AD-A227 796

CONTRACT NO.: DAMD17-88-C-8124

TITLE: EARLY DIAGNOSIS AND TREATMENT OF OPPORTUNISTIC
MYCOBACTERIAL INFECTIONS IN HIV SEROPOSITIVE
AIDS PATIENTS

PRINCIPAL INVESTIGATOR: I.M. Orme

PI ADDRESS: Colorado State University
Fort Collins, Colorado 80523

REPORT DATE: August 31, 1990

TYPE OF REPORT: Final

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

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ELECTE
OCT.2 3, 1990
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90 10 2 14

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
Unclassified	

2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT
	Approved for public release;
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	distribution unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
Colorado State University		

6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
Fort Collins, CO 80523	

8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
		DAMD17-88-C-8124

8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
Fort Detrick Frederick, MD 21702-5012	PROGRAM ELEMENT NO. 63105A	PROJECT NO. 3M2-63105DH29	TASK NO. AC	WORK UNIT ACCESSION NO. 091

11. TITLE (Include Security Classification)
Rapid diagnosis and treatment of opportunistic mycobacterial infections in HIV-seropositive AIDS patients

12. PERSONAL AUTHOR(S)
I. M. Orme

13a. TYPE OF REPORT	13b. TIME COVERED FROM 6/15/88 TO 6/14/90	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final		1990 August 31	42

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Mycobacterium avium; AIDS. RA 1
06	03		Serodiagnosis.
06	13		Glycopeptidolipid antigens.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
The purpose of this project was to construct a sensitive and specific serodiagnostic assay designed to identify individuals previously exposed to the serovars of Mycobacterium avium, a major opportunitic pathogen occurring as a disseminated disease in patients with AIDS. The assay was based upon the use of highly purified glycopeptidolipid (GPL) antigens, which occur in the cell wall of M. avium in copious amounts, and which are serovar-specific immunodominant inducers of the humoral responses in the infected host.

The primary results of the project were as follows: whereas control sera had very low reactivity to GPL antigens, a significant proportion of both HIV-negative and HIV-positive homosexual men possessed substantially elevated anti-GPL antibody levels, leading us to hypothesize that the conventional wisdom that M. avium is a terminally acquired infection by AIDS patients is incorrect, and that lifestyle practices not common in the control group may account for the widespread exposure of both healthy and HIV-positive homosexuals to this bacterium. Secondly, in a study involving patients with multiple reactivity to M. avium

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86   Previous editions are obsolete.   SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract. (Continued) ...serovars, a number of serovars were recognized that do not usually give rise to disease, exemplifying the role of parasite pathogenic factors in the establishment of disseminated disease.

In further work, it was found that a correlation existed between the expression of the fully glycosylated polar form of the GPL antigens, and the morphotype and virulence of the isolate. In addition the ability to undergo morphotype reversion from domed to transparent was identified as a potentially critical factor in the ability of the organism to survive in vivo, whereas a central role for the GPL antigens in such mechanisms was suggested by the observation that GPL could interfere with the capacity of gamma interferon to mediate macrophage activation.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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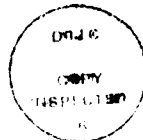

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INTRODUCTION.

Before the epidemic of acquired immunodeficiency syndrome (AIDS), Mycobacterium avium complex (MAC) infections were of clinical importance in the United States as a rare form of pneumonia in patients with underlying chronic lung disease (1). In fact, disseminated disease due to MAC had been reported in only 37 patients (2). MAC has, however, emerged as the most common cause of bacterial infection complicating AIDS in the United States (3,4). Over 99% of nontuberculous mycobacterial infections in patients with AIDS is due to M.avium complex (5); isolates are predominantly of serovars 1, 4, and 8 (6,7), and should be classified as M.avium on the basis of DNA homology and G+C content (5,8). Disseminated MAC infection has been detected antimortem in 18-27% of patients with AIDS (9,10), with higher prevalence (47-53%) in most series based upon autopsy findings (10-12). The frequency of this opportunistic infection varies little with the geographic region of the United States, but is less common in patients with Kaposi's sarcoma (4). The occurence of M.avium is, however, unaffected by age, sex, race, and sexual preference. Such data has led to the general contention that infection with M.avium follows recent exposure to the organism, for example, in water or food, and is more evenly distributed in this country than previously recognised. However, as will be argued below, this contention may not be correct.

The complaints of patients with disseminated M.avium infection are fever, anorexia, marked weight loss, and weakness; some patients also have lymphadenopathy, diarrhea and abdominal pain (3,9,10). Overall, approximately 25-40% of febrile AIDS patients with a consistent clinical syndrome hospitalised at New York's Sloan Kettering Center have had M.avium bacteremia, whilst the prevalence of disseminated M.avium infection increases to over 70% once infections such as Pneumocytis, cytomegalovirus and diarrheal pathogens have been excluded as the cause of fever and other symptoms (3). Bacteremia is continuous and high-grade with quantitation at 20-30,000 CFU/ml (13,14). Tissue loads are one million times higher, at about 10^9 CFU/g tissue (13).

As noted above, M.avium is believed to disseminate after colonization of the gastrointestinal tract. This is believed to occur as a result of exposure to the organism in food and water, although exposure as a result of homosexual intercourse has also been forwarded as a possibility (15). Regardless of transmission, it is clear that many patients have extensive

infection of the lower gastrointestinal tract, with concurrent isolation of M.avium in stools (15,16). In contrast, a respiratory portal seems less likely in view of the limited pulmonary pathology associated with disseminated M.avium (17,18). In about 75% of patients in whom the initial isolation of M.avium is from local sites, such as respiratory secretions and urine, disseminated disease will subsequently occur (9,10). Overall, 40-67% of patients with AIDS and disseminated M.avium infection will have the organism recovered from cultures of bone marrow, bronchial secretions, or sputum before bacteremia can be documented (19). Thus, the bone marrow smear and blood cultures are the most sensitive ways to establish the diagnosis (10,12,19).

The significance of M.avium infection in terms of morbidity and mortality is controversial. Patients with disseminated infections with M.avium have an extremely poor prognosis with a mean life expectancy of 3 to 7 months (10,20). In 96-97% of patients, infection with M.avium is in the second wave of opportunistic infections, occuring a mean of 9 months after the diagnosis of AIDS (16). Moreover, all patients with M.avium present at autopsy had at least one other concurrent infection (18). In view of this, M.avium has come to be regarded as a "terminal" type of opportunistic infection, acquired by the patient only when their immune defences are almost completely destroyed. This contention, however, ignores the basic biological fact that M.avium is a very slow growing organism, which surely most take a long period of time to reach the extraordinary tissue and blood borne CFU levels recorded in such patients. Indeed, it is because of such phenomena that the tendency to discount M.avium as a major pathogen must be tempered. Inanition, interference with macrophage function, and immunosuppression associated with disseminated M.avium may predispose to other infections (18,21). It is also of theoretical but great concern that M.avium may exacerbate HIV disease; a hypothesis that is strengthened by the observations in the current project that lead this investigator to believe that M.avium infection can sometimes occur at a much earlier stage than currently proposed, even prior to HIV infection.

When this project was originally formulated and proposed, in the winter of 1987, we adhered to the conventional wisdom that M.avium infection occurred at a stage in AIDS patients when immunocompetence was declining, but still potentially at a time when the presence of a humoral immune response to the organism could be detected. If a diagnostic test could be developed to specifically measure such a response, it was reasoned that this might be helpful as a guide to rapidly initiate

chemotherapeutic intervention, thus slowing or preventing the infection from reaching the huge numbers at which organ dysfunction must surely be inevitable. Thus, at that time, we indulged in the speculation that the patient would have very low, background levels of reactivity to M.avium whilst HIV-negative, and during the early Walter Reed stages of HIV-positivity. At some point herein, however, the patient would be exposed to M.avium and unable to resist colonization (presumably in the gut), giving rise to an active infection. The patient would then mount an immune response, which would remain elevated above that of normal controls until the declining immune response fell to T cell levels which would be unable to sustain the response. Thus, we reasoned, by examining the immune status of HIV-negative and HIV-positive homosexual men in terms of their reactivity to M.avium, we might be able to develop criteria allowing for the identification of those individuals at potentially increased risk of developing disseminated M.avium disease.

To detect a specific humoral immune response to M.avium in such individuals, we exploited the knowledge that serotypes of this species possess a completely unique, serotype-specific immunodominant complex carbohydrate-containing group of C-mycoside antigens, now called the glycopeptidolipids (GPL) following the full elicidation of their general structure.

All M.avium serovars contain a family of singly glycosylated apolar GPL structures:

Fatty acyl-NH-D-Phe-D-aThr-D-Ala-L-Alaninol-O-S

where S= 3,4 diMe-Rha or 3-Me-Rha, and where the threonine is linked by a sidechain to 3-Me-dTal or 6-dTal.

In addition, each serovar contains its own polar, multiglycosylated GPL (attached to the dTal) in which the oligosaccharide hapten is characteristic of the particular serovar and responsible for its singular antigenicity. At this time the full structures of the oligosaccharide haptens of 12 of the 28 known serovars have been identified, whilst the key features of the rest are known (Table 1).

Based upon our knowledge of this technology, we proposed that fully purifed GPL isolates of the major representative serovars of M.avium species would provide ideal specific antigens for which to test for serum

reactivity in patients. To achieve this, therefore, we painstakingly constructed and tested an ELISA based diagnostic system with which to attain this goal. A large number of control and patient groups were tested using this approach over the two year project period, and the results of these analyses are shown below.

In addition to this central objective, the project also attempted to address other related issues. As shown below, a small but important percentage of isolates of M.avium are of rough morphotype. As will be demonstrated here, these isolates are nonglycosylated, that is, they are devoid of the GPL antigens, as determined by gas chromatography. In view of this we attempted to determine if different isolates of rough morphology could be typed by their relative expression of certain protein antigens, as has been claimed by Abe's group using the protein marker Avi-3. As will be reported, this approach was largely unsuccessful.

In addition to the usefulness of the GPL antigens as major targets of the antibody response in infected individuals, these structures may be also important barriers to drugs, occuring as they do in copious amounts in the bacterium external cell wall. As reported in the Quarterly Report of 12/89, unfortunately too close to the end of the funding period, we made the fascinating observation that certain M.avium clinical isolates appear to undergo substantial colony morphotype reversion upon infection into mice or within murine cultured bone marrow macrophages. (A similar finding has just appeared using human cells [ref 22]). In addition, by applying chemical methods of analysis, we will show here that a direct correlation can be made between colony morphotype, nature of polar or apolar GPL content, and virulence of the organism. This data is in direct agreement with the clinical experience that smooth-transparent morphotypes, which are rich in the polar form of GPL, are the most troublesome to treat. Moreover, these antigens themselves may further contribute to pathogenicity, in that we have made some preliminary observations that suspensions of GPL may interfere with the gamma interferon-mediated activation of macrophages.

