CHANGES IN THE SERUM OF MICE INFECTED WITH *STREPTOCOCCUS PNEUMONIAE* THAT STIMULATE IN-VITRO MULTIPLICATION OF VIRULENT BUT NOT AVIRULENT STRAINS

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SUMMARY. During pneumococcal infection of mice, nucleic-acid products, including deoxynucleotides, may be released into the serum from cellular disintegration in at least three organs, the lungs, spleen and liver. The serum, after sterilisation to remove contaminating pneumococci, stimulated multiplication of virulent but not avirulent pneumococci *in vitro*. It also stimulated growth of virulent pneumococci in serum from uninfected animals and could be replaced, at least in part, by certain nucleic-acid degradation products at concentrations found in infected serum. The effects of the serum were lost after dialysis or dilution.

INTRODUCTION

The pathogenicity of many microbes depends on their ability to proliferate *in vivo* (Dubos, 1954). We and others (Braun and Firshein, 1967; Firshein, 1960; Firshein and Zimmerman, 1964; McKee and Braun, 1962) have observed that cell components capable of stimulating DNA synthesis and multiplication of gram-positive cocci, notably pneumococci, *in vitro* also stimulate their multiplication and virulence *in vivo*. The “supplement” which consists of oligomers of ribonucleic and deoxyribonucleic acid and all eight naturally occurring deoxynucleosides and deoxynucleotides could exist in the serum of infected hosts. With other factors, notably a polysaccharide capsule, these components, if present in sufficient amounts, could affect the course of a pneumococcal infection.

Other investigators have reported changes in various liver components in animals infected with pneumococci. These changes include increased uptake of amino acids and zinc (Wannemacher *et al.*, 1971; Pekarek, Wannemacher and Beisel, 1972), increased synthesis of nucleic acid and protein (Lust, 1966; Thompson and Wannemacher, 1973), and changes in metabolism of carbohydrate and lipid (Fiser *et al.*, 1971; Guckian, 1973). Some of these events were related to the synthesis of plasma proteins and could represent a specific host response to the infection (Little, 1978). In none of these investigations was the possible influence of such changes on growth rates of the infecting pathogen considered. This paper describes the results of our investigations into this possibility.

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MATERIALS AND METHODS

Organisms. Encapsulated (virulent) cultures of *Streptococcus pneumoniae* type 3 and type 1 (strains A66 and SVI, respectively), and their non-capsulated (avirulent) mutants (strains A66R and RI, respectively), were used. They were cultured in Bacto Brain Heart Infusion Broth (Difco) containing Bacto Neopeptone (Difco) 0.1% (w/v) (BHI-Neo) and maintained on Bacto Brain Heart Infusion Agar (Difco) supplemented with defibrinated rabbit-blood 5% (v/v).

In the experiments, BHI-Neo was diluted with 0.5 volumes of water and 1 volume of sterile serum obtained from infected and uninfected mice (see below). Water alone, 1.5 volumes, was used in control cultures. The rather large dilution of medium was made to test the effects of the serum to a greater extent than was possible with full-strength medium. As a result, the viable counts shown in the figures and tables are not as high as in normal medium, and the lag phase is longer.

Viable counts were performed by serial-dilution methods after inoculation of approximately 2×10^2 cells/ml of culture (standard inoculum). They were checked by microscopy to ensure that the cocci were not agglutinated by the serum and that chains of avirulent cocci were not interfering with estimates of viable counts. Although significant clumping was not observed with either cell type, chains of avirulent cocci were observed. They were broken by vigorous mixing (vortexing) of each dilution sample before plating. Virulent pneumococci did not form chains, but were treated in the same way.

