Br. J. exp. Path. (1978) **59**, 48

THE SIGNIFICANCE OF SECONDARY GRAM-NEGATIVE COLIFORM INFECTION OF THE LUNGS OF MICE WITH INFLUENZAL PNEUMONITIS

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Received for publication September 5, 1977

Summary.—Influenza-virus-induced pneumonia of mice was consistently shown to be associated with secondary infection with coliform organisms. Treatment with gentamicin effectively sterilized the lungs of these mice but had no effect on either mortality or extent of the pneumonic process. The significance of these findings is discussed.

THE importance of secondary bacterial infection of acute respiratory viral disease is well established. Staphylococcal pneumonia following influenza is a good example of the phenomenon as seen in man and in experimental systems it has been shown that the deliberate secondary infection of the harmless Sendai virus infection of the lungs of mice with *Haemophilus influenzae* results in a fatal pneumonic illness (Degré and Glasgow, 1968).

The role and significance of Gram-negative coliform organisms other than *Klebsiella aerogenes* in respiratory infection has been less well established. Preliminary experiments had shown that the lungs of mice with viral pneumonia induced in various ways were frequently colonized with Gram-negative coliform organisms. In this study we have determined the frequency with which secondary infection with these organisms occurs in mice with influenzal pneumonia. We have further attempted to determine the signifiance of this secondary bacterial infection on the pathogenesis of the viral disease.

MATERIALS AND METHODS

The virus.—Two variants of the Kunz influenza A virus were used. The virulent variant (M25) was obtained by 25 serial passages in the lungs of mice of the original avirulent egg-adapted (MO) virus as described by Raut *et al.*, (1975). Both variants were propagated in the allantoic cavity of embryonated hens' eggs.

Infectivity of virus suspensions was assayed in monkey kidney tissue cultures.

Infection of mice.—20–25 g CD1 male mice were lightly anaesthetized with ether and were inoculated intranasally with 0·1-ml volumes of virus suspension. The mice were killed at the time after infection when they were showing signs of severe pneumonic illness. Their lungs were removed, examined for degree of consolidation and then homogenized in broth saline for the culture of bacteria.

Lung suspensions obtained from uninfected controls that were killed both before infection and at the termination of the experiment were cultured in a similar manner.

Lung lesions were scored by the method of Horsfall (1939).

Bacteriology.—The lungs of each mouse were suspended in 4 ml of chilled broth, homogenized and then centrifuged. Samples (0·1 and 0·001 ml) of the supernate were then cultured aerobically on blood agar and MacConkey plates and incubated at 37° overnight. Bacterial counts were then made and have been recorded as the number of organisms per lung.

In some experiments, coliform organisms were identified using the methods of Cowan and Steel (Cowan, 1974).

Occasionally small numbers of *Staphylococcus albus* (<10 organisms/lung) were isolated from the lung supernatants. These were presumed to be contaminants and are not recorded in the tables.

Antibiotic treatment.—Gentamicin (50 mg/kg) was administered to the mice s.c. twice daily. Treatment began 24 h after virus inoculation and was terminated 24 h before surviving mice were killed.

A control group of non-infected mice, subjected to the same antibiotic regimen, was included in each experiment.

RESULTS

The standard egg-passaged Kunz strain of influenza virus had previously been shown to cause an infection of mice which is restricted to the airways and from which the mice almost invariably recover. Passage of this virus in the lungs of these animals eventually results in the appearance of a virulent variant which consistently induces a fatal pneumonic illness (Raut *et al.*, 1975). These two variants of the Kunz strain were used to determine the role of secondary bacterial infection in the disease process.

In the first experiments, groups of 8 mice were infected with $10^{3\cdot0}$ TCD_{50} of either the avirulent (MO) or virulent (M25) variant of this virus. A further group of 8 mice were retained in the same environment but were not infected. By the sixth day after infection, the uninfected controls and the mice infected with the MO virus appeared healthy but those infected with the M25 virus were showing signs of a severe pneumonic illness. All the mice were killed at this time, their lungs were removed and examined for consolidation and then cultured for the presence of bacteria.

From Table I it can be seen that the lung cultures from mice infected with the MO virus and from the uninoculated controls were sterile. However, all but one of the lungs from mice infected with the M25 virus contained appreciable numbers of either *Proteus vulgaris* or *Kl. aerogenes*. It can be seen that the lungs from the mice infected with the virulent M25 variant showed almost complete consolidation, whereas those obtained from the other groups were either normal or showed only trivial changes.

The association between consolidation and bacterial colonization raised the possibility that the latter was playing some role in the pneumonic process. To investigate this possibility a group of 40 mice was infected with $10^{4\cdot5}$ TCD_{50} of the virulent

TABLE I.—*Secondary Coliform Infection of the Lungs of Mice Infected with Virulent and Avirulent Strains of Influenza Virus*

Experimental group	No. of mice	Mean lung score of survivors (Day 6)	Bacterial culture (Day 6)	
			No. sterile	No. with $>10^2$ coliforms/lung
Uninfected controls	8	0	8	0
Infected with avirulent (MO) virus	8	0·8	8	0
Infected with virulent (M25) virus	8	3·9	1	7*

* 3 mice were infected with *Kl. aerogenes* ($1\text{–}4\times10^3$ organisms/lung) and 4 were infected with *Prot. vulgaris* ($1\times10^2\text{–}2\cdot6\times10^3$ organisms/lung).