Finally, although the funding for this project was primarily directed towards diagnostic assays, whilst chemotherapeutics was regarded (in 1988) as a side issue, we used some spare animals to develop a new model of immune deficiency, the thymectomized CD4 T cell-deficient mouse (TxCD4- mouse). The results of these experiments are also briefly given, as a potential new model for the testing of experimental chemotherapies, should the evaluator of this report deem it of interest to the Army Medical Research Command.

MATERIALS AND METHODS.

1. Bacteria.

Serovars representative of each substrain of M.avium, M.intracellulare, or M.scrofulaceum (old classification; now grouped as M.avium complex) were obtained from the authenticated collection maintained at the National Jewish hospital, Denver. In addition, a small number of representative serovars were originally from the Trudeau Mycobacterial Culture Collection, Saranac Lake, New York. In addition 12 clinical isolates were kindly provided by A. Tsang, National Jewish Center, as well as the widely used strains 571-8 (from Dr P. Gangadharam, NJH) and strain 101 (from Dr. L. Young, Kuzell Institute, San Francisco). Strains used in morphotype cloning studies were strain 2-151 (Dr. Tsang) and strain "Crowle", from Dr. A. Crowle, Webb-Waring Institute, Denver.

2. GPL extraction procedures.

Bacteria (M. avium serotypes 1 to 28, 41, 42, and 43) were grown to confluence in 1 liter of Middlebrook 7H11 nutrient broth. They were autocalved for 30 minutes at 120° C, and then centrifuged at 2,000g for 10 minutes. The supernatant was discanted and the pellet freeze-dried. A mixture of chloroform : methanol (2:1) was added to dried samples (20 ml per gram dry cell weight) and incubated in a water bath (50°C) for 18 hours. The extracted material was then centrifuged for 10 minutes at 2,000g and dried under nitrogen. For every 10 mg of intact lipid,1.5 ml of 0.2 N NaOH and 1.5 ml chloroform : methanol (2:1) was added, vortexed well, and incubated at 40°C in a water bath for 30 minutes. Glacial acetic acid was then immediately added to neutralize the NaOH, and the residual material dried under nitrogen. The now deacylated lipid was then given a Folch wash by adding twice the amount of chloroform : methanol (2:1), 0.2 N NaOH as in the preceding step to each tube, and distilled H_2O at 1/6th the amount of the C/M added, followed by vortexing and centrifugation. The lower organic phase was then extracted from under the aqueous phase and once again dried under nitrogen.

Purity of the isolated GPL antigens was then assessed by reactivity with a panel of serotyping antibodies and by thin-layer chromatography, as well, when necessary, by HPLC and GC/MS methods.

3. Construction of the anti-GPL ELISA assay.

Deacylated GPL antigen was dissolved in absolute ethanol to a concentration of 50ug/ ml and sonicated for 2 minutes before applying to 96-well polystyrene microtiter plates (Dynatech). The plates were then left at room temperature to allow the ethanol to evaporate, and then blocked with PBS + 0.1 % Tween 80 (200 ul per well) at room temperature for 30 minutes. Patient's sera were then diluted 1:100 using PBS + 0.1 % Tween 80 as diluent and then added to each well in volumes of 100ul. Plates were then incubated at 37° C in humidified air for 1 hour. Following four-fold PBS washes, goat anti-human polyvalent immunoglobulin peroxidase conjugate was diluted 1:1000 in PBS + 0.1 % Tween 80 and applied to each well in 100ul volumes. Plates were again incubated at 37° C in humidified air for 30 minutes, and then washed four times with PBS. Chromogen was then added (25 ml of citrate phosphate buffer, 10 ul of 30% H_2O_2, and 10 mg of o-phenylenediamine dihydrochloride) to wells in 100ul volumes, and plates further incubated in the dark for 5-10 minutes. A 100 ul volume of 2.5 N H2SO4 was then added to each well to stop the reaction, and plates read at 490nm in an ELISA reader.

4. Protein analysis of GPL-negative isolates.

In these experiments, MAC isolates were grown to confluence and then autoclaved at 80° C for 1 hour. After harvesting of cells by centrifugation at 10,000g for 30 minutes, the culture supernatant was filtered through a 0.45 um filter and lyophilized. The remaining cell pellet was washed with several changes of deionized water, and suspended in 2 % SDS for 2 hours at 56° C with constant stirring. Following centrifugation at 2,000g for 30 minutes, the procedure was repeated twice more using fresh SDS. The extracted supernatant was then mixed with 8 volumes of acetone and incubated at -20° C overnight This material was then centrifuged at 10,000g for 30 minutes, the supernant decanted, and the protein pellet dissolved in a small amount of water.

For analysis of protein profiles, protein concentrations were determined by the hot Lowry protein assay method. Suitable amounts of protein samples were then run on SDS-PAGE, followed in addition by Western blotting procedures using defined monoclonal antibodies to M. avium proteins produced as described below.

5. Monoclonal antibodies to M.avium GPL serovars.

As positive controls for the ELISA assay systems, we prepared monoclonal antibodies to GPL serovars 1-14,16,19,20 and 25 in collaboration with B. Riviore and C. Bozic in Dr Patrick Brennan's laboratory which is adjacent to mine. All are reactive to the oligosaccharide sidechain of individual serovars; none react to the core aminoacyl or terminal glycosyl constituents of the core region.

6. Patient and control groups.

A large pool of sera were obtained from graduate students attending Veterinary School in Fort Collins, CO. All were healthy, and between 23 and 30 years of age. Domicile was from a large catchment area, including Hawaii, California, Arizona, and Wyoming. 55% of the group were female; we did not however see any evidence of sex influencing the data obtained from this group. 75 individuals were male; they were not questioned as to their sexual preference.

Serum samples were obtained on a regular basis throughout the project from a small number (n=39) of HIV-negative homosexual men attending an AIDS prevention program at the Denver Disease Control Service. These individuals were part of a longitudinal cohort study of homosexual and bisexual men designed to monitor AIDS prevention and risk reduction. These individuals received counciling each six months, as well as a physical examination.

For HIV-positive patients, we examined sera under storage at Fitzsimons Army hospital. These consisted of sera from both Army personnel and patients in various study groups in collaboration with the Denver Public Health Service. In addition to Walter Reed staging, these patients were tested for antibody to cytomegalovirus (as a separate evaluation of humoral immune status) using a commercial Fluorimetry kit, and for T cell subsets by cell fluorocytometry.

7. Characterization of morphotypes.

Two clinical isolates, 2-151 and Crowle, were grown on Middlebrook 7H11 nutrient agar and examined carefully for multiple morphotypes. Each colony type was identified on these primary plates and then picked and subcultured two or three times to ensure lack of reversion. At this point

we were confident that stable forms of the rough morphotype (R) , and smooth-domed (SmD) and smooth-transparent (SmT) types for each of these strains could now be investigated individually.

The amount of GPL expression on each of these colony forms was determined as follows. Following the standard extraction procedure detailed above, the extracted lipids were hydrolysed in 2M trifluoroacetic acid to liberate monomeric sugars. These sugars were then reduced and acetylated to form alditol acetates that could be detected by gas chromatography. The ratio of apolar to polar GPL was quantitated using the relative amounts of 3-methyl-6-dTal and 6-dTal.

8. Behaviour of morphotypes in vitro.

To test the infectivity of morphotypes in vitro, C57Bl/6 mice were euthanized and bone marrow washouts performed using a 19-gauge needle. The aspirate was collected and washed, and the cell suspension incubated at 5×10^6/ml in 1ml volumes in 24-well culture plates. The medium used was RPMI 1640 supplement with 10% fetal calf serum and 10% L929 fibroblast cell line conditioned medium containing various colony stimulating growth factors (GM-CSF, multi-CSF, etc). After about 10-14 days of culture contaminating fibroblasts die off leaving a monolayer of about 10^5 bone marrow-derived macrophages.

Each well was then infected with approximately 10^6 CFU of a M.avium morphotype, then washed thoroughly 90min later to remove extracellular bacteria. Growth of the bacterial inoculum was then followed against time by lysing wells using Triton-X detergent (0.01%) following by plating on 7H11 agar and counting colony formation after 21 days incubation in humidified air.

9. Inhibition of macrophage activation by GPL.

In preliminary experiments performed in the Summer of 1990, we have identified a potential mechanism of M.avium pathogenesis, based upon the ability of serovar 4-purified GPL to inhibit gamma interferon-mediated activation of macrophages.

Peritoneal macrophages were elicited in C57Bl/6 mice using 2ml of 4% casein, or 30ug of E.coli lipopolysaccharide. Four days later peritoneal cells were collected using ice-cold medium, washed once and plated in

24-well culture plates at a cell density of 106/well. After 2h incubation at 37°C, nonadherent cells were washed out with warm medium.

Cells were incubated in medium containing 20ug/ml serovar 4 GPL or in medium alone for 4h. Cells were then washed and further incubated for 48h in medium containing 100U of recombinant gamma interferon. Supernatants were then removed for tumor necrosis factor (TNF) determinations, whilst the cells were triggered for superoxide production using medium containing 0.5ug/ml phorbol myristate acetate. Superoxide anion release was measured by its capacity to cause reduction of a 0.08mM ferricytochrome c solution over a 45min period.

Tumor necrosis factor production was measured as the ability of supernatants to cause the lysis of L929 fibroblast cells following incubation for 18h. Recombinant TNF was added to control wells at a range of doses as a positive control. Cell lysis was measured by adding 50ul of a 5mg/ml stock solution of 3-[4,5-dimethylthiazol-2-yl]-2,5--diphenyltetrazolium bromide dye to each well and incubated for a further 5h. The color reaction was then developed using acid/alcohol and read at 550nm.

10. A thymectomized, CD4 T cell-deficient mouse as a potential model for the evaluation of anti-M.avium chemotherapy.

We have recently been able to evaluate a new model of immune deficiency in the mouse, which could be used to test new chemotherapeutic regimen for the treatment of M.avium infection. This model is included here should the evaluator of this project report deem it of interest to the future research directions of the Army Medical Research Command.

Four week old C57Bl/6 mice were anesthetized with ketamine and rhompin and thymectomized by light suction through a small incision just above the sternum. One week later they were infused with 200ug of monoclonal anti-CD4 antibody (hybridoma line GK1.5). This antibody was purified from confluent tissue culture medium containing the hybridoma line by passing the supernatant through a Gamma-G column, and then eluting off the bound antibody. A few days after treatment of mice there is a profound drop in circulating CD4+ T cells, as determined by flow cytometry using a Coulter Epics II.

RESULTS.

