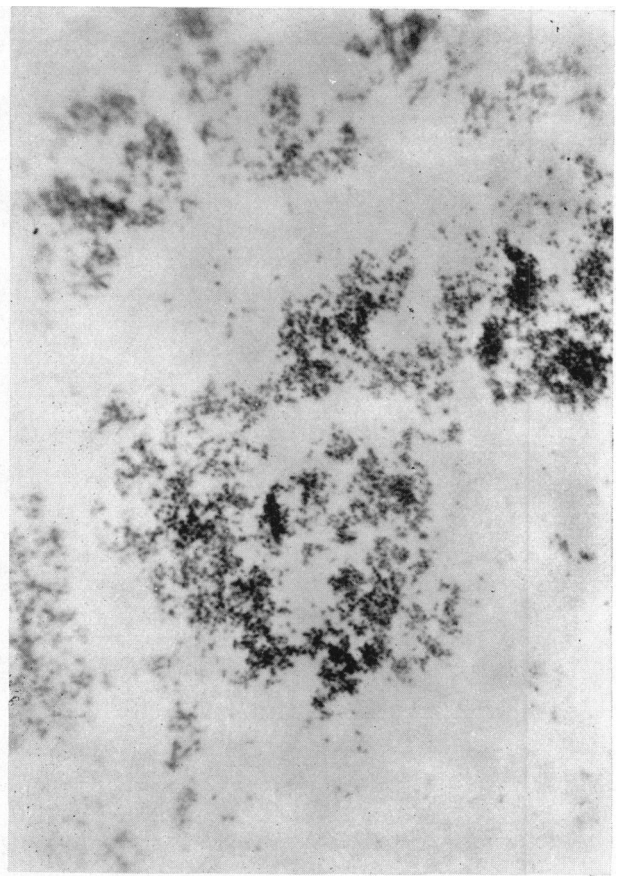

FIG. 1. A TWO-DAY-OLD SERUM GLUCOSE BROTH CULTURE STAINED WITH GIEMSA. × 1510.

field mice (*Microtus californicus*). White leghorn chickens of 2 and 10 weeks of age were used. All animals were inoculated separately by the intranasal, intracerebral, and intraperitoneal routes. Some of the chickens were sacrificed at 8 days and some were allowed to go for more than 30 days. The chickens sacrificed at 8 days were normal. However, of 8 chickens allowed to go for a longer period, 5 that had had intracerebral, intranasal, and intramuscular injections developed definite weakness of the legs 26 days after inoculation. One of the 2 chickens inoculated intranasally developed, in addition to weakness of the legs, tremors above the shoulders and ataxia. This last chicken and one of

the former were sacrificed at this time, but they showed no gross pathological changes. Attempts to recover the organism from various organs failed. The other 3 chickens recovered their usual appearance after 3 to 5 days and appeared perfectly normal thereafter.

The animal that showed the greatest susceptibility was the cotton rat, when inoculated by the intranasal route only. Cotton rats thus inoculated generally died in 2 to 3 days with extensive red edematous consolidation of the lungs extending to all lobes. Subsequent passages of a broth suspension of these lungs intranasally into cotton rats have failed to produce similar lesions. Both embryo suspensions and cultures of the organism produced similar lung lesions in cotton rats. Smaller pulmonary lesions without death were seen in 3 out of 7 white rats inoculated intranasally and sacrificed at 3 days. Inoculations by intracerebral and other routes were without effect.

Further investigation revealed that a Seitz filtrate of a 48-hour horse serum broth culture caused similar pulmonary consolidation in cotton rats, with death of the animals. However, the Seitz filtrate when inoculated into horse serum glucose broth failed to produce growth. Intranasal inoculation of cotton rats with a Seitz filtrate of a suspension of infected amnion caused the same phenomena, and the filtrate was shown to be sterile by three passages in chick embryos.

In preliminary experiments neutralization of this lung poison with hyperimmune rabbit serum was not accomplished when the serum culture mixtures were inoculated intranasally into cotton rats. Animals that recovered from sublethal doses did not survive reinoculation with a larger, lethal dose. The poison was not appreciably inactivated by heating to 90 C for 30 minutes. The lack of antigenicity and the stability to heat indicate that this substance is not a true toxin.

Serological Experiments: Methods and Results

Agglutination tests were done with the serum of a rabbit hyperimmunized by intraperitoneal inoculation at intervals of 3 to 4 days with a suspension of the organism grown in rabbit serum glucose hormone broth. An antigen was made from a 48-hour culture by centrifuging at a high speed and resuspending the sediment in saline to the proper turbidity. In setting up the agglutination test, 0.2 ml of this antigen was added to 0.2 ml of serum dilution and incubated for 2 hours at 37 C. The test was then allowed to stand at 4 C for 12 hours before it was read. A fine granular type of agglutination occurred to a titer of 1:80 with the hyperimmune rabbit serum.

