INVASIVE ASPERGILLOSIS ANIMAL MODELS (IAAM)

Critical Needs and Future Directions Workshop

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Executive Summary

Invasive aspergillosis is a major cause of morbidity and mortality in immunosuppressed hosts. The diagnosis of this infection remains very difficult and there are limited treatment options. Animal models have been utilized to evaluate both the diagnosis and treatment of infection and to assess the pathogenicity and virulence of the organism. However, these models have not been standardized and have been used in only a limited fashion for genomic evaluation in this disease. Extensive efforts are underway that will significantly expand the genomic information regarding *Aspergillus*.

The **overall objective** of this proposal is to establish and standardize animal models of invasive aspergillosis that can be used to evaluate genomic information on the pathogenicity and virulence of *Aspergillus* in order to improve diagnosis and therapy of invasive aspergillosis. The **specific aims** of this contract are to:

- Establish and standardize animal models of invasive aspergillosis including murine and larger animal species with both pulmonary and disseminated infection;
- Develop molecular tools utilizing pre- and post-genomic information that will provide standardized procedures for conducting genetic manipulation of *Aspergillus* strains that can be tested in animal model systems and to allow assessment of pathogenicity and virulence of *Aspergillus* in animal models of infection, in order to establish new diagnostic targets or tools.
- Determine gene expression of both whole cell and individual target genes of *Aspergillus* in experimental infection.
- Utilize post-genomic data to develop novel diagnostic and immunologic approaches in the management of invasive aspergillosis.
- Provide a mechanism for disseminating knowledge and skills obtained from this effort to other interested scientists and laboratories and effectively interact with the extended *Aspergillus* community (academia as well as commercial interests) to develop new diagnostic tools.

Investigators have been assembled with extensive experience with animal models of invasive aspergillosis (including both murine models and larger animal species) as well as research groups with established molecular and genetic programs in *Aspergillus* that can provide genetic tools for evaluating this disease. These investigators will work closely with the extended community (including academia and industry) to develop new diagnostic targets and tools to answer key questions in this disease. The goal of this work is to evaluate these genetic tools in animal models in order to improve the diagnosis and treatment of patients with invasive aspergillosis that will ultimately result in improved outcomes of patients with this frequently fatal infection.

Standardized animal models are essential in the development of new diagnostic techniques, although no single model will be able to provide a definitive reference standard for all applications. Murine models have the advantage that numerous animals can be tested under identical conditions simultaneously, and as such should be used as a first screen to identify diagnostic candidates which are suitable for testing in larger animal models, and potentially in the clinical setting. Such models should also be used to define the minimum criteria for identification of new targets for diagnostic techniques. Diagnostics will be most valuable in the clinical setting if they are able to identify infections at the earliest opportunity, and for this reason, experimental systems should provide a good model for early stages of infection. A number of variables will affect the performance of a model, including host (mouse strain, inbred or outbred lines, housing conditions, degree and duration of immunosuppression) and pathogen-related factors (*Aspergillus* strain, culture conditions, route of exposure).

Larger animal models (guinea pigs, rabbits) have the advantages that they allow serial sampling, and as such could be used both for the detection of early infection, and for monitoring the course of disease; these models could also be used to generate data to attract commercial interest in the new diagnostic. Small partners may initially be a more likely source of commercial backing than larger companies.

The molecular tools under investigation need continued development, and their utility in serial assessments of outcomes recognized. An effective typing system is critical for determining the identity of *Aspergillus* strains. The 2 key challenges for collection and use of post-genomic data are 1) differentiating fungal and host genetic material and 2) the ongoing annotation of the genome. The development of an effective database for these data is vital, as this will be a primary means of disseminating information and tools to the broader community involved in *Aspergillus* research.

Background

Overview of the contract

NIH-NIAID-N01-AI-30041 Invasive Aspergillosis Animal Models (IAAM)
To run from August 15, 2003 – August 14, 2010
To establish and standardize animal models of invasive aspergillosis

- Using post-genomic data define surrogate markers of infection and disease progression
- Allow reliable quantification of infectious burden
- Improve diagnosis and therapy of invasive aspergillosis

Timelines

- Phase 1 (years 0 – 2): Development and standardization of model(s)
    - Establish Steering Committee and Expert Panel
    - Workshop for critical needs
    - Community input
    - Develop animal models
    - Molecular tools
- Phase 2 (years 3 – 7): Further development, community training and support
    - Provide resources/skills for answering up to 100 "key questions" over 7 years
    - Genetic approaches
    - Animal models
    - Training/dissemination of information

Critical issue: In other areas where detailed guidelines/models are available (eg, the NCCLS documents), interlaboratory variability in results is still apparent. A realistic target should be set for this contract, given that the model is due to be developed in the first 2 years of the contract.