A. Summary of Midterm report.

The Midterm report dealt with the incidence of anti-GPL antibody levels in the sera of various test and control groups. Although we have had the opportunity to examine further sera since that time, the results remain equivalent. These can be summarized as follows:

1. In the control group of sera, absorbance values fell in 90% of cases within the 0-0.3 range for reactivity to serovars of M.avium most commonly associated with AIDS patients (when corrected for background negative controls for the assay procedure itself). Reactivity to less "pathogenic" serovars was in general much lower, whilst a similar scatter to the first group was seen for ELISA assays run against predominantly M.scrofulaceum-type serovars. Making the assumption that the gathered data fell into a normal distribution, we calculated 95% confidence limits for the data, and operationally defined anv values falling above these limits as raised or high. By this criterion. ,pproximately 3% of these sera tested possessed raised levels (see MIDTERM report for further details).

2. In the group of HIV-negative homosexual men tested, 33% possessed raised anti-GPL antibody levels to the "pathogenic" M.avium serovars.

3. In 150 serum samples now tested from HIV-positive men, 44% possessed raised anti-GPL antibody levels. In addition, there was no apparent correlation between T cell numbers and anti-GPL absorbance values, thus indicating no direct association between exposure and colonization with M.avium and the immune status of the individual. (See Appendix A).

B. New data.

1. Analysis of sera exhibiting reactivity to multiple serovars of M.avium.

In this study, we selected 19 HIV-negative individuals whose sera exhibited reactivity to multiple serovars of M.avium. Sera from HIV-positive men were not tested since we could not distinguish which serovar reactivities were established prior to the onset of immune deficiency.

The basic question asked was whether multiple reactivity profiles still mirrored the prevalence of the major serovars seen in AIDS patients (1,4,8,etc), or whether other serovars, not predominantly associated with disseminated disease might also be represented.

It was found (see Appendix B for details) that serotypes 1,6,8,9,10, and 12 were the targets of the anti-GPL antibody response in this group of patients. The range of multiple reactivities was from 2 to 7, with a mean of 3.0.

Whilst most of the serum samples tested reacted to serovar 8, there was also reactivity to serovars 10 and 12. This is of interest because clinical isolates with these serovars are very uncommon. Equally of note was the observation that reactivity to serovar 4, one of the most commonly isolated clinical serovars, was not detected in this group of patients.

2. Failure to type rough variants using protein profiles.

In this study we attempted to address a fundamental issue; namely, is there a simple diagnostic procedure that can be constructed that could *specifically* detect immunological reactivity to the 25-30% of clinical M.avium isolates that cannot be typed by seragglutination as a result of their rough (i.e. spontaneously agglutinating) morphotype (Table 2).

As an initial approach, we questioned whether culture filtrate proteins from different isolates of known serovars exhibited certain patterns against which the protein profile of a rough isolate could be compared. This attempt was spured by the knowledge that monoclonal antibodies against specific proteins have been widely used in bacxterial taxonomy, and, closer to home, by the observation of Abe et al (23) that the monoclonal antibody Avi-3 reacts specifically with M.avium culture filtrate protein (actually, probably one the fibronectin-binding proteins) but not M.intracellulare (i.e. any serovar above 10).

To address this, therefore, we examined the profiles of each the representative serovars (Figs 1-6). As can be seen, they were all very similar, so this line of investigation was abandoned at this point as probably unproductive.

3. Relationship between morphology and GPL expression.

Although the above episode was unproductive, our recent work with the phenomenon of morphotype reversion may suggest that M.avium isolates may actually assume a rough morphology under resting conditions, but revert to the fully glycosylated form as a survival mechanism in vivo (and thus conveniently providing us with the anti-GPL response as a diagnostic tool).

As evidence of this, the growth of three morphotypes of strain 2-151 in C57BL/6 mice is shown in Fig 7. It can be seen, firstly, that SmD variants fail to survive in the infected host, whereas the fully glycosylated SmT form grows progressively. There is also evidence of progressive bacterial growth in mice inoculated with the rough variant, but here we made the startling finding that 95% of colonies *recovered* from the host were of the SmT morphology.

In view of the wide dichotomy in terms of survival of the SmD and SmT forms, we then set out to formally show that each of the morphotypes *differed directly in the total* expression of GPL antigens. The results of these chemical analyses are shown in Fig 8. Moreover, analysis of polar to apolar ratios for each of the strains further confirmed these differences (Fig 9).

4. Behaviour of morphotypes in bone marrow macrophages.

To examine the behaviour of morphotypes in vitro, cultured bone marrow macrophages were infected with the three cloned strains of 2-151 and strain Crowle. The growth of each organism is shown in Fig 10. It was found that both the rough and smooth-transparent forms of 2-151 were able to persist in the macrophage cultures, whilst the smooth-domed variant was progressively eliminated.

In the case of strain Crowle, a slightly different picture was observed, in that the smooth-transparent form gave rise to a persistent infection, whilst both the rough and smooth-domed forms appeared to be very slowly cleared.

This type of in vitro model thus seems to partially reflect in vivo behaviour of these isolates, with the exception being that progressive

growth of the organism was not seen over the (seven day) culture period. With longer cultures better growth might be seen, although it proves difficult to maintain the cell culture itself over much longer periods. Nevertheless, this type of model may prove useful in the testing of chemotherapeutic agents under in vitro conditions.

5. Inhibition of macrophage activation by GPL.

In these experiments, we determined if GPL antigens themselves might contribute to the pathogenicity of GPL-rich smooth-transparent forms of M.avium. It was found (Figs 11-15) that peritoneal exudate macrophages produced high quantities of toxic oxygen radicals when triggered by phorbol myristate actetate, whilst the addition of a range of doses of GPL to the culture medium did not affect this activity. However, in contrast, preincubation of the macrophages with GPL reduced the radical production, with maximal inhibition occuring at 10ug/ml.

Moreover, using both peritoneal exudates and cultured bone marrow macrophages as sources of cells, preincubation with 100U of gamma interferon induced substantially enhanced production of oxygen radicals. Here again, preincubation with GPL inhibited this enhanced activity, and, in addition also substantially reduced the capacity of such cells to secrete tumor necrosis factor. Similar activity was observed using the macrophage cell line P388D1.

6. The TxCD4-deficient mouse model.

Although this project was mainly concerned with diagnosis of HIV-positive individuals would might be at risk of developing active M.avium disease, the treatment of this infection can never be far from our minds, given the intractibility of these organisms to conventional chemotherapies (3,16).

To date, the only model available for testing drugs against M.avium has been the "beige" mouse, a mutant strain derived from the C57BL/6 mouse (24) which is more susceptible to infection than its parent strain. This model has been heavily touted by some (25,26) as a "model of AIDS" despite the fact that the primary lesion is defective phagocytosis by neutrophils whilst T cell and monocyte activity is normal. In view of this, in our opinion, this model is inappropriate.

To attempt to design a better animal model of disseminated infection, we reasoned that removal of CD4 T cells would be an obvious goal if we were to simulate the effects of HIV-positivity. To achieve this therefore, mice were infused intravenously with anti-CD4 monoclonal antibody, and T cell depletion monitored by flow cytometry (Fig 16). Moreover, to prevent new generation of CD4 T cells, each animal was thymectomized prior to antibody treatment.

As shown in Fig 17, there was little difference between the growth of a moderately virulent serovar 4 strain (5-417) in the spleens of thymectomized CD4 deficient (TxCD4-) mice, beige mice, or normal sham-thymectomized controls. However, the infection gave rise to an uncharacteristic persistent infection in the livers of the TxCD4- mice, and also gave rise to an approximately ten-fold greater dissemination into the bone marrow. Only in the lungs did the beige mice show evidence of greater susceptibility as compared to the other test groups.

This experiment illustrates that much can still be done to improve animal models of M.avium infection. Moreover, given the recent advances in the field of immunosuppressive murine retroviruses, there is promise that highly specific murine simulations of the events in AIDS patients may be mimicked.

DISCUSSION.

The primary goal of this project, namely to construct a highly specific ELISA diagnostic method that could be used to detect evidence of exposure and colonization to serovars of the Mycobacterium avium complex, was quickly achieved. We then set about the task of collecting serum samples from various groups of interest, and analysing these samples for the presence of specific antibody to the immunodominant GPL antigens of M.avium.

As noted above, at the time this project began, we were swayed by the conventional wisdom that M.avium was an infection only contracted at a time when the immune response had been badly damaged by the presence of the HIV virus. Thus, we anticipated that control groups would have only background reactivity to the organism whilst a small percentage of HIV-positive individuals would have evidence of exposure to M.avium, and hence could be considered at increased risk of developing active, disseminated disease as their immune status worsened.

The findings of the study, however, bear no resemblance to the above hypothesis. Whilst the reactivity of a large group of control sera were indeed close to zero absorbance, a significant percentage (33%) of HIV-negative actively homosexual men had substantially raised anti-GPL antibody levels. In addition, of 152 HIV-positive sera tested, 67 (44%) again had substantially raised levels.

How can these data be explained ? Although not a particularly popular hypothesis amongst the clinical fraternity, Damsker and Bottone (15) have suggested that the high prevalence of M.avium as a gastrointestinal infection in AIDS patients may become established as a result of transmission through the act of homosexual intercourse. Against this argument is the observation that non-homosexual HIV-positive individuals also contract M.avium infections.

The contradictions above can be explained if one allows that the etiology of disease may be different in these two situations. In non-homosexuals, M.avium may well be a terminal type of illness, arising as a result of prolonged immune depression. In homosexuals, however, there may be some transmission occuring prior to HIV infection as a result of sexual practices, which then reemerge (in much the same way as tuberculosis lesions recrudesce in senescent individuals) when the immune system is damaged.

The data obtained in this project are consistent with this hypothesis. If the reactivity of HIV-negative homosexual men to GPL antigens were merely a result of environmental exposure to M.avium, then surely a similar level of reactivity would be expected to occur in the graduate student control group. Since it did not, and since such reactivity in the healthy homosexual group cannot be ascribed to immune depression, we must conclude that the prevalence of anti-GPL reactivity in these individuals came about as a result of lifestyle practices not common amongst the non-homosexual control group.

It should also be noted that most of the HIV-positive individuals tested were asymptomatic at the time, and yet nearly half of these patients had elevated anti-GPL antibody levels. This we regard as further evidence that a significant number of the homosexual community may have been exposed to a local infection with M.avium, sufficient to generate a detectable humoral response. Furthermore, in patients in which we were able to assay helper T cell numbers, it was clear that the percentages of GPL-reactive

patients had no correlation to T cell levels. In other words, there was no evidence of increased numbers of patients with anti-GPL reactivity as T cell numbers fell.

A further aspect that was studied within this project concerned those individuals that exhibited multiple reactivity to several serovars of M.avium GPL antigens. It was clear, from the results of this study, that in patients with evidence of multiple exposures, certain serotypes were represented that are not usually associated with active disease.

This, then, sheds some light on the pathogenesis of the disease. It indicates that many M.avium serovars have the capacity to give rise to colonization of the host, but that only certain serovars (types 1, 4, and 8 being the most common) possess (? virulence) factors that allow them to give rise to active disease. As an initial speculation, this may reflect the capacity of certain serovars to persist within infected macrophages, whilst others are destroyed (but still processed and GPL antigens presented to the immune system).

