Parallel Production of Altered Infectivity of a Streptococcus and Related Filtrable Agents Isolated from Outdoor Air

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IN PREVIOUS STUDIES, it has been shown that streptococci isolated in studies of influenza and other milder respiratory infections and from outdoor air in winter have a "pneumotropic" distribution curve of cataphoretic velocity, pneumotropic virulence and corresponding serologic properties. Also streptococci isolated in studies of epidemic hiccup, encephalitis and poliomyelitis and from outdoor air in summer have a "neurotropic" distribution curve of cataphoretic velocity, neurotropic virulence and corresponding serologic properties.[7,9,12,13]

It is the purpose of this communication to describe the methods employed and record the results obtained in a study of a streptococcus as isolated from outdoor air in winter during a severe epidemic of influenza and as isolated six months later in summer from a chick-embryo culture that had been stored in the dark at room temperature meanwhile.

METHODS

The streptococcus studied was obtained from one of sixteen tubes of dextrose-brain broth, inoculated with wood applicators that had been exposed during the epidemic to outdoor air in test tubes with open ends screened with a double layer of surgical gauze and that revealed pure cultures of a streptococcus eighteen hours after incubation at 35° C. Exposure to air was made on an improvised weather vane for eight days of sunshine atop a 10-foot pole on the roof of a four-story building situated in a city in the center of the region during the epidemic. The remaining fifteen tubes of dextrose-brain broth, similarly inoculated, revealed the streptococcus in mixture with Gram-positive or Gram-negative bacilli. Blood-agar platings inoculated with washings of the exposed wood applicators revealed a moderate number of colonies of B. subtilis, but no streptococci.

The dextrose-brain broth as used in tall columns affords a gradient of oxygen tension, being aerobic at the top and anaerobic at the bottom. This condition is of the greatest importance for the primary isolation of specific types of streptococci and for maintenance of their specific properties. To each test tube, 1.7 by 15 cm., was added 20 c.c. of 0.2 per cent dextrose broth and three to four pieces of fresh calf brain, representing approximately 1 part of brain substance to 6 or 7 parts of broth before autoclaving. Serial dilution cultures in this medium

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were made from tube to tube in rapid succession with a nichrome wire to which there adhered approximately 2 cubic millimeters of culture, representing a dilution of 1:10,000 or 10^{-4} at each transfer. The wire was sterilized in a Bunsen flame immediately before the first transfer and between each successive transfer.

Three consecutive serial dilution cultures were made in four tubes of dextrose-brain broth from one of the tubes that revealed a pure culture of the streptococcus. Blood-agar plates made of the initial tube and from the end point of growth of the three serial dilution cultures represented a total dilution of original inoculum of 10^{-32} and yielded pure growths of a slightly green-producing streptococcus. A single colony of a twelve-hour blood-agar plating from the end point of growth of the third serial dilution culture was then inoculated into an additional tube of dextrose-brain broth. The streptococcus that grew in eight hours at 35° C. was then inoculated into eighteen mice and into a tube of chick-embryo medium layered with liquid petrolatum and which was sealed with a rubber-lined screw cap. The chick-embryo culture was incubated overnight at 35° C. and then stored at room temperature in the dark from January 10 to July 2, 1948.

The chick-embryo medium consisted of the mash (1 part), obtained by passing through a meat chopper nineteen-day hatching chicken eggs, including the shell, and distilled water (7 parts). The mixture of ground mash and water was kept in the refrigerator for twenty-four hours, and stirred at intervals meanwhile, after which it was placed in tall columns in test tubes or bottles and autoclaved at 17 pounds pressure for twenty minutes. Titration was not necessary since the reaction was always at approximately pH 7. The medium in the tubes was layered with sterile liquid petrolatum and autoclaved for a few minutes at 17 pounds pressure to insure sterility. The tubes and bottles were then sealed with cotton plugs, rubber stoppers, or rubber-lined screw caps.

The chick-embryo medium was inoculated because it was found in previous studies[7] to be highly favorable for rapid growth of streptococci and for maintenance of viability[13] that cultures did not turn acid and that seasonal changes in the streptococci occurred in accord with streptococci associated with or as the cause of current epidemics, in winter of respiratory infection and in summer of poliomyelitis or encephalitis, on prolonged storage at room temperature.[11]

A subculture in dextrose-brain broth made July 2 from the stored chick-embryo culture was inoculated July 3 into twelve mice. Successive mouse passages were made in winter and summer with emulsions or filtrates of emulsions of pneumonic lungs or polio-encephalitic brains respectively.