Of special interest was the observation that agglutination of chicken erythrocytes and those of other species occurred with horse serum broth cultures. Hemoagglutination was attempted by the method of Hirst (1942) using chicken, guinea pig, human, horse, sheep, hamster, and cotton rat washed erythrocytes with untreated horse serum broth cultures of the organism. The results are shown on table 1.

Hyperimmune rabbit serum caused definite inhibition of agglutination to a

titer of 1:80 using washed chicken erythrocytes, whereas the normal serum from the same rabbit failed to inhibit agglutination. These same rabbit sera failed to exhibit any difference in the inhibition of hemoagglutination with the viruses of influenza A or B. The results are shown on table 2.

Experiments were done to determine what part of the culture agglutinated erythrocytes. It was found that the high-speed supernatant as well as the

TABLE 1

Agglutination of erythrocytes from various species

TYPE OF ERYTHROCYTE	DILUTION OF CULTURE				
	1:2	1:4	1:8	1:16	1:32
Human	+	+	+	±	−
Chicken	+	+	+	±	−
Guinea pig	+	+	+	±	−
Horse	−	−	−	−	−
Sheep	+	+	−	−	−
Rabbit	+	+	−	−	−
Cotton rat	+	±	±	−	−
Hamster	+	+	+	±	−

TABLE 2

Results of direct agglutination and hemoagglutination inhibition tests done with normal and hyperimmune rabbit sera

ANTIGEN	NORMAL RABBIT SERUM DILUTIONS							HYPERIMMUNE RABBIT SERUM DILUTIONS						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:10	1:20	1:40	1:80	1:160	1:320	1:640
(Direct agglutination)														
Pleuropneumonialike agent	0	0	0	0	0	0	0	+	+	+	+	±	0	0
(Inhibition of hemoagglutination)														
Pleuropneumonialike agent	+	+	+	+	+	+	+	0	0	0	0	+	+	+
Influenza A	0	0	0	0	+			0	0	0	0	+		
Influenza B	0	0	0	0	+	+	+	0	0	0	0	+	+	+
Glucose horse serum broth control	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note: + indicates strong agglutination and 0 indicates no agglutination.

Seitz filtrate had no agglutinating power, whereas the sediment, when resuspended in saline, agglutinated chicken cells as strongly as the untreated broth culture. Hemoagglutination also occurs with ground suspensions of infected amniotic membranes, but not noticeably with allantoic fluid. Unlike influenza hemoagglutination the chicken cells remain agglutinated for 12 hours at 20 C.

In order to determine the specificity of the hemoagglutination test, 22 additional rabbit sera were examined. Eight of these were hyperimmune to influenza A and B; 7 were hyperimmune to viruses of cotton rat, hamster, and mouse

origin (P.V.M. of Horsfall and Hahn, 1939); 7 were from rabbits inoculated with material from cases believed to be primary atypical pneumonia (Meiklejohn, Eaton, and van Herick, 1945). Of this last group, 2 rabbit sera were proved hyperimmune to the primary atypical pneumonia virus. Also tested were 14 human sera, 6 of which had neutralizing antibodies to the primary atypical pneumonia virus; 27 hamster sera; and 8 ferret sera, all of which showed an appreciable hemoagglutination inhibition titer to influenza A. The results on all these sera when tested with this pleuropneumonialike organism by the hemoagglutination inhibition test proved to be entirely negative.

Since this agent was isolated from developing eggs, the possibility that it could be of chicken origin called for further investigation. Sera from a total of 110 chickens from various sources were tested. They were, for convenience, split into two groups. The first group, for the largest part, consisted of sera

TABLE 3

Results of hemoagglutination inhibition tests done on chicken sera from various sources

SOURCE OF SERA	NUMBER TESTED	TITERS				
		<1:10	1:10	1:20	1:40	1:80
Group 1						
Immune to avian pneumoencephalitis...	60	60	0	0	0	0
Normal sera.....	14	10	3	1	0	0
Total, in percentage		95	4	1	0	0
Group 2						
Hatchery which was the source of eggs..	36	10	15	7	4	0
Total, in percentage.....		28	41	20	11	0

from chickens with varying degrees of immunity to the avian pneumoencephalitis (Stover, 1942; Beach, 1942) virus, later found to be serologically identical with the virus of Newcastle disease (Beach, 1944). These sera were furnished through the courtesy of Professor J. R. Beach, Division of Veterinary Science, University of California. The second group of sera were taken from chickens at the hatchery furnishing eggs to our laboratory. These chickens were approximately a year old and the sera were collected about one year after the organism had been isolated from the eggs. Table 3 shows the results of hemoagglutination inhibition tests run on these two groups of chickens. There is a marked difference between the groups. The second group shows a fairly high percentage of the normal chickens with an appreciable antibody level to this organism.