Moreover, in addition to the serovar and the potential pathogenic factors it may or may not possess, there is also the question of the particular morphology of the organism. Towards the end of this project we began to realize how important this might be, and some experiments performed this Summer have confirmed these realizations.

As a spin-off from our unsuccessful attempts to construct a strategy to deal with the problem of non-typable rough variants of M.avium, we were able to confirm by formal chemical methods that rough morphotypes are non-glycosylated. In addition, however, an extension of this finding was that by carefully cloning the three major morphotypes from first one, and then a second single clinical isolate, we were able to show that the (typable) smooth-domed and smooth-transparent forms also differed in their glycosylation, in that SmD variants had only an apolar GPL structure, whilst SmT forms were fully polar.

We were then able to show, further, that degree of glycosylation correlated directly with the ability of smooth morphotypes to persist in the animal, or indeed in vitro. Thus, whilst SmT forms gave rise to progressive, eventually fatal disease in immunocompetent C57BL/6 mice, the SmD form was always progressively cleared from host tissues. A similar situation could be simulated using cultured bone marrow macrophages.

A possible molecular mechanism to explain the persistence of GPL-rich smooth variants was then provided by in vitro experiments in which it was shown that GPL antigens could interfere with macrophage activation processes. In this study, it was found that highly purified (serovar 4) GPL antigen did not in itself induce superoxide or TNF secretion by macrophages, but instead substantially reduced the subsequent capacity of gamma interferon to activate these cells to release such mediators.

These observations provide an explanation for the apparent paradox in which certain virulent strains of M.avium grow progressively in the host despite evidence for substantial macrophage activation within the granulomas of the infected organ. Within fixed sections of granulomas forming at the sites of bacterial implantation, it is clearly apparent that only a minority of mononuclear cells are actively infected with M.avium, whilst the majority of cells form a surrounding mantle.

The findings of the present study now allow us to hypothesize that whilst most cells in the granuloma will be activated by the various T cell mediators, the macrophages actually containing the bacteria may be inhibited by the presence of the M.avium GPLs. Thus, whilst a classical assay such as a Listeria challenge infection may provide evidence of widespread systemic macrophage activation (27), cells actually containing the M.avium organism may be prevented from expressing acquired resistance.

A further paradox to all these observations, however, is the knowledge that rough variants of certain strains can also give rise to active disease, even though we now know these bacteria to be devoid of GPL antigens. We must hypothesize, therefore, that rough morphotypes possess different mechanisms that allow them to give rise to persistent or progressive disease. In a sense, these findings convey the fact that virtually nothing substantial is known about the molecular and cellular biology of the M.avium organism.

In this regard, if the connection between GPL expression and virulence of smooth variants is real and not circumstantial, then identification of the biosynthetic pathways involved may offer potential new, specific, molecular targets for chemotherapeutic intervention. Although these pathways are not known, it is clear that glycosyl transferases and methyltransferases must be involved. We are beginning, under another program, to probe for the genes that may encode for these enzymes.

Finally, regarding the problem of effective chemotherapy, it is clear from the clinical experience to date that the limited number of drugs with activity against M.avium are only marginally effective. Whilst we provide here a new animal model which may be of use in screening new potential agents, it is also apparent from our data that prophylactic chemotherapy of both HIV-negative and HIV-positive asymptomatic homosexual men with raised anti-GPL levels may also be of benefit.

CONCLUSIONS.

1. The observation that significant numbers of both HIV-negative and HIV-positive homosexual men have raised anti-GPL antibody levels allows us to refute the existing hypothesis that M.avium is only contracted as a terminal disease in AIDS patients.

2. The above observations allow us, in addition, to hypothesize that the high incidence of apparent exposure to M.avium in HIV-negative men may arise as a result of lifestyle practices not shared by a control population.

3. In individuals with exposure to multiple serovars, pathogenic mechanisms associated with the infecting organism determine whether it can persist in the host, and give rise to disseminated disease following destruction of the immune system.

4. In the case of smooth variants, the degree of glycosylation correlates directly with the ability of the organism to resist host defence mechanisms.

5. The presence of the fully glycosylated GPL antigen may contribute to the pathogenesis of the organism, by interfering with macrophage activation mechanisms.

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PERSONNEL

The following personnel were funded by this project:

Ian M. Orme, P.I. 20% effort.
Bai-Yu Lee, Graduate student, 100% effort.
Cindy Skaggs, Laboratory assistant, 100% effort from 9/88 to 6/89.
Cynthia Bozic, Research Associate, 50% effort from 6/88 to 9/88.

Ms. Lee is a Ph.D candidate in this Department who devoted full time to this project. She is now working on more fundamental aspects of M.avium biochemistry and molecular biology, and should complete her studies in 1991.

Table 1. Structures of Ose Haptens from the GPL Antigens of Serovars of the M. avium Complex

Serovar No.	Comment	Structure
1	Tentative	α-L-Rhap(1→2)6-d-L-Tal
2	Firm	2,3-Me$_2$-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
3	Firm	Same as 9 except for absence of terminal 4-Ac group.
4	Firm	4-Me-α-L-Rnap(1→4)2-Me-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
5	Glycosyl comp. only	Glc, Gal and 3MeRha (instead of Rha), 6dTal
6	Tentative	2-Me-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
7	Glycosyl comp. only	Novel amino sugar, 2 x Rha, 6dTal
8	Firm	3-Me-4,6-Pyr-β-D-Glcp(1→3)-α-L-Rhap(1→2)6d-L-Tal
9	Firm	4-Ac-2,3-Me$_2$-α-L-Fucp(1→4)-β-D-GlcpA(1→4)-2,3-Me$_2$-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
10	Glycosyl comp. only	Glc,2,3-Me$_2$-Gal, 3-Me-Rha (instead of Rha), 6d-L-Tal
11	Glycosyl comp. only	Indistinguishable from serovar 10
12	Firm	3-Me-β-Quip4NLa(1→3)-4-Me-α-L-Rhap-(1→3)-α-L-Rhap(1→3)-α-L-Rhap(1→2)6d-L-Tal
13	Glycosyl comp. only	Novel sugar, 4-Me-Rha, 2 x Rha, 6dTal
14	Firm	Kanp4NFo-(1→3)-2-Me-α-D-Rhap(1→3)-2-Me-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
15	Glycosyl comp. only	3-Me-Quip4NLa, 2-Me-Fuc, 4-Me-Rha, Rha, 6dTal
16	Glycosyl comp. only	Novel amino sugar, 4-Me-Rha, Rha and 6dTal
17	Firm	β-Quip3NHHB-(1→3)-4-Me-α-L-Rhap(1→3)-α-L-Rhap(1→3)-α-L-Rhap(1→2)6d-L-Tal
18 (st. 4990)	Glycosyl comp. only	May be same as serovar 6
18 (st. Melnick)	Glycosyl comp. only	6-Me-Glc, 2,3-Me$_2$-Fuc, 4-Me-Rha, Rha, 6dTal
19	Firm	3,4-Me$_2$-β-D-GlcpA(1→3) 4-Me$_2$-α-Funo(1→3)-α-L-Rhap(1→3)-α-L-Rhap(1→2)6d-L-Tal
20	Firm	2-Me-α-D-Rhap(1→3)2-Me-α-L-Fucp(1→3)-α-L-Rhap-(1→2)6d-L-Tal
21	Firm	4,6-Pry-β-D-Glcp(1→3)-α-L-Rhap-(1→2)6d-L-Tal
22	Glycosyl comp. only	Ara, 2 x Rha, and 6dTal
23	Glycosyl comp. only	2 x Rha, 6dTal
24	Glycosyl comp. only	Indistinguishable from serovar 23
25	Firm	2-Me-α-Fucp4NAc-(1→4)-β-D-GlcpA-(1→4)-2-Me-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
26	Firm	2,4-Me$_2$-α-L-Fucp(1→4)-β-D-GlcpA-(1→4)-2-Me-α-L-Fucp(1→3)-α-L-Rhap(1→2)6d-L-Tal
27	Glycosyl comp. only	Novel Me$_2$-hexose, Glc, 2 x Rha units, 2-Me-Fuc, 6dTal
28 (st. 6845)	Glycosyl comp. only	Same as serovar 12
28 (st. 9055)	Glycosyl comp. only	Novel amino sugar, 4-Me-Rha, 2 x Rha and 6dTal

All of these oligosaccharides are attached to the alloThr residue in the peptide moiety of the fatty acyl peptide core.

Standard sugar abbreviations are used except for Qui4N, 4-amino-4-deoxyquinovose (4-amino-4,6-dideoxyglucose); Kan4N, kansosamine (4-amino-4-deoxy-3-C-methyl-2-O-methylrhamnose); Eva, evalose (3-C-methylrhamnose); Ac, acetyl; MHB, 2'-methyl-3'-hydroxybutanoyl; A, lactyl; Me, O-methyl; Pyr, pyruvyl [4,6-O-(1'-carboxyethylidene)], 6dTal, 6-deoxy-talose.

CSU December '87 to June '88

Typed by seragglutination, TLC, or ELISA

n = 297

Serotype	#	%	Serotype	#	%
1	14	4.7	14	7	2.4
2	6	2.0	15	2	0.7
4	43	14.5	16	10	3.4
6	17	5.7	17	2	0.7
7	2	0.7	18	3	1.0
8	45	15.2	19	2	0.7
9	1	0.3	21	1	0.3
10	10	3.4	25	2	0.7
11	5	1.7	42	1	0.3
12	1	0.3	43	1	0.3

4/8 mix--23   (7.7%)
6/8 mix--6   (2.0%)

GPL-neg--39   (13.1%)
GPL-weak--54   (18.2%)

--not typable by ELISA.....
--93 (31.3%)

Distribution of M. avium Serotypes.