Mice, weighing from 10 to 15 grams, were inoculated under deep ether anesthesia alternately with .03 ml. cerebrally only and with .03 ml. cerebrally and .06 ml. nasally of the undiluted dextrose-brain broth cultures in the first mouse passage and with like amounts of 10 per cent emulsions or filtrates of emulsions of pneumonic lungs or brain in winter and emulsions and filtrates of emulsions of polio-encephalitic

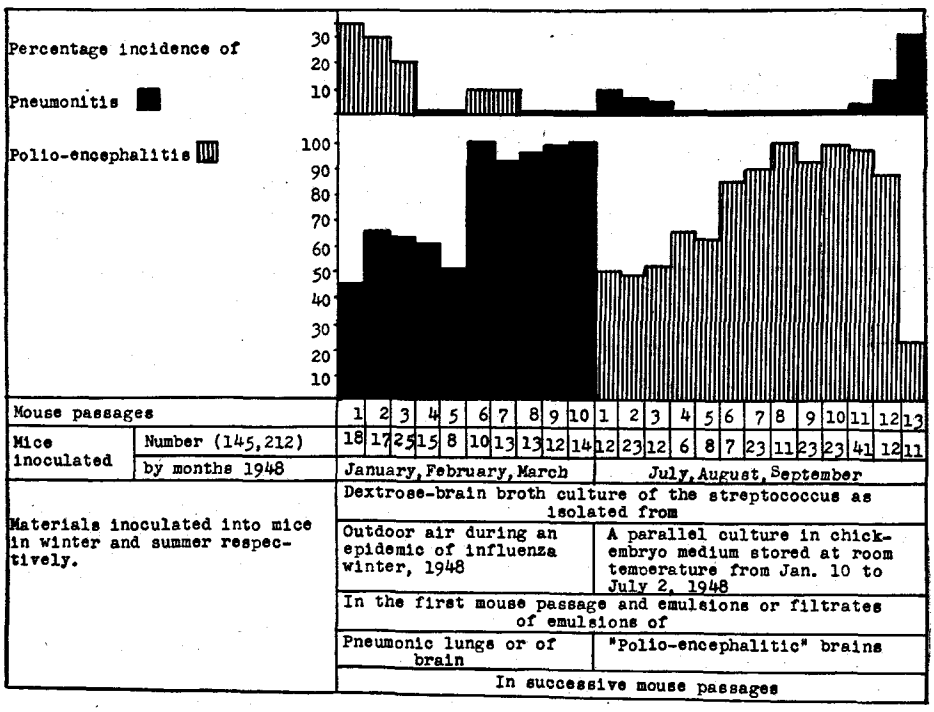

Fig. 1. Incidence of pneumonitis and of polio-encephalitis in mice following cerebral and/or nasal inoculation of a streptococcus, as isolated from outdoor air in winter during a severe epidemic of influenza and from a parallel culture in chick-embryo medium after storage at room temperature from January 10 to July 2, 1948, for the first mouse passage, and following inoculation of emulsions or filtrates of emulsions of pneumonic lungs and of "polio-encephalitic" brains, respectively, in successive mouse passages.

brains in summer on respective mouse passages.

Control mice were similarly inoculated with filtrates of dextrose-brain broth cultures, with emulsions and filtrates of emulsions of the lungs and brain of normal mice. The inoculated mice were observed two or more times daily, when notes of symptoms, if any, were made. Mice that died were examined as soon after death as possible. Those that survived were anesthetized to death with ether after from five to ten days. Cultures of emulsions and filtrates of emulsions of lungs and brain were made on blood-agar and into dextrose-brain broth.

RESULTS IN MICE

The results of inoculations of the streptococcus, as isolated in winter and as isolated from the chick-embryo cultures after storage into summer in the first mouse passage and in successive mouse passages of emulsions and filtrates of emulsions, respectively, of pneumonic lungs and brain in winter and of emulsions and filtrates of emulsions of "polio-encephalitic" brains in summer, are shown graphically in Figure 1.

