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SOME IMPLICATIONS FOR BIOMEDICAL RESEARCH

by

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LABORATORY RAT AND MOUSE COLONIES: SOME IMPLICATIONS FOR BIOMEDICAL RESEARCH

J.R. McNeill

Abstract

The health of laboratory rodents has a significant bearing on experimental results; the use of healthy animal stock allows experiments to be completed without the complication of premature mortality. The first part of the report describes the implementation and management of germ-free and specific-pathogen-free rodent facilities at the AAEC Research Establishment.

The second part is a comparative histological study of the lungs of germ-free rats and those reared conventionally. In animals autopsied over the period of 2 to 18 months it was found that there was little or no pathological change in the lungs of the germ-free rats whereas a progressive and serious deterioration was observed in the lungs of conventionally reared rats examined at similar time intervals. Some barrier-housed ex germ-free rats were similarly examined and found to be free from chronic respiratory disease.

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GERM-FREE ANIMALS; LABORATORY ANIMALS; RATS; MICE; AAEC; LUNGS

CONTENTS

1. INTRODUCTION

The animals in most frequent use for biological experiments, and for quality control studies of pharmaceutical and other materials intended for human use, are the small rodents, rats and mice. This report deals particularly with the use of rats, but the arguments and conclusions generally apply also to mice. Rats which are bred and kept in conventional animal houses are seldom healthy, even when free from the common pathogenic bacteria and fungi, species-specific viruses, and parasitic worms and arthropods. The principal reason for this is the almost universal presence of a chronic respiratory disease in rats more than a few months old; this disease is a well-defined pathological entity and its primary cause is almost certainly an infectious agent[1]. Rats affected by this form of respiratory disease are poor subjects for almost any kind of experimental procedure and practically useless for studies which involve the lung. Although there is no evidence that tumour induction rates differ in healthy and affected animals[2], the latter have a reduced life span and are, therefore, unsatisfactory for studies of carcinogenesis.

It is possible to eliminate chronic respiratory disease, along with other undesirable infections, from laboratory rodent colonies. This has been accomplished at the AAEC Research Establishment by converting the existing animal house at Lucas Heights to a barrier-controlled isolation facility, which was then restocked with healthy specific-pathogen-free (SPF)* animals. In the case of rats, these were obtained by handrearing caesarian-derived, germ-free, infant rats in sterile isolators; from these rats a germ-free colony was bred. At the time, this was the only such colony in Australia and it has provided the basis for the major colonies of healthy laboratory rats in Australia. The wanted strains of SPF mice were originally imported from other countries.

The principal purposes of this report are to describe how the GF and SPF rodent facilities were set up and managed, and to make a pathological and histological comparison of the lungs of conventional and healthy rats.

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* This and other terms are explained below.

2. TERMINOLOGY

In this paper, conventional (CV) refers simply to the usual type of animal house, without isolation facilities, and to animals maintained in such quarters. Conventional animal houses may have adequate ventilation and temperature control, and animals may be free of parasites and the common bacterial pathogens, but they are likely to have chronic respiratory disease and may harbour other latent infections. As noted above, the chronic respiratory disease (CRD) common in these animals is a specific entity. Conventional animals are sometimes referred to as 'dirty', or 'diseased', but these inexact terms are best avoided. Germ-free (GF), or axenic, animals are free of bacteria, fungi, protozoan and metazoan parasites, and specified viruses. The germ-free state can be maintained only by complete isolation from the external environment. Specific-pathogen-free animals are free of known pathogens, including that of chronic respiratory disease, and parasites, but may carry non-pathogenic organisms which may be characterised. Specific-pathogen-free animals may be referred to as 'clean', but this is another inexact term. The SPF state can be maintained for any length of time only by some form of 'barrier isolation', in which human access is controlled and only sterile materials may cross the barrier.

3. DISADVANTAGES OF CONVENTIONAL ANIMALS

It has often been shown that much laboratory animal research has been performed on stressed or diseased animals of poor quality, while using elaborate or expensive facilities to examine the experimental effects[3-13]. Infectious agents, with effects varying from mild to catastrophic, may be present in a colony; some 60 infections and infestations have been listed[11]. In fact, in all conventional animal houses, there are likely to be chronic viral or bacterial diseases which may cause specific organ changes, especially in the lung, or may shorten expectation of life. These effects may obscure organ changes produced by an experimental agent, invalidate studies of the distribution of a radioisotope, and may invalidate long-term studies[14], e.g. of carcinogenesis or respiratory function, which commonly require that the experimental animals be observed for the whole of their natural life-span[10,15-18].

Other problems arise from the presence of unsuspected and uncontrolled associations of microflora[7,19,20]. An example is the simultaneous presence of mouse hepatitis virus and Eperythrozoon coccoides; separately, either may cause little noticeable effect but, when present together, they result in

severe hepatitis[20,21]. Colonies of conventional animals can harbour low grade or asymptomatic infections which can be activated to a disease state by an experimental agent or procedure, and the resultant pathological change may be attributed wrongly to the experimental agent or procedure[22]. Rickettsial (Bartonella) infections of rodents are in this category[23]. Rickettsiae may haemolyse red cells both *in vitro* and *in vivo*[24-28], and this illustrates how a hidden infection may severely disturb even short-term studies of the haemopoietic system, particularly with respect to membrane fragility and red cell turnover rate.

Experiments in which laboratory rodents are used for radiobiological and oncological studies are especially at risk from dormant or subclinical infections and infestations[29,30]. Latent infections may be activated in either rats or mice of conventional status by experimentally imposed stress [31-33]. Infection and toxic shock have been identified as immediate causes of death in lethally irradiated animals[23,34-42]. Reduction of immunocompetence in conventional animals following irradiation may be followed by a dramatic microbiological invasion or by septicaemia[43-53].

If animals are insufficiently standardised, it may not be possible to duplicate described physiological or pathological studies from one laboratory to another[54-61]. For example, cellular immune reactions may be inhibited by the presence of lactic dehydrogenase virus, and lung mucopolysaccharide levels may be increased by the presence of chronic respiratory disease[62,63]. The presence of CRD makes it difficult to differentiate between chronic lesions of spontaneous disease and effects produced by experimental agents, a difficulty which increases progressively with the age of the animals. Clearly, CRD-affected rats should not be used for studies of chronic inflammatory changes in the lung[55,64-66] and, in fact, CRD in rats and mice, because of its high incidence, progressive nature and destructive characteristics, is the most serious obstacle to effective use of conventionally-reared laboratory rodents for biological studies[67-69]. Full descriptions of CRD have been published [64,68,70]. One characteristic is that there may often be few signs of disease, even when advanced pathological changes are present in the lungs. Incidence rates are variable, but often above 50 per cent at 6 months of age, and increase with age. Postulated causal agents include bacteria, viruses and food or bedding particulates[64,68,71,72]. However, comparative studies[73-80] indicate that control of infectious agents effects a marked reduction in morbidity. Dietary factors have been investigated but, in controlled

experiments, no dietary cause could be identified[81,82]. The pathology of the rat lung in CRD will be discussed in Section 7.4.

4. ADVANTAGES OF SPECIFIC-PATHOGEN-FREE ANIMALS

The use of healthy animal stock[180], free from respiratory disease and from identifiable pathogenic organisms for other diseases, allows experiments to be completed without the complication of premature mortality unrelated to the agents and procedures under test, and without the risk that pre-existing chronic illness may be confused with experimental effects[73,83,84]. A parallel may be drawn with historical human demography[15,85-87]. Public health practice with respect to quarantine, vaccination, sanitation and hygiene has brought very significant changes to the age distributions of human populations; mortality trends in long-term experimental animal colonies are likely to be even better because both genetic and environmental factors should be under control. Simply by reducing the incidence of infectious disease in rat or mouse laboratory colonies we can give the animals a longer life-span and they are, therefore, more satisfactory for the study of carcinogenesis or other long-term hazards[18].

