THE INFLUENCE OF A PRE-EXISTING RESPIRATORY INFECTION ON THE COURSE OF ANOTHER SUPERIMPOSED BY THE RESPIRATORY ROUTE

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THERE is a large body of experimental evidence concerning alteration in the resistance of a host to infectious disease by modification of its diet, hormone balance, physical environment, etc. On the other hand, knowledge about the effect of pre-existing infection on the reaction of a host to a superimposed infection of another kind is very limited. Certainly there is little known about the mechanisms that may lead either to a synergic type of action of the two invaders or to increased resistance on the part of the host to one or the other.

From at least as early as the 16th century claims were made that " one ill cureth another ". However, during the late 19th century and early in the present one a mass of literature accumulated on the subject of inhibitory action by one organism on another. An extensive review of this work is given by Florey and others (1949). While the greater part of this effort seems to have been devoted to antagonisms demonstrated by *in vitro* technique, there is also much writing on attempts to inhibit a pre-existing infeetion by exposing the host by various means to another pathogen. In this second field it seems that success or failure followed the experimenter, in good measure at least, according to his general beliefs supported by his " clinical impressions ". Perhaps for this reason alone, efforts eventually ceased and there seems to have been no serious attention given to the problem from the experimental angle until the classical researches of Shope (1931 : 1936) on swine influenza. He clearly established that *Haemophilus influenzae* can precipitate acute infection with the virus of swine influenza but the bacterium produces no pathological change when acting alone. Subsequently a large body of evidence has accumulated concerning the activation of latent or superimposed bacterial infection by the influenza or pneumonia viruses. This can be exemplified by the work of Brightman (1935) and Glover (1941) with *Streptococcus pyogenes* in the ferret, and that of Volkert, Pierce, Horsfall and Dubos (1947) with *Mycobacterium tuberculosis* in the mouse. There has also been a reawakening of interest in the possible inhibition of one infection by the action *in vivo* of a second pathogen. But attention has been directed principally to such interference phenomena as occur in virus infections (reviewed by Lennette, 1951). A few sporadic findings in the field of bacterial disease clearly indicate that antagonisms can also occur but the researches have not been primarily designed to investigate the general problem. Thus, for example, Pullinger (1936; 1938) showed that when a combined injection of living tubercle bacilli and brucella organisms was made in the guinea-pig, the tubercle bacilli induced a local non-specific immunity response which impeded or completely prevented the

establishment of brucella infection. Again in a study of the efficacy of staphylococcal vaccines and their specificity, Cowan (1939) showed that a killed suspension of *Pasteurella pseudotuberculosis rodentium* gave the same order of protection against virulent staphylococci as any of the specific vaccines tested. Much more recently Mika, Goodlow, Victor and Braun (1954) and Nyka (1956) have shown that in the guinea-pig a primary infection with *Brucella* sp. confers a non-specific resistance in the host to a superimposed infection with *Coxiella burneti* (Mika *et al.*) or *Myco. tuberculosis* (Nyka).

Our interests have been centred mainly on problems connected with experimental respiratory infection. So far the guinea-pig has been the only host used. The anatomy of its respiratory tract is particularly well suited to studies on the influence of inhaled particle size on infectivity. Thus, for example, Harper and Morton (1953) have shown that the percentage retention of particles of different size found in the various areas of the respiratory tract of the guinea-pig follows closely that found by other workers on retention of particles in the human tract. This is why we selected the guinea-pig for earlier studies, accepting as a result serious limitation on the number and type of pathogens that could be tested, for the species seems susceptible to relatively few of the respiratory diseases of major interest. Nevertheless, it seemed that by such studies certain broad principles concerning modes of initiation of respiratory disease might emerge; and it now seemed of interest to examine the effect of those same pathogens acting together.

In the present work five species of pathogen have been studied. *Brucella suis* was used to initiate a relatively chronic form of disease and *Myco. tuberculosis* on a much smaller scale. *Pasteurella pestis*, *Bacillus anthracis* and *Str. pyogenes* (Group C) which normally can produce a fatal septicaemia by the respiratory tract, were used as the secondary invaders in animals suffering from either brucellosis or tuberculosis. In addition, some experiments were made in which *Br. suis* was superimposed on tuberculosis and *vice versa*. Finally, a few experiments were made using a relatively avirulent strain of *Str. pyogenes* (Group C) as the primary invader.

METHODS

Techniques for cloud production and assessment

These were as previously described (Henderson, 1952; Druett and May, 1952; Druett, Henderson, Packman and Peacock, 1953).

Organisms and their preparation for use

Earlier records give details concerning the strains of *Br. suis*, *P. pestis* and *B. anthracis* that were used, as well as methods for the production of suspensions (Druett, Henderson and Peacock, 1956*a*; Druett, Robinson, Henderson, Packman and Peacock, 1956*b*; Druett *et al.*, 1953).

One bovine strain (Vallée) of *Myco. tuberculosis* kindly supplied by Dr. Pierce of the Rockefeller Institute was used. Details for the preparation of suspensions for spraying, cloud collection and assessment have been given by Albericci and Fletcher (1956).

