MODELS OF INFECTION

Experimental Legionnaires' disease in SCID-Beige mice reconstituted with human leucocytes

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Summary. A new small animal model of experimental Legionnaires' disease is described in which the reconstitution of SCID-Beige mice with human peripheral blood leucocytes permits the in-vivo growth of *Legionella pneumophila* in the lungs of aerosol-challenged mice. Following infection, viable bacterial counts within the lungs of mice increased from 10^5 cfu/lung at the time of inoculation to a maximum of 10^{10} cfu/lung by 48 h post-inoculation. Two types of disease were detected in the lungs of infected SCID-Beige mice. An acute exudative bronchiolitis and bronchopneumonia were seen in the most severely affected mice and, in the less severely affected mice, lesions of subacute or chronic disease were seen with thickening of alveolar walls and consolidation of lung tissue. Human cells did not appear to be involved directly in the pathology but were required for the establishment of infection. Immunohistological staining of lung tissue revealed substantial amounts of bacterial antigen distributed in a pattern similar to that seen in human Legionnaires' disease.

Introduction

Legionella pneumophila, a facultatively intracellular bacterium, is the causative agent of Legionnaires' disease, an acute pneumonia in man.¹ This environmental pathogen enters the host by the nasopharyngeal route and can produce a fatal pneumonia in elderly and immunocompromised patients. Consequently, there is a requirement for an animal model to study the mechanisms of infection, the processes leading to disease and approaches to prophylaxis and therapy. Various animal species have been used to assess virulence and pathogenesis of *L. pneumophila*, including marmosets, rhesus macaques, vervet monkeys² and guinea-pigs.³ Despite the fact that in all these species the responses seen have been shown to closely mimic the human disease, none of these models requires immune deficiency, a factor which usually predisposes human patients to Legionnaires' disease.⁴ Furthermore, the guinea-pig model, the most cost-effective model, is not suitable for investigating antibiotic therapies based on derivatives of penicillin. Unfortunately, *L. pneumophila* cannot proliferate in many strains of mice including C57BL/6, CeH/HeN, AKR and BALB/c mice,^{5, 6} although it can proliferate in macrophages from A/J mice.⁷ Thus, in the current absence of more appropriate models, the exact

mechanisms of pathogenesis remain obscure and the full potential for disease control are unproven.

Recently, a small animal model, the severe combined immunodeficient (SCID) mouse, has been shown to be susceptible to infection with various micro-organisms to which normal immuno-competent mice are refractory.^{8, 9} Mice homozygous for the SCID defect fail to develop mature T and B cells and in a closely related mouse strain, SCID-Beige, there is also a defect in natural killer (NK) cell function. Due to these immunological defects it has been found that these mouse strains are also capable of accepting human xenografts¹⁰ and, furthermore, it has been possible to infect these human-mouse chimeras with agents which are restricted to replication in human cells.¹¹ In this report the infection of Human-PBL-SCID-Beige mice with *L. pneumophila* is described and the possible mechanisms leading to the development of experimental Legionnaires' disease are discussed.

Materials and methods

Mice

A breeding colony of *scid/scid.bq/bq* (termed SCID-Beige) and intercross progeny derived from C.B-17 *scid/scid* (backcross 6) mice (termed CB17-SCID-Beige) was established from mice originally supplied by Dr Anne Croy, University of Guelph, Ontario, Canada. Animals were maintained in filter boxes

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within HEPA filtered flexible isolators. The original breeding stock were checked for the absence of mature T and B cells by in-vitro H^3 -thymidine incorporation into mitogen (Con A and LPS)-stimulated spleen cells. The experimental mice were screened for low mouse serum immunoglobulin production ($< 5 \mu\text{g/ml}$) with an ELISA assay (see below) and for the beige marker by Sudan black staining of blood films which stains giant granules within anomalous neutrophil polymorphonuclear leucocytes (PMNLs). BALB/c mice were used as immunocompetent controls.

