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Abstract

This study was undertaken to compare bacterial clearance and immune responses in a mouse model of pneumonia and a rat model of otitis media following parenteral or mucosal immunisation in both models. For the purposes of this study the immunisation schedules for pneumonia (mice), otitis media (rats) and route of immunisation (parenteral or mucosal) were held constant. The animals were challenged intratracheally for pneumonia or directly into the middle ear space through the middle ear bulla for otitis media. Both animal models gave highly reproducible disease and provided high levels of sensitivity for testing the efficacy of candidate vaccine antigens. Parenteral and mucosal immunisations were equally as effective in inducing bacterial clearance from the lung in the mouse pneumonia model. Both routes upregulated the recruitment of white cells and lead to an increase in the concentration of TNF-α, IL-1β and specific antibody in the bronchoalveolar lavage. In the rat otitis media model both routes of immunisation enhanced clearance of bacteria from the middle ear, with parenteral immunisation being the most effective overall in enhancing bacterial clearance and recruiting white cells to the middle ear. Both immunisation routes significantly suppressed the levels of TNF-α in the middle ear lavage and mucosal immunisation induced a *Streptococcus pneumoniae*-specific IgA antibody response in the middle ear. This study has found that both animal species provide suitable models for studying vaccine-induced immune protection, but differences were observed in some of the measures of inflammatory responses that could either be related to the differences in the type of infection or associated with the animal species.

Keywords: *Streptococcus pneumoniae*, animal models; otitis media; pneumonia; immunisation

1. Introduction

Reliable animal models have formed the basis of research in infectious disease since the early 1880s, when Louis Pasteur demonstrated that heat-attenuated anthrax bacilli and chicken cholera provided protection against subsequent disease from more virulent strains of those organisms [1]. Animal models provide the opportunity to study disease caused by infection that resembles that which occurs in humans. New vaccine and therapeutic candidates can be tested, immunisation regimes and routes trialled, correlates of protection assessed and disease pathogenesis studied. However, animal models can ever only provide a guide to the real human situation and final observations and trials need to be ultimately conducted in human studies. The advantage of pre-human studies in animals is that human studies can be more effectively designed and focused towards a desired outcome.

Streptococcus pneumoniae normally accesses the host via mucosal surfaces of the upper respiratory tract where initial colonisation is established. We have established standardised rodent models of acute otitis media and pneumonia. Whilst there are a diverse array of animal model variables for *S. pneumoniae*, no studies have been conducted which compare mucosal and parenteral immunisation routes in models of pneumonia or otitis media and in different rodent species.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions.

S. pneumoniae strain NC12695 (serogroup 3) was used in this study. The bacteria were revived from stock cultures and prepared for live challenge by culture overnight at 37°C on blood agar in 5% CO_2.

2.2. *Animals*

Specific pathogen free (SPF) male BALB/c mice aged 6 to 8 weeks and Sprague Dawley rats aged 8 to 10 weeks were purchased from the Animal Resources Centre (Perth, Western Australia, Australia) and were maintained under SPF conditions. They were removed from behind the barrier for immunisation at which time they were contained within filtered cages and returned to SPF conditions until bacterial challenge. Food and water were available *ad libitum.*

2.3. *Immunisation regimes*

All animal work was approved by the University of Canberra, Canberra, Australia, Animal Ethics Committee. The animals were immunised by either a mucosal or parenteral regime.

The mucosal regime involved an intra-Peyer's patch (IPP) immunisation, which has been described previously for mouse and rat models [2, 3], on day 0 followed by an intratracheal (IT) boost on day 14. Essentially, the immunisation inoculum was prepared by emulsifying formalin killed bacteria in a 1:1 ratio with incomplete Freund's adjuvant (IFA) with mice receiving $1x10^8$ colony forming units (CFU)/dose and rats $5x10^8$ CFU/dose. Mice were anaesthetised by a subcutaneous injection of 0.25 ml ketamine/xylazine (5 mg/ml ketamine hydrochloride {Troy Laboratories, Smithfield, NSW, Australia}; 2 mg/ml xylazine hydrochloride {Bayer, Pymble, NSW, Australia}) in phosphate buffered saline (PBS) whereas the rats were lightly sedated with halothane and further anaesthetised by intraperitoneal injection of pentobarbital sodium (Nembutal; Rhone Merieux Australia Pty Ltd) at a concentration of 10 mg/mL in PBS and a dosage of 40 mg/kg rat weight. The small intestine was exposed through a midline incision in the abdominal wall and the inoculum was delivered subserosally to each Peyer's patch using a 26G needle resulting in a total inoculum volume of 10 µl per mouse and 50 µl per rats. The intestines

