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A Rodent Model of Concurrent Respiratory Infection with Influenza Virus And Gram-Negative Bacteria: Synergistic Infection and Protection by Oral Immunization

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ABSTRACT

A mouse model of dual influenza/bacteria infection has been established to investigate the severity of infection (measurement of virus and bacteria levels in bronchoalveolar lavage fluid [BAL] and lung tissue), and effect on the host immune response. The effect of oral pre-immunization against bacteria on these parameters was also examined.

Dual infection with influenza A/Qld/6/72, H3N2 (AQld) and *Haemophilus influenzae* (Hi) gave a 10-fold increase in viral titre in lung homogenate compared to infection with AQld alone, and a 10-100 fold increase in Hi in BAL and 5-10 fold increase in Hi in lung homogenate when compared to infection with Hi alone. Oral pre-immunization against Hi cleared the infection of Hi alone, suppressed the Hi in dual AQld/Hi infection, and reduced the AQld in the AQld/Hi infection. In mice with a pre-existing, underlying bacterial lung infection with *Bordetella bronchiseptica* bacteria were increased 10-fold in BAL and 1000-fold in lung homogenate. This infection was unaffected by pre-immunization against Hi. Recruitment of neutrophils induced by Hi was inhibited in the presence of AQld. These results suggest a deleterious effect of AQld on host clearance mechanisms which are important in clearance of Hi and *B. bronchiseptica* .

INTRODUCTION

Secondary bacterial pneumonia is the most common pulmonary complication of influenza virus infection and is the major cause of influenza A - related morbidity and mortality, particularly in the elderly, and those with compromised lung function[1-3]. Certain bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureas* and *Haemophilus influenzae* are commonly isolated from the lung following natural or experimental influenza infection in man[1,4] suggesting that influenza infection is making conditions more favourable in the lung for survival of these bacteria. The mechanism(s) by which influenza virus infection enhances bacterial infection is not clear, although a reduction in neutrophil function has been observed clinically and *in vitro*[5]. The severity of influenza infection in dual influenza-bacteria infections is often enhanced. This appears to be due to a direct effect of bacteria on the influenza virus and several studies have now demonstrated a role for bacterial proteases and

host proteases induced by bacteria, in increasing the infectivity of influenza virus by cleaving hemagglutinin[6,7].

While secondary bacterial infection can often be successfully treated with antibiotics, antibiotic resistance levels of bacteria are increasing[8] and preimmunization against bacteria as well as influenza may therefore be of increasing importance, particularly in high-risk groups. An understanding of the mechanism by which influenza virus infection predisposes the host to bacterial infection is desirable if such prophylactic vaccination is to be effective. Why some bacteria are preferentially selected by influenza infection is not yet known. The current study describes an animal model of dual influenza/bacteria infections which will allow study of the mechanisms involved in the development of such infections and determine the usefulness of prophylactic immunization against bacterial infection in minimising the severity of mixed virus/bacteria infections.

MATERIALS AND METHODS

Mice

Male Balb/c specific pathogen free (SPF) mice approximately eight weeks of age were obtained from the Central Animal House, The University of Newcastle, or the Animal Resource Centre, Murdoch, Western Australia. Mice were held in isolator cages both prior to and during the experiments. All experiments were approved by the University of Newcastle Animal Care and Ethics Committee.

Pathogens

Influenza virus A/Qld/6/72, H3N2 (AQld) was grown in embryonated chicken eggs for 72h at 33-34°C. Virus was harvested and purified by centrifugation on sucrose gradients. Activity was determined by plaque assay in confluent cultures of a Madin-Darby canine kidney (MDCK) cell line. Virus was stored at -70°C until required. *Haemophilus influenzae* (Hi; biotype 1, serotype B) was grown on chocolate agar, and harvested into phosphate-buffered saline. Formalin-killed bacteria for immunization studies were prepared by incubation of bacteria at $5x10^9$/mL in 1% (w/v) formalin in PBS, at 37°C for 2h. Bacteria were washed three times in saline and resuspended to 10^{10}/mL in PBS.