Two strains (K.159 and D.181) of *Str. pyogenes* (Group C) were kindly supplied by Dr. Lancefield of the Rockefeller Institute. Strain K.159 (relabelled S1) is relatively avirulent and was isolated from guinea-pigs during an outbreak of cervical lymphadenitis. Strain D.181 (relabelled S2) is the original strain 4 of Seastone (1939) and is virulent. They were laid down as freeze-dried cultures. Surface growth was obtained on 10 per cent horse blood agar (tryptic meat base). This medium maintained the strains in mucoid form. In preparing suspensions for spraying, a tryptic meat broth was used to which was added 1 per cent

Marmite, 0·6 per cent K_2HPO_4, 1 per cent xylose and 5 per cent horse serum, pH 7·4. Isolated mucoid colonies from an overnight growth at 37° were inoculated into 10 ml. of liquid medium and incubated for 7 hr. at 37°. A loopful of this culture was streaked on blood agar to check colonial form and the remainder used to inoculate 100 ml. of liquid medium which was then incubated at 37° for 18 hr. Pure mucoid forms were then obtained with a viable count of about 10^8 units per ml. For the production of clouds of the virulent strain, dilutions of this material were made in 10 per cent CCY broth (Gladstone and Fildes, 1940) in phosphate buffer, pH 7·2. Several drops of olive oil were added before spraying to avoid foaming. Cloud samples were collected in CCY diluent to which 0·05 per cent sodium alginate was added to avoid aggregation of particles during the collecting process (Henderson, 1952); a drop or two of olive oil was also added. On spraying, a sharp rise in viable count occurred owing to disaggregation of chains of cocci. After 4 min. the count became practically constant at a level of about eleven times that of the starting material and microscopic examination of the suspension showed organisms in groups of 2 to 5. Viable counts were made on tryptic meat agar containing 5 per cent horse serum. There was no loss in viability in suspensions as a result of prolonged refluxing during spraying and the undiluted culture could be stored satisfactorily at 0°–4° for at least a week.

Animals

Guinea-pigs weighing 350–400 g. were used. In all experiments they were allocated to their groups by random sampling. After exposure they were housed four to a cage, measuring approximately 40 × 40 × 24 cm. high. During much of the work they received a so-called complete diet in pellet form. This was done to avoid dust and insects, but it was later found that the diet was deficient and some deaths occurred some weeks after the animals had been exposed to a primary infection with *Br. suis*, an otherwise very rare event. Good hay of timothy grass was used as a supplement in later experiments and the trouble ceased. The results obtained in these latter experiments in no way invalidated the earlier findings.

All animals were autopsied at death or at appropriate times after infection. Various organs were cultivated and as necessary a selective medium was used for any particular organism involved.

Selective culture media

In experiments involving *Br. suis*, *P. pestis*, *B. anthracis* and *Str. pyogenes*, it was fortunate that highly selective media for the first three organisms were available. That for *B. anthracis* has already been described (Morris, 1955); those for *Br. suis* and *P. pestis* are to be described later by Mr. E. J. Morris of this Department. Where *Str. pyogenes* was involved, incorporation of 1/100,000 brilliant green to tryptose agar medium allowed ready growth of the streptococcus but completely inhibited *B. anthracis*. Where *Br. suis* and *P. pestis* were involved with the streptococcus they were grown on the respective selective media and the more rapid growth of *Str. pyogenes* on tryptic meat agar made the incorporation of inhibitors for *Br. suis* and *P. pestis* unnecessary. Experiments involving *Myco. tuberculosis* with other pathogens called for no selective media. Under the selected conditions of test macro- and microscopic evidence of infection with tubercle bacilli were adequate guides and because of the slowness of growth of *Myco. tuberculosis* no problem arose in detecting the presence of any accompanying pathogen.

RESULTS

Br. suis *as the Primary Invader*

Earlier work (Druett *et al.*, 1956*a*) showed that an estimated respiratory retention of about 60 single cells of *Br. suis* produced about 90 per cent infection. Harper (1955) has shown that 2–3 days after exposure to such small doses there is already an increase in the number of organisms recoverable from the lung. Infection of bronchial glands can be detected as early as the 4th day. From the 8th to 10th day practically all animals that will become infected yield brucella from bronchial glands and a few animals already give a positive culture from the spleen. At about 21 days there is a well marked generalised infection which reaches its

peak towards the end of the 4th week. Thereafter it declines so that by about the 18th week very few organisms are recoverable from the spleen.

More recently we have shown that the cellular response in the lung is primarily in lymphoid and lymphatic tissue and that the substance of the lung is not involved—at least not until very late in the disease. The first histological change is found about the 10th day after exposure. There is then an infiltration of mononuclear cells round the various lymphatics and foci of lymphoid tissue along the course of the main bronchi and blood vessels. Most of the cells are lymphocytes, macrophages and reticular cells. This process steadily increases until about 5 weeks after exposure, during which time there is a great increase in lymphoid tissue but there is no evidence of necrosis. The bronchial lymph nodes undergo a similar sequence of changes but these are followed by necrosis shown in the form of abscess formation with well marked peripheral fibrosis. By the 16th week after infection the whole lymphatic system is rapidly returning to normal. A few abscesses in lung tissue have by then developed and, as was shown by Harper (1955), this is accompanied by a sharp recurrent rise in the number of viable brucella organisms recoverable from the lung but the animals appear healthy and steadily gain weight.