The presence of CRD in laboratory rodent colonies can lead to specific problems in the interpretation of experimentally produced lung disease. It may be necessary to observe the reactions of respiratory tract macrophages and goblet cells to inhaled particulates or to irritant gases[19,88], but airway cell ratios are known to be dependent on the bacteriological status of the animals[89-91]. It has been said that a diagnosis of experimentally induced bronchitis cannot be made with confidence unless the lung tissue of research animals is known to be free from respiratory tract disorder[92], and that the dirty rat lung state is so different in histology that "reactions of the lung to experiment are of no value whatsoever unless they have been carried out on rats shown to be free of disease"[93]. Workers in the field of cardiovascular research have found wide differences in results and an inability to confirm other studies when undefined experimental animals have been used; one comment is that "in SPF animals we are able to study the reaction of the uncomplicated cardiovascular system to a new stimulus rather than the reaction of an already compensated system, the degree of modulation of which is unknown"[57].

The National Health and Medical Research Council of Australia (NHMRC) considers that SPF animals are the animals of choice for most experimental work, and that the use of specially bred disease-free animals and the provision of special animal quarters can prevent the frustration of failed

experiments and also save time and money[94]. Furthermore, at the 16th International Symposium on Laboratory Animals, there were several recommendations to the effect that, because of the superior results obtainable, animals which are of germ-free origin and maintained as barrier-controlled stock should be used, wherever possible, for experimental work[95]. Healthy rats are, in fact, suitable for studies of respiratory effects and diseases; SPF rats have been shown to possess sufficient goblet cells and alveolar macrophages to allow accurate quantitative analysis of respiratory tract irritation[69], although it has been observed that the presence of an infective agent may so increase goblet cell numbers and macrophage phosphatase[65] that the animals are useless for further study.

There are five age-related diseases found in laboratory rats, whose incidence is not substantially reduced in well-maintained colonies[96-98]. These are: chronic nephrosis, myocardial degeneration, periarteritis, skeletal muscle degeneration and pituitary adenoma. The possible presence of these diseases should always be taken into account when planning experiments[83] which may affect the pertinent organs since, at present, these diseases cannot be eliminated. In the planning and assessment of longterm studies[14], it should be remembered that life span is increased by a reduced nutritional intake[99], which may also affect the latent periods of induced tumours[100].

5. REQUIREMENTS FOR SPECIFIC-PATHOGEN-FREE ANIMALS

Techniques are now available which allow animals to be bred and maintained free of the troublesome infections found in conventional animal colonies. However, in addition to controlling the entry of infectious agents into animal breeding and holding units, it is necessary to control the presence of toxic gases or vapours[101,102], and undesirable airborne particulates[103] by providing adequate ventilation and filtration. One noxious gas, ammonia[104], is formed from excreted urea in the presence of the enzyme urease. This enzymatic breakdown is enhanced in the warm and moist 'shoe-box' type of cage in common use. It can be reduced by 'filter rack' ventilation[105] and, in barrier-controlled units, the exclusion of urease-producing microorganisms, such as Proteus and Klebsiella, will reduce the formation of ammonia. Experience gained with the unit suggests that suspended wire cages should be used for rats held over long periods and, as noted elsewhere[103,106,107], finely particulate pinewood or cedarwood bedding should not be used.

Insects may transmit infectious agents to animal colonies[108-110], and for this reason, as well as for the potential effect on occupational and public health from zoonoses[111-116], animal colonies should be protected against intrusion by flies, cockroaches, mites and other arthropods. This can be done by the use of mechanical barriers and acceptance of suitable standards of hygiene for laboratory animal management[117-120]. Even when the presence of infectious agents has been adequately controlled, it remains absolutely necessary to control other variables of housing and management[121, 122], including those of genetics[181]. Specific-pathogen-free rodents possess the usual susceptibility to infectious agents[123-126], and it is clearly undesirable to use for serious chronic study, animals with a rapidly changing microbial association[127] or animals subjected to acclimatisation stress[128].

Dietary standards have been very well documented[129-131], and this aspect of rodent care must be given proper attention. Microbiological contamination of commercial rodent food preparations is often excessive. A recent British recommendation[132] sets an upper limit of 5000 organisms per gram, with a zero count for Eschericia coli type 1 which indicates the possibility of faecal contamination. However, counts of 350 000 organisms per gram have been recorded for dry, pelleted rat and mouse diets supplied to this laboratory[133]. The US National Research Council's committee on the management of laboratory rodents advises[117] that succulent feeds should not be provided as a dietary component because they are potential sources of biological and chemical contamination, and may also lead to variations in the amounts of nutrients consumed. There may also be residues of chemical contaminants, such as insecticides used during grain storage, over which there seems to be little control by the manufacturers of pelleted diets. Residual insecticide may be responsible for problems associated with the use of sawdust as a bedding material[134]. Another potentially serious problem is the presence of aflatoxin[135-137] produced by the fungi Aspergillus flavus or (ergot) Claviceps purpurea[138], both of which may affect cereals.

Germ-free animals may present special problems. For example, it has been suggested that the absence of gut microflora may be associated with a vitamin B6 deficiency in a particular rat strain; this deficiency is, in turn, responsible for a peripheral nerve-related insufficiency of the respiratory muscles[139].

Effective management procedures for rodent colonies must include microbiological evaluation and control measures[117-120], and barrier maintenance. The status of established SPF colonies needs to be monitored routinely using established guidelines[11,96] to detect pathogens according to whatever category of exclusions is required[140,141]. In addition to bacteriological control, histological screening of at least the more likely target organs for bacteriological or chemical attack, e.g. lung, kidney or liver, should be undertaken.

The UK Medical Research Council (MRC) has introduced a grading scheme for the quality control of laboratory animals. Those of the lowest category (Grade 1) should be free from all evidence of infectious disease, especially those communicable to man, with special reference to Salmonellae, Shigellae, Pasteurellae, pathogenic dermatotrophic fungi, and Sarcoptes scabei. This basic grade of animal is regarded as suitable for schools and colleges which teach biology. Animals of category 2 have additionally been checked for the absence of the intermediate stages of cestodes, parasitic arthropods of all kinds, and species-specific viruses (such as ectromyelia in mice). These animals are considered suitable for most short-term studies if kept under high standards of management. Animals in categories 3 and 4 are derived by caesarian section and bacteriologically controlled; these are likely to become the grades acceptable for use in research laboratories. At present, it is unfortunately the case that most Australian holdings of laboratory rodents do not satisfy even the lowest of the MRC grading standards.

6. METHODS USED AT LUCAS HEIGHTS

The animal house at Lucas Heights supplies animals for radiobiological research (which includes oncology), for quality control testing of radiopharmaceuticals, and experimental work with new radiopharmaceuticals. For the reasons indicated above, it was decided, in 1972, to institute a scheme to upgrade the quality of the animal product. It was evident that the most satisfactory means of accomplishing this was to set up a barrier-controlled SPF system in the main animal house, and to provide its initial stock from a nucleus of GF animals. This project has been completed, and the present study reports on the comparative health, with particular reference to the lung, of three categories of animal - GF, SPF and CV. Only rats have been considered in this study but it is likely that the results apply equally to mice.