Immunization of Mice

Mice were anaesthetised with 0.25-0.3 mL 5% chloral hydrate administered i.p. An incision was made in the abdomen to expose the intestine. The duodenum was located and the mice immunized by injection of 0.3mL of formalin-killed Hi directly into the duodenum. The abdomen was closed and the mice kept warm during recovery from anaesthesia. This procedure is considered to be the equivalent to administration of an enteric-coated oral vaccine.

Respiratory Infection Model

Mice were anaesthetised with 0.25-0.3 mL 5% chloral hydrate administered i.p. The trachea was exposed by cutting the skin and gently pushing aside the salivary glands and soft tissue

using cotton buds. Fifty microliters of the bacteria and virus preparations were injected directly into the trachea. The skin wound was sutured and the mice kept warm during recovery from anaesthesia. Bacteria were used at a dose of 0.625-1.25x10^6 and influenza virus at 1.25x10^7 as previously determined in a mouse influenza infection model[9, 10]. Mice were killed 24-48 h after infection by i.p. overdose of sodium pentobarbitone. The lungs were lavaged with 2 x 1 mL of PBS to obtain pooled bronchoalveolar lavage fluid (BAL), and the lung tissue was homogenised in 10 mL of PBS. A total leukocyte count was performed on the BAL and a cytospin prepared for a differential count. Samples of BAL and lung homogenate (LH) were serially diluted (10-fold dilutions) and 20μL plated onto chocolate agar plates. After overnight culture the colonies were counted and the total CFU in BAL and LH determined. Samples of BAL and LH were also tested in the MDCK plaque assay to quantitate the number of plaque-forming units (PFU) of influenza virus in the samples.

Statistics

Groups of mice with different treatments were compared by unpaired student t test (MacIntosh Systat). Differences between groups were considered significant if $P<0.05$.

RESULTS

Combined H. influenzae/AQld Infection in Balb/c Mice

Mice were infected with Hi, AQld or a mixture of the two, and bacteria and influenza levels measured at 24h and 48h (Fig. 1). The presence of AQld led to a 10-fold increase in Hi in BAL at 24h compared to Hi infection alone ($P<0.05$), and a 10-fold increase in LH which was not statistically significant (Fig. 1a, 1b). A 100-fold increase in Hi in BAL in mice with the dual infection compared to Hi infection alone was observed at 48h ($P<0.05$). Similarly, the presence of Hi increased the level of AQld compared to the AQld only infected group (Fig. 1c, 1d). At 24h, although there was no difference in BAL, there was a 5-fold increase in virus in the LH of mice with dual Hi/AQld infection compared to mice infected with AQld only ($P<0.05$). By 48h virus was only detectable in the mice with dual infection. Thus there is synergistic infection when both Hi and AQld are present in the lung. The BAL leukocyte count and total number of macrophages, polymorphonuclear cells (PMN) and other cells is shown in Fig. 2. Th leukocyte count is highest in mice infected with Hi only, and lowest in mice infected with AQld only. PMN make up the majority of the BAL leukocytes, and both PMN and macrophages are significantly decreased when AQld is present together with Hi compared to Hi infection only ($P<0.05$).