A truly remarkable difference in localizing and disease-producing properties occurred. The streptococcus, as isolated from outdoor air during the

epidemic of influenza in winter in the first mouse passage and emulsions and filtrates of emulsions of pneumonic lungs and brain on nine successive passages, produced a high and increasing incidence of pneumonitis and at first a lower and later no incidence of polio-encephalitis. The same strain of streptococcus isolated from the chick-embryo culture after storage of the chick-embryo culture at room temperature into summer in the first mouse passage and emulsions of "polio-encephalitic" brains on successive mouse passage produced instead a very high and increasing incidence of polio-encephalitis and a very low or no incidence of pneumonitis throughout eleven mouse passages. Then during the twelfth and thirteenth passages the latter part of September, there was a sharp "seasonal" drop in incidence in inoculated mice of polio-encephalitis and a significant rise in the incidence of pneumonitis. This occurred as the incidence of poliomyelitis in human beings in and surrounding Cincinnati declined and as the incidence of respiratory infections sharply increased.

Important data, in addition to those shown in Figure 1, in the experiments on successive passage of emulsions and filtrates of emulsions of pneumonic lungs and of polio-encephalitic brains after initial inoculation of the streptococcus in winter while pneumotropic and after it had changed to neurotropic type on storage in chick-embryo medium into summer, are summarized in Table I. It will be seen that the incidence of pneumonitis and of isolations of the streptococci from pneumonic lungs on the initial injection of the streptococcus and on successive mouse passages in winter of emulsions and filtrates of emulsions of pneumonic lungs and brain, when the streptococcus injected initially was pneumotropic, were uniformly high: 100 per cent for the streptococcus, 85 per cent for emulsions of pneumonic lungs, 74 per cent for emulsions of brains and 72 per cent for filtrates, or a total average of pneumonitis of 75 per cent and of polio-encephalitis of 12 per cent or 145 mice inoculated. The streptococcus was isolated from pneumonic lungs cultured in 100 per cent, 93 per cent and 82 per cent, respectively, following inoculation of the streptococcus and emulsions, and 68 per cent following inoculation of filtrates, or a total average of 86 per cent of 102 cultured. In sharp contrast, the incidence of pneumonitis following inoculation of the streptococcus, of unfiltered and filtered emulsions of polio-encephalitic brain was but 5 per cent, while that of polio-encephalitis was 50 per cent for the streptococcus, 84 per cent for emulsions and 63 per cent for filtrates, and isolations of the streptococcus from brain was 82 per cent of 135 cultured on successive mouse passage in summer after the streptococcus initially injected had changed to neurotropic type on storage of the chick-embryo culture. The mortality was twice as great during the experiments made in winter (35 per cent) as those made in summer (18 per cent). The streptococcus was isolated in dextrose-brain broth from but three (not shown in Table I) of the twenty-one filtrates of emulsions of pneumonic lungs and of polio-encephalitic brains that were inoculated into mice. Cultures in dextrose-brain broth from sixteen pneu-

TABLE I. EXPLANATORY DETAILS OF EXPERIMENTS SUMMARIZED GRAPHICALLY IN FIGURE 1.

Time and type of experiments and materials inoculated				Number specimens inoculated	Mice					
					Number inoculated	Per cent that died	Percent in which there developed		Isolation of streptococci from lungs in winter; from brain in summer respectively	
							Pneumonitis	Polioencephalitis	Number cultured	Percent positive
Winter	Animal passage in mice	First	"Pneumotropic streptococcus on isolation	1	18	61	45	35	11	100
		Subsequent	Emulsions — Pneumonic lungs	14	56	32	85	7	41	93
			Emulsions — Brain	18	46	35	74	13	34	82
			Filtrates of emulsions of pneumonic lungs	6	25	20	72	8	16	68
			Total	39	145	35	75	12	102	86
			Control materials*	17	45	0	0	0	27	7
Summer	Animal passage in mice	First	After the streptococcus had changed to "neurotropic" type	1	12	40	9	50	5	100
		Subsequent	Emulsions of brain and spinal cord — Unfiltered	39	138	17	5	84	92	84
			Emulsions of brain and spinal cord — Filtered	15	62	15	5	63	38	76
			Total	55	212	18	5	77	135	82
			Control materials*	20	72	0	0	0	35	0