Reconstitution of SCID-Beige and CB17-SCID-Beige mice with human peripheral blood leucocytes

Human peripheral blood leucocytes (PBL) were separated from buffy coat preparations by density gradient centrifugation (Lymphoprep; Nycomed, Birmingham). Cells were counted with a Coulter ZM counter (Coulter Electronics Ltd, Luton) and viability was assessed by trypan blue exclusion. Mice were reconstituted by intra-peritoneal injection of 2×10^7 PBL in 0.5 ml of serum-free RPMI 1640 medium.

Measurement of mouse and human immunoglobulin levels

Immunoglobulin levels in sera were monitored by ELISA before inoculation with *L. pneumophila*. Maxisorb 96-well plates (Nunc, Gibco BRL, Paisley) were coated with a 1 in 5000 dilution of rabbit anti-human or anti-mouse IgG + IgM (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) in PBS for 18 h at 4°C. Plates were blocked with PBS containing Tween 20 0.05% (PBST) and fish gelatin (Sigma) 0.5% for 1 h at 37°C. Blood samples were collected in capillary tubes from the sub-orbital sinus and the serum was diluted in blocking solution in two-fold steps from 1 in 100. Purified human and mouse Ig (Jackson ImmunoResearch Lab. Inc.) of known immunoglobulin concentration were included as standards. The plates were incubated for 90 min at 37°C, washed and then bound antibodies were detected with a 1 in 10000 dilution of rabbit anti-human or anti-mouse Ig conjugated to horse-radish peroxidase (HRP; Jackson ImmunoResearch Lab. Inc.) by incubation for 30 min at 37°C. Plates were developed for 10 min at room temperature with TMBblue (TSI-CDP, Milford, MA, USA) and the reaction was stopped with 2 M H_2SO_4 . Plates were read at 450 nm.

Bacteria and media

An isolate of *L. pneumophila* serogroup 1 of human origin was kindly provided by Dr R. A. Swann (John Radcliffe Hospital, Oxford). This strain was the cause of an outbreak of disease in Corby, Northamptonshire and was shown to be highly virulent in a guinea-pig model of Legionnaires' disease.¹² *L. pneumophila* cultures were maintained on buffered charcoal yeast extract (BCYE) agar.¹³

Cells from lung washings were maintained in modified Eagles' medium (MEM; Imperial Laboratories, Andover) supplemented with sodium bicarbonate 0.075%, 20 mM HEPES buffer and 20 mM L-glutamine.

Exposure of mice to aerosols of L. pneumophila

SCID-Beige (n = 20), BALB/c (n = 4) and human PBL reconstituted SCID-Beige (n = 20) and CB17-SCID-Beige (n = 22) mice (3 weeks post-reconstitution) were challenged with a lethal aerosol (determined in guinea-pigs) of *L. pneumophila* by methods described previously.³ A Henderson apparatus connected to a 3-jet collision spray was used to generate aerosols from a water suspension (1×10^{10} cfu/ml) of the bacteria and deliver it to the snouts of the mice. The apparatus was adjusted to produce aerosols with a particle size of $< 5 \mu\text{m}$ at a relative humidity of 65%. The animals were exposed to the aerosol for 5 min after which time two mice from each group were killed and their lungs were removed for counts of viable *Legionella*. Mice were observed daily for symptoms of infection, such as laboured breathing. Animals showing signs of distress were killed by intra-peritoneal injection of 0.5 ml of Euthatal (May and Baker, Dagenham) and their lungs were removed for assessment of bacterial load and histopathology. If no signs of distress were evident, mice were killed at random for comparative bacterial counts and immunohistology.

Viable counts of L. pneumophila

Lungs from infected mice were macerated in 5 ml of distilled water and serial, 10-fold dilutions of lung homogenates were prepared by transfer of aliquots through sterile distilled water. Duplicate BCYE plates were inoculated with (0.2-ml) volumes of dilutions of bacteria and incubated for 3 days at 37°C before counting bacterial colonies.

