COMBINED VIRAL AND BACTERIAL INFECTION

An in Vitro Analysis of the Population Dynamics and Factors Influencing the Enhancement of Virulence of Hemophilus influenzae in Combined Infection with Influenza Virus in Embryonated Eggs

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Previous publications[1,2] from this laboratory have reported studies concerned with the problems presented by the experimental combined infection of embryonated eggs with Influenza C virus and *Hemophilus influenzae,* type b. The marked increase in virulence of the combined infection as compared with that observed when either virus or bacterium alone was the infecting agent was found to reflect certain manifestations of the population dynamics of the bacterial component. In the early stages of infection there was a much faster growth rate of the bacteria in the combined process than in the absence of an established virus infection. Furthermore, in the virus infected host the original composition of the bacterial population in the superimposed inoculum in respect to the proportion of potentially pathogenic (encapsulated) to nonpathogenic (non-encapsulated) members was maintained. In the host without virus infection the proportion of potentially pathogenic bacteria decreased.

Subsequent experiments have explored the possibility of applying *in vitro* methods to the discovery of factors that enhance bacterial virulence in combined infection. It was found that Hemophilus could be cultured in a nutrient medium in which amniotic fluid from virus infected or from normal embryonated eggs was combined with Levinthal's broth. In these media differences in growth rate and population composition of Hemophilus were observed comparable to those encountered in embryonated eggs with and without influenza virus infection. With the *in vitro* system it was possible to devise experiments to determine whether or not the

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enhancement of bacterial virulence in the combined infection could be attributed to the virus *per se,* to an increase of protein in the amniotic fluid of virus infected embryonated eggs or to other factors. This report will present the results obtained in these studies.

METHODS

Influenza Virus and Hemophilus influenzae

Influenza A, PR8 strain was used instead of the J.J. strain of Influenza C previously utilized. Type A was used because it was considered to be more injurious than type C. Thus, presumably, more protein and other nutritive substances would be released from infected cells. The PR8 strain obtained originally from Dr. Thomas Francis, Jr., and maintained in this laboratory through numerous allantoic passages was readily adapted to this growth method. Pools of bacteria-free fluid to which inactivated normal rabbit serum was added to 5 per cent by volume stored at 5° C served as the stock virus suspension.

A strain of *Hemophilus influenzae* type b was obtained by culture of the spinal fluid from a case of purulent meningitis admitted to the contagious unit of Charity Hospital in New Orleans. The primary isolate was transferred to Levinthal's agar and the 24-hour growth brought to a heavy suspension in inactivated normal rabbit serum diluted to 20 per cent in sterile distilled water. The suspension was distributed in 1 ml amounts to ampules and lyophilized from the frozen state. The flame-sealed ampules stored at −20° C provided a uniform source for the bacterial component.

Determination of in Vitro Growth Curves of Hemophilus

Considerable preliminary experiment indicated that the best medium for the determination of the growth curve and other *in vitro* qualities of Hemophilus consisted of a mixture of equal parts by volume of Levinthal's broth and amniotic fluid. In all of the experiments amniotic fluid from virus infected embryonated eggs was collected during the 13th incubation day 36 hours after inoculation with 0.1 ml 10^{-8} dilution of the stock virus suspension. Hemagglutination titers of pools of bacteria-free amniotic fluid were consistently 1:256 or 1:512. Normal amniotic fluid used for control was collected from uninfected embryonated eggs during the 13th incubation day. The nutrient media were dispensed in 25 ml amounts to 125 ml Erlenmeyer flasks and inoculated immediately after preparation.