were rinsed with sterile PBS and the abdominal cavity sutured. The non-immune groups consisted of a mix of sham-immunised animals, which were subjected to, the same surgical procedure but injected with an emulsion of IFA and PBS or were left untreated. An IT boost was given on day 14 post-IPP immunisation. To achieve this in mice, they were sedated by intravenous Saffan anaesthesia (0.15 ml; 20 mg alphadone in PBS /kg body weight; Pitman-Moore, Nth Ryde, NSW, Australia) and a 20 µl volume comprising $1x10^7$ CFU killed bacteria in PBS was delivered into the lungs via a 22.5G catheter (Terumo, Tokyo, Japan) inserted orally into the trachea. This was dispersed with two 0.3 ml volumes of air. The rats were sedated with halothane and boosted with a 50 µl volume comprising $5x10^9$ CFU killed bacteria in sterile PBS to the lungs via insertion of a 20G catheter orally into the trachea. The inoculum was dispersed with two 5 ml volumes of air.

The parenteral regime involved an intramuscular (IM) immunisation on day 0 followed by an IM booster on day 14. Animals received 10^8 CFU (mice) or $5x10^8$ CFU (rats) in 100 µl of a 1:1 ratio of IFA:bacteria in PBS.

2.4. Bacterial challenge

Bacteria were grown on blood agar plates overnight, harvested and washed three times in PBS. The concentration was estimated by measuring the optical density at 405 nm and was confirmed by counting CFU of the overnight culture by duplicate plating of serial dilutions of the inoculum.

Lung challenge in the mice was achieved by sedation with Saffan (described above) and a 20 µl bolus inoculum of live *S. pneumoniae* ($2x10^5$ CFU) in PBS was introduced into the lungs as described for IT boosts. Mice were killed by an intra-peritoneal injection of pentobarbital sodium

2h, 5h, 8h and 24 h for bacterial clearance and cytokine measurements. Blood was obtained by heart puncture from animals euthanised at 2 hr post challenge and allowed to clot for collection of serum. The trachea was exposed through the neck and bronchoalveolar lavage (BAL) was obtained by instilling and recovering 0.5 ml of PBS into the lungs via a cannula. The BAL was assessed for bacterial clearance by duplicate plating of serial dilutions for CFU determination. Serum was separated by centrifugation at 4°C and 450*g* for 10 min (Jouan BR3.11, St Nazaire, France). Serum and BAL were stored at -80 °C until required for further analysis.

Middle ear bacterial challenge was performed with rats as previously described [4]. Essentially, the rats were sedated with Nembutal (described above) and the middle ear infection administered to the animal's right ear through the tympanic bulla that had been exposed by a small incision in the neck. A 26G needle was used to pierce the bulla and inject a 20 µl volume of the live *S. pneumoniae* in PBS. The skin wound was sutured and the animal allowed to recover.

Bacterial clearance, cytokine measurements and white cell recruitment were undertaken at 0, 8, 22, 42 and 72 h post challenge for mucosally immunised and non-immunised animals. For parenterally immunised animals, groups were studied at 0, 8 and 22 h only. Animals were killed by an intra-peritoneal injection of Nembutal. Blood was collected by heart puncture from animals euthanised at 0 h post challenge and allowed to clot for collection of serum as described above.

Middle ear lavage (MEL) was collected by injection and recovery of 3 x 50 µl volumes of PBS into the middle ear through the tympanic membrane. The MEL was assessed for bacteria (CFU) and serum prepared as described above. Serum was stored at -80°C until required for

further analysis.

2.5. *BAL and MEL cell counts*

The BAL or MEL were centrifuged at 1500*g* for 10 minutes, the supernatant removed and stored at -80°C until required for further analysis. The cell pellet was resuspended in PBS and methylene blue for counting the total number of cells recovered. The cell count was corrected for the prior removal of aliquots. An aliquot was also prepared for differential cell counts as previously described [5].