Intracellular fate of L. pneumophila in SCID-Beige alveolar macrophages

The lungs of eight SCID-Beige mice were washed out with MEM containing heparin 5U/ml, 4 h after aerosol challenge with *L. pneumophila*. Viable extracellular bacteria were counted in the lavage fluid before and after removal of alveolar macrophages by centrifugation at 200 g for 10 min. The presence of intracellular bacteria was determined by removing a portion of the cell pellet and disrupting the macrophages by adding 200 μl of digitonin 0.8% and incubating for 10 min at 37°C. The cell lysate was diluted in distilled water and plated on to BCYE to determine the number of viable, intracellular *L. pneumophila*. The remainder of the cells were re-suspended in MEM containing newborn calf serum 5% and were counted in a Neubauer haemocytometer chamber. The volume was adjusted to give a concentration of 1×10^5 – 10^6 macrophages/ml and 200- μl

samples were added to wells of a 96-well tissue culture plate (Nunc, Gibco BRL) and allowed to settle for 30 min at 37 °C. Non-adherent cells and extracellular bacteria were then washed away. The final wash was plated on to BCYE to check that all bacteria had been removed. Viable bacteria were counted in the tissue culture medium and in washed, disrupted cells after incubation for 48 h at 37°C.

Immunohistological staining of lung tissue for human cells

Lungs were removed immediately after death and immersed in neutral phosphate-buffered formalin 10%. Blocks of fixed tissue were dehydrated in a graded series of alcohols, embedded in paraffin wax, and sections were cut at 5 µm. Similarly prepared human tonsil tissue was used as a control of specific monoclonal antibody (MAb) staining. Paraffin sections were de-paraffinised, rehydrated and sections to be subsequently stained for the human macrophage/monocyte marker (CD68) were trypsinised by incubation for 30 min at room temperature with trypsin 0.1% in 20 mM Tris buffer, pH 7.6, containing CaCl₂ 0.1%. Endogenous peroxidase was blocked by treating all sections with H₂O₂ 3% in methanol for 5 min. Sections were washed in PBS and incubated with either mouse MAbs (Dako, High Wycombe, Bucks) to human leucocyte common antigen, CD20 (human B cells), CD43 (human T cells) or CD68 (human macrophages/monocytes) for 45 min at room temperature. Normal mouse serum (Dako) was used as a negative control serum. Sections were washed in PBS and non-specific binding was blocked by incubation with a 1 in 10 dilution of normal rabbit serum in PBS for 20 min at room temperature. Sections were incubated immediately with a 1 in 300 dilution in PBS of rabbit anti-mouse IgG-Biotin (Dako) for 45 min at room temperature and, after washing in PBS, were incubated with a 1 in 500 dilution in PBS of streptavidin-peroxidase conjugate (Jackson ImmunoResearch Lab. Inc.) for 45 min at room temperature. After washing in PBS, the sections were developed with DAB (3 mg/ml in PBS containing H₂O₂ 3%) for 5 min at room temperature and counterstained with haematoxylin, dehydrated and mounted.

Immunohistological staining for L. pneumophila

Paraffin sections (5 µm) of lung were prepared and rehydrated and endogenous peroxidase was blocked as described above. Sections were washed with water for 5 min, incubated in 20 mM Tris-buffered saline, pH 7.4 (TBS) for 10 min and non-specific binding was blocked by incubation with undiluted newborn calf serum for 20 min at room temperature. Sections were drained and the following primary antibodies added: (a) rabbit anti-*L. pneumophila* serogroup 1 lipopolysaccharide IgG diluted 1 in 500 in TBS; (b) control

rabbit serum diluted 1 in 500 in TBS; (c) TBS only; and (d) rabbit anti-*L. pneumophila* serogroup 1-HRP conjugate (10 µg/ml). The sections were incubated for 18 h at 4°C, washed in TBS, and biotinylated swine anti-rabbit IgG (Dako) diluted 1 in 300 in TBS was added to (a), (b) and (c) for 30 min at room temperature. The sections were washed as above and incubated with Streptavidin-Biotin-Complex (Dako) diluted 1 in 200 in TBS for 30 min at room temperature. After washing with TBS, the sections were developed with amino-ethyl-carbazole (AEC) substrate (Zymed, Cambridge Bioscience, Cambridge), washed in water, counterstained with haematoxylin and mounted in glycerol.

