Specificity of Antibodies to Immunodominant Mycobacterial Antigens in Pulmonary Tuberculosis

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A serological survey was performed in groups of patients with active sputum smear-positive or smear-negative pulmonary tuberculosis, healthy household contacts, and controls. Sera were tested for titers of antibodies which bound to each of five purified mycobacterial antigens by enzyme immunoassay and for competition of binding to single epitopes, using six radiolabeled monoclonal antibodies directed toward corresponding molecules. The evaluation of diagnostic specificity was based on a positive score represented by titers above the cutoff point of 2 standard deviations above the mean titer of a control group. For smear-positive samples, the best sensitivity (83%) was achieved by exclusive use of the 38-kilodalton (kDa) antigen or its corresponding monoclonal antibodies. For smear-negative samples, levels of antibodies binding to the 19-kDa antigen showed a lower sensitivity of 62% compared with the control group or 38% compared with the contact group. Titters of antibody binding to the 14-kDa antigen were raised in Mycobacterium bovis BCG-vaccinated contacts, indicating that the greatest potential of this antigen may be in the detection of infection in a population for which tuberculin testing is unreliable. The results demonstrated the differing antibody responses to each of the tested antigens and distinct associations with the stage of infection or disease.

The diagnosis of pulmonary tuberculosis depends on the culture of Mycobacterium tuberculosis from sputum samples. Acid-fast bacilli in a sputum smear permit the confident introduction of therapy (17) (although subsequent identification of nontuberculous mycobacteria may alter the drug regimen), while contributory evidence obtained from chest radiography and tuberculin testing is helpful. Smear test positivity is an indicator of infectious advanced disease; when the smear is negative, the delay of several weeks for a culture result, even when available, often means that the patient will become infectious. Therefore, a specific serological test, able to detect early disease, could speed up the start of therapy and reduce transmission of the infection.

Previous investigations into tuberculosis serology have been faced with problems of both sensitivity and specificity (3, 4). Antigen preparations have contained moieties present in many microbial species that lead to serological cross-reactivity (1, 15). Recently, mouse monoclonal antibodies (MAbs) to specific epitopes on antigens of M. tuberculosis (2) have been used in a solid-phase antibody competition test (SACT) to measure antibody titers to these determinants in human sera (5, 6, 9). Although the SACT was successful in identifying smear-positive tuberculosis, it was less valuable in "paucibacillary" disease (8). If there were immunodominant epitopes on the antigens other than those defined by the MAbs, an improved rate of detection of smear-negative tuberculosis cases might be achieved by using the purified whole molecules to measure antibody levels. In this paper we have compared the antibody titers in human sera to six epitopes of mycobacterial antigens with antibody titers to the corresponding whole-antigen molecules with respect to their potential value for the diagnosis of tuberculosis.

MATERIALS AND METHODS

Sera. Sera were collected from patients at the London Chest Hospital at the start of treatment for suspected tuberculosis. A total of 46 patients with sputum smear-positive pulmonary disease were included. For 13 smear-negative patients, treatment was commenced on the basis of radiographic evidence, although diagnosis was subsequently confirmed in 5 subjects by sputum culture. Sera were obtained from 18 contacts who lived in households each containing a person with smear-positive pulmonary tuberculosis. Control sera were obtained from 38 healthy residents of the United Kingdom. Mycobacterium bovis BCG vaccination status was confirmed by the presence of a visible scar.

MTSE. To prepare M. tuberculosis soluble extract (MTSE), strains H37Rv and H37Ra were grown as surface cultures on Sauton medium for 8 weeks. The medium was separated from the bacilli by centrifugation and rendered safe for use by filtration (Durapore 0.45-μm-pore-size filter). The bacilli were irradiated (2.5 megarads, 30Co) and disrupted as described previously (11). Briefly, bacilli were suspended 1:1 (wt/wt) in ice-cold phosphate-buffered saline (Dulbecco A without Ca2+ or Mg2+; PBS) and placed in a bottle containing glass beads (diameter, 0.1 mm). The suspension was then agitated at 4,000 rpm for 2 min in a homogenizer (B. Braun, Melsungen AG, Melsungen, Federal Republic of Germany). The soluble extract was separated from the cell debris by centrifugation at 47,000 × g for 1 h at 4°C (SS-34 rotor; Ivan Sorvall, Inc., Norwalk, Conn.). The optimal coating concentrations of the bacterial extracts and filtrates for immunoassays were estimated after measurement of the protein content (phenol reagent method, procedure 690; Sigma, Poole, Dorset, England). Unless otherwise stated, MTSE refers to the soluble extract of strain H37Rv.