*Control material inoculated in winter consisted of 8 filtrates of dextrose-brain broth cultures of the streptococcus having pneumotropic virulence and of emulsions and filtrates of emulsions of the lungs and brains of 9 normal mice; in summer of 6 filtrates of dextrose-brain broth cultures of the streptococcus after it dissociated into neurotropic type and of emulsions and filtrates of emulsions of the brain of 12 normal mice.

monic lungs of mice that had received filtrates of pneumonic lung emulsions yielded the streptococcus in ten (68 per cent and from twenty-nine (76 per cent) of thirty-eight polio-encephalitic brains cultured, or a total isolation in thirty-nine (72 per cent) of fifty-four specimens cultured. All cultures of filtrates on blood-agar proved sterile.

There were no deaths and no instances of pneumonitis or polio-encephalitis in altogether 117 mice inoculated with thirty-seven specimens of control material consisting of fourteen filtrates of dextrose-brain broth cultures of the streptococcus, of nine filtrates of emulsions of normal mouse lungs and of twelve filtrates of emulsions of the brains of normal mice. A small Gram-negative streptococcus was isolated from two (7 per cent) of twenty-seven normal lungs cultured, following inoculation of control lung material, and all cultures made of brain of thirty-five control mice proved sterile.

The symptoms in mice following inoculation of the streptococcus, while having high pneumotropic virulence on isolation, and of corresponding emulsions and filtrates of emulsions of pneumonic lungs, varied greatly from no apparent effects to rapidly increasing respirations, often followed by death in two or three days. The lesions of lungs in the early stages and especially in mice that died consisted of a striking distention from interspersed widely disseminated areas of emphysema atelectasis and hemorrhagic edema and later of patchy areas of hemorrhagic consolidation.[14] Exudative pleuritis and spontaneous lesions of lungs were not observed. Inoculat-

ed mice, seemingly well, often revealed surprisingly extensive lesions of lungs when etherized and examined after death. The symptoms in mice receiving the streptococcus and emulsions and filtrates of emulsions in summer, after the streptococcus inoculated initially had changed to neurotropic type, varied widely from no apparent effect to tremors, sometimes clonic spasms and slight or moderate weakness of one or more extremities or to early occurring and rapidly progressing flaccid paralysis with death from respiratory failure.[7] There was a striking sameness in symptoms following respective different types of inocula. The gross and microscopic lesions of the lungs following inoculation of the "pneumotropic" material, especially filtrates, resembled those following inoculation of influenza virus.[15] Those of the brain and spinal cord were in close harmony with the symptoms noted during life. Suppurative meningitis and abscess formation at the site of injection were not observed. Edema and degeneration of anterior horn cells in medulla and spinal cord with minimal infiltration by round cells were roughly proportional to the degree of paralysis. Perivascular leukocytic and round cell infiltration and hemorrhage in the cortex and basal ganglia characterized the microscopic lesions in mice in which symptoms of encephalomyelitis were dominant.

ILLUSTRATIVE EXPERIMENTS AND PROTOCOLS

Mouse 1 was inoculated intranasally February 3, 1948, with .06 ml. of a Berkefeldt V filtrate of a 10 per cent emulsion of the pneumonic lung of a mouse that succumbed to pneumonitis following inoculation of the streptococcus (referred to in Figure 1) isolated from outdoor air during the epidemic of severe influenza. The animal had remained seemingly well until February 7 when anesthetized to death with ether. Examination revealed a large area of hemorrhagic bronchopneumonia in the left apical and a small area in the right diaphragmatic lobe. The brain and other viscera were normal. Cultures of emulsion of the pneumonic lung in dextrose-brain broth yielded a pure culture of the streptococcus and no growth on blood agar. Two of three other mice inoculated with this filtrate developed pneumonitis and results of cultures were similar to cultures of Mouse 1.

Mouse 2 was inoculated intranasally under deep ether anesthesia February 8, 1948, with .06 ml. of a 10 per cent emulsion of the pneumonic portion of the lung of Mouse 1. On February 10, the respirations were increased. On February 11, the mouse was found dead. Extreme hemorrhagic edema and hemorrhagic bronchopneumonia, involving almost the entire greatly distended lungs, were found at necropsy. The brain and other vital organs were normal. Cultures in dextrose-brain broth of pipettings of the hemorrhagic lung yielded a pure culture of the streptococcus. One other of three mice similarly inoculated revealed extreme pneumonitis, and the remaining two, moderate pneumonitis.