Experiments with B. anthracis *as secondary invader*

In the first experiments guinea-pigs were exposed to a cloud of single *Br. suis* organisms estimated to give a 90 per cent infection rate. Eight days later they were exposed to a cloud of anthrax spores which for normal animals was estimated to give a 90 per cent death rate. The anthrax ran a normal course and there was no indication of reduction in the number of animals dying over those in control groups. In other experiments the initial dose of *Br. suis* was 100-fold higher but thereafter the animals were similarly treated; this procedure also failed to influence the course or degree of anthrax infection.

When experiments were made on exactly similar lines but exposure to anthrax spores was delayed for 21 days after exposure to *Br. suis* a marked reduction in death rate from anthrax was observed. The expected degree of about 90 per cent infection with brucella was found and there was a 95 per cent death rate in anthrax controls. In contrast, in the combined group only about 18 per cent of animals died with combined anthrax and brucella infection, one animal found negative for brucellosis died of anthrax, and one died having a positive brucellosis infection but culture of heart's blood and spleen was negative for anthrax. Survivors were autopsied 24 days after exposure to anthrax spores and none showed evidence of infection with *B. anthracis* on culture of spleen, liver, cervical or bronchial glands; all showed positive generalised brucellosis. Results of this type are readily reproducible. Table I summarises the combined data from three experiments of which one is that described above; the findings are self-evident.

Effect of route of infection

It was of interest to examine whether the route of primary or secondary infection altered the general course of events just described. In some experiments animals were first infected with *Br. suis* by the subcutaneous route and later exposed to a cloud of anthrax spores. In others, animals were exposed to a cloud of *Br. suis* and later challenged with anthrax spores by the intracutaneous route.

In one experiment guinea-pigs received an estimated dose of 22 cells of *Br. suis* subcutaneously in the flank. This was known to be about 4 ID_{50} and in the present experiment it was shown that of 10 controls receiving approximately 4 cells subcutaneously 6 showed generalised brucellosis 21 days later and 4 were negative. At this time an estimated LD_{90} of anthrax spores was superimposed by the respiratory route on the animals that had received 22 brucella organisms. The results are summarised in Table II ; it happens that they form an almost exact replica of those given in Table I, thus indicating that primary infection by the respiratory route with brucella is not a necessary condition for the development of resistance to anthrax.

TABLE I.—*Combined Data from 3 Experiments showing the Inhibitory Action of Active Brucellosis on Superimposed Respiratory Infection with* B. anthracis

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors.
B*	30	0	27+B
B plus A	60	10+B+A	42+B
		5+A−B	2−B
		1+B−A	..
A	60	54+A	Not examined

Animals exposed to anthrax spores 21 days after respiratory infection with *Br. suis*.
*B = *Br. suis*. A = *B. anthracis*. + = positive culture. − = negative culture.
Experiments terminated 24 days after challenge with anthrax spores.

TABLE II.—*Influence of Route of Primary Infection with Brucella by Subcutaneous Route on Incidence of Anthrax Superimposed by the Respiratory Route*

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors 31 days after exposure to A.
B*	10	0	9+B
B plus A	20	3+B+A	14+B
		1+A−B	1−B
		1+B−A	..
A	20	17	Not examined

Infections : *Br. suis ca.* 4 ID_{50} subcutaneously.
B. anthracis spores *ca.* LD_{90} as a single spore cloud.
Anthrax superimposed 21 days after infection with *Br. suis*.
* B = *Br. suis*. A = *B. anthracis*. + = positive culture. − = negative culture.

The experiments designed to test the effects of respiratory brucellosis on the course of anthrax induced intracutaneously failed to show any significant degree of protection. The strain of *B. anthracis* used was highly virulent by this route ; probably one or two spores were enough to kill. In one experiment the test group of animals first received an estimated ID_{90} dose of *Br. suis* and 21 days later each received a dose of 16 spores. All (20) control animals and 14/20 test animals died of anthrax within 23 days after challenge when the experiment was terminated. This result seemed suggestive but subsequent experiments showed a completely negative response although the challenge dose of spores was kept low (10 spores). These findings are discussed later.

Attempts at passive transfer of protection

Even the most sensitive type of serological test, as for example the agar diffusion precipitation technique of Ouchterlony, fails to show any antigenic relationship between *Br. suis* and *B. anthracis*. Nevertheless, it seemed important to test if the serum from animals with brucellosis could protect normal animals against respiratory infection with *B. anthracis*. It does not do so. In one experiment animals with brucellosis of 21 days standing were anaesthetised and bled from the heart. The blood from each was allowed to clot for 30 min. at 37°; the tubes were then "ringed" and placed at 0–4° overnight. The serum was decanted and spun to remove blood cells. Immediately thereafter 4 ml. of each sample (still heavily infected with *Br. suis*) was given subcutaneously to a corresponding normal guinea-pig. Twenty-four hours later these animals were exposed to an estimated LD_{90} of anthrax spores by the respiratory route. Concurrently a group of animals each receiving 4 ml. of normal guinea-pig serum in like manner and a group with acute brucellosis of 21 days' duration were similarly exposed to anthrax. The results are summarised in Table III. It is clear that no protection was given by serum from animals with brucellosis but the usual protective effect was evident in animals suffering from this disease. This type of experiment was repeated using a "pooled" and filtered brucella serum, each test animal receiving 8 ml. subcutaneously 24 hr. before challenge with anthrax spores. There was no evidence of passive protection, although again animals with active brucellosis exposed to the challenge dose showed the usual degree of resistance. This finding is discussed later.