Preimmunization with Killed Hi

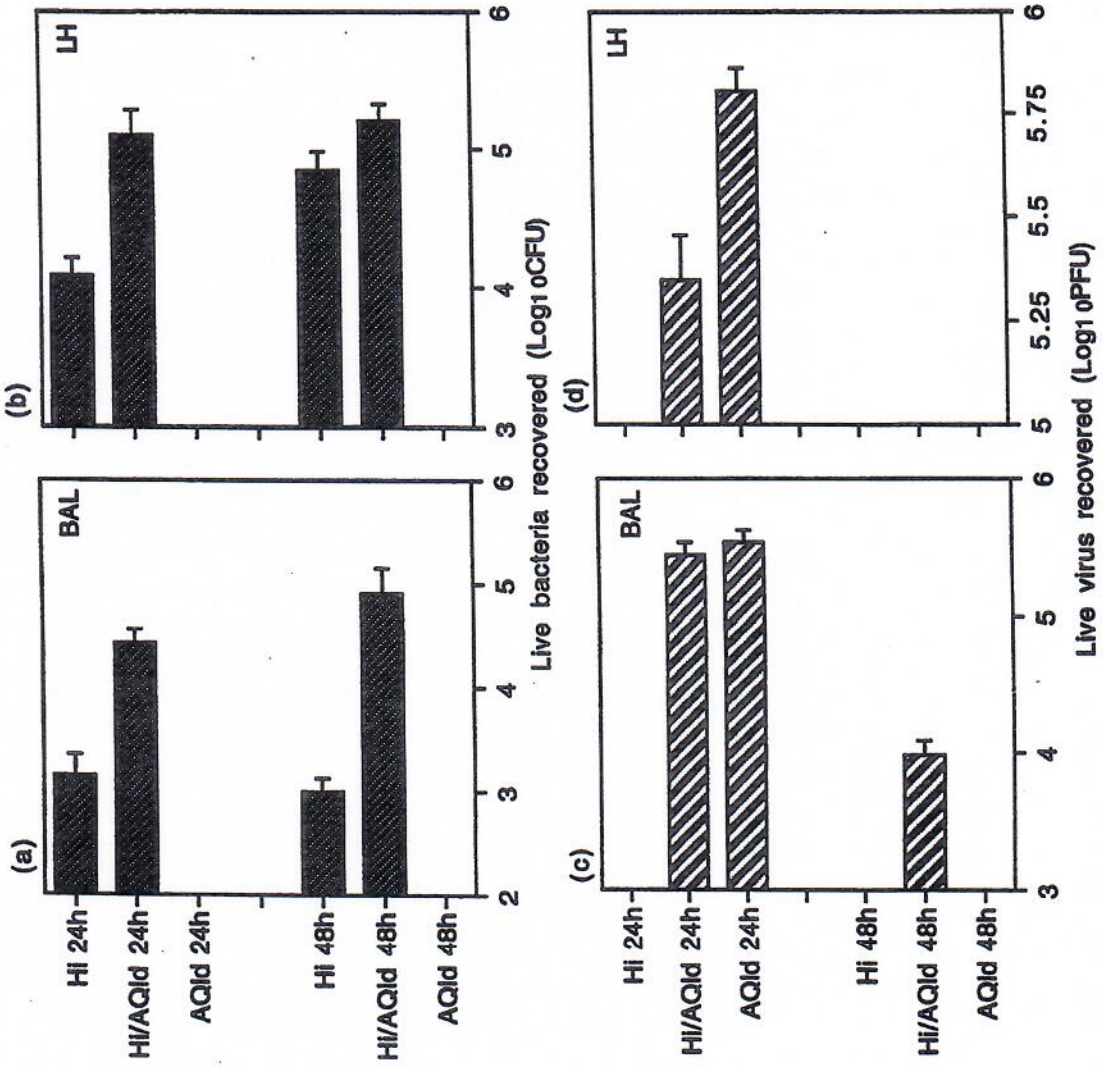
Mice preimmunized with killed Hi have a decreased Hi infection compared to unimmunized mice, both in mice with Hi infection and mice with Hi/AQld infection (Fig. 3a, 3b). There is also a reduction in AQld in the immunized mice (Fig. 3c). These differences, however, are not statistically significant.

Effect of AQld on Bacteria *in vitro*

Hi was culture alone or in the presence of AQld at 37°C for 4h. Hi concentration was 9.0x10^5

when cultured alone and $3.3x10^5$ in the presence of AQld. Thus there was a small decrease in Hi in the presence of AQld.