Table.2. Demonstration that, in a six month period, our typing laboratory were unable to identify the serovars of 31% isolates as a result of weak or absent GPL antigens.
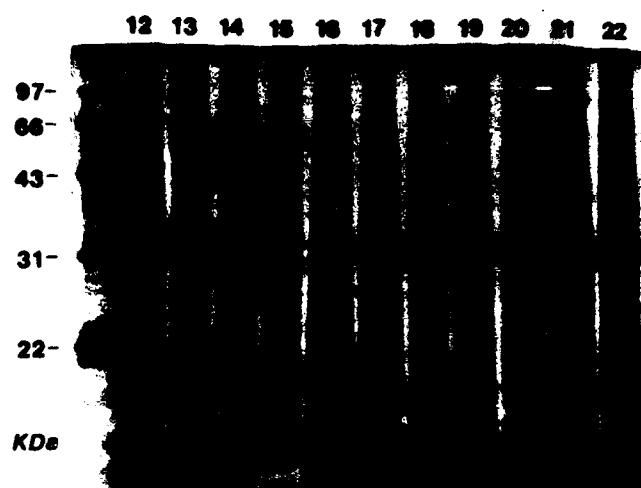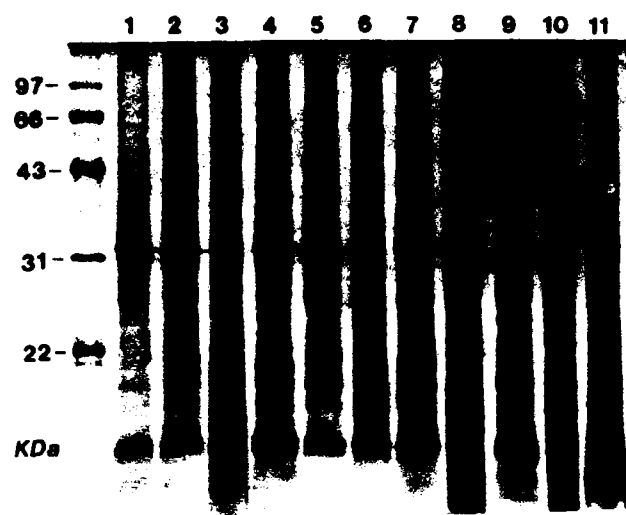
Figs 1&2. <u>M.avium</u> serovar protein profiles.
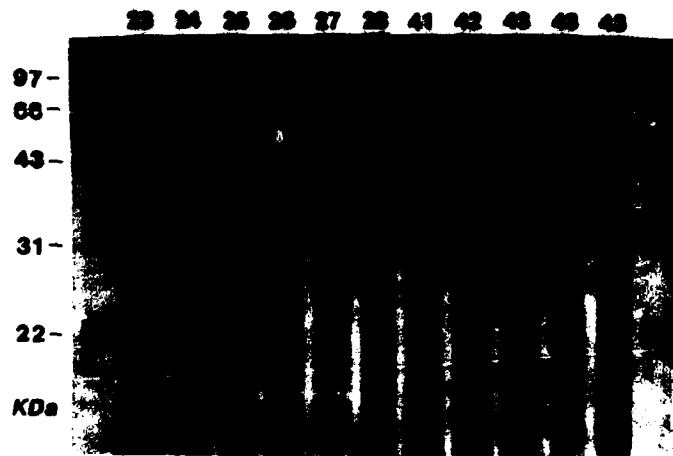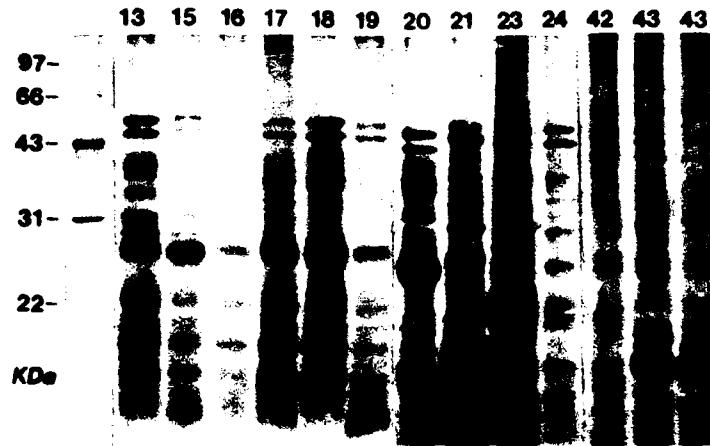
Figs 3&4. M.avium serovar protein profiles.
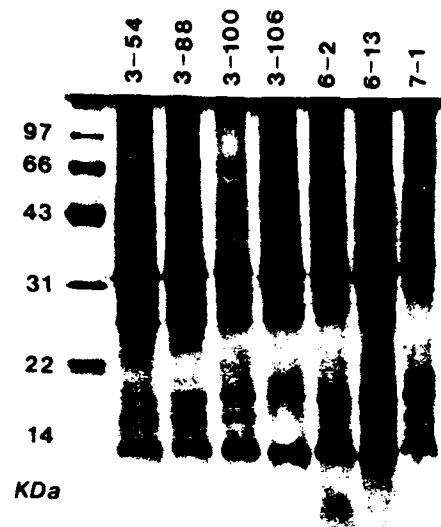
Figs 5&6. M.avium serovar protein profiles.