Infection of mice. Group of 10 mice (BALB/cIbg) weighing approximately 25 g each were given intraperitoneal inoculation of 10^4 viable cells in 0.2 ml and were kept in an isolation chamber, with food and water, for various periods of time. At the end of each time period, the mice were weighed to ensure that there was no loss of body weight, some were killed, and the spleen, liver, and lungs were removed. The size of an organ, particularly the spleen or the lungs, can change during the course of an infection. To control this variable, samples of 2 g (wet weight) of the organs were minced and heated to dryness at 80°C at each of the three time periods (0, 12 and 19 h). If there was a difference in the water content, the concentration of each sample was adjusted during the processing described below. Blood was removed by eye puncture with a 0.1-ml haematocrit tube (Sherwood Medical Industries). Eight samples were taken from each mouse after light anaesthesia with ether during the course of 30 min, and the blood was pooled. After 12 h at 0°C, the serum was separated by centrifugation at 4000 rpm and 4°C for 15 min. Serum from uninfected mice was obtained in the same way.

Analysis of organs and tissues. A complete Schneider (1957) procedure was used to separate acid-soluble nucleotide components from acid-insoluble nucleic acids and proteins. The appropriate organ concentration, usually 2.0–5.0 g wet weight determined as described above and obtained from a specific group of mice at a particular time, was homogenised with an equal volume of 0.9% (w/v) sterile saline to give a final volume of 4–10 ml, and immediately mixed with an equal volume of ice-cold 10% (w/v) trichloroacetic acid (TCA) to yield a 5% final concentration of TCA. The treated material was processed and assayed for nucleic acids and proteins by colorimetric assay with orcinol (Schneider, 1957) or Folin's phenol reagent (Lowry *et al.*, 1951), respectively. Deoxynucleotides were determined by colorimetric assay with diphenylamine (Schneider, 1957).

The serum fraction of the blood was sterilised by filtration through Millipore filters (HA, 0.45 μ m). A sample of each serum filtrate was streaked on BHI blood-agar plates to check for contamination before analysis. The same procedure for extracting serum and separating nucleic acids and proteins from nucleotides was used as described above for the organs except that initially, to 2 ml of serum, an equal volume of ice-cold 10% (w/v) TCA was added before processing and assay.

RESULTS

Changes in macromolecular components and nucleotides during infection

Groups of mice were infected with either type-1 or type-3 virulent pneumococci;

and at various times during the course of infection, animals were killed and organs and tissues were removed and analysed for nucleic acids, proteins, nucleotides and deoxynucleotides. One complication was the contribution of the infecting pneumococci to the total amounts of the components but it was not possible to remove contaminating bacteria by sterile filtration of organ homogenates. Nevertheless, it was possible to sterilise the serum by filtration before the assays. When comparisons were made between filtered and unfiltered serum, there was an increase in the concentration of components depending upon the postinfection time, presumably due to the proliferating cocci (results not shown).

Table I shows the results as a composite from both pneumococcal types; there was little difference in the amounts of material produced *in vivo* between the two cell types. In each of the organs examined—lungs, spleen, and liver—there was a significant decrease in the concentration of total nucleotides, including deoxynucleotides, during the course of infection. This change was paralleled by a significant increase in the concentration of these components in serum from infected animals. In the case of nucleic acids and proteins, however, only the serum demonstrated a similar trend; i.e., an increase in concentration of these components with duration of infection. In contrast, in the various organs, there was an initial increase in the content of nucleic acids and proteins 12 h after infection as compared to the zero-time control followed by a decrease after 19 h to levels less than the zero-time value.

Effect of serum derived from infected and uninfected mice on multiplication of virulent pneumococci in vitro

Serum was removed from groups of mice infected with either type-1 or type-3 pneumococci after 12 or 19 h, sterilised by filtration, added to BHI-Neo broth, and seeded with a standard inoculum of type-1 or type-3 virulent cells, respectively. Serum from untreated animals was also tested. Viable counts were made at various times during growth (fig. 1). For both types, the viable count in cultures containing serum

TABLE I
Relative concentration of nucleic-acid products and proteins in organs and tissues during pneumococcal infection