TABLE II.—*Effect of Gentamicin on Mice with Viral Pneumonitis Secondarily Infected with Coliform Organisms (Experiment 1)*

Experimental group	No. of mice	No. of mice surviving (Day 4)	Mean lung score of survivors	Bacterial culture (Day 4)	
				No. sterile	No. with $>10^2$ coliforms/lung
Uninfected controls	5	5	0	5	0
Uninfected controls treated with gentamicin	5	5	0	5	0
Infected with M25 virus but untreated	20	4	2·5	0	4*
Infected with M25 and treated with gentamicin	20	5	3·0	5†	0

* Concentrations or organisms ranged from $1\cdot6\times10^2$ to $2\cdot8\times10^4$/lung.

† The lungs of one of these mice contained $3\cdot4\times10^5$ β haemolytic streptococci/lung.

M25 virus and half of these were treated with gentamicin. A further 10 uninfected mice were used as controls and half of these were treated with gentamicin. This dose of virus produced a rapid mortality in the mice so that over 70% of each group were dead by the fourth day after infection. There was no significant difference between the cumulative mortality curves of the treated and untreated groups (see Fig.). The dose of antibiotic used had no untoward effect on the uninfected controls.

All surviving mice were killed on the fourth day and their lungs removed for estimation of lung consolidation and for bacterial culture. From Table II it can be seen that the lungs from the 4 surviving mice of the untreated group were all colonized with coliform organisms and these were present in concentrations similar to those seen in the previous experiments. All of the isolated organisms were shown to be sensitive to gentamicin. The antibiotic treatment was successful, in that coliforms could not be isolated from any of the lungs of the 5 surviving mice of the treated group. The lung suspension prepared from one of these mice contained a very high concentration of a β haemoloytic streptococcus but this may have been a contaminant since we had never previously isolated this organism from mouse lungs. All the lungs from the uninfected controls were sterile.

A further experiment was carried out using a less concentrated virus inoculum (10^3 TCD_{50} mouse) to produce a more delayed lethal effect. The results are shown in Table III and the Figure. Fifty

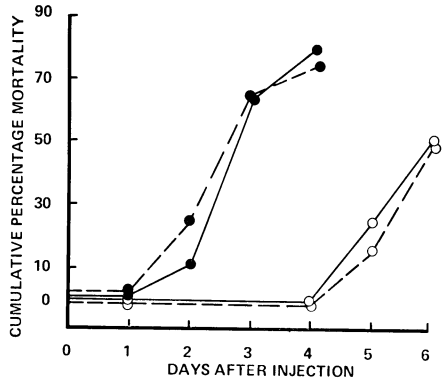

FIG. 1.—Cumulative mortality of mice infected with $10^{4.5}$ TCD_{50} (●) and $10^{3.0}$ TCD_{50} (○) of the M25 strain of Kunz virus.
The broken line shows the mortality of the mice that were treated with gentamicin and the continuous line the mortality of those that were untreated.

per cent of both the gentamicin-treated and untreated mice survived to the sixth day after infection. The cumulative mortality curves and the mean lung scores of surviving mice of both groups were again virtually identical.

All of the lungs from the infected but untreated mice contained appreciable numbers of coliforms and these organisms were again shown to be sensitive to gentamicin. In contrast only one of the 10 infected mice that were treated with gentamicin was colonized with coliforms and these organisms were shown to be still sensitive to the antibiotic.

No deaths occurred in the uninfected group of mice and lungs removed from killed animals on the sixth day were sterile.

TABLE III.—*Effect of Gentamicin on Mice with Viral Pneumonitis Secondarily Infected with Coliform Organisms (Experiment 2)*

Experimental group	No. of mice	No. of mice surviving (Day 6)	Mean lung score of survivors	Bacterial culture (Day 6)	
				No. sterile	No. with $>10^2$ coliforms/lung
Uninfected controls	5	0	0	5	0
Infected with M25 virus but untreated	20	10	3·7	0	10*
Infected with M25 virus and treated with gentamicin	20	10	3·8	9	1†

* Concentrations of organisms ranged from $1{\cdot}2\times10^2$ to $1{\cdot}2\times10^6$/lung.
† The lungs of this mouse contained $3{\cdot}2\times10^4$ gentamicin-sensitive coliforms.

DISCUSSION

The lungs of mice that had been infected with a pneumonia-inducing strain of influenza virus were consistently shown to be secondarily infected with coliform organisms. This bacterial infection was effectively prevented by gentamicin but this treatment had no effect on either mortality or the extent of the pneumonic process. Clearly the secondary bacterial infection has no significant role in the pathogenesis of this pneumonic disease. In this respect coliform organisms differ from respiratory pathogens such as *Staphylococcus aureus* (Janssen, Chappell and Gerone, 1963) and *Haemophilus influenzae* (Degré and Glasgow, 1968) which increase the mortality of experimental animals infected with respiratory viruses.

It would appear that lung tissue damaged by viral replication provides a suitable medium for the growth of coliform organisms. It is also possible that lung damage caused by any other means would do the same. We failed to obtain any evidence to suggest that the coliform infection was having any additional adverse effect on the rapidly fatal viral infection. It is, however, possible that when the underlying lung disease is less acute, secondary coliform infection could be harmful, perhaps by delaying healing or by the inception of septicaemia. These possibilities are worthy of further investigations in experimental systems since the isolation of a coliform from the sputum of a patient is a major dilemma in clinical practice (Philp and Spencer, 1974).

The technical assistance of Ms Linton-Brooks is gratefully acknowledged.

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