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THE PATHOGENESIS OF PNEUMOCOCCAL INFECTION IN MICE

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DEATH in mice infected with pneumococci has been attributed to a rapid proliferation of organisms in the bloodstream. In human lobar pneumonia the degree and persistence of bacteraemia rather than the local condition in the lungs is considered to determine the outcome of the infection (Wilson and Miles, 1964). Death in pneumococcal infections has also been attributed to intravascular haemolysis (Shumway and Pollock, 1965) and to physiological imbalance due to bacterial multiplication *in vivo* (Dubos, 1954). With the discovery of bacterial toxins that reproduce disease syndromes attempts were made to show that a toxin is involved in pneumococcal infection. Evidence has been put forward for the production *in vitro* of a leucocidin (Oram, 1934), a purpura-producing substance (Julianelle and Reimann, 1926) and an oxygen-labile haemolysin, pneumolysin (Cohen, Halbert and Perkins, 1942). Halbert, Cohen and Perkins (1946) showed that cell-free preparations of the pneumolysin were rapidly lethal to mice, although Cowan (1934) had failed to observe this earlier.

Similarities exist between the pathological changes in mice infected with pneumococci and guinea-pigs infected with *Bacillus anthracis*; both infections are characterised by a rapidly progressing bacteraemia. Anthrax has been shown to be toxin-dependent (Smith, Keppie, Stanley and Harris-Smith, 1955), and production of the toxin has been demonstrated both *in vivo* (Smith, Keppie and Stanley, 1955) and *in vitro* (Harris-Smith, Smith and Keppie, 1958).

We investigated the importance of the bacteraemia at death and the physiological changes associated with pneumococcal infection in mice. Indirect evidence was obtained that a toxin is elaborated in the course of the infection.

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MATERIALS AND METHODS

Bacteria

Throughout these experiments *Streptococcus pneumoniae* type III (no. NCTC7978) was used. Overnight cultures on horse blood agar were emulsified in a 1 in 2 dilution of peptone water in saline (Ross and McAllister, 1965) and used for injection. The virulence of the organisms was maintained by monthly intraperitoneal passage in mice, and stock cultures were kept in Robertson's meat broth to which 10 per cent. (v/v) horse blood was added.

Mice

Male Porton white mice (20–25 g in weight) supplied by Messrs Tuck, Essex, were used. They were starved for 12 hr before infection with pneumococci.

Injection of pneumococci

Pneumococci were injected intraperitoneally. The infecting dose was of the order of 10^7 – 10^9 viable organisms or 10^4 – 10^5 viable organisms, estimated by the method of Miles and Misra (Miles, Misra and Irwin, 1938). With the higher inoculum, death occurred in 12–14 hr; the smaller inoculum led to death after about 30 hr. Blood samples were taken every 2 hr as described below.

Estimation of the number of bacteria in the blood

Blood was collected by tail-clipping from the live mice, and by complete exsanguination after death. Serial ten-fold dilutions of blood were made in physiological saline and counts of viable bacteria were performed by the technique of Miles *et al.* on 10 per cent. (v/v) horse blood agar plates. The bacterial count was estimated during the final 10 hr of the experiment, as the values are very consistent during this period no matter what the size of the inoculum has been.

Biochemical estimations

Blood was collected in heparinised capillary tubes. It was centrifuged in a micro-haematocrit centrifuge (Measuring and Scientific Equipment Ltd, England) and the plasma obtained from five mice was pooled and diluted 1 in 5 to give a convenient volume on which to carry out biochemical estimations. Five groups of ten normal mice were killed and their blood was used to estimate the standard values of the plasma components under study; controls were also included in each experiment. Biochemical estimations were performed as follows.

Transaminases. Glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activity was estimated by the colorimetric method of King (1960) with 2:4-dinitrophenylhydrazine as colour reagent. The reagents were supplied in kit form by Tests Schweizerhall (Pye Unicam, England).

Glucose. Glucose estimations were performed by the glucose oxidase method with uranyl acetate as deproteinising agent and *o*-dianisidine as colour reagent. The reagents were supplied in kit form by Merck Laboratories (Darmstadt, Germany).