Results

Infection of SCID-Beige and BALB/c mice

To determine whether SCID-Beige mice would be a suitable model for Legionnaires' disease, they were challenged with aerosols of *L. pneumophila* and the establishment of infection was compared with similarly exposed immunocompetent BALB/c mice. Despite having severe immunodeficiency, the SCID-Beige mice were able to clear a lethal dose of *L. pneumophila* from their lungs over a 7-day period as efficiently as BALB/c mice. Neither of these mouse strains showed signs of disease and a steady decrease in bacterial counts was seen in the SCID-Beige mice lungs when examined at days 2, 3, 4 and 7 after infection. *L. pneumophila* was not recovered from the lungs of either SCID-Beige or BALB/c mice 7 days after infection.

To investigate the resistance of SCID-Beige mice to *L. pneumophila* infection, the intra- and extra-cellular environments of the lungs were examined 4 h after aerosol challenge. Similar numbers ($c. 2 \times 10^4$ cfu/ml of lung homogenate) of viable *L. pneumophila* were present in the whole lavage as were in the lavage supernate which contained no cells. This indicated that the majority of the viable *L. pneumophila* were extracellular and, to confirm this, the lavage cells were incubated in tissue culture for 48 h. The cultured cells did not contain viable *Legionellae* despite the presence of bacteria in the tissue culture medium surrounding the cells. Therefore, it is likely that the SCID-Beige mice do not succumb to disease following aerosol challenge because their macrophages do not support the growth of *L. pneumophila*.

Reconstitution of SCID-Beige and CB17-SCID-Beige mice

The concentration of human immunoglobulin in sera of mice reconstituted with human PBL was measured by ELISA 2 weeks after reconstitution. SCID-Beige mice (n = 20) had detectable but very low levels of human immunoglobulin with an average value of 1 µg/ml whereas the levels in CB17-SCID-

Fig. 1. Multiplication of *L. pneumophila* in the lungs of SCID-Beige mice after aerosol challenge: □, animals in which a subacute or chronic type tissue response was observed; ■, animals in which an acute bronchopneumonia was observed; ▨, animals that died.

Fig. 2. Multiplication of *L. pneumophila* in the lungs of CB17-SCID-Beige mice after aerosol challenge.

Fig. 3. Lung section from a SCID-Beige mouse reconstituted with human PBMC and infected with *L. pneumophila*. Neutrophilic PMNLs are present in the bronchial epithelium (B), the lumen of a bronchiole (BL) and in alveoli (arrow heads). Alveoli also contain amorphous eosinophilic material, fibrin and erythrocytes. Haematoxylin and eosin $\times 255$. Bar = 0.05 mm.

Fig. 4. Lung section from a SCID-Beige mouse reconstituted with human PBMC and infected with *L. pneumophila*. Lung tissue is consolidated with macrophage-like cells in alveolar walls and lumens. Neutrophilic PMNLs are seen occasionally (arrow heads). Haematoxylin and eosin $\times 255$. Bar = 0.05 mm.

Beige ($n = 22$) were *c.* 100 times higher, with an average value of 106 $\mu\text{g/ml}$.

Infection of human PBL reconstituted mice

SCID-Beige and CB17-SCID-Beige mice which had been reconstituted with human PBL were challenged

with an aerosol of *L. pneumophila* and then killed at regular intervals to perform counts of viable *L. pneumophila* in the lungs. Multiplication of *L. pneumophila* occurred only in the SCID-Beige mice with viable counts increasing from 10^5 up to a maximum of 10^{10} cfu/lung (figs. 1 and 2). These mice also became ill and the hatched bars indicate those

Fig. 5. Lung section from a SCID-Beige mouse reconstituted with human PBMC and infected with *L. pneumophila*. Immunohistochemical staining for *L. pneumophila* lipopolysaccharide antigen; *L. pneumophila* antigen can be seen in the lumen of bronchi (B), bronchioles (Br) and alveoli (A). Immunoperoxidase $\times 70$. Bar = 0.09 mm.

animals that died (these were not used for histological examination). In mice that did not become overtly ill (open bars), the numbers of viable bacteria decreased with time (fig. 1). In contrast, viable counts on the lungs of all CB17-SCID-Beige mice did not increase following infection (fig. 2) and overt signs of illness were not seen. The number of viable bacteria showed a decrease 4 days after infection.