TABLE III.—*Experiment on Non-specific Passive Protection against Respiratory Anthrax with Serum from Guinea-pigs with Brucellosis*

Preliminary treatment of animals.	Animals exposed to :	Number of animals : Tested.	Died.
4 ml. B* serum	A	20	19+A
4 ml. normal serum	A	20	16+A
Nil	B then A at 21 days	20	3+B+A
Nil	B	10	10+B
Nil	A	20	18+A

* B = *Br. suis*. A = *B. anthracis*. + = positive culture.

The state of immunity to anthrax in animals with brucellosis after surviving exposure to anthrax spores

It seemed of interest to test if a specific anthrax immunity response could be detected in these circumstances. Two groups of animals with brucellosis of 3 weeks' duration were, as in previous experiments, exposed to anthrax spores (*a*) by the respiratory route and (*b*) intracutaneously. Fig. 1 summarises the results which fully confirm the earlier findings; no effective protection against intracutaneous challenge was found, but a high degree of protection against challenge by the respiratory route was obtained. Five weeks later (*i.e.*, 8 weeks after the initiation of brucellosis) the survivors in the group exposed to respiratory challenge with anthrax spores were injected with spores by the intracutaneous route. The results, summarised in Fig. 2, at least suggest that some animals had acquired specific immunity to anthrax. Thus, while 17/20 control animals (with brucellosis

of 8 weeks' duration) died of anthrax, only 8/19 in the test group so died. Attempts were made to show the presence of circulating antitoxin in both test groups, but the method used (Belton and Henderson, 1956) failed to detect it; however, as noted in the paper quoted, the technique is known to have limited sensitivity.

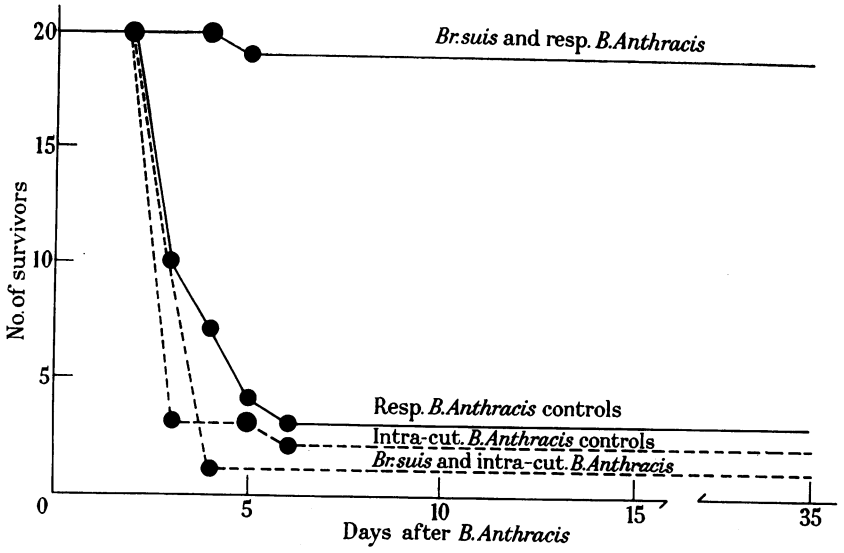

FIG. 1.—Effect of a primary respiratory infection of *Br. suis* on a secondary infection of *B. anthracis* given by the respiratory and intracutaneous routes.

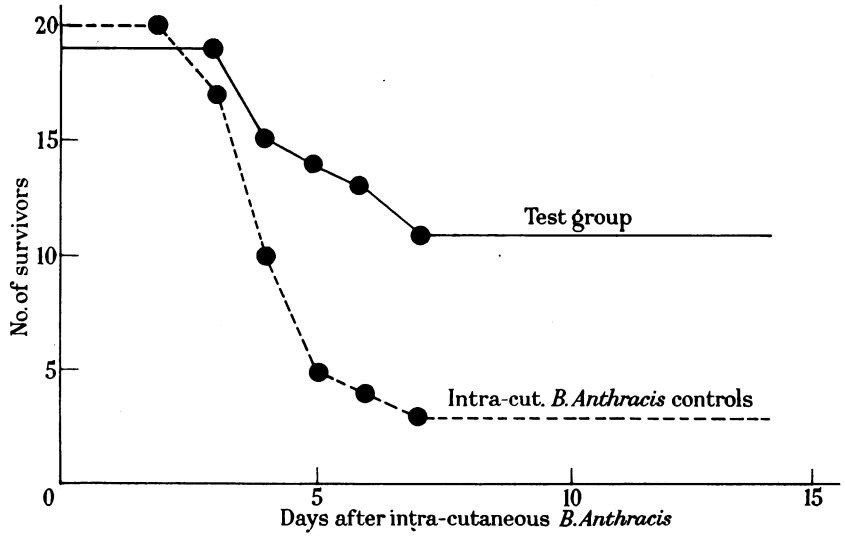

FIG. 2.—Experiment showing that animals with brucellosis which exhibit non-specific resistance to respiratory challenge with anthrax spores thereafter develop a specific immunity to anthrax.