6.1 Germ-free Animals

An effective method of obtaining animals *de novo* which are free from infective disease, is by aseptic caesarian section. The immature, sterile young may then be cross-fostered to an SPF or 'clean' strain kept under some form of isolation[142-145]. In this way the problems of vertical transmission or herd-associated (horizontal) transmission of infective agents are overcome with only a few exceptions; these are mainly viral, e.g. mouse leukaemia viruses. This system has been well established internationally[142,146]. A still more effective procedure is to hand-rear the sterile, newborn animals derived by caesarian section, although this approach presents difficulties in timing the caesarian intervention and in rearing the very delicate young by manual methods. An alternative is to sterilise, in the bacteriological sense, parent animals by the use of antibiotics, anthelmintics and pesticides, etc., but this approach is less certain. Both of the latter methods were attempted at Lucas Heights, the starting point being a remote Wistar rat line which had been housed in an open, diseased system for some years previously. Both approaches appeared to be successful but, since the hand-reared animals were more likely to meet criteria for GF stock, they were adopted for all future work. Since it is unlikely that many other workers will wish to repeat this arduous and difficult task, details of the hand-rearing process[147,148] are not given here but are available on application to the author. Details of the antibiotic sterilisation regime used in the alternative approach are also available[149].

Stainless steel and Perspex isolators (Figure 1) were designed and built at Lucas Heights[150] and used to rear the initial litter of caesarian-derived GF rats. Subsequently, a small colony of GF animals was bred from the original axenic litter and has been housed in similar isolators. These isolators, which in essence are glove-boxes with provision for the interior to be sterilised and fitted with ports to allow for the exchange of animals and the introduction of food or other materials without compromising sterility, may be housed in any convenient laboratory with adequate temperature control. The sterilising agent for the isolators and their port systems is peracetic acid, used as a 2 per cent aerosol[151]; this has proved perfectly satisfactory. All mature animals are maintained on a commercially available pelleted diet which is made in accordance with recommendations from the Walter & Eliza Hall Institute of Medical Research, Melbourne[152]. The pellets are bagged and

heat-sealed in two separate polythene containers, then sterilised by irradiation at 3.5 megarads in the Lucas Heights gamma irradiation pond. Drinking water from the mains supply is bottled in one-litre units, then sterilised by steam at 120°C for 30 minutes. Bedding material, of mixed hard and softwood shavings, is also double-bagged, heat-sealed and gamma-sterilised at 3.5 megarads.

These animals have been monitored weekly for contaminants in samples of faeces and hair as well as bedding and diet. Aerobic and anaerobic liquid and solid culture media are used at 20 and 37°C, using the general guidelines of the US National Academy of Sciences (NAS)[153]. When spare animals became available, usually one per month, they were sacrificed and the tissues inoculated into the same variety of media. Stained films of blood and faeces, and wet films of faeces, were examined for evidence of microbial or parasitic contamination. Whole animals were referred to a reference laboratory[154] for detection of mycoplasma[70,155], and serum samples from older animals were tested for virological profiles at 12 month intervals by Microbiological Associates (USA)[156]. The results of monitoring indicate that the rats have remained free of metazoan, protozoan, fungal, bacterial and the specified viral agents.

6.2 Barrier-controlled Animals

The Lucas Heights animal house has been used for conventional rodent colonies (in which CRD was prevalent), but its original design was such that its conversion to completely barrier-controlled SPF operation was feasible. In 1975, this conversion was undertaken, and the final layout is shown in Figure 2. Conversion required effective sealing of all animal rooms and access corridors against the intrusion of insects or other animals, modification to and improved filtration in the air conditioning system, restriction of access to sterile food and other materials (via port systems which do not compromise asepsis), provision of removal and waste disposal facilities, and a changing room for the admission of essential staff. Before the modified facility was re-occupied, it was thoroughly fumigated. It was then restocked with GF rats from the colony prepared at Lucas Heights, and with SPF mice of suitable strains obtained by courtesy of the Walter & Eliza Hall Institute. Entry is restricted to the regular staff who can enter only through the changing room.

The SPF animals receive the same diet as the GF colony, the food being radiation-sterilised at 2.5 megarads, and are subject to similar bacteriological control[157]. As would be expected, because there is some human access to the animals, GF stock eventually acquires a bacteriological flora when transferred to SPF conditions. However, the presence of a limited non-pathogenic microflora is not detrimental to the health of the animals and CRD is not seen. It may even be desirable for optimum animal health; SPF and GF animals are not physiologically identical, the latter having, for example, an enlarged caecum. It is of course essential that SPF animals be adequately monitored to ensure that they remain, as the name implies, free of pathogenic organisms. Large-scale experiments may be carried out conveniently and satisfactorily with SPF animals, reserving GF animals for use in restocking SPF colonies and in experiments where the GF status is essential.

6.3 Experimental Observations

To determine if satisfactory animals were produced by the new regimes, and to verify that CRD was no longer present, three categories of rat were examined in detail - GF, SPF and CV. The CV animals, of the same strain, were maintained on the same but unsterilised diet and bedding as the GF and SPF rats. In all three categories, the diurnal illuminated time was 12 hours and room temperature was held at 22 ± 2°C.

The principal comparison undertaken was that of CV and GF animals. Two males and two females of each category were examined, killed with chloroform and subjected to autopsy at 2, 3, 4, 6, 9, 12 and 18 months, representing a total of 56 animals. After removal, the lungs were reflated with 10 per cent formal saline via the trachea, fixed in formalin and embedded. For examination, the major (left) lobe of each lung was sectioned longitudinally in a frontal plane to display the bronchial tree. In addition to these two groups, separate groups of GF rats were transferred to conventional maintenance (20 animals) and to SPF, i.e. barrier-controlled conditions (20 animals), to determine whether or not respiratory or other diseases would develop under those conditions in previously healthy animals.

7. RESULTS

7.1 Gross Appearance of Whole Animal

None of the GF animals showed any evidence of abnormality at any age. The animal coat was normal in appearance, and there was no sign of encrustations about the eyes or nares even in the oldest group.

The CV animals at 2 months of age were virtually indistinguishable from the GF rats. However, the CV group from 3 months of age onwards, and with age-related incidence, showed typical CRD associated characteristics. In most cases, these included encrustations about the nares and eyes and roughened coats, and wheezing and snuffling breathing was noticed in one animal at 3 months of age[158]. Again, only one of the CV group at 4 months was found to be audibly wheezing, whereas none of those at 6 months displayed clinical signs. All four of the 9 months of age group were breathing audibly, varying from snuffling to wheezing, but only two were recorded as wheezing at 12 months of age. The final age group of 18 months contained one very sick animal, displaying hunched posture, dyspnoea and roughened coat; one other of this group displayed prominent brownish-red eye encrustations, but the remaining two animals of this group did not exhibit any clinical symptom.

Two CV animals of those set aside for the study died before the completion of term, and were not included because of autolysis (16 and 17 months). (In anticipation of this event, a larger number of ageing animals had been maintained than was needed.)

7.2 Gross Appearance of Lungs

No GF lung at any age exhibited atypical appearance and all were light pink and fully crepitant throughout. In contrast to these findings, the CV lungs showed early lesions in the form of patchy discolouration and spotting of grey or brownish-red foci varying in size to about 1 mm in diameter in three out of four cases in the 3 months of age group. Only one of the 4 months of age CV group displayed macroscopic lesions; these were of the same order as those found in the 3 months of age group. At 6 months of age, the CV series contained one seriously affected lung which included underlying nodular processes and bulges and multiple foci of brownish discolouration; two others in this group showed multiple minor spotting and the remaining one appeared normal. All four lungs at 9 months displayed multiple spotted surfaces while only two of the next CV group at 12 months of age showed lungs of spotted and distorted appearance, exhibiting multiple disseminated foci of discolouration varying in size up to 2 mm in diameter. Of the 18 months of age group, one lung from the sick animal was markedly distorted by nodular masses and several indurated discoloured areas were generally brownish in colour, the whole lung displaying a cobbled misshapen appearance. Two other lungs in this group showed generalised spotting of varying degree, and the fourth lung appeared to be superficially healthy.