Ultimate goal

Identify new targets for the development of a licensed diagnostic technique with the following characteristics:

- Can be used to **detect** infection early, and to **monitor** the subsequent course of disease (including the effect of therapeutic interventions, although this is not a specific aim for this contract)
- Be **standardized**
    - To allow comparisons of data between laboratories
    - To provide validated baseline responses, against which other techniques/modifications can be compared
- Be fully **reproducible**
- Be **appropriate** (cost/equipment) for exporting to laboratories around the world
- Some degree of correlation to clinical events would be desirable, but this is not essential

The new diagnostic test could have application to:

- Clinical trial design
    - To ensure that patients recruited do genuinely have aspergillosis
- Drug development
- Clinical practice

Critical issue: It is unlikely that a single model or combination of animal/Aspergillus strains will provide all the information required. The contract does not require a model for every combination of Aspergillus and mouse strains – the aim should be to develop a single, robust, reproducible model against which other models can be compared.

Challenges

As a proportion of current healthcare expenditure in the USA, development of diagnostics accounts for less than that spent on administrative costs. Expenditure on prevention and treatment of infections greatly outstrips that on diagnostics.

Because of the perceived commercial limitations of a new diagnostic test for aspergillosis, there has been relatively limited interest from industry historically. However, with the team assembled for this contract, and the NIH funding, the main barriers to the development of a new diagnostic are now scientific issues, rather than limitations in expertise or finance resources.

Terminology needs to be clearly defined from the start of the contract. This is essential if the protocols developed are to fully standardized – ie,

- Differentiating "infection" from "disease"
- Use of "reference standard", rather than "gold standard", as the latter sounds permanent

Animal Models

The development of standardized animal models is essential for the development of a licensed diagnostic test.

- Appropriate targets/surrogate markers for the infection need to be defined for this process.
    - At this early stage of development, it is not known exactly what the marker for detecting/monitoring infection will be – it could be a completely new marker, linked in some way to the biology of the pathogen – but it is hoped that the utility of the marker will be apparent immediately when it is identified
- Detecting fungal pathogens, estimating fungal burden, and differentiating colonization from infections all remain key challenges.

One issue of increasing importance, particularly in Europe, is the goal of reducing the number of animals used in medical research (the "3Rs": **r**eplacement, **r**efinement, and **r**eduction).

- The current NIH contract has the potential to have strong input into the definition of endpoints in future studies.
    - Is death a necessary endpoint?
    - Do animals suffer unnecessarily because of this?
- If standard operating procedures (SOPs) can be defined during the development of the models as part of the current NIH contract, this could reduce the number of animals needed in future studies. By defining the course of disease under closely defined conditions, this may allow fewer controls in subsequent studies.

Critical issue: Consider approaches to minimize variation in morbidity (and hence in the time that the animals reach the experimental endpoints). This will help statistical analyses, should result in a reduction in the number of animals required per experiment, and would minimize the effect of changes in the degree of immunosuppression.

The goal of such models is to provide a robust definition of the course of infection, which is reproducible within and between laboratories.

Murine Models

Introduction

Murine models are widely used and are an invaluable research tool, although the techniques used for these models vary considerably between laboratories.

- As the size of the community working on *Aspergillus* infections continues to grow, this lack of standardization limits the amount of meaningful communication between centers, as direct comparisons of data are made very difficult.

Advantages of murine models

- Relatively large numbers of animals can be studied simultaneously, making statistical analyses more robust.
- Such models could be used as an initial screening technique to identify those which would be most suitable for transfer to rabbit/guinea pig models, and ultimately to the clinical setting.

Disadvantages of murine models

- Invasive procedures limited to single application; this does not mimic the likely clinical use of diagnostics (repeated bloodstream sampling).

Basic considerations

- Define the virulence characteristics of the *Aspergillus* strains involved.
- Consider the impact of the route of administration:
    - IV route represents an unnatural process, compared with inhalation followed by dissemination
    - Intranasal administration deposits conidia in the trachea, limiting penetration to the alveoli
        - Aerosolization and inhalation more closely mimics the natural disease process in humans
- Ideally, the host factors predisposing the animals to infection should mimic clinical situations.
    - Inbred mice are good models for studying specific questions, but can they be used as models for patient populations?

Critical issue: For a model to be used in the development of diagnostics, responses at an early stage of infection are most important. Even if an animal model does not mimic the whole course of the infection in humans, standardized models are still critical – it just needs to be recognized that they do not necessarily mimic the entire situation in the clinical setting.