Antigen purification. Purification of the 14-, 19-, and 38-kilodalton (kDa) protein antigens of tubercle bacilli was based on the use of MAb affinity chromatography as

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previously described (20). The initial preparatory steps differed for each antigen. For the 14-kDa protein, MTSE at a protein concentration of approximately 2.5 mg/ml was dialyzed extensively against half-strength PBS containing 0.05% Triton X-100. The 19-kDa antigen was similarly prepared from the culture filtrate (0.5 mg/ml of protein) of M. tuberculosis H37Ra. For purification of the 38-kDa antigen, the culture filtrate of strain H37Rv was subjected to lyophilization and the redissolved protein was precipitated by ethanol at 80% of total volume. The precipitate was resuspended in a minimal volume of PBS, after which the suspension was centrifuged at low speed to remove insoluble material. The resultant supernatant was dialyzed overnight against PBS.

Affinity chromatography was done by first coupling the globulin fraction precipitated by 18% sodium sulfate from ascitic fluid from mice injected with hybridomas TB68, TB23, and TB71, respectively, to CNBr-activated Sepharose 4B as recommended by the manufacturer (Pharmacia, Uppsala, Sweden) at 5 to 10 mg of protein per ml of gel. The columns were washed thoroughly with elution buffers and equilibrated with PBS. The culture filtrate or soluble extract was then passed through the affinity column at a flow rate of approximately 30 ml/h, followed by a washing of the column with PBS until the eluate showed no significant absorbance at 280 nm. After further washing of the column with 1 M NaCl (10 to 15 ml for a 10-ml column), the antigen was eluted from the column with 0.1 M glycine-HCl, pH 2.5, followed by the same buffer containing 10% (vol/vol) dioxan. A typical column would yield 0.5 mg of purified antigen from approximately 50 mg of starting material.

The eluted fractions were collected in tubes containing 2 M tris(hydroxymethyl)methyamine to adjust the pH immediately to near neutrality and then extensively dialyzed against PBS over a period of 48 h. Mouse antibody fragments leached from the column during elution were eliminated by passing the eluate through a second CNBr-Sepharose 4B column (2 ml) coupled with rabbit anti-mouse immunoglobulins. Finally, the fractions were dialyzed against PBS overnight, divided into equal portions, and stored at −20°C. All chromatography was performed at room temperature, and all dialysis steps were done at 4°C.

The 65-kDa antigen from M. bovis BCG, purified from overproducing recombinant Escherichia coli K-12 cells (16), was kindly supplied by J. van Embden, and purified lipoprotein fraction (LAM) (7) from M. tuberculosis was obtained from P. Brennan.

Assessment of antigen purity as applied to enzyme-linked immunosorbent assay (ELISA) serology. Purified antigens diluted in 0.1 M sodium bicarbonate buffer, pH 8.5, were coated onto microtiter plates (Immunol M129B; Dynatech, Billingshurst, Sussex, England) by overnight incubation at 4°C. Plates were washed with PBS containing 0.05% Tween 20 (PBST), and nonspecific binding was blocked with PBST containing 3% (wt/vol) bovine serum albumin. In all assays involving ML34 antibody (for LAM determinations), the bovine serum albumin was omitted. MABS were used at 0.05 μg/ml diluted in PBST. The MABS used were TB68, TB23, TB71, TB72, TB78, ML30, and ML34, whose properties have been fully described previously (10). After incubation with the primary antibody for 1 h at 37°C, the plates were washed with PBST and bound antibody was detected by using a peroxidase-conjugated goat anti-mouse immunoglobulin antibody. Peroxidase activity was assayed by using tetramethylbenzidine and hydrogen peroxide substrates as described fully elsewhere (11).