Experiments with P. pestis *as secondary invader*

Earlier work (Druett *et al.*, 1956*b*) had shown that two forms of plague, both originating in the respiratory tract of the guinea-pig, can develop according to the size of the particle carrying *P. pestis* presented to the host. Small particles initiate a broncho-pneumonia which eventually leads to septicaemia and death. Large particles establish a septicaemia and death results more quickly without the development of pneumonia.

In the first experiment on combined infection, therefore, both small (single cell) and large (about 12μ diameter) particle clouds of *P. pestis* were used. As in the experiments with *B. anthracis*, it was found that 8 days after infection with single cell clouds of *Br. suis*, guinea-pigs had failed to develop any resistance to moderate doses (LD_{80-90}) of *P. pestis* delivered either as small or as large particles; this applied even if the initial dose of *Br. suis* was high (*ca.* 100 ID_{90}). However, 21 days after exposure to *Br. suis* protection against superimposed plague could be demonstrated.

With a high infective dose (100 ID_{90}) of *Br. suis*, animals exposed to a cloud of single cells of *P. pestis* showed delay in death time over that in controls but there was no significant degree of protection. If 12μ diameter particles of *P. pestis* were used to challenge similarly infected animals there was about a 75 per cent survival rate. The results of a typical experiment are shown in Table IV.

TABLE IV.—*Effect of Brucellosis Established by the Respiratory Route on the Development of Infection with* P. pestis *Similarly Applied*

Animals exposed to :	Number of guinea-pigs. Tested.	Number of guinea-pigs. Died.	Autopsy of survivors (21 days after *P. pestis* challenge).
B*	17	0	15+B
B + P (single cell particles)	30	26+P+B	1+B−P
		3+P−B	
B + P (12 μ diam. particles)	30	5+P+B	11+B
		2+P−B	2−B−P
P (single cell particles)	25	25+P	..
P (12 μ diam. particles)	25	23+P	2−P

Initial infecting dose *Br. suis* by respiratory route = 100 ID_{90}.

Animals exposed to *P. pestis* 21 days after infection with *Br. suis* (single cell cloud).

* B = *Br. suis*. P = *P. pestis*. + = positive culture. − = negative culture.

In an isolated experiment it was found, as might now be expected, that if animals were exposed to a cloud of 12μ diameter *Br. suis* particles and later to a similar one of *P. pestis* then a high degree of protection resulted. Groups of 20 animals were used; 95 per cent of brucella controls were found infected and 85 per cent of plague controls died, whereas in the combined infection group 85 per cent of animals survived the plague challenge.

Inhibitory action of plague-infected tissue on the growth of Br. suis

At death or at autopsy of animals on the termination of an experiment various tissues were cultivated on selective media mentioned earlier to confirm the presence or absence of active infection with the selected pathogen. The procedure was to rub a cut tissue surface over selective agar. Animals with brucellosis of 3 weeks' duration yield a confluent growth on selective agar when tissues such

as lung and spleen are used. It was soon noticed, however, if such animals died of superimposed plague the growth of brucella was never confluent, frequently very scanty and confined to discrete colonies at the periphery of the smear, or entirely absent. These characteristics followed no regular pattern, except perhaps the order of inhibition in any animal was probably most strongly noticeable in lung, less so in bronchial and cervical lymph glands and least in spleen; but exceptions occurred. The phenomenon was further investigated. First it was shown that an *in vitro*-grown culture of the strain of *P. pestis* used had no inhibitory action on the growth of *Br. suis* when taken from *in vitro* culture. (The tests were performed by the simple procedure of streaking plates of selective agar with *Br. suis* and cross-streaking with *P. pestis*—which, of course, was itself inhibited in growth.) Likewise it was shown by the use of a medium highly selective for *P. pestis* that *Br. suis* had no inhibitory action on this organism. Then it was found that if the lung of an animal dead from uncomplicated plague was streaked at right angles across a similar smear of either *in vitro* or *in vivo*-grown *Br. suis* on its selective medium an area of complete inhibition of growth occurred at the crossing zone, but here the converse did not hold; *in vivo*-grown brucella failed to show any trace of inhibition for *P. pestis* (*in vitro*- or *in vivo*-grown) on selective medium for the latter organism.

It would seem from these observations that the factor(s) suppressing the development of plague in animals with brucella infection is distinct from the plague factor(s) which suppresses growth of *Br. suis*. However, this particular phase of the work requires special treatment which it has not yet been given. It is mentioned primarily to draw attention to the manifold factors probably at work in processes of combined infection.