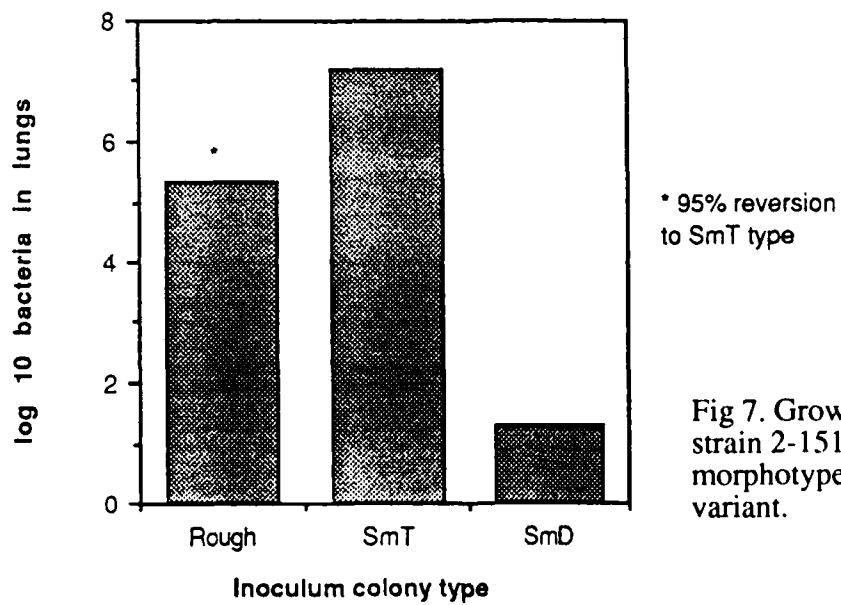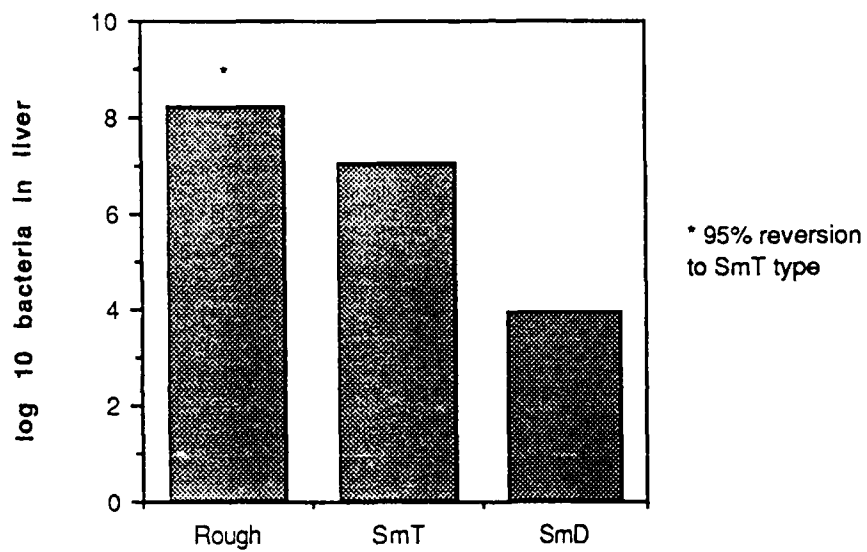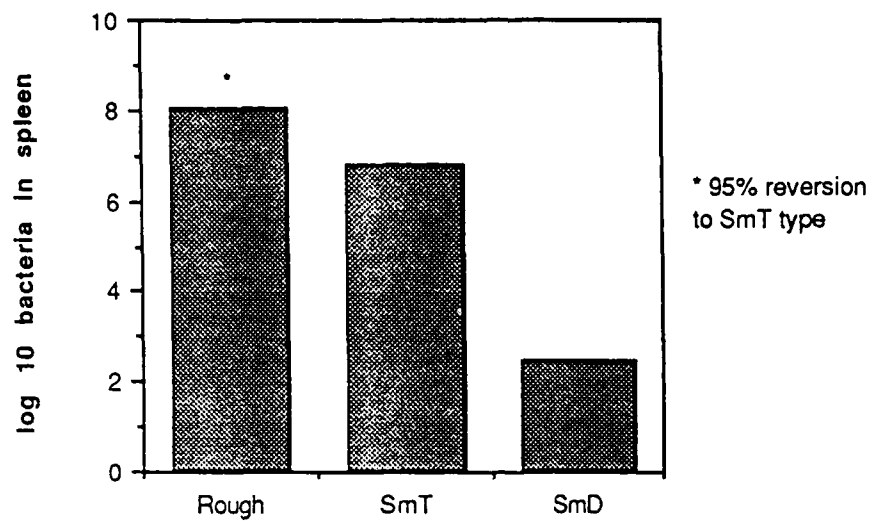
Fig 7. Growth of morphotypes of
strain 2-151 in C57BL/6 mice. Note
morphotype reversion of the rough
variant.
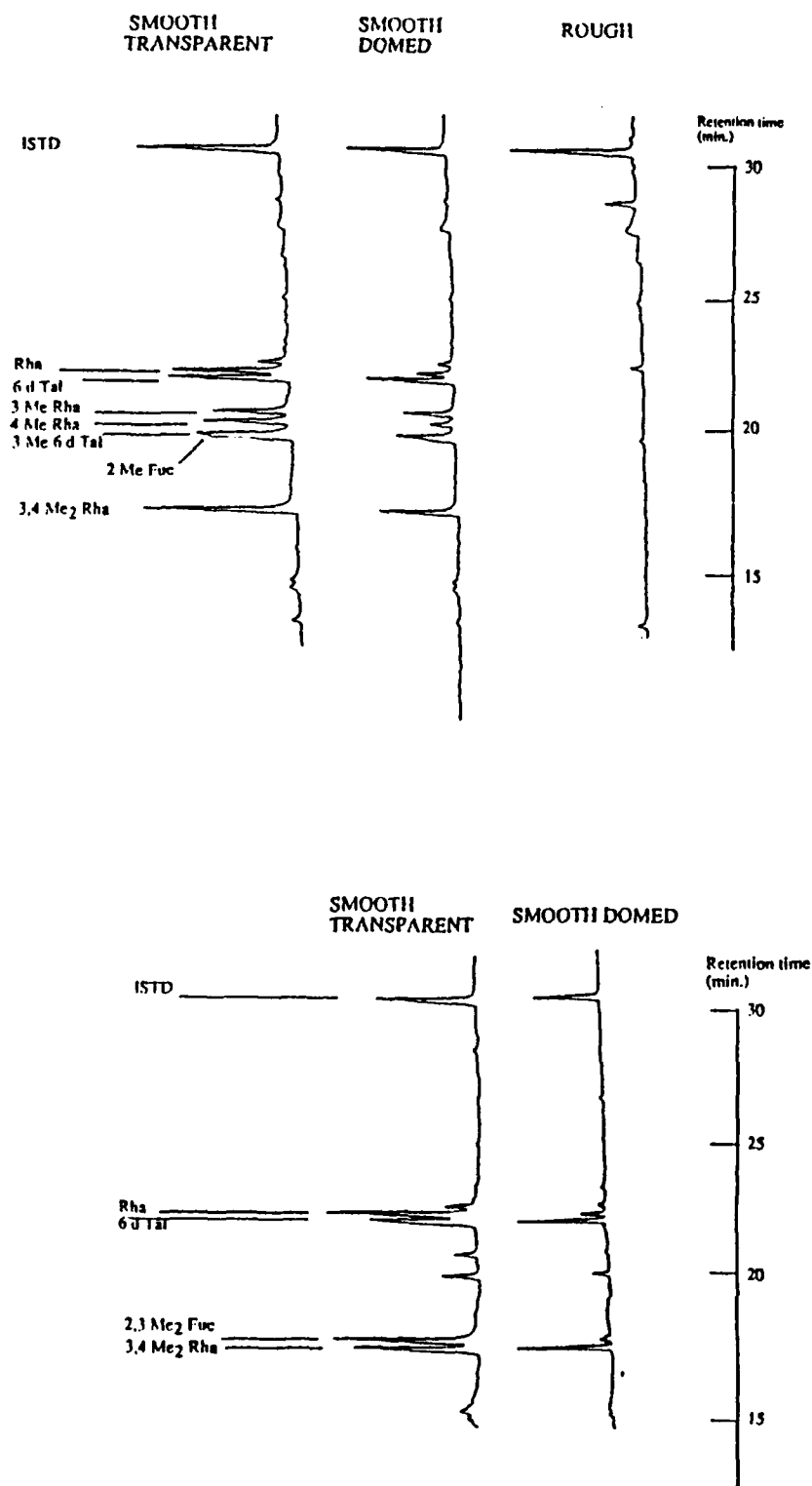
Fig 8. Gas chromatography analysis of monomeric sugars released from the morphotypes of 2-151 and strain Crowle.
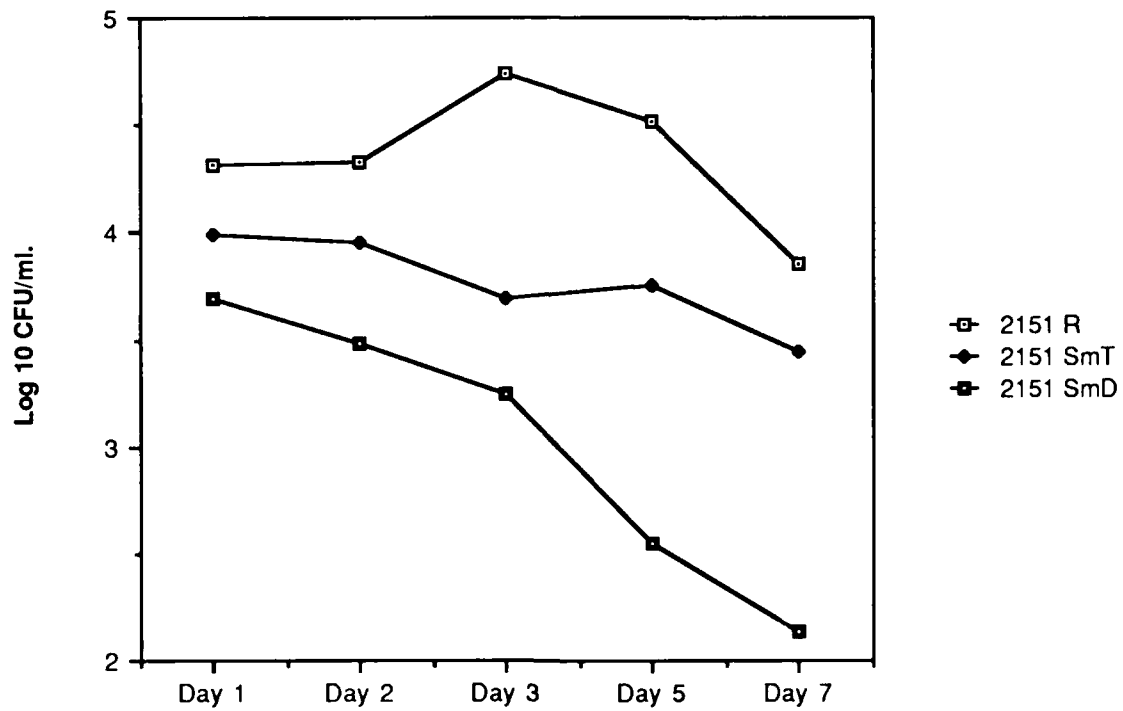
Molar ratios of SmT and SmD variants
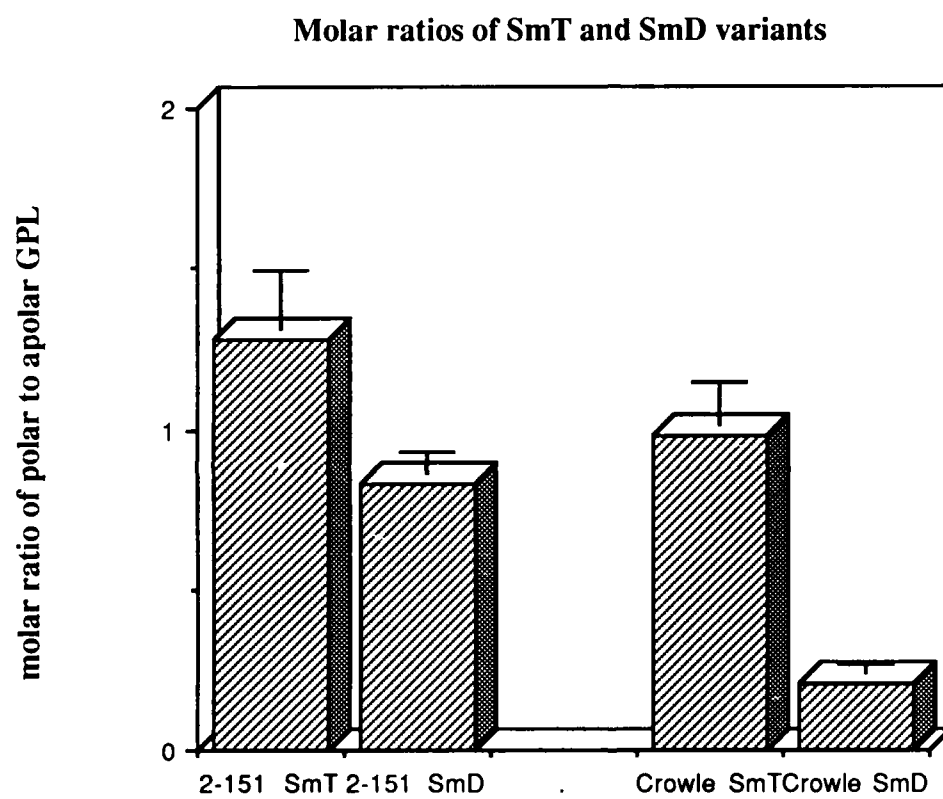
Fig.9