Each purified antigen bound only to its complementary MAB (optical density values greater than 0.4 U), with background absorbance values of less than 0.1 optical density units with the other MABS. The coating concentration used for this test of purity for each antigen was equal to or greater than that used in subsequent assays with human sera.

SACT. The radioimmunoassay technique used is fully described elsewhere (5, 6). Briefly, polyvinyl microdilution plates (M24; Dynatech) were coated with 50 μl of 30-μg/ml MTSE diluted in PBS overnight at 4°C. Plates were washed with PBS and then blocked with PBS containing 3% (wt/vol) bovine serum albumin. Human sera diluted in bovine serum albumin were incubated for 4 h at 20°C and then without washing incubated further with 125I-labeled MAB overnight at 4°C. After being washed and dried the wells were counted in a 1260 Multigamma II counter (LKB-Wallac, Milton Keynes, United Kingdom).

Solid-phase antibody binding test (ELISA). Antibodies in human sera binding to solid-phase bound purified antigens of M. tuberculosis were developed by using peroxidase-conjugated anti-human immunoglobulin G (IgG). Microdilution plates (M129B) were coated with either 14-kDa, 19-kDa, 38-kDa, 65-kDa, or LAM antigens or with PBS for subsequent serum blank correction. The optimal coating concentration for each antigen, established individually, was 0.2 μg/ml for the 14- and 19-kDa molecules, 0.35 μg/ml for the 38-kDa molecules, 1 μg/ml for the 65-kDa molecules, and 0.1 μg/ml for LAM. The plates were incubated overnight at 4°C. They were then washed once with PBST, and nonspecific binding was blocked by the addition to each well of 150 μl of a solution of semiskimmed dried milk (J. Sainsbury, London; 1%, wt/vol) in PBST, followed by 1 h of incubation at 37°C. After the blocking solution was tipped off, 50-μl portions of dilutions of human sera in the milk-PBST solution were added to plates individually coated with each of the antigens or with PBS alone. Plates were incubated for 1 h at 37°C and washed four times with PBST; 50 μl of affinity-purified goat anti-human IgG peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) diluted 1:3,000 in milk-PBST was then added to each well, followed by a further incubation for 1 h at 37°C. After six washes with PBST, bound peroxidase activity was estimated as described earlier (11) and optical densities were corrected by subtracting the nonspecific binding to PBS-coated wells at corresponding serum dilutions. Results were calculated by expressing the binding of the test serum at each dilution as a percentage of the binding of a selected “standard” serum (high control).

Statistical analyses. Cutoff titers for antibodies binding to each antigen and antibodies competing with each MAB were defined as the log mean titer ± 2 standard deviations (SD) of the control group. Sera with titers of ≤1 were all given a value of 1, and those with titers of >700 were given a value of 700. Correlations between responses to each pair of antigens were analyzed by using the Spearman rank correlation coefficients (r ± 95% confidence intervals). Comparison of the distributions of antibody titers between two groups was by the Mann-Whitney U test, giving a P value and 95% confidence intervals for the difference in mean rank (d). Comparison among several groups was by the Kruskal-Wallis analysis, where H values indicate the heterogeneity between groups and z values indicate the difference in mean rank for each group compared with the whole sample.
RESULTS

Definition of cutoff points between patient and control groups. The titers of IgG antibodies in human sera were determined in groups of patients with active smear-positive or smear-negative pulmonary tuberculosis, in healthy contacts, and in healthy controls (Fig. 1). Cutoff values to afford discrimination between controls and other groups were calculated as 26, 10, 1, and 354 for the 14-kDa, 19-kDa, 38-kDa, and LAM antigens, respectively. The calculated value for the 65-kDa antigen (1,158) did not permit any discrimination between the groups.
TABLE 1. Binding specificity of antibodies in individual patients with smear-negative pulmonary tuberculosis

<table>
<thead>
<tr>
<th>Group</th>
<th>Titer of ELISA binding to given antigen</th>
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<tr>
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<td>14 kDa</td>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>Mean</td>
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<tr>
<td>Cutoff*</td>
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<tr>
<td>Smear negative</td>
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<tr>
<td>Culture positive</td>
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<tr>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>Culture negative</td>
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* Log mean titer + 2 SD of healthy controls.