Pathology of L. pneumophila-infected SCID-Beige mice

Two types of disease were detected in the lungs of reconstituted SCID-Beige mice infected with *L. pneumophila*. An acute bronchopneumonia was seen in mice in which the number of cfu was increased (fig. 1, solid bars). The acute disease was characterised by acute inflammation of bronchioles, terminal bronchioles and respiratory bronchioles (fig. 3). Mouse PMNLs were seen within the bronchiolar epithelium and in the lumen. In some instances, the epithelial cells lining the bronchiole were necrotic. The alveoli adjacent to affected bronchioles were inflamed and PMNLs and eosinophilic material (probably fibrin) filled the alveolar lumina.

Lesions of subacute or chronic disease were associated with a decreasing bacterial count (fig. 1, open bars). Alveolar walls were thickened by

macrophage-like cells and lung tissue was consolidated (fig. 4). Small foci of acute inflammation were also detected in three of these mice.

Immunohistochemical staining of lung sections for *L. pneumophila* lipopolysaccharide revealed substantial amounts of immunostained antigen in those animals with lesions of acute disease (fig. 5). Immunostained antigen was distributed focally through the lung tissues in alveolar spaces and in cells in the walls of alveoli, bronchi and bronchioles. In the animals with lesions of subacute or chronic disease in which alveolar walls were thickened by macrophage-like cells, the amount of antigen was much less and often appeared to be located within large mononuclear, macrophage-like cells (fig. 6). These macrophage-like cells were numerous in alveolar walls but only a minority contained immunostained antigen.

Immunohistological analysis of lung tissue from all groups of animals for the presence of human cells with a panel of MABs was universally negative. There was no correlation between the total human antibody levels before challenge and the type of lesions observed.

Pathology of L. pneumophila-infected CB17-SCID-Beige mice

In contrast to SCID-Beige mice, lesions in CB17-

Fig. 6. Lung section from a SCID-Beige mouse reconstituted with human PBMC and infected with *L. pneumophila*. *L. pneumophila* lipopolysaccharide antigen immunostained within macrophage-like cells (arrow heads). Immunoperoxidase $\times 510$. Bar = 0.02 mm.

Fig. 7. Lung section from a SCID-Beige mouse reconstituted with human PBMC. Human T cells immunostained with MAb anti-CD43 (arrow heads). Immunoperoxidase $\times 510$. Bar = 0.02 mm.

SCID-Beige mice infected with *L. pneumophila* were mainly restricted to the major airways of the lung and, generally, alveoli were not involved in the disease process. In the early stages of infection, individual bacteria could not be identified when immunostained with anti-serogroup 1 rabbit sera, but amorphous particulate material was concentrated within inflamed

airways, usually within PMNLs. Four days after infection, the immunostained material was seen mainly within large mononuclear cells with a macrophage morphology but these cells did not react with the CD68 specific MAb and were, therefore, of mouse origin.

Of the 19 CB17-SCID-Beige mice reconstituted with

human PBL and examined by immunohistology, seven mice had human T or B cells, or both, present within the lung tissue (fig. 7) as determined by specific MAbs (a further four mice had human cells present within spleen tissue). One mouse also had human B and T cells within the bronchus-associated lymphoid tissue (BALT). In only one mouse were large numbers of human B and T cells associated with foci of inflammation but in this and all other mice no human macrophages were detectable.