Growth om M. avium #2151 Morphotypes in BMM

Macrophages infected on Day 0 for 1 hour with 1.0E+06 CFU's

Growth of M. avium Crowle Morphotypes in BMM

Macrophages infected at Day 0 for 1 hour with 1.0E+06 CFU's

Time Point

Fig.10.

16 days BMMØ − PMA-triggered O2− prod.
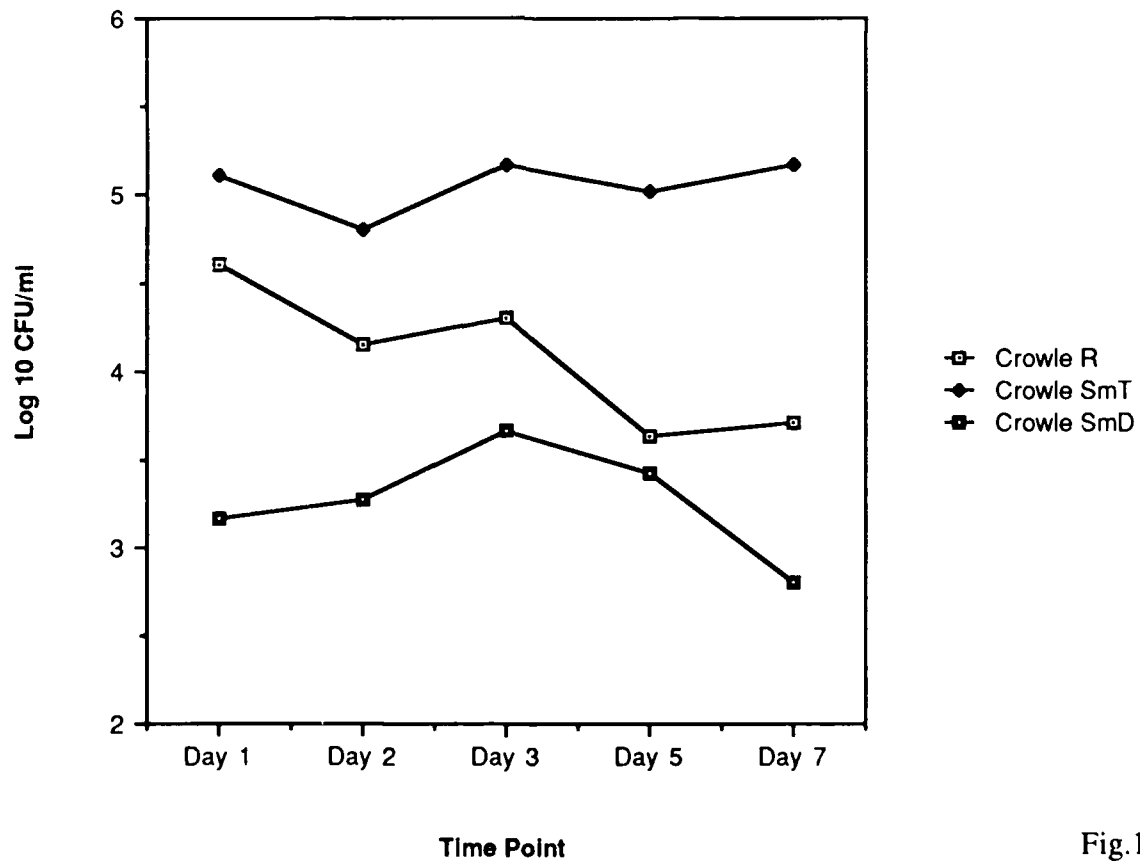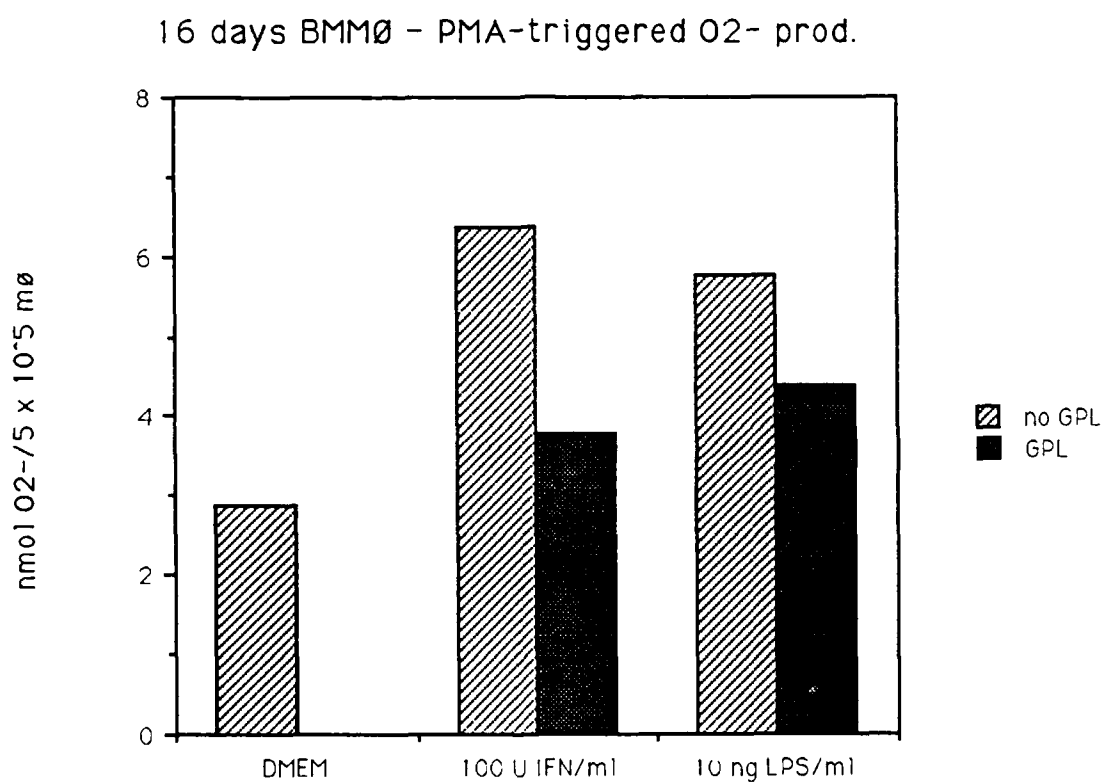
Inhibition of toxic oxygen radical production by gamma interferon-activated
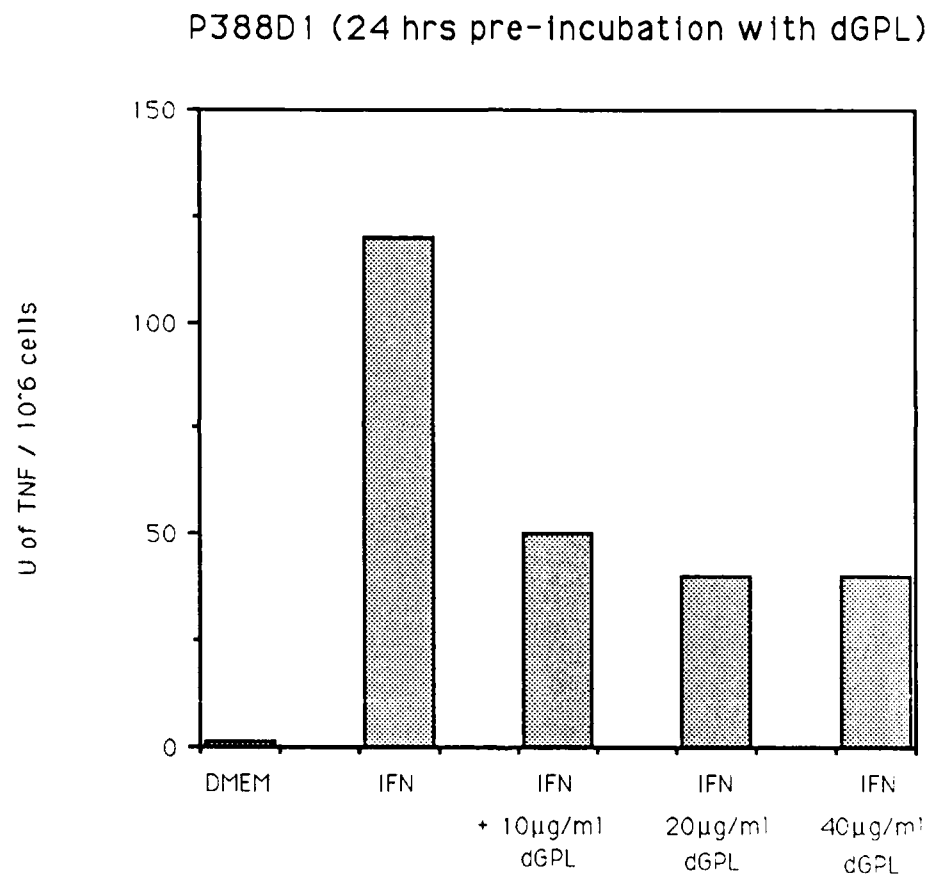bone marrow macrophages following incubation with GPL antigen.

Fig. 11.

Casein-mø – PMA-triggered O2- prod.
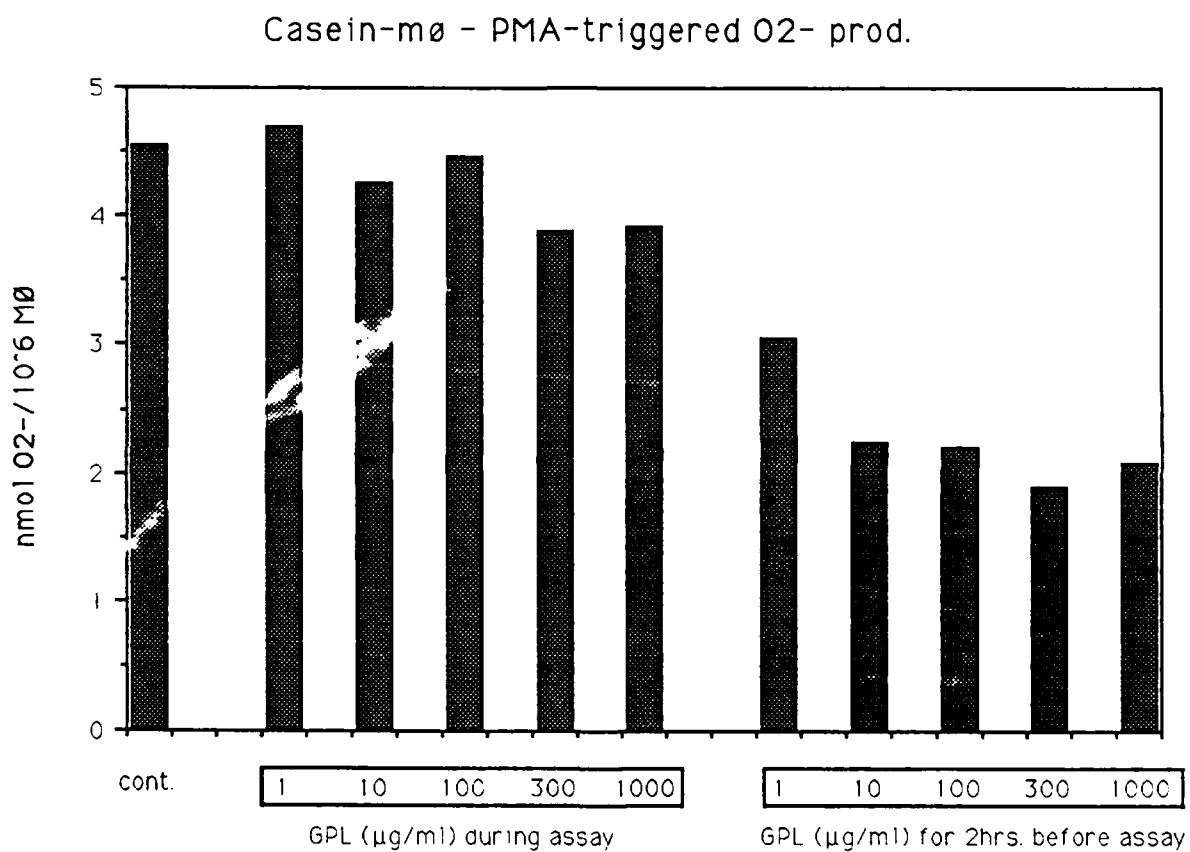
Preincubation, but not coculture of macrophages with GPL inhibits
toxic oxygen radical generation.

Fig. 12

EXP.12 – Cas MØ
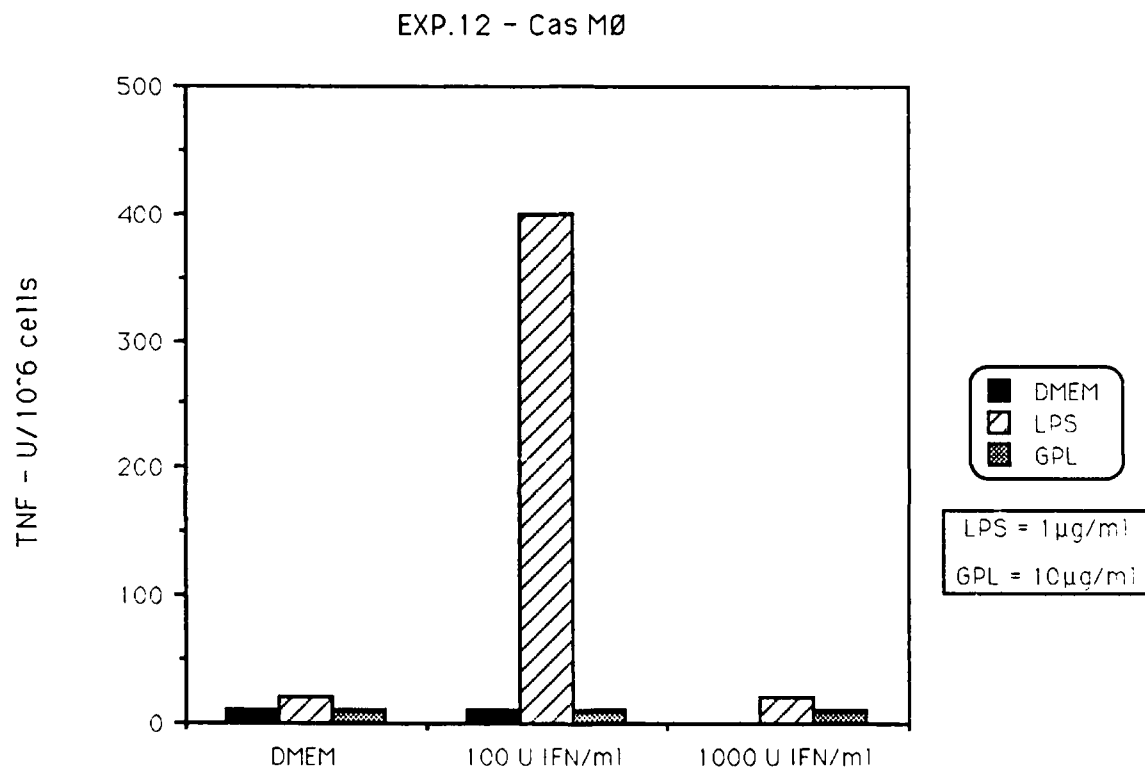
GPL antigen fails to trigger TNF release from casein-induced peritoneal macrophages.