Fig. 1: Dual Hi/AQld infection

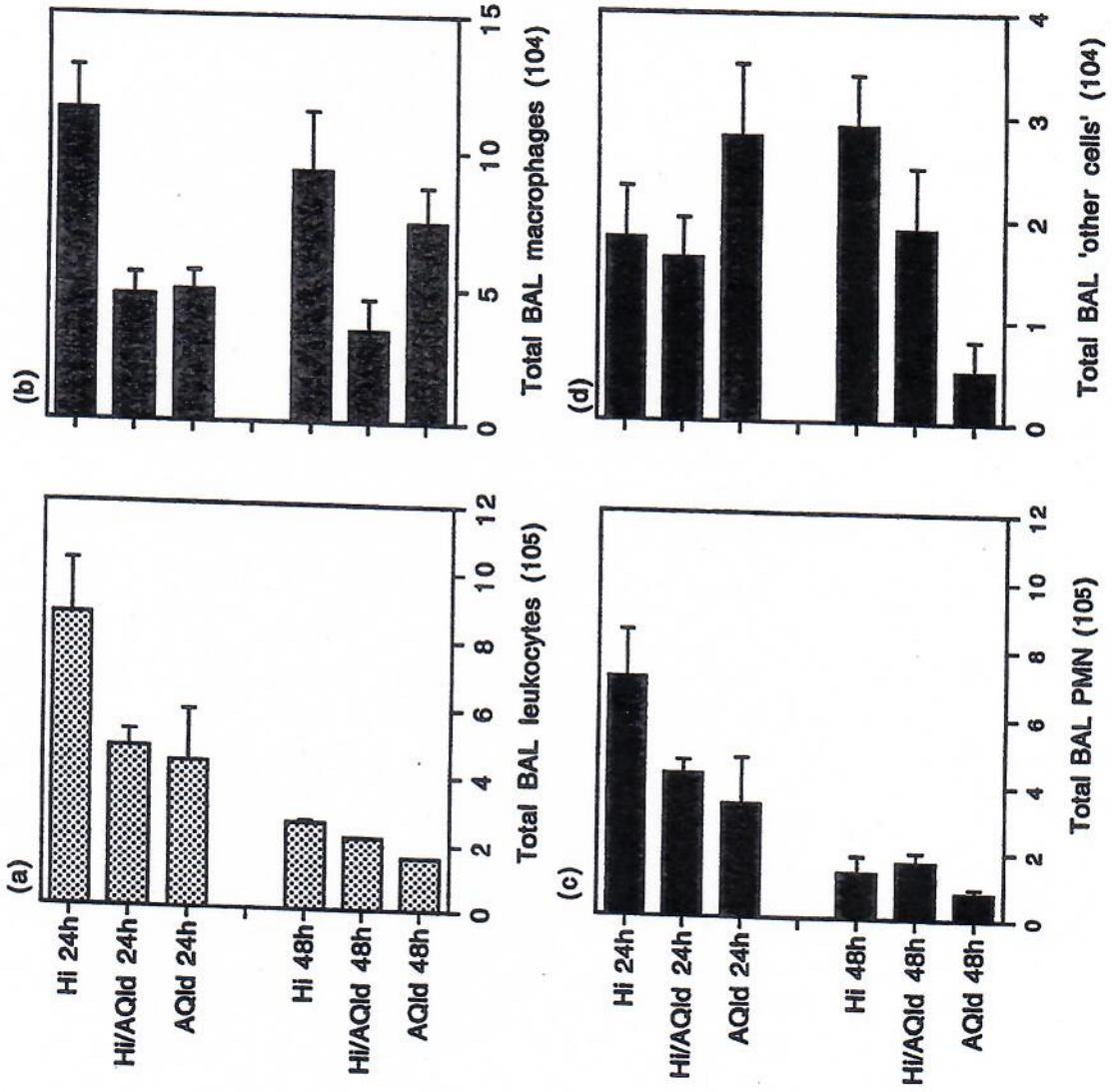


Live Hi in BAL (a) and LH (b) and live AQld recovered from BAL (c) and LH (d) are shown. Bars represent the mean±SEM of the pooled data from 3 experiments and a total of 20 mice per group for 24h bacteria data, 2 experiments and 10 mice per group for 48h Hi data and one experiment and 5 mice per group for AQld data. Mouse groups are presented on the y axis. Experimental infection status is: Hi alone (Hi), Hi plus AQld (Hi/AQld), or AQld alone (AQld). Infection duration is 24h or 48h.

Effect of AQld Infection on an Underlying Infection with *Bordetella bronchiseptica*