Discussion

Morphologically and culturally this organism must be distinguished from ordinary bacteria, and because of its behavior it appears to belong to the pleuro-

pneumonia group of organisms. The criteria that are commonly employed by many workers (Sabin, 1941; Warren, 1942) are fulfilled. In general, these criteria are as follows: (a) growth in cell-free media with a high concentration of horse serum (10 to 20 per cent); (b) filtrability; (c) poor staining with the common aniline dyes, but good staining with Giemsa; (d) slow rate of growth in fluid media as well as solid media, the latter showing only minute colonies; (e) pleomorphism with such structures as globules, filaments, rings, elementary bodies, etc. However, not until more work has been done to determine the possibility of any cross relationship between existing members of the pleuropneumonia group, especially the "L1", "L3", and "L4" rat strains (Klieneberger, 1935; Klieneberger and Steabben, 1937; Woglom and Warren, 1938), will the matter of classification be illuminated.

The serological evidence and the fact that the organism was isolated from eggs suggest that it is carried by chickens as a latent infection and could possibly be transmitted to the fertilized egg, either as an infection of the embryo itself or as a contamination on the shell. Further experimentation certainly would be necessary to establish the manner in which the chicken harbors the organism and the mode of transmission.

The possibility that this agent has any relation to the primary atypical pneumonia virus seems to have been excluded by (a) its growth in cell-free media and its microscopic appearance, (b) the failure to recover the organism again from chick embryos of other passages and strains of primary atypical pneumonia virus, (c) its greater virulence for eggs and lack of infectivity for hamsters, (d) the extensive and rapidly fatal nature of the pulmonary lesions produced in cotton rats by a heat-stable nonneutralizable poison as distinguished from the small lesions produced by the labile and neutralizable atypical pneumonia virus, and (e) the negative results of chicken cell agglutination inhibition tests with rabbit sera and convalescent human sera which neutralized the primary atypical pneumonia virus.

The agglutination of erythrocytes by a pleuropneumonialike organism indicates another similarity of this group of agents to the filterable viruses. Hemoagglutination by a virus was first described by Hirst (1942) with allantoic fluid infected with influenza virus. Since that time other viruses have been found to exhibit the property of agglutinating chicken erythrocytes. It has been shown with the virus of Newcastle disease by Burnet (1942), with vaccinia virus by Nagler (1942), and with fowl-plague virus by Lush (1943). Recently, Mills and Dochez (1944) have shown that the pneumonitis virus of mice (Horsfall and Hahn, 1939) will cause specific agglutination of murine erythrocytes. The agglutination is apparently produced by the pleuropneumonialike organism itself and not by any soluble product of the organism. The large particle size does not seem to preclude this phenomenon because hemoagglutination has been observed with vaccinia virus which is of comparable size. By the hemoagglutination inhibition test this unknown agent exhibits no antigenic relation to influenza, Newcastle disease, and the pneumonitis virus of mice.

Sabin (1938) found that the type A pleuropneumonialike organism from

mice produced a true neurotropic exotoxin which gave rise to choreiform nervous signs, and Nocard *et al.* (1898) had previously obtained evidence for the production by pleuropneumonia bovis of a soluble substance toxic for rabbits. The toxin described by Sabin was thermolabile, antigenic, and neutralizable, thus differing from the substance elaborated by the pleuropneumonialike organism from eggs which produced pulmonary hemorrhage and edema in cotton rats, was thermostable, and, as indicated by preliminary experiments, nonantigenic.

Summary

An organism, pleuropneumonialike in its characteristics, has been isolated during passages of the primary atypical pneumonia virus in chick embryos. This agent was shown to be unrelated to the primary atypical pneumonia virus and was considered to be a contaminant.

Characteristic pleuropneumonialike growth occurs in 10 per cent horse serum beef heart hormone infusion broth and on horse serum agar plates.

Hemoagglutination occurs with cultures of the organism. This agglutination is specifically inhibited with homologous hyperimmune rabbit sera as well as with a high percentage of sera from the hens at the hatchery which furnished the eggs from which the organism was isolated.

The organism elaborates a substance which causes edematous pulmonary consolidation and death in cotton rats by intranasal instillation. This poison can be separated from the organism by Seitz filtration of broth cultures or suspensions of chick embryo material. It is not neutralized with homologous hyperimmune rabbit serum, and it is heat-stable.

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