IMMUNOASSAYS OF FIELD ISOLATES OF *MYCOBACTERIUM BOVIS* AND OTHER MYCOBACTERIA BY USE OF MONOCLONAL ANTIBODIES

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SUMMARY. Antigen extracts obtained by sonication of 22 strains of *Mycobacterium bovis* from cattle and badgers together with extracts of strains of *M. tuberculosis*, *M. paratuberculosis*, *M. avium*, *M. africanum*, *M. kansasii*, *M. leprae* and BCG were examined with a panel of 10 monoclonal antibodies to *M. tuberculosis* or *M. leprae*. Antigen extracts were coated in aqueous solution (wet coating) and the extracts were also dried on to the polyvinyl plates (dry coating). When dry coating was compared to wet coating, there was a major increase in the binding of monoclonal antibody ML03 to *M. avium* and *M. paratuberculosis*, monoclonal antibody ML02 to *M. paratuberculosis*, and monoclonal antibodies TB71 and TB72 to the majority of *M. bovis* isolates.

The study confirmed that on wet-coated plates, monoclonal antibodies TB71 and TB72 bind poorly or not at all to *M. bovis* and that monoclonal antibodies TB68, TB78, TB77 and TB23 each bind to field strains of *M. bovis* while TB23 binds poorly to BCG in wet-coating conditions. Antibodies TB72 and TB71, originally thought to be specific for *M. tuberculosis*, each reacted with *M. africanum*. Antibody TB78 bound to *M. paratuberculosis* but did not react with *M. avium*, and *M. avium* and *M. paratuberculosis* were distinguished from *M. bovis* and *M. tuberculosis* by the binding of antibody ML03 to dry-coated plates. When wet-coated plates were used, ML03 bound strongly only to *M. leprae*. The panel of monoclonal antibodies did not demonstrate distinct serotype differences between the field isolates of *M. bovis*.

INTRODUCTION

Many of the antigenic determinants of mycobacteria are present on different species and, therefore, antibodies to one strain of *Mycobacterium* will often react with another species. Consequently, the detection of mycobacterial infections by serological means is of limited value (Daniel and Baum, 1968; Freedman, 1976; Morris *et al.*, 1979). Many workers have attempted to isolate species-specific antigens (Kniker and

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La Borde, 1964; Turcotte, 1975; Daniel and Anderson, 1978) but these have yet to be used successfully in the epidemiology and diagnosis of mycobacterial diseases.

Monoclonal antibodies offer a unique opportunity to identify single antigenic determinants on mycobacteria and to study their distribution between different species. Coates *et al.* (1981) described monoclonal antibodies that reacted only with *M. tuberculosis* and *M. bovis*; others reacted only with *M. tuberculosis* and one monoclonal antibody appeared to differentiate between isolates of the same species. Ivanyi *et al.* (1983b) produced monoclonal antibodies to *M. leprae* and described four soluble antigens produced by this organism. One antigen was not detectable in extracts from any of the other 20 species of *Mycobacterium* examined, monoclonal antibodies to a second antigen exhibited marginal cross reactivity with *M. avium*, *M. kansasii* and *M. paratuberculosis*, and two other antigens were shared by several species of *Mycobacterium* including *M. bovis* and *M. tuberculosis*. In the present study the monoclonal antibodies described in both of the previous reports have been used to examine 20 field isolates of *M. bovis* to determine (a) whether the specificity of these antibodies claimed initially still held when a large number of isolates from the same species was examined, and (b) whether the panel of monoclonal antibodies could differentiate between strains of *M. bovis* isolated from cattle and from badgers.

MATERIALS AND METHODS

Mycobacteria. A total of 22 strains of *M. bovis* was examined. Ten were isolated from badgers and 10 from cattle. The laboratory strain AN5 was provided by the Biological Products and Standardisation Department, Ministry of Agriculture, Fisheries and Food, Weybridge and the Vallée strain by Dr A. R. M. Coates, MRC Unit, Hammersmith Hospital. Two strains of *M. tuberculosis* were examined; strain C was from Weybridge and strain H37Rv was supplied by Dr A. R. M. Coates who also supplied *M. africanum*, BCG (Glaxo) and *M. avium* strain NCTC8559. *M. paratuberculosis* strains III and V were from Weybridge.