2.6. *Enzyme Linked Immunosorbent Assay (ELISA)*

ELISA measured levels of IgG and IgA specific for the bacteria. The test wells of 96 well polysorb plates (Nunc, Intermed, Denmark) were coated with 100 µl of bacterial extract at a concentration of 40 µg/ml in carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) as previously described [2] and incubated for 1 h at 37°C. Quantitation of IgG and IgA was achieved by coating wells with serial dilutions of known concentrations of mouse or rat IgG or IgA standards (Calbiochem-Novabiochem) in carbonate-bicarbonate coating buffer. Plates were washed between each assay step five times in PBS/Tween (6 mM disodium hydrogen phosphate, 2 mM sodium dihydrogen phosphate, 0.27 M sodium chloride, pH 7.2 and containing 0.05% v/v Tween 20). The wells were blocked for 90 min at room temperature with 100 µl 2% (w/v) skim milk in PBS/Tween, then incubated with dilutions of BAL or sera in 1% (w/v) skim milk in PBS/Tween for 90 min. After washing, the wells were incubated with either horseradish peroxidase conjugated anti-mouse IgG (1/3000), anti-mouse IgA (1/250) (Sigma), anti-rat IgG (1/2000) or anti-rat IgA (1/1000) (Nordic Immunological Laboratories, Tilberg, Netherlands) for

90 min. The plates were developed with TMB (3',3',5',5'-tetramethylbenzidine in 0.05 M citric acid, 0.1 M sodium hydrogen phosphate, 0.05% (v/v) hydrogen peroxide) for 10 min and the reaction stopped with 0.5 M H_2SO_4. The plates were read at 450 nm on a Microplate reader model 3550 and analysed using the microplate reader data analysis software (BioRad, Regents Park, NSW, Australia).

2.7. TNF-α and IL-1β

Commercially available kits (Biosource International, Mount Waverley, Vic Australia) were used to determine the TNF-α and IL-1β concentrations in BAL and MEL. The kits were used in accordance with the manufacturer's instructions.

2.8. Statistical Analysis

Each experiment is terminal. Hence, each observation point represents the mean results of a group of at least 5 animals. The bacterial clearance data, cytokine and total numbers of white cells were compared for statistical significance between groups by a multi-factorial ANOVA and differences in the antibody responses by a student's t-test (Macintosh Systat).

3. Results

3.1. Bacterial clearance

3.1.1. Bacterial clearance from the middle ear

By 22 h post challenge the number of bacteria remaining in the MEL was significantly less ($p<0.05$) in both the mucosally and parenterally immunised groups of rats compared with the non-immune group (Fig 1A). The parenterally immunised animals tended to clear the bacteria

faster than the mucosally immunised animals but the difference in this observation was not statistically significant. By 42 h there were no detectable viable microbes in the MEL of mucosally immunised animals compared with 72 h for the non-immune animals.

3.1.2. Bacterial clearance from the lung

Over the 24 h observation period both the mucosally and systemically immunised mice cleared all bacteria from their lungs and were statistically different from non-immunised animals ($p<0.05$) (Fig. 1B). Enhanced clearance was only apparent after 8 h post challenge. There was no statistical difference between mucosally and parenterally immunised mice.

3.2 TNFα and IL-1β

3.2.1. Middle ear

In the non-immune rats the level of TNFα in the MEL increased over a 1000 fold compared with 0 h and 8 h after infection (Fig. 2A). These levels were maintained to 22 h and returned to baseline levels by 72 h. In mucosally immunised animals, a TNFα peak response was observed only at 8 h post infection. This peak was significantly less than that observed in the non-immune animals ($p<0.05$). No TNFα response was observed in the animals that were parenterally immunised. No IL-1β could be detected in any of the experimental groups (Fig 2B).

3.2.2. Lung

Following infection the levels of TNFα in the BAL increased 3 fold in non-immune mice to 8 h (Fig. 2C). By 24 h the levels had returned to those observed 2 h post-infection. The pattern of the TNFα response in mucosally and parenterally immunised animals was similar to

the non-immune animals but significantly greater ($p<0.05$) levels were observed at 8 h post infection. Levels observed in parenterally immunised animals were significantly higher ($p<0.05$) than those observed in mucosally immunised animals at 8 h. The levels of IL-1β in BAL rose significantly ($P<0.05$) over the 24 h observation period (approximately 2-fold) in both immune and non-immune animals (Fig. 2D). Mucosally immunised animals were lower than both the parenterally immunised animals and non-immune animals at 8 h post infection but this difference was not statistically significant. Otherwise there was no difference between the groups.