** Boldface values are those above the cutoff titer.

Antibodies to the 38-, 19-, and 14-kDa protein antigens. The pattern of binding to the 38-kDa antigen was very similar to that shown by TB71 (r = 0.742 ± 0.068; P < 0.001) and TB72 (r = 0.843 ± 0.045; P < 0.001) competition. However, only 2 serum samples among the 38 controls gave measurable titers of binding to the 38-kDa antigen. At 99% specificity (control log mean + 3 SD), 38-kDa antigen binding identified more sputum-positive cases (38 of 46, or 83%) than did binding with either TB71 (26 of 46, or 57%) or TB72 (25 of 46, or 54%). Both ELISA (P = 0.12) and TB72-SACT (P = 0.23) failed to distinguish smear-negative patients from controls. Although TB71-SACT was able to detect 31% of smear-negative samples, discrimination from vaccinated controls was much less than for smear-positive patients (P = 0.02, 1.0 < d < 3.0).

Titters of binding toward the whole 14-kDa antigen and the TB68 epitope competing titers were significantly raised in all groups relative to titers for the healthy controls (Kruskal-Wallis analysis: 14-kDa antigen, H = 50.79, z = -6.10, -1.20, 1.05, and 6.10 for controls, contacts, smear-negative group, and smear-positive group, respectively; TB68, H = 61.73, z = -6.65, -0.84, -0.30, and 7.21 for controls, contacts, smear-negative group and smear-positive group, respectively). Values in the smear-positive group were highest, although 5 of the 18 healthy contacts demonstrated titers of binding to the 14-kDa antigen that were above the cutoff. Antibody levels to the 19-kDa molecule or in competition with TB23 did not distinguish between contacts and controls (P = 0.68 and 0.95, respectively). However, sera from 8 of 13 patients with smear-negative disease did show increased binding to the 19-kDa antigen (P = 0.002; 0.19 < d < 1.15, Mann-Whitney U test; Table 1), whereas none showed high competition titers of TB23 specificity.

Antibodies to the 65-kDa protein and LAM. High antibody titers of binding to the 65-kDa antigen were observed in all groups, precluding the possibility of any discrimination between diseased groups and controls. However, titers of antibodies with specificity for the TB78 epitope were higher in smear-positive samples than in controls (12 of 46 versus 0 of 38). A wide range of individual titers was seen in binding to the LAM antigen in all groups, with an accompanying increase in titers for controls and contacts through smear-positive tuberculosis (log mean titer ± SD: controls, 0.94 ± 0.80; contacts, 0.80 ± 0.86; smear-negative group, 1.66 ± 0.92; smear-positive group, 2.10 ± 0.82). This increase was apparent through all groups with ML34 competition (controls, 1.16 ± 0.20; contacts, 1.27 ± 0.21; smear-negative group, 1.41 ± 0.32; smear-positive group, 1.79 ± 0.45). In view of the polysaccharide structure of the LAM antigen, sera were tested also for IgM antibodies. Although these were present in the majority of sera, the IgG responses presented in the figure discriminated between smear-positive patients and controls better than did IgM responses. Thus, 54% of patients had greater IgG than IgM titers, compared with only 29% in the control group (data not shown).

**Correlations between antibody specificities.** The antibody titers obtained from the 46 smear-positive patients were analyzed for possible correlations between responses to each pair of antigens. This analysis revealed the strongest correlation (r = 0.717 ± 0.072; P < 0.001) between titers of binding to the 14-kDa and LAM antigens. Binding to the 19-kDa antigen accompanied binding to the 38-kDa and 14-kDa antigens (r = 0.574 ± 0.099 and 0.523 ± 0.107, respectively; P < 0.01), although these correlations were less than that between the 14-kDa and LAM antigens. There was no significant correlation (r = 0.160) between titers of binding to the 19- and 65-kDa antigens.