Myco. tuberculosis *as secondary invader*

Two experiments were made in which *Myco. tuberculosis* was superimposed by the respiratory route on animals already suffering from brucellosis initiated by the same route. Unfortunately, at the time, the ID_{50} with single cell clouds of our strain of *Myco. tuberculosis* had not been defined. It is now known that the dose given contained about 500 ID_{99+} and was sufficiently severe to produce the first deaths among control guinea-pigs about 4 weeks after infection. All animals in the combined infection groups developed tuberculosis but in the early stages of the experiment there was clear macro- and microscopical evidence of a marked retardation in the development of tuberculosis over that in control groups. Thus it was not until about 5 weeks after infection with *Myco. tuberculosis* that the lesions in control and test animals showed about the same degree of severity.

More recently an experiment was made in which guinea-pigs with brucellosis were exposed to a cloud of single units of tubercle bacilli so that each animal had an estimated intake of 24 units. This probably represents about 20 I.D. As in the earlier experiments, tuberculosis was detected by culture in all animals by the 5th week after exposure but again there was a very definite delay in development over that in controls. Thus, for example, at the 4th and 5th week after exposure to tubercle 10/10 control animals on each occasion showed macroscopic evidence of tubercle formation in lung tissue, whereas only 2/10 were similarly affected in each of the brucellosis groups. By the 6th week 7/10 test animals showed evidence of tubercle formation whereas the controls were not only positive

but much more advanced. The experiment is still in progress 15 weeks after exposure but no deaths from tuberculosis have yet occurred.

Str. pyogenes *as secondary invader*

The virulent Group C strain is highly invasive by the respiratory route when presented to the guinea-pig as a cloud of small particles. In exposed animals abscesses began to form by the second day after exposure. These are preceded by cellular infiltration in the alveoli at the edge of the lung. Septicaemia later develops, very frequently accompanied by a pericarditis. Death supervenes between the 6th and 10th day after exposure.

Guinea-pigs with pre-existing brucellosis induced by the respiratory route proved to have no detectable alteration of response to a superimposed streptococcal infection of this group: a finding reminiscent of the results obtained with single cell clouds of *P. pestis*. Unfortunately we have been unable to extend the experiment as with *P. pestis* to the use of clouds of large particle size; highly irregular response to infection so far obtains with such clouds.

Myco. tuberculosis *as Primary Invader*

It is now known that an estimated intake of the order of 10 single cell units of our strain of bovine *Myco. tuberculosis* will produce about 100 per cent infection in guinea-pigs. This fact emerged after primary experiments on combined infection with *Myco. tuberculosis* and *Br. suis* or *B. anthracis* had been planned. It is now known that in these experiments a high dose (order of 500 ID) of tubercle bacilli was given. This massive primary infection may somehow have influenced results, but it remains that the protective effect produced by the primary tuberculous infection three weeks after exposure was closely similar in degree to that obtained with brucellosis against anthrax or plague. Table V summarises the results of a typical experiment with anthrax and Table VI of one in which *Br. suis* was the secondary invader. First it is seen that the dose of *B. anthracis* given killed 90 per cent of controls but only 15 per cent of those in the combined group died with an anthrax septicaemia. Again the dose of *Br. suis* was sufficient to infect 85 per cent of controls but in the combined group, tested at the second, third, fourth and fifth week after superimposing brucellosis, only 12·5 per cent of animals were found to suffer from this infection.

TABLE V.—*Effect of Established Pulmonary Tuberculosis on the Development of Anthrax by the Respiratory Route*

Animals exposed to:	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors.
Tb*	20	2+Tb	18+Tb
Tb plus A	20	3+Tb+A	16+Tb
		1+Tb−A	..
A	20	18+A	..

Animals exposed to anthrax 21 days after infection with *Myco. tuberculosis*.
* Tb = *Myco. tuberculosis*. A = *B. anthracis*. + = positive culture. − = negative culture.
Experiment terminated 21 days after challenge with spores.

TABLE VI.—*Effect of Established Pulmonary Tuberculosis on the Development of Brucellosis by the Respiratory Route*

Animals exposed to :	Guinea-pigs examined : weeks after exposure to *Br. suis*. 2.	3.	4.	5.	Deaths from Tb.
Tb*	10/10+Tb	10/10+Tb	10/10+Tb	10/10+Tb	8/10 (8–14 weeks after exposure to Tb)
Tb plus B	10/10+Tb (1/10+B)	10/10+Tb (1/10+B)	10/10+Tb (1/10+B)	10/10+Tb (2/10+B)	*Nil*
B	*Nil*	*Nil*	*Nil*	17/20+B	*Nil*

Animals exposed to *Br. suis* 21 days after respiratory infection with *Myco. tuberculosis*.
* Tb = *Myco. tuberculosis*. B = *Br. suis*. + = positive culture.

Str. pyogenes (*Group C*) *as Primary Invader*

Experiments of this nature were made for two reasons. First the organism is a natural pathogen of the guinea-pig, producing a chronic lymphadenitis. Second, Seastone (1939) observed in an infected colony of guinea-pigs what appeared to be a spontaneous variation to high virulence of the otherwise lowly virulent invader, but most important he showed that active infection with a strain of low virulence induced a high degree of resistance to superimposed infection with a virulent one. However, this resistance could not be passively transferred by serum nor would various types of killed vaccine protect. The possibility remained, therefore, that while the two strains were probably genetically related the resistance conferred on guinea-pigs against infection with the virulent strain was of the same type as that reported in the present paper. In fact, Seastone states that the type of resistance he was dealing with might be " an uncomplicated form of so-called tissue immunity and should furnish a favourable approach to that important problem."