A Berkefeldt V filtrate of emulsions of the brain of nine mice in which polioencephalitis had developed following inoculation of filtrates of emulsions of the brain of mice in the fifth, sixth and seventh mouse passages, respectively, was inoculated intracerebrally on August 31 and September 1, 1948, into eight mice. All developed symptoms resembling poliomyelitis or encephalitis, and the streptococcus was isolated in dextrose-brain broth from the brain of each after death, while cultures on blood-agar proved sterile. Cultures on blood-agar and in dextrose-brain broth of the filtrate inoculated remained sterile. The protocols of Mice 3, 4 and 5 will serve to illustrate. Mouse 3 remained seemingly well until September 4, 7:00 a.m., when it had developed marked weakness of extremities, moderately accelerated respira-

tion and tremors especially on prodding. At 7:00 p.m. it had continuous fine and coarse tremors and mild clonic spasms, and great weakness of right hind extremity. On September 5, 7:30 a.m., it had almost complete flaccid paralysis of both hind extremities and marked weakness of fore extremities. Respiration was labored, indicating respiratory failure, of which it died one hour later. Necropsy revealed a moderately congested brain and normal lungs and other viscera. Cultures of emulsions of the brain and spinal cord on blood-agar remained sterile; those in dextrose-brain broth yielded a pure culture of the streptococcus.

Mouse 4 remained well until September 9, when tremors and generalized weakness and spells of great excitability were noted at 7:00 a.m. At 9:30 a.m. it had an attack after slight prodding, in which generalized twitchings of muscles occurred as it dashed about in wildlike fashion. At 8:00 p.m. a similar attack was seen to occur. On September 10, 8:00 a.m., moderate weakness of extremities, severe tremors, twitchings, tonic and clonic spasms were noted, and at 10:00 a.m., September 11, it was found dead with outstretched hind extremities, indicating that it died from generalized spasms. The brain was severely congested, there was no mark at the point of injection and the meninges were moist and shiny. The lungs and other viscera were normal. Cultures of the brain emulsion on blood-agar proved negative, and those in dextrose-brain broth yielded a pure culture of the streptococcus.

Mouse 5 remained well until September 3, when fine tremors were noted. On September 4 the animal was lethargic or semi-comatose, lying on its side with outstretched extremities in greatly increased tonus. On September 5, it was still comatose and the extremities were held outstretched in increased tonus. Fine and coarse tremors but no spasms were noted. On September 6, it was found dead. Necropsy revealed severe congestion of the brain, no mark at the site of injection, and the meninges were moist and shiny. The lungs and other viscera were normal. Cultures of the emulsions of the brain on blood agar were negative; those in dextrose-brain broth yielded a pure culture of the streptococcus.

CONTROL EXPERIMENTS

In order to be certain that the great difference in the incidence of pneumonitis and polio-encephalitis in the mice inoculated in winter and summer respectively was due to differences in the streptococcus and corresponding emulsions and filtrates of emulsions on successive passages, and not due to a possible increased susceptibility of mice to streptococci and to corresponding filtrable agents of the respiratory tract in winter and of the nervous system in summer, mice were inoculated in winter with streptococci shown to have neurotropic virulence and in summer with streptococci shown to have pneumotropic virulence. The respective pneumotropic and neurotropic streptococci inoculated had been preserved meanwhile at 5° to 10° C., either in dehydrated form in dense suspensions in glycerin, 2 parts, and saturated NaCl solution, 1 part, or in pneumonic lungs or polio-encephalitic brains of mice in 50 per cent glycerin. Of seventy-five mice thus inoculated in summer with twenty-four strains that had pneumotropic virulence, pneumonitis developed in forty-six (61 per cent), polio-encephalitis in only six (8 per cent), and gastroenteritis in but one. The streptococcus was isolated from pneumonic lungs in thirty-five (85 per cent) of forty-one cultured. In sharp contrast, of fifty-six mice inoculated in winter with twenty strains that had neurotropic virulence, polio-encephalitis developed in thirty (54 per cent), pneumonitis in nine

(16 per cent), and gastroenteritis in one. The streptococcus was isolated from the brain in twenty-seven (60 per cent) of forty-five cultured. Moreover, during the course of these and other experiments no instance of deaths from pneumonitis or symptoms of and deaths from polio-encephalitis occurred among large numbers of uninoculated mice in mouse stocks.