Organ or tissue	Duration of infection (h)	Mean* concentration ($\mu\text{g/ml}$ of diluted tissue $\pm$ SE) of			
		nucleotides	deoxynucleotides	nucleic acids	protein
Spleen	0	23.5 $\pm$ 3.1	3.8 $\pm$ 0.11	19 $\pm$ 1.4	680 $\pm$ 8.1
	12	18.1 $\pm$ 2.9	3.1 $\pm$ 0.01	30 $\pm$ 2.1	1600 $\pm$ 29
	19	8.2 $\pm$ 0.93	1.4 $\pm$ 0.07	9 $\pm$ 0.6	189 $\pm$ 14.2
Lung	0	70.0 $\pm$ 9.2	7.1 $\pm$ 0.74	10 $\pm$ 2.1	230 $\pm$ 14.2
	12	28.2 $\pm$ 3.2	3.8 $\pm$ 0.42	13 $\pm$ 1.4	420 $\pm$ 21
	19	18.1 $\pm$ 4.1	2.3 $\pm$ 0.51	5.2 $\pm$ 0.9	138 $\pm$ 23
Liver	0	220 $\pm$ 23	31 $\pm$ 4.2	18 $\pm$ 2.2	1050 $\pm$ 17
	12	180 $\pm$ 31	26 $\pm$ 1.8	37 $\pm$ 2.0	2414 $\pm$ 90
	19	85 $\pm$ 9.1	13 $\pm$ 2.2	9 $\pm$ 0.84	805 $\pm$ 72
Serum (filtered to sterilise before analyses)	0	20.5 $\pm$ 3.4	4.2 $\pm$ 0.6	16 $\pm$ 1.4	600 $\pm$ 41
	12	39.0 $\pm$ 5.2	10.6 $\pm$ 0.6	43 $\pm$ 3.8	1775 $\pm$ 94
	19	92.5 $\pm$ 10.2	30.0 $\pm$ 1.3	68 $\pm$ 3.9	2810 $\pm$ 104

* Mean of eight experiments.

derived from mice infected for 12 or 19 h was significantly greater than in cultures supplemented either with water or serum from uninfected animals. The extent of multiplication was greater and the lag was shorter for both types in cultures containing serum from animals infected for 19 h than those infected for 12 h. However both serum supplements (12 and 19 h) showed 1.5–2.5 log differences in viable counts over controls with both types from 8 to 32 h. Serum from uninfected animals also stimulated multiplication of both types, almost 0.5–2 log difference, when compared with the water control. Whether this enhancement reflects a basal level of the same components found in “infected” serum is unknown, but results for avirulent cells (see fig. 2 and table V) suggest that other stimulating components may be present.

To determine whether serum from infected animals could enhance the growth-promoting activity of normal serum from uninfected animals, various ratios of serum from mice infected with type-3 pneumococci were added to normal mouse serum in BHI-Neo broth and their ability to enhance multiplication of type-3 pneumococci was compared. Table II shows that after 24 h, the viable count was proportional to the amount of serum added from infected mice.

Effect of serum from infected and uninfected mice on multiplication of avirulent pneumococci in vitro

Serum from infected or from uninfected mice was added to BHI-Neo cultures and seeded with avirulent strains. The results are shown in fig. 2. Multiplication of

FIG. 1.—Viable counts of virulent pneumococci in cultures supplemented with: serum preparation derived from mice infected for 19 h (□), serum preparation from mice infected for 12 h (○), serum preparation from uninfected mice (■), water control (●). A = experiment with *S. pneumoniae* type 3, and B with type 1. The results shown are representative of several replicate experiments. The mean and standard error are not shown but they were not significantly different from those in tables II–V.

TABLE II

*Effect of various ratios of serum from infected and normal mice on in-vitro multiplication of virulent pneumococci**

Percentage of each serum from		Mean† viable count (10 ⁵ cells/ml ± SE) at 24 h
infected mice	normal mice	
0	100	45 ± 6.1
25	75	193 ± 18.0
50	50	475 ± 32.4
75	25	632 ± 44.3
100	0	1205 ± 194

* The two sera were sterilised by filtration, mixed in the indicated proportions and added to BHI-Neo broth cultures. Each culture was inoculated and counted as described in *Methods*. The serum from infected mice was taken 19 h after infection.