The inoculum for all growth curve determinations consisted of 0.1 ml 10^{-7} dilution of an 18- to 24-hour Levinthal's broth culture started with the lyophilized stock of Hemophilus. Measured samples from the cultures at 37° C were withdrawn at regular intervals and set up in appropriate dilutions in Levinthal's broth. Colony counts were obtained by inoculating 0.1 ml from each of 4 appropriate ascending dilutions into each of 6 Levinthal's agar pour plates. Growth curves were drawn by utilizing the mean logarithm (Log-e-mean) of the logarithm of the mean of the number of bacilli per ml derived from the colony counts obtained at specified time intervals in a given number of successive experiments. Analysis of the growth curves was made by methods described by Snedecor.[3]

Determination of the Precipitinogen Producing Capacity of Hemophilus Clones by Spectrophotometry

Previously [2] the composition of Hemophilus populations on the basis of the precipitinogen producing capacity of individual clones was determined by the gel stabilized double diffusion method as described by Gispen.[4] Comparative determination demonstrated that good distinction could be made by spectrophotometry between clones producing precipitinogen titers by the double diffusion test from 1:8 to 1:64 and those producing titers of 1:4 or less.

Clone samples for the determination of the population composition of the inoculum or at intervals following inoculation of cultures were obtained by transferring 0.1 ml of appropriately diluted measured samples to the surface of each of 6 Levinthal's agar plates. After 24 hours at 36° C a plate was chosen from which each of 30 adjacent separate colonies could be transferred for individual culture in 50 ml Erlenmeyer flasks containing exactly 10 ml Levinthal's broth. After 24 hours at 36° C the broth cultures were centrifuged at 3,000 rpm at 5° C for 40 minutes and the supernate decanted into sterile chemically clean tubes.

To 0.1 ml of the supernate 0.2 ml hyperimmune *Hemophilus influenzae,* type b rabbit antiserum obtained as previously described[2] was added and the mixture incubated for 5 minutes in the water bath at 37° C. The mixture was then brought to a final volume of 2.8 ml by the addition of normal saline and reincubated for 5 more minutes. It was then transferred to an absorbing cell and shaken vigorously to obtain a uniform dispersion of the precipitate. Turbidity readings were made immediately in a Coleman Junior Spectrophotometer at wave length 445 mμ.

Clones producing turbidity readings comparable to those determined by the double diffusion tests to have precipitinogen titers of 1:8 or more are hereafter arbitrarily referred to as potentially pathogenic (PP); those producing readings comparable to those with precipitinogen titers of 1:4 or less will be referred to as nonpathogenic (NP).

Determination of the Protein Content of Amniotic Fluid from Normal and Virus Infected Embryonated Eggs

The protein content of amniotic fluid from normal and virus infected embryonated eggs was determined by the biuret method. Each sample consisted of a pool of equal amounts of amniotic fluid collected from 2 eggs. The protein was precipitated with 10 per cent trichloracetic acid and the precipitate redisolved in 1N sodium hydroxide. Upon the addition of the biuret reagent the optical densities of the developed purple complex in the samples were compared against a blank consisting of 4 ml biuret reagent plus 1 ml normal saline in a Coleman Junior Spectrophotometer transmitting at 500 mμ. The protein content expressed in mg per ml was calculated against a previously established standard curve.

Preparation of Protein Free Amniotic Fluid

Amniotic fluid from normal and virus infected embryonated eggs was dispersed to test tubes in 15 ml amounts and immersed in boiling water for 10 minutes. After cooling the coagulated protein was removed by centrifugation at 3,000 rpm for 1 hour at 5° C. Equal amounts of the sterile supernate and Levinthal's broth were combined to serve as nutrient medium.

Observations and Results

Comparison of the Growth Curve of Hemophilus in Levinthal's Broth with that in Levinthal's Broth and Normal Amniotic Fluid