Plasma electrolytes. Sodium and potassium levels were estimated by means of a flame-photometer (Evans Electro-Selenium Ltd, England). Plasma chloride was estimated by the mercuric nitrate method of Schales and Schales (1941) with a reagent kit supplied by Sigma Chemical Company, Ltd (London).

Inorganic phosphorus. Inorganic phosphorus compounds in plasma react with molybdc acid and ascorbate to give molybdenum blue. This was measured colorimetrically by means of a reagent kit supplied by Tests Schweizerhall.

Alkaline phosphatase. Plasma alkaline phosphatase was estimated by the method of King and Armstrong (1934) with a reagent kit supplied by Tests Schweizerhall.

The biochemical results were found to be most constant during the 8 hr before death and these are the results reported.

Penicillin

Penicillin G (Cristapen, Glaxo Laboratories Ltd, England) was made up at a concentration of 5000 units per ml in sterile pyrogen-free saline, and injected intravenously in 0.2 ml amounts into the infected mice. Antibiotic therapy was used only in the experiments in which the infecting dose was of the order of 10^7 – 10^9 viable pneumococci. Penicillin was injected at 4, 6, 7, 9 and 10 hr after initial infection. Since these mice, if untreated, would die in an average time of 12 hr, penicillin was administered at 8, 6, 5, 3 and 2 hr before the expected time of death.

Pneumolysin

Pneumolysin was produced by a modification of the method of Cohen *et al.* Three litres of Holt's basal medium (Holt, 1962) was seeded with 100 ml of an overnight culture of pneumococci in the same medium and incubated for 18 hr in an atmosphere of carbon dioxide gas at 37°C with constant stirring. A total of 3 ml per l of sterile 50 per cent. (w/v) glucose was added at 2 and 6 hr and 5 ml per l of sterile 20 per cent. (w/v) Na_2CO_3 was added at 4 hr. Extra Na_2CO_3 was added at a later stage if the pH of the culture fell below 6.8. After incubation the pH of the culture was adjusted to pH 4.0 and the cells were deposited by centrifugation. They were then washed, in the proportion of 1 g per 100 ml in 0.1M acetate buffer, pH 4.0, containing 0.3 m.mole cysteine hydrochloride per 100 ml. They were then resuspended in the same proportion in distilled water containing cysteine. The pH was adjusted to 7.8 and the mixture was stirred for 3 hr at 4°C. 0.5 ml 0.1M basic lead acetate was then added and the mixture was centrifuged for 1 hr at 10,500 r.p.m. at 0°C in an MSE 18 centrifuge (Measuring and Scientific Equipment, Ltd, England).

The supernatant was collected and the pH was adjusted to 4.0 with cold N-acetic acid. At this point the precipitate, which began to form at pH 4.8, became floccular. After the mixture had stood for 1 hr at 0°C the precipitate was removed by centrifugation at 18,000 r.p.m. for 20 min. at 0°C. After it had been washed with ten times its own volume of water acidified to pH 4.0, the precipitate was freeze-dried to give a light-brown powder, which was stored at 4°C.

The protein content of the preparation was assayed by the method of Lowry *et al.* (1951). Its haemolytic activity against rabbit erythrocytes was assayed by titrating serial doubling dilutions of 0.1-ml volumes of a 1 mg per ml solution of pneumolysin against rabbit erythrocytes in a WHO plate.

Pneumolysin was injected intravenously into mice in doses of 500 μg (5000 MHD) in 0.5 ml Hendry's phosphate buffer, pH 7.2 (Hendry, 1948).

RESULTS

Development of bacteraemia

The bacterial count per ml of blood during the final 10 hr of the infection is shown in fig. 1. The points shown are means of all the observations made on 200 mice, of which 80 had received an inoculum of 10^4 – 10^5 pneumococci and 120 had received 10^7 – 10^9 pneumococci. When the counts were plotted "backwards" for 10 hr from the time of death there was no significant difference between the counts obtained after the high and the low inoculum. The bacteria are multiplying rapidly at this stage of the infection. The generation time during the most rapid phase of growth (i.e., between 2 and 6 hr before death) is 13 min. In calculating the generation time in this way one is assuming that the pneumococci are either evenly distributed between the blood and

tissues or are localised in the blood. The second assumption appears to be nearer the truth as we have been unable in preliminary experiments to detect marked localisation in any organs.