Specific models

Flask Model

- Under the conditions described at the workshop, all animals died within 3 days of infection with *A fumigatus.*
    - Not a suitable model for long-term studies or investigations of intervention
- If *A flavus* (a less-virulent species) was used, this was extended to 7 days.
- Tissue burden assessments indicated 10^5 – 10^6 CFU in the lungs 1h after exposure, remaining relatively stable until the mice died at Day 5 (10^5 CFU).
    - This contrasts with *Candida*/*Cryptococcus* infections, where fungal burdens increase steadily following exposure
- Interlaboratory reproducibility in these data differed by approximately 1 log in CFU counts. This was thought to be due to different conidiation patterns, due to non-uniform culture conditions.

Madison Chamber

Advantages:

- Allows large numbers of mice to be challenged simultaneously.
- Would permit very useful studies defining the course of disease under specific conditions, for example the use of the same *Aspergillus* strain, dose, conditions, and timings, but different mice strains.
    - No-one has undertaken these comparisons yet

Disadvantage:

- Very specialized equipment; costly.

Acrylic Chamber

Advantages:

- Inexpensive to produce, and thus likely to be more widely available than the Madison chamber.
- Allows accurate quantification of inoculum.
- The addition of a sampling port (to allow quantification of conidia in the chamber) would be relatively easy.

Disadvantages:

- Specific nebulizers may not be available in all countries- may need standardization.
- There are some indications that dissemination of infection is very limited using this system (some brain involvement in 10-20% of mice, but very low CFU counts).

Need fairly strong immunosuppressive regimen:

- 2 applications of cyclophosphamide + cortisone acetate (Day -2 and Day 3), resulting in immunosuppression for approx 7 days
- Typical conditions (12 ml of 10^9 conidia/mL, 40 min aerosolization, 1 hour exposure) result in approx 3000 conidia in the lungs, with reasonable reproducibility

Residual conidia in the chamber between exposures are unlikely to affect results, as relatively heavy inoculations (3000 conidia per lung) are required for infection and subsequent death.

- Although these conidia can be cleared between experiments (eg, with glutaraldehyde), separate chambers could be used for different *Aspergillus* strains.

Variables tested

- Antibiotic use (subcutaneous ceftazidime vs oral tetracycline).
    - Local conditions/microflora may influence this choice
    - Tetracycline does not provide strong Gram –ve coverage, and its spectrum is not equivalent to ceftazidime: an ideal agent would give the spectrum of ceftazidime, but could be administered via drinking water
        - Would ciprofloxacin be an option?
- *Aspergillus* culture period prior to preparation of inoculum.
    - No apparent difference in virulence between *Aspergillus* cultures maintained for 10 or 24 days, so 10-day cultures are preferable
    - In this model, there was no apparent decline in spore viability as cultures aged, although declines have been reported for some other fungal strains
- Number of doses per exposure.
    - Nebulizing 3 doses of *Aspergillus* into the chamber resulted in higher lung CFU counts and earlier deaths than 2 doses, although overall mortality was not different
        - Early death is not desirable in this context, so 2 doses are probably preferable

- The "final" model (2 doses of conidia from 10-day cultures; mice given tetracycline PO) resulted in a workable latency period of approximately 8 days between exposure and death.

Assessing fungal burden

- Although CFU counts were seen to decline in the first 3 days after exposure, this probably does not reflect a reduction in fungal burden, but rather the relatively poor recovery of hyphae from tissues, compared with conidia.
- Fungal burden did not increase after Day 3, when assessed by cultures (CFU).
- Other techniques are being investigated for quantifying this:
    - Quantitative PCR (qPCR)
        - This was unsuccessful, due to insufficient extraction of DNA
        - In the clinical setting, samples may have been fixed prior to calling for an ID consult. This is likely to impact on DNA recovery, but the use of alcohol in place of formaldehyde should reduce the detrimental effect of fixation on recovery
    - Antigen quantification will be attempted using other markers: galactomannan, β-glucan

- Histopathology indicated that neutrophil recovery occurred at approximately the same time as death.
    - At Day 9, hyphal degradation was observed, and by Day 11 no hyphae were present
        - The effect of these observations on fungal burden assessments using antigenemia or qPCR is unknown

Issues to resolve

Ideal parameters for the model

Options to consider

- By reducing the degree of immunosuppression, could an infection be maintained without developing into overt disease?
- By extending the period of neutropenia, could the mortality rate be made to reach 100%?
- Would calcineurin inhibitors (mimicking transplant recipients) affect the model?
- In an ideal model, pathology would probably mimic that seen in human infections (vascular invasion, infarction, necrosis).
    - For diagnostic uses, this mimicry would be most important for the early phases of disease progression
    - Once a model is developed for this type of pathology, it may be possible to adapt it to encompass other disease types, if necessary (eg, nodular infections)

Critical issue: A model used in the development of diagnostic procedures need not be the same as one used for testing therapeutic interventions. Once a suitable model is prepared, and there is confidence in the data is produces, it should be used to select early candidates for diagnostic tests which could be further studies in patients.