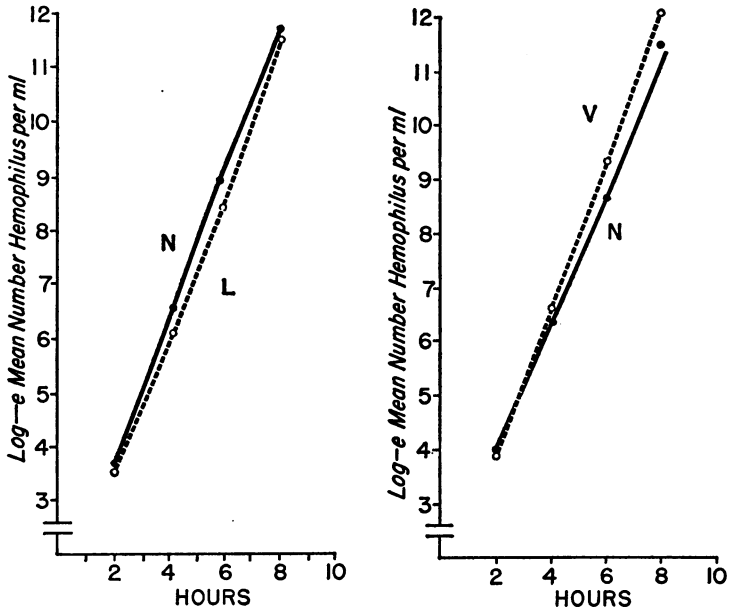
It was first ascertained that the *in vitro* system consisting of equal parts of Levinthal's broth and amniotic fluid of normal 13-day embryonated eggs (medium N) was equal to Levinthal's broth (medium L) as a nutrient medium for Hemophilus. Text-figure 1 presents the growth curves obtained from 3 successive determinations at the 2-, 4-, 6- and 8-hour periods.

In medium N the slope for the best fitting straight line to the growth curve was 2.65 log units and for medium L it was 2.62 log units per 2-

hour time period. There appeared to be no real difference in the growth rate of Hemophilus in the two media.

Comparison of the Growth Curves and Population Composition of Hemophilus in Levinthal's with Normal Amniotic Fluid and in Levinthal's with Amniotic Fluid from Virus Infected Embryos

Text-figure 2 presents the growth curves of Hemophilus obtained from 10 successive determinations at the 2-, 4-, 6- and 8-hour periods in a



TEXT-FIG. 1. The growth curves of Hemophilus in Levinthal's medium only and in Levinthal's medium with normal amniotic fluid.

TEXT-FIG. 2. The growth curves of Hemophilus in Levinthal's medium with normal amniotic fluid and in Levinthal's medium with amniotic fluid from virus infected embryos.

medium containing normal amniotic fluid (N) and in a medium containing amniotic fluid from virus infected embryos (V).

For medium N the slope of the best fitting straight line was 2.49 log units and the corresponding slope for medium V was 2.72 log units per 2-hour time period. The F value associated with variability between the slopes was 5.19. The difference between the slopes of the curves for media N and V appeared to be real ($P<.05$).

The population composition of Hemophilus at the 3-, 6-, 9- and 12-hour intervals of growth in media N and V was compared in a series of 3 experiments. The population of the inoculum consisted of 69 per cent

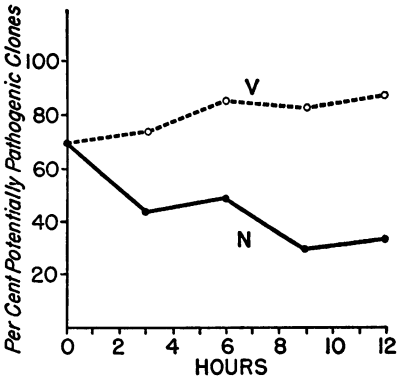
PP and 31 per cent NP clones. Text-figure 3 presents the composite results of these experiments.

In medium V the proportion of PP clones increased from 69 to 87 per cent during the first 12 hours of the growth curve and in medium N there was a decrease to 33 per cent PP clones during the same period.