Gross physiological changes

Around 6 hr before death the infected mouse begins to show ruffling of the coat and a tendency to huddle in the corner of the cage along with the other mice. During the next few hours its gait becomes progressively unsteady

FIG. 1.—Mean bacterial count per ml of blood during the last 10 hr of fatal pneumococcal infection in mice.

and it tends to walk on the toes of its hind legs. Immediately before death it becomes very lethargic and these periods of lethargy alternate with periods of intense muscular spasm which often cause it to jump wildly about the cage with intense kicking of its hind legs. Death of the mouse follows one of these periods of spasm. As the infection progresses it becomes more difficult to obtain blood by tail-clipping. This may be due to a fall in blood pressure, to reduced cardiac output or to peripheral venous collapse, but such physiological changes cannot be measured easily in mice. Haemoconcentration does not appear to occur as there is no significant change in the haematocrit reading.

Biochemical changes

The average values for some of the chemical constituents of the blood plasma are given in table I. The activity of transaminases increases markedly

during the 8 hr before death. The activity of GOT and GPT is raised to approximately five times the control value at death (means of observations on 150 mice, 105 of which had received the larger inoculum). Plasma potassium (on 75 mice receiving the larger inoculum) is increased to around six times normal and blood glucose (on the same mice as the transaminase tests) falls to less than 50 per cent. of normal. All these values are of high significance ($P \leq 0.05$ by a modified Student's *t* test). Inorganic phosphorus, alkaline phosphatase and sodium (on the same mice as the potassium tests) show no significant changes. Standard deviations are given for all values obtained by analysing the blood from more than 50 mice.

TABLE I

Amount of glucose oxalacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), glucose and potassium in plasma during the terminal 8 hr of a pneumococcal infection in mice

Time (hr) before death	Mean concentration ($\pm$ SD) in plasma of			
	GOT (IU per ml)	GPT (IU per ml)	glucose (mg per 100 ml)	potassium (m.equiv. per l)
8	113 (± 40)	53.5 (± 38)	52.3 (...*)	4.1 (± 1.6)
6	158.5 (± 76.5)	58.5 (± 32)	46.25 (± 1)	4.05 (± 1)
4	336 (± 178)	71.4 (± 53)	26.1 (± 2.2)	7.3 (± 2.3)
2	217 (± 72)	106 (± 77)	... (...)	11.7 (...*)
0	509 (± 384)	196 (± 163)	22.6 (± 8)	... (...)
...†	73.29 (± 7.7)	16.3 (± 5.2)	66.5 (± 11)	1.9 (± 0.39)

SD = Standard deviation.

IU = International unit.

* Estimations performed on 20–30 mice, so SD not calculated.

† Control mice (see text).

The qualitative tests on mouse urine showed no significant change in the levels of glucose, protein or pH.

Effect of penicillin on the bacteraemia

By analogy with the methods used in the study of the mechanism of pathogenicity of anthrax it was decided to assess the effect of reducing the bacteraemia by penicillin therapy. Fig. 2 shows the general picture obtained after intravenous injection of 2500 units of penicillin G into 50 mice that had received the higher dose of pneumococci, and the bacterial count in the blood after injection of penicillin 8 hr before the expected time of death without therapy. The bacterial numbers in the blood begin to fall as soon as penicillin is injected and reach $3 \cdot 10^2$ pneumococci per ml blood 8 hr later. Control mice, untreated with penicillin, died at this time. Administration of penicillin

at other time-intervals before death led to a similar fall in the numbers of viable pneumococci.

We felt that a clearer picture of the effect of penicillin would be obtained if the time to death of mice treated with penicillin at various stages of the

FIG. 2.—The effect of intravenous injection of 2500 units penicillin G on the number of bacteria in the blood of mice infected with pneumococci. O---O---O Untreated controls; ●—●—● penicillin-treated mice.

infection was studied. The results are presented in table II. The time to death of penicillin-treated mice depends on the bacterial count per ml blood at the time of injection of penicillin. The striking point is that penicillin does not prevent death by lowering the number of bacteria in the blood.

These results suggest that death of penicillin-treated mice is due to the action of a pneumococcal product.