Bacterial infections

- Oral administration of antibiotics may be suboptimal – intake of water by mice could reduce as their infection progresses.
    - Evidence from murine models of cryptococcal infections treated with 5-FC in drinking water suggest that this need not be the case, but this may need investigating for aspergillosis models
- Antibacterials can have a number of effects, in addition to those on bacteria, including host effects (eg, altered cytokine expression, disruption to normal microflora) and effects on fungi.
    - For this reason, a model not requiring antibiotics may be preferable to ensure that the model accurately reflects disease progression under "normal" conditions

        - However, such a model may not be clinically relevant, as most neutropenic patients will be receiving antibacterials
- A key determinant of infections seems to be the choice of supplier of the mice.
    - Specific pathogen free (SPF) animals are an option, but the availability and cost of these may make the model less easy to export to all centers
    - Local microflora effects also need to be considered, as this will vary between centers

Use of antifungal agents

- Even in the setting of early diagnosis, many patients will be receiving antifungal agents. In that regard, the standardized model will assess impact of antifungal therapy on diagnostics, but not be developed to test antifungal therapies per se.
    - Should consideration of this fact by included in the model?
        - Given that amphotericin B has immunomodulatory effects, would other agents (azoles?) be more appropriate for use in the model?

Choice of *Aspergillus* strain

- Although AF293 is the standard strain, it is not particularly virulent.
    - In the acrylic chamber model, animals start to die around the time of leukocyte recovery. This change in host status is likely to additionally complicate analyses
        - Could reconstitution of the immune system result in a cytokine storm, and contribute to mortality?
    - Would the use of a more virulent strain of *Aspergillus* result in 100% mortality rate before the end of the period of neutropenia?
        - Or, could the period of neutropenia be extended, as has been achieved in rabbit models?
- It is arguable whether 100% mortality is desirable in the context of diagnostics.
    - For evaluation of therapeutic interventions, 100% mortality rates would be ideal, to test survival benefits
    - However, for diagnostic models, prolonged, early-phase infections may be more appropriate

Standardization of inocula

- Culture conditions (temperature, culture conditions) and processing (choice of diluent, timing of plate/hemocytometer counts) must also be standardized.

Define the disease course under specified conditions

The full course of aspergillosis is relative poorly understood in humans and animal models. To help define the disease in specific models, experimentation is necessary:

- Undertake a series of experiments under carefully defined conditions (mouse strain, *Aspergillus* strain, culture conditions, inoculation method, etc).
- Conduct the experiments in a sufficiently large number of animals (large enough for statistical analysis) to allow a proportion to be killed regularly throughout the course of the infection for quantification of fungal burden (CFU counts/antigenemia/qPCR).
- Allow 4-6 months to develop this model (ie, methods for extending the period of neutropenia).
- Such a model could be used:
    - As a reference for other studies using similar conditions in different laboratories (effectively acting as a control group)
    - To improve the study of dissemination from the lungs
    - To study the impact of other *Aspergillus* strains or different immunosuppressive regimens

Quantification of fungal burden

- Limited data suggest that CFU counts and qPCR are correlated in some models (eg, preliminary studies with *Scedosporium* burden in kidneys). If this is true for aspergillosis, CFU counts may be a more practical option, given that the technology required is relatively non-specialized, affordable, and exportable.
    - qPCR may be additionally complicated if *Aspergillus* strains develop altered numbers of nuclei per hyphal segment
        - This is usually seen in mutant *Aspergillus* strains, but, given the morphological effects of some antifungal agents on *Aspergillus*, this may also occur in clinical samples from patients receiving antifungal therapy
- The correlation between CFU counts and tissue specimens (ie, liver, lung, kidney, brain) is unknown.
- Could steroids also affect CFU counts, given that fungi have steroid receptors?
- For accurate standardization, CFU per gram body weight is probably the most appropriate unit, rather than CFU per animal or CFU per organ.
- Processing of tissue samples also needs standardization – too much will result in low CFU counts; too little, and large colonies will result.

Choice of animal strain

- Given that there are no hypervirulent strains of *Aspergillus*, host factors are the key determinant of the course of disease.
    - For this reason, the choice of mouse strain is critical
    - The key requirement of the model is its reproducibility, so the use of outbred mice should probably be discouraged (although inbred strains are considerably more expensive)
- Variables to consider in the choice of mouse strain include:
    - Country
        - DBA2 and BALB/c strains in the UK appear to differ from those in the USA
    - Supplier
        - Over time, differences are likely to emerge between mice from individual suppliers, particularly for inbred mice strains
        - Would this necessitate recalibration of the model over time?
    - Size
        - Weight can have a profound impact on mortality rates
        - Weight matching between mice is therefore important to ensure reproducibility of results
    - Immunosuppression regimen (steroid use)
        - Dexamethasone appears less effective for establishing a pulmonary model, compared with triamcinolone or hydrocortisone
    - Housing conditions
        - Choice of bedding, handling, and the use of acidified water can all have an impact on the development of bacterial infections, and should be standardized

Critical issue: It is arguable that animal models can never be fully standardized so that data they produce are 100% reproducible. Given the large number of variables in the development of this model, a realistic goal would be to define the minimum criteria for measuring the utility of a new diagnostic which would give us the confidence to test it in the clinical setting.