† Mean of three experiments.

avirulent cells of both types was greater when serum from uninfected mice was added to the broth than when water was added. This result is similar to what was seen with virulent cells (compare fig. 1.). Of great significance, however, is the fact that serum from infected animals did not enhance multiplication of either type any more than normal mouse serum alone. It is interesting to note that avirulent cultures derived from both types proliferated to a greater extent than the parent cultures in the artificial medium when either water or normal serum was added. However, when serum from infected animals was added, this trend, particularly with the 19-h serum, was reversed; that is, the extent of multiplication of virulent cultures was as great or greater, tenfold in the case of type 3 pneumococcus, than that of avirulent cultures (compare figs 1 and 2).

One possible explanation for the inability of serum from infected animals to affect multiplication of avirulent mutants is that the rate and extent of proliferation is already high in medium containing normal serum. To check this possibility with the avirulent mutant derived from type 1, the medium was diluted further to decrease the growth rate before it was added to the sera. Viable-cell counts were made after incubation for 12 and 24 h (table III). Despite the decrease in viable-cell counts of the normal-serum control, sera from mice infected for 19 h did not yield viable-cell counts higher than those of the control.

Effect of dialysis and dilution on the stimulatory activity of serum from infected mice

Sera from infected and normal mice were either dialysed for various periods of time or diluted with sterile physiological saline, resterilised, and added to BHI-Neo broth. The media were seeded with virulent type-3 cells and incubated for 12 or 24 h. Viable counts were made at the end of each time period and the results are shown in table IV. Dialysis and dilution each depressed the stimulatory activity of serum from infected animals.

FIG. 2.—Viable counts of avirulent pneumococcal cultures supplemented with serum preparations from uninfected and infected mice. Symbols and comments as for fig. 1.

Effect of adding artificial supplements to normal serum on multiplication of virulent and avirulent pneumococci

If our hypothesis concerning the stimulatory effect of nucleic-acid degradation products on multiplication of virulent, but not avirulent pneumococci is valid, the addition of such components to normal serum should stimulate the multiplication of virulent but not avirulent cells. Table V shows that the addition of nucleic-acid degradation products to normal serum did have this effect on type-3 virulent, but not avirulent, cells up to levels that were two-thirds of that caused by serum from infected animals after incubation for 24 h. None of the other supplements exhibited the same

TABLE III

Effect of dilution of culture medium on the ability of serum from uninfected or infected mice to influence multiplication of avirulent pneumococci in vitro

Dilution of original BHI-Neo medium*	Mean† viable count (10^5 cells/ml $\pm$ SE) after 12 h in medium supplemented with serum from		Mean viable count (10^5 cells/ml $\pm$ SE) after 24 h in medium supplemented with serum from	
	infected animals	uninfected animals	infected animals	uninfected animals
None	$22 \pm 2.8^\dagger$	20 ± 2.8	214 ± 32	325 ± 41
Twofold	5.1 ± 0.7	4.8 ± 0.5	24 ± 4.0	38 ± 3.5

* The original medium (see *Methods*) was made two-thirds of full strength then supplemented with an equal volume of serum.

† Mean of four experiments.

TABLE IV
Effect of dialysis or dilution on the stimulatory activity of infected serum on multiplication of virulent pneumococci in vitro

Duration of dialysis (h)	Dilution of serum	Mean* viable count (10^5 cells/ml $\pm$ SE) after 12 h in broth supplemented with serum from		Mean* viable count (10^5 cells/ml $\pm$ SE) after 24 h in broth supplemented with serum from	
		infected animals	uninfected animals	infected animals	uninfected animals
None		422 $\pm$ 30	4.6 $\pm$ 0.61	937 $\pm$ 105	72 $\pm$ 6.5
1	None	188 $\pm$ 19	4.2 $\pm$ 0.42	162 $\pm$ 17	74 $\pm$ 7.0
2	None	114 $\pm$ 15	4.8 $\pm$ 0.44	141 $\pm$ 21	61 $\pm$ 6.8
6	None	46 $\pm$ 6	3.6 $\pm$ 0.39	62 $\pm$ 7.4	70 $\pm$ 6.9
24	None	43 $\pm$ 6	3.9 $\pm$ 0.39	66 $\pm$ 7.2	60 $\pm$ 5.8
48	None	44 $\pm$ 2.5	3.6 $\pm$ 0.32	56 $\pm$ 6.4	65 $\pm$ 5.8
None	2	196 $\pm$ 21.6	3.9 $\pm$ 0.31	481 $\pm$ 70	65 $\pm$ 5.4
None	4	110 $\pm$ 11.5	3.7 $\pm$ 0.28	155 $\pm$ 18	47 $\pm$ 5.0
None	10	76 $\pm$ 9.5	1.8 $\pm$ 0.14	101 $\pm$ 12	26 $\pm$ 3.1
None	50	24 $\pm$ 3.0	1.4 $\pm$ 0.23	40 $\pm$ 5.1	26 $\pm$ 2.1
None	100	24 $\pm$ 2.8	0.8 $\pm$ 0.12	34 $\pm$ 4.4	16 $\pm$ 1.9

* Mean of three experiments.

TABLE V

Effect of various supplements to normal serum on multiplication of virulent and avirulent type-3 pneumococci in vitro

Supplement to normal serum	Mean* viable cell count (10 ⁵ cells/ml) $\pm$ SE at 24 h for	
	virulent cells	avirulent cells
None	52 $\pm$ 7.3*	218 $\pm$ 37
Nucleic-acid degradation products†	802 $\pm$ 94	150 $\pm$ 31
Yeast extract‡	101 $\pm$ 24	480 $\pm$ 70
Casitone§	101 $\pm$ 23	590 $\pm$ 81
Serum from infected mice	1150 $\pm$ 185	185 $\pm$ 15

* Mean of two experiments.

† The degradation products consisted of all eight of the naturally occurring deoxynucleosides and deoxynucleotides (each 5 μ g/ml) plus acid-soluble oligoadenylates (90 μ g/ml), obtained by treating polyadenylic acid with ribonuclease for 5 min.

‡ Yeast Extract (Difco) was added to give a final concentration of 0.1%.

§ Casitone (Difco), a pancreatic digest of casein, was added to give a final concentration of 3 mg/ml.

differential response for virulent and avirulent cells. In addition, when there was a stimulatory effect, both cell types were affected by the supplement but not nearly to the levels observed with the nucleic-acid products.

DISCUSSION

The ability of exogenous factors related to low molecular-weight ribo- or deoxyribo-oligomers and some DNA precursors to stimulate multiplication of pneumococcal types *in vitro* and *in vivo* has been known for many years (Braun and Firshein, 1967). These factors act by enhancing DNA synthesis in virulent cells without affecting such processes in avirulent mutants. If such factors were released during a pneumococcal infection they could stimulate the multiplication of the proliferating pneumococci further as long as the capsule protected the pneumococci from phagocytosis.

Several observations support the idea that such components exist *in vivo* and that they can affect the multiplication of the infecting virulent pneumococci. First, an increase in the concentration of acid-soluble nucleic-acid products, including deoxynucleotides, occurs in the serum of mice infected with pneumococci. The longer the duration of infection, the greater the increase in these components. Second, serum from infected animals stimulates multiplication of virulent cocci *in vitro* in proportion to the duration of infection, and the greater the concentration of these components in the serum, the greater the stimulatory effect on multiplication. The stimulatory effect is lost after dialysis or dilution of the serum and the enhancement can be replaced, at least in part, by known nucleic-acid products, but not to the same extent by yeast extract and

protein-degradation products. Third, multiplication of avirulent pneumococci is not affected by serum from infected animals, nor do known nucleic-acid products affect such multiplication when added to serum from uninfected animals *in vitro*.

An alternative explanation for the effect of serum from infected mice on virulent cells is that the infectious process is removing an inhibitor, such as beta lysin, from the serum (Skarnes and Watson, 1957). However, if this were the case, it would be difficult to explain why avirulent cells are similarly not affected, and why nucleic-acid degradation products can augment normal serum for virulent cells.

An important question to ask is how simply the acquisition or loss of a capsule results in the profound changes observed between virulent and avirulent pneumococci. The relationship between capsular synthesis and the effects of the serum components on multiplication, which may be related to DNA replication, is unknown. We are now attempting to probe this relationship as well as identify the specific nucleic-acid products in the infected serum.

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