The Effect of Influenza Virus on the Growth Curve and Population Composition of Hemophilus

Influenza virus was removed from pools of amniotic fluid from virus infected embryonated eggs by hemadsorption. It was then eluted in normal amniotic fluid to 10 times its original concentration. Four types of nutrient media consisting of equal parts of Levinthal's media and amniotic fluid were prepared in which the growth curve of Hemophilus was determined. Medium SO contained amniotic fluid from which the virus had been removed. In medium SV the virus was added to restore the original hemagglutination titer. Medium NV contained normal amniotic fluid to which the virus had been added to the same hemagglutination titer as in medium SV. Medium N contained normal amniotic fluid. Text-figure 4 presents the growth curves of Hemophilus in these media representing 6 successive determinations at the 2-, 4-, 6- and 8-hour periods.

In medium SO the slope for the best fitting straight line to the growth curve was 3.04 and for medium SV, 2.98 log units per 2-hour time period.

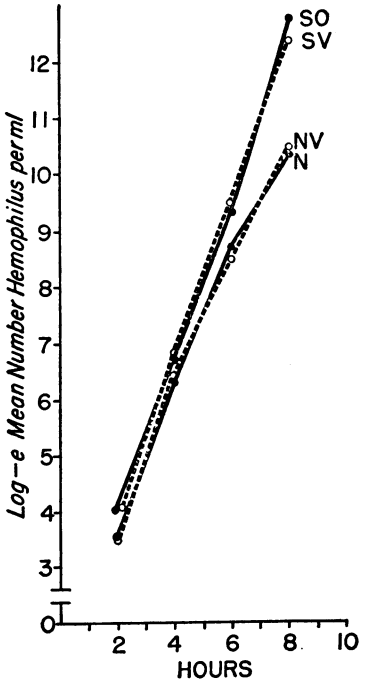


TEXT-FIG. 3. The population composition of Hemophilus in Levinthal's medium with normal amniotic fluid and in Levinthal's medium with amniotic fluid from virus infected embryos.

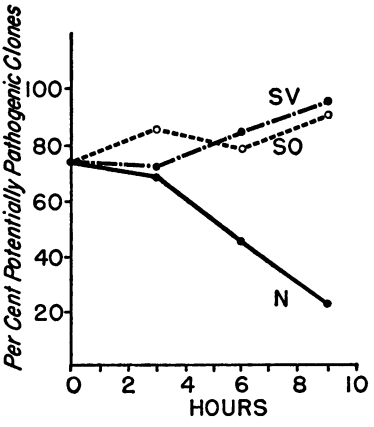
For medium N the slope was 2.17 and for medium NV, 2.23 log units per 2-hour time period. There appeared to be no real difference between the value for the slopes in media SO and SV or for media N and NV. The presence or absence of influenza virus apparently had no appreciable effect on the growth rate of Hemophilus in the *in vitro* system.

The effect of the virus *per se* on the population composition of Hemophilus was determined in media SO, SV and N. Text-figure 5 presents the composite results of 2 successive determinations. The population of the inoculum was composed of 73 per cent PP and 27 per cent NP clones.

The presence or absence of influenza virus had no appreciable effect on the population composition of Hemophilus during the first 9 hours of



TEXT-FIG. 4. The effect of influenza virus on the growth curve of Hemophilus.



TEXT-FIG. 5. The effect of influenza virus on the population composition of Hemophilus.

growth. In media SO and NV the proportion of PP clones present in the inoculum was maintained at approximately equal levels during the first 6 hours of growth and was markedly increased by the 9-hour period. In the medium containing normal amniotic fluid there was a steady decrease from 73 to 22 per cent PP clones during the 9-hour period.

Comparison of the Total Protein Content of the Amniotic Fluid from Normal and Virus Infected Embryonated Eggs

The total protein content determinations on 15 samples each of amniotic fluid from normal and virus infected embryonated eggs are presented in Table I.