7.3 Macroscopic Appearance of Lung Section

Macroscopically, the lung sections of all four GF rats examined at 3, 4, 6, 9 and 12 months of age were free of lesions (Figures 3a, 3c, 4a, 4c and 5a). Lungs of three of the four rats examined at 18 months of age also appeared normal; the remaining rat lung from this group showed a single isolated lesion accumulation < 1 mm diameter (Figure 5c).

Lungs from conventionally reared rats examined at 3 months were somewhat variable in appearance. The lung of one rat of this group showed the major airway to be extensively cuffed, extending even to secondary bronchioles (Figure 3b). In addition, patchy parenchymal foci were also present. Lungs from two other rats exhibited minor multifocal lesions and the remaining lung from this group of rats appeared normal.

At 4 months of age, the lungs of three rats showed extensive multiple foci of the parenchyma and the remaining lung showed moderate cuffing of the hilar region airway (Figure 3d). There were extensive multiple foci of the parenchyma and the airways exhibited moderate cuffing (1.5-3 mm diameter) in one of the lungs from the group of CV rats examined at 6 months of age. This lung was also enlarged to about twice normal size. The remaining three lungs from this group exhibited extensive cuffing, mainly at the major bifurcation, ranging from 2-4 mm (Figure 4b).

Two of the lungs from CV animals sacrificed at 9 months of age also displayed extensive multiple foci of the parenchyma and, for the first time, the major airways were heavily sleeved (Figure 4d). The lungs were enlarged in both of these rats. The remaining two rats of this group exhibited moderate to heavy cuffing along the major bronchioles (Figure 4b).

At 12 months of age, lungs from two rats were extensively affected to an extent similar to that of the first two rats at 9 months of age. The lungs from the remaining two rats of this group, however, showed no macroscopic evidence of CRD.

The lungs of one CV rat, autopsied at 18 months, showed extensive multiple foci of the parenchyma, massive cuffing, considerable enlargement in size and one cyst. In the lungs of two other rats the major airway was completely cuffed and parenchymal foci were present. No conclusion could be reached concerning the lung from the remaining rat of this group; although macroscopically normal on the slide examined, the bronchiolar tree had been inadvertently lost on sectioning.

7.4 Microscopic Appearance of Lung Section

Microscopically, the haematoxylin and eosin stained sections of all GF lungs exhibited peribronchiolar lymphoid accumulations of between three and six loci[80] per section, over the whole age range from 2 to 18 months.

The single greatest lymphoid accumulation in the GF sequence appeared in one section at 4 months of age; it was 1.5 mm long, located at the major airway bifurcation, and was in addition to four other round cell accumulations (RCA) of length 0.2 to 0.6 mm, found along the major airway.

Of the other GF sections of the sequence, one at 18 months of age also exhibited four lymphoid aggregations, of which all were peribronchial and less than 1 mm in diameter. The remaining twenty six sections of the germ-free series all displayed round cell accumulations of less than 0.8 mm, generally at one to four loci and none of peripheral distribution. No slide from the lungs of the GF series was found to contain macrophages in other than normal tissue proportions and no mucus plugs or any other abnormality were detected.

The sections from 2 months of age CV and GF rats exhibited similar minor lymphocyte aggregations at the hilar airway bifurcation. With two exceptions (one at 12 months and one at 18 months), all of the remaining sections of the conventional animal lung tissues were found to contain heavier and more frequent distributions of lymphoplasia. At 3 months of age, two sections showed RCA of greater than 2 mm diameter at major airway bifurcations, not restricted to proximal areas; in addition there were smaller multifollicular distributions to be found throughout the parenchyma. The second two sections at 3 months of age contained several RCA foci of smaller dimensions both along the major airway and in the parenchyma. These secondary accumulations, though of lesser size, contained an appreciable percentage of neutrophils.

All slides from the lungs of the CV rats of the 4 months of age group were affected by peribronchial, perivascular and parenchymal lymphoid hyperplasia in addition to varying degrees of neutrophil and macrophage associations. At 6 months of age, two sections showed moderate airway cuffing at the hilar region; both of these also showed perivascular accumulation of microscopic proportions only. The second two slides of the 6 month grouping exhibited both peribronchiolar and perivascular multifocal sleeving, and included macrophage associations. Massive continuous lymphoid airway sleeving was a feature of two sections of the 9 months of age CV group which also exhibited early necrotic follicles or intrusions from heavy submucosal

bronchiolar lymphocyte collections. Extensive continuous lymphoid sleeving was seen in the remaining two slides of the 9 months of age group, in which, additionally, germinal centres were observed. Again, the 12 month's of age CV group exhibited heavy to massive peribronchiolar and perivascular cuffing in two sections, whereas a third showed moderate lymphocytic involvement proximal to the major airway (two areas extending > 2 mm in length), together with some diffuse interstitial cellular infiltration which included macrophages, lymphocytes and plasma cells. The fourth example from this group showed little lymphoid aggregation (three areas of < 0.2 mm located at the major airway hilar region), although a diffuse interstitial neutrophil infiltration was noted.

Of the 18 month CV group, two slides exhibited germinal and massive lymphoid hyperplasia showing some necrotic cells, together with obliteration and replacement of much of the mucosa and submucosa. Some perivascular round cell collections were observed, together with a macrophage and neutrophil presence. One slide showed little sign of lymphocyte collections, but this section had been taken past the bronchiolar arborisation plane at microtomy. The fourth and final slide from this group showed extensive bronchiolar lymphoid hyperplasia as a continuous sleeve invading and replacing much of the bronchiolar mucosa. Heavy multiple perivascular lymphoid cuffing as well as focal parenchymal lymphopurulent necrotic areas were seen. There were some minor cysts containing caseonecrotic material, as well as a massive caseopurulent plug in a saccular bronchiectatic extension abutting the hilar bronchiolar bifurcation.

7.5 Germ-free Animals Transferred to CV or SPF Conditions

The twenty GF animals which were transferred to a CV environment all displayed some evidence of chronic respiratory disease within six months. The lesions observed included multifocal lung lesions and associated cuffing of the airways, perivascular cuffing and infiltration by neutrophils and eosinophils. The appearance of the lungs of these animals differs little from those of conventionally maintained rats with chronic respiratory disease.

On the other hand, GF rats transferred to barrier (SPF) maintenance remained healthy. No evidence of CRD was apparent in any of the twenty animals tested at 10 months of age, when the experiment was terminated, either macroscopically or in histological sections. It was noted that, as has been

seen in other laboratories[73], these rats have rather more lymphoid tissue than the GF category. However, in the histology of these lungs, although free from CRD stigmata, there were two sections which displayed microscopic disease states. One of these was an acute peribronchial pneumonitis characterised by associated goblet cell hyperplasia and intrusive histiocytic peribronchial cellulitis, while the other case showed multifocal epithelioid associated giant cell 'islet' granulomas of up to 0.2 mm diameter. These granulomas were distributed throughout the parenchyma. There was no direct round cell association; the largest RCA was < 0.8 mm and located in the proximal major airway submucosa. The giant cells were of the Langhans type. Such lesions have been reported previously[159].

Although no frank pathogens have been isolated from the unit up to this time, and the disease incidence is obviously much less than that under conventional conditions, it points to the fact that quality control of SPF and GF animals requires routine histological appraisal.