**Antibody titers in patients with smear-negative disease.** The titers of antibodies which bound to each of the purified antigens in the sera of patients with smear-negative pulmonary tuberculosis are shown in Table 1. Compared with results from healthy controls, binding to the 14-kDa antigen identified 7 of 13 cases, and binding to the 19-kDa antigen identified 8 of 13. Together, they were able to identify 11 of 13 cases, including all 5 culture-positive cases. No useful discrimination between smear-negative disease and controls was possible with the other antigens.

**Effect of vaccination with M. bovis BCG on anti-14-kDa antigen titers in healthy contacts and controls.** The ranked titers of binding in sera are shown in Fig. 2. All tested controls had been BCG vaccinated and, like the nonvaccinated contacts, showed low or undetectable titers of binding. However, an increased response to this antigen was observed in the BCG-vaccinated contact population. The dis-

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**FIG. 2.** Effect of BCG vaccination on titers of binding to the 14-kDa antigen in sera from healthy household contacts. For serum samples from the control group, n = 38; for those from BCG-vaccinated contacts, n = 9; for those from nonvaccinated contacts, n = 9.


tation of antibody titers to the 14-kDa antigen differed significantly between vaccinated and nonvaccinated contacts ($P = 0.04$; Mann-Whitney U test). Furthermore, comparison of the number of sera with titers of $\geq 1$ with those having titers of $>1$ revealed a highly significant difference between the vaccinated contacts and the vaccinated controls ($P = 0.006$; Fisher exact test) but not between nonvaccinated contacts and controls ($P = 0.523$).

**DISCUSSION**

The development and application of a SACT technique using mouse MABs directed against specific epitopes on mycobacterial antigens advanced the potential for the serological diagnosis of smear-positive tuberculosis (5). Its principal advantage is the inherent epitope specificity of MABs, with TB68, TB71, and TB72 restricted to tubercle bacilli (10). However, a possible loss of sensitivity could have resulted from the limited repertoire of antibodies to single epitopes. Therefore, we have analyzed the binding of antibodies in human sera to purified antigens which carry the epitopes recognized by the mouse MABs. We wished to see if (i) the specificity previously demonstrated with the MABs was retained with the whole molecules and (ii) greater sensitivity was achieved by the contribution of additional immunodominant epitopes.

All previous surveys using the SACT assay had compared antibody titers in patients with those in healthy controls who had not knowingly been in contact with active tuberculosis. We therefore compared the ELISA with the SACT on that basis first. However, we also considered that in population screening, particularly in areas where tuberculosis is endemic, a test should be able to discriminate between diseased and healthy people, even where the latter have been infected through contact with people with active tuberculosis. We therefore included a study group of individuals who lived in the same domiciliary unit as a person known to have smear-positive tuberculosis but who did not show any symptoms of the disease for 1 year after the blood sample was taken. Groups of patients were also compared with this group of contacts for both assays.

Studies of the 38-kDa antigen expanded the previous evidence that the TB71 and TB72 epitopes were restricted to tubercle bacilli (2, 10) and immunodominant in tuberculosis (5, 6, 9). No loss of specificity was observed by widening the test window to include the whole molecule. Indeed, the fact that the observed sensitivity of 83% for the 38-kDa antigen was almost identical to that for the combination of TB71 and TB72 (84%) suggests either that these two may represent the total of immunodominant epitopes on this antigen for humans or that the response to any other presumed epitopes is similar in specificity and magnitude to that of the TB71- and TB72-like responses. This interpretation agrees with the description of other MAB-defined epitopes on this molecule (12, 18). Antibody titers (30% antigen-binding titer, $>5$) to the 38-kDa molecule were found only in smear-positive patients, not in contacts or in smear-negative patients. The absence of titers in the contact group is a particularly favorable pointer to the potential use of this antigen in a simple test for the diagnosis of smear-positive disease in areas in which the disease is endemic. The absence of antibodies in the smear-negative group indicates that antibody production to the 38-kDa antigen is a reflection of either bacterial load or certain pathological events associated with the advanced stage of the disease. Perhaps the kinetics of release of this antigen in vivo from live or dead, intra- or extracellular bacilli determine its immunogenicity.