TABLE I

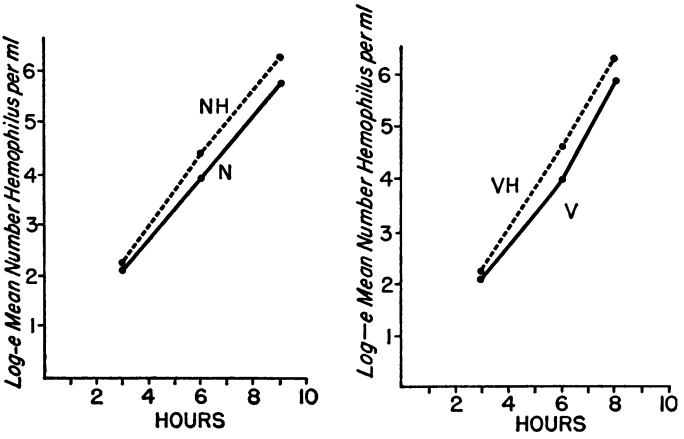
COMPARISON OF THE TOTAL PROTEIN CONTENT OF THE AMNIOTIC FLUID FROM NORMAL (N) AND VIRUS INFECTED (V) EMBRYONATED EGGS

No.	Mg/Ml Protein	
	N	V
1	0.08	0.61
2	0.24	0.96
3	0.08	0.70
4	0.16	0.62
5	0.14	0.46
6	0.16	0.93
7	0.08	0.93
8	0.08	0.59
9	0.24	0.70
10	0.14	0.59
11	0.18	0.96
12	0.09	0.46
13	0.08	0.88
14	0.20	0.42
15	0.25	0.80
Mean	0.15	0.71

Amniotic fluid from virus infected embryonated eggs had a considerably higher total protein content than the amniotic fluid from uninfected normal controls. The protein content in fluid from infected eggs ranged from 0.42 to 0.96 mg with a mean of 0.71 mg per ml while that in normal fluid ranged from 0.08 to 0.25 mg with a mean of 0.15 per ml. There appeared to be a real difference between the mean values obtained ($P<.01$).

The Effect of Removal of Total Protein from Amniotic Fluid on the In Vitro Growth Curve of Hemophilus

In a series of 4 consecutive experiments the growth curve of Hemophilus was determined at the 3-, 6- and 9-hour period in a medium containing unheated amniotic fluid and compared with that in a medium containing amniotic fluid from which the total protein was removed.



TEXT-FIG. 6. The effect of the removal of the total protein content from normal amniotic fluid on the growth curve of Hemophilus.

TEXT-FIG. 7. The effect of the removal of the total protein content from the amniotic fluid from virus infected embryos on the growth curve of Hemophilus.

Text-figure 6 presents the growth curves in the medium containing unheated amniotic fluid (N) and in the medium containing amniotic fluid from which the total protein had been removed (NH). Text-figure 7 presents the growth curves determined at the same periods in the medium containing unheated amniotic fluid from virus infected embryos (V) and in medium containing amniotic fluid from which the total protein had been removed (VH).

For medium N the slope of the best fitting straight line was 1.78 and for medium NH, 1.84 log units per 3-hour time period. The F value for variation among slopes was about 0.18. For medium V the slope of the best fitting straight line was 1.91 and for medium VH, 2.00 log units per 3-hour perod. The F value for variation among slopes was 0.437. There appeared to be no real difference among the slopes for medium N and NV or for medium V and VH. Removal of the coagulable protein from the amniotic fluid from normal or virus infected embryos did not seem to effect any marked difference in the growth rate of Hemophilus during the first 9 hours in the *in vitro* system.

Discussion

It is generally accepted, as stated by Burnet,[5] that "The damage done by the virus to the epithelial lining of the respiratory tract with associated functional and chemical changes, simply changes the local environment, making it a suitable ecological niche for a variety of bacteria." The experiments described in the foregoing were attempts to ascertain the nature of some of these changes.

Comparative determination demonstrated that during the first 8 to 12 hours in media containing amniotic fluid from embryonated eggs infected with influenza virus the growth rate of Hemophilus was significantly faster than in media containing amniotic fluid from normal control eggs. Furthermore the proportion of potentially pathogenic members of the population in the bacterial inoculum increased in media containing fluid from virus infected embryos while in media containing normal fluid this proportion decreased.