8. DISCUSSION

No macroscopic or microscopic difference was discernible in the lungs of GF or CV rats at 2 months of age. From 3 months of age and becoming increasingly apparent with age, the presence of disease processes in the lungs of CV animals was shown by an initial proximal peribronchiolar lymphoid hyperplasia, becoming both germinal and disruptive of airway mucosa and submucosal stroma. The appearance of perivascular and other parenchymal foci of round cell accumulations are early indications of an expanding destructive multifocal lymphoid hyperplasia characteristic of CRD[160]. Often, in conjunction with these processes, there may be an accompanying intrusive interstitial infiltration of macrophages and lymphocytes and the development of follicular caseonecrosis together with continuous airway sleeving or cuffing, both major and minor, by lymphoid hyperplasia. The lung parenchyma became increasingly displaced and replaced with round cell metaplastic consolidation, and bronchiectatic mucopus abscesses were superimposed on the distal histology of mucopurulent bronchiolar plugs. Finally the lung lost elasticity and became indurated and inactive.

The sequence of changes described in the CV series of rats has the characteristics which are typical of CRD, as reported in the extensive literature on the subject[3-9,161-163]. The disease becomes evident in an increasing proportion of animals at ages from 3 months onwards. The changes

of CRD were not seen in the GF animals or SPF animals of GF origin which had been maintained under barrier control.

There can be no doubt that infective agents are involved in CRD aetiology; however, there is evidence that gaseous[164] or particulate irritants may also be contributory factors. Although the presence of minor lymphocyte accumulations at the major airway bifurcations of rats has been questioned[69,165,166], several authors have provided evidence to support the thesis that such a lymphocyte presence may be a normal species characteristic[76,78,167,178]. There is no basis in the findings of this GF series of lungs for thinking that these lymphocyte accumulations are progressive or that they are indicative of early CRD.

Viruses have been proposed as aetiological agents of rodent CRD[67,168] and ultrafiltrates have been shown to produce the abundant 'CRD type' of lymphoid hyperplasia. Virus elicited cellular responses[169] in the lungs include the presence of giant cells and macrophages, as well as epithelial proliferation followed by necrosis and regeneration, epithelialisation of alveoli, intra-alveolar oedematous exudate, and possibly intra-alveolar erythrocytes and macrophages, in addition to lymphomononuclear cell hyperplasia. The histology of the CRD in this series of CV animals does not resemble those classic cellular changes, and it is felt that a virus aetiology is not proven.

Clearly, the dissociation of these GF rats from infective agents throughout life has resulted in markedly altered lung histology, differing from that of the CV animals which initially displayed the same appearance as GF animals at 2 months of age, but which progressively developed the debilitating multifocal consolidating lesions of CRD.

Antigenic stimuli from dead bacteria in diet or bedding material may be responsible for low grade activity of lymphoid cells in the respiratory system[170,171], as well as for the strain-dependent autoimmune disease[171,172] that has been observed in some GF rodents. Another possibility that perhaps should be kept in mind is that the presence of materials with mitogenic activity may evoke such responses. Certain materials of vegetable origin, e.g. phytohaemagglutinin[173] and Pokeweed mitogen[174], are able to stimulate lymphocyte division *in vitro* and may possess that property *in vivo*.

It has been suggested that inhaled particulate matter[175,176] contributes to the pathogenesis of rat CRD. However, rat CRD has no histological similarity to any of the human pneumoconioses[169], and the conditions of the

work reported here with respect to particulate inhalation, e.g. from food or bedding, were identical for GF rats and rats with CRD. Particulates have been shown to increase goblet cell activity and associated mucopolysaccharide production, after which bacterial infection may superimpose focal or interstitial pneumonitis[65,71,72]. It is therefore possible that inhaled particulates (or ammonia) play some role in the development of CRD but, on the evidence presented here, it does not seem to be a major one. On the other hand it seems clear that infectious agents are of primary importance in the pathogenesis of CRD.

Other reports have suggested that microscopic lymphocytic aggregations[177] may be present in both GF[64,75,178] and SPF[68,75,78,80] rodent colonies. Throughout the present series of undoubtedly GF animals there was some lymphocyte presence. This took the form generally of submucosal accumulations in the hilar regions, usually less than 0.8 mm in length and resembling those reported from other laboratories[75,76,79,80] (Figures 3-5).

Demonstration of the absence of CRD from a rat colony appears to require histological examination of aged animals, for macroscopic inspection at ages from 6 to 9 months will not always demonstrate existing disease. The primary criterion of the disease-free state is the absence of age-related lymphocytic hyperplasia in the respiratory system, ranging from minimal peribronchial aggregations at about 2 months of age to expanding perivascular foci at 6 months or less. The presence of other than small ($\sim$ 1 mm) proximal peribronchial round cell accumulations at up to 18 months of age is likely to signify some unusual response of the respiratory system's immune mechanism - whether to an unusual antigenic load or to infection. The low numbers of lymphocytes usually present in these 'clean' animals is regarded as a characteristic of this rat strain under the maintenance standards practised, and it is probable that these lymphocytes may be stimulated into frank germinal areas given the appropriate antigenic stimuli.

The presence of some lymphocytic aggregation may be accounted for by local, non-infective antigenic stimulation and strain variation. It is the progressive massing of lymphocytes and associated progressive desquamation and epithelial metaplasia of the cells lining the airway lumen together with alterations in numbers and distribution of goblet cells and macrophages, becoming evident at about 4 to 6 months of age, which suggest CRD. This is especially so where perivascular accumulations of lymphocytes are present, as well as increase in both size and number of foci usually followed by necrotic

caseation (see Figure 6) and induration of affected peripheral areas. Mucoid plugs may be present in the bronchioles, and both lumenal and interstitial macrophage infiltration may be followed by massive consolidation of a lobe while occasionally leaving an adjacent lobe relatively unaffected[179]. Compounding this may be the distal advance of multiple loci of lymphocyte collections to form a continuous sleeve around the airway lumen as the disease progresses. Bronchiectatic lesions may follow and be accompanied by formation of mucopus and epithelioid encystments of caseonecrotic material [167,179]. The conventional animal sections showed increased numbers of goblet cells, the distribution of which may be extended to distal bronchioles; however, the developing lymphoid sleeving eventually desquamates even these regions, in conjunction with which may be associated atelectasis and consolidation. In confirmation of the work of Innes *et al.*[160], squamous metaplasia was not a feature of the CRD syndrome examined in this study.

9. CONCLUSIONS

These findings demonstrate several points:

- The original Wistar rat colony held under 'open' or 'conventional' housing conditions was seriously diseased.
- By introducing GF caesarian delivery and the subsequent GF holding of the progeny, every sign of progressive chronic respiratory disease had been removed.
- Some lymphocytic aggregations of the lungs of this existing remote Wistar 'clean' stock is a non-progressive typical finding.
- The pre-existing chronic respiratory disease syndrome of the diseased colony was probably caused by infective agents.

10. SOURCES AND ACKNOWLEDGEMENTS

Appendix A lists a number of national and international biological research organisations which have established extensive specific-pathogen-free rodent colonies for use in research. All of these bodies have been consulted and have provided advice or assistance on the management of the SPF facilities at the AAEC Research Establishment. The author and the Commission are grateful for valuable assistance obtained in this way.

The Lucas Heights GF rat colony has become the basis for the major holdings of SPF rats in Australia. Australian organisations which have been supplied with GF or SPF animals are also listed in the Appendix.