Guinea Pig Models

Introduction

Many of the issues discussed in the context of the murine models are also applicable to larger animal models, including guinea pigs and rabbits.

Advantages over murine models

- Serial sampling possible.
- Potentially useful for PK/PD analyses for azoles (voriconazole).
- Evidence from tuberculosis models in guinea pigs suggest that inhalational exposure can result in dissemination and extrapulmonary disease, making them a good model of human infection.
    - Recent data using the Madison chamber indicate that good lung tissue *Aspergillus* burdens can be achieved in guinea pigs immunosuppressed with cyclophosphamide and triamcinolone
        - Although pulmonary infection was established in this model, no extrapulmonary infection was detected

Basic considerations

- Although guinea pig models are more expensive than murine models, they are less costly than rabbits.
- Standardization of the inoculum and infection are essential if a reference standard is to be established.
- Is it possible to establish an early model of exposure which is analogous to that resulting from inhalation of *Aspergillus* conidia in humans?

Rabbit Models

Introduction

Considerable work has already been conducted at the Immunocompromised Host Section of the National Cancer Institute to develop 14 models of mucosal, pulmonary, and disseminated fungal infections in immunocompromised rabbits.

Specific Models of Invasive Pulmonary Aspergillosis

Non-neutropenic model

- Immunosuppressed with cyclosporine A and methylprednisolone to simulate the post-engraftment immune-impaired conditions of bone marrow transplant recipients.
    - This model may be useful for producing long-term, “smoldering” infections (typically approx 30d)

Profound, persistent neutropenic model

- Profound, persistent neutropenia (immunosuppression induced by cytosine arabinoside) to simulate the neutropenic conditions of acute leukemia.
    - Histologically, infections in this model are indistinguishable from those in profoundly neutropenic patients, including in the development of halo signs and air-crescent signs on CT scans

These models have been particularly useful in characterizing the expression of *Aspergillus*-related antigens, metabolites, and nucleic acid markers, and for investigating the utility of serial diagnostic imaging techniques.

Markers

Six markers are in use at the NCI for characterizing the course of infection:

- Residual fungal burden (CFU/g).
- Mean pulmonary infarct score.
- Mean total lung weight.
- Survival.
- Pulmonary CT scan infarct score.
- Galactomannan antigenemia.

Issues relating to larger animal models

Serial sampling

- If early detection of infection is critical for an effective diagnostic, serial sampling of larger animals has the potential to provide data before an infection develops into full-blown disease.
    - In guinea pigs, daily bronchoscopy is possible, so serial analyses could be conducted in the first 48h of infection from single animals
    - In rabbits, daily bronchoscopy is a possibility, although this would probably be associated with an increased mortality rate
- Serial sampling would also probably result in the need for smaller numbers of animals per experiment.
    - One challenge with serial bronchoscopy would be differentiating between infection and full disease following inhalation or intranasal challenge

General discussion arising from animal models presentations

Galactomannan

- As the body temperature of rabbits is slightly higher than that of humans, it would be interesting to determine whether galactomannan release is influenced.
- In the clinical setting, there are 2 uses of diagnostics, including galactomannan
    1. Detect fungal infection (sensitivity, specificity, time to detection)
    2. Therapeutic monitoring
        - Galactomannan has limited prognostic value (ie, how many physicians would stop antifungal therapy on the basis of a drop in galactomannan value alone?)
- What information could a new diagnostic test produce that would differentiate it from the galactomannan assay?

Diagnostic marketplace

- It was agreed that, although the technology exists for better diagnostics, the major players in this field do not regard fungal infections as a priority market, so development has been limited.
- A reliable animal model of aspergillosis could be used to validate new diagnostics, and to compare these with existing standard approaches.
    - The market for a reliable diagnostic method validated in this way would be considerable, and this could be used to raise corporate interest

Basic research

- Basic research also has enormous potential in the development of new diagnostics.
    - Advances in the detection of other infectious organisms have been very impressive (eg, 1000x increase in the affinity of monoclonal antibodies to *Bacillus anthracis*)
- NIAID grants are available which could be used to support such research, including the R01 (Research Project Grant), R03 (Small Research Grants), and R21 (Exploratory/Developmental Grants) mechanisms.