Preparation of extracts. *M. tuberculosis* strain C and *M. bovis* strain AN5 were grown in Reid's medium and in Sauton's medium. The other mycobacteria were grown on Lowenstein-Jensen medium containing added pyruvate. All bacteria were cultured for 4-6 weeks at 37°C. When appropriate, mycobacteria were harvested in saline, centrifuged at 1000 *g*, resuspended to 250 mg/ml (wet weight) in saline and disrupted by ultrasonication with an MSE ultrasonic disintegrator fitted with a 0.5-inch titanium probe operated at a frequency of 12 μ m peak to peak. The samples were immersed in an ice-water slurry and the disintegrator was switched off after alternate 30-s intervals to reduce heating. After sonication for a total of 15 min, suspensions were centrifuged at 10 000 *g* for 30 min at 4°C and the supernates sterilised by filtration through 0.45- μ m filters. Antigen extracts of *M. avium* JALMA (provided by the Central JALMA Institute, Agra, India), *M. kansasii* (provided by Dr J. L. Stanford) and *M. leprae* (provided by Dr R. J. W. Rees) were also examined.

Radioimmunoassays. Mycobacterial sonicates were diluted to a protein concentration of 100 μ g/ml as estimated by the method of Lowry *et al.* (1951) and 50- μ l volumes were applied to polyvinyl microtitration plates (Dynatech). Plates were incubated overnight at either 4°C (wet-coating) or allowed to dry at room temperature (dry-coating). Subsequently both types of plates were washed once in phosphate buffered saline (PBS), blocked by incubation for 2 h with bovine serum albumen (BSA) 3% in PBS at room temperature and washed again. Hybridoma culture medium diluted 1 in 5 in normal RPMI medium (Gibco Ltd) containing fetal calf serum 10% was applied in 50- μ l volumes in duplicate and incubated overnight at 4°C. Plates were then washed four times with Tween 0.05% in PBS, incubated with 50 μ l of ¹²⁵I-rabbit F(ab)₂ antibody to mouse immunoglobulin diluted in 3% BSA (40 000 cpm) overnight at 4°C, again washed in Tween-PBS and the radioactivity counted. Binding to wells coated with mouse globulin at 50 μ g/ml was taken as 100% (10890 cpm) and binding to uncoated blocked wells as 0% (20 cpm). Figures given represent means from duplicate samples.

RESULTS

The binding of the 10 monoclonal antibodies to sonicates of the various species of *Mycobacterium* is summarised in the table. Monoclonal antibodies TB71 and TB72 bound poorly (1–7%) or not at all (<1%) to the sonicates of all the strains of *M. bovis* and BCG when the conventional procedure of antigen coating was used (wet-coating). When the antigen was dried on to the wells (dry-coating) there was more pronounced binding of both antibodies to sonicates from the majority of *M. bovis* strains and the BCG strain (2–23%). However, the increase in the degree of binding was variable from strain to strain (0–20%) when the two coating procedures were compared. Antibodies TB71 and TB72 bound strongly to sonicates of *M. tuberculosis* when wet-coating was used (18–35%) and the binding was intensified when the antigen was dried onto the plate (12–32% increase). Wells coated with *M. africanum* sonicate showed significant binding of both antibodies (19–24%) which was enhanced in dry-coated wells (6–20% increase). There was no significant binding of antibodies TB71 or TB72 to sonicates of *M. paratuberculosis*, *M. avium*, *M. kansasii* or *M. leprae* irrespective of the method used to coat the wells ($\leq 3\%$). Monoclonal antibody TB78 bound strongly to all sonicates of *M. bovis* and BCG (25–79%) and *M. tuberculosis* (28–53%). With the exception of the sonicate of *M. bovis* strain AN5 grown in Sauton's medium (4% binding), TB68 exhibited the same specificity as TB78 with these strains. There was a tendency for the binding to be enhanced when dry-coating was used but this was more pronounced with the TB68 antibody (5–25% increase). In contrast to TB68 ($\leq 3\%$), TB78 bound to sonicates of *M. paratuberculosis* (10–21%). Marginal binding to *M. africanum* was observed with TB68 when wet-coating was used (6%) but the reaction was stronger with dry-coated plates (14%). TB68 and TB78 did not bind significantly to any of the other sonicates examined regardless of the method used for antigen coating (1–3%).