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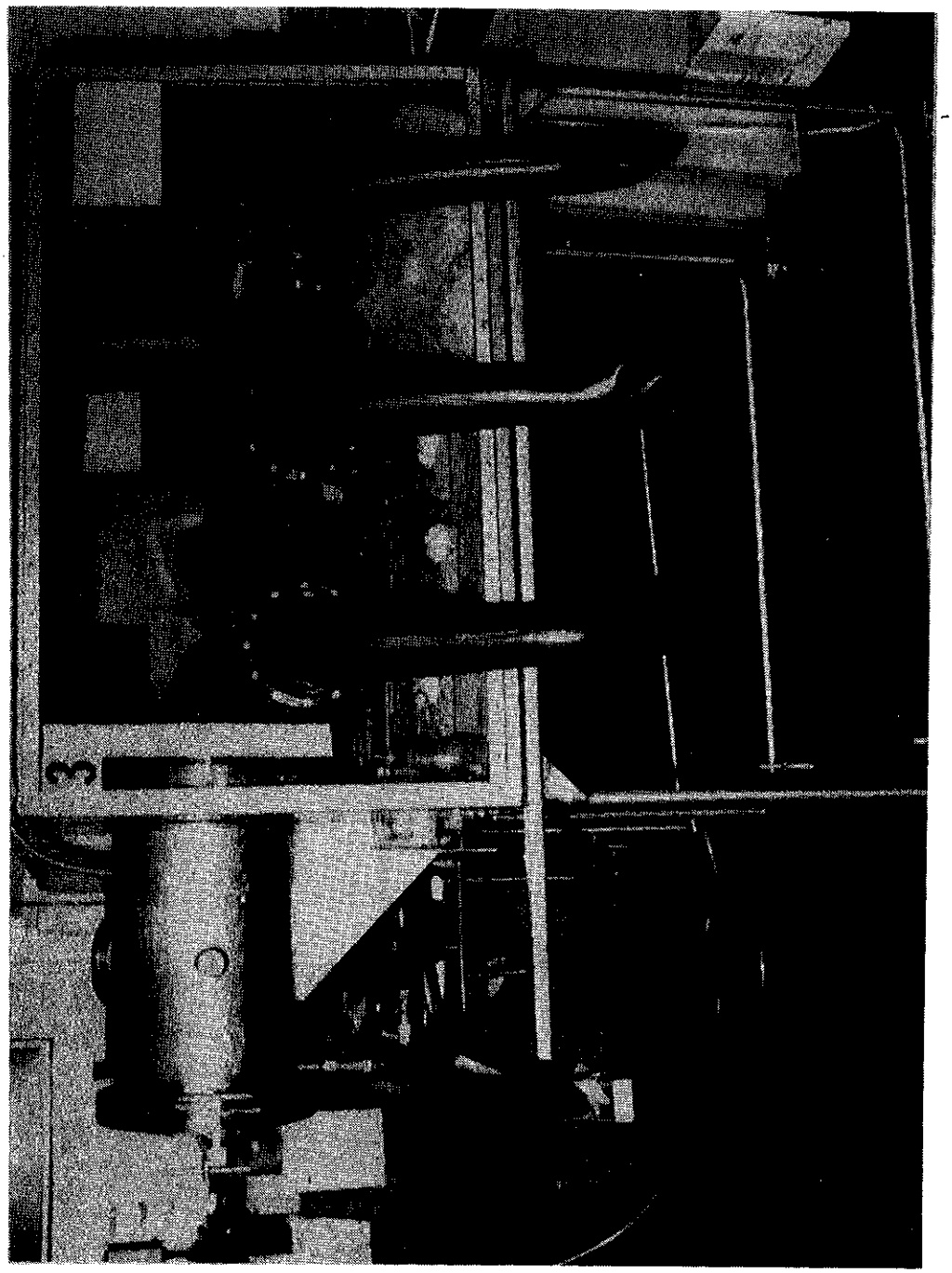

Figure 1. Stainless steel and Perspex isolator. Model E, AAEC design, used for the housing of germ-free 'foundation' stock and from which 'clean' animals are taken to colonise a barrier maintained animal house.

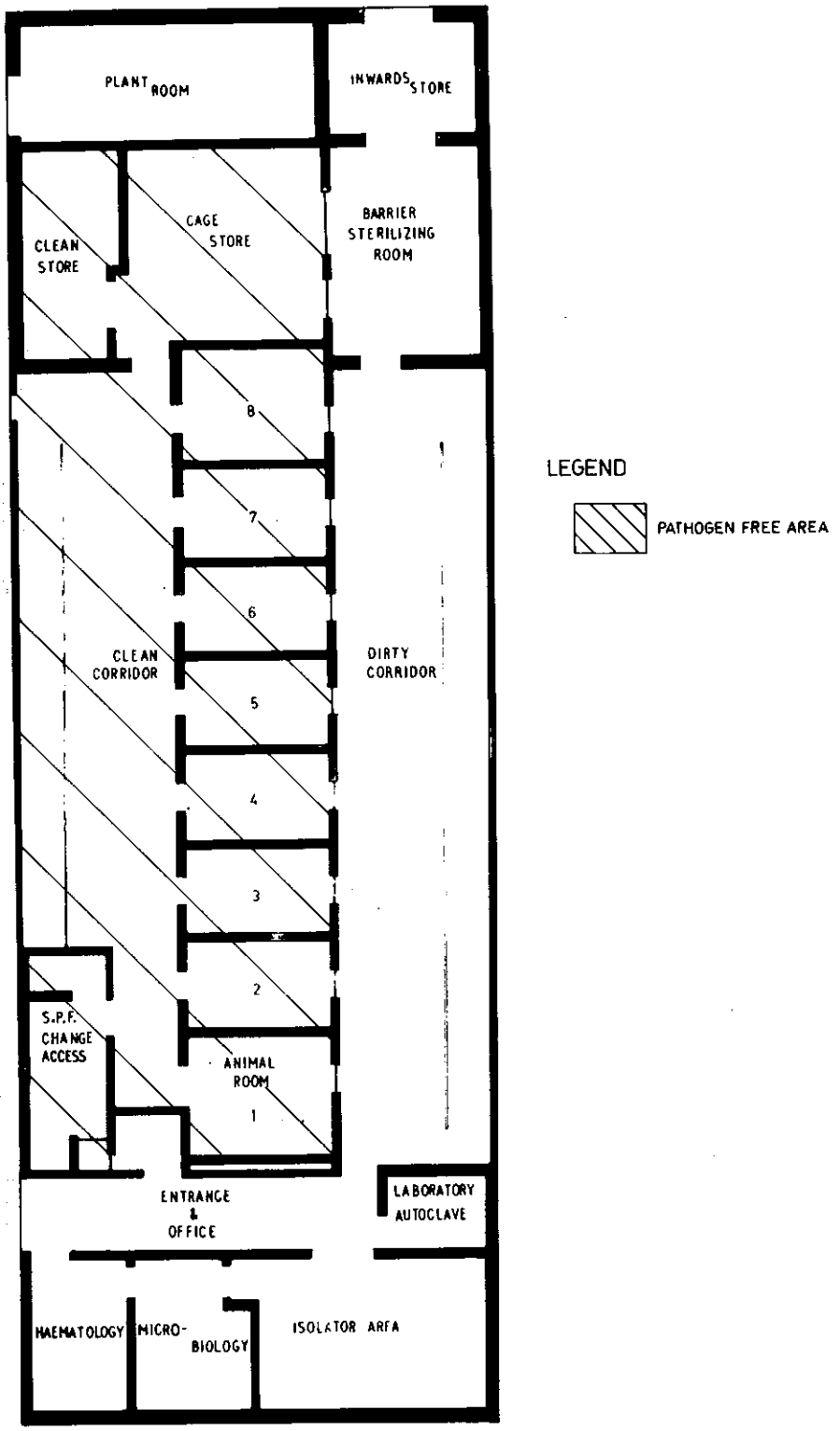

Figure 2. Specific-pathogen-free animal breeding unit at Lucas Heights. The barrier sterilising room contains a double-doored autoclave and a germicidal dunk tank. The animal rooms, though sealed from the 'dirty' corridor, are fitted with individual stainless steel double-doored ports to allow cages of animals to be passed out.