Molecular Toolbox

Introduction

The goal of this section of the contract is to develop and standardize an extensive collection of tools, reagents, and strategies for manipulating *A fumigatus* at the molecular and genetic level.

Goals/milestones

- Obtain or create, and confirm at least two counterselectable mutants; evaluate virulence role
  - pyrG, niaD, sC
- Isolate and confirm one chromosomal element for a shuttle plasmid
  - ars, tel, cen
- Prepare plasmid constructs of both inducible and constitutive promoters, with appropriate MCS and terminator sequences
  - GpdAp, alcAp
- Develop and standardize at least one typing system
  - RAPD, fingerprinting, rDNA, AFLP; pilot test on clinical strains
- Prepare libraries as needed (ie, support of genome project)

Transformation

Several techniques exist, although electroporation is probably the preferred option.

- Low-tech methods, such as the use of lithium acetate, are attractive future options, as they would be relatively easy to export to other laboratories.
  - Although this technique has been used to transform yeasts, it has yet to be studied with *Aspergillus*

Markers

Markers of transformation include dominant and nutritional (auxotrophic) factors.

- Dominant markers are generally preferred, as they can be used for wild type strains, and they avoid some of the variables associated with auxotrophic makers.
  - Examples include *hph* (hygromycin) and *ble* (phleomycin)
- The role of auxotrophic mutations in affecting virulence needs to be investigated.

Vectors

Three main types exist containing chromosomal elements:

- *A fumigatus* ARS-like sequences.
- *A fumigatus* telomere-like sequences.
- *A fumigatus* centromere-like sequences.

Promoters

- Constitutive:
  - gpdAp (glyceraldehyde-3-phosphate dehydrogenase)
  - ACTp (actin)
  - TEF1p (translation elongation factor 1)
- Inducible:
  - alcAp (alcohol dehydrogenase) ethanol and threonine induced, glucose repressed
  - TetOp (tetracyclin/doxycyclin regulatable)

Reporters

- Substrate dependent:
    - LacZ (β-galactosidase)
    - GUS (β-glucuronidase)
- Substrate independent:
    - GFP (Green Fluorescent Protein)

There is the potential for reporters to be used in conjunction with non-invasive imaging techniques.

- Luciferase can be used in whole-body imaging, which could allow the course of infection to be followed closely, although:
    - There are considerable costs associated with acquiring the necessary equipment
    - Such studies would probably be limited to murine models, as infections in larger animals may be too deep to allow detection of the reporter
- GFP may be suitable for this type of study, although it is less sensitive, and has a longer half-life than luciferase.

Typing methods

The characteristics for a suitable typing method would include:

- Rapid turnaround: 24-48 hrs.
- Ease of completion: requiring only basic microbiology/molecular skills.
- Inexpensive: requiring only routine laboratory equipment.
- Accuracy and reproducibility: unambiguous results in different labs.
- The ability to be conducted "in house": no need to ship samples to dedicated laboratory for analysis.

Five major candidates:

RAPD (random amplification of polymorphic DNA)

- Advantages: no sequence knowledge, basic skills, simple.
- Disadvantages: highly variable, poor interlaboratory agreement.

RFLP (restriction fragment length polymorphism) + fingerprint probe

- Advantages: no sequence knowledge, basic lab skills, simplified analysis.
- Disadvantages: isotopic, requires characterized probe, laborious.

rDNA sequencing

- Advantages: reproducibility, sensitive, high throughput.
- Disadvantages: requires sequencing, expensive, molecular biology software.

MLST (multilocus sequence typing)

- Advantages: archived data, reproducible, sensitive
    - Used for *C albicans* and bacteria
- Disadvantages: requires sequence knowledge, expense

MSRT (microsatellite repeat typing)

- Advantages: simple, qualitative, inexpensive, potentially quantitative
    - Given these advantages, this technique could become a preferred option, once it has been shown to be appropriate for *Aspergillus*
- Disadvantages: potentially ambiguous interpretation

Issues

- Should molecular approaches be restricted to *A fumigatus* alone?
    - Would creative design of primers for highly conserved regions of DNA allow typing methods to be used for other species too?

Gene Expression in Models & Data Management

Work is progressing on sequencing the genomes of a number of *Aspergillus* species:

- *A fumigatus*, *A nidulans*, and *A oryzae* are due to be submitted for publication in the near future.
- *A flavus* and *A fischeri* (syn *A fischerianus*) are in progress.
- A proposal has been developed to sequence *A terreus.*
- *A sydowii* has been identified as a species which should be sequenced in the near future.

The University of Manchester has been funded to provide a public resource for the long-term annotation and analysis of genomic data from *Aspergillus* species. This resource is maintained as CADRE (Central *Aspergillus* Data Repository) at www.cadre.man.ac.uk.