No evidence was obtained to indicate that influenza virus *per se* constituted a factor in the enhancement of bacterial virulence in combined infection. Removal of the virus by hemadsorption from amniotic fluid of infected embryos or the addition of eluted virus to a comparable titer in normal fluid had no appreciable effect on the *in vitro* growth rate of Hemophilus. This might have been anticipated in considering that the virus comprised a relatively minute constituent of amniotic fluid of infected embryos. There was, however, the possibility that viral nucleic acid or protein was utilized by the bacteria as a growth stimulating factor.

The amniotic fluid of virus infected embryos was found to contain approximately 5 times more coagulable protein than that of normal controls. Most of this extra protein was presumably derived from virus damaged cells. Its removal from amniotic fluid of normal or virus infected embryos did not result in a decrease but promoted a slight but not significant increase in bacterial growth rate.

In the previous studies [1,2] Influenza C, J.J. strain, instead of Influenza A was found to promote the enhancement of bacterial virulence in the combined infectious process. It is noteworthy that subsequent comparative determinations revealed no significant difference in the total protein content of the amniotic fluid from J.J. strain infected and from normal control embryonated eggs. The coagulable proteins as such did not appear to constitute an important factor in the increase in bacterial growth rate in combined infection. It is well recognized that few pathogenic bacteria utilize whole protein directly for their nutrition.

The results of the present studies provide new impetus and direction

to the search for factors that operate in the enhancement of bacterial virulence in combined infection with influenza virus and *Hemophilus influenzae*. The virus *per se* and coagulable proteins appear to be ruled out. It is realized that neither trichloracetic acid precipitation nor coagulation by boiling will remove all of the proteins or protein components from amniotic fluid from normal or virus infected embryos. Other smaller molecular species apparently remain behind. Although small in amount these simpler molecular complexes must be identified as possible factors that stimulate the growth rate and enhance the virulence of the bacterial component. As yet unidentified carbohydrate complexes may also contribute to this effect.

Further studies are being directed to a comparative determination of the nonprotein nitrogen content and analysis of the amino acids, peptides and polypeptides in coagulable protein-free amniotic fluid from normal and from virus infected embryonated eggs. Differences in the type and quantity of various carbohydrate components should also come under scrutiny.

More attention must also be directed to the possibility that virus infection interferes with or inhibits the production of natural bactericidal or bacteriostatic substances, for example lysozyme. Preliminary efforts by conventional methods have thus far provided no satisfactory answer to this question. Many refinements in the technical approach to this problem are feasible.

Eventually it may be demonstrated that the enhancement of bacterial virulence in combined infection is effected by a combination of the production of growth promoting factors on the one hand and the suppression of inhibitory factors on the other.

Summary

A nutrient medium composed of equal parts of amniotic fluid from virus infected or normal control embryonated eggs and Levinthal's broth provided an *in vitro* system in which the population dynamics and some of the factors in the enhancement of virulence of the bacterial component in combined infection with influenza virus and *Hemophilus influenzae*, type b, could be analyzed.

The presence or absence of influenza virus *per se* in the *in vitro* system produced no appreciable effect on the growth rate of Hemophilus.

Approximately 5 times more coagulable protein was present in amniotic fluid from virus infected embryos as compared with normal controls. Removal of the coagulable protein from the amniotic fluid from normal or virus infected embryos effected a slight but not significant increase in the *in vitro* growth rate of Hemophilus.

Continuing studies are concerned with an analysis of the amino acid, peptide and polypeptide composition of amniotic fluid from virus infected embryos from which the coagulable protein has been removed in a search for factors that enhance bacterial virulence in combined infection. Attention is further directed to determining whether or not virus infection inhibits or interferes with the production of natural bactericidal or bacteriostatic substances, e.g. lysozyme.

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