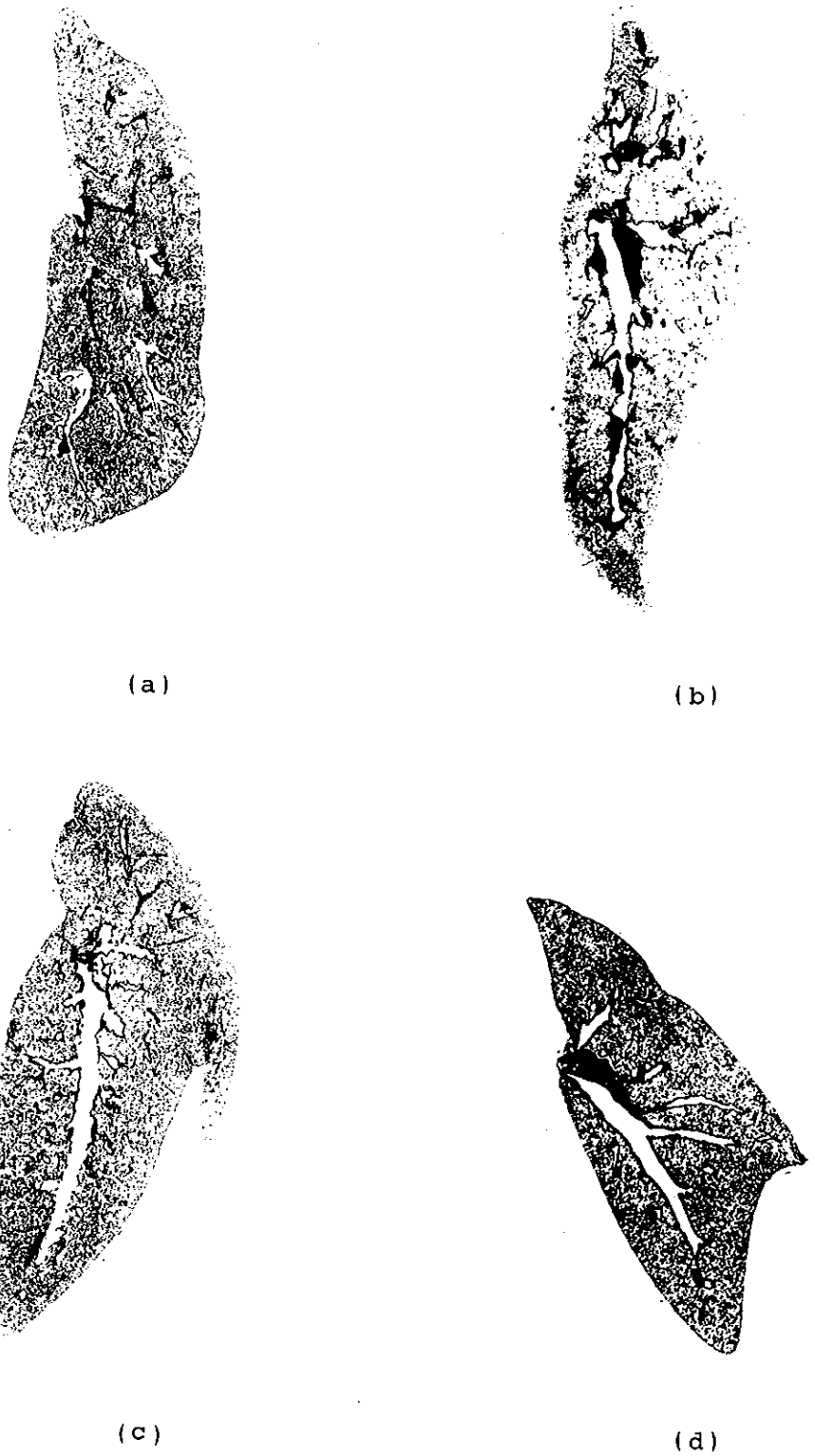

Figure 3. Rat lungs, left lobe, frontal plane, longitudinal section (x 4 magnification).

(a) 3 months germ-free, (b) 3 months 'conventional', showing multifocal lymphoid accumulations, (c) 4 months germ-free, (d) 4 months 'conventional' again showing early airway 'cuffing'.

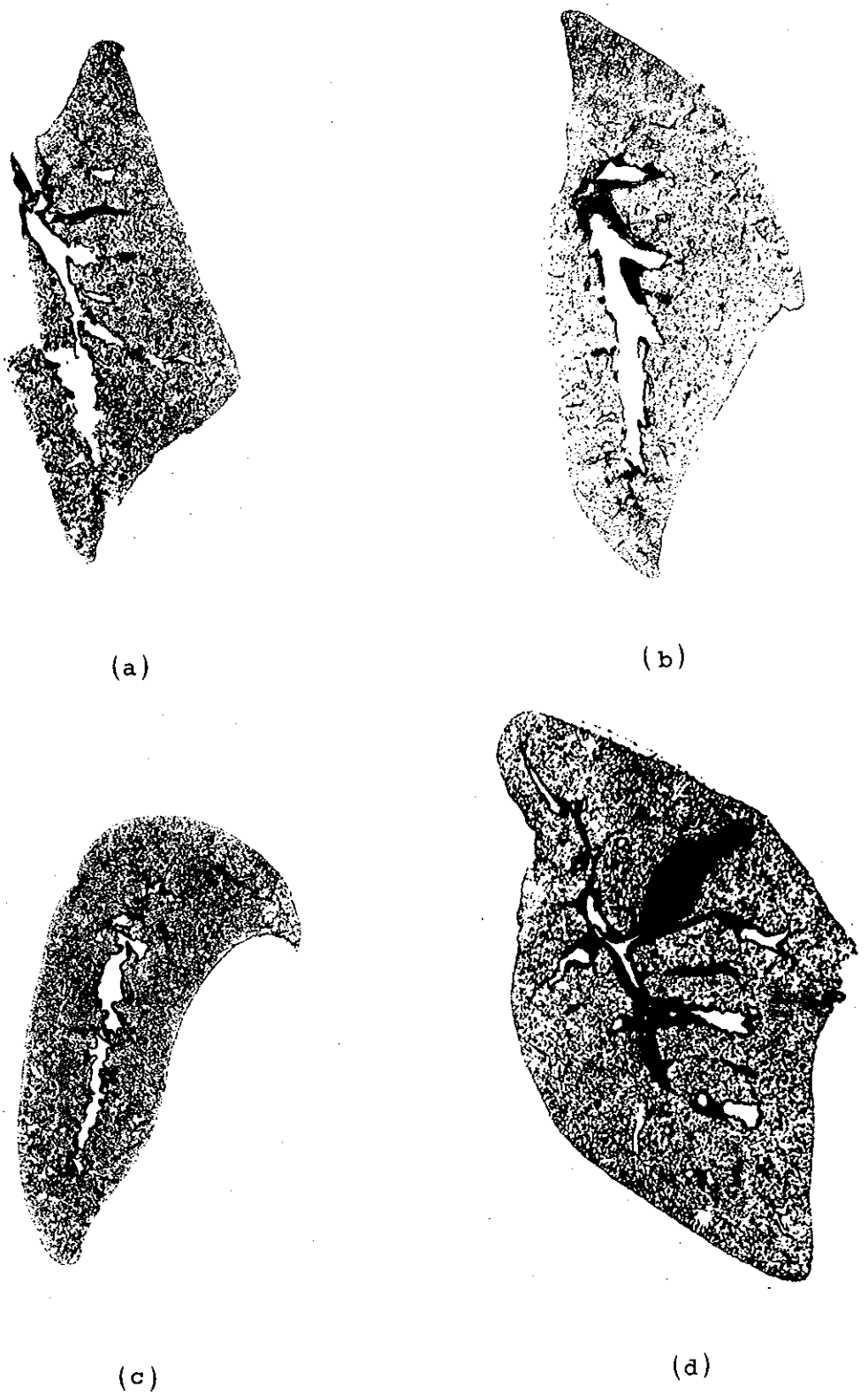

Figure 4. Rat lungs, l ..c lobe, frontal plane, longitudinal section (x 4 magnification).