Introduction

There are 3 main objectives of the team in Manchester:

- To conduct studies on in vivo gene expression (phospholipase genes initially).
- To assist in standardizing murine models, and participate in interlaboratory studies.
- To develop a common means of tracking strains, relevant data pertaining to each, and laboratory details, to be displayed on the internet.

In vivo gene expression

Initial studies of in vivo gene expression have focused on phospholipase genes; phospholipases destroy bilipid membranes and are key virulence determinants of *Clostridia.*

- In *A fumigatus*, differences are seen in whether these genes are expressed extracellularly: phospholipase C is secreted, phospholipase D is not secreted, and phospholipase B is secreted in some instances.
- Gene expression studies have focused on phospholipase B and C, and the following progress has been made:
    - Primers have been designed for putative/known phospholipases and lipases
    - PCR has been completed, and the products sequenced or primers redesigned. The design of a “miniarray” will be finalized soon)
    - Training has been completed in the use of LightCycler® for qPCR.
    - Planning of experiments using miniarrays, full arrays, and mouse arrays.

Standardizing murine models

Details of studies correlating growth rate of cultures of a number of strains of *Aspergillus* with their in vivo virulence will be published in the near future (Paisley D et al, *Med Mycol* 2004 in press).

Web-based data management

- Databases in development using MySQL.
    - High speed
    - Based on common programming language
    - Numerous clients can connect to database simultaneously
    - Database can be accessed interactively using several interfaces
- Four main databases planned, linked by strain/mutant number.

- Strain-specific information
- Typing information
- Data from models
- Other data (MICs, etc)
- Specifics (scope, content) for these databases are being developed; drop-down menus will be used wherever possible to avoid regional differences in nomenclature

- Requires some scripting work and will require some new code to integrate different aspects of the databases.

Key Messages: Critical Needs and Future Directions

Murine Models

- Recognition of number of factors impacting diagnostics
  - Mouse strain selection (inbred/outbred—differences in strains over time; outbred—increased variability but utility of survival/quantification
  - Aspergillus strain selection (inoculum preparation, storage/culture, virulence)
  - Immunosuppressive regimen/state of immunosuppression
- Quantification of tissue burden: impact of alternative measures of tissue burden measurement identified—including qPCR, glucan, chitin, histopathology
  - Recognition that mutants may have altered nuclear number impacting utility of methods
  - Correlation with cultures also good (in some settings) and may be useful as a screen

Larger Species

- Recognition of significance for serial assessments
  - Early detection/detection following exposure
  - Therapeutic monitoring correlating with residual fungal burden/pulmonary infarct score/metabolites/antigens/etc.

Molecular Toolbox

- Develop data in larger systems to attract newer technologies for use in humans
- Importance of tools to assist in animal model assessments
- Develop tools as need identified
- Role of typing system in strain identity
  - Role in database development

Gene Expression and Database

- Promise of post-genomic identification of new diagnostic targets
- Importance of database to identify key elements in strain characteristics and model development
- Significance in identifying industry partners early in process

Appendices

Participants:
Jack Bennett, NIAID, Bethesda, MD
Matthias Brock, Universität Hannover, Hannover, Germany
Arturo Casadevall, Albert Einstein College of Medicine, New York, NY
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Bruce Klein, University of Wisconsin, Madison, WI
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Kieren Marr, Fred Hutchinson Cancer Center, Seattle, WA
Greg May, MD Anderson Cancer Center, Houston, TX
Bill Nierman, TIGR, Bethesda
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John Perfect, Duke University, Durham, NC
David Perlin, Public Health Research Institute, Newark, NJ
Judith Rhodes, University of Cincinnati, Cincinnati, OH
Don Shepherd, Harbor-UCLA, Los Angeles, CA
Tom Patterson, UTHSCSA, San Antonio, TX
Tom Walsh, NCI, Bethesda, MD
Paul Verweij, University Medical Center, Nijemgen, Netherlands
Brian Wickes, UTHSCSA, San Antonio, TX

Attendees:
Yun C. Chang, NIAID, Bethesda, MD
Neil Clancy, University of Florida, Gainesville, FL
William Kirkpatrick, UTHSCSA, San Antonio, TX
Connie LaBeaux, UTHSCSA, San Antonio, TX
Laura Najvar, UTHSCSA, San Antonio, TX
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Peter Warn, University of Manchester, Manchester, UK
Aimee Zaas, Duke University, Durham, NC

Agenda

Friday, May 21, 2004
08:30 - 15:00

Welcome & Introductions

Welcome/Meeting Expectations	Dennis Dixon Rory Duncan Tom Patterson	08:30 – 08:45