RESULTS OF AGGLUTINATIVE EXPERIMENTS

The streptococcus inoculated initially in the first mouse passage, as isolated during the severe epidemic of influenza, and the streptococci isolated from the pneumonic lungs of mice on successive mouse passages of emulsions and filtrates of emulsions of pneumonic lungs and brains in winter were agglutinated maximally by thermal antibody and antiserum prepared with the streptococcus isolated in studies of influenza and by the serums of persons convalescing from influenza.[11] After the streptococcus had changed to neurotropic type in the chick-embryo culture on storage into summer and as isolated from polio-encephalitic brains of mice on successive passages of emulsions and filtrates, it was agglutinated maximally by thermal antibody and antiserum prepared with streptococci isolated in studies of poliomyelitis and by the serums of persons convalescing from poliomyelitis.[8,19]

SUMMARY AND COMMENTS

The *in vitro* dissociation or mutation of a streptococcus having high pneumotropic virulence, as isolated from outdoor air in winter during an epidemic of influenza currently considered as of virus X origin, into a streptococcus having high neurotropic virulence and the *in vivo* genesis of respective pneumotropic and neurotropic filtrable phases of the streptococcus are reported.

The isolation in high incidence of streptococci having respective specific serologic properties from the pneumonic lungs and polio-encephalitic brains of mice following inoculation of emulsions of pneumonic lungs and polio-encephalitic brains and corresponding filtrates of emulsions, respectively, which often proved sterile to dextrose-brain broth, the absence of lesions of lungs, brains and streptococci in control mice studied in parallel with test mice in this study and a much larger number of control mice in other similar studies,[11,19] and the total absence of pleuropneumonia-like organisms and of B. bronchisepticus in the cultures of lung and brain indicate that the pneumotropic and neurotropic filtrable phases developed *in vivo* from the streptococcus.

The great difference in incidence of pneumonitis in winter and polio-encephalitis in summer in the inoculated mice was shown to be due to changes in the localizing and other properties of the streptococcus inoculated and not due to a seasonal differential susceptibility to streptococci of lungs and brains, respectively, of mice.

Through the use of methods especially favorable for the isolation of specific types of streptococci from material containing pneumotropic or neurotropic viruses and for mutation of specific types of streptococci into fil-

trable form, this and other studies[15,16] indicate perhaps that the filtrable pneumotropic viruses from mouse stocks,[2,3,6] from upper respiratory infections,[5] and from influenza in human beings, and the filtrable neurotropic viruses from mouse stocks[15,22] and from encephalitis[19] and poliomyelitis[20] may initially be derived from indigenous pneumotropic and neurotropic streptococci, respectively. The experimental production of dwarf and filtrable forms from staphylococci and B. proteus by Tulasne,[23,24] the observations on the importance of a nonhemolytic streptococcus in atypical or virus pneumonia,[21] and the climatic changes of races of Achillea[1] are in accord with such concept.

The data emphasize the importance of the phenomena of dissociation or mutation of streptococci and perhaps filtrable agents in the pathogenesis of disease.[4,8,12,18] They indicate that the high incidence of epidemic respiratory infections in winter months associated with pneumotropic streptococci and pneumotropic viruses, and of infective diseases of the nervous system in summer associated with neurotropic streptococci and neurotropic viruses, may be attributable in part to changes in the indigenous streptococcal flora in the throats and respiratory tracts of human beings and in outdoor air, which for reasons still obscure tends to acquire pneumotropic properties in temperate climates in winter and neurotropic properties in summer and to dissociate into respective filtrable phases or viruses.[11] The data further indicate the importance of making bacteriologic studies by adequate methods in addition to the purely viral methods, especially of the initial material suspected of harboring causative agents. The nature of the extrinsic influence responsible for seasonal changes of nonhemolytic streptococci is under study.[10]

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