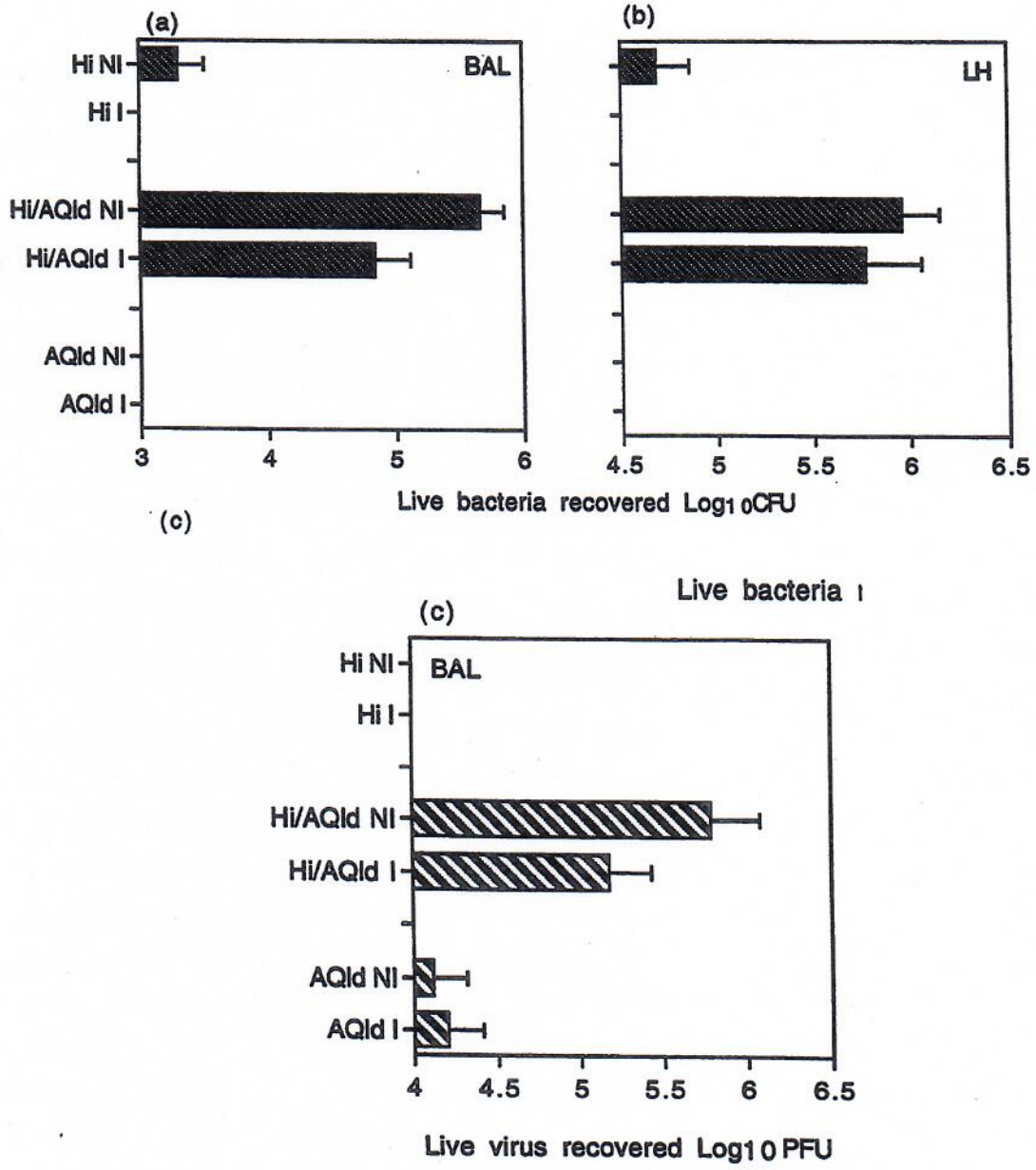
In one experiment mice were found to have an underlying *B. bronchiseptica* infection. The level of *B. bronchiseptica* in animals dosed with a low level of Hi was minimal but in the presence of AQld the level of infection increased 10-fold in BAL and 1000-fold in LH (Fig. 4). On occasions it has been observed that infection with AQld increases low-level background infection with other bacteria including *Pasteurella pneumotropica* and *Moraxella phenylpyruvica* (data not shown).