Session 1
MODEL STANDARDIZATION

IAAM Organization	Tom Patterson	08:45 – 09:00
Murine Models	Scott Filler Dick Graybill	09:00 – 09:30
Panel Discussion		09:30 – 10:30
Break		10:30 – 10:45
Guinea Pigs Rabbits	Tom Patterson Tom Walsh	10:45 – 11:05
Panel Discussion		11:05 – 11:35

Session 2
TOOLS

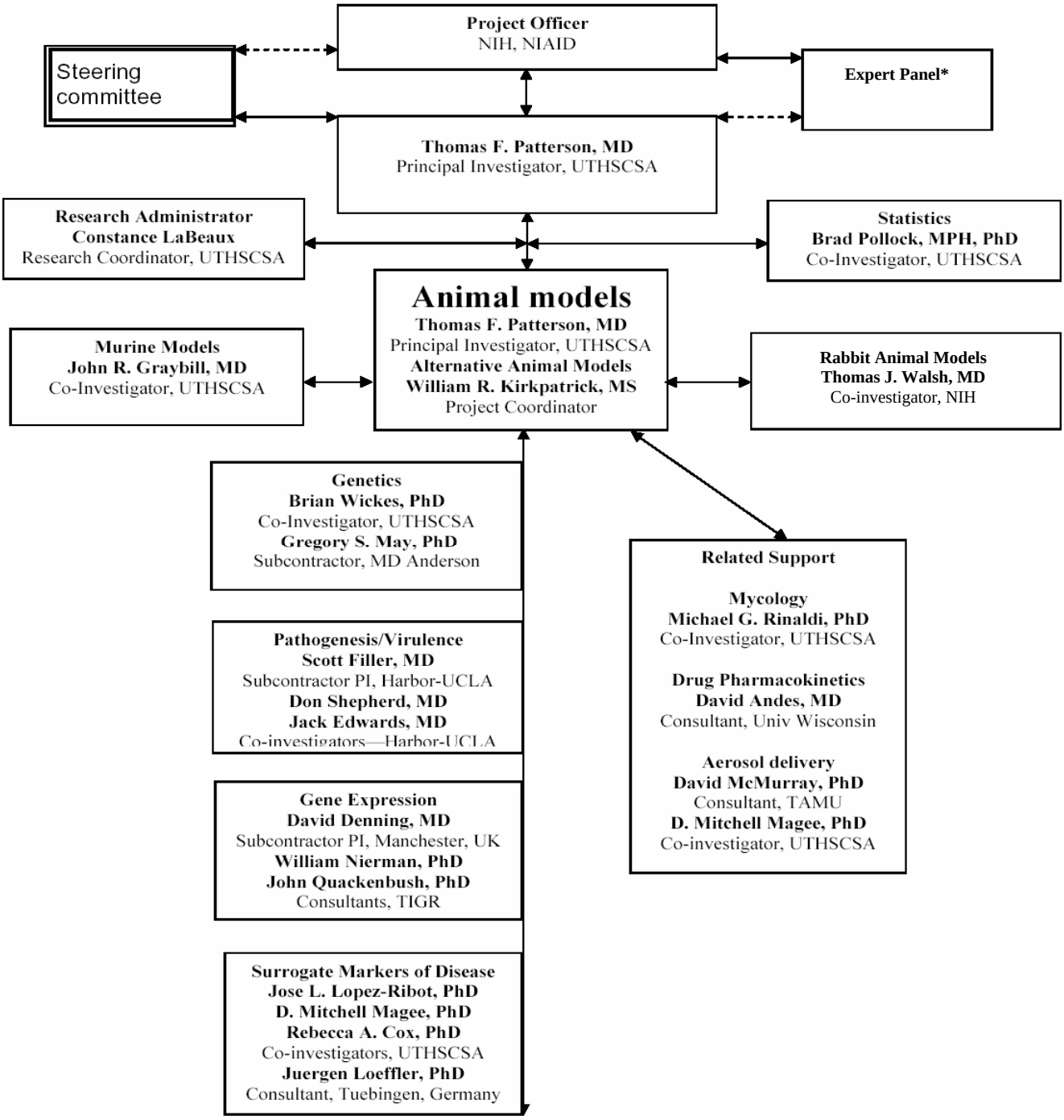
Molecular Toolbox	Brian Wickes	11:35 – 11:55
Panel Discussion		11:55 – 12:30
Lunch		12:30 – 13:15
Gene Expression in Models & Data Management	David Denning	13:15 – 13:45
Panel Discussion		13:45 – 14:15

Session 3
CRITICAL NEEDS

Open Discussion & Public Comments		14:15 – 14:45
Conclusions & Future Directions	Tom Patterson	14:45 – 15:00
Close		15:00

Organization chart

Figure 27. NIH-NIAID-DMID-03-09 part B, Organization chart



*** A. Casadevall (Chair)**
J. Rhodes
F. Odds