(a) 6 months germ-free, (b) 6 months 'conventional', increased sizes of lymphoid aggregations,
(c) 9 months germ-free, (d) 9 months 'conventional', exhibiting massive cuffing of bronchioles.

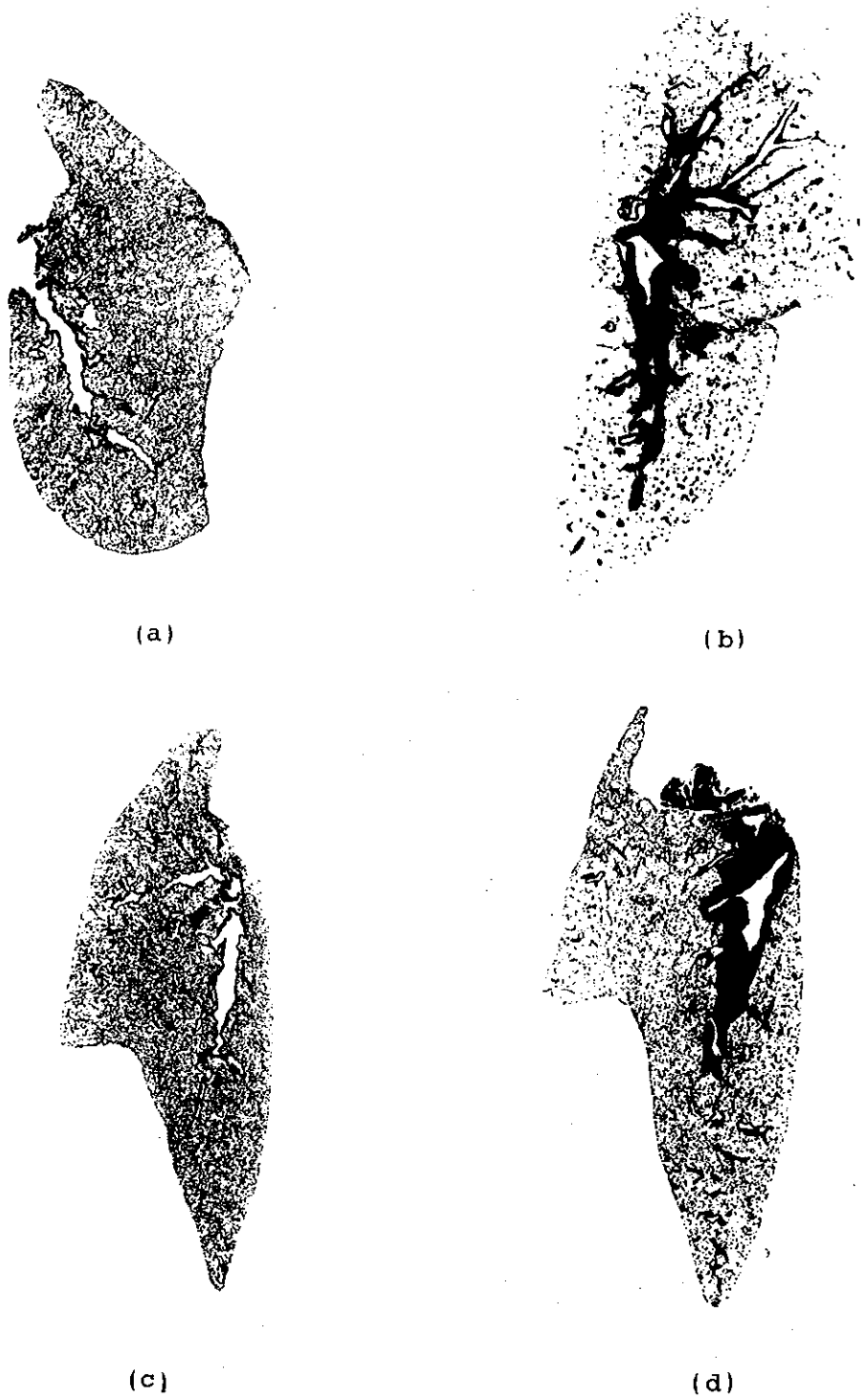

Figure 5. Rat lungs, left lobe, frontal plane, longitudinal section (x 4 magnification)

(a) 12 months germ-free, (b) 12 months 'conventional', (c) 18 months germ-free, (d) 18 months 'conventional'

Figure 6. Rat lung, left lobe, frontal plane, longitudinal section, 21 months 'conventional' or 'dirty' colony.

This is a classic example of the dramatic effect which may be expected in an ageing rat colony which has not been caesarian derived and barrier maintained. The obvious disadvantages of using such animals for any long-term work are very much in evidence. Most of the lung tissue has been replaced with bronchiectatic cavities filled with caseous necrotic material. A disease state of this type would never be found from a 'clean' colony of rats or mice.

APPENDIX A

After development of axenic rat holdings on site but before commissioning of the barrier controlled animal unit at Lucas Heights, a limited survey of relevant national and international bodies was undertaken (all have established barrier facilities). These included

National Biological Standards Laboratories, Canberra.
The John Curtin School of Medical Research, Australian National University, Canberra.
The Walter & Eliza Hall Institute of Medical Research, Melbourne.
Biology Division, Oak Ridge National Laboratory, Tennessee, USA.
National Institute of Health, Maryland, USA.
Central Institute for Laboratory Animals, Hanover, Germany.
The Radiobiological Institute (TNO), The Netherlands.
Laboratory Animals Centre, Medical Research Council, Carshalton, UK.
Huntington Research Centre, Alconbury, Huntingdon, UK.
Lilly Research Laboratories, Indianapolis, USA.
Microbiological Research Establishment, Porton Down, UK.

In many instances, operational methods in use at these institutes have been incorporated in the upkeep of research grade animals at Lucas Heights.

The animals from the isolators or from the barrier facility have been used either for foundation of 'clean' stock or for direct use at many Australian research centres. The following list shows those Australian organisations which have arranged for the use of animals prepared at Lucas Heights. Some have extensive facilities and may be available to provide advice on SPF animal management or on the value of animals of SPF standards.

The Walter & Eliza Hall Institute of Medical Research, Melbourne.
The John Curtin School of Medical Research, Australian National University, Canberra.
The Ludwig Institute of Cancer Research, University of Sydney.
Division of Animal Health, CSIRO, Sydney.
Kanematsu Institute of Pathology, Sydney Hospital.
NSW Institute of Technology, Gore Hill, Sydney.
Macquarie University, Ryde, Sydney.
NSW State Cancer Council, Prince of Wales Hospital, Sydney.
University of Sydney, Sydney.

Institute of Clinical Pathology and Medical Research, Westmead, NSW.

Gastroenterology Department, Prince of Wales Hospital, Sydney.

University of NSW, Kensington, Sydney.

Sir Charles Gairdner Hospital, Perth, WA.

The Institute of Medical and Veterinary Science, Gilles Plains, SA.

Other major Australian organisations which maintain barrier holdings of rodents and from which advice on relevant methodology could be obtained are:

Adelaide University, Adelaide, SA.

National Biological Standards Laboratory, Canberra.

Commonwealth Serum Laboratories, Parkville, Vic.

Peter McCallum Institute for Medical Research, Melbourne.