Monoclonal antibody TB77 bound to all sonicates of *M. bovis* and BCG (11–26%) and with two exceptions (each 5% binding) antibody TB23 reacted similarly. One strain was a field isolate of *M. bovis* and the other was the vaccine strain BCG. When dry-coated plates were used both extracts reacted strongly with antibody TB23 (25% and 31% respectively) and these reactions were comparable to those with other extracts of *M. bovis*. In general, dry-coating increased the binding (1–45% increase) but in some reactions with TB77 there was no increase and with eight sonicates the binding values were reduced when dry-coated antigens were used (1–7% reduction). Antibody TB23 bound strongly to sonicates of *M. tuberculosis* (19–55%), *M. africanum* (80%) and *M. kansasii* (59%). The values were enhanced by dry-coating (19–45% increase) but there was no reaction with other mycobacteria irrespective of antigen coating (<1%). Antibody TB77 bound to *M. paratuberculosis* (9–15%), *M. africanum* (14–15%), *M. tuberculosis* H37Rv (21–23%) and *M. avium* NCTC8559 (20–17%) but the binding was only 4% (wet and dry) with *M. avium* JALMA. This antibody bound poorly to *M. kansasii* (5–7%) and not at all to *M. leprae* (2–3%).

Monoclonal antibodies ML30 and ML34 each bound to all sonicates of *M. bovis* and BCG (17–100%), *M. tuberculosis* (20–89%), *M. paratuberculosis* (30–79%), *M. africanum* (25–37%) and *M. leprae* (43–58%). Both antibodies also bound strongly to *M. avium* NCTC8559 (26–41%) but the reaction of ML30 was weaker with *M. avium* JALMA (8%) and ML34 failed to react with this strain (2%). Antibody ML30 gave 9% binding with *M. kansasii* and ML34 gave 5% binding. Dry-coating had little effect on the binding of these two antibodies.

TABLE
Binding patterns of monoclonal antibodies to mycobacteria

Species	Number of sonicates	Degree of binding to the given species in wet-coating conditions by antibody									
		TB71	TB72	TB68	TB78	TB23	TB77	ML30	ML34	ML02	ML03
<i>M. bovis</i>	22	-(+ +)*	-(+ +)	++(+++)	++++(++++)	++(+++)	++(+++)	+++(++++)	++(+++)	PR	-(-)
BCG	1	-(+ +)	-(+ +)	+++(++++)	++++(++++)	+(+ +)	++(++++)	+++(++++)	++(+++)	PR	-(-)
<i>M. tuberculosis</i>	4	++(++++)	++(++++)	+++(++++)	++(+++)	++(++++)	++(+++)	+++(++++)	++(+++)	++(+++)	-(-)
<i>M. africanum</i>	1	++(++++)	++(+++)	++(+++)	-(-)	+++(++++)	++(+++)	++(+++)	++(+++)	++(+++)	-(-)
<i>M. paratuberculosis</i>	2	-(-)	-(-)	-(-)	++(+++)	-(-)	++(+++)	+++(++++)	++(+++)	++(+++)	-(+ +)
<i>N. avium</i>	2	-(-)	-(-)	-(-)	-(-)	-(-)	±(±)	±(+++)	±(±)	++(+++)	-(+ +)
<i>M. kansas</i>	1	-(-)	-(-)	-(-)	-(-)	+++(++++)	+(+)	++(+++)	++(+++)	++(+++)	++(+++)
<i>M. leprae</i>	1	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	+++(++++)	+++(++++)	+++(++++)	++(+++)

* Results in parenthesis refer to dry-coating conditions; - = <5% binding, ± = binding to one sonicate only, + = 5% to 10% binding, ++ = 11% to 40% binding, +++ = >40% binding. PR = poor reproducibility.

There was individual variation in the relatively weak binding of monoclonal antibody ML02 to different strains of *M. bovis* and BCG (1–10%) but this was poorly reproducible in subsequent assays (data not given). This antibody also reacted with *M. tuberculosis* (6–33%), *M. avium* (10–27%), *M. africanum* (12%), *M. kansas*i (22%) and *M. leprae* (40%). Binding to *M. paratuberculosis* was 5% (both samples). Binding was consistently enhanced when dry-coated plates were used (4–16% increase).

Monoclonal antibody ML03 reacted strongly with *M. leprae* (23%), showed marginal binding with *M. kansas*i (7%) and bound poorly or not at all to all other sonicates examined (1–3%) when wet-coated plates were used. However when dry-coated plates were used, antibody ML03 reacted strongly with *M. leprae* (71%), *M. kansas*i (37%), *M. avium* (33%) and *M. paratuberculosis* (22–24%). The binding to other mycobacteria did not exceed 1% (wet-coating) and 5% (dry-coating).