The pattern of antibody response to the 19-kDa antigen was similar to that of the TB23 epitope, enabling discrimination of 61% of smear-positive patients relative to the control population. The observation that this figure was similar (62%) for patients with smear-negative disease may be indicative of an earlier response to this antigen than to the 38-kDa antigen, which had only 15% sensitivity in this same group of patients. Although there was no significant difference in the distributions of the antibody titers to the 19-kDa antigen between the contact and control groups, in terms of discriminatory cutoff values based on 2 SD above the log mean titer value, antibody titers to the 19-kDa protein were slightly increased in the contact group. This increase only marginally affected the sensitivity in smear-positive disease but reduced to a greater extent the discrimination of the smear-negative patients because of their lower overall titers. However, 38% (5 of 13), including 3 of 5 culture-positive patients, were still diagnosed successfully by binding to this antigen.

The loss of sensitivity found by comparing diseased groups with the contact group rather than the healthy controls was even more pronounced with the 14-kDa antigen. The effect on the discriminatory potential of TB68 by making the same comparison was similar, reducing the diagnostic smear-positive disease from 61 to 36. However, in contrast to the responses to the whole 38-kDa molecule and the specific TB71 and TB72 epitopes, the presence of high titers to the 19- or 14-kDa antigen in sera with low titers against the TB23 or TB68 epitopes suggests the presence of immunodominant epitopes as yet undefined by MABs. However, the low titers of binding to whole 19- and 14-kDa antigens in the control group suggest that the
additional epitopes are of restricted specificity (unlike the 65-kDa protein).

It was reported previously that hospital workers having contact with tuberculosis patients had elevated TB68 antibody titers (6). This was corroborated by the finding that titers to the 14-kDa antigen in the contact group were enhanced by prior BCG vaccination. Although the increase in titers of binding to the 14-kDa antigen in vaccinated contacts appears to be specific to sensitization by BCG, cross-reactivity at the T-cell level with other mycobacteria (13) might contribute to the response in areas with abundant environmental mycobacteria.

Even if the results from the contact group were used to establish the cutoff titers, a combination of the 14- and 19-kDa antigens successfully diagnosed four of the five smear-negative, culture-positive cases, whereas only one of eight culture-negative cases was correctly identified in this manner. Despite the small number of cases, these data warrant further study of smear-negative pulmonary tuberculosis. Even if success were limited to the culture-positive group, this finding would still be a valuable aid in the decision of whether to treat a patient pending the outcome of the culture and hence reduce the spread of infection.

The lack of discrimination afforded by the 65-kDa antigen was expected and in general agreement with recent data. It is known that the 65-kDa antigen is a member of a family of stress proteins whose presence is ubiquitous among bacteria thus far examined (16, 19; J. Ivanyi, K. Sharp, P. S. Jackett, and G. H. Bothamley, Springer Semin. Immunopathol., in press). Therefore, individuals certainly encounter this antigen within commensal organisms, resulting in high natural antibody levels as shown in this study. Although the ML34 antibody is specific for the genus Mycobacterium, similar lipopolysaccharide structures are prevalent in other microorganisms, indicating that some epitopes may be cross-reactive with other genera (14). This fact may explain the wide range of antibody titers to this antigen in control subjects.

In conclusion, our results indicate that a raised level of antibody to the 38-kDa antigen is the potentially most useful serological test for smear-positive tuberculosis. Antibodies to the 19- and 14-kDa molecules are the most relevant specificities for the detection of smear-negative disease, although anti-14-kDa antigen responses occur also in a proportion of sensitized healthy subjects. In contrast, the excessive cross-reactivity of the 65-kDa and LAM antigens may be reduced only at the epitope level, currently as found by using the SACT and in the future perhaps by use of binding assays to prospective peptides and oligosaccharides.

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LITERATURE CITED