Fig. 13

P388D1 (24 hrs pre-incubation with dGPL)

Preincubation of P388D1 macrophages with GPL reduces the capacity of these cells to become activated by gamma interferon, and to subsequently release TNF upon triggering with LPS.

Fig. 14

P388D1 (48 hrs pre-incubation with dGPL
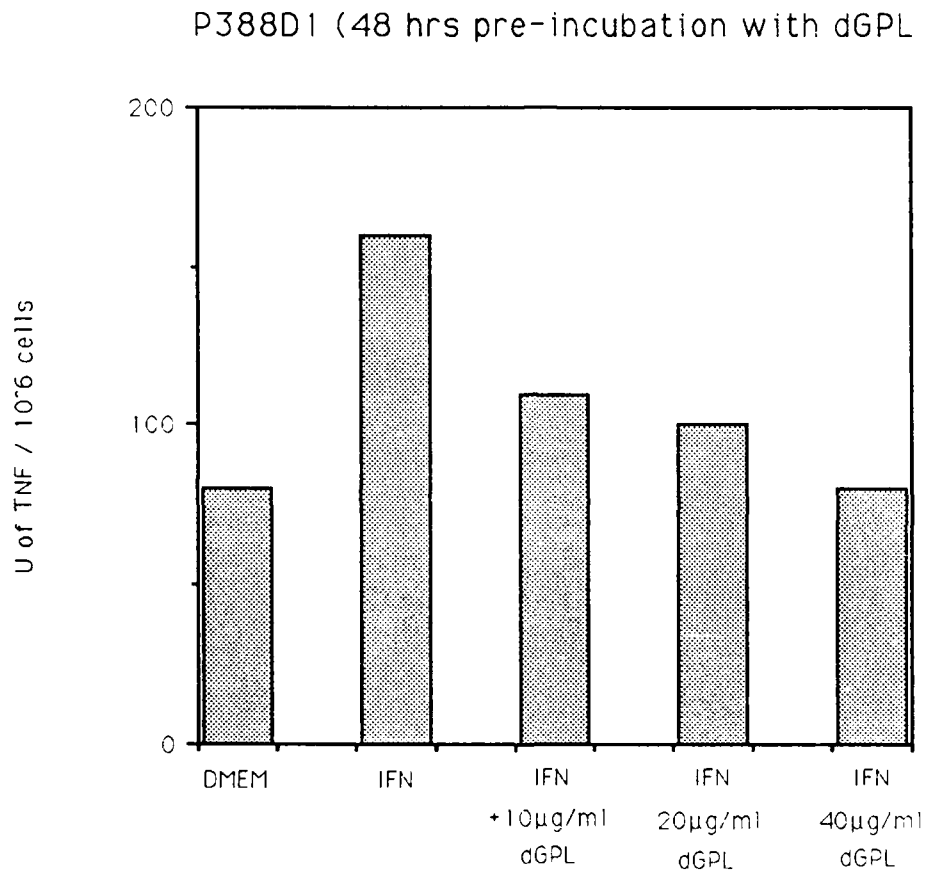
A similar result to the one shown above is achieved if preincubation with GPL is extended to 48 hours.
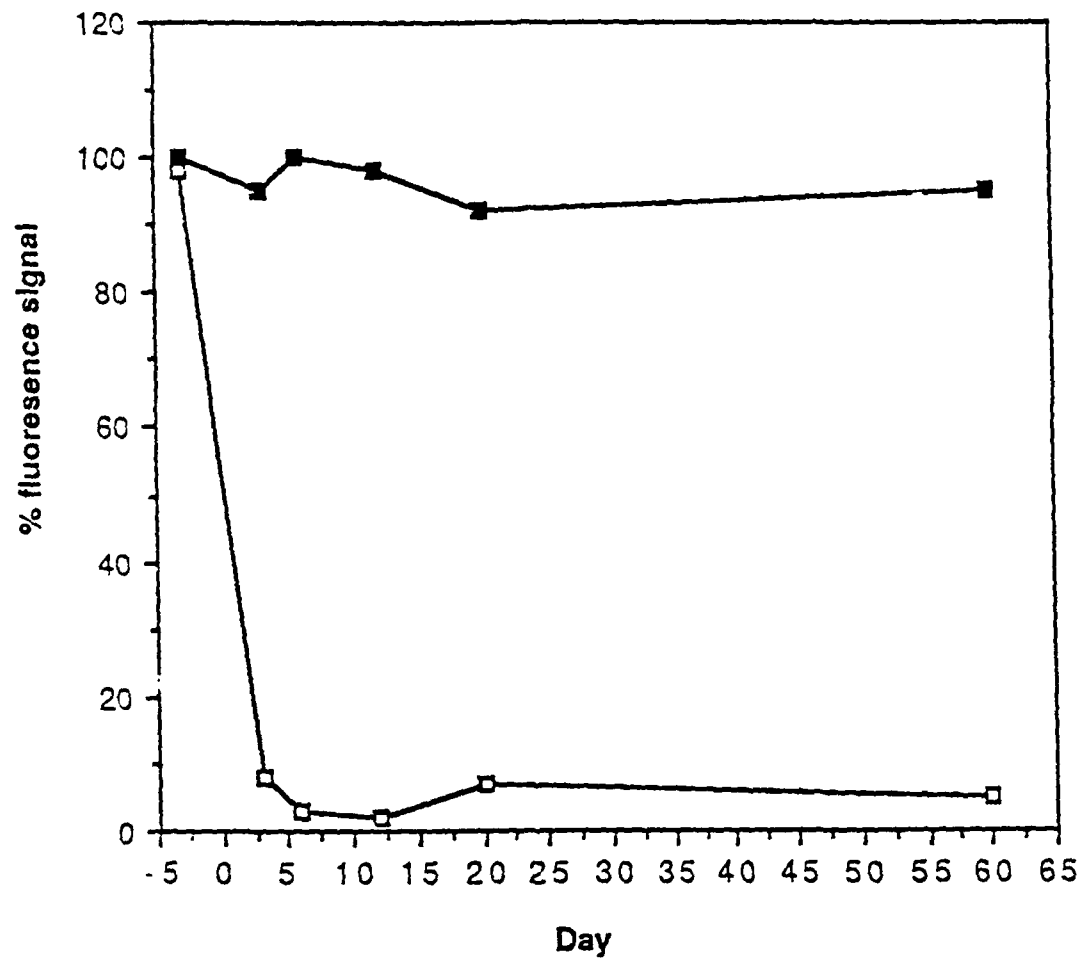
Fig. 15

Fig. 16. Summary of flow cytometry data showing depletion of CD4 T cells in GK1.5 monoclonal antibody treated mice. Control mice (closed squares), antibody treated mice (open squares).
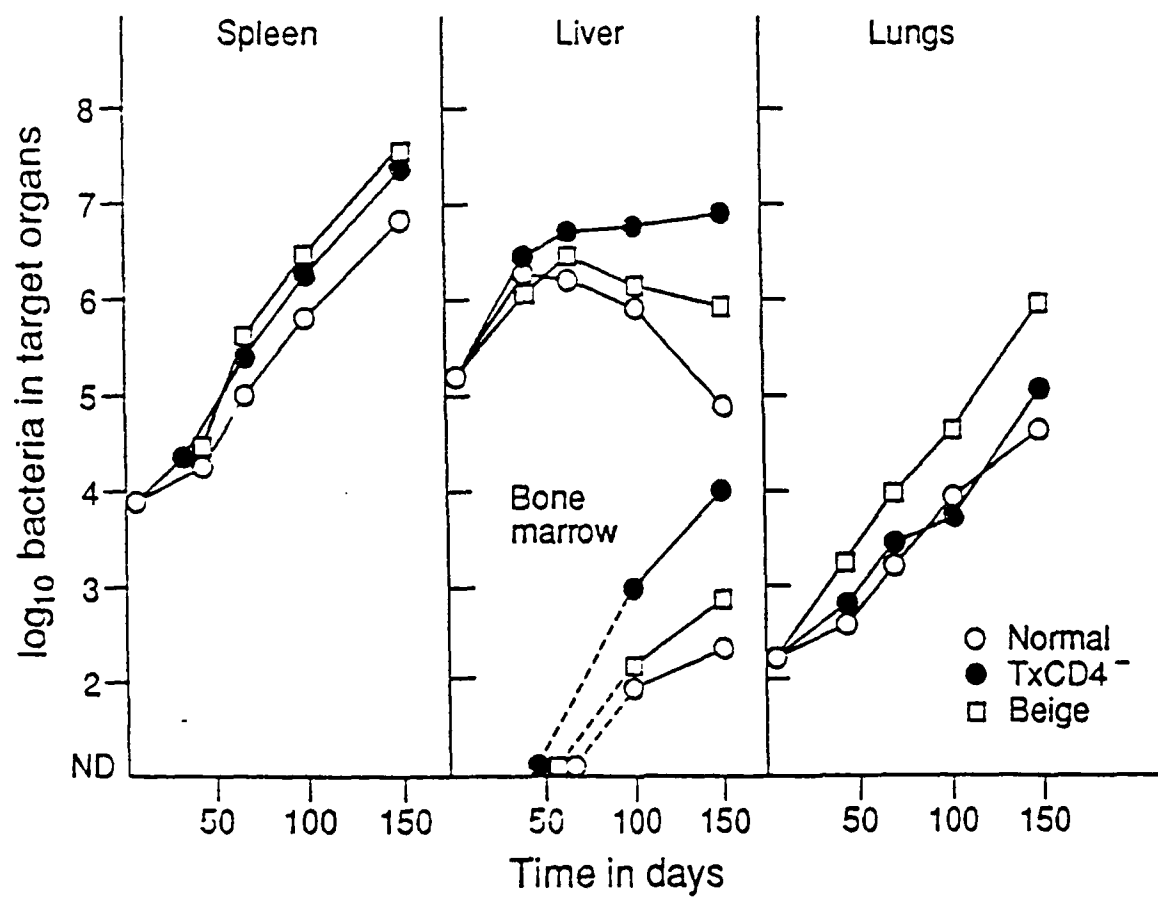
Fig.17. Growth of <u>M.avium</u> in the target organs of normal, beige, or thymectomized CD4 T cell-deficient mice.