Attempts were made to infect guinea-pigs by the respiratory route with the strain of low virulence (S1); the results were erratic even when high doses were used and attention was turned to infection by the parenteral route (intracutaneous, as used by Seastone). Regular and non-lethal infection (within the period of the experiments) was obtained with an inoculum of about 1×10^8 chains of cocci in 0·2 ml. Twenty-one days later readily palpable induration of the superficial inguinal and axillary lymphatic glands had developed. At this stage animals were exposed by various routes to a secondary infection of either the virulent streptococcus Group C, *B. anthracis*, *Br. suis* or *P. pestis*.

Virulent streptococcus as secondary invader

In the first instance we attempted to repeat Seastone's (1939) work by re-infecting with the virulent strain (S2) of streptococcus; but, in addition to intracutaneous challenge, other groups of animals were tested by the intraperitoneal and inhalation routes. The results are summarised in Table VII. Confirmation is given to the finding that infection with S1 confers a high degree of protection against S2 given intracutaneously. A not significantly different degree of protection was observed when the animals were challenged by the intraperitoneal or inhalation route. The surviving animals were autopsied 27 days after secondary challenge. Cultures were made from inguinal and axillary lymph glands, heart's blood, spleen and lung tissue. All inguinal and axillary

glands were positive and yielded a growth of the S1 strain (which, with experience, could be differentiated from the S2 strain). Only 3 animals (from the group exposed to respiratory challenge with S2) yielded positive but scant growth of S2 from the heart's blood. Lung, cervical and bronchial lymph glands and spleen gave no growth.

TABLE VII.—*Effect of a Primary Low Grade Group C (S1) Streptococcal Infection in Guinea-pigs on Subsequent Exposure to a Virulent Strain of the Same Group (S2).*

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	% deaths.
S1 with S2 given I.C.	30	2	7
S1 „ „ „ I.P.	40	1	2·5
S1 „ „ „ Inhal.	40	6	15
S2 given I.C.	20	18	90
S2 „ I.P.	20	17	85
S2 „ Inhal.	30	26	86

S1 given intracutaneously 21 days before applying S2. I.C. = intracutaneously. I.P = intraperitoneally. Inhal. = inhalation

B. anthracis, Br. suis *or* P. pestis *as secondary invaders*

The low grade streptococcal infection induced by the intracutaneous route, as described above, produced no signs of non-specific protection against any of these secondary invaders introduced by the respiratory route; this applied to *P. pestis* whether presented as a cloud of single cells or as a cloud of large particles. In addition, an experiment was made in which, 21 days after infection with the streptococcus, an estimated ID_{90} of *Br. suis* was injected intracutaneously at a site in close proximity to that used for injecting the streptococcus. At autopsy 4 weeks later there was no evidence of inhibition of infection with *Br. suis* but this finding may be akin to that observed with *B. anthracis* given intracutaneously to animals with brucellosis.

DISCUSSION

In earlier work, detailed study had been made of factors determining the initiation of infection by the respiratory route with *B. anthracis*, *Br. suis* and *P. pestis* (Druett *et al.*, 1953, 1956*a*, 1956*b*; Ross, 1957; Harper and Morton, 1953; Harper, 1955). The information thus obtained has been of great assistance in analysing the results of the present experiments on combined infection.

A point of first importance is that none of the experiments showed any evidence of enhanced pathogenicity as a result of superimposing one infection on another. Secondly, there was no evidence to support the records from early times that a pre-existing infection could be suppressed by another superimposed. It is, of course, possible that these negative findings are a matter of chance related to the host species and the combination of pathogens chosen for experimentation. Only two main positive findings emerged. First a pre-existing infection with one pathogen can induce a high degree of non-specific resistance to attack by a second and wholly unrelated one, while under other conditions using the same organisms no trace of resistance is observed.

Essential conditions for the development and manifestation of non-specific resistance seem to be, first, the ability of the primary invader to produce early non-fatal generalised infection of the lymphatic system, and second that the pathogen superimposed will be obliged to attempt entry and multiplication in

the host primarily through the lymphatic system. This is very clearly indicated in the experiments with *Br. suis* as primary invader and *B. anthracis* or *P. pestis* as secondary pathogens. The situation is in some measure reminiscent of the numerous observations on local tissue immunity. It differs, however, by the fact that a resistance can be demonstrated when the secondary invader is given by an entirely different route than that chosen for initiating primary infection; thus, for example, *Br. suis* given subcutaneously induces resistance to infection with *B. anthracis* or *P. pestis* secondarily given by the respiratory route.

From present experiments and earlier work noted above, there is good evidence to show that *Br. suis* given by the respiratory route invades primarily through the local lymphatic tissue. This is true also for *B. anthracis* (Barnes, 1947; Ross, 1957). The evidence is that, once brucellosis is thus established, a high proportion of animals are resistant to infection with *B. anthracis* imposed by the same route. On the other hand, if *B. anthracis* is superimposed by, say, the intracutaneous route no trace of non-specific resistance is detected. There is evidence to support the view that in this latter circumstance *B. anthracis* finds a nidus for continued multiplication external to the lymphatic system. This, of course, was Besredka's opinion; he mistakenly insisted that the only portal of entry for this organism was through the skin. Under these circumstances, therefore, it is probably not surprising that the non-specific resistance of the lymphatic system is eventually overcome.