Discussion

In human Legionnaires' disease and in the guinea-pig model, *L. pneumophila* is phagocytosed by pulmonary macrophages where the bacteria multiply and eventually are released in large numbers after cell lysis. An initial interpretation of the results with the Hu-PBL-SCID-Beige mice is that introduced human monocytes/macrophages enabled the bacteria to multiply within the lung tissue. Indeed, the most surprising result from these studies was the multiplication level of *L. pneumophila* found in the lungs of some Hu-PBL-SCID-Beige mice after challenge. The number of cfu/lung seen in the mice with lesions of acute type disease approached the total numbers of organisms that have been recovered from the lungs of *L. pneumophila*-infected guinea pigs.¹² However, this interpretation does not seem to be correct because neither human macrophages nor human B and T cells were detected by immunohistological staining in the lungs of reconstituted SCID-Beige mice. Furthermore, the levels of human immunoglobulins in these mice, although detectable at 2 weeks after reconstitution, were extremely low, being of the order of 1 µg/ml. Interestingly, SCID-Beige mice that had been backcrossed to the CB.17 *scid.scid* parent strain (but still retain the beige marker) were more readily reconstituted with human cells. Human antibodies reached c. 100 µg/ml after reconstitution and human B and T cells were detected in the lung tissue of a proportion of the mice. These mice, although infected with *L. pneumophila*, did not become ill and bacterial counts in lung tissue did not increase. Lesions in backcrossed mice did not resemble those seen in the human disease and human cells within the lung tissue of backcrossed mice did not appear to be associated with the disease process.

Resistance to infection with *L. pneumophila* may be controlled by non-specific immune functions that may be genetically segregated from the genes controlling the SCID and Beige phenotypes. Comparison of the strain distribution of *L. pneumophila* susceptibility-resistance genes in A/J and C57BL/6 mice, with recombinant inbred mice, suggests that this gene may be located in the proximal part of chromosome 15.¹⁵ Since both SCID-Beige mouse strains are severely immunocompromised with respect to B and T cell functions and have defective NK cell function, this

genetic difference, if present, is most likely to involve the activity of mouse macrophages or PMNL. The effects of reconstitution with human PBL on susceptibility is unknown but it is possible that the human cells may have an immunosuppressive effect on the SCID-Beige mice, perhaps involving cytokines, enabling multiplication of bacteria within mouse macrophages by a mechanism similar to that seen in A/J mice.^{14, 15} Indeed it has been demonstrated that both murine and human cytokines can act across the species and stimulate human and murine T cells in xenogenic mixed leucocyte cultures.¹⁶ Murine antigen-presenting cells (APC) are capable of presenting xeno-antigens to purified human T cells, ruling out the possibility that there is an absolute cytokine incompatibility between these species.¹⁶ Alternatively, activation of mouse macrophages or PMNL may occur to a greater extent in reconstituted CB17-SCID-Beige mice, enabling them to control infection before it can become established.

The involvement of human monocytes in the disease process seen in SCID-Beige mice cannot be excluded, because these cells may have been present in sites not examined in this study but which were accessible to circulating bacteria. This may apply to the peritoneal cavity, since this has been shown to be a site in which human macrophages are harboured.¹⁷ Multiplication of *L. pneumophila* may have been restricted to a relatively small number of human macrophages within lung tissue which were destroyed subsequently during the disease process. It is possible that a few permissive macrophages lead to the development of lesions, since *L. pneumophila* exoproducts have been shown to deplete host defences in numerous ways.¹⁸⁻²² A protease produced by *L. pneumophila*, the principal mediator of inhibition of host defences, inhibits the antibacterial properties of human neutrophils, monocytes and NK cells^{18, 19, 20} and degrades α -1-antitrypsin,²¹ interleukin 2²² and γ -interferon (A. Williams, unpublished observation). Therefore, the production of exoproducts may have depleted both mouse and human non-specific defence mechanisms.

The extremely high numbers of *L. pneumophila* found in some reconstituted SCID-beige mice strongly suggests active multiplication of bacteria within human macrophages/monocytes because no mouse strain is known to support bacterial growth to this level. It remains to be determined what mechanisms contribute to the high level of bacterial multiplication seen in this model. Furthermore, various parameters, such as the period of reconstitution prior to infection and the age of mice, need to be optimised.

Notwithstanding these preliminary observations, this new animal model of Legionnaires' disease should be a useful aid in the study of the pathology and in the prevention and control of a disease with serious health and economic implications.

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