Fig. 2: Dual Hi/AQld infection



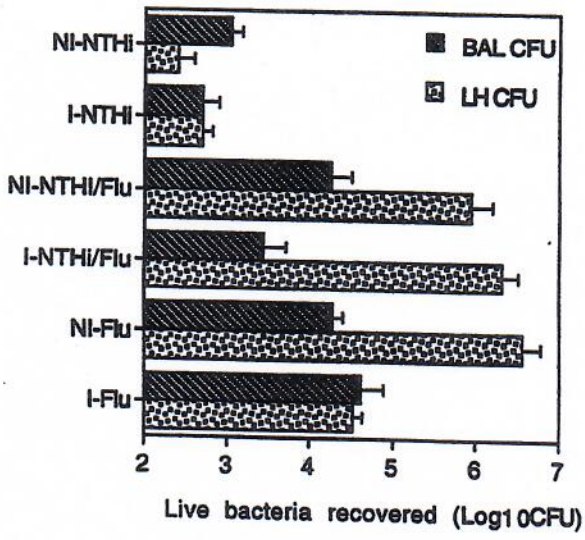
Total BAL leukocytes (a), macrophages (b), PMN (c) and other cells (d). Bars represent mean±SEM of pooled data from 3 experiments and a total of 20 mice per group for 24h data, and 2 expts and 10 mice per group for 48h data. Mouse groups are presented on the y axis. Experimental infection status is: Hi alone (Hi), Hi plus AQld (Hi/AQld), or AQld alone (AQld). Infection duration is 24h or 48h.

Fig. 3: Pre-immunization with killed Hi



Live Hi recovered from BAL (a) and LH (b). Live AQld recovered from BAL (c). Bars represent mean±SEM of pooled data from 2 experiments and 4-8 mice per group. Mouse groups are presented on the y axis. Experimental infection status is: Hi alone (Hi), Hi plus AQld (Hi/AQld), or AQld alone (AQld). Immune status is non-immune (NI) or immunized against Hi (I).

Fig. 4: Effect of AQld on underlying *B. bronchiseptica* infection



B. bronchiseptica recovered from BAL and LH is shown. Bars represent mean±SEM of data from one experiment and 4 mice per group. Mouse groups are presented on the y axis. Experimental infection status is: Hi alone (Hi), Hi plus AQld (Hi/AQld), or AQld alone (AQld). Immune status is non-immune (NI) or immunized against Hi (I).

DISCUSSION

A mouse model has been developed that allows the study of mixed bacteria-virus respiratory infection. The combination of AQld and Hi leads to an increased infection level of both virus and bacteria. The increase in Hi is not due to a direct effect of the AQld on the Hi, as co-culture of AQld and Hi *in vitro* results in a small decrease in bacteria concentration compared to bacteria cultured alone. Previously reported *in vitro* studies have demonstrated that some bacteria such as Hi release proteases that result in increased release of influenza virus from the cell in which it is replicating, whereas other bacteria may increase the influenza infection by inducing the release of host proteases which also have the effect of enhancing virus infectivity[6,7]. This mechanism may account for the increase in AQld observed in these mixed influenza/bacteria infection experiments. However, the mechanism by which influenza virus increases the bacterial infection is not known although it has been observed that influenza infection diminishes PMN function, and this could contribute to the enhanced Hi infection. The increase in Hi infection could also be due in part to the decreased recruitment to the lung of both PMN and macrophages as observed in this study. As many different immune mechanisms combine in elimination of bacteria from the lung[11, 12] examination of other

immune parameters including macrophage, T cell function, and cytokine levels may clarify the mechanism for enhancement of selected bacterial infections during influenza infection.

Prophylactic oral immunization with killed bacterial vaccines has been shown to enhance clearance of an acute bacterial infection in rodents[13, 14]. Therapeutic oral immunization with killed bacteria vaccines has been demonstrated to reduce antibiotic usage in chronic bronchitis patients[15, 16] and to induce an antigen-specific T cell response in blood and reduce the mean sputum bacterial count and sputum leukocyte count in bronchiectasis patients (Dunkley et al, submitted for publication). Immunization against bacterial infection may be useful not only in decreasing the bacterial infection but also in preventing or reducing the enhanced influenza infection seen in mixed influenza/bacteria infection. In the present study, preimmunization of the mice with a killed Hi vaccine leads to a decrease in Hi and AQld in the mixed infection compared to that in unimmunized mice, indicating that vaccination against bacteria will be important in contributing to protection against severe respiratory disease caused by mixed influenza/bacteria infection.

Further study of the immune parameters affected by dual influenza/bacteria infection will allow optimisation of immunotherapy against respiratory infection. Possibly the activation state of the phagocytes may be more important than the number recruited and measurement of phagocyte activation state may shed light on the mechanism of synergistic infection in the case of Hi/AQld as opposed to the decreased infection in the case of Pa/AQld.

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