3.3. White cell counts

3.3.1. Middle ear

Infection of the middle ear with *S. pneumoniae* resulted in a rapid recruitment of white cells to the middle ear fluid (Fig 3A). Immunisation tended to enhance this recruitment, with a significantly greater ($p<0.05$) number of cells in the MEL of rats who had been parenterally immunised compared with non-immunised animals. Mucosally immunised animals tended to have higher white cell counts than non-immunised animals but these were not significantly different. White cells were recovered in the MEL at these high levels for the 72 h observation period. Neutrophils were the predominant cell type (data not shown) across all experimental groups.

3.3.2. Lung

The number of white cells in the BAL of immunised mice was significantly greater ($p<0.05$) than in non-immunised animals at 8 h post challenge (Fig 3B). At 24 h post challenge white cell recruitment to the BAL was further enhanced in parenterally immunised animals and

the number of white cells in the BAL of these animals was significantly greater ($p<0.05$) than both the mucosally immunised and non-immunised animals. At 24 h there was no difference between the non-immunised and mucosally immunised animals. The predominant cell types observed in this BAL post challenge were neutrophils (approximately 75-85%) and macrophages (approximately 20%). Immunisation did not alter this distribution (data not shown).

3.4. S. pneumoniae specific antibody responses

Low levels of IgG antibody were observed in both serum and MEL of non-immune animals (Table 1). Mucosal immunisation induced a specific IgA response in serum and MEL, whereas none was detected in rats immunised by the parenteral route. Both parenteral and mucosal immunisation resulted in a significant increase ($p<0.05$) in IgG specific antibody in both serum and MEL. IgG specific antibody levels observed in parenterally immunised animals were greater than those observed in mucosally immunised animals.

In the mouse model, there was approximately a 50 fold increase in the specific IgG titre in the sera of parenterally immunised mice compared with those that were mucosally immunised ($p<0.001$) (Table 2). Both routes induced a specific IgA in the sera but only the mucosally immunised mice had detectable specific IgA in the BAL and the parenteral group had detectable levels of specific IgG.

4. Discussion

The animal models and immunisation routes tested in this study provide evidence of robust systems for studying potential vaccine candidates for *S. pneumoniae* and assessing host immune responses. The models produced remarkably consistent results that easily discriminated between immune and non-immune groups with clearly defined differences associated with the route of immunisation. The advantages of the rat and mouse models of infection are that: they

are cost effective with respect to the acquisition and housing of sufficient numbers of animals to provide statistically reliable data; reagents are readily available; and finally genetic variants, particularly of mice, are available to enhance the study of pathogenesis and immune correlates of protection. However, a major drawback remains that pneumococcal infection is not natural in either the mouse or rat and lethal infection models are very bacterial-strain specific, as well as, animal model specific [6]. Hence, even non-immune animals will rapidly clear most infections, particularly from their lungs. The rate of clearance is less rapid in the rat model of middle ear infection for both immune and non-immune animals.

There was remarkable consistency between the observations in the lung and middle ear with the notable exception of the TNF-α response in the middle ear following immunisation and the total lack of an IL-1β response in the middle ear following challenge with live bacteria. It will be important to determine if these observations are simply a result of the different animal species or reflect a difference in the manner in which the middle ear responds to infection, post immunisation. If the latter is so, it may in part explain the modestly beneficial outcome of pneumococcal conjugate vaccination against otitis media in children compared to other sites of pneumococcal infection [7-13].

From the current study it is concluded that:

(i) The animal models described are robust, reproducible and provide high levels of sensitivity;

(ii) Both parenteral and mucosal immunisation are equally effective at enhancing bacterial clearance from the lung;

(iii) Immunisation enhances bacterial clearance from the middle ear and there is a suggestion that parenteral immunisation might be marginally superior to

mucosal immunisation in this model. Further studies need to be conducted.

(iv) Immunisation up-regulates the TNF-α response in the lung following bacterial challenge. IL-1β was significantly upregulated in all groups following challenge.

(v) In contrast to the lung, immunisation down-regulates the TNF-α response in the middle ear following bacterial challenge. Further studies are needed to determine the significance of this observation. IL-1β was not detected.

(vi) Rapid recruitment of neutrophils is the hallmark of the inflammatory response to infection in both the middle ear and lung. Immunisation tends to enhance this cellular infiltrate while bacteria are present.

(vii) It appears that the recruitment of white cells to both the lung and the middle ear, in particular, may be independent of TNF-α.

(viii) Immunisation induces a significant specific antibody response in the lung and middle ear. Only mucosal immunisation induces an IgA response detectable in lavage fluids.