When Sauton's medium and Reid's medium were used to culture *M. bovis* strain AN5 there were differences in binding values with antibody TB68 (4% in Sautons medium, 38% in Reids medium with wet-coating; 13% and 57% with dry-coating) and antibody ML02 (12% and 3% wet-coating; 25% and 8% dry-coating). These possible medium-related differences were not observed with *M. tuberculosis* strain C. The panel of monoclonal antibodies did not differentiate between *M. bovis* isolates from cattle and badgers.

DISCUSSION

Mycobacterial antigen extracts are often coated in aqueous solution on to polyvinyl microplates, a method we have referred to as wet-coating. In this study the extracts were also dried on to the plates (dry-coating) initially with the intention of economising on the coating antigen. However, the results indicate that dry-coating affects the binding of some monoclonal antibodies to the extracts. Using dry-coating, we found a major increase in the binding of monoclonal antibody ML03 to *M. avium* and *M. paratuberculosis*, monoclonal antibody ML02 to *M. paratuberculosis* and monoclonal antibodies TB71 and TB74 to the majority of *M. bovis* isolates. The increase was most significantly represented when the binding to wet-coated plates was low or undetectable. When low concentrations of target antigens are present in sonicates, dry-coating might be expected to concentrate antigen on to the polyvinyl wells and, because antigen was limiting in these assays, dry-coating might therefore be expected to increase sensitivity. Nevertheless, the increase in the degree of binding of antibody TB71 and TB72 was variable between strains of *M. bovis* and the binding values of TB77 to many of these isolates were either unaffected by dry-coating or reduced. Thus, these differences may represent selective changes in binding patterns that might not be attributed entirely to changes in sensitivity.

The results with monoclonal antibodies TB71 and TB72 on wet-coated plates confirm that these antibodies bind poorly or not at all to isolates of *M. bovis* (Coates *et al.*, 1981). The study has also confirmed that monoclonal antibodies TB68, TB78, TB77 and TB23 each bind to field strains of *M. bovis*. Coates *et al.* (1981) found that antibodies TB23, TB77 and TB78 bound to *M. bovis* strain Vallée but not to BCG. The present study with wet-coating confirmed that antibody TB23 binds poorly to BCG but we found that antibodies TB77 and TB78 bound significantly to our preparations of this strain. In contrast, the binding of antibody TB77 to our sonicates of *M. kansas*i

was poor whereas Coates *et al.* (1981) demonstrated strong binding of this antibody to their extract of *M. kansasii*. These workers also found that antibody TB77 did not bind to *M. avium* and of the two strains of *M. avium* examined in this study only one of the extracts reacted strongly with antibody TB77. A difference between the two extracts of *M. avium* was also evident when the binding of antibodies ML30 and ML34 was examined. These disparities need further investigation, because Coates *et al.* (1981) used a French press to prepare their antigenic extracts and we used an ultrasonic disintegrator for ours. It is possible that the differences may be associated with the potencies of the various extracts.

M. africanum was not included in earlier reports and the significant binding in both assays of antibodies TB71 and TB72 to this organism demonstrates that while these antibodies are capable of differentiating between *M. bovis* and *M. tuberculosis* they cannot differentiate between *M. tuberculosis* and *M. africanum*. Similarly *M. paratuberculosis* has not been examined until now and the binding of antibody TB78 to these sonicates was interesting because this reaction differentiates *M. paratuberculosis* from *M. avium*. *M. avium* and *M. paratuberculosis* were distinguished from *M. bovis* and *M. tuberculosis* by the binding of ML03 to dry-coated but not to wet-coated plates. This antibody also binds to *M. kansasii* when dry-coated antigen is used but when antibody ML03 binds to wet-coated plates the major reaction is only with *M. leprae* (Ivanyi *et al.*, 1983*b*).

The number of *M. bovis* strains examined by monoclonal antibodies has now been extended to 22 comprising laboratory strains and isolates from naturally infected cattle and badgers. The panel of antibodies did not yield distinct "serotype" differences between the field strains of *M. bovis* examined. Nevertheless it is possible to discriminate serologically between isolates of *M. bovis* and *M. tuberculosis* by their differential reaction with antibody TB68 and, for example, antibody TB72. Furthermore, the use of these antibodies as tracers in serodiagnostic immunoassays (Hewitt *et al.*, 1982; Ivanyi *et al.*, 1983*a*) or as immunoabsorbants for the purification of target antigens may be of value in the immunodiagnosis of *M. bovis* infection.

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