The findings with *P. pestis* as secondary invader are not dissimilar. When this organism is presented to the host in the form of a cloud of single cells there is a primary infection of lung tissue leading consecutively to broncho-pneumonia, septicaemia and death. Under these circumstances there is no evidence (other than some delay in time of death) of non-specific resistance in animals with brucellosis. On the other hand, when *P. pestis* is superimposed as a cloud of large (*ca.* 12μ diameter) particles in animals with brucellosis a high degree of non-specific resistance to this secondary invader is observed. If the hypothesis outlined above is correct, these findings might be predicted from our knowledge of the pathogenesis of plague according to the route of infection. Thus we have shown (Druett *et al.*, 1956*b*) that if a cloud of single organisms of *P. pestis* is used, direct penetration of lung tissue occurs and generalised infection *viâ* the lymphatic system is by-passed. If a cloud of large particles is used, local penetration of the upper respiratory mucosa occurs but the main path for systemic invasion is through the local lymphatic system.

In experiments where *Br. suis* was the primary invader and *Myco. tuberculosis* or virulent *Str. pyogenes* Group C was superimposed by the respiratory route using clouds of small particle size, there was no evidence of effective non-specific resistance; both pathogens, of course, can invade lung tissue directly. However, there was marked delay in development of infection (over that in controls) when *Myco. tuberculosis* was superimposed on animals with brucellosis. So far we are at a loss to account for this but it is hoped that the detailed histological studies now in progress on the general development of tuberculosis in such animals may provide a clue.

Myco. tuberculosis as a primary invader led to a high degree of non-specific resistance against *B. anthracis*. It is also interesting that *Br. suis* used as secondary invader was almost completely inhibited. These findings are in general agreement with our hypothesis. Histological examination showed that under the conditions

we used, *Myco. tuberculosis* had stimulated a very marked hyperplasia of the lung lymphatic tissue by the time the potential secondary invaders were applied.

The results with *Str. pyogenes* Group C as primary invader are interesting in two respects. First, the lymphadenitis established completely failed to induce non-specific resistance to secondary infection with *P. pestis*, *B. anthracis* or *Br. suis* when these organisms were presented by the respiratory route under optimal conditions for the demonstration of resistance in the presence, say, of pre-existing brucellosis or tuberculosis. Second, we confirmed and extended Seastone's findings that such streptococcal lymphadenitis conferred a high degree of protection against superimposed infection with a fully virulent *Str. pyogenes* Group C. It seems clear, therefore, that this resistance is much more probably specific rather than non-specific as Seastone argued. The reasons for the failure to demonstrate non-specific resistance are not yet clearly understood. However, the pathological and bacteriological findings so far indicate that the low grade streptococcus infection may induce only strictly localised infection and lymphatic tissue response; this could be the determining factor. On the other hand, more recent experiments show no evidence of non-specific resistance to infection with *Br. suis* when the latter is superimposed by the intracutaneous route at a site in close proximity to one inoculated three weeks previously with the streptococcus of low virulence. Here again, however, parenteral injection of *Br. suis* may lead to conditions closely approximating those observed with *B. anthracis* in so far, at any rate, as failure to demonstrate non-specific resistance is concerned.

More attention could profitably be given to the finding that guinea-pigs with brucellosis which have survived a respiratory challenge with anthrax spores show evidence of the development of specific active immunity. The fact that this was demonstrated, as it were, by subterfuge is probably not for the present important, for the species is notoriously difficult to immunise against the disease under any circumstances. The results do not show complete protection and they are probably not statistically significant, but anyone with experience of this form of disease in the guinea-pig, and its prevention, might nevertheless regard the issue as highly significant.

The evidence which shows that tissue extract from animals dead from plague is strongly inhibitory to the growth of *Br. suis in vitro* is of interest in two respects. First it may offer a link with past history when claims were made that " one ill cureth another ". If this be so, it is here just unfortunate that the host died, as it were, at the hands of its potential saviour. Second, the phenomenon could not be shown to operate in reverse, *i.e.*, extract of tissues from animals with brucellosis failed to inhibit the growth of *P. pestis in vitro* and yet, of course, under certain circumstances the host with brucellosis was highly resistant to infection with *P. pestis*. As already noted, all these observations do is to indicate the manifold complexities in processes of combined infection. They also help to focus attention on the fact that the present experiments do nothing more than expose some of these complexities. So far they have not contributed to an understanding of the mechanisms involved.

SUMMARY

A primary infection with a pathogen such as *Br. suis* or *Myco. tuberculosis* which induces a marked generalised lymphatic response has been shown to produce non-specific protection against secondary infection with organisms

whose normal attack is through the lymphatic system. No protection is observed against secondary invaders of this nature if presented in such a way that they attack the host otherwise than primarily through lymphatic tissue. The mechanisms involved in the resistance process have not been elucidated but some of the factors determining whether the process will develop or manifest itself have been disclosed.

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