Further studies can be undertaken with such models described above to examine the functional antibody responses, new vaccine candidates, as well as differences resulting from challenge with different serotypes of *S. pneumoniae.* It will be important to explore the intranasal route of mucosal immunisation as an alternative to the intra-Peyer's patch primary and intratracheal boost described in this study. Recent studies have shown that intranasal immunisation induces a specific immune response in the respiratory tract. [14] If successful,

intranasal immunisation would provide a simpler mucosal immunisation route that is closer to what might be used in humans.

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Table 1.

Specific antibody in the serum and MEL of rats following mucosal and parenteral immunisation with whole killed cell *S. pneumoniae*. Antibodies were measured against a whole cell sonicate preparation. Values presented are the mean ± SE of a minimum of 5 animals

Immunisation Group	MEL		Serum	
Specific *S. pneumoniae* antibody class	IgG (ng/ml)	IgA (ng/ml)	IgG (μg/ml)	IgA (μg/ml)
Non-immune	100±30	n.d.	3.1± 1.6	n.d.
Mucosal	540±190*	6±6	123±51*	2.5±1.1
Parenteral	980±150*	n.d.	190±52*	n.d.

n.d. None detected at the lowest dilution

* $P<0.05$ compared with non-immune animals

Table 2.

Specific antibody in the serum and BAL of mice following mucosal and parenteral immunisation with whole killed cell *S. pneumoniae*. Antibodies were measured against a whole cell sonicate preparation. Values presented are the mean ± SE of a minimum of 5 animals

Immunisation Group	BAL		Serum	
Specific *S. pneumoniae* antibody class	IgG (ng/ml)	IgA (ng/ml)	IgG (μg/ml)	IgA (μg/ml)
Non-immune	n.d.	n.d.	n.d.	n.d.
Mucosal	n.d.	185±56	5.9±0.5	3.4±0.3
Parenteral	0.7±0.6	n.d.	352±30*	6.2±1.5

n.d. None detected at the lowest dilution

* $P<0.05$ compared with non-immune animals

Figure Legends

Figure 1.

The clearance of *S. pneumoniae* from the middle ear and lung of animals challenged with live bacteria. (A) MEL – rats. (B) BAL – mice. Each time point represents the mean ± SE of a minimum of 5 animals.

Non-immune Mucosally immunised Parenterally immunised

Figure 2.

TNF-α and IL-1β in the MEL and BAL of animals challenged with live *S. pneumoniae*. (A) TNF-α MEL – rats; (B) IL-1β MEL – rats; (c) TNF-α BAL - mice (C) IL-1β– mice. Each time point represents the mean ± SE of a minimum of 5 animals.

Non-immune Mucosally immunised Parenterally immunised

* indicates significantly different from non-immune animals ($P<0.05$)

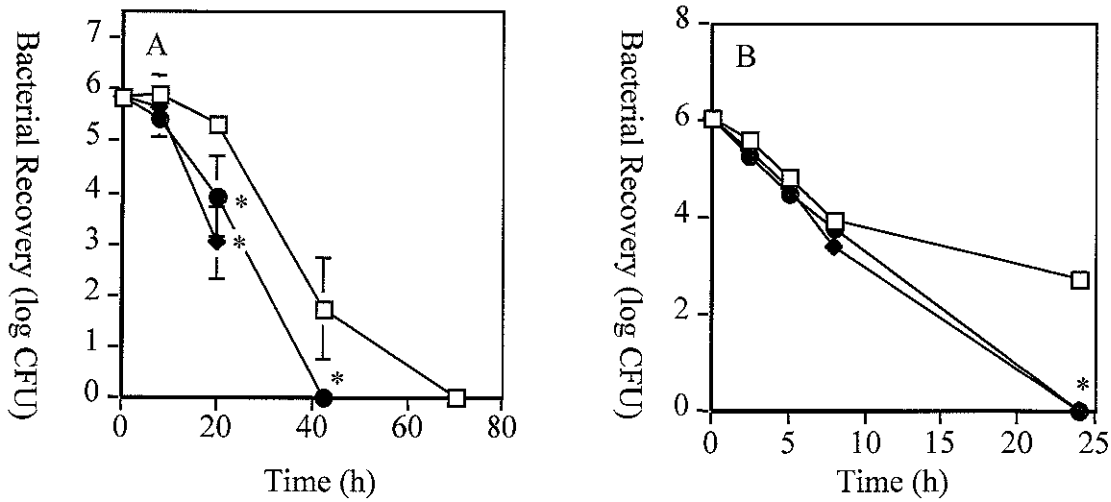
† indicates significantly different from parenterally immunised animals ($P<0.05$)

Figure 3.

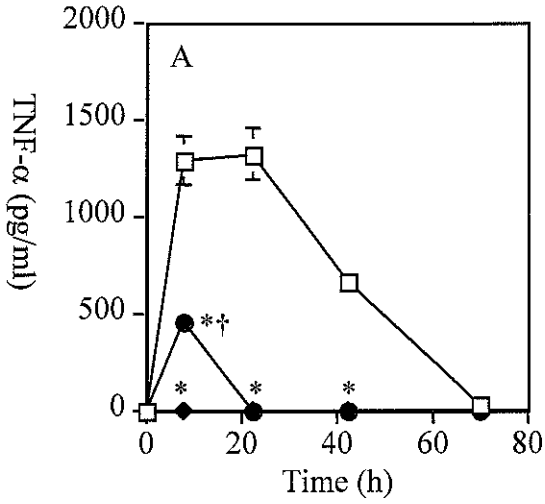
White cell counts in the MEL and BAL of animals challenged with live *S. pneumoniae*. (A) MEL – rats (B) BAL – mice. Each time point represents the mean ± SE of a minimum of 5 animals.

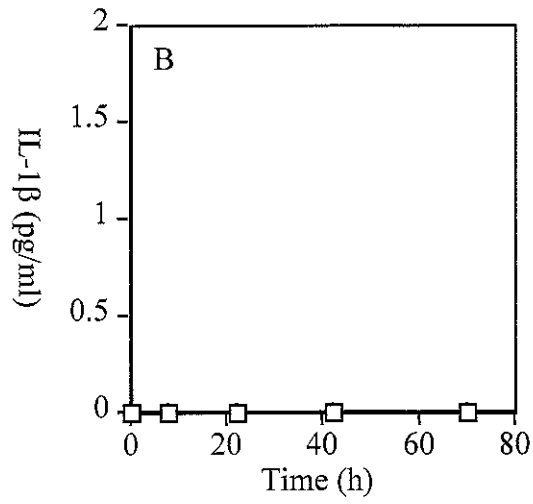
Non-immune Mucosally immunised Parenterally immunised

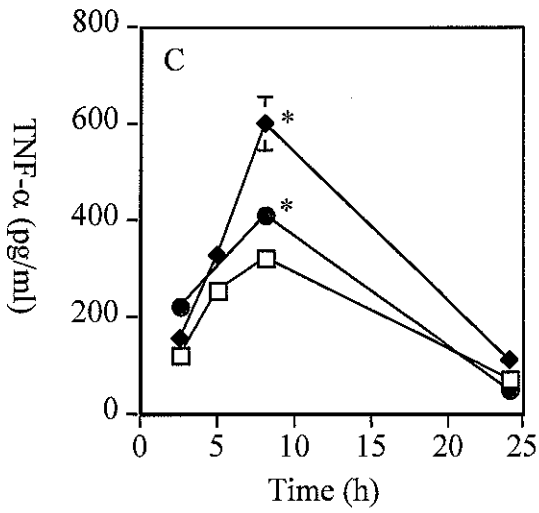
VACCINE, Cripps & Kyd, Fig. 1A and 1 B

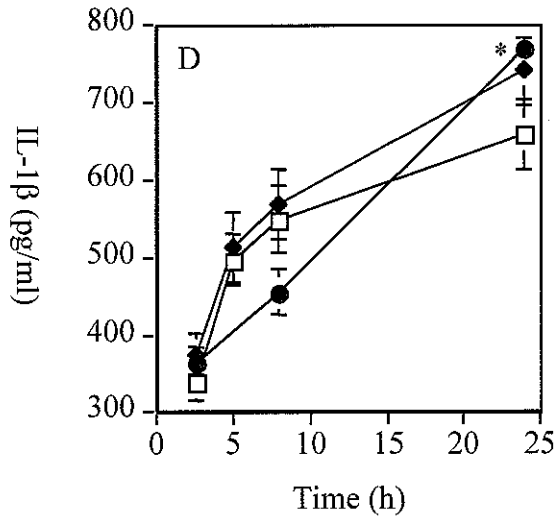


VACCINE, Cripps & Kyd, Fig. 2A, 2B, 2C, 2D









VACCINE, Cripps & Kyd, Fig. 3A and 3B