The effect of penicillin treatment on the levels of plasma components

Table III shows the amounts of selected plasma components in the 8 hr following injection of penicillin. These figures are from experiments in which

TABLE II

The effect of penicillin therapy on mice infected with pneumococci

Time of administration of penicillin (hr before expected time of death without therapy)	Number of mice	Bacterial count per ml blood at time of injection of penicillin	Mean time to death ($\pm$ SD) of treated mice (hr)
8	50	1×10^8	24.8 ± 18
6	20	4.5×10^8	23.5 ± 11.5
5	10	1×10^{10}	17.6 ± 11
3	10	1.12×10^{12}	11.6 ± 6.5
2	10	5×10^{12}	11 ± 3

SD = Standard deviation.

penicillin was administered 8 hr before the expected time of death in an untreated infection to 50 mice that had received the larger inoculum of pneumococci. After penicillin therapy, GOT and GPT activities continue to rise at

TABLE III

Effect of injection of 2500 units penicillin G on the concentration of certain plasma components of mice infected with pneumococci

Time after injection of penicillin (hr)	Mean concentration ($\pm$ SD) in plasma of				
	GOT* (IU per ml)	GPT* (IU per ml)	Na ⁺ (m. equiv. per l)	K ⁺ (m. equiv. per l)	glucose (mg per 100 ml)
2	215 (± 35)	107.5 (± 37.5)	135 (± 5)	4.5 (± 0.8)	39 (...)
4	310 (...) [†]	130 (...)	115 (...)	5.0 (...)	... (...)
8	1303 (± 844)	693 (± 73.5)	149 (± 1)	12.5 (± 2.2)	29.17 (± 1.3)
... [‡]	509 (± 384)	196 (± 163)	130 (...)	11.7 (...)	22.6 (± 8)

SD = Standard deviation.

IU = International unit.

* See table I.

[†] Where no SD is included the estimations were performed on the blood of 20–30 mice.

[‡] At time of death in untreated mice.

a more rapid rate than they do in an untreated infection. There is a slight increase in the amount of plasma potassium compared with that in untreated animals and there is a slowing down in the rate of fall of blood glucose. Having established that physiological changes can occur without the presence of intact bacteria, we decided to study the effect of one pneumococcal product, pneumolysin, on the levels of serum enzymes.

The effect of pneumolysin on plasma enzyme activity

Intravenous injection of 5000 MHD pneumolysin proved to be non-lethal. Blood was removed 1, 2, 3 and 6 hr after injection of pneumolysin into 25 mice and plasma GOT and GPT activities were determined (table IV). These enzymes show a transient increase in activity to levels similar to those found during the bacterial infection.

TABLE IV

*Effect of intravenous injection of 500 µg pneumolysin on plasma enzyme levels in mice**

Time (hr) after injection	Mean concentration ($\pm$ SD) in plasma of	
	GOT† (IU per ml)	GPT† (IU per ml)
1	207 ($\pm$ 36)	60 ($\pm$ 5)
3	278 ($\pm$ 161)	135 ($\pm$ 85)
6	149 ($\pm$ 31)	67.5 ($\pm$ 17.5)
...‡	73.29 ($\pm$ 7.7)	16.3 ($\pm$ 5.2)

SD = Standard deviation.

IU = International unit.

* Each estimation performed on the blood of 25 mice.

† See table I.

‡ Control mice (see text).

DISCUSSION

It had previously been thought that the main cause of death in pneumococcal infections in mice was the overwhelming bacteraemia. Our work has substantiated that a rapid multiplication of organisms does occur in the terminal 10 hr of the infection and that counts of 10^{12} pneumococci per ml of blood are recorded at death.

With this in mind, the metabolic response of the mouse to infection with pneumococci was studied, and the results were compared with the biochemical changes that occur during other septicaemias (table V).

Many of the changes that we have recorded occur also in infections with *B. anthracis* (Smith, Keppie, Stanley and Harris-Smith, 1955) and *Staphylococcus aureus* (Smith, 1965). However, the over-all picture appears to be characteristic for pneumococcal infection. This bears out the conclusions of Dubos (1945) that it is through a disturbance of the physiological processes of the host that pathogenic bacteria cause the symptomatic and pathological manifestations that characterise each individual disease. In experimental bacterial infections it is very difficult to explain individual changes in the host's metabolism. In an acute infection such as pneumococcal pneumonia there is too little time for marked histological changes to occur and one can only

speculate on the possible reasons for physiological imbalance. Many of the changes occurring in the mouse are probably secondary manifestations of toxæmia. Elevated enzyme activity and raised potassium levels might indicate general cytotoxicity caused by the pneumococcus. The high standard deviations recorded in the transaminase estimations may be attributed to the fact that fluctuations in the amount of these enzymes in the blood may not always be directly correlated with the severity of the disease, but our over-all results

TABLE V

*Comparison of the physiological changes occurring during infection with Streptococcus pneumoniae with those occurring during infection with Staphylococcus aureus and Bacillus anthracis**

Organism and experimental animal	Amount of increase or decrease, in the disease shown, of the plasma content of						
	glucose: decrease	inorganic phosphate: increase	alkaline phos- phatase: increase	trans- aminase: increase	potassium: increase	sodium: decrease	chloride: decrease
<i>Streptococcus pneumoniae</i> : mouse	+++	—	—	+++	+++	—	—
<i>Staphylococcus aureus</i> : mouse	++	+	++	+	—	—	—
<i>Bacillus anthracis</i> : guinea-pig	+++	+++	+++	NA	+	±	++

+ = 0-25 per cent. control value; ++ = 25-50 per cent. control value; +++ = >50 per cent. control value; — = no change.

NA = Not available.

* The results for *Staph. aureus* are taken from the work of Counts *et al.* (1961) and those for *B. anthracis* from the results of Smith (1960).

are statistically significant. Pneumococcal haemolysin, when injected intravenously, causes increases in plasma enzyme activity. This indicates that the physiological changes are not solely due to the presence of intact bacteria.

Mice infected with pneumococci continue to die after sufficient antibiotic therapy to reduce the number of bacteria to *c.* 300 per ml blood. Death, therefore, is not due solely to the presence of pneumococci in the blood, which is virtually sterile 6-8 hr after administration of penicillin.

The time at which penicillin is injected into the infected mice determines their survival time: the later the administration of the drug the shorter the survival time. This time is directly proportional to the logarithm of the number of pneumococci per ml of blood at the time penicillin is given. Such results are suggestive of the production of a toxin during the logarithmic phase of bacterial growth *in vivo*.

It is also of significance that the level of plasma transaminase activity continues to rise during therapy, but that the blood glucose levels are stabilised, indicating that the fall in blood glucose may in part be due to its utilisation by the pneumococcus.

These findings bear a strong resemblance to those obtained by Keppie, Smith and Harris-Smith (1955) in infections of guinea-pigs with *B. anthracis*. Streptomycin therapy at various stages of anthrax infection did not prevent death of the animals although the survival time was prolonged. A toxin was implicated in this disease, and these workers were later able to isolate it both *in vivo* (Smith, Keppie, Stanley and Harris-Smith, 1955) and *in vitro* (Harris-Smith *et al.*, 1958).

So far we have been unable to demonstrate a toxin in the plasma of infected mice. This may be due to inactivation of the toxin during attempts to sterilise the plasma *in vitro* or to the fact that the toxin may be rapidly fixed by the tissues *in vivo*. *Strep. pneumoniae* produces an oxygen-labile haemolysin *in vitro* (Cowan, 1934; Cohen *et al.*, 1942). Cowan reported that pneumococcal haemolysin (pneumolysin) is fixed by erythrocytes. This toxin may play a part in acute pneumococcal infection because it causes an increase in plasma enzyme activity, but it is probably only one of several factors contributing to death. The pneumococcus may produce a number of potentially toxic substances some of which may be produced only *in vivo*. This might account for the fact that this organism must be frequently passed through experimental animals in order to maintain or enhance its virulence. Attempts are being made at present to detect such substances *in vivo*.

SUMMARY

Pneumococcal infection in mice was studied by following physiological changes and the course of the bacteraemia during the infection. Significant increases in the activity of plasma glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were recorded, along with a rise in potassium and a fall in blood glucose. There was no significant change in plasma alkaline phosphate, inorganic phosphorus or sodium. Similar changes in GOT and GPT were recorded after injection of a cell-free pneumococcal product. These changes were compared with those occurring in other septic-aemias. Antibiotic therapy dramatically reduced the bacterial numbers without preventing death. Such results suggest that the pneumococcus produces a toxin or